High frequency of spontaneous helicase-primase inhibitor (BAY 57-1293) drug-resistant variants in certain laboratory isolates of HSV-1

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Introduction

For several decades nucleoside analogues have been almost the only drugs used for suppression or treatment of herpes simplex virus (HSV) lesions (Coen & Schaffer, 2003; Eizuru, 2003; Knipe & Howley, 2001). Acyclovir (ACV) is regarded as the gold standard and more recently valaciclovir, the oral prodrug of ACV, and famciclovir, the prodrug of penciclovir, have also entered the clinical arena (reviewed, De Clerq & Field, 2006). The mechanism of action of these compounds is well understood and depends on initial phosphorylation of the nucleoside analogue by HSV thymidine kinase (TK) and subsequent inhibition of HSV DNA polymerase. Drug-resistant variants occurring as a result of spontaneous mutations in TK or DNA polymerase are selected at relatively high rates ($10^3$ to $10^4$) in tissue culture (Bacon et al., 2003; Parris & Harrington, 1982), the majority having defective TK. However, this high frequency of drug resistance has not translated into clinical resistance. In clinical practice, resistance to ACV and penciclovir remains extremely rare among immunocompetent patients and this might, in part, be due to the attenuation of many TK-defective mutants (Harris et al. 2002). Resistance is recognized as a problem in immunocompromised patients, where the rate is reported to be ∼5% of isolates, and in some cases this represents a clinical problem (Christophers et al., 1998; Field, 2001).

Helicase-primase inhibitors (HPI) comprise a new class of antivirals which appear to have the advantage of higher potency in cell culture and in animal models (Duan et al., 2003; Betz et al., 2002; Kleymann et al., 2002; Liuzzi et al., 2004; Spector et al., 1998). BAY 57-1293, a representative HPI (Kleymann et al., 2002; Kleymann, 2003, 2004) appears to be markedly superior in preclinical profile to current HSV antiviral compounds (Betz et al., 2002; Kleymann et al., 2002). Indeed, we have confirmed the superiority of BAY 57-1293 in comparison with famciclovir in a murine skin infection model in both immunocompetent and immunocompromised BALB/c mice (Biswas et al., 2007). Another claimed advantage of the HPI is a low level of drug resistance selection; furthermore, in some cases, the drug-resistant variants were shown to have reduced growth rates in vitro (Kleymann et al., 2002). This manuscript describes...
methods for the ready isolation of HSV-1 variants that have acquired high levels of resistance to BAY 57-1293 in cell culture. Contrary to some previous reports, evidence is presented here that HPI-resistance can occur at high frequency in virus working stocks. The resistant viruses form plaques in high concentrations of BAY 57-1293 and acquire co-resistance to a different member of the HPI class, BILS 22 BS (Liuzzi et al., 2004). However, the resistant viruses, as expected, retain sensitivity to the nucleoside analogue, ACV. It is speculated that resistance might be an important issue in relation to this class of inhibitors.

Materials and methods

Virus
The strains of HSV-1 used in the present study were HSV-1 SC16 and PDK, originally known as Cl (101) (Dubbs & Kit, 1964; Field & Wildy, 1978). The strain SC16 has been used extensively for studying antiviral compounds (Boyd et al., 1988; Field et al., 1979, 1995; Hill et al., 1975; Sutton & Boyd, 1993). Working stocks of virus were prepared at a low multiplicity of infection (MOI) and titrated in African green monkey kidney (Vero) cells and termed SC16 crude and PDK crude respectively. The working stocks have been passaged at least ten times in this laboratory in BHK-21 cells and Vero cells since they were originally plaque-purified. The working stocks have been stored in the freezer from a time that predates the introduction of HPI into our laboratory. This eliminated the possibility that the working stocks might have been contaminated with the HPI-resistant variants under investigation. Plaque-purified sub-strains, namely HSV-1 SC16 cl-2 and PDK cl-1, were derived recently from the HSV-1 SC16 and PDK working stocks by three successive single-plaque isolations in Vero cells.

Antiviral compounds
BAY 57-1293 (molar mass: 402 g/mol) and BILS 22 BS (molar mass: 449 g/mol) were provided as powders by Arrow Therapeutics, London and their structures are illustrated (Figure 1). ACV was obtained from commercial sources.

