After several decades during which nucleoside analogues (especially acyclovir and penciclovir and their prodrugs) have benefited many patients suffering from herpes simplex virus (HSV) infections, the discovery of the helicase–primase inhibitors (HPIs) represents an interesting new approach. Although antiviral resistance has not been a major problem for nucleoside analogues in immunocompetent patients, the problem of acyclovir resistance in immunocompromised patients is well documented. Several HPIs are extremely potent antiviral compounds and may, therefore, offer an important alternative therapy in these patients. The potential for synergy, not just for the inhibition of virus replication but also to delay the appearance of drug-resistant virus, needs to be thoroughly investigated. The study of resistance to HPIs has been important towards understanding the mechanism of action of these compounds and confirming the target function. However, during the course of our studies on HPI resistance, we have made a number of interesting observations that may be relevant to their clinical use. This article draws attention to the major observations on HPI resistance reported by others and to our own recently published observations that have extended this expanding area of antiviral research.

Herpes simplex virus (HSV) research may have been less prominent in recent years, but the virus remains as a major cause of disease. Approximately 70% and 20% of western adult populations are seropositive for HSV-1 and HSV-2, respectively, many of whom suffer recurrent oral or genital lesions.

The search for novel antiviral drugs against herpes continues because the symptoms of HSV infection usually are not reduced unless therapy with nucleoside/nucleotide analogues is prophylactic (for example, long-term suppressive therapy) or initiated very early during primary or recurrent disease [1]. There is a more serious problem in immunocompromised patients (including HIV patients) – lesions may become chronic and ≥5% of isolates have evidence of resistance to therapy with nucleoside analogues [2]. Moreover, the existence of Herpes genitalis lesions may increase the risk of acquiring HIV infections [3]. Finally, current HSV therapy does not eradicate latent virus or prevent recurrences after treatment cessation.

The helicase–primase inhibitors (HPIs) represent a class of non-nucleoside/non-nucleotide antiviral, which are extremely active against HSV in cell culture. They target the virus helicase–primase complex (Figure 1), which is involved in DNA replication. The UL5 helicase is a 5′-3′ helicase that unwinds the DNA double helix at the replication fork and UL52 primes single-stranded DNA [4]. The single-stranded DNA is then extended by HSV DNA polymerase, the main target, to date, for nucleoside analogue antivirals such as acyclovir (ACV; Figure 1). HPIs are also highly effective in laboratory animal infection models [5–10]; their efficacy has been highlighted in at least one comprehensive review [8] and a previous article in this journal [7]. When tested in murine infection models of disseminated or zosteriform herpes and a guinea pig model of genital herpes, HPIs showed potent antiviral activity [5,11,12]. Representative HPIs were claimed to significantly reduce time to healing, prevent immediate recurrence of disease after treatment cessation and reduce the frequency and severity of recurrences. Furthermore, HPIs are suggested to be more efficacious than current therapies when treatment initiation was delayed.
Unexpectedly high frequency of HPI resistance in certain laboratory and clinical isolates

The 50% effective concentration of BAY 57-1293 against HSV-1 in Vero cells is approximately 0.03 µM. In general, the passage of virus in the presence of a subinhibitory concentration of an antiviral compound yields resistant virus after several passages. However, even after a single passage in an inhibitory concentration of BAY 57-1293 (for example, 3.0 µM, which is 100 times the 50% effective concentration), it was possible to detect resistant viruses in laboratory working...
Drug resistance to helicase–primase inhibitors

stocks of HSV-1. This occurred even when the drug was continuously present from before virus inoculation and throughout the selection process. It was, therefore, suggested that the mutant viruses were already present in the virus population [14]. This led to the observation that two laboratory working stocks [14] and 2/10 recent clinical isolates of HSV-1 [15] were shown to contain BAY 57-1293-resistant variants at $10^{-4}$–$10^{-5}$ plaque-forming units (PFU), which is 10–100 times the rate previously reported ($10^{-6}$). The resistance mutations were defined by sequencing the HSV-1 helicase (UL5) and primase (UL52) genes. The most common resistance mutations found by ourselves and reported by others are confined to three amino acid residues, namely G352, M355 and K356 (position of amino acids as in HSV-1 UL5), which are immediately downstream from the functional motif IV predicted by Zhu & Weller [16] in the UL5 protein.

