Review

**Herpesviridae and novel inhibitors**

George Siakallis¹, Demetrios A Spandidos¹ and George Sourvinos¹*

¹Department of Virology, Faculty of Medicine, University of Crete, Crete, Greece

*Corresponding author: e-mail: sourvino@med.uoc.gr

**Herpesviridae** comprises a large family of double-stranded DNA viruses that infect both animals and humans. Eight herpesviruses are known to infect humans: herpes simplex virus type-1 and -2, varicella zoster virus, human cytomegalovirus, Epstein–Barr virus, human herpesvirus 6 type-A and -B, human herpesvirus type-7 and -8 or Kaposi's sarcoma virus.

Despite the fact that the past two decades have been evolutionary in the development of antiviral agents, therapeutic choices are restricted by limited efficacy and toxicity. Viral infections remain the cause of significant mortality worldwide, thus indicating the high medical need for the introduction of novel promising compounds in the antiviral chemotherapy era.

This review focuses on recent data regarding several novel groups of agents that have proved to be effective as antitherpetic drugs. The agents mentioned are those considered to be the most likely candidates for entering clinical trials and those in the process of being granted approval by the US Food and Drug Administration. The diversity in their molecular mechanism of action highlights the different perspectives currently encountered in the era of antiviral therapy.

**Benzimidazole ribonucleosides as anti-HCMV agents**

Over 50% of the population worldwide is seropositive for human cytomegalovirus (HCMV). This ubiquitous herpesvirus is the cause of widespread infections in humans and, although benign in immunocompetent hosts, patients with immature (for example, neonates) or compromised (for example, AIDS patients and organ transplant recipients) immune systems suffer from life-threatening complications [1]. Currently available drugs that have been approved by the US Food and Drug Administration (FDA) include ganciclovir, foscarnet, cidofovir, fomivirsen and valganciclovir [2].

A novel class of compounds called benzimidazole ribonucleosides (Table 1) is currently being developed for the treatment of HCMV infections. A representative agent of this group is 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl)-benzimidazole (BDCRB). Although BDCRB is efficacious in inhibiting HCMV replication (Table 2), it is rapidly metabolized to the inactive and toxic aglycone. The more stable 1-riboside analogue, maribavir (1263W94), was developed as the most promising candidate and is the leading compound of this group for further clinical studies.

**Maribavir**

Results from plaque reduction assays have shown that maribavir was fivefold more active than ganciclovir in inhibiting viral replication of the HCMV AD19 laboratory strain [3]. Similar results were obtained using clinical viral isolates of wild-type and ganciclovir-resistant HCMV. Additionally, maribavir has proved to be effective even against multiple drug-resistant mutants (ganciclovir, foscarnet and cidofovir), supporting the notion of a different mechanism of action compared with the currently used agents [3,4].

It has been shown that BDCRB, the parental agent of benzimidazole ribonucleosides, inhibits HCMV replication at the stage of viral DNA maturation as resistance conferring mutations were mapped to UL89 and UL56 genes (the small and large subunit of HCMV terminase, respectively) [5,6]. Surprisingly, maribavir has a unique mode of action. Biron et al. [3] demonstrated that maribavir inhibited viral DNA synthesis in HCMV-infected cells without direct inhibition of the viral DNA polymerase. Selection of drug-resistant mutants mapped resistance conferring mutations in the UL97 open reading frame (ORF); thus, indicating the role of maribavir as a potent inhibitor of UL97 HCMV protein kinase. It has been shown that the HCMV pUL44 DNA polymerase processivity factor interacts with and is phosphorylated by the UL97 kinase, explaining viral DNA inhibition in the presence of maribavir [7,8]. Although the specific role of this unusual kinase is yet to be defined,
UL97 deletion mutants exhibit severe replication defects in cell cultures [9]. Krosky et al. [10] showed that infection of cells with UL97 null mutants or wild-type HCMV in the presence of maribavir resulted in defective accumulation of cytoplasmic capsids, thereby suggesting a possible role for this kinase at the stage of nuclear egress. The functions of the UL97 kinase have recently been reviewed [11] (Figure 1).

Acute and chronic toxicological studies conducted in mice and monkeys highlight a favourable safety profile for maribavir. Furthermore, oral administration of the drug demonstrated excellent bioavailability [3,12], allowing maribavir to enter clinical trials as a potent therapeutic option against HCMV.

Phase I clinical trials evaluating the pharmacokinetics, safety profile and anti-HCMV activity of maribavir in healthy and HIV-infected individuals showed that the drug was generally safe and reasonably well tolerated [13,14]. Maribavir reduced HCMV viral titres in urine and semen samples of asymptomatic HIV-seropositive males to levels comparable with those reported for the approved anti-HCMV chemotherapeutics [13].

In a recent randomized double-blind placebo-controlled study, the anti-HCMV activity and safety of orally administrated maribavir was evaluated as a prophylactic agent for the prevention of HCMV disease in HCMV-seropositive allogeneic stem cell transplant recipients [15]. The study established excellent results for the use of maribavir as a prophylactic agent. HCMV antigenaemia and plasma DNAemia was reduced by 70%, levels similar to those observed in previous randomized trials for the use of ganciclovir. Besides its antiviral efficacy, the drug was safe and well tolerated, indicating its potential use in preventing HCMV recurrences in transplant recipients. The promising results of this study led Viropharma, Inc. (the pharmaceutical company that was involved in the development of maribavir) to announce the initiation of Phase III clinical trials evaluating the drug for prophylaxis against HCMV disease.

