Detection of HSV-1 variants highly resistant to the helicase–primase inhibitor BAY 57-1293 at high frequency in 2 of 10 recent clinical isolates of HSV-1

Subhajit Biswas1, Christopher Smith2 and Hugh J. Field1*

1Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK; 2Division of Virology, Department of Pathology, University of Cambridge, Laboratories Block Level 5, Addenbrooke’s Hospital, Hills Road, Cambridge CB2 2QQ, UK

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Objectives: BAY 57-1293 is a helicase–primase inhibitor (HPI) from a new class of antivirals that are highly efficacious in herpes simplex virus (HSV)-1 animal infection models. Resistant mutants with point mutations in the helicase (UL5) were reported to be present in laboratory isolates at a low frequency of approximately 10^{-6}. In contrast, we have shown elsewhere that some laboratory isolates contain resistant variants at higher frequency (10^{-4}). Therefore, we screened 10 recent clinical isolates of HSV-1 for BAY 57-1293-resistant virions.

Methods: Clinical isolates were screened by a plaque reduction assay in Vero cells to determine the frequency of occurrence of BAY 57-1293-resistant variants. The helicase gene for the resistant variants was sequenced.

Results: One isolate contained highly resistant variants at 10^{-4} and another at 10^{-5}. Both variants contained a previously reported BAY 57-1293 resistance mutation (K356N) in UL5 and were >5000-fold resistant.

Conclusions: Occurrence of HPI-resistant viruses at high frequency in a clinical isolate is intriguing. Two alternative hypotheses are proposed to explain this phenomenon. It is also surprising that two unrelated clinical isolates contain an identical HPI resistance mutation. These results have important implications for HPI drug-resistant monitoring during subsequent clinical trials.

Keywords: HSV, antivirals, resistance

Introduction

Helicase–primase inhibitors (HPI) represent a novel class of herpes simplex virus (HSV) inhibitors that are active in tissue culture,1–5 efficacious in animal infection models,1–3,5 and several are candidates for clinical development.6 Previous studies reported the rate of resistance to HPI to be low; 0.5–4.5 × 10^{-6} for BAY 57-1293,1–3 10^{-6} for BILS 22 BS1 and 10^{-7} for T157602,4 compared with approximately 10^{-4} for nucleosides, e.g. aciclovir or penciclovir.7 In contrast, we observed that working stocks of two laboratory isolates (HSV-1 SC16 and PDK) contained BAY 57-1293-resistant (15–40-fold) virions at relatively high frequency (10^{-4}–10^{-5}), although the rate was lower in recently plaque-purified clones, e.g. SC16 cl-2 and PDK cl-1.8 These observations prompted us to test recent clinical isolates for sensitivity to BAY 57-1293 and frequency of HPI-resistant variants.

Materials and methods

Viruses
A series of 10 anonymous clinical isolates of HSV-1 (A–J) were procured from the Health Protection Agency (HPA), Addenbrooke’s Hospital, Cambridge, UK during 2005–06 from apparently immunocompetent patients from skin, oral or genital sites. Swabs were placed in virus-transport medium, inoculated onto MRC-5 cells and the medium was changed after 24 h. When cytopathic effect (CPE) was noted within 48 h, the monolayer was suspended in 1 mL of

*Corresponding author. E-mail: hjf10@cam.ac.uk

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HPI resistance in HSV-1 clinical isolates

minimal essential medium and a drop was typed as HSV-1 using a standard indirect fluorescent antibody test with monoclonal antibody obtained from the Division of Virology, Department of Pathology, University of Cambridge. The remaining sample was stored at −70°C.

Antiviral (BAY 57-1293) used for screening

BAY 57-1293, a thiazole amide (molecular mass: 402 Da), was provided as a dry powder by Arrow Therapeutics (London, UK), constituted in DMSO and stored at 1 mg/mL in Dulbecco’s modified Eagle’s medium (DMEM) at −20°C.

Plaque reduction assays

The 10 isolates (A–J) cultured from MRC-5 cells were tested by a plaque reduction assay (PRA). Aliquots of the specimens were diluted to contain 100 pfu/well and inoculated into 12-well tissue culture plates (Nunc, Denmark) containing approximately 2 × 10^5 Vero cells/well. After adsorption for 45 min at 37°C in humidified 5% CO₂, a DMEM-overlay, thickened with carboxymethylcellulose (CMC) and containing dilutions of BAY 57-1293, was added to each well. Plaques were enumerated after 48 h.

