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Effects of therapy using a helicase–primase inhibitor (HPI) in mice infected with deliberate mixtures of wild-type HSV-1 and an HPI-resistant UL5 mutant

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ABSTRACT

Point mutations in the HSV-1 *UL5* (helicase) gene confer resistance to helicase–primase inhibitors (HPIs), e.g. BAY 57-1293. Such mutations normally occur at a frequency of $\leq 10^{-6}$ PFU. However, individual HSV-1 laboratory strains and some clinical isolates contained resistance mutations (e.g. UL5: Lys356Asn) at 10^{-4} PFU. To address the possibility that pre-existing mutants at high frequency might have an impact on therapy using HPIs, deliberate mixtures were prepared to contain the SC16 UL5: Lys356Asn mutant in SC16 wild-type in the proportion of 1/500 or 1/50 PFU. Mice were infected in the neck-skin with 5×10^4 PFU/mouse of wt alone, mutant alone, or the respective mixture. The mutant could not be detected in infectious virus yields from mice inoculated with the 1/500 mixture. However, resistant mutant was recovered from some treated mice inoculated with the 1/50 mixture. All mice inoculated with mixtures remained responsive to BAY 57-1293-therapy with no increase in clinical signs compared to treatment of wt-infected mice.

1. Introduction

Herpes simplex virus (HSV) causes life-long infection in 50–99% of the human population world-wide and many of infected people suffer recurrent disease (Kleymann, 2005). Therapy with nucleoside analogues (e.g. Acyclovir, ACV) is widely used. While resistance to ACV in immunocompetent patients is not regarded as an important clinical problem, HSV infections resistant to ACV are a major cause of concern in immunocompromised patients. Here, the prevalence of resistance is about 5% and rates as high as 14–30% in allogenic bone marrow transplant patients have been reported (Frobert et al., 2008). Because of resistance development in immunocompromised individuals and due to shortcomings of therapy with nucleoside analogues in immunocompetent individuals, there is a need for effective alternatives to nucleoside analogue inhibitors for improved therapy and to counteract resistance. The helicase–primase inhibitors (HPIs), e.g. BAY 57-1293, are promising candidates. These compounds target the viral helicase–primase (HP) enzyme complex and are more potent inhibitors of HSV replication than commonly used nucleoside analogues in cell culture and *in vivo* (Baumeister et al., 2007; Betz et al., 2002; Biswas et al., 2007a; Crute et al., 2002; Kleymann et al., 2002).

The HP complex, comprising the HSV gene products UL5, UL8 and UL52, is essential for viral DNA replication. The *UL5* gene product has 5′–3′ helicase activity to unwind the DNA double helix at the replication fork whereas the *UL52*-encoded primase enzyme primes single-stranded DNA (Crute et al., 1989). The latter is then extended by the HSV DNA polymerase. The accessory protein, UL8 has been shown to stimulate the primase activity of the HP complex (Cavanaugh et al., 2009) besides interacting with other HSV replication proteins, including UL9, UL30, and ICP8 (Weller, 2006).

HSV helicase–primase inhibitors (HPI) represent a new class of non-nucleoside antivirals with potential to improve HSV therapy. Two HPIs are currently undergoing clinical trials. AIC316 has been analyzed in three phase I trials and the drug was generally well-tolerated and showed high and long-lasting exposures in human subjects (www.aicuris.com [09 November 2009]). ASP2151, another novel HPI was previously reported to be efficacious in a murine infection model (Katsumata et al., 2009) with activity against both HSV and VZV (Suzuki et al., 2009) and has been in phase II clinical trials.

To date, the majority of reported HPI-resistance mutations discovered *in vitro* by applying a selective pressure have been located in *UL5* (Biswas et al., 2009; Biswas and Field, 2008) and, rarely, in the *UL52* gene (Biswas et al., 2008a; Kleymann et al., 2002). In plaque-

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purified strains the frequency of HPI-resistance mutations appears to be low (in the range 10^{-6} to 10^{-7} PFU) (Biswas et al., 2007b; Kleymann et al., 2002; Liuzzi et al., 2004; Spector et al., 1998). However, using plaque-based assays with high input inocula, we reported that HPI-resistant HSV-1 may pre-exist at a frequency of up to 10^{-4} in some HSV-1 isolates (Biswas et al., 2007b,c). The UL5: Lys356Asn mutation had been reported to confer >1000-fold HPI-resistance (Betz et al., 2002; Liuzzi et al., 2004; Spector et al., 1998) while retaining near wild-type (wt) pathogenicity in murine infection models (Betz et al., 2002; Liuzzi et al., 2004).

For the present study the above mutation was created by means of marker transfer in HSV-1 SC16 cl-2, a well-characterized laboratory strain of HSV-1. This mutant was solidly resistant to BAY 57-1293-therapy in the murine neck-skin HSV-1 infection model. Deliberate mixtures were created to test the effects of HPI therapy on the presence of low levels of pre-existing resistant virus *in vivo*. In order to match or even exceed the natural situation, mixtures up to 1 in 50 PFU were used, i.e. concentrations up to 200 times higher than seen in some clinical isolates (Biswas et al., 2007c).

