A mutation in helicase motif IV of herpes simplex virus type 1 UL5 that results in reduced growth in vitro and lower virulence in a murine infection model is related to the predicted helicase structure

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A variant was selected from a clinical isolate of herpes simplex virus type 1 (HSV-1) during a single passage in the presence of a helicase–primase inhibitor (HPI) at eight times the IC₅₀. The variant was approximately 40-fold resistant to the HPI BAY 57-1293 and it showed significantly reduced growth in tissue culture with a concomitant reduction in virulence in a murine infection model. The variant contained a single mutation (Asn342Lys) in the UL5 predicted functional helicase motif IV. The Asn342Lys mutation was transferred to a laboratory strain, PDK cl-1, and the recombinant acquired the expected resistance and reduced growth characteristics. Comparative modelling and docking studies predicted the Asn342 position to be physically distant from the HPI interaction pocket formed by UL5 and UL52 (primase). We suggest that this mutation results in steric/allosteric modification of the HPI-binding pocket, conferring an indirect resistance to the HPI. Slower growth and moderately reduced virulence suggest that this mutation might also interfere with the helicase–primase activity.

The herpes simplex virus type 1 (HSV-1) helicase–primase (UL5/8/52) complex is required continuously during HSV DNA synthesis (Zhu & Weller, 1988). The UL5 component of this replication complex has six predicted functional motifs (I–VI) that are essential for the helicase activity during viral DNA unwinding (Zhu & Weller, 1992). Site-directed mutagenesis supported this model, showing that substitutions at highly conserved residues within predicted motifs I and IV produce non-viable progeny (Zhu & Weller, 1992). Furthermore, biochemical data from the expressed mutant proteins showed that certain mutations within motif III–VI decreased DNA-dependent ATPase activity by three- to six-fold. However, mutations in motifs III, IV, V and VI did not eliminate DNA binding or ATP hydrolysis independently, suggesting that the residues might be involved in the coupling of these processes to DNA unwinding (Graves-Woodward et al., 1997). Two helicase–primase inhibitor (HPI) resistance mutations just downstream of the UL5 functional motif IV (Gly352Arg and Met355Thr) were previously shown to be associated with slower growth in tissue culture (Biswas et al., 2007a). These mutations were primarily described as isolate F (Biswas et al., 2007b) but have been renamed isolate pF to avoid possible confusion with the well-known HSV-1 strain F. Here we report a novel HPI resistance mutation (Asn342Lys) located at the upstream boundary of helicase functional motif IV, as defined by Zhu & Weller (1992). Moreover, we show that this residue is important for virus replication and function of the inhibitor.

The HPI BAY 57-1293 (molecular mass 402.5 Da) was kindly provided by Arrow Therapeutics, London. The IC₅₀ values were determined by plaque reduction assays (PRA) in Vero cells using standard methods (Biswas et al., 2007b). Virus strain PDK cl-1 was derived from HSV-1 PDK (originally Cl-101; Dubbs & Kit, 1964; Field & Wildy, 1978) by three plaque-purification steps in Vero cells. An anonymous clinical isolate of HSV-1, isolate pF, was provided by the Health Protection Agency (HPA), Addenbrooke’s Hospital, Cambridge, UK. The same isolate was previously described as isolate F (Biswas et al., 2007b) but has been renamed isolate pF to avoid possible confusion with the well-known HSV-1 strain F.

An HPI-resistant plaque was selected from isolate pF in Vero cells using 0.8 μM BAY 57-1293 (eight times IC₅₀) as follows: approximately 2 × 10⁵ p.f.u. HSV-1 (isolate pF) was inoculated into a T75 tissue culture flask (approx. 10⁷ cells per flask). Cells were pre-incubated with 0.8 μM BAY 57-1293 for 2 h and overlaid with fresh drug after virus
adsorption. Plaques were not clearly defined after 48 h; however, when infected cells were harvested at 72 h post-infection (p.i.), low levels of infectious virus (2 x 10^4 p.f.u. ml^-1) were detected. Approximately 30% of this yield was resistant to BAY 57-1293. One resistant plaque was plaque-purified three times and named BAY-pF-r3.