It was of interest that when HSV-1 laboratory working stocks (which contained a high frequency of BAY 57-1293 resistance) were plaque purified, the frequency of resistance selection by BAY 57-1293 was low ($\leq 10^{-4}$ PFU) and within the published range. Moreover, there was no difference in the high frequency of resistance to ACV between the virus populations before and after plaque purification [14]; the frequency of ACV resistance for laboratory and clinical isolates is about $10^{-4}$ PFU for nucleoside resistance resulting from mutations in virus thymidine kinase [17].

As mentioned above, evidence was obtained during our studies that the selection of drug-resistant variants did not require virus replication in the presence of the compound; variants appeared to pre-exist within the virus population. Indeed, to our knowledge, there has been no evidence reported that HPIs increase virus mutation rate. Like the nucleoside analogue ACV, the generation of resistant variants by HPIs appeared to result from the selection of pre-existing resistance mutations that have occurred spontaneously.

Is the K356N mutation favoured over other HPI-resistant mutations?

It is intriguing to note that the HPI resistance mutation observed in 2/10 HSV-1 clinical isolates was identical (UL5: K356N), conferring >5,000-fold resistance to BAY 57-1293. Interestingly, it was pointed out previously [10] that following selection using an alternative HPI (T157602) the K-N mutation was more frequently encountered in both HSV-1 and -2 (two of three HSV-1 variants and two of four HSV-2 variants). It was also reported in the same paper that this mutation was the most efficient of those tested in achieving marker rescue of wild-type virus in presence of an inhibitory concentration of HPI (T157602) [10].

The same K-N mutation was also observed in HSV-1 F and KOS strains [6,9] following selection with BAY 57-1293 and BILS 22 BS, respectively.

The K-N mutation confers the highest resistance to HPIs among all observed resistance mutations studied to date. Furthermore, variants carrying this mutation have been shown by others to have similar growth properties in cell culture [6,9] and pathogenicity in murine infection models compared with wild type [11]. This is in contrast with the most commonly encountered mutants resistant to nucleoside analogues, such as ACV and penciclovir. Viruses resistant to such compounds are most frequently thymidine kinase defective and these variants are usually less pathogenic in vivo [18,19]. It is evident that >10 different mutations involving five different amino acid residues in UL5 confer HPI resistance (Table 1). Among these it is not clear why the K-N mutation is frequently selected. This could be because of its relatively high resistance to HPIs or perhaps the mutation is favoured for some other reason?

High frequency HPI resistance – an unsolved enigma or phenomenon

It is open to speculation as to why virus isolates from two patients should contain the same HPI resistance mutation at 10–100 times the expected frequency and poses the question as to whether this and possibly other ‘polymorphisms’ exist naturally at relatively high frequency. This is currently under investigation.

The HPIs (including BAY 57-1293) are experimental drugs in early development and it is extremely unlikely that patients could have had access to BAY 57-1293 or other HPIs. However, BAY 57-1293 has been previously reported to have structural and functional similarity to the diuretic drug Diamox (acetazolamide) [6]. It is just conceivable (although extremely unlikely) that the patients were exposed to compounds, for example, antibiotics, with partial structural similarity to HPIs.

Perhaps a more likely possibility is that a component of the isolation procedure (a single passage in MRC-5 cells) provided some selective pressure. Following from this, it would be interesting to evaluate the normal medium constituents that have some partial structural similarities to HPI (for example, antibiotics, antifungals or growth factors) for their contribution to HPI resistance selection in laboratory working stocks of virus. The possibility remains, however, that laboratory working stocks (after several passages) and clinical isolates may be more heterogeneous than recently plaque-purified viruses and could contain ‘precursor’ or ‘founder’ viruses more prone to accumulate mutations. If so, defects in proof-reading mechanism(s) [20] involved during virus replication could lead to resistance mutations. However, to date, no evidence for this has been obtained from
our study. Another suggestion proposed to explain the appearance of this mutant is that K356N could be an escape mutation providing an immunological advantage in the patient; however, we know of no evidence to support this supposition.

