Antiviral Research 80 (2008) 81–85

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Short communication

Mutations close to functional motif IV in HSV-1 UL5 helicase that confer resistance to HSV helicase–primase inhibitors, variously affect virus growth rate and pathogenicity

Subhajit Biswas[a,*], Laurence S. Tiley[a], Holger Zimmermann[b], Alexander Birkmann[b], Hugh J. Field[a]

[a] Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, United Kingdom
[b] AiCuris GmbH and Co. KG, Wuppertal, Germany

ARTICLE INFO

Article history:
Received 11 January 2008
Accepted 15 April 2008

Keywords:
Herpes simplex virus
BAY 57-1293
Antiviral
Resistance
Mutant

ABSTRACT

Herpes simplex virus (HSV) helicase–primase (HP) is the target for a novel class of antiviral compounds, the helicase–primase inhibitors (HPIs), e.g. BAY 57-1293. Although mutations in herpesviruses conferring resistance to nucleoside analogues are commonly associated with attenuation *in vivo*, to date, this is not necessarily true for HPIs. HPI-resistant HSV mutants selected in tissue culture are reported to be equally pathogenic compared to parental virus in animal models. Here we demonstrate that a slow-growing HSV-1 mutant, with the BAY 57-1293-resistance mutation Gly352Arg in UL5 helicase, is clearly less virulent than its wild-type parent in a murine zosteriform infection model. This contrasts with published results obtained for a mutant containing a different HPI-resistance substitution (Gly352Val) at the same location, since this mutant was reported to be fully pathogenic. We believe our report to be the first to describe an HPI-resistant HSV-1 mutant, that is markedly less virulent *in vivo* and slowly growing in tissue culture compared to the parental strain. Another BAY 57-1293-resistant UL5 mutant (Lys356Gln), which showed faster growth characteristics in cell culture, however, was at least equally virulent compared to the parent strain.

The herpes simplex virus (HSV) helicase–primase (HP) complex is a hetero-trimer comprising UL5, UL8, and UL52 proteins (Crute et al., 1988). The amino acid sequence of UL5 protein contains six conserved ATP-binding and helicase motifs required for DNA unwinding at the replication fork. Site-specific mutagenesis within each of the six motifs showed that all are critical for helicase function (Zhu and Weller, 1988, 1992). HSV-1 UL52 protein contains a two-aspartate motif, common to many primases which, when altered, abolishes primase (but not ATP-ase and helicase activity) (Dracheva et al., 1995; Klinedinst and Challberg, 1994).

The HSV HP-complex is the target of a class of HSV inhibitors known as helicase–primase inhibitors (HPIs), including BAY 57-1293. HPIs are extremely potent and reportedly superior to nucleoside analogues in animal infection models (Betz et al., 2002; Biswas et al., 2007a; Crute et al., 2002; Duan et al., 2003; Kleymann, 2003, 2004; Kleymann et al., 2002). Substitutions conferring resistance to HPIs most frequently occur in the UL5 protein, particularly at residues G352, M355 and K356 (Biswas et al., 2007b; Kleymann et al., 2002; Liuzzi et al., 2004; Spector et al., 1998), just downstream of motif IV. Previous reports had suggested that the frequency of HPI-resistance mutations is 10^{-6} to 10^{-7}. However, certain laboratory strains and recent clinical isolates of HSV-1 contained drug-resistant mutants at a frequency of 10^{-4} to 10^{-5} (Biswas et al., 2007c,d). With a few notable exceptions (e.g. Grey et al., 2003) nucleoside analogue-resistant HSV mutants have commonly been reported to be less fit *in vivo* compared to the wild-type; these viruses usually contain resistance mutations in their thymidine kinase (TK) (Field and Wildy, 1978) or, rarely, DNA polymerase (Field and Coen, 1986). However, HPI-resistant mutants tested in animal models, to date, were equally virulent compared to the corresponding wild-type strains (Betz et al., 2002; Biswas et al., 2007b; Liuzzi et al., 2004). Given these facts, it is important to characterize HPI-resistant mutants in regard of their pathogenicity.