Screening protocols

The conventional PRA, which tests approximately 100 pfu of sample, could not detect resistant variants at lower frequency (<1%). Therefore, we screened aliquots from the stored MRC-5 virus isolates (titres ~10^2–10^7 pfu/mL) directly for BAY 57-1293 resistance using three different protocols. All isolates were tested using 6- or 12-well plates (protocols I and II). Isolates D–H were also tested using 75 cm² tissue culture flasks (T75) (Nunc) (protocol III). Approximately 10^2–10^6 pfu were screened for each sample (limited by the number of Vero cells) for BAY 57-1293-resistant plaques.

Stringent conditions were used: 2 h pre-incubation with BAY 57-1293 (0.8 μM BAY 57-1293 in DMEM) and overlay with 0.8 or 3 μM BAY 57-1293 (4- or 15-fold above ID₉₀, respectively); conditions designed to prevent replication of sensitive virus and rule out the spontaneous mutants. Infected monolayers were examined and clearly discernible plaques at 48 h post-infection or 72 h post-infection were marked, enumerated, and the infected cells were aspirated from individual plaques. In the case of T75 flasks, the whole cell sheet was suspended in supernatant, stored at −70°C and retested using PRA in Vero cells. The three screening protocols differed in the following details.

Protocol I. Twelve-well plates and 10-fold dilutions of virus were used. The first six wells of each plate received no drug; three wells received 0.8 μM each and the remaining three wells received 3 μM BAY 57-1293 per well. All drug-treated wells were pre-incubated with 0.8 μM drug in CMC-free medium for 2 h before virus inoculation then overlaid with fresh compound in CMC medium after virus adsorption (Figure 1a).

Protocol II. In this case, 6-well plates (approximately 10^5 cells/well) were used and a single dilution of virus (confirmed by back titration). As mentioned earlier, wells were pre-incubated with 0.8 μM BAY 57-1293 for 2 h and medium containing 0.8 or 3 μM drug was added in the overlay after virus adsorption as for protocol I (Figure 1b).

For isolates F and G, the inoculum was divided among multiple wells and screened for resistance at 3 μM. For the remaining isolates (A–C, I, J), neat virus was inoculated into a single well and compound added at 0.8 μM. In the latter cases, the 48 h yield was also tested for drug resistance by PRA.

Protocol III. In a third experiment, 2 × 10^5 pfu each of several isolates (D–H) were inoculated into separate T75 tissue culture flasks (approximately 10^7 cells/flask), pre-incubated with 0.8 μM BAY 57-1293 for 2 h, and overlaid with fresh drug-containing medium after virus adsorption. By this method, plaques were not clearly defined because of CPE from the inoculum virus. However, when the cells were harvested at 72 h post-infection, reduced levels of infectious virus were detected (Figure 1c).

Sequencing of BAY 57-1293-resistant variants

The HSV-1 helicase gene (UL5) was sequenced for BAY 57-1293-resistant variants using a set of eight pairs of overlapping HSV-1-specific primers (design on the basis of HSV-1 strain 17 sequence; RefSeq accession no. NC 001806). A touchdown PCR was adapted to amplify the UL5 gene in overlapping fragments with a high specificity. PCR amplicons of the expected size were purified and sequenced (both directions using the same primers as for PCR) at the Cambridge Department of Biochemistry by Sanger’s dideoxy chain-termination method. The nucleotide and amino acid sequences of UL5 were aligned by ClustalW v 1.82.

Results and discussion

All 10 isolates were sensitive to BAY 57-1293 (Table 1) with ID₉₀ < 0.05 μM and ID₉₀ ≤ 0.2 μM. No plaques were observed from any clinical isolate at inhibitor concentrations ≥0.3 μM from approximately 100 pfu tested in each well. The results are similar to laboratory isolates previously tested and lie within the published range.1

When larger numbers of pfu were screened, isolate F yielded resistant plaques by three different methods. Using 12-well plates (protocol I), an estimated 2 × 10^3 pfu/well was inoculated into the first row; the subsequent two rows received serial 10-fold lower dilutions. Plaques could not be distinguished because of confluent CPE in the first row. However, at 48 h post-infection, one plaque was visible (the well containing 3 μM drug) out of a putative 5.2 × 10^2 pfu. The number of pfu was deduced from the number of plaques in the first two columns which received no inhibitor. This plaque (BAY-F-r1) was picked for further analysis.