2. Materials and methods

2.1. Viruses

The parental strain was HSV-1 SC16 that had been three-times plaque-purified (SC16 cl-2). The single resistance mutation UL5: Lys356Asn was transferred to SC16 cl-2 to produce the recombinant cl-2-r2-Rec following a standard protocol (Biswas et al., 2007d). Briefly, a 2.1 kb product, containing the target mutation was amplified by PCR. Transfection of the DNA was carried out in 293T cells, which were subsequently infected with SC16 cl-2. Resistant plaques were selected using 30.0 μM BAY 57-1293 at a frequency approx. 50-fold above background (10^{-6} PFU). The marker transfer of the required mutation was confirmed by sequencing as previously described (Biswas et al., 2007d).

2.2. Antiviral compound

BAY 57-1293 was provided by AiCuris and Co. KG, Wuppertal, Germany. It was dissolved in distilled water containing 1% carboxymethylcellulose (CMC) facilitated by sonication for 5 min. Stocks containing 1.0 mg/ml were stored in aliquots at −20 °C and thawed immediately before use.

2.3. Murine experiments

Female BALB/c mice were obtained from Harlan UK at approximately 16 g. The mice were acclimatised for one week before use. The neck-skin infection (zosteriform) model was used as described previously (Biswas et al., 2007a,d, 2008b). Mice were inoculated with wt or mutant (cl-2-r2-Rec) or a mixture of both and a control group was mock-infected. Briefly, the skin on the right neck was shaved and two days later a dose of 5×10^4 PFU/mouse in 10 μl DMEM or DMEM alone was applied to the skin which was then scarified in a crossed-hatch pattern.

Two virus inoculum mixtures were prepared: (i) the 1/500 mixture comprised 10^2 PFU of cl-2-r2-Rec in 5×10^4 PFU of wt and (ii) the 1/50 mixture comprised 10^3 PFU of cl-2-r2-Rec in 5×10^4 PFU of wt. Observation groups of 5 or 6 mice were inoculated with wt or mutant alone or the mixtures. Separate groups for sampling comprised 10–12 mice corresponding to each observation group. Thus, mice inoculated with (i) or (ii) each received 100 or 1000 PFU of the resistant mutant respectively. Parallel groups of mice, which received inoculum (i) or (ii) were treated by means of BAY 57-1293 at 5 mg/kg *per os* from day 1 (24 h) p.i. for 4 days. Infected or mock-infected control mice were given the vehicle only (1% CMC in distilled water). One group of wt-infected mice was treated with BAY 57-1293 to confirm the effectiveness of therapy.

In a pilot experiment of similar design with a target inoculum of 5×10^4 PFU/mouse for both wt and the mutant, 17 mice infected with cl-2-r2-Rec were found to be refractory to therapy at 60 mg/kg OD. This dose was 12 times higher than the dose used in the present experiment. The pilot experiment also contained uninfected (5 mice), vehicle-treated infected wt (18 mice) and mutant (11 mice) controls. A treatment group ($n = 11$) of wt-infected mice, using 5 mg/kg BAY 57-1293 was also included as described above. Therefore, in order to minimize the use of animals, no treatment group for the mice infected with the mutant only, was included in the experiment containing virus mixtures. It should be noted that an oral dose of 60 mg/kg BAY 57-1293 *per os* (TID) has been used for treating HSV-1 in mice before and did not produce weight-loss or other signs of toxicity (Betz et al., 2002).

The general appearance of all mice and specific clinical signs (e.g. lesion score, body-weight and mortality) were noted at the same time each day. Mice were numbered so that changes could be related to individual animals to facilitate a two-way ANOVA with repeated measures (see below). Assessment of clinical signs and scoring was done as before (Biswas et al., 2008b) based on an arbitrary scale adapted from that proposed by Nagafuchi et al. (1979). Briefly, lesions were scored first at the primary inoculation site then at the secondary site (ipsilateral ear-pinna) according to the following arbitrary scale: 0: no clinical signs; 1: one vesicle and swelling; 2: more than one vesicle; 3: local erosion; 4: ulceration of the local lesion; 5: primary lesions plus isolated zosteriform lesion; 6: mild ulceration of confluent zosteriform lesions; 7: moderate ulceration of confluent zosteriform lesions; 8: severe ulceration of confluent zosteriform lesions.

On days 1, 3, 5 and 8 p.i., groups of three mice for each condition were euthanatized and their tissues sampled. The skin local to the inoculation site and the ipsilateral ear-pinna were homogenized and tested for infectious virus by plaque-formation in tissue-culture (Biswas et al., 2007d, 2008b). DNA from homogenized tissue samples was tested for the mutant virus-specific sequence using a PCR-based method (see below). Animal experiments were performed according to the Home Office (UK) guidelines. Mice *in extremis* or those showing irreversible neurological signs or rapid weight-loss (>15%) were culled and deaths of these mice were recorded as having died 1 day later.

In order to detect the presence of resistant mutants by plaque-formation, tissue homogenates were inoculated into replicate Vero cell cultures that contained none, 0.8 or 3 μM BAY 57-1293 in the overlay.