Table 1. Chemical entities of the most important novel molecules with antiherpetic activity

<table>
<thead>
<tr>
<th>Category</th>
<th>Antiviral agent</th>
<th>Chemical entity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzimidazole ribonucleosides</td>
<td>Maribavir</td>
<td>(5,6-Dichloro-2-(isopropylamino-1-( β-ribofuranosyl)benzimidazole)</td>
</tr>
<tr>
<td></td>
<td>BDCRB</td>
<td>2-Bromo-5,6-dichloro-1-( β-ribosylnucleosyl)benzimidazole</td>
</tr>
<tr>
<td>Aminothiazoles</td>
<td>BAY 57–1293</td>
<td>(N-[5-(aminosulfonyl)-4-methyl-1,3-thiazol-2-yl]-N-methyl-2-[4-(2-pyridinyl)phenyl]acetamide)</td>
</tr>
<tr>
<td></td>
<td>BILS 179-BS</td>
<td>N-[N-[4-[2-aminothiazole-4-ylphenyl]carbamoylmethyl]-N-[1-phenylethyl]pyridine-4-carboxamide</td>
</tr>
<tr>
<td>BCNA</td>
<td>FV-100</td>
<td>3-[2-(Deoxy-β-ribofuranosyl)-6-[p-(pentoxyphenyl)-2,3-dihydrofuro[2,3-d]pyrimidin-2-one, 50-valyl ester, HCl salt (10 HCl)</td>
</tr>
<tr>
<td>4-Oxo-dihydroquinolines</td>
<td>PNU-183792</td>
<td>(N-[4-chlorobenzyl]-1-methyl-6-(4-morpholinylmethyl)-4-oxo-1,4 dihydro-3-quinolinecarboxamide)</td>
</tr>
</tbody>
</table>

BCNA, bicyclic nucleoside analogue.

Table 2. Novel antiherpetic drugs under development and their characteristics

<table>
<thead>
<tr>
<th>Antiviral agent</th>
<th>In vitro studies</th>
<th>Animal studies</th>
<th>Developmental status in humans</th>
<th>Mechanism of antiviral action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1263W94 (maribavir)</td>
<td>HCMV and EBV</td>
<td>HCMV</td>
<td>Phase III (currently on hold)</td>
<td>Inhibition of HCMV UL97 kinase</td>
</tr>
<tr>
<td>BDCRB</td>
<td>HCMV</td>
<td>None</td>
<td>Halted (pharmacokinetic limitations)</td>
<td>Inhibition of HCMV terminase (UL89 and UL56)</td>
</tr>
<tr>
<td>BAY 57–1293 and BILS 179-BS</td>
<td>HSV-1 and HSV-2</td>
<td>HSV-1 and HSV-2</td>
<td>Have not entered clinical trials</td>
<td>Helicase–primase complex (UL5, UL8 and UL52) inhibition</td>
</tr>
<tr>
<td>FV-100</td>
<td>VZV</td>
<td>VZV</td>
<td>Phase II (first results in 2010)</td>
<td>Still unknown (activation via VZV TK phosphorylation)</td>
</tr>
<tr>
<td>PNU-183792 and PNU-182171</td>
<td>HSV-1, HSV-2, HCMV, VZV, EBV and HHV-8</td>
<td>HCMV</td>
<td>Have not entered clinical trials</td>
<td>DNA polymerase inhibition</td>
</tr>
<tr>
<td>ART</td>
<td>HCMV, HSV-1, EBV and HHV-6</td>
<td>HCMV</td>
<td>Case report for HCMV</td>
<td>Reduced NF-κB, Akt activity (7)</td>
</tr>
<tr>
<td>PCIs</td>
<td>HCMV, HSV-1, EBV and HHV-8</td>
<td>None</td>
<td>Clinical trials for cancer</td>
<td>CDK inhibition</td>
</tr>
</tbody>
</table>

ART, artesunate; CDK, cyclin-dependent kinase; EBV, Epstein–Barr virus; HCMV, human cytomegalovirus; HHV-6, human herpesvirus type-6; HHV-8, human herpesvirus type-8; HSV-1, herpes simplex virus type-1; HSV-2, herpes simplex virus type-2; NF-κB, nuclear factor-κB; PCIs, pharmacological CDK inhibitors; TK, thymidine kinase; VZV, varicella zoster virus.
trials. The prevention of HCMV disease in allogeneic stem cells or bone marrow transplant recipients was set as a primary efficacy end point. Results obtained were not as expected. There was no statistically significant difference between maribavir and the placebo in reducing the rate of HCMV disease 6 months post-transplant. The incidence of HCMV disease in the maribavir-treated group was 4.4% versus 4.8% in the placebo-treated patients ($P=0.79$). Following this, the company announced discontinuation of Phase III clinical trials evaluating maribavir as a prophylactic agent against HCMV disease in liver transplant patients (Table 2). Viropharma, Inc. announced that further development of the drug would be on hold, although future studies might be conducted should the FDA grant regulatory approval for maribavir exploitation.

Aminothiazoles as anti-HSV agents
Herpes simplex virus type-1 (HSV-1) and -2 (HSV-2) are responsible for a wide spectrum of diseases in humans and, although usually not life-threatening, HSV infections can be the cause of significant mortality (mainly in immunocompromized patients and newborns) and recurrences of the disease can affect the quality of life of the infected individuals.