One BAY 57-1293-resistant mutant, BAYr1 was previously isolated from a plaque-purified well-characterized HSV-1 laboratory isolate, SC16 cl-2 (Biswas et al., 2007b,d). BAYr1 was shown to contain two mutations (A4V and K356Q) in UL5 helicase and was consistently shown to replicate faster in cell culture. Marker-transfer experiments showed that only one mutation (K356Q) in the recombinant virus (SC16 cl-2-BAYr1-Rec, in short, cl-2-r1-Rec)

* Corresponding author. Tel.: +44 1223 330 810; fax: +44 1223 337 610.
E-mail addresses: sb507@cam.ac.uk (S. Biswas), lst21@cam.ac.uk (L.S. Tiley), Holger.Zimmermann@aicuris.com (H. Zimmermann), Alexander.Birkmann@aicuris.com (A. Birkmann), hjf10@cam.ac.uk (H.J. Field).

0166-3542/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2008.04.005

accounted for drug resistance as well as faster growth (Biswas et al., 2007b). In contrast, a different mutant (BAYr2) from the same parent with a single substitution (G352R), three residues upstream, proved to be slower growing in cell culture (Biswas et al., 2007b). In the present study, we compared the virulence of cl-2-r1-Rec and BAYr2 in comparison with the virulence of the wild-type virus using the murine zosteriform HSV-1 infection model.

The strain of HSV-1 used in the present study was HSV-1 SC16 (Hill et al., 1975). Working stocks of virus were prepared at a low multiplicity of infection and titrated in African green monkey kidney (Vero) cells. A plaque-purified sub-strain, namely HSV-1 SC16