2.4. Intentional mismatch primer (IMP)-PCR

The Lys356Asn mutation was detected by IMP-PCR based on a method described by Wilkins et al. (2006). This method was adapted to detect HPI-resistant target mutations in the background of an excess of wt HSV-1 as described elsewhere (Sukla et al., 2010). The method has a sensitivity to detect 10 PFU of cl-2-r2-Rec in the presence of up to 10^5 PFU wt.

2.5. Statistics

A two-way ANOVA with repeated measures of each clinical parameter (lesion score, body-weight 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 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.

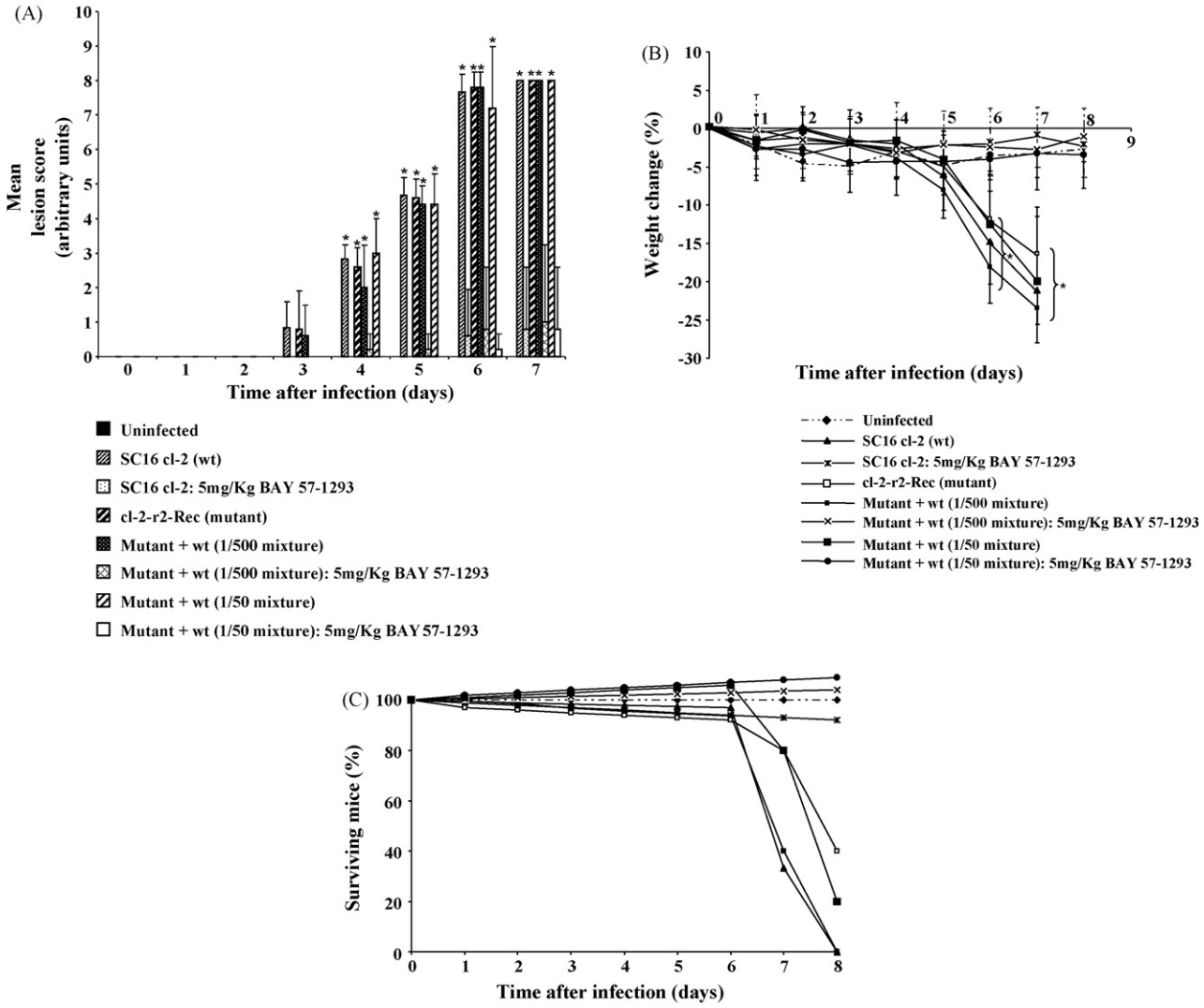


Fig. 1. Effects of BAY 57-1293-therapy on clinical signs in mice infected with wild-type (wt) alone or mixtures of wt and the BAY 57-1293-resistant UL5: Lys356Asn mutant, cl-2-r2-Rec. Mice were inoculated by means of scarification with 5×10^4 PFU/mouse of HSV-1 in the skin of the neck. Treatment with 5 mg/kg BAY 57-1293 by oral gavage OD was commenced on day 1 p.i. and was continued until day 4. Uninfected control mice were mock-inoculated with medium alone and infected control mice were treated with vehicle. (A) Lesion score. *Mice that did not receive BAY 57-1293-therapy showed significantly higher ($P \leq 0.002$) lesions compared to uninfected or BAY 57-1293-treated virus-infected mice on days 4–7 p.i. (B) Weight change. *Mice that did not receive BAY 57-1293-therapy showed significant ($P \leq 0.02$) loss of body-weight compared to uninfected or BAY 57-129-treated virus-infected mice on days 6 and 7 p.i. (C) Mortality. Values actually indicating zero mortality i.e. 100% survival on the graph are varied slightly above or below the 100% survival value to make the symbols for different groups visible on the graph.