Plaque reduction assays (PRA)
The 50% effective dose (ED$_{50}$) and 90% effective dose (ED$_{90}$) values were determined by PRA in Vero cells. Approximately 100 plaque-forming units (PFU) were inoculated into 12-well plates containing $\sim 2 \times 10^5$ Vero cells/well. After adsorption for 45 min at 37°C in a humidified atmosphere of 5% CO$_2$, DMEM overlay containing 1% new-born calf serum and high density carboxymethyl-cellulose (CMC-DMEM) and different concentrations of antiviral compounds were added to each well. The plaques were fixed, stained and enumerated after 48 h incubation.

Isolation of BAY 57-1293 resistant variants from HSV-1 working stocks
Defined numbers of Vero cells were inoculated with a given number of PFU of the designated virus working stock and the cultures were exposed to inhibitory concentrations of BAY 57-1293 in virus adsorption and/or overlay media. In all cases the inoculum was confirmed by back-titration. Experiments with crude and plaque-pure clones of each working stock were carried out simultaneously under identical conditions. The particular conditions, which resulted in the isolation of drug-resistant plaques, are shown in the corresponding figures. When plaque formation was observed in inhibitory concentrations of BAY 57-1293 these were marked under a microscope and the infected cells from individual plaques were carefully aspirated and stored at -70°C. Harvested plaques were either tested by titration and PRA for drug sensitivity or three-times plaque-purified in Vero cells in the absence of inhibitor prior to further analysis. The rates for BAY 57-1293-resistance were calculated based on the number of resistant plaques from a given inoculum. In some cases, when no plaques were visible at 48 h, the cells were homogenized and tested for the presence of infectious virus.

Figure 1. Structural formulae for antiviral compounds

A

![Structural formula of BAY 57-1293](image)

B

![Structural formula of BILS 22 BS](image)
High frequency BAY 57-1293 resistance in HSV-1 laboratory isolates

Similar methods were used to determine the rate of resistance to the nucleoside analogue ACV.

Sequencing HSV-1 helicase (UL5)
The entire HSV-1 UL5 gene (2649 nucleotides) from the virus strains used in this study (including the BAY 57-1293-resistant variants) was sequenced for comparative analysis. A touchdown PCR (Hecker & Roux, 1996) was adapted to amplify the UL5 gene in overlapping fragments with high specificity. A set of eight pairs of overlapping HSV-1-specific primers (designed on the basis of HSV-1 strain 17 sequence; Genbank accession number NC001806) was used (McGeoch et al., 1988). PCR amplicons of the expected size were purified and sequenced (both directions using the same primers) at the University of Cambridge Department of Biochemistry, by Sanger’s di-deoxy chain-termination method. The nucleotide and predicted amino acid sequences of UL5 were aligned by ClustalW v 1.82 (available from http://www.ebi.ac.uk/clustalw/).

Results
Antiviral sensitivity of parental viruses and derived plaque-pure clones
The parental viruses used in this study were tested for sensitivity to BAY 57-1293 by PRA in Vero cells. The results (Table 1) showed that the virus HSV-1 PDK was less sensitive than HSV-1 SC16 to BAY 57-1293 (ED$_{50}$: 0.04 μg/ml and 0.02 μg/ml, respectively; Figure 2A) as well as to ACV (Figure 2B). The ED$_{50}$ values for the two

### Table 1. ED$_{50}$ and ED$_{90}$ inhibitory concentrations for BAY 57-1293, BILS 22 BS and acyclovir for herpes simplex virus (HSV)-1 parental strains determined by plaque reduction assay in Vero cells

<table>
<thead>
<tr>
<th>Molecular weight, g/mol</th>
<th>BAY 57-1293</th>
<th>BILS 22 BS</th>
<th>Acyclovir</th>
</tr>
</thead>
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<tr>
<td></td>
<td>ED$_{50}$</td>
<td>ED$_{90}$</td>
<td>ED$_{50}$</td>
</tr>
<tr>
<td>HSV-1 F</td>
<td>0.004–0.008*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SC16 crude</td>
<td>0.02–0.03†</td>
<td>0.06–0.08†</td>
<td>0.3†</td>
</tr>
<tr>
<td>SC16 cl-2</td>
<td>0.04†</td>
<td>0.08†</td>
<td>n/d</td>
</tr>
<tr>
<td>PDK crude</td>
<td>0.05†</td>
<td>0.1†</td>
<td>n/d</td>
</tr>
<tr>
<td>PDK cl-1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All data are expressed as μg/ml. *Data reported by Kleymann et al. (2002); †from Figure 2A; ‡from Figure 3; § from Figure 2B; ‡‡ from Figure 8A; $not reported. ED$_{50}$ 50% effective dose; ED$_{90}$ 90% effective dose; n/d, not done.