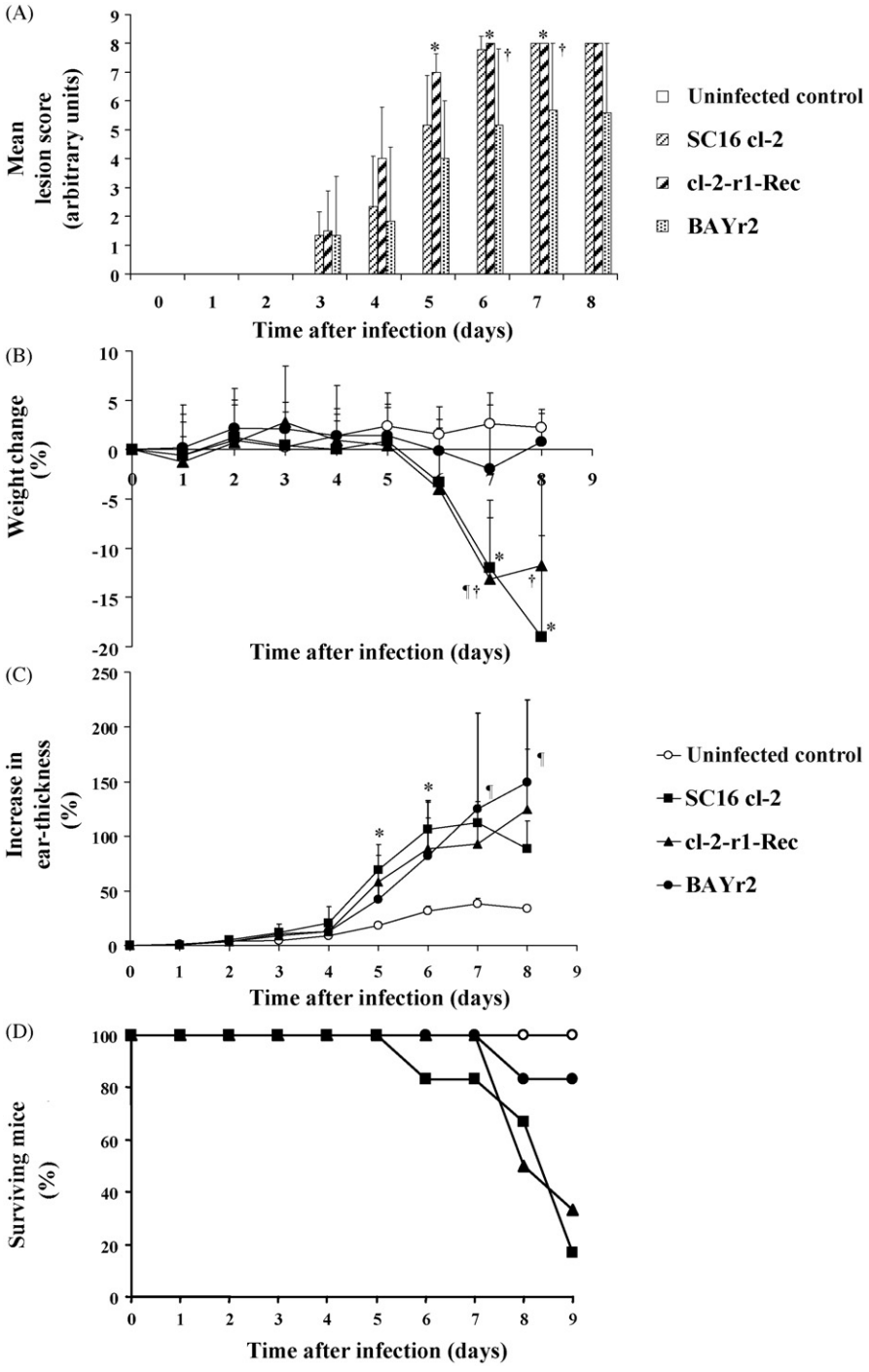

Fig. 1. Clinical signs in BALB/c mice infected with SC16 cl-2 or cl-2-r1-Rec or BAYr2 at a standard inoculation titer. (A) Skin lesion scores. Mean lesion scores (+S.D.) are shown. BAYr2 showed significantly ($P < 0.05$) less (†) lesion scores compared to wild-type SC16 on days 6 and 7 p.i. Lesion scores due to cl-2-r1-Rec were significantly greater (*) than those due to slowly growing virus, BAYr2 on days 5, 6 and 7 p.i. (B) Body weight. Change in mean body weight (+S.D.) are shown. Weights of mice, infected with the wild-type were significantly lower than those of the uninfected mice or mice infected with BAYr2 on days 7 and 8 p.i. (*). Weights of mice, infected with cl-2-r1-Rec were significantly lower than those of the uninfected mice on days 7 and 8 p.i. (†), whereas compared to BAYr2, weight-loss was significant on day 7 p.i. only (¶). (C) Inflammation on the ipsilateral ear pinna. Changes in mean thickness (+S.D.) of the ipsilateral ear pinna caused by inflammation are shown. Mice inoculated with wild-type or cl-2-r1-Rec showed a significant increase in ear thickness compared to the uninfected mice on days 5 and 6 p.i. (*). Ear thickness of mice infected with BAYr2 significantly exceeded that of the uninfected control on days 7 and 8 p.i. (¶). (D) Mortality. Number of mice (%) dead or culled *in extremis* following infection is shown.

cl-2 was derived from the HSV-1 SC16 laboratory working stock by three times single-plaque isolation in Vero cells. Details of mutant selection, growth characteristics in Vero cells, and drug-resistance profiles to HPIs and acyclovir have been published elsewhere (Biswas et al., 2007b).

Six groups of sixteen BALB/c mice were inoculated in the neck skin at 4.7 log 10 p.f.u./mouse with either of the mutants (cl-2-r1-Rec or BAYr2) or with wild-type HSV-1, SC16 cl-2, and one group of six mice remained uninfected. Sub-groups of six infected mice were used for observation only, the remainder for sampling. Mice were numbered and data from individual mice were recorded. The experiment was carried out as described before (Biswas et al., 2007b) according to Home Office (UK) guidelines.

Clinical signs and infection parameters were noted daily for 8 days as described previously (Biswas et al., 2007a,b). Mice showing irreversible neurological signs or rapid weight-loss (>15%) were culled and recorded as having died 24 h later. On 1, 3, 5 and 8 days post-infection (p.i.), three mice per group were killed and skin from the inoculation site, right ear pinna and brainstem were tested for infectious virus by plaque titration in Vero cells.

