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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

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Abstract

BAY 57-1293 represents a new class of potent inhibitors of herpes simplex virus (HSV) that target the virus helicase primase complex. The present study was conducted using the zosteriform infection model in BALB/c mice. The helicase primase inhibitor, BAY 57-1293 was shown to be highly efficacious in this model. The beneficial effects of therapy were obtained rapidly (within 2 days) although the onset of treatment was delayed for 1 day after virus inoculation. The compound given orally, or intraperitoneally once per day at a dose of 15 mg/kg for 4 successive days was equally effective or superior to a much higher dose of famciclovir (1 mg/ml, i.e. approximately 140–200 mg/kg/day) given in the drinking water for 7 consecutive days, which, in our hands, is the most effective method for administering famciclovir to mice. In contrast to the vehicle-treated infected mice, all mice that received antiviral therapy looked normal and active with no mortality, no detectable loss of weight and no marked change in ear thickness. BAY 57-1293 and famciclovir reduced the virus titers in the skin to below the level of detection by days 3 and 7 post infection, respectively. In both BAY 57-1293 and famciclovir-treated mice, infectious virus titers in the ear pinna and brainstem remained below the level of detection. Consistent with these findings, BAY 57-1293 also showed a potent antiviral effect in an experiment involving a small number of severely immunocompromised athymic-nude BALB/c mice.

Keywords: HSV; Helicase primase; BAY 57-1293; Famciclovir; Antiviral

1. Introduction

Herpes simplex virus (HSV) infections cause recurrent cold sores, keratoconjunctivitis and genital herpes and occasionally, life-threatening herpes encephalitis. HSV lesions may be chronic in immunocompromised patients and in the latter the infections are prone to become resistant to existing nucleoside and pyrophosphate analogues used for therapy. Approximiately 5% of the isolates from such patients have evidence of resistance (Field, 2001) and there is a particular need in this case to provide alternatives to existing therapy.

To date, clinical management of HSV disease comprises chemotherapy using nucleoside or nucleotide or pyrophosphate analogues that exclusively target the viral enzymes thymidine kinase (the product of the HSV-1 *UL23* gene) and DNA polymerase (UL30 and the accessory factor UL42). Recently, experiments in immunocompetent animal infection models have suggested that several different compounds which have the novel target, the viral helicase primase complex (the products of HSV-1 *UL5*, *UL8* and *UL52* genes) are highly efficacious; indeed, they may be superior to the "gold standard", acyclovir (Betz et al., 2002; Crute et al., 2002; Duan et al., 2003; Kleymann et al., 2002; Liuzzi et al., 2004; Spector et al., 1998).

Numerous rodent infection models exist for both immunocompetent and immunocompromised hosts in practice, the latter being most important for the development of HSV-resistance in man (reviewed by Field and Brown, 1989). In the present study, we observed that BAY 57-1293, one of the promising helicase primase inhibitors (HPIs) developed to date, is highly effective against HSV-1 infection in immunocompetent BALB/c mice and appeared to be superior to famciclovir, which, in our hands was the most effective compound we had studied to date (Field, 1996). These results confirm and extend previous publications

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describing HPIs in different murine models (Betz et al., 2002; Kleymann et al., 2002).

A small experiment with a limited number of athymic-nude BALB/c mice provided strong evidence that BAY 57-1293 also had potent antiviral activity in immunocompromised animals in a dose-dependent manner.

2. Materials and methods

2.1. Virus

The strain of HSV-1 used in the present study was HSV-1 SC16. This virus has been extensively characterized in mice (Hill et al., 1975) and has been used previously for studying antiviral compounds (Boyd et al., 1988; Field et al., 1979; Field et al., 1995; Sutton and Boyd, 1993). 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 cl-2 was derived from the HSV-1 SC16 laboratory working stock by three times single-plaque isolation in Vero cells.

2.2. Antiviral compounds

Penciclovir (molar mass: 253.3 g/mol) and the penciclovir prodrug, famciclovir powders were supplied by SmithKline Beecham Pharmaceuticals. BAY 57-1293 (molar mass: 402.5 g/mol) powder was kindly provided by Arrow Therapeutics, London.