For the infectious virus titres in the neck-skin and ear-pinna, data points are geometric mean titres (+SD) from three mice sampled at each time point with standard deviation. The limit of detection was 0.7 log 10 PFU/sample for all tissues; differences between titres of the wt and mutants at particular time points were compared by one-way ANOVA. Virus titres below the level of detection of 0.7 log 10 PFU/tissue were taken as 0.6 log 10 PFU/tissue for statistical calculations.

3. Results

3.1. Sensitivity of viruses to the helicase–primase inhibitor, BAY 57-1293 in tissue-culture

The EC_{50} for SC16 cl-2 in Vero cells by plaque-reduction assay was 0.03 μM as previously reported (Biswas et al., 2008a). The mutant, cl-2-r2-Rec (UL5: Lys356Asn) was created by marker transfer into HSV-1 SC16 cl-2 background. The mutant exhibited an EC_{50} of >248 μM and was therefore, more than 5000-fold resistant to BAY 57-1293.

3.2. Pathogenicity of wild-type (wt) and BAY 57-1293-resistant mutant in vivo

Mice were inoculated with wt or cl-2-r2-Rec using a target inoculum of 5×10^4 PFU/mouse which was confirmed after inoculation by back-titration. Without treatment, both viruses produced a vigorous disease response with clinical signs apparent from day 3 or 4 p.i. The lesion scores (taking account of the zosteriform spread of lesions from the site of inoculation to the ear-pinna) reached maximum by day 6 or 7 p.i. (Fig. 1A). Ear-swelling occurred in all wt and mutant-infected animals (data not shown) and weight-loss was observed (Fig. 1B). By day 8 p.i., the mortality for wt-infected mice was 100% while that in the mutant-infected mice was 60% (Fig. 1C), suggesting a lower pathogenicity of the mutant, although such difference was not statistically significant. This was consis-