Despite the fact that it has been over 20 years since the guanosine analogue acyclovir was first approved, it is still considered as the gold standard therapy for HSV.
Indeed, currently used therapeutic agents are based on modified nucleoside analogues (for example, valaciclovir and famcyclovir) that target the DNA polymerase; however, although these drugs are effective in treating primary and recurrent HSV infections, a delay in the initiation of treatment can significantly reduce therapeutic efficacy. Additionally, a growing number of immunocompromised patients currently encountered results in nucleoside-analogue-resistant HSV strains.

The HSV-1 and HSV-2 genome encodes for seven viral proteins that are essential for DNA replication. Three of these proteins (UL5, UL8 and UL52) form a heterotrimeric complex with both helicase and primase function [16,17]. Although the specific role of each individual subunit has yet to be defined, strong evidence suggests that the UL5 protein contributes to helicase activity of the complex; UL52 is responsible for primase activity, whereas the UL8 gene encodes for a protein that interacts with other viral replication proteins of the HSV. Because the helicase-prime complex is essential for viral replication, it represents an attractive target for the development of novel anti-HSV drugs.

Recent data suggest that aminothiazoles (helicase primase inhibitors), a novel class of compounds, represent promising candidates for the initiation of clinical trials in humans (Table 1). By targeting the heterotrimeric helicase-prime complex of HSV, aminothiazoles have proved to be quite effective as antiherpetic drugs.

In vitro studies have proven the efficacy of BAY 57-1293 in inhibiting replication of HSV-1 and HSV-2 in the nanomolar range [20]. Indeed, compared with acyclovir, BAY 57-1293 was more potent in vitro with regards to its inhibitory effect on HSV-1 and HSV-2 clinical isolates and laboratory strains. Furthermore, acyclovir-resistant viral strains carrying mutations in the thymidine kinase (TK) or pol genes remained susceptible to the inhibitory effect of the compound; thus, indicating the difference in the molecular target [20].

Using various rodent models of HSV infection, including a murine lethal challenge model, Betz et al. [21] confirmed that BAY 57-1293 was superior to valaciclovir in treating acute disseminated or cutaneous HSV infection, even with the once-daily dosing regimen. The compound retained its efficacy even when treatment was initiated after the onset of symptoms and proved to be more efficacious in reducing viral titres in various organs of HSV-infected animals. Moreover, recurrence of the infection after cessation of the therapy was found to be less frequent compared with animals receiving valaciclovir [21]. Kleymann et al. [20] showed that BAY 59-1293 retained its superiority in treating HSV-infected mouse models over all compounds currently in clinical use. In another study, Baumeister et al. [22] observed that HSV-2 genital infection in a guinea pig model was susceptible to the therapeutic action of BAY 59-1293 even when the drug was administered after the initiation of symptoms. Compared with valaciclovir, the drug tested retained its superiority in suppressing acute symptoms of HSV-2 infection and reducing latency reactivation. Finally, using severe immunocompromised athymic nude BALB/C mice, Biswas et al. [23] recently showed the potent antitherpetic effect of the compound in immunocompromised hosts.

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initiated 65 h post-infection, compared with equivalent doses of acyclovir. Similarly, in an HSV-2 genital mouse disease model, BILS 179-BS retained its superiority over acyclovir in reducing mortality.

Because BILS 179-BS activity against acyclovir-resistant HSV disease was not evaluated, Duan et al. [24] used acyclovir-resistant strains and found that orally administered BILS 179-BS almost completely abolished HSV-1-mediated topical lesions, whereas acyclovir was profoundly ineffective.

The above evidence support the potential use of these agents as a therapeutic choice for humans infected with HSV strains resistant to currently used antiviral drugs. Compared with the gold standard therapeutic choice, acyclovir, helicase–primase inhibitors have many advantages. Firstly, they can be used for the treatment of acyclovir-resistant HSV infections. Secondly, they are effective even after the initiation of symptoms and their use can be limited to the once-daily dosing regimen. Thirdly, their antiviral activity and bioavailability properties are superior to acyclovir and their use can also limit HSV viral spread in the population because they successfully block viral shedding in infected individuals and reduce the likelihood of recurrences. Finally, the fact that these agents bind to two targets simultaneously resembles combination therapy; thus, reducing the risk of emerging resistant viral strains.

Bicyclic pyrimidine nucleoside analogues as anti-VZV agents

Varicella zoster virus (VZV) is a member of the neurotropic herpesviruses that establish latency in dorsal root ganglia for the entire lifespan of the host. VZV is the causative infectious agent of varicella (chickenpox), a mild to moderate disease that occurs mainly during childhood. Although benign in immunocompetent patients, involvement of the central nervous system or secondary bacterial infections of the lung might lead to severe life-threatening complications. Immunocompromised individuals, pregnancy and old age are the main risk factors associated with a bad prognosis. In the past few years, routine vaccination against VZV has led to a significant reduction of varicella infection cases [25,26].

Reactivation of the latent virus in the dorsal root ganglia causes herpes zoster (shingles), a condition that usually affects patients over 60 years of age. Impaired cell-mediated immunity is considered to be the main reason for VZV reactivation; thus, HIV infection and bone marrow transplant recipients represent high-risk patients for herpes zoster.