A two-way ANOVA with repeated measures of each clinical parameter (lesion score, bodyweight or ear-thickness) was performed to determine statistically significant overall differences among the groups of mice ($P < 0.05$). When a significant difference was detected, the Tukey (*post hoc*) test (widely used for comparing means that are significantly different by ANOVA) was performed to confirm which group(s) contributed to such difference. On a given day, the statistically significant difference among the groups was determined by one-way ANOVA.

The disease caused by the three viruses (SC16 cl-2, cl-2-r1-Rec or BAYr2) was compared using an equal inoculum. The faster growing mutant (cl-2-r1-Rec) produced higher lesion scores than wild-type at early time points, notably days 4 and 5 p.i. (Fig. 1A) although these differences did not reach significance. For weight-loss (Fig. 1B), ear-thickness (Fig. 1C) and mortality (Fig. 1D), this mutant resembled the wild-type.

In contrast, the slower-growing mutant (BAYr2) showed lower lesion scores (Fig. 1A); no detectable weight-loss (Fig. 1B) and less mortality (1 out of 6; 17%) compared with 83 and 67% for SC16 cl-2 and cl-2-r1-Rec, respectively (Fig. 1D). The mutant, BAYr2 produced an ear thickness response whose maximum was greater than for the wild-type although the progression of thickness-increase was slower (Fig. 1C).

All three viruses multiplied in the skin local to the inoculation site with no significant differences in infectious virus titers on sampling at days 1 (geometric mean titer = 4 log 10 p.f.u./tissue) and 3 p.i. (4.6 log 10 p.f.u./tissue) (Fig. 2A). However, the slower virus produced significantly lower titers on days 5 (1.3 log 10 p.f.u./tissue less than wild-type) and 8 p.i. (2 log 10 less than wild-type). Infectious virus was detected in the ear tissues from the faster growing mutant, cl-2-r1-Rec from day 3 p.i. (2 out of 3 sampled mice). The wild-type and BAYr2 ear-tissue samples were below the level of detection on day 3 p.i. (<0.7 log 10 p.f.u./tissue). On subsequent sampling days, the ear-tissue samples from cl-2-r1-Rec did not show significant difference from wild-type values. However, as before, the slower mutant produced lower in the ear tissues at all time points and this reached significance ($P < 0.01$) on sampling day 8 p.i. (1.7 log 10 p.f.u./tissue lower than wild-type) (Fig. 2B). In brainstem, the infectious virus remained below the level of detection for BAYr2 but was detected by day 8 (geometric mean titer = 2.1 log 10 p.f.u./tissue) in all mice inoculated with wild-type or the faster growing virus cl-2-r1-Rec (Fig. 2C).

Compared to the wild-type parent, the HSV-1 mutant, BAYr2 (previously shown to replicate more slowly in tissue culture) showed less severe clinical signs, no loss of body weight and reduced mortality in a murine infection model. Furthermore, infectious virus titers in skin, ear and brainstem were lower compared to the wild-type parent. Thus, BAYr2 was clearly less virulent than w/t SC16. We believe this is the first report of an HPI-resistant HSV-1 variant, containing a single defined drug resistance mutation, which is markedly less virulent at a standard inoculation titer *in vivo* and slowly growing in cell culture compared to the parental strain. However, the mutant retained the ability to undergo zosteriform spread as indicated by the ear thickness response and presence of infectious virus in the ear. This contrasts with certain TK-deletion mutants, which are attenuated and where zosteriform spread was shown to be restricted (Harris et al., 2003).

To date, we know of one published example of a BAY 57-1293-resistant HSV-1 mutant (UL5: M355T) with impaired growth rate and titers in cell culture (Kleymann et al., 2002). However, the pathogenicity of this mutant in mice was not reported. Another mutant (UL5: K356N) selected from HSV-1 KOS using a different HPI (BILS 22 BS) was reported to cause significantly less mortality compared to wild-type at low inoculation titers (3 log 10 and 2 log 10 p.f.u.). However, it was also reported that at higher inocula (4 log 10 and 3 log 10 p.f.u.) the mean time to death due to this mutant was similar as for the wild-type. Furthermore, the mutant showed a similar growth rate as wild-type in cell culture (Liuzzi et al., 2004). The same mutation (K356N) in a different background (HSV-1F) produced mortality almost equal to wild-type in mice following intranasal inoculation at 5.5 log 10 p.f.u. (Betz et al., 2002).