2.3. Plaque reduction assays (PRA)

The ED_{50} and ED_{90} values (drug concentrations inhibiting 50 and 90% plaque formation) of BAY 57-1293 and penciclovir were determined by PRA in Vero cells. Approximately 100 p.f.u. virus were inoculated into 12-well plates (Nunc, Denmark) containing approximately 2×10^5 Vero cells/well. After adsorption for 45 min at 37 °C in a humidified atmosphere of 5% CO_2, Dulbecco's modified Eagle's medium (DMEM)-overlay containing 1% new-born calf serum and high-density carboxymethylcellulose (CMC-DMEM) and different concentrations of antiviral compounds, was added to each well. The plaques were fixed, stained and enumerated after 48 h incubation.

2.4. Murine infection model

Female BALB/c mice were purchased (Harlan UK Ltd., Blackthorn, Bicester, UK) at 4 weeks of age and were inoculated after 1 week of acclimatization. Anaesthetized mice were shaved on the right side of the neck 3 days before inoculation, when 10 μl drop of virus suspension containing 5×10^4 p.f.u. was placed onto an approximately 1 cm^2 skin site, 0.5 cm lateral to the ventral mid-line on day 0 (i.e. day of inoculation). The skin was lightly scarified through the drop of virus suspension (12 light strokes of a hypodermic needle) in a crossed-hatch pattern at the base of the right ear.

BAY 57-1293 was dissolved in water containing 1% carboxymethylcellulose (CMC) and administered by means of oral gavage or intraperitoneally (i.p.) once per day commencing 1 day after virus inoculation and continued with once daily dosing for 4 days. Famciclovir was administered by means of the drinking water (1 mg/ml) also starting from day 1 post infection (p.i.) but continuing for 7 days (Field and Thackray, 1995). Previous studies showed that volumes consumed per day by BALB/c mice of similar weight and age ranged from 2.2 to 3.3 ml/mouse, with a mean of 2.6 ± 0.4 ml/mouse (Thackray and Field, 2000). It was noted that water consumption was not affected by the presence of famciclovir corresponding to a calculated mean dose of 140–200 mg/kg/day for all groups.

For the experiment using immunocompetent mice, a total of 79 animals were used for the experiment and were grouped i–v as shown (Table 1). Each of groups ii–v was sub-divided into smaller groups of four to five mice and, in each case, one subgroup of five mice was kept for observation and measurements only. Sample mice were drawn from other sub-groups.

Mice were carefully examined each day. Clinical signs were noted and each animal was weighed. The skin thickness of each right ear was measured using an engineers' micrometer screw gauge. On selected days, mice were killed and tissue samples taken approximately 3 h after dosing. Skin from the inoculation site, right ear pinna and brainstem were collected in virus isolation medium on days 1, 3, 5 and 7 p.i. and stored at −70 °C.

For the experiment using immunocompromised mice, only 12 animals were available. MF1-Nude HsdOla.MF1-Foxn1nu female BALB/c mice (mean weight: 27 g; age 8 weeks) were supplied from Harlan, UK. The mice were inoculated as above except that SC16 cl-2 was used in this case and the mice were treated from day 3 p.i. with a single daily dose of either 1.5 or

Table 1
Grouping and therapeutic regimes for the experiment with immunocompetent BALB/c mice

Group	Number of mice	HSV-1 infection[a]	Treatment regime		
			Therapy	Route (once per day)	Daily intake (mg/kg)
i	5	uninfected	Vehicle[b]	Oral gavage	None
ii	20	+	Vehicle[b]	Oral gavage	None
iii	20	+	BAY 57-1293	Oral gavage	15
iv	14	+	BAY 57-1293	Intraperitoneal	15
v	20	+	Famciclovir	Drinking water @ 1 mg/ml	Approximately140–200[c]

[a] Infected with HSV-1 SC16 at 5×10^4 p.f.u./10 μl drop.
[b] Vehicle is 1% carboxymethylcellulose (CMC) in water.
[c] Estimated dose based on a 20 mg mouse drinking 2–3 ml water in 24 h.

15 mg/kg of BAY 57-1293 (dissolved in 1% CMC and administered in 100 μl volume/animal) using the i.p. route either once only on day 3 or once on days 3 and 4 p.i. All mice were culled on day 6 p.i. and relevant tissues were sampled and tested as before.

In consideration of cost and to minimise the use of animals, the experiments with immunocompetent or immunocompromised BALB/c mice were each conducted once only.