Figure 2. Sensitivity of parental viruses to antiviral compounds by plaque reduction assay (PRA)
plaque-purified derivatives, PDK cl-1 and SC16 cl-2 were similar to the parental working stocks (Figures 2A, 2B). It was apparent from the PRA that BAY 57-1293 was more active (about tenfold, in general) than either BILS 22 BS or ACV (ED$_{90}$∼0.2 μg/ml for SC16 crude) (Figure 3). Given the respective molecular weights (Table 1) this superiority is even greater when considered at μM concentrations.

**Detection of BAY 57-1293 resistant variants**

For each of the four viruses PRA were carried out using inocula of ∼100 PFU/well. It is important to note that, under these conditions, no plaques were visible at concentrations >0.3 μg/ml of BAY 57-1293 at 48 h post inoculation. In order to detect drug-resistant variants, increasing titres of inoculum were tested for the presence of spontaneously occurring drug-resistant plaques and these were apparent for the crude working stocks of PDK and SC16 at virus titres >10$^7$ PFU/ml. The frequency of drug-resistant variants (defined as ED$_{90}$ >0.08 μg/ml) was then investigated quantitatively.

**Frequency of BAY 57-1293 resistance in virus isolates**

Selection of drug-resistant variants was attempted using a variety of conditions for exposure to the inhibitor. When >10$^5$ PFU were inoculated, plaques were sometimes observed in concentrations of BAY 57-1293 as high as 1 μg/ml by 48 h post inoculation. The numbers of plaques that developed under various conditions are shown for PDK crude (Figures 4A, 4B), PDK cl-1 (Figure 4C) and SC16 cl-2 (Figure 5) and the particular conditions are defined in the respective figure legends. The formation of resistant plaques was reproducible and this was true even under conditions of pre-incubation with BAY 57-1293 and its replenishment after 24 h to ensure that inhibitory conditions were maintained throughout several virus replication cycles (Figures 4 & 5). Over a series of experiments the number of plaques that developed from the two crude working stocks suggested that ~1 in 4×10$^6$ for PDK crude and >1 in 10$^3$ PFU for SC16 crude (data not shown) were pre-existing drug-resistant variants. These values are summarized (Table 2) with conditions (Table 3). Experiments were carried out in parallel with the plaque-pure clones, SC16 cl-2 and PDK cl-1; notably, in these cases, no plaques were seen unless the inoculum was increased to ≥10$^6$, confirming earlier observations that a lower proportion of resistant variants were present in the clones.

**Frequency of resistance at different levels**

The most common resistant variants in SC16 crude were subsequently shown to have relatively low resistance (Figure 6A). These resistant variants were detected by studying the virus yield after one passage in BAY 57-1293 and estimating the frequency of resistant plaques therein. When ≥10$^5$ PFU were inoculated into a Vero cell culture in the presence of BAY 57-1293, the yield of infectious virus was found to contain resistant variants (present as 5–12% of the yield) and this was reproducible over at least four independent experiments. An example of such a variant was E cl-2, which was shown to have 15-fold greater resistance than wild-type. These were relatively difficult to detect because a lower selection concentration was required (0.1 μg/ml compared with the ED$_{90}$ of 0.08 μg/ml), and so residual sensitive plaques visible at 48 h confused the observations. No difference in the resistant yield was observed even when the cells were pre-incubated with BAY 57-1293 before virus inoculation and the drug was replenished during incubation.

BAY 57-1293-resistant variants with higher levels of resistance were also obtained from both SC16 crude (for example, SC16 C-cl-2, 125-fold, Figure 6A, Table 3) and SC16 cl-2 (for example, BAY r2, Figure 6B, Table 3). However, it was estimated that such drug-resistant viruses occur less frequently, at ~1 in 10$^6$ in both strains (Table 2), which is within the range previously reported (Kleymann et al., 2002).