Interestingly, Liuzzi et al. (2004) described a mutant K22^{r}5 derived from HSV-1 KOS, selected for resistance to another HPI, BILS 22 BS and containing the UL5 mutation G352V. Compared to the wild-type, K22^{r}5 showed no difference in single-step growth in BHK-21 tissue culture. Furthermore, there was no difference in neurovirulence or pathogenicity following intracranial or ocular inoculation or reactivation characteristics following ocular inoculation. These results clearly contrast with the *in vivo* characteristics of our UL5 G352R mutant. However, it must be kept in mind that the G352V mutant was in a different parental background (HSV-1 KOS) with respect to the G352R mutant (HSV-1 SC16 background). Moreover, the pathogenicity of the G352V mutant was tested in different BALB/c mice models (intracerebral and ocular inoculation models). Notwithstanding, it appears that different amino acid substitutions at the same location may have a different impact on virus growth and pathogenicity.

Previously we showed that the double UL5 mutant, BAYr1 (A4Vand K356Q) is fully virulent in the murine model (Biswas et al., 2007b) and produced higher lesion scores. Here we conclusively show that the mutant containing only the second mutation is pathogenic and we have ruled out the possibility that A4V might have had a compensating role. This mutant (cl-2-r1-Rec), containing a single drug-resistance mutation also produced somewhat higher lesion scores at early time points.

Another recent publication reports that an HSV-1 UL6 mutant selected for resistance to a thiourea compound was more virulent compared to wild-type, based on greater mortality and morbidity (Pesola and Coen, 2007).

Taken together, these results demonstrate that not all HPI-resistant mutants are highly pathogenic in mice. Because this has important implications regarding the clinical use of these compound we suggest that further *in vivo* characterization should be carried out on a larger panel of HPI-resistant mutants. The drug-resistant HSV mutants, to date, encountered (mostly in immunocompromised patients resulting from selection due to nucleosides, e.g. acyclovir, are most commonly TK-deficient mutants (Bacon et al., 2003). The majority of the latter are known to have lower pathogenicity and impaired ability to reactivate from latency (Efstathiou et al., 1989; Field and Darby, 1980; Field and