2.5. Analysis of tissue samples for infectious virus

Skin, ear and brainstem tissue samples were processed before virus titration. Skin or ear was cut into small chips by means of a sterile pair of scissors; manually homogenized using a glass tissue homogenizer in 1 ml virus isolation medium; subjected to ultrasonic vibration for 15 s and spun at 1540 rpm for 5 min to remove tissue debris. The supernatant was collected for virus titration. Brainstem was collected in 0.5 ml virus isolation medium and homogenized with a sterile plastic homogenizer. The homogenized mass was allowed to sediment and the supernatant was carefully collected without centrifugation. The virus titer was determined for each sample by plaque titration in Vero cells. In some cases the sensitivity of the virus to the inhibitors was tested by plaque reduction assay.

2.6. Statistical analysis

For the experiment with immunocompetent mice, statistical differences for changes in body weight and ear thickness were determined by one-way ANOVA followed by the Tukey test for multiple comparisons and $P < 0.05$ was considered to be statistically significant. Further details are provided in the relevant figure legends. Virus titres were calculated as geometric mean ± S.D. and differences were tested using an un-paired two-tailed *t*-test.

For immunocompromised BALB/c mice, fewer animals were available and virus titers are presented as geometric mean ± range.

3. Results

3.1. Activity of compounds in cell culture

The ED_{50} values, determined by PRA in Vero cells were 0.05 and 1.0 μM for BAY 57-1293 and penciclovir, respectively, with ED_{90} values of 0.2 and 4.0 μM, respectively. Both HSV-1 SC16 and the three times plaque-purified clone, SC16 cl-2 gave identical values. As expected, famciclovir itself was inactive when tested in tissue culture.

3.2. Immunocompetent murine model

BALB/c mice in the vehicle-treated infected-control group first showed signs of disease (ruffled fur, and reddening at base of the ears) from the second day p.i. These signs became more prominent by day 3 p.i. and, by day 4 p.i., all vehicle-treated mice were ruffled and hunched and these signs worsened with a typical zosteriform distribution of lesions up to day 7 p.i. when four out of five were culled *in extremis* (Fig. 1A). There was little change in bodyweight up to day 5 p.i.; however, marked weight-loss was observed in the vehicle-treated control group from days 6 to 7 and weight loss on day 7 was statistically significant compared to that on days 0–5 (Fig. 1B). The ear thickness on the inoculated side increased progressively from day 2 p.i., and on day 7 p.i. it was significantly more than that on days 0–4 p.i. (Fig. 1C).

By contrast, all mice that received antiviral therapy from day 1 p.i. (BAY 57-1293 p.o. or i.p. or famciclovir in the drinking water) looked normal and active with no mortality, no detectable loss of weight and no marked change in ear thickness (Fig. 1), and in all cases this was significant. Furthermore, apart from slight reddening at the inoculation site, no zosteriform distribution of lesions was visible in animals which received either treatment.