It is possible that the K-N mutation affects virus growth in the patient. We only have indirect evidence to support this. We have seen that a different mutation at the same residue (for example, K356Q) clearly results in faster growth of virus in tissue culture [21]. However, Luizzi et al. [9] and Kleymann et al. [6] both reported that K356N shows near wild-type growth at high multiplicities of infection in tissue culture. Given our more recent observation of the presence of this mutant in clinical specimens, we suggest that further research should be focused on possible subtle effects on the growth properties under various conditions for viruses containing this mutation.

**Residues other than K356 that can confer HPI resistance**

Among a series of randomly selected mutants, apart from K356, the two most common residues where single substitutions confer resistance to HPIs were G352 and M355. Individual substitutions, for example, G352V and G352R in HSV-1 F confer 400-fold and approximately 3,000-fold resistance, respectively [6,21]. Interestingly, G352V produces a virus of normal growth (HSV-1 KOS background [9]), whereas G352R produces a virus of slower growth (HSV-1 SC16 background [21]). It was established in our laboratory and elsewhere [6,10] that M355T produces slow-growing viruses, which form small plaques. Thus, particular amino acid substitutions in this critical region just downstream of HSV-1 helicase–primase motif IV can confer significant drug resistance and at the same time have a definite effect on the rate of virus replication in tissue culture. This could provide evidence for a molecular role for herpes helicase as a ‘biological motor’ in controlling the efficiency of the replication cycle. The 3-D crystal structure of HSV UL5 helicase has not yet been published, but structural data exist for homologous enzymes in other species [22]. Therefore, a comparative approach to determine the UL5 helicase structure may help to elucidate biological data and may ultimately lead to rational improvements in drug design and therapeutic strategies. A summary

Table 1. HSV helicase and/or primase mutations conferring resistance to various HPIs

<table>
<thead>
<tr>
<th>HSV strain</th>
<th>Drug</th>
<th>Fold resistance</th>
<th>Nucleotide substitution</th>
<th>Nucleotide position (sense strand)</th>
<th>Amino acid change UL5</th>
<th>Amino acid position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 KOS (K22r1)</td>
<td>BILS 22 BS</td>
<td>2,500</td>
<td>AAG→AAT</td>
<td>1068</td>
<td>K→N</td>
<td>356</td>
<td>[9]</td>
</tr>
<tr>
<td>HSV-1 KOS (K22r5)</td>
<td>BILS 22 BS</td>
<td>316</td>
<td>GGT→GTG</td>
<td>1055</td>
<td>G→V</td>
<td>352</td>
<td></td>
</tr>
<tr>
<td>HSV-1 KOS (K138r3)</td>
<td>BILS 138 BS</td>
<td>38*</td>
<td>GGT→GTG</td>
<td>1054</td>
<td>G→C</td>
<td>352</td>
<td></td>
</tr>
<tr>
<td>HSV-1 (Two of three resistant isolates)</td>
<td>T157602</td>
<td>n/r</td>
<td>AAG→AAT</td>
<td>1068</td>
<td>K→N</td>
<td>356</td>
<td>[10]</td>
</tr>
<tr>
<td>HSV-2 (G) R1</td>
<td>T157602</td>
<td>n/r</td>
<td>ATG→ACG</td>
<td>1061</td>
<td>M→T</td>
<td>354*</td>
<td></td>
</tr>
<tr>
<td>HSV-2 (G) R4; R6 (Two of four resistant isolates)</td>
<td>T157602</td>
<td>n/r</td>
<td>AAG→AAT</td>
<td>1065</td>
<td>K→N</td>
<td>355*</td>
<td></td>
</tr>
<tr>
<td>HSV-2 (G) R3</td>
<td>T157602</td>
<td>n/r</td>
<td>GAG→GAT</td>
<td>1197</td>
<td>E→D</td>
<td>399*</td>
<td></td>
</tr>
<tr>
<td>HSV-1 F</td>
<td>BAY 44-5138</td>
<td>&gt;333</td>
<td>ATG→GTG*</td>
<td>1063</td>
<td>M→V</td>
<td>355</td>
<td>[6]</td>
</tr>
<tr>
<td></td>
<td>BAY 54-6322</td>
<td>&gt;500,000</td>
<td>GGT→GTG*</td>
<td>1984</td>
<td>V→I</td>
<td>662</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAY 54-6322</td>
<td>&gt;2,000</td>
<td>GCG→ACG (UL52)*</td>
<td>2689 (UL52)</td>
<td>(A→T)</td>
<td>897 (UL52)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAY 57-1293</td>
<td>&gt;400</td>
<td>GGT→GTG</td>
<td>1055</td>
<td>G→V</td>
<td>352</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAY 57-1293</td>
<td>&gt;5</td>
<td>ATG→ACG</td>
<td>1064</td>
<td>M→T</td>
<td>355</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAY 57-1293</td>
<td>&gt;150</td>
<td>AAG→cAG</td>
<td>1066</td>
<td>K→Q</td>
<td>356</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAY 57-1293</td>
<td>&gt;1,000*</td>
<td>AAG→AAT*</td>
<td>1068</td>
<td>K→N</td>
<td>356</td>
<td>[6,11]</td>
</tr>
<tr>
<td>HSV-1 SC16 (BAYr1)*</td>
<td>BAY 57-1293</td>
<td>100</td>
<td>AAG→cAG</td>
<td>1066</td>
<td>K→Q</td>
<td>356</td>
<td>[21]</td>
</tr>
<tr>
<td>HSV-1 SC16 (BAYr2)*</td>
<td>BAY 57-1293</td>
<td>3,333</td>
<td>GGT→CGT</td>
<td>1054</td>
<td>G→R</td>
<td>352</td>
<td></td>
</tr>
<tr>
<td>HSV-1 SC16 (C-1c-2)</td>
<td>BAY 57-1293</td>
<td>124</td>
<td>AAG→ACG</td>
<td>1067</td>
<td>K→T</td>
<td>356</td>
<td>[14]</td>
</tr>
</tbody>
</table>