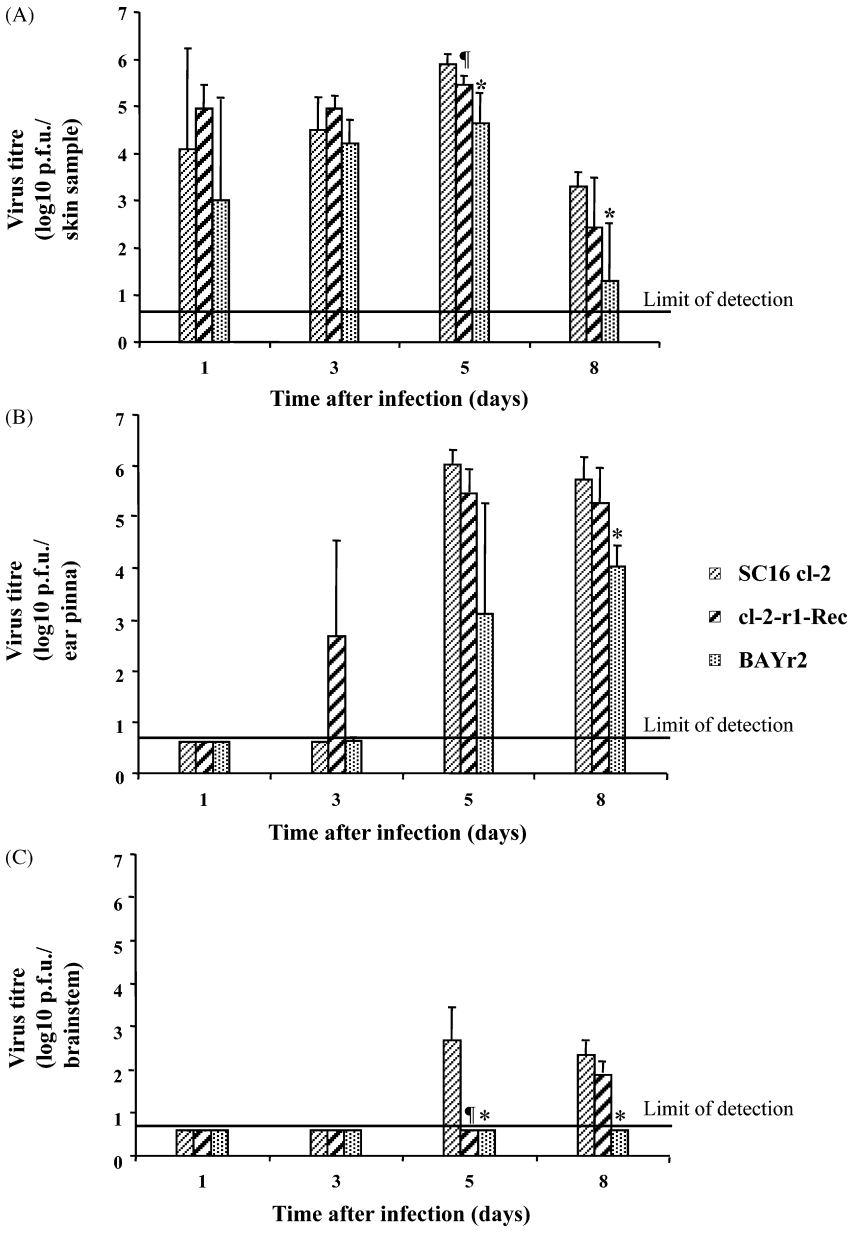

Fig. 2. Infectious virus in the tissues of BALB/c mice inoculated with w/t HSV-1 SC16 cl-2 or the BAY 57-1293-resistant mutants (cl-2-r1-Rec or BAYr2). Data points are geometric mean titers (+S.D.) from three mice sampled at each time point with standard deviation. The limit of detection was 0.7 log 10 p.f.u./sample for all tissues; differences between titers of the w/t and mutants at particular time points were compared using Student's *T*-test (two-tailed for unpaired data) and the variances of the data at each time point were measured by the *F*-test. Virus titers below the level of detection of 0.7 log 10 p.f.u./tissue were recorded as 0.6 log 10 p.f.u./tissue for statistical calculations. (A) Infectious virus titers in skin tissue. BAYr2 titers were significantly ($P < 0.05$) lower on day 5 p.i. (*). The difference was close to statistical significance ($P = 0.05$) on day 8 p.i. (*). Cl-2-r1-Rec titers were similar to w/t except on day 5 p.i., when the mutant was significantly ($P < 0.05$) lower (¶). (B) Infectious virus titers in ear pinna. BAYr2 titers were lower than the w/t on all sampling days, reaching statistical significance ($P < 0.01$) on day 8 p.i. (*). (C) Infectious virus titers in brainstem. BAYr2 titers remained below the level of detection and were therefore significantly ($P < 0.05$) lower than w/t on days 5 and 8 p.i. and cl-2-r1-Rec on day 8 p.i. (*). Cl-2-r1-Rec titers were similar to w/t except on day 5 p.i., when the mutant was significantly ($P < 0.001$) lower (¶).

Wildy, 1978). This may, in part, account for the low incidence of nucleoside-resistant mutants except in immunocompromised patients. In contrast to previously characterized HPI-resistant mutants, the relatively low pathogenicity in mice of one HPI-resistant mutant described here suggests that some HPI-resistant mutants may be attenuated and less likely to emerge in the clinical setting.