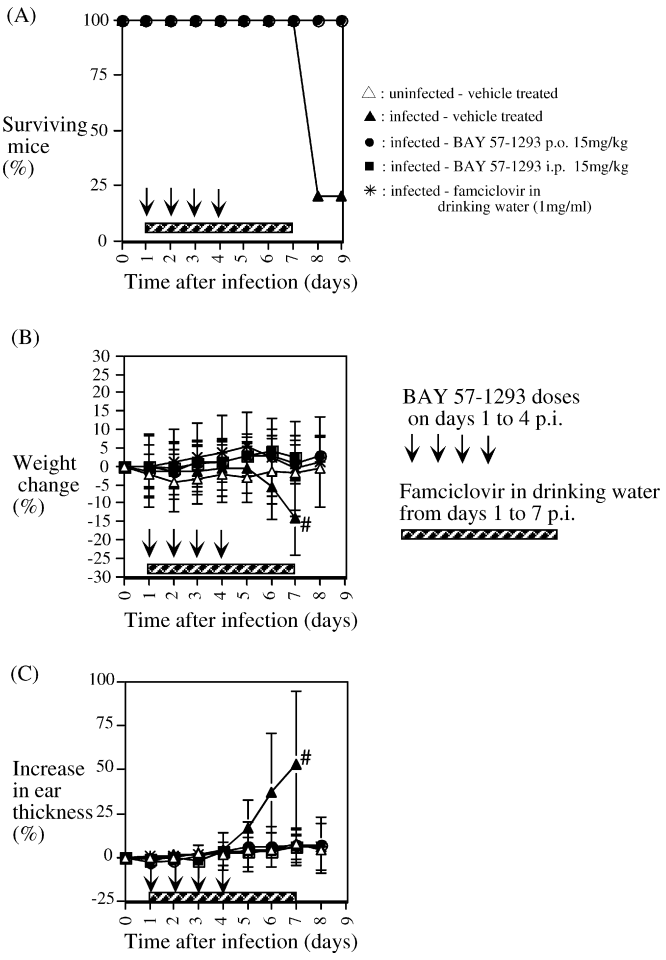

Fig. 1. Effect of BAY 57-1293 vs. famciclovir on HSV-1 infection in immunocompetent BALB/c mice: (A) survival; (B) change in body weight; #significantly different from days 0–5 ($P < 0.01$); (C) increase in thickness of right (ipsilateral) ear pinna; #significantly different from days 0–4 ($P < 0.001$); mice were inoculated by scarification on a small patch of shaved skin on the right neck using 5×10^4 p.f.u. of HSV-1 SC16 crude suspended in 10 μl. Mice were given BAY 57-1293 p.o. or i.p. at 15 mg/kg once per day on the days indicated by vertical arrows. The horizontal box indicates the period that mice received famciclovir at 1 mg/ml in their drinking water. The points are arithmetic mean values from five mice examined at each time point with standard deviation.

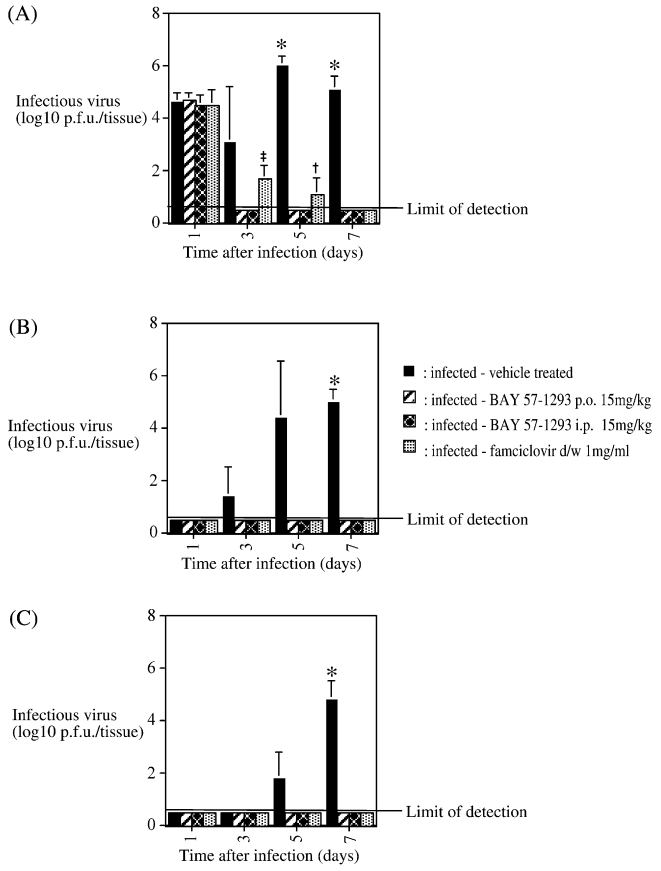

Fig. 2. Effect of BAY 57-1293 vs. famciclovir on infectious virus titers in the target tissues: (A) infectious virus in skin at site of inoculation; (B) infectious virus in right (ipsilateral) ear pinna; (C) infectious virus in brainstem; mice were infected and treated as for Fig. 1. Tissue samples were taken from mice from each treatment group on days 1, 3, 5 and 7 p.i. Data points are geometric mean titers from three mice sampled at each time point with standard deviation. The limit of detection was 5 p.f.u./sample. Differences between titres 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 was measured by the *F*-test. *Significantly different ($P < 0.05$) from treated groups; †significantly different from infected vehicle control ($P < 0.001$). ‡Famciclovir appeared to have less effect on reducing virus titer than BAY 57-1293 by either route but this inferiority did not quite reach significance ($P = 0.08$).