For PDK, the situation was similar except that the common resistant variants (Figures 4A, 4B) in PDK crude were somewhat more resistant (50-fold or higher, for example, PDK crude-rm-1, Figure 6C) than those seen in SC16 crude and highly resistant viruses (>2,000-fold, for...
Figure 4. Scheme for determining frequency of variants resistant to BAY 57-1293 in herpes simplex virus-1 PDK

A  PDK crude*
1.6x10^5 PFU

Approximately 10^6 Vero cells (3 replicates/condition)

1 μg/ml – not replenished

1 μg/ml – replenished at 24 h intervals

Cells were pre-incubated and replenished with 1 μg/ml fresh drug at 24 h interval

(4.1±2 plaques)/1.6x10^5 PFU visible at 48 h p.i.

Plaques were sampled at random and confirmed resistant to 1 μg/ml BAY 57-1293 by titration and PRA

Three times plaque-purified in absence of inhibitor

PDK crude-rm-1

B  PDK crude*

i) 1.6x10^6 PFU or ii) 1.6x10^7 PFU

Approximately 10^6 Vero cells (3 replicates/condition)

1 μg/ml – not replenished

1 μg/ml – replenished at 24 h intervals

Cells were pre-incubated and replenished with 1 μg/ml fresh drug at 24 h interval

i) 0.3 plaque/1.6x10^4 PFU visible at 48 h p.i.

Plaques were sampled at random and confirmed resistant to 1 μg/ml BAY 57-1293 by titration and PRA

ii) No plaques visible by 48 h p.i.

Yield: sensitive

C  PDK cl-1*
1.6x10^5 PFU or 1.6x10^6 PFU or 1.6x10^7 PFU

Approximately 10^6 Vero cells (3 replicates/condition)

1 μg/ml – not replenished

1 μg/ml – replenished at 24 h intervals

Cells were pre-incubated and replenished with 1 μg/ml fresh drug at 24 h interval

No plaque detected at 48 h p.i.

Total yield (48 hrs p.i.) of cells and supernatant sampled from at least two wells for each condition

Sensitive to 1 μg/ml BAY 57-1293

Titration & PRA

Schemes show PDK crude (high inoculum), the origin of PDK crude-rm-1, (A); PDK crude (low inoculum) (B); and PDK cl-1 (high and low inoculum) (C). Vero cell monolayers (10^6 cells/well in 6-well plates) were infected with 1.6x10^-1-1.6x10^2 plaque-forming units (PFU)/well (multiplicity of infection =0.002-0.2), confirmed by back-titration. Cells were overlaid with carboxymethylcellulose-DMEM, containing 1 μg/ml BAY 57-1293 (∼15× 90% effective dose). At 48 h post inoculation (p.i.) visible plaques were marked using a microscope and the cells from two or more individual plaques for each condition were selected at random, carefully aspirated and stored at -70°C. Harvested plaques were tested later by plaque reduction assay (PRA) directly or plaque-purified three times in Vero cells in the absence of inhibitor prior to further analysis. For wells in which plaques were visible, the cells were fixed, stained and all plaques enumerated. However, for wells where no plaques were visible, the cells were harvested (multiple wells for each condition) and the virus yield was tested for resistance by PRA. *Frequency of BAY 57-1293 (≥1 μg/ml)-resistant virus in PDK crude working stock is approximately 1 in 4x10^4 PFU. †Frequency of BAY 57-1293 (≥1 μg/ml)-resistant virus in PDK cl-1 working stock is <1 in 1x10^6 PFU.
example, BAY-Pr2 from PDK cl-1, Figure 6D) were detected at low frequency (<1 in 10^6) in PDK cl-1 (Figure 4C, Table 2). The most common variants from PDK crude readily formed plaques in 1 μg/ml BAY 57-1293 whereas those from SC16 crude were only able to form plaques in 0.1 μg/ml. The named viruses are three-times plaque-purified representative examples of these commonly occurring variants.

Two other moderately drug-resistant variants BAYr1 from SC16 cl-2 and BAY-Pr1 from PDK cl-1 (Figure 7) were found to be ~80-fold and 50-fold resistant respectively when compared to their respective parents (Figures 6B, 6C & 6D).