Fortunately, mutants resistant to one HPI are not always co-resistant to another HPI (Biswas et al., 2008). Furthermore, HPI-mutants studied, to date, are sensitive to other inhibitors, e.g. nucleoside analogues (Biswas et al., 2007b,d; Kleymann et al., 2002). This raises the prospect for combination therapy to improve treatment and reduces the likelihood of selecting drug-resistant viruses, particularly in immunocompromised patients.

Note added in proof

The sensitivity of infectious virus collected on day 5 p.i. from the ear pinnae of mice, infected with wild-type or either mutant to BAY 57-1293 was consistent; BAYr2 and cl-2-r1-Rec retained their respective resistance to the inhibitor. Furthermore, both the mutants showed positive reactivation from latency.

Acknowledgements

S.B. gratefully acknowledges support from the Cambridge Commonwealth Trust by means of a Cambridge Nehru Scholarship. We thank Mrs Liz Lay for expert technical assistance. We are also grateful to Dr Stacey Efstathiou, University of Cambridge, for helpful advice and discussion of the data. Financial support for these studies was provided by AiCuris GmbH and Co. KG, Wuppertal, Germany.

References

Bacon, T.H., Levin, M.J., Leary, J.J., Sarisky, R.T., Sutton, D., 2003. Herpes simplex virus resistance to acyclovir and penciclovir after two decades of antiviral therapy. Clin. Microbiol. Rev. 6, 114–128.

Betz, U.A.K., Fischer, R., Kleymann, G., Hendrix, M., Rübsamen-Waigmann, H., 2002. Potent in vivo antiviral activity of the herpes simplex virus primase–helicase inhibitor BAY 57-1293. Antimicrob. Agents Chemother. 4, 1766–1772.

Biswas, S., Jennens, L., Field, H.J., 2007a. The helicase primase inhibitor, BAY 57-1293 shows potent therapeutic antiviral activity superior to famciclovir in BALB/c mice infected with herpes simplex virus type 1. Antiviral Res. 75, 30–35.

Biswas, S., Jennens, L., Field, H.J., 2007b. Single amino acid substitutions in the HSV-1 helicase protein that confer resistance to the helicase–primase inhibitor BAY 57-1293 are associated with increased or decreased virus growth characteristics in tissue culture. Arch. Virol. 152, 1489–1500.

Biswas, S., Smith, C., Field, H.J., 2007c. Detection of HSV-1 variants highly resistant to the helicase–primase inhibitor BAY 57-1293 at high frequency in two of ten recent clinical isolates of HSV-1. J. Antimicrob. Chemother. 60, 274–279.

Biswas, S., Swift, M., Field, H.J., 2007d. High frequency of spontaneous helicase primase inhibitor (BAY 57-1293) drug-resistant variants in certain laboratory isolates of HSV-1. Antiviral Chem. Chemother. 18, 13–23.

Biswas, S., Kleymann, G., Swift, M., Tiley, L.S., Lyall, J., Aguirre-Hernández, J., Field, H.J., 2008. A single drug-resistance mutation in HSV-1 UL52 primase points to a difference between two helicase–primase inhibitors in their mode of interaction with the antiviral target. J. Antimicrob. Chemother. 61, 1044–1047.

Crute, J.J., Grygon, C.A., Hargrave, K.D., Simoneau, B., Faucher, A.-M., Bolger, G., Kibler, P., Liuzzi, M., Cordingley, M.G., 2002. Herpes simplex virus helicase–primase inhibitors are active in animal models of human disease. Nat. Med. 8, 386–391.

Crute, J.J., Mocarski, E.S., Lehman, I.R., 1988. A DNA helicase induced by herpes simplex virus type 1. Nucleic Acids Res. 16, 6585–6596.

Dracheva, S., Koonin, E.V., Crute, J.J., 1995. Identification of the primase active site of the herpes simplex virus type 1 helicase–primase. J. Biol. Chem. 270, 14148–14153.

Duan, J., Liuzzi, M., Paris, W., Liard, F., Browne, A., Dansereau, N., Simoneau, B., Faucher, A.-M., Cordingley, M.G., 2003. Oral bioavailability and in vivo efficacy of the helicase–primase inhibitor BILS 45 BS against acyclovir-resistant herpes simplex virus type 1. Antimicrob. Agents Chemother. 47, 1798–1804.