High virus titers were observed in the infected, vehicle-treated mice in the skin samples at all time points (Fig. 2). Virus was detected in the ear pinna from day 3 and brainstem from day 5 with maximum titers on day 7 of approximately 10^5 p.f.u. per tissue sample.

BAY 57-1293 administered per os (p.o.) or i.p. from days 1 to 4 p.i. inclusive reduced the virus titers in the skin from approximately 10^5 p.f.u./ml on day 1 to below the level of detection by day 3 p.i., i.e. after 2 days of treatment (Fig. 2A). Famciclovir, in drinking water from days 1 to 7 p.i. reduced the virus titers in the skin to $<10^2$ p.f.u./sample by day 3 (approximately 3 $\log_{10}$ reduction) and to approximately 10^1 p.f.u./sample by day 5 p.i., and finally below the level of detection on day 7 p.i. While both compounds had a significant inhibitory effect on the level of infectious virus in the skin on days 5 and 7 p.i., BAY 57-1293 appeared to be slightly superior to famciclovir. However, this superiority did not quite achieve statistical significance (e.g. $P = 0.08$ on day 3 p.i.; Fig. 2A). Both BAY 57-1293 and famciclovir reduced infectious virus in the ear ($P < 0.01$; Fig. 2B) and brainstem ($P = 0.01$; Fig. 2C) to below the level of detection by day 3 p.i.

3.3. Immunocompromised mice model

Nude (athymic) BALB/c mice were infected by scarification of a neck skin site with the SC16 cl-2. Clinical signs became visible within 2 days of inoculation. These were assessed subjectively and recorded; however, given the very small number of animals available these results are not documented here. It became evident that the inoculation site was slightly posterior to the normal position and the zosteriform distribution of lesions that were prominent at 5 days p.i. in the two untreated animals barely reached the right ear pinna. Notwithstanding, virus translocation to the ipsilateral ear was readily detected at high titers on day 6 p.i. in both infected, untreated control mice (Fig. 3A).

Treatment of the two infected, athymic BALB/c mice with a single dose of 15 mg/kg i.p. on 2 successive days, reduced virus-yield in the skin of the inoculation site to $\log_{10}$ 2.4 p.f.u. per tissue measured on day 6 p.i. which represents almost a 4 $\log_{10}$ reduction compared to that of the untreated infected mice in

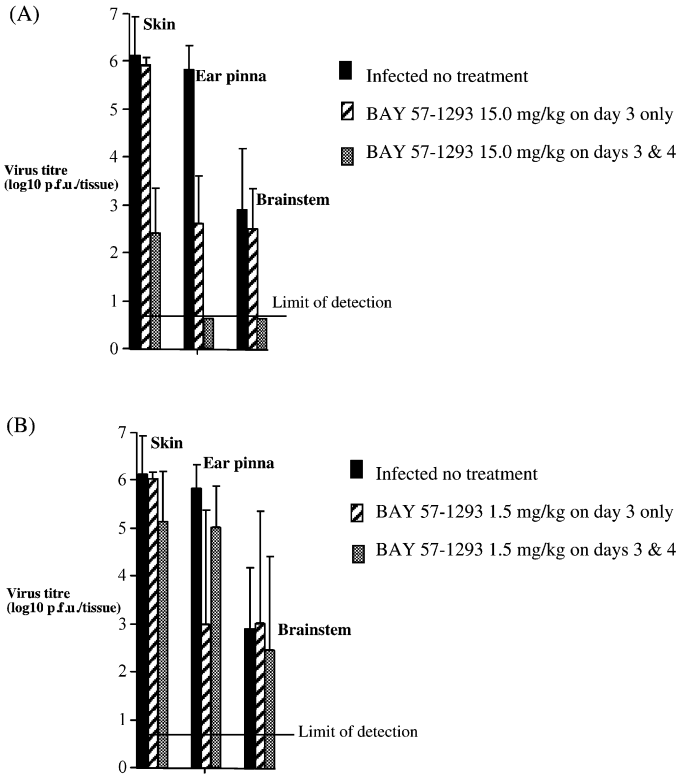

Fig. 3. Effect of BAY 57-1293 on HSV-1 infection in immunocompromised (athymic nude) BALB/c mice showing reductions of infectious virus in target tissues at 6 days post infection: (A) higher dose (15 mg/kg); (B) lower dose (1.5 mg/kg); mice were inoculated by scarification on a small patch of shaved skin on the right neck using 1×10^4 p.f.u. of HSV-1 SC16 cl-2 suspended in 10 μl. Groups of two mice were given BAY 57-1293 i.p. once only on day 3 or once on each of days 3 and 4. Tissues were sampled on day 6 p.i. and the data are geometric mean values from two mice at each time point with the range shown.