Currently available therapeutic agents for herpes zoster are acyclovir, valaciclovir and famciclovir. The pyrimidine nucleoside analogue brivudine (BVDU) and its arabinosyl derivative sorivudine (BVaraU) are novel anti-VZV therapeutics that have been approved for clinical use in Europe, but not in the US [27,28].

During the past decade, a novel class of compounds called bicyclic nucleoside analogues (BCNAs) has emerged as the most potent anti-VZV agents reported thus far (Table 1). Optimization of the parental compound Cf 1368 has led to the development of Cf 1743 and Cf 1742 substituted furopyrimidine derivatives [29,30], the most potent and selective anti-VZV agents to enter further clinical development.

BCNAs are structurally related to BVDU, although they differ significantly in their biological and biochemical properties. In contrast to the anti-HSV-1 activity of BVDU, BCNAs presented with an unprecedented selectivity in their antiviral spectrum, solely inhibiting VZV.

Using 17 different VZV clinical isolates, Andrei et al. [31] reported that Cf 1742 and Cf 1743 were markedly more potent in inhibiting viral replication in vitro, when compared with the currently used drugs. More specifically, the compounds were 10–25-fold more active against wild-type VZV clinical isolates than BVDU, and had up to 4,000–7,800-fold higher efficacy compared with acyclovir and penciclovir. These results are comparable with those reported for Oka and YS laboratory strains [29,32]. Even more astonishing was the unprecedented high selectivity index of these agents, which was calculated to be >100,000 [31].

VZV mutant strains resistant to BCNAs showed cross-resistance to BVDU, BVaraU and acyclovir. Conversely, acyclovir-, BVDU- and BVaraU-resistant strains proved to be no longer sensitive to BCNAs. Because the above therapeutic agents depend on the viral TK for their phosphorylation and therefore activation, it seems reasonable to assume that BCNA-resistance-conferring mutations are caused by alterations in the viral TK. The crucial role of this enzyme in the metabolic activation of BCNAs has been demonstrated in previous studies [29,32], where TK-deficient VZV strains lost their sensitivity to the inhibitory effect of these agents. Moreover, Cf 1742 and Cf 1743 were unable to inhibit viral replication against two TK-deficient VZV clinical isolates [33].

Indeed, Sienaert et al. [34] demonstrated that VZV TK selectively phosphorylates BCNAs to their 5′-diphosphate derivatives in contrast to the HSV-1 TK and thymidylate kinase (dTMP), neither of which recognize BCNAs as substrates. These data might also explain the high selectivity index of BCNAs because the structurally related BVDU is recognized and phosphorylated by both VZV and HSV-1 TK/dTMP kinase. In addition, further studies have revealed that neither cytosolic nor mitochondrial TKs are able to phosphorylate and activate BCNAs; thus, explaining the low toxicity of these agents [34].

Despite the fact that the above data highlight the vital role of VZV TK in activating BCNAs, the exact
molecular target of their antiviral activity has yet to be
defined and is currently under investigation. Sienaert
et al. [34] observed that after the addition of human
erthrocyte nucleoside 5′-diphosphate kinase to VZV
TK in the presence of BVDU or BCNAs, there was no
conversion of BCNA-diphosphates to their triphosphate
derivatives, whereas a considerable amount of the anti-
virally active BVDU-triphosphate was readily formed.
This is in agreement with previous studies reporting no
traces of BCNA-triphosphate in BCNA-exposed VZV-
infected cell lines. These data virtually rule out viral
DNA polymerase as the molecular target, suggesting
that BCNAs exert their inhibitory effect through their
monophosphate or diphosphate derivatives.

Compared with the majority of the potent anti-VZV
agents currently being used in clinical practice (BVDU
and BVaraU), BCNAs are characterized by their strik-
ing differences in their catabolic pathways, which pro-
vide them with significant advantages.

Pyrimidine nucleoside analogues are susceptible to
pyrimidine catabolic enzymes (for example, uridine
phosphorylase or thymidine phosphorylase [TPase]) and
are hydrolysed to their free base metabolites that lack
antiviral activity. More specifically, BVDU is converted
to its free base (E)-5-(2-bromovinyl) uracil (BVU) by
both human and Escherichia coli TPase [35]. Human
dihydropyrimidine dehydrogenase (DPD), the catabolic
enzyme involved in the degradation of pyrimidines and
pyrimidine analogues, has proved to be prone to the
inhibitory effect of BVU free base [36]. This has led to
devastating results in the past. Cancer patients suffering
from VZV infection were coadministrated oral BVaraU
and fluorouracil (5-FU), a chemotherapeutic pyrimidine
analogue. Bacterial TPases of the intestine microflora
hydrolysed BVaraU to its BVU free base; thus, block-
ing the catabolic inactivation of 5-FU by DPD. The
uncontrolled increase in plasma levels of 5-FU caused
severe toxicity and led to a number of deaths resulting
in discontinuation of further clinical trials [37–40].

Interestingly, Balzarini et al. [41] demonstrated that
BCNAs are not recognized as substrates by human or
E. coli TPases and are therefore not converted to their
inactive free base derivatives. Furthermore, the free
bases of BCNAs did not inhibit human DPD activity

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**Figure 2.** Pathways implicated in the anabolism and catabolism of BCNAs

[Diagram showing pathways involving BCNAs, nucleoside kinases, and catabolic enzymes]
and had no effect on 5-FU plasma levels in 5-FU-treated mice [41] (Figure 2).