Efstathiou, S., Kemp, S., Darby, G., Minson, A.C., 1989. The role of herpes simplex virus type 1 thymidine kinase in pathogenesis. J. Gen. Virol. 70, 869–879.

Field, H.J., Coen, D.M., 1986. Pathogenicity of herpes simplex virus mutants containing drug-resistance mutations in the viral DNA polymerase gene. J. Virol. 60, 286–289.

Field, H.J., Darby, G., 1980. Pathogenicity in mice strains of herpes simplex virus which are resistant to acyclovir in vitro and in vivo. Antimicrob. Agents Chemother. 17, 209–216.

Field, H.J., Wildy, P., 1978. The pathogenicity of thymidine kinase-deficient mutants of herpes simplex virus in mice. J. Hyg. (Lond.) 81, 267–277.

Grey, F., Sowa, M., Collins, P., Fenton, R.J., Harris, W., Snowden, W., Efstathiou, S., Darby, G., 2003. Characterization of a neurovirulent acyclovir-resistant variant of herpes simplex virus. J. Gen. Virol. 84, 1403–1410.

Harris, W., Collins, P., Fenton, R.J., Snowden, W., Sowa, M., Darby, G., 2003. Phenotypic and genotypic characterization of clinical isolates of herpes simplex virus resistant to acyclovir. J. Gen. Virol. 84, 1393–1401.

Hill, T.J., Field, H.J., Blyth, W.A., 1975. Acute and recurrent infection with herpes simplex virus in the mouse: a model for studying latency and recurrent disease. J. Gen. Virol. 28, 341–353.

Kleymann, G., 2003. New antiviral drugs that target herpesvirus helicase primase enzymes. Herpes 10, 46–52.

Kleymann, G., 2004. Helicase primase: targeting the Achilles heel of herpes simplex viruses. Antiviral Chem. Chemother. 15, 135–140.

Kleymann, G., Fischer, R., Betz, U.A., Hendrix, M., Bender, W., Schneider, U., Handke, G., Eckenberg, P., Hewlett, G., Pevzner, V., Baumeister, J., Weber, O., Henninger, K., Keldenich, J., Jensen, A., Kolb, J., Bach, U., Popp, A., Mäben, J., Frappa, I., Haebich, D., Lockhoff, O., Rübsamen-Waigmann, H., 2002. New helicase–primase inhibitors as drug candidates for the treatment of herpes simplex disease. Nat. Med. 8, 392–398.

Klinedinst, D.K., Challberg, M.D., 1994. Helicase–primase complex of herpes simplex virus type 1: a mutation in the UL52 subunit abolishes primase activity. J. Virol. 68, 3693–3701.

Liuzzi, M., Kibler, P., Bousquet, C., Harji, F., Bolger, G., Garneau, M., Lapeyre, N., McCollum, R.S., Faucher, A.-M., Simoneau, B., Cordingley, M.G., 2004. Isolation and characterization of herpes simplex virus type 1 resistant to aminothiazolylphenyl-based inhibitors of the viral helicase–primase. Antiviral Res. 64, 161–170.

Pesola, J.M., Coen, D.M., 2007. In vivo fitness and virulence of a drug-resistant herpes simplex virus 1 mutant. J. Gen. Virol. 88, 1410–1414.

Spector, F.C., Liang, L., Giordano, H., Sivaraja, M., Peterson, M.G., 1998. Inhibition of herpes simplex virus replication by a 2-amino thiazole via interactions with the helicase component of the UL5–UL8–UL52 complex. J. Virol. 72, 6979–6987.

Zhu, L., Weller, S.K., 1988. UL5, a protein required for HSV DNA synthesis: genetic analysis, overexpression in *Escherichia coli*, and generation of polyclonal antibodies. Virology 166, 366–378.

Zhu, L., Weller, S.K., 1992. The six conserved helicase motifs of the UL5 gene product, a component of the herpes simplex virus type 1 helicase–primase, are essential for its function. J. Virol. 66, 469–479.