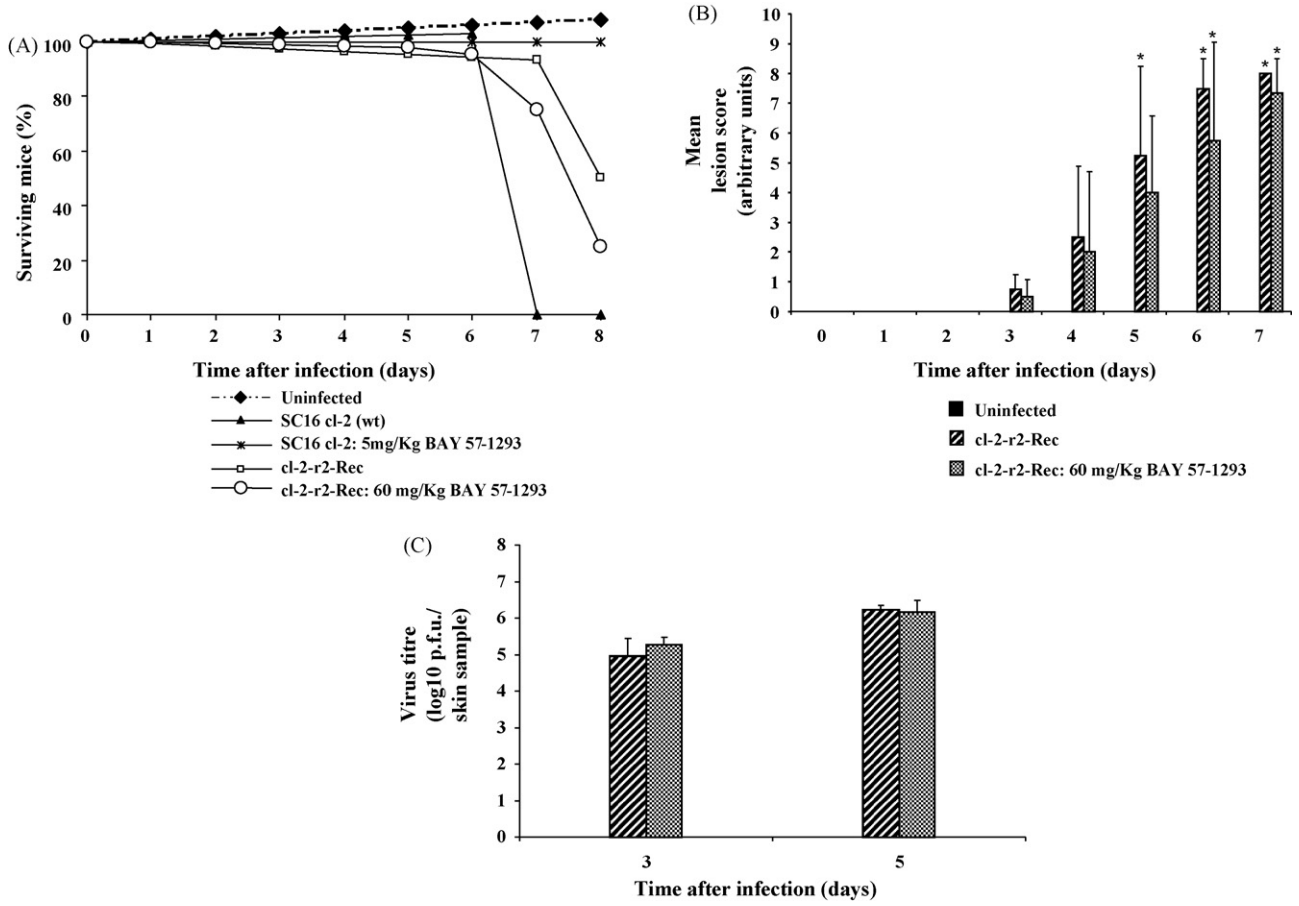


Fig. 2. Effects of BAY 57-1293-therapy on clinical signs and infectious virus titres in mice infected with the wt or BAY 57-1293-resistant mutant, cl-2-r2-Rec. Mice were infected as in Fig. 1 with a target inoculum of 5×10^4 PFU/mouse for wt or the mutant virus. One group of mutant-infected mice was treated with 60 mg/kg BAY 57-1293 by oral gavage OD, commencing on day 1 p.i. and continued until day 4. Uninfected control mice were mock-inoculated with medium and infected control mice were treated with vehicle. A further wt-infected group of mice was treated with 5 mg/kg BAY 57-1293 to confirm effectiveness of treatment. (A) Mortality. The values on the graph are slightly varied for reasons of clarity as described previously (see legend to Fig. 1C). (B) Lesion score for mutant-infected mice, treated with 60 mg/kg. *Significantly higher ($P \leq 0.01$) progression of lesion scores compared to uninfected controls. (C) Infectious virus in neck-skin of mice infected with the mutant and treated with 60 mg/kg.

tent with the results of the pilot experiment of similar design using same inoculum i.e. 5×10^4 PFU/mouse, in which the mutant caused 50% mortality and the wt 100% by day 8 p.i. (Fig. 2A).

The infectious virus titres observed in the target tissues (skin of inoculation site and ear-pinna), were consistent with the clinical signs. The infectious virus titres were similar for wt and the resistant mutant (Fig. 3A and B).

3.3. *Effects of therapy with BAY 57-1293 on wt or mutant-infected mice*

The wt-infected mice were treated with 5 mg/kg OD *per os* from days 1-4 p.i. (inclusive). This was a moderate dose equivalent to approx. twice the ED_{50} (Biswas, S., unpublished data; Betz et al., 2002) and as expected, the clinical signs were absent in almost all cases, or markedly reduced. This effect was statistically significant (see relevant figure legends). Only one out of five mice in the observation group ($n = 5$) that received 5 mg/kg for 4 days developed a few small vesicles on neck-skin at the site of inoculation on day 6 p.i. (2 days post-cessation of therapy), which progressed to ulceration of local erosion of skin by day 7 p.i. However, the lesions did not show zosteriform spread and were limited to a maximum lesion score of 4. The treated wt-infected mice showed no loss of bodyweight (Fig. 1B), no significant increase in ear-thickness (data not shown), and no mortality (Fig. 1C). Moreover, therapy reduced the titres of infectious virus in the tissues in all target sites to below the level of detection in most cases as determined by titration in tissue-culture (Fig. 3A and B). Low titres of infectious virus were recorded in skin of the mouse that showed slight clinical signs but this was about 3 log 10 PFU/tissue sample lower than the mean titre of untreated controls (5.2 log 10 PFU/tissue sample).

The low levels of infectious virus from wt-infected treated mice were screened for increased frequency of HPI-resistant variants in Vero cells but none could be detected. Thus, screening in the presence of 0.8 μM (30-fold EC_{50}) or 3 μM (100-fold EC_{50}) BAY 57-1293 fully prevented the formation of plaques. Furthermore, the samples were also negative using the Lys356Asn-specific IMP-PCR confirming that the target resistant mutant was not present at or above the level of detection equivalent to 10 PFU mutant in 10^5 PFU wt.

In the pilot experiment in which mice were infected with the resistant mutant, cl-2-r2-Rec, therapy with 60 mg/kg/day for 4 days had no significant effects on clinical signs, e.g. lesion scores (Fig. 2B) and virus titres (Fig. 2C) in the mutant-infected mice compared to untreated infected controls. The mean lesion scores of the mutant-infected mice that received 60 mg/kg BAY 57-1293-therapy were slightly lower compared to the infected but vehicle-treated mice however, the reductions were not significant (Fig. 2B).