DNA from virus-infected Vero cells was amplified and bidirectionally sequenced in the HSV-1 UL5 (full) or UL52 (partial) genes, using multiple pairs of overlapping HSV-1-specific primers (Biswas et al., 2007a, 2008b). Full details of the PCRs and sequencing primers can be obtained in the supplementary online data associated with our previous paper (Biswas et al., 2008b; http://jac.oxfordjournals.org/cgi/content/full/dkn057/DC1). BAY-pF-r3 contained a single substitution (Asn342Lys) at the upstream boundary of the UL5 functional motif IV. The putative resistance mutation in BAY-pF-r3 was reconstructed in a PDK cl-1 background by PCR-based site-directed mutagenesis. For this, forward (PF: 5'-CGCACCGTGTGTTTTATAA-3') and reverse (PR: 5'-TTATTAAAAACAAACGGTTGCG-3') primers were designed based on the PDK cl-1 sequence but containing the target mutation. PCR products [obtained using UL5-2F (Biswas et al., 2007a) and PR, and PF and UL5-7R (Biswas et al., 2007a)] were used as templates for a further PCR using primers UL5-2F and UL5-7R. The reconstructed mutation was then transferred to wild-type (w/t) PDK cl-1 using a standard protocol (Biswas et al., 2007a). Putative drug-resistant recombinants occurred at 10^-4 p.f.u., more than 100-fold over the background of 10^-6 p.f.u. PDK Rec-1 is a recombinant containing the same resistance mutation in the PDK cl-1 background.

The growth of BAY-pF-r3 and PDK Rec-1 was compared, at an m.o.i. of 3, with that of their respective w/t viruses, isolate pF and PDK cl-1. Infected Vero cells and supernatants were harvested together from four wells independently at each time point, stored at -80° C and then thawed and disrupted by ultrasonic vibration. Virus yields were measured by plaque titration in Vero cells.

The amino acid sequence and 3D structure of DNA helicase II from _Escherichia coli_ were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/) (pdb-id: 2I56) (Lee & Yang, 2006) and used as templates for comparative modelling. Initial alignments between the target protein and its templates were obtained using the program FUGUE (Shi et al., 2001), which produces alignments by comparison of sequence profiles against structural profiles of homologous protein families taken from the HOMSTRAD database (de Bakker et al., 2001). Models were produced using the program MODELLER (Sali & Blundell, 1993), which produces comparative models by satisfaction of spatial restraints with simultaneous optimization of CHARMM energies (Brooks et al., 1983); this employs methods of conjugate gradients and molecular dynamics with simulated annealing (Sali & Blundell, 1993). Comparative models were verified using PROCHECK (Laskowski et al., 1993), VERIFY3D (Lutyh et al., 1992) and JOY (Mizuguchi et al., 1998). The alignments were manually modified as required and the modelling and validation processes were repeated. The process of modelling, validation and realignment was repeated until models with satisfactory geometry and conformation had been obtained.

Small molecule docking was carried out using GOLD (Jones et al., 1997). This program uses a genetic algorithm to explore the full range of ligand conformational flexibility with partial flexibility of the protein, and satisfies the fundamental requirement that the ligand must displace loosely bound water on binding. The docking experiment was driven for BAY 57-1293 to bind to an interacting area of UL5, previously predicted by Biswas et al. (2008b). A total of eight complexes showing higher scores were considered for this study. The complex showing the best interactions between BAY 57-1293 and the predicted interacting residues was finally selected. Contact residues were defined as the residues that possessed an interface solvent accessible surface area (ASA) that decreased (ΔASA) by more than 1 Å on formation of a complex (Jones & Thornton, 1996). The ASA was calculated using the Lee and Richards algorithm (Lee & Richards, 1971) and developed by Richmond (1984). HBPLUS was used for hydrogen bond definition (McDonald & Thornton, 1994). The atomic coordinates of the predicted HSV-1 UL5–BAY 57-1293 complex are available as supplementary data in JGV Online.

The virulence of isolate pF and BAY-pF-r3 was compared using a murine neck–skin zosteriform infection model (Biswas et al., 2007a, 2008a), using 29 5–6-week-old female BALB/c mice (Harlan UK). Two groups of five mice were observed for clinical signs while two groups of seven mice provided tissue samples. Mice were inoculated in the neck with 5 x 10^4 p.f.u. per mouse and five control mice were mock-infected. Clinical signs were noted and infection parameters were measured daily, including weight, right ear pinna thickness and mortality. Lesions on the neck (primary site) and pinna (zosteriform spread) were scored according to an arbitrary scale (Nagafuchi et al., 1979). On days 3, 5 and 7 p.i., mice were killed and skin from the inoculation site and the right ear pinna and brain stem were stored at -70° C. Infectious virus from the skin and ear collected on days 5 and 7 p.i. were also screened for drug resistance using 0.8 μM BAY 57-1293. Animal work was carried out in accordance with Home Office (UK) guidelines.