*Fold-resistance to BILS 22 BS. The virus was selected to BILS 138 BS. †Herpes simplex virus (HSV)-1 has one leucine more at position 20 in UL5 protein. *Three nucleotide substitutions in one resistant variant. ‡G Kleymann, personal communication. §The virus was selected to BAY 57-1293. n/r, not reported.
of the HPI resistance mutations reported, to date, by others and ourselves are shown in Table 1.

**Cross-resistance among various HPIs and sensitivity to alternative compounds**

As expected all the HPI-resistant mutants, to date, retain sensitivity to ACV, which is a nucleoside analogue that interacts with different target proteins (thymidine kinase and DNA polymerase) [6,14]. Among HPIs, the single substitutions K356Q and G352R have been shown to confer coresistance to both BAY 57-1293 and BILS 22 BS; however, it should be noted that G352R confers approximately 3,000-fold resistance to BAY 57-1293, but only 360-fold resistance to BILS 22 BS. There is a similar difference between the compounds for the K356Q mutation (Table 1). These results point to an interesting difference in the precise mechanism of the two different HPIs [23].

**Conclusions**

After more than a decade during which the herpes simplex chemotherapy field has been rather quiet, the HPIs represent an exciting new avenue in the development of antivirals active against herpesviruses. Combination therapy using BAY 57-1293 and nucleosidic drugs has been reported to be synergistic in vivo, but, at present, little data on potential synergism have been published [24]. At least one of these new compounds (ASP2151) is now in clinical development - patients are being recruited in Phase II clinical trials in Japan and USA for oral treatment of herpes zoster and recurrent genital herpes, respectively [25,26].

HPIs may be particularly helpful, either alone or in combination with existing compounds or other HPIs, in combating infections in immunocompromised hosts that have acquired resistance to the current nucleoside analogues, such as ACV. However, whether or not HPI drug resistance will become a problem in its own right only time will tell. The research documented in this article suggests that there is still much to learn. Furthermore, the study of resistance mutations will be key to understanding the mechanism of action of these new compounds, at the same time providing insight into this key enzyme complex that plays such an essential role in HSV DNA replication.

**Note added in proof**

During the reviewing process of this article, a mutation conferring resistance to BAY 57-1293 has been confirmed in HSV-1 UL52 primase (A899T). Interestingly, this primase mutation does not confer resistance to BILS 22 BS [23].

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