which the titer was 6.1 $\log_{10}$ (Fig. 3A) and infectious virus was reduced in the ear pinna and brainstem to levels below detection by PRA. The two mice treated with the higher dose for 2 days had no visible lesions and showed a gain in body weight (figure not shown).

The two mice, which received a single dose of 15 mg/kg i.p. for 1 day or the 2 given the lower dose of 1.5 mg/kg for 1 or 2 successive days all showed a trend to reduced virus titers in skin, brain and ear pinna (Fig. 3B), however, the small number of animals mean that these results were not significant; furthermore these mice showed variable zosteriform lesions. Notwithstanding, the number of animals was small, it appeared that two single doses of BAY 57-1293, 15 mg/kg by i.p. given on successive days markedly reduced clinical lesions and reduced infectious virus at the secondary sites of infection (ear and brainstem) in immunocompromised animals.

In mice that received the lower dose treatments, there was considerable virus replication with high levels detected at 6 days p.i. Virus isolated from mice that had received no therapy or a single high dose or one or two doses at the lower level was analyzed for the presence of BAY 57-1293 drug-resistant mutants. Drug resistance was defined as a >1.25-fold increase in ED_{90} by means of PRA. No resistant plaques were detected in any of the infected tissue samples at the level of 10^{-3} to 10^{-5} depending on quantity of virus in the tissue. Moreover, when tissue virus was analyzed for the development of resistance no increased rate was observed in comparison with inoculum virus, SC16 cl-2.

4. Discussion

The main findings from this study were that (i) The helicase primase inhibitor, BAY 57-1293 is highly efficacious in a murine HSV-1 infection model. The beneficial effects of therapy were obtained rapidly (within 2 days) although the onset of treatment was delayed for 1 day after virus inoculation. (ii) The compound given orally, or intraperitoneally at 15 mg/kg just once per day was equally effective or superior to famciclovir given in the drinking water (1 mg/ml) at a much higher dose, which in our hands, is the most effective method for administering famciclovir to mice (Field et al., 1995). (iii) The compound was effective in athymic-nude mice which are severly immunocompromised. (iv) No evidence was obtained for the development of drug resistance in this study although it is acknowledged that this experiment was limited to a relatively small number of animals and was of short duration. Furthermore, it would be interesting to investigate whether or not resistance would develop in vivo if immunocompromised mice were to be subjected to multiple exposures of sub-optimal concentrations of BAY 57-1293 over a prolonged period (e.g. >2–4 weeks).

Our results confirm those published by others (Betz et al., 2002; Kleymann et al., 2002). In those studies, C3H/Tif Bom-hr female mice were infected with 10^6 p.f.u. of HSV-2G. Subsequent treatment with 15 mg/kg BAY 57-1293, three times daily (from days 3 p.i. to 7 p.i.), per os proved superior (disease score: >0–1 by day 30 p.i.) to treatment with 240 mg/kg valaciclovir ($P < 0.011$, disease score: 4–5 by day 30). However, in our case the beneficial effects were obtained with less frequent dosing. Although our experiment using athymic-nude mice involved a limited number of animals available for this study, we nevertheless obtained clear evidence of a marked antiviral effect. It is in immunocompromised patients that HSV drug resistance has clinical importance; therefore, the availability of the new class of compounds that target the helicase primase of HSV is a welcome addition to the therapeutic armoury to combat such infections which are likely to have developed resistance mutations in the DNA polymerase and or thymidine kinase genes.

No rapid development of drug resistance in mice was detected even at low levels in either immunocompetent or immunocompromised mice. However, studies in vitro on the nature and dynamics of resistance selection from a variety of different strains including some clinical isolates of HSV suggests that resistance is an issue that should be further addressed (Biswas and Field, manuscripts in preparation). Clearly the problem of counteracting resistance with this new class of inhibitor is an important area of work for the future.

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