Statistical differences for changes in body weight and ear thickness within each group over the days were determined by one-way ANOVA followed by Tukey's (post-hoc) test for multiple comparisons. For differences between two groups on a given day, including virus titres, Student's t-test (two-tailed for unpaired data) was applied and variances were measured by the F-test. The significance of differences in lesion scores between isolate pF and BAY-pF-r3 were tested by a non-parametric rank test (Mann–Whitney test). In all cases, P<0.05 was considered to be statistically significant.
BAY-pF-r3 and PDK Rec-1 were shown to be approximately 40-fold more resistant to BAY 57-1293 compared with their respective w/t viruses by PRA (data not shown). Both mutants were equally sensitive to the nucleoside analogue, acyclovir (data not shown). DNA sequencing of full UL5 and partial UL52 confirmed that BAY-pF-r3 and PDK Rec-1 contain the single mutation Asn342Lys. There were no mutations in the region containing Gly352, Met355 and Lys356, where UL5 HPI resistance mutations have been described previously (reviewed by Biswas & Field, 2008). Furthermore, neither BAY-pF-r3 nor PDK Rec-1 contained the UL52 Ala899Thr mutation, which is also known to confer resistance to BAY 57-1293 (Biswas et al., 2008b).

Under one-step conditions, both mutants showed decreased growth in Vero cells compared with their respective w/t viruses (Fig. 1). Mutant yields were approximately 0.4 log₁₀ p.f.u. lower in the exponential phase of growth (6–12 h p.i.) and this difference was significant (see Fig. 1 legend). BAY-pF-r3 was then tested in a murine infection model and showed moderately reduced virulence compared with the w/t. All infected mice developed clinical signs. However, the onset of vesicles on the skin at the inoculation site and progression of lesions and clinical signs to zosteriform spread was delayed and significantly less severe for BAY-pF-r3 (Fig. 2a). Mice infected with isolate pF became hunched and ruffled by day 6 p.i., with red ears. Although BAY-pF-r3 produced less severe signs, the infected mice showed some reddening of the ipsilateral ear pinnae. By day 7 p.i., two of five mice infected with w/t virus developed severe clinical signs and were culled. All mice lost weight (Fig. 2b) compared with the uninfected controls; however, in comparison with the uninfected control, weight loss in BAY-pF-r3-infected mice was not significant, whereas w/t-infected mice showed significant weight loss on day 7 p.i. Mean ear thickness (a quantitative indication of zosteriform spread) of mutant-infected mice remained lower compared with the w/t-infected mice (Fig. 2c). Tissue samples of skin from the inoculation site showed high titres of infectious virus on day 3 p.i. for mutant and w/t viruses. Later, the mutant-inoculated mice yielded lower titres by approximately 1 log₁₀ p.f.u. per tissue and this was significant in skin from the inoculation site (Fig. 2d) and the ear pinna (Fig. 2e) on days 5 and 7 p.i. Infectious virus obtained 5 or 7 days p.i. from skin or ear pinnae of BAY-pF-r3-infected mice retained resistance to BAY 57-1293, whereas samples from pF-infected mice remained sensitive to the inhibitor.