**Cross-resistance to alternative HPI and ACV**

Two well-characterized variants derived from SC16 were tested for sensitivity to an alternative HPI. Thus, BAYr1 (80-fold) and BAYr2 (4,000-fold resistant to BAY 57-1293) were shown to be ~30- and 400-fold co-resistant, respectively, to BILS 22 BS compared to their parent, SC16 cl-2 (Figure 8A). As expected, both these BAY 57-1293-resistant variants were sensitive to ACV (Figure 8B), showing no cross-resistance to the nucleoside analogue.

**Experimental mixtures of wild-type and resistant variants**

In order to confirm our ability to detect relatively low proportions of drug-resistant variants in the virus population, the plaque-pure working stock SC16 cl-2 was deliberately contaminated with either a low resistant (E cl-2) or moderately resistant variant (C cl-2), which are 15-fold and 125-fold resistant respectively. Approximately 100 PFU of each variant were independently mixed with 3 x 10^5 PFU SC16 cl-2. Back titrations showed the actual additions were 71 and 43 PFU respectively. The mixture was inoculated onto Vero cells, pre-incubated and replenished at 24 h intervals with 0.1 or 1 μg/ml BAY 57-1293 and the plaques counted after 48 h. The results reflected the expected composition of the mixtures in each case; yielding 50% in 0.1 μg/ml and 25% in 1 μg/ml input plaques respectively.

Given the unexpectedly high proportion of BAY 57-1293 in both crude working stocks, SC16 crude was compared with the plaque-purified derivative for frequency of resistance to ACV. SC16 crude and SC16 cl-2 were tested in the presence of 1 μg/ml ACV (just above the ED_{90}) and this revealed the selection of ACV-resistant plaques at the previously reported frequency of 10^{-3} to 10^{-4}. Furthermore, there was no difference between the crude working stock and the plaque-purified clone. Resistant plaques selected under these conditions were individually tested and found to be resistant to 1 μg/ml ACV.

**Sequence analysis**

The helicase protein, HSV-1 UL5, of the helicase-primase complex is thought to be the target of BAY 57-1293 and all previously reported BAY 57-1293-resistant variants contain point mutations producing single amino acid substitutions in the UL5 protein (Kleymann et al., 2002).
High frequency BAY 57-1293 resistance in HSV-1 laboratory isolates

Sequence data revealed that the majority of BAY 57-1293-resistant variants described in this study have amino acid substitutions located close to and downstream of the functional domain IV in the UL5 gene (amino acids 342-350 as defined by Zhu & Weller, [1992]). Amino acid substitutions were observed at positions G352, M355 or K356 (Table 2). There were some interesting exceptions: PDK crude-rm-1 and BAY-Pr1 showed no known HPI resistance mutations in UL5.

**Discussion**

The main findings to emerge from this study were as follows: (i) confirmation that BAY 57-1293 is superior in...
potency to ACV and BILS 22 BS (an alternative HPI) against a laboratory strain of HSV-1 (SC16 crude) tested at low MOI in Vero cells; (ii) the selection of drug-resistant variants does not require virus replication in the presence of compound; variants appeared to be pre-existent in the virus population; (iii) both laboratory working stocks contained BAY 57-1293-resistant variants at a frequency 10–100× higher than previously reported. For example, for PDK crude the mean frequency from nine replicate cultures was ∼1 in 4×10³; (iv) plaque-purified clones derived from these working stocks contained a relatively low frequency of BAY 57-1293-resistant variants (within the published range). There was, however, no difference in the frequency of ACV resistance; (v) individual resistant variants showed different levels of resistance from ∼15-fold to 4,000-fold; (vi) two well characterized variants (one moderately and one highly resistant to BAY 57-1293) were shown to be co-resistant to BILS 22 BS while they remained sensitive to ACV. To date,
Figure 7. Scheme for isolation of BAY 57-1293-resistant variant, BAYr1 from herpes simplex virus-1 SC16 cl-2