Further studies need to be conducted to evaluate the efficacy and safety of BCNAs in vivo. In a recent study, McGuigan et al. [42] administrated mice intraperitoneally with Cf 1743. No side effects were observed when mice were examined 20 days after the treatment. So far, the major obstacle in administrating BCNAs seems to be their high lipophilicity. Low water solubility of these agents also give poor oral bioavailability. McGuigan et al. [42] synthesized a prodrug designated FV-100 that has an improved oral bioavailability, making this the most promising candidate for further study.

In 2007, the FDA granted Inhibitex, Inc. approval to initiate a Phase I clinical trial in order to evaluate the safety profile and pharmacokinetic properties of FV-100 in healthy volunteers (Table 2). The trial was completed a year later and Inhibitex, Inc. reported no significant side effects for orally administered FV-100. In May 2009, the company announced the initiation of a Phase II clinical trial. This double-blind study involved patients suffering from herpes zoster. Patients were treated either with FV-100 or the currently approved therapy, valaciclovir. Primary objectives involved evaluation of the safety profile and the therapeutic efficacy of FV-100 in reducing the duration and severity of shingles-associated pain, the incidence of post-herpetic neuralgia and the time to skin lesion healing. Study duration is set to be for 9 months and first results are expected in the first half of 2010.

4-Oxo-dihydroquinolines as broad-spectrum anti-herpetic agents

Recently, a novel class of compounds named naphthalene carboxamides was reported by Vaillancourt et al. [43] to act as inhibitors of the HCMV DNA polymerase. Through high-throughput screening and structure-activity relationship studies, several groups have modified the initial lead templates, which has resulted in the development of 4-oxo-dihydroquinolines (4-oxo-DHQs) [44–48]. 4-Oxo-DHQs proved to have broad-spectrum antiviral activity via specific inhibition of the herpesvirus DNA polymerase (Table 1).

Herpesvirus polymerases share a high degree of homology and are classified in the family of B DNA polymerases along with human α and β DNA polymerases [49]. Several in vitro studies proved that 4-oxo-DHQs inhibit herpesvirus DNA polymerases, leaving human α, γ and β polymerases unaffected [46–48,50] by targeting conserved domains shared solely among herpesviruses.

Using standard plaque reduction assays, PNU-183792, PNU-182171 and several other 4-oxo-DHQs demonstrated inhibitory activity against HSV-1, HSV-2, HCMV, VZV, Epstein–Barr virus (EBV) and human herpesvirus type-8 (HHV-8) [45–48,50]. More specifically, these agents proved to have comparable antiviral activity to the licensed antiherpetic drugs currently used. With VZV being the most susceptible to the inhibitory effect of PNUs, these agents were found to be active against clinical isolates as well as ganciclovir/cidofovir double-resistant mutant HCMV variants and acyclovir-resistant HSV strains. The compounds tested did not show signs of cytotoxicity in the concentrations used for achieving antiviral effect [46,50].

4-Oxo-DHQs showed a high specificity index in inhibiting DNA polymerases belonging to the herpesviridae family because unrelated DNA and RNA viruses were not susceptible to their inhibitory effect [46,47].

Using animal models of cytomegalovirus (CMV) infection, Brideau et al. [46] tested the efficacy of PNU-183792 in vivo. Indeed, PNU-183792 was efficacious as a prophylactic as well as a therapeutic agent. The drug showed comparable activity to ganciclovir when administered 24 h post-infection [46]. In the same study, PNU-183792 presented with good oral bioavailability, achieving blood levels above the cell culture antiviral 90% inhibitory concentration.

In order to clarify their exact mechanism of action Oien et al. [47] demonstrated that 4-oxo-DHQs act as competitive inhibitors of substrate binding to the viral polymerase. Given the fact that acyclovir-resistant HSV and ganciclovir-resistant HCMV isolates retain their susceptibility to 4-oxo-DHQs, it seems possible that this novel class of compounds does not bind to identical sites as acyclovir-triphosphates and ganciclovir-triphosphates do. Mutational analysis of PNU-182171-resistant HSV-1 and HSV-2 variants revealed that 4-oxo-DHQs exert their inhibitory effect via interaction with a viral DNA polymerase site that is less important for the binding of deoxynucleoside triphosphates [48].

Resistance to the drug links to the conserved domain III and, more specifically, to a point mutation that results in a V823A change in the viral polymerase gene. V823 is highly conserved among DNA polymerases of HHV, except HHV type-6 (HHV-6) and HHV type-7 (HHV-7), which contain an alanine at this amino acid [49]. The vital role of this particular residue in the molecular mode of action of 4-oxo-DHQs is highlighted by the fact that in vitro studies using PNUs as anti HHV-6 agents proved to have no antiviral activity [50]. Thomsen et al. [44] changed V823 to A823 in the HSV-1, HHV-6 and HCMV polymerases, proving that the engineered viral strains were less sensitive to the inhibitory action of PNU-183792. Conversely, changing the equivalent A823 of the HHV-6 polymerase to valine rendered the mutant
variant sensitive to the drug. Finally, with regards to the susceptibility to the currently approved nucleoside analogues, HSV-1-, HSV-2- and HCMV PNU-resistant mutants remained sensitive to their inhibitory effect.