3.4. *Effect of BAY 57-1293-therapy on mice inoculated with mixtures containing resistant mutant*

Mice infected with mixtures of cl-2-r2-rec mutant in wt in the proportions of 1/50 or 1/500 developed similar clinical signs to

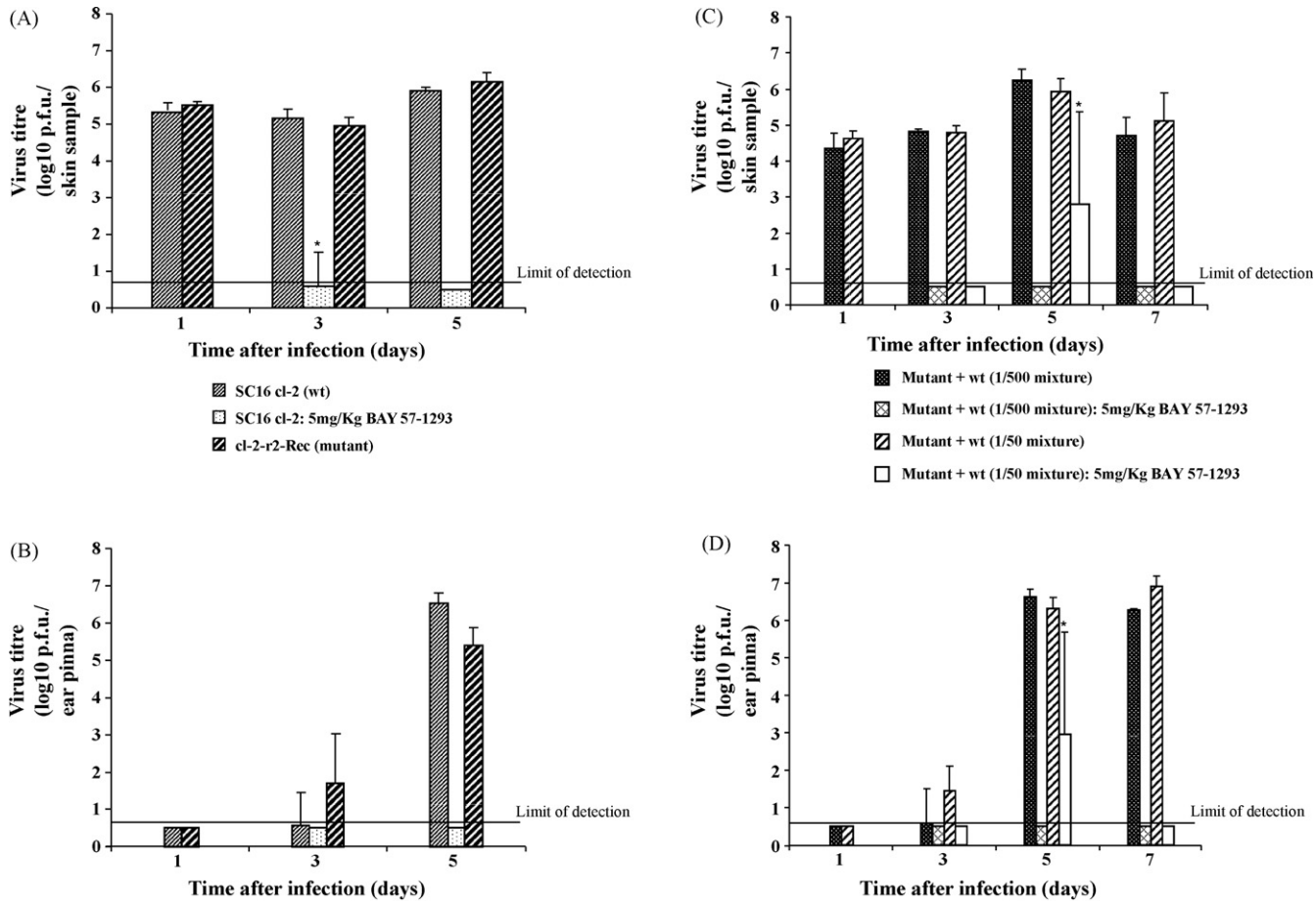


Fig. 3. Effects of BAY 57-1293-therapy on infectious virus titres in mice infected with wt alone or its mixtures with the BAY 57-1293-resistant mutant, cl-2-r2-Rec. Mice are from same groups as shown in Fig. 1. (A and B) Infectious virus in neck-skin and ear-pinna respectively of vehicle or BAY 57-1293-treated wt and vehicle-treated mutant-infected mice. *Mice that received BAY 57-1293-therapy showed significantly lower ($P \leq 0.0001$) or no detectable infectious virus titres compared to vehicle-treated wt virus-infected mice. (C and D) Infectious virus in neck-skin and ear-pinna respectively of vehicle or BAY 57-1293-treated mice infected with mixtures of wt and the mutant. *Mice that received BAY 57-1293-therapy showed significantly lower ($P = 0.03$) infectious virus titres compared to vehicle-treated 1/50-mixture-infected mice on day 5 p.i.

those inoculated with wt only (Fig. 1). Therapy was equally effective compared with the treatment of mice infected with wt alone. All clinical signs, including mortality, were fully prevented, except for one of five mice infected with each of the two mixtures in which there was some small lesion development. This was similar to treated mice infected with wt alone. Consistent with our knowledge of this infection model, it appears that this inoculum was on the threshold for productive infection (10^3 PFU/mouse) for the mutant had it been inoculated alone.

Using the cell culture-based method, no resistant mutant could be detected in the skin or ear tissues of mice infected with the 1/500 mixture with or without treatment. Furthermore, the resistant mutant (Lys356Asn) could not be detected by IMP-PCR in the DNA from any of the tissue samples from these mice.