Screening 1.4 × 10^4 pfu (using 6-well plates, protocol II) produced one drug-resistant plaque (BAY-F-r2) at 48 h post-infection which was also aspirated for further analysis.

In the 75 cm² flasks (protocol III), 72 h yields (~2 × 10^3 pfu/mL) for isolate F or G contained BAY 57-1293-resistant virus (at 30% and 5%, respectively). For the remaining isolates (D, E and H), the infectious virus yields remained sensitive to the drug (0% resistant plaques) and no evidence of resistance was obtained.

Thus, isolates F and G yielded resistance to BAY 57-1293 among the 10 isolates screened. Specimens F and G came, respectively, from a vulval swab (female, 25 years) and a swab from an unrelated male (33 years) with penile ulcers. Specimen F was obtained on 11 April 2006 at Addenbrooke’s Hospital, Cambridge, whereas specimen G was obtained on 13 April 2006 at West Suffolk Hospital and sent to Addenbrooke’s for analysis.
**Figure 1.** Schematic representations of the three protocols used for screening clinical isolates for BAY 57-1293 resistance. (a) Protocol I: used for all isolates (A–J). Vero cells (approximately $2 \times 10^5$ well) were pre-incubated with BAY 57-1293 (in CMC-free medium) for 2 h before inoculation. After adsorption, infected cells were overlaid with fresh compound in CMC medium. Plaque formation was observed after 48 h. The number of pfu inoculated into the $10^2$ wells ranged from $3.5 \times 10^3$ (isolate A) to $5 \times 10^4$ (isolate D). (b) Protocol II: used for isolates A–C, F, G, I and J. Vero cells (approximately $10^6$ well) were pre-incubated with 0.8 μM BAY 57-1293 (in CMC-free medium) for 2 h before virus inoculation and overlaid with fresh compound (in CMC medium) as for Protocol I. Each isolate was inoculated at neat or higher dilution into one (isolates A–C, I and J) or four wells (isolates F and G) depending on the titre and volume of isolate available. The inoculum ranged from $3.5 \times 10^3$ (isolate A) to $4.4 \times 10^5$ (isolate J) pfu/well. Plaque formation was assessed at 48 h post-infection. (c) Protocol III: used for isolates D–H. Vero cells (approximately $10^7$ T75 flask) were treated with BAY 57-1293 as above, then inoculated with D–H with input as shown in the diagram and at 72 h post-infection, the cells were harvested and the yields were tested for sensitivity to BAY 57-1293 by PRA.
HPI resistance in HSV-1 clinical isolates

Table 1. Sensitivity to BAY 57-1293 in Vero cells and the frequency of drug-resistant variants in recent clinical isolates and laboratory strains of HSV-1