SC16 cl-2 was inoculated onto Vero cell monolayers into each of two 175 cm² tissue culture flasks (T175, Nunc, Denmark). Following virus adsorption for 45 min at 37°C, the infected cells were overlaid with a sub-inhibitory concentration of BAY 57-1293 (90% effective dose \( [ED_{90}] = 0.2 \mu M \)) in DMEM (without carboxymethylcellulose) and incubated for further 72 h post inoculation (p.i.), cytopathic effect (CPE) developed and cells were harvested, sonicated and the yields tested for plaque-formation in presence or absence of an inhibitory concentration of BAY 57-1293 (twofold above ED 90). The yields were \( \sim 10^8 \) plaque-forming units (PFU)/ml and from one flask all plaques were sensitive to BAY 57-1293, however from the second flask 0.3% (that is, about \( 2 \times 10^5 \) PFU) of the yield formed plaques in 0.1 \( \mu g/ml \) BAY 57-1293, suggesting drug-resistance. A putative BAY 57-1293-resistant plaque was selected and purified by single plaque isolation three times in the absence of drug and named BAYr1. A similar protocol was followed to select BAY 57-1293-resistant viruses from PDK cl-1 (BAY-Pr1 and BAY-Pr2) (data not shown). MOI, multiplicity of infection.

Figure 8. Plaque reduction assay (PRA) showing cross-resistance/sensitivity of the BAY 57-1293-resistant variants BAYr1 and BAYr2 to alternative antiviral compounds

Figures show cross-resistance to BILS 22 BS (A) and sensitivity to acyclovir (B). Fifty percent effective doses (ED 50 ) of various inhibitors were measured by means of PRA in Vero cells. Plaques were counted after 48 h incubation. Data points are the mean of three replicate counts with standard deviation.
all BAY 57-1293-resistant variants appear to be co-resistant to the alternative HPI.

Our best evidence that resistant variants are already present in the virus population is based on the observation that plaque formation is unaffected by the conditions of selection. This includes using high concentrations and pre-incubation and/or replenishment of drug at 24 h intervals. Furthermore, resistant plaques are already discernable at 24 h post inoculation. Finally, deliberate mixtures of sensitive virus and the higher or lower drug-resistant variants, when subjected to drug-pressure, revealed the anticipated proportion of drug-resistant plaques at 48 h post inoculation.

One of the resistant variants isolated from SC16 cl-2 (BAYr1, 80-fold) was shown to grow significantly faster than its parent in cell culture and was pathogenic in a murine infection model (Biswas et al., unpublished data). Presence of 'faster' viruses in the population could account for gradual enrichment with subsequent passages of such populations at low MOI in cell culture. This proposition is currently being tested using appropriate experiments (for example, serial blind passages of known mixtures of resistant and parent viruses and analysing yields for deviations from starting frequency).

The results described above were obtained from well characterized laboratory strains of HSV-1. It is of interest to know how recent clinical isolates would behave regarding the selection of resistance to BAY 57-1293. Surprisingly, among ten recent clinical isolates studied to date, one has already been identified as containing a high frequency (similar to PDK crude) of highly resistant variants (Biswas et al., unpublished data).

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References


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Correction

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The authors wish to draw attention to the following errors in their article:

On page 15 an incorrect sequence database was referenced. The sentence 'A set of eight pairs of overlapping HSV-1-specific primers (designed on the basis of HSV-1 strain 17 sequence; Genbank accession number NC001806) was used (McGeoch et al., 1988).’ should read 'A set of eight pairs of overlapping HSV-1-specific primers (designed on the basis of HSV-1 strain 17 sequence; RefSeq accession number NC001806) was used (McGeoch et al., 1988).’

On Table 2 an incorrect symbol was used: ‘’ reported by Liuzzi et al., 2004’ should be replaced by ‘‘ reported by Liuzzi et al., 2004’. A corrected table can be found below.

On Table 3 the 5th column heading is incorrect: ‘ BAY 57-1293 concentration, mg/ml’ should read ‘ BAY 57-1293 concentration μg/ml’. A corrected table can be found below.