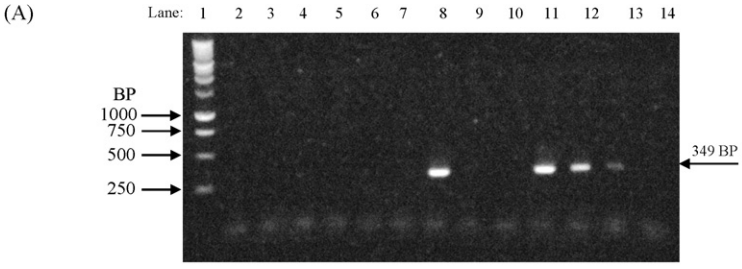
For mice inoculated with the 1/50 mixture, the mutant was detected in the skin sample from one of three mice sampled on day 1 (before commencement of treatment). In this case the mutant was present at about 1% of the infectious virus yield of 4.7 log 10 PFU/sample. Resistant virus was also detected in both skin and ear samples taken from two treated mice sampled on day 5 p.i. (Fig. 3C and D) When these samples were tested by plaque assay in the presence of 3 μM BAY 57-1293 the yield was shown to comprise exclusively mutant virus. However, no infectious virus was recovered from treated mice sampled on days 3 and 7 p.i. and the mutant was not detected in virus samples obtained from any of the untreated mice at any time point. The isolates from day 1 (Fig. 4A) and day 5 p.i. (Fig. 4B) that showed HPI-resistance in cell culture were also confirmed to be positive by IMP-PCR of the extracted DNA. All remaining samples in which no HPI-resistant plaques were detected using the cell culture method, were also negative by the IMP-PCR (Fig. 4). It should be noted that the IMP-PCR readily detected the presence of Lys356Asn mutants in both 1/500 and 1/50 mixtures prior to inoculation.

4. Discussion

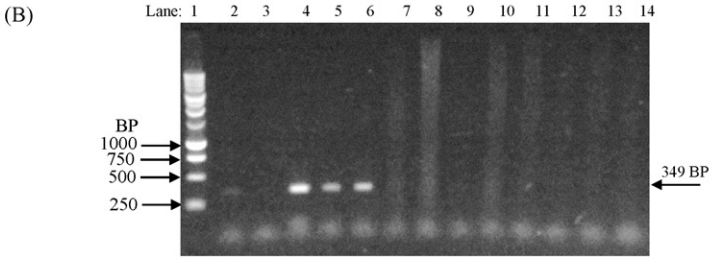
This study was carried out in order to investigate the *in vivo* impact of HPI-resistant virus in an infectious dose of wt HSV-1 in the face of therapy with an HPI. The proportions of mutant (chosen to be artificially high) were 20 or 200 times higher than the highest rates observed in clinical isolates (Biswas et al., 2008). The dose chosen for therapy, 5 mg/Kg OD from 1 day post-infection for 4 days, was considered to be just above the threshold of the effective dose for wt (approx. twice the ED_{50} of 3 mg/kg) (S. Biswas, unpublished data; Betz et al., 2002) and was expected to allow a low level of virus replication during therapy.

The main findings from this study can be summarized as follows:

(i) The SC16 cl-2 wt virus was sensitive to once daily oral dosing at 5 mg/kg BAY 57-1293 for 4 days p.i. The slight lesions and low level of infectious virus occasionally detected in some of the sampled mice in this and earlier studies at this low drug concentration showed no evidence of HPI-resistance-selection/enrichment.



Lane 2: SC16 cl-2 (wt): PCR from DNA, extracted from 6 x 10^4 PFU.
Lanes 3, 4: Mutant + wt (1/500 mixture) PCR from DNA, extracted from (3, 8) x 10^4 PFU (day 5 ear homogenates).
Lanes 5-7: Mutant + wt (1/50 mixture) PCR from DNA, extracted from (1, 2, 5) x 10^4 PFU (day 5 ear homogenates).
Lane 8: Mutant + wt (1/50 mixture): 5 mg/Kg BAY 57-1293: PCR from DNA, extracted from 1 x 10^3 PFU (day 1 skin homogenate).
Lanes 9-10: SC16 cl-2 : PCR from DNA extracted from 10^6 and 10^5 PFU respectively (negative controls).
Lanes 11-14: Mutant: PCR from DNA extracted from 10^3, 10^2, 10 and 1.0 PFU respectively (positive controls).



Lane 2-3: SC16 cl-2 : PCR from DNA extracted from 10^6 and 10^5 PFU respectively (non-specific amplification and negative control respectively).
Lanes 4: Mutant: PCR from DNA extracted from 10^2 PFU (positive control).
Lanes 5-6: Mutant + wt (1/50 mixture): 5 mg/Kg BAY 57-1293: PCR from DNA, extracted from (0.002,1) x 10^4 PFU (day 5 ear homogenates).
Lane 7-9: Mutant + wt (1/50 mixture): PCR from DNA, extracted from (0.3, 0.5, 1) x 10^3 PFU (day 3 skin homogenates).
Lanes 10-12: Mutant + wt (1/50 mixture): PCR from DNA, extracted from (5, 7, 20) x 10^4 PFU (day 7 ear homogenates).
Lanes 13-14:Mutant + wt (1/50 mixture): PCR from DNA, extracted from (0.2, 1, 6) x 10^4 PFU (day 8 ear homogenates).

