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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.