The above data provide substantial evidence for the different mechanism of action between 4-oxo-DHQs and nucleoside analogues, despite the fact that they both target viral polymerase. Their use for the treatment of resistant herpesviruses infections in combination with their broad-spectrum antiherpetic activity makes them attractive candidates for further development. Although preliminary data point to a favourable pharmacokinetic profile and profound in vivo activity, we cannot speculate their use in humans. More studies need to be conducted along with clinical trials before these compounds can enter clinical use (Table 2).

**Targeting cellular proteins**

Over the past few years, drug discovery in the antiviral era has been focused on targeting viral proteins. This approach ensures advantages, such as minimum host cell side effects and maximum safety profile for the drugs tested; however, there have been several limitations. Drugs targeting specific viral proteins act as selective pressure against viruses, which results in the emergence of drug-resistant viral strains. Moreover, the antiviral potential of these agents is rather limited given the fact that viruses encode for a small number of proteins. In addition, drugs targeting viral encoded proteins tend to have a narrow spectrum of activity against only a few related viruses.

In an effort to overcome the above limitations, several recent studies discuss the exploitation of cellular proteins as potential targets for the development of novel antiviral agents. Targeting cellular proteins will provide us with a useful tool against mutant strains resistant to conventional therapeutics. Considering the fact that a given cellular protein is often required for the replication of many unrelated viruses, a drug design that targets such proteins could lead to the development of wide-spectrum antiviral agents. However, deregulation of the host cell signalling pathways might result in significant undesirable effects, counteracting any antiviral advantages. In the following section we will focus on the inhibitory activity of artesunate (ART), a well-known antimalarial agent, and pharmacological cyclin-dependent kinase (CDK) inhibitors (PCIs) against herpesviruses.

**Antimalarial endoperoxides: artesininin and its derivatives**

Artemisinin and its derivatives, such as ART, demonstrate overwhelming antiparasitic activity against *Plasmodium* and are currently used as antimalarial agents. During the past few years, several studies have shown that the true potential of these agents extends far beyond their current clinical use.

*In vitro* studies conducted by Efferth *et al.* [51] demonstrated that ART exhibited a strong inhibitory effect against HCMV laboratory strains and clinical isolates. Even when the authors used ganciclovir and ganciclovir/cidofovir double-resistant mutants, ART retained its antiviral efficacy, indicating a novel mechanism of action. Moreover, HSV-1 and EBV also proved to be susceptible to the drug [51]. Although Naesens *et al.* [52] reported that artesininin had no antiviral activity against HHV-6, recent data [53] provide evidence that the semisynthetic derivative ART is active against HHV-6A. In general, ART has so far proved to have a broader spectrum of action and higher efficacy as an antivireric agent compared with the parental compound artesininin.

Using immunocompromised rats as a model for CMV infection, Kaptein *et al.* [54] demonstrated that animals treated with ART coadministered with Fe²⁺ presented with significant lower levels of viral DNA copies and infectious viral particles, compared with the untreated control group.

The use of ART as an antimalarial agent over the past few years has established this agent as a well tolerated drug with minor side effects [55]. In addition, plasma levels of ART administrated for malaria are three orders of magnitude higher than those required for HCMV inhibition *in vitro* [51]; therefore, ART satisfies all the criteria for entering clinical trials. Recently, the first case that reports the use of ART as an anti-CMV agent was published [56]. The authors presented the first clinical use of the drug as a therapeutic option for a patient with foscarnet/ganciclovir double-resistant HCMV infection after haematopoietic stem cell transplantation. After the completion of therapy, there was no rebound viraemia. The drug was very well tolerated and effectively suppressed viral replication. The same centre announced that a clinical trial for ART in haematopoietic stem cell recipients is currently ongoing.

Little is known regarding the antiviral mechanisms of action of ART; however, preliminary data suggest that the drug inhibits HCMV replication by interfering with cellular activation pathways that are vital for viral replication. It is tempting to assume that ART acts as antiviral agent through a mechanism similar to that of its antiprotozoan action by producing reactive oxygen intermediates (ROIs). It is known that ROIs affect the activity of cellular factors such as nuclear factor-xB (NF-xB), diminishing their DNA binding activity via oxidation [57]. This correlates with the early findings of Efferth *et al.* [51], who demonstrated that HCMV-infected cells treated with ART showed reduced levels of stimulatory protein-1 (Sp1) and NF-xB transcription factors with diminished
DNA binding activity. HCMV infection normally up-regulates these factors [58], which is crucial for the onset of viral replication because the HCMV immediate early promoter–enhancer contains Sp1/NF-κB binding sites [59–61]. As expected, western blot analysis of HCMV-infected cells in the presence of ART verified decreased levels of IE72 protein, confirming the inhibitory effect of the drug at immediate early steps of gene expression [51]. This appears rather oversimplified considering that data from the same study indicate that ART inhibits HCMV-induced Akt and p70S6K activation of the PI3K pathway, essential for viral DNA replication [62].

Indeed, ART makes an attractive candidate for entering clinical trials and we can expect to have an effective drug against resistant HCMV infections (Table 2). It would be of high interest to further investigate the drug efficacy against HSV-1, EBV and HHV-6 in vivo as most studies conducted so far refer to HCMV.

Pharmacological CDK inhibitors
CDKs comprise a family of serine/threonine kinases that are required for the regulation of several cellular functions, such as the cell cycle (CDK1, -2, -3, -4, -6 and -7), transcription (CDK7, -8 and -9) and development and maintenance of neuronal cytoskeleton (CDK5). Their active form consists of a complex composed of a catalytic subunit (the CDK) and a regulatory subunit termed cyclin [63]. Despite the fact that some human viral pathogens encode their own kinases, viruses in general have evolved to employ various cellular proteins [64] such as CDKs. In this review, we will focus on the antiviral properties of PCIs towards herpesviruses.