<table>
<thead>
<tr>
<th>HSV-1 isolate</th>
<th>ID$_{50}$ (μM)</th>
<th>ID$_{90}$ (μM)</th>
<th>N$^a$; number of pfu screened in ≥0.8 μM BAY 57-1293</th>
<th>Minimum frequency of BAY 57-1293-resistant plaques (pfu/N)</th>
<th>Screening protocols used</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>≤0.05</td>
<td>≤0.2</td>
<td>3.5 × 10$^4b$</td>
<td>&lt;1</td>
<td>I, II</td>
</tr>
<tr>
<td>B</td>
<td>≤0.05</td>
<td>≤0.2</td>
<td>5.4 × 10$^4b$</td>
<td>&lt;1</td>
<td>I, II</td>
</tr>
<tr>
<td>C</td>
<td>≤0.05</td>
<td>≤0.2</td>
<td>1.3 × 10$^5b$</td>
<td>&lt;1</td>
<td>I, II</td>
</tr>
<tr>
<td>D</td>
<td>≤0.05</td>
<td>≤0.2</td>
<td>5.0 × 10$^5$</td>
<td>&lt;1</td>
<td>I, III</td>
</tr>
<tr>
<td>E</td>
<td>≤0.05</td>
<td>≤0.2</td>
<td>1.5 × 10$^5b$</td>
<td>&lt;1</td>
<td>I, III</td>
</tr>
<tr>
<td>F</td>
<td>0.05</td>
<td>0.2</td>
<td>5.2 × 10$^3$</td>
<td>1</td>
<td>I</td>
</tr>
<tr>
<td>F</td>
<td>0.05</td>
<td>0.2</td>
<td>1.4 × 10$^4$</td>
<td>1</td>
<td>II</td>
</tr>
<tr>
<td>F</td>
<td>0.05</td>
<td>0.2</td>
<td>2.6 × 10$^5$</td>
<td>&gt;1</td>
<td>III</td>
</tr>
<tr>
<td>G</td>
<td>0.05</td>
<td>0.2</td>
<td>1.7 × 10$^5$</td>
<td>≥1</td>
<td>I–III</td>
</tr>
<tr>
<td>H</td>
<td>≤0.05</td>
<td>≤0.2</td>
<td>2.2 × 10$^5$</td>
<td>&lt;1</td>
<td>I, III</td>
</tr>
<tr>
<td>I</td>
<td>≤0.05</td>
<td>≤0.2</td>
<td>2.8 × 10$^5$</td>
<td>&lt;1</td>
<td>I, II</td>
</tr>
<tr>
<td>J</td>
<td>≤0.05</td>
<td>≤0.2</td>
<td>4.4 × 10$^5$</td>
<td>&lt;1</td>
<td>I, II</td>
</tr>
<tr>
<td>SC16</td>
<td>0.05</td>
<td>0.2</td>
<td>1.0 × 10$^5$</td>
<td>&gt;1$^c$</td>
<td>II, III$^f$</td>
</tr>
<tr>
<td>SC16 cl-2</td>
<td>0.05</td>
<td>0.2</td>
<td>1.0 × 10$^5$</td>
<td>1$^c$</td>
<td>II, III$^f$</td>
</tr>
<tr>
<td>PDK</td>
<td>0.1</td>
<td>0.2</td>
<td>4.0 × 10$^4$</td>
<td>1$^c$</td>
<td>II$^f$</td>
</tr>
</tbody>
</table>

$^a$The data in this column indicate the number of pfu screened in order to obtain an estimate of the frequency of BAY 57-1293-resistant variants.

$^b$In these cases, the maximum number of pfu screened was limited by the actual infectious virus titre in the original specimens.

$^c$Adapted; details published elsewhere.$^8$

The estimated frequency of drug resistance in isolate F was approximately 10$^{-4}$, which is >10 times the highest incidence for BAY 57-1293-resistant viruses reported (4.5 × 10$^{-6}$). This frequency is similar to aciclovir resistance. Isolate G contained an identical resistance mutation towards the higher limit of previously reported frequency. It is highly unlikely that this mutant was generated spontaneously during the 72 h incubation in BAY 57-1293 for two reasons: (i) the stringent conditions (discussed earlier) meant that virus replication was inhibited below the level of 1% input titre and (ii) using the same protocol, no drug-resistant variants were detected in isolates E, H or even D with the highest titre of all (5 × 10$^{6}$ pfu/mL) (Table 1 and Figure 1c).

The substitution K356N close to UL5 helicase motif IV$^{1,11}$ (Figure 2) has previously been reported to be an HPI resistance mutation conferring a high level of resistance.$^1–^3$ It is noteworthy that a BAY 57-1293-resistant variant from HSV-1P with the same mutation in UL5 was reported to have similar growth rate and produced equal mortality when compared with wild-type in BALB/c mice.$^5$ The same Lys-356 residue in UL5, when mutated to Gln$^3$ or Thr (S. Biswas and H. J. Field, unpublished results), has also been shown to confer resistance to BAY 57-1293. Furthermore, the same substitution in HSV-1 KOS conferred 2500-fold resistance to another HPI, BILS 22 BS,$^2$ suggesting that the mutants from our clinical isolates are likely to be co-resistant to other HPIs. Interestingly, the K356N KOS mutant was also a fit virus in tissue culture and pathogenic in mice.$^3$

It is intriguing that isolates F and G contain a mutant with the identical K356N change in UL5 given that >10 different mutations involving 5 different amino acid residues in UL5 confer HPI resistance.$^1,^3–^5$ (S. Biswas and H. J. Field, unpublished results). Lys-356 of the GNLMK sequence adjacent to motif IV is conserved across herpesvirus helicases and appears to be important for helicase activity.