Table 2. Observed frequency of BAY 57-1293 and other HPI-resistant variants in various laboratory isolates of HSV-1 and mutations in the helicase (UL5) protein

<table>
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<tr>
<th>HPI</th>
<th>HSV-1 parental strain</th>
<th>Frequency*</th>
<th>Level of resistance, fold increase in ED_{50} (example)</th>
<th>Amino acid substitution in HSV-1 UL5</th>
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</thead>
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<tr>
<td>BAY 57-1293</td>
<td>F</td>
<td>0.5–4.5 in 10^6</td>
<td>&gt;5 (n/s)</td>
<td>M355→T</td>
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<td></td>
<td>F</td>
<td>–</td>
<td>&gt;150 (n/s)</td>
<td>K356→Q</td>
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<tr>
<td></td>
<td>F</td>
<td>–</td>
<td>&gt;400 (n/s)</td>
<td>G352→V</td>
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<tr>
<td>BAY 57-1293</td>
<td>SC16 crude</td>
<td>&gt;1 in 10^{11}</td>
<td>15 (E cl-2)</td>
<td>M355→T</td>
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<td>SC16 crude</td>
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<td>125 (C cl-2)</td>
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<td>50 (BAYr1)</td>
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<tr>
<td>BILS 22 BS*</td>
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<td>38 (K138R3)</td>
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<td>T1576022†</td>
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<td>– in 10^7</td>
<td>n/s (R1, R2 &amp; R3)</td>
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</tr>
</tbody>
</table>

*Frequency expressed as observed number of drug-resistant plaque-forming units (PFU) in the total number of PFU screened; ‘reported by Kleymann et al., 2002; ‘SC16 crude stock reproducibly contained >10^11 more drug-resistant variants than that observed for plaque-purified SC16 cl-2; ‘novel substitutions, not previously reported as BAY 57-1293-resistant mutations; ‘PDK crude stock reproducibly contained >70x more drug-resistant variants than that observed for plaque-purified PDK cl-1; ‘reported by Liuzzi et al., 2004; ‘reported by Spector et al., 1998. Bold type indicates the BAY 57-1293-resistant variants occurring at high frequency and the corresponding laboratory isolates in which they were detected. ED_{50}, 50% effective dose; HPI, helicase-primase inhibitor; HSV, herpes simplex virus; n/s, not specified.
Table 3. A summary of BAY 57-1293-resistant variants showing the methods used for their selection

<table>
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<tr>
<th>Parent virus</th>
<th>Variant clone</th>
<th>$\text{ED}_{50}$ $\mu$g/ml</th>
<th>Fold-resistance</th>
<th>BAY 57-1293 concentration, $\mu$g/ml</th>
<th>Method of selection (protocol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC16 crude</td>
<td>E-cl-2</td>
<td>0.3*</td>
<td>15</td>
<td>0.1</td>
<td>Pre-incubated and replenished:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24 h interval × 1 day</td>
</tr>
<tr>
<td>SC16 crude</td>
<td>C-cl-2</td>
<td>2.5*</td>
<td>125</td>
<td>1.0</td>
<td>Pre-incubated and replenished:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24 h interval × 1 day</td>
</tr>
<tr>
<td>SC16 cl-2</td>
<td>BAYr1</td>
<td>0.8†</td>
<td>80</td>
<td>0.1</td>
<td>One exposure</td>
</tr>
<tr>
<td>SC16 cl-2</td>
<td>BAYr2</td>
<td>40†</td>
<td>4,000</td>
<td>1.0</td>
<td>Pre-incubated and replenished:</td>
</tr>
<tr>
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<td></td>
<td>24 h interval × 5 days</td>
</tr>
<tr>
<td>PDK crude</td>
<td>PDK cr-rm-1</td>
<td>2†</td>
<td>50</td>
<td>1.0</td>
<td>Pre-incubated and replenished:</td>
</tr>
<tr>
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<td></td>
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<td>24 h interval × 1 day</td>
</tr>
<tr>
<td>PDK cl-1</td>
<td>BAY-Pr1</td>
<td>$2^{&lt;1.5}$†</td>
<td>50</td>
<td>0.3</td>
<td>One exposure</td>
</tr>
<tr>
<td>PDK cl-1</td>
<td>BAY-Pr2</td>
<td>79†</td>
<td>2,000</td>
<td>0.3 &amp; 10</td>
<td>Two exposures</td>
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</tbody>
</table>

Fifty percent effective dose ($\text{ED}_{50}$) data was determined by plaque reduction assay in Vero cells and was significantly different from respective wild-type in an un-paired 2-tailed t-test ($P<0.001$). *From Figure 6A; †from Figure 6B; ‡from Figure 6C; § from Figure 6D.