The fact that herpesviruses can replicate their genome, even when viral proteins are not expressed (latent infection), is indicative of the requirement for S-phase cellular proteins, such as CDKs [65–68]. In addition, many viruses of this particular family seem to modulate CDK activity. More specifically, HHV-8 encodes for a cyclin leading to activation of CDKs, whereas EBV induces the expression of cyclins [69–72]. EBV-associated cell lymphoma and Kaposi’s sarcoma related to HHV-8 are partially pathophysiologicaly connected with the activation of CDK2 and the resulting abnormal cell growth.

PCIs have been studied in the past decade in an effort to identify novel chemotherapeutics against cancer. Since the development of olomoucine (the first PCI to be discovered), many other agents inhibiting CDKs have been synthesized [73]. These inhibitors are divided into three categories according to their specificity. Non-specific PCIs inhibit CDKs with the same potency as those inhibiting other kinases. Pan-specific PCIs, such as flavopiridol, selectively inhibit CDKs but with no further discrimination among them. Finally, oligo-specific PCIs, such as roscovitine, inhibit only a few CDKs allowing further subclassification of this category to transcription-specific PCIs (inhibition of CDKs involved in transcription regulation) and cell cycle-specific PCIs (inhibition of CDKs involved in the cell cycle) [73].

The first original article that confirmed the antiviral efficacy of PCIs was published by Bresnahan et al. [74] in 1997, demonstrating that inhibition of the cellular CDK2 resulted in the blockade of HCMV replication. Since then, the inhibitory action of these agents against several unrelated viruses has been shown.

Following Bresnahan et al. [74], Schang et al. [75,76] published a series of papers proving in vitro the antiviral potency of PCIs against herpesviruses at concentrations already proven safe in clinical trials against cancer (Table 2). More specifically, PCIs exerted strong antiviral activity against HSV-1, HSV-2, VZV, HCMV, EBV and HHV-8 [74–78]. In agreement with the idea that PCIs exert their inhibitory effect on viral replication by targeting cellular proteins, no resistant mutants were isolated despite extensive efforts.

With regards to HCMV, several studies attributed a crucial role for CDKs in the productive infection. Using roscovitine, these studies proved that during the early phase of infection, viral transcript processing and early gene expression requires CDK activity [74,79], whereas during the late phase, CDKs are implicated in virion maturation [80,81].

In the case of HSV-1, roscovitine inhibited viral replication during transcription and DNA synthesis. Although much work has been done, the exact mechanism of this inhibitory effect has not been clarified [82–86]. Moreover, roscovitine was effective in preventing latent HSV-1 infection from reactivation [66].

The antiviral potency of roscovitine against VZV seems to be mediated via inhibition of CDK1 and CDK2, although similarly to HCMV and HSV-1, the mechanism of action has not yet been fully elucidated. VZV infection induces CDK2 activity [87], whereas CDK1 phosphorylates the VZV Fc receptor component of viral glycoprotein I [88] and the VZV IE63 protein [89]. In addition to these, Leisenfelder et al. [90] recently demonstrated that CDK1/cyclin B phosphorylates viral IE62 protein and is incorporated into virions. It appears that the role of CDKs is more complex and involves several stages of VZV replication, as roscovitine (CDK1 inhibitor) blocked the transcription of ORF 62 and ORF 71 (which encode for IE62).

Although PCI antiviral efficacy has not yet been evaluated in vivo, they are considered excellent candidates for entering clinical trials because they have already been tested for cancer, showing a favourable safety and pharmacokinetic profile [91,92]. Additionally, in vitro studies showed that PCIs exerted their antiviral effect at concentrations lower than those used...
in trials for cancer. In a recent review paper, Schang [93] proposed three pathways to test PCIs as antitherpeptic agents in clinical trials. According to the author, cancer patients infected with herpesviruses could benefit from the anti-proliferative effect of these agents in parallel with their antiviral potency.

Although there has been much progress over the past few years in understanding the mechanism by which PCIs exhibit their antiviral effect, it is still unknown exactly how they discriminate between cellular and viral transcription blockade. Moreover, their effect in vivo for doses that are non-toxic for humans has not yet been evaluated. Still, PCIs are excellent candidates for entering clinical trial and we expect more data in the near future.

The future of antiviral drug discovery

Antisense oligonucleotides

It has been almost 30 years since the first report that antisense oligonucleotides (AODs) are effective in inhibiting gene expression in a sequence-specific way [94]. In 1998, the FDA approval of fomiviren (ISIS 2922; the first antisense oligonucleotide to be granted such approval) proved that AODs have a role to play in every day clinical practice. Over 50 antisense molecules are currently in clinical trials for the treatment of cancer, cardiovascular diseases and HIV, to name a few, suggesting that this therapeutic approach still has a lot to offer.

AODs are synthetic single-stranded oligonucleotides that bind specifically to the complementary messenger RNA (mRNA) leading to translational arrest or degradation of the corresponding RNA target [95].