It is therefore open to debate whether the K356N mutation had some selective advantage in the patient or during the single cell passage during isolation.
An alternative and more likely explanation could be that the K356N mutation is ‘neutral’ according to Kimura’s neutral theory of molecular evolution.12,13 The latter proposes that protein and DNA polymorphisms constitute a transient phase of molecular evolution and rejects the notion that the majority of such polymorphisms result from adaptive responses and are actively maintained in the species by some form of natural balancing selection. However, this still begs the question as to why the frequency of this mutation in isolate F should be approximately 100 times the expected range for spontaneous mutations.

The UL5 protein is highly conserved; notwithstanding, we detected a drug-resistant mutant in 2 of 10 independent clinical isolates, and furthermore, a similar high frequency was reported in two of our laboratory working stocks.8 Our hypotheses to explain these high frequency areas are as follows. (i) Under certain conditions, helicase mutations accelerate growth rate providing an advantage. We have evidence that certain HPI-resistant UL5 mutations can increase or decrease viral growth characteristics in tissue culture.14 This should be testable more rigorously using appropriate tissue culture and animal infection models. For instance, deliberate reconstructions comprising mixtures of parental virus and known HPI-resistant mutants derived from it (e.g. one with faster growth kinetics) will be investigated under defined growth conditions in order to study whether there is an increase in the proportion of the faster-growing mutant over several virus replication cycles. (ii) Virus was exposed to HPI or similar compounds in the patient or during the isolation procedure.

We favour (i) because HPI is at an early stage of development and unlikely to have been available to the patients. However, BAY 57-1293 has structural and functional similarity to the diuretic drug Diamox (acetazolamide).1 We and others have shown that the HPI BAY 57-1293 is highly efficacious in a

Table 2. Nucleotide and amino acid polymorphisms in the HSV-1 helicase (UL5) gene (2469 nt) in SC16 cl-2, BAY-F-r1 (from isolate F) and BAY-G-r1 (from isolate G) in comparison with the published sequence of HSV-1 strain 179

<table>
<thead>
<tr>
<th>Nucleotide positions</th>
<th>SC16 cl-2 isolate F</th>
<th>isolate G</th>
<th>Amino acid positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A41G</td>
<td>A41G</td>
<td>A41G</td>
<td>AAA (Lys-14)→AGA (Arg)</td>
</tr>
<tr>
<td>A200G</td>
<td>A200G</td>
<td>A200G</td>
<td>CAT (His-67)→CTG (Arg)</td>
</tr>
<tr>
<td>A477G</td>
<td>A477G</td>
<td>A477G</td>
<td>silent</td>
</tr>
<tr>
<td>A483G</td>
<td>A483G</td>
<td>A483G</td>
<td>silent</td>
</tr>
<tr>
<td>T614C</td>
<td>T614C</td>
<td>T614C</td>
<td>TTG (Leu-205)→TCG (Ser)</td>
</tr>
<tr>
<td>C984G</td>
<td>C984G</td>
<td>C984G</td>
<td>silent</td>
</tr>
<tr>
<td>C1158A</td>
<td>C1158A</td>
<td>C1158A</td>
<td>silent</td>
</tr>
<tr>
<td>G1347T</td>
<td>G1347T</td>
<td>G1347T</td>
<td>silent</td>
</tr>
<tr>
<td>T2069G</td>
<td>T2069G</td>
<td>T2069G</td>
<td>GTC (Val-690)→GCC (Gly)</td>
</tr>
<tr>
<td>C2484T</td>
<td>C2484T</td>
<td>C2484T</td>
<td>silent</td>
</tr>
</tbody>
</table>
variety of animal models, e.g. showing superiority to famciclovir. However, our present study suggests that it will be particularly important to monitor drug resistance during clinical trials of HPI and further development of these compounds: ‘forewarned is forearmed’.

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Transparency declarations

H. J. F. is a Virology Editor for JAC and held a consultancy with Arrow Therapeutics during the period of these studies.

References