Fig. 4. Screening of DNAs extracted from skin or ear tissues of mice for the presence of mutant by means of K356N-mutation specific intentional mismatch primer (IMP) PCR (representative results). DNA ladder (lane 1) and PCR products are run on 0.9% agarose gel and visualized by ethidium bromide staining under UV-transilluminator. The numbers to the left correspond to DNA ladder size and the number shown on the right represents the PCR product size. The specificity of the above PCR is at 10^5 PFU equivalent of wt sequence while the sensitivity is at 10 PFU equivalent of the mutant (see Sukla et al., 2010 for details). (A) Testing DNAs extracted from tissues of mice, infected with wt or mutant or mixtures (lanes 2–8) using the K356N-specific IMP-PCR, alongside negative (lanes 9–10) and positive (lanes 11–14) controls. Lane 8 shows positive result for a skin tissue homogenate, which also produced resistant plaques when screened by tissue-culture method. (B) Testing DNAs extracted from tissues of mice, infected with wt or mutant or mixtures (lanes 5–14) using the K356N-specific IMP-PCR alongside non-specific amplification (lane 2); negative (lane 3) and positive (lane 4) controls. Lanes 5 and 6 show positive results for ear tissue homogenates which also produced resistant plaques when screened by cell culture method.

(ii) Based on the results of weight-loss and mortality, the UL5: Lys356Asn (K356N) mutant cl-2-r-2-Rec appeared to be slightly less virulent compared to wt, although the reductions were not statistically significant. Nonetheless, the mutant was clearly pathogenic in the murine model.

(iii) As expected, mice infected with the cl-2-r-2-Rec-mutant alone were refractory to treatment using up to 60 mg/kg which is approx. 20 times the ED50 of BAY 57-1293 for wt infection.

(iv) When a mixture containing 100 PFU resistant mutants in 5×10^4 PFU, i.e. a proportion of 1/500 PFU, was inoculated into mice, there was no evidence of resistance-selection on treatment with HPI.

(v) When the higher mixture (1/50) was inoculated, resistant virus could be detected in the skin at the inoculation site (before the commencement of therapy) and ear tissues in several treated mice although the infection was cleared. Therefore, under treatment, both wt and mutant viruses can be regarded as separate entities with different susceptibility to treatment. The proportion of each virus population determines if a critical titre has been reached to seed a productive infection. A better understanding of the dynamics of infection and pathogenesis *in vivo* by the wt and mutant viruses could be obtained by titration of the infective dose with varying number of viruses in the mixed inoculum. Such experiments would give an accurate estimation of infectious dose for a productive infection for both the wt and mutant viruses. However, we know from previous data that the threshold for productive infection of SC16 cl-2 in BALB/c mice in case of the zosteriform model is lower than 5×10^3 PFU/mouse. At this inoculum, we have observed lesion scores and mortality in approx. 40% of the infected mice ($n = 5$) (S. Biswas, unpublished observations). Consistent with the above result, it appears that the mutant virus inoculum (10^3 PFU/mouse) in the higher mixture (1/50) was on the threshold for a productive infection for the mutant had it been inoculated alone. Accordingly, in mice infected with the 1/500 mixture the proportion of the mutant (100 PFU/mouse) most likely did not reach the titre required to establish a productive infection had it been present alone.

Resistance is important for long term suppression therapy or in the immunocompromised patient (Bacon et al., 2003). Also, treatment in the clinic usually commences when disease symptoms occur. In the present study, effective therapy was initiated 1 day post-infection. So it would be worthwhile to do similar experiments with defined mixtures of wt and mutant viruses in the face of delayed therapy or in immunocompromised mice. Interestingly, HPIs have been shown to be more efficacious than current therapies (e.g. valaciclovir) in case of immunocompromised mice (Betz et al., 2002; Katsumata et al., 2009) and also when the initiation of treatment was delayed in mice (Betz et al., 2002; Crute et al., 2002), guineapig (Baumeister et al., 2007) or rabbits (Kaufman et al., 2008).

Although HPI-resistance mutations at low level have been detected in clinical isolates (Biswas et al., 2007c; Sukla et al., 2010) the frequency of resistant virus in none of the isolates tested to date has been higher than 10^{-4} which is 20-fold below the 1/500 mixture tested in the present study. Thus, according to data from our experimental model we do not expect that these

virus isolates harbouring resistant mutants will be refractory to treatment.

For ACV the presence of pre-existing mutants in clinical isolates has been reported at a similar frequency (Parris and Harrington, 1982). Moreover, the rate of spontaneous ACV-resistance-mediating mutations within plaque-purified strains is two orders of magnitude higher than reported for HPI-resistance mutations (Kleymann et al., 2002). Nevertheless, HSV infection can be treated in immunocompetent subjects with ACV and breakthrough of resistant mutants occurs only very infrequently in this population (Bacon et al., 2003). It is therefore reasonable to assume that treatment of subjects infected with HSV that possibly contains low levels of pre-existing HPI-resistant mutants with an HPI should be feasible and that the presence of these mutants may not result in treatment-resistance in immunocompetent subjects in the clinic.

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