In a previous study, the relative inhibitory concentrations required to overcome particular drug resistance mutations in UL5 or UL52 (including double mutants) suggested that BAY 57-1293 interacts with both components of the helicase–primase (HP) complex to achieve maximum potency (Biswas et al., 2008b). It was also postulated that BAY 57-1293 interacts simultaneously with UL5 and UL52, possibly involving residues Gly352, Met355 and Lys356 of UL5 and Ala899 of UL52 (Biswas et al., 2008b). In the present study, homology modelling (based on E. coli helicase) was used to predict a model for the 3D structure of HSV-1 UL5. The objective was to determine whether a single molecule of BAY 57-1293 can interact with UL5 residue Asn342 while simultaneously interacting with UL5 residues Gly352, Met355 and Lys356. For this, BAY 57-1293 was docked onto the surface of the predicted structure of HSV-1 UL5 in order to bind to the residues previously suggested to interact with the inhibitor (Biswas et al., 2008b). This model of the HSV-1 UL5–BAY 57-1293 complex predicted that an α-helical region of UL5, formed by Phe351-Gly-Asn-Leu-Met-Lys356 (Fig. 3a), appears to be the centre of interaction with BAY 57-1293. This region is just downstream from the predicted helicase motif IV [Asn342-Glu350 (Zhu & Weller, 1992) or Ala338-Ile-Phe-
Ile-Asn-Asn-Lys-Arg345 (Gorbalenya & Koonin, 1993). Computer docking predicted that BAY 57-1293 binds to HSV-1 UL5 by strong hydrophobic interactions with Met355, Phe351 and Phe375, by cation–π interaction with Arg874, by hydrogen bonds with the side chains of Lys356, Glu367 and His368, and polar interaction with Glu359 (Fig. 3b, c). Docking experiments also predicted that if BAY 57-1293 interacts with residues Gly352, Met355 and Lys356 of HSV-1 UL5, as suggested previously (Biswas et al., 2008b), the resultant drug interaction pocket is spatially apart from the predicted motif IV, including residue Asn342 (Fig. 3a). The Phe351-Gly-Asn-Leu-Met-Lys356 region forms a groove for BAY 57-1293 (Fig. 3a). Lys356 (positively charged) probably forms a salt bridge with the Glu359 downstream (distance between them is 3.49 Å) and a strong hydrogen bond with the carbonyl oxygen of BAY 57-1293 (Fig. 3c).

Furthermore, BAY 57-1293 has three aromatic rings to involve in aromatic interaction with Phe351 and Phe375 and cation–π interaction with Arg874. As mentioned previously, it was proposed that HSV-1 Ala899 of UL52 comes in spatial proximity to UL5 Gly352-Asn-Leu-Met-Lys356, so that a single BAY 57-1293 molecule(s) can simultaneously interact with UL5 and UL52. We believe that UL5 and UL52 have a dynamic interaction and can move about each other. HPIs (e.g. BAY 57-1293) possibly block the relative movement of the two molecules of the dynamic HP complex and prevent further enzyme activity on viral double-stranded DNA. The mutation Asn342Lys could be anticipated to cause considerable modification/distortion of the local structure of HSV-1 UL5; but as Asn342 was predicted to be on the surface of the structure of UL5 and exposed to the solvent, the local structural modification is actually likely to be small. Thus, the
mutation Asn342Lys possibly results in steric/allosteric hindrance to the UL5–UL52 interaction caused by the large side-chain of Lys342. This is supported by slower growth and reduced virulence caused by this motif mutation. The mutation may obstruct BAY 57-1293 from simultaneously interacting with both the components of the enzyme complex, reflected by the 40-fold resistance to the inhibitor. Further biochemical studies will be conducted to elucidate the effect of this motif mutation on viral HP activity. This drug–enzyme interaction study has been

Fig. 3. Predicted structure (ball model) of HSV-1 UL5 based on homology with the known structure of E. coli helicase and the position of BAY 57-1293 on the surface of HSV-1 UL5. (a) Space-fill representation of the structure of the HSV-1 UL5 component of the HP complex showing the positions of the Phe-Gly-Asn-Leu-Met-Lys region, Asn342 and Lys344. Asn342 is spatially apart from the Phe-Gly-Asn-Leu-Met-Lys region, interacting with helicase inhibitors. Green, grey and light blue denote different domains of the structure of HSV-1 UL5. (b) BAY 57-1293 on the surface of HSV-1 UL5. The HSV-1 UL5 surface is coloured to show electrostatic potential, i.e. positive charges (blue) and negative charges (red). The positions of Met355 (yellow) and Asn342 (black) are shown. Ball-and-stick representation of BAY 57-1293 is shown with carbon (green), oxygen (red), nitrogen (blue) and sulphur (yellow) atoms. (c) Ball-and-stick representation of the predicted interactions between BAY 57-1293 and the amino acid residues of HSV-1 UL5. BAY 57-1293 is shown with carbon (grey), nitrogen (blue), oxygen (red) and sulphur (yellow) atoms. UL5 residues showing hydrophobic interactions with BAY 57-1293 are in yellow and their interactions are shown as yellow lines. Residues that have hydrogen bonds or polar interactions are shown in cyan; the hydrogen bonds are shown by dark cyan lines and the polar interaction is shown by a light cyan line. The black line indicates a salt bridge. Arg874 (red), which produces a cation–π interaction (red line) with BAY 57-1293, is also shown.
carried out using a theoretical model and docking approach. Structural data on the HSV-1 UL5–BAY 57-1293 complex would be required to confirm the present study.

Acknowledgements


References