Despite the fact that many antisense molecules have proved to be active as antiviral agents in vitro, only fomiviren was able to fulfill all of the criteria required for FDA approval. This molecule is composed of 21 phosphorothioate-linked nucleosides complementary to the RNA of the HCMV IE2 gene [96]. It is active against drug-resistant mutants of CMV and shows a 30-fold greater efficacy in inhibiting viral replication compared with ganciclovir. Fomiviren is administered intraocularly for dealing with cases of AIDS-related HCMV retinitis, when conventional drugs fail to exhibit a therapeutic outcome.

Several reports have demonstrated that fomiviren might exert an inhibitory effect via both sequence-dependent and sequence-independent mechanisms. Anderson et al. [97] showed that the agent reduced not only IE2 protein levels, but also those of IE1 in HCMV-infected cell cultures, pointing to a sequence-independent mode of action. Additionally, when a fomiviren-resistant HCMV strain was sequenced, there were no alterations in the DNA region corresponding to the drug’s target sequence [98]. Despite these, the viral mutant variant retained its resistant phenotype towards a modified derivative of fomiviren, but with the same nucleotide sequence.

The first study demonstrating that AODs are able to block viral replication was published in 1978 by Zamecnik and Stephenson [94]. Following these, several articles were published describing HSV-1 inhibition. Smith et al. [99] showed that methylphosphonate oligonucleotides complementary to the splice junction of HSV-1 IE4/IE5 pre-mRNAs were able to block viral growth in vitro. These data were further verified by Kulka et al. [100], proving that a possible target for inhibiting HSV-1 could be the translation initiation site of the viral IE4 mRNA [101]. Using AODs bearing different types of backbone modifications, several other groups also reported the antiviral effect of antisense HSV-1 IE4 pre-mRNA targeting [102,103]. In 1995, Peyman et al. [104] used many different oligonucleotides against a variety of HSV-1 genes, in an effort to identify the optimal target that confers maximum antiviral effect. The translation start region of IE110 mRNA rendered the virus more susceptible to the inhibitory action of the AODs tested.

Recent publications regarding the antiviral action of antisense technology against herpesviruses have mainly focused on EBV and HHV-8 and their associated malignancies. In 1993, Yao et al. [105] reported that 28-mer phosphorothioate oligonucleotides complementary to EBV genes were effective inhibitors of viral growth in vitro in a dose-dependent manner. A number of research groups have studied antisense molecules for silencing EBV latent membrane protein-1 (LMP-1) in the notion of modulating the course of EBV-related lymphoproliferative disorders as it is vital for transformation of B-cells. As expected, silencing LMP-1 expression rendered the EBV-positive lymphoblastoid cell lines susceptible to chemotherapeutic agents by abrogating Bcl-2 up-regulation and consequently enhancing apoptosis [106–109]. These effects were sequence-specific to the nucleosides tested and were present only in EBV-positive cell lines. Supporting the idea that AODs targeting LMP-1 can modulate the prognosis of EBV-associated lymphomas, Galletti et al. [110] tested the efficacy of lipid-based and receptor-mediated delivery systems for antisense molecules. According to the authors, exploitation of the transferrin receptor pathway internalized active molecules for silencing LMP-1 expression.

Dealing with the same problem but from a different perspective, other researchers applied antisense technology to target Bcl-2 expression. As expected, the results were similar to those of LMP-1 silencing [111–113]. Indeed, a fully phosphothioate Bcl-2 antisense oligonucleotide called G3139 (Genasense) is currently in clinical trials for cancer [114,115].

The targets of antisense oligonucleotides can be truly unlimited. In a recent publication, Xia et al. [116]
fascinating results in the near future. Entering clinical trials up to Phase III, verifying that The past decade has seen many antisense molecules development of fomivirsen seems to prove otherwise. Back clinical employment of antisense mechanisms, the biostability, pharmacokinetics and cellular uptake) hold cellular genes implicated in the disease pathophysiology. Nancies can also be therapeutically approached, either antisense technology proved that HHV-8-related malignancies can be applied for silencing viral encoded miRNAs, thereby modulating pathogenesis. In this case, targeting BHFRF could serve as an immunomodulating tool to promote immune response against EBV-induced lymphomas. Similar to findings on EBV, many reports employing antisense technology proved that HHV-8-related malignancies can be therapeutically approached, either by silencing viral products [117–119] or by targeting cellular genes implicated in the disease pathophysiology. Although quite a few limitations (for example, biostability, pharmacokinetics and cellular uptake) hold back clinical employment of antisense mechanisms, the development of fomivirsen seems to prove otherwise. The past decade has seen many antisense molecules entering clinical trials up to Phase III, verifying that much remains unknown on this class of compounds. Much work needs to be done and we can expect more fascinating results in the near future.

Conclusions

The growing number of immunocompromised patients currently encountered (transplant recipients and AIDS patients) have made the otherwise benign herpesviruses infections a significant threat in everyday clinical practice. In addition, currently used agents have, over the past few years, led to the emergence of resistant viral strains, resulting in ineffective conventional therapies. After the completion of the human genome sequence and the discovery of novel techniques of global gene monitoring, such as microarrays, we are now in a position to alter our perspectives with regards to the era of antiviral drug development. It is now clear that viral protein inhibition is not the only pathway. Targeting cellular proteins or even silencing viral genes will provide us with novel tools for limiting viral threats; however, despite exceptional progress over the past two decades, further investigations and novel therapeutic approaches must be developed for the establishment of effective antiviral therapies.

Disclosure statement

The authors declare no competing interests.

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