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Isolation of equine herpesvirus-1 mutants in the presence of (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine: demonstration of resistance in vitro and in vivo

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Summary

The compound (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) had been previously shown to be highly effective in treatment of EHV-1 in a murine model for the equine disease. This paper describes the isolation of a series of mutants resistant to the drug. Resistance was demonstrated in cell culture and one mutant was tested in a murine model. The resistant mutant was pathogenic for mice; infectious virus was recovered from respiratory tissues and blood at levels similar to the parental virus. However, the mutant showed a significant degree of resistance in vivo, thus proving the virus-specific mode of action of the antiviral compound.

Equine herpesvirus-1, EHV-1; Resistance; Phophonyl; Nucleoside analog; Chemotherapy

Introduction

One of the most important virus infections of horses is equine herpesvirus-1 (EHV-1) (Allen and Bryans, 1986). The virus is transmitted by the respiratory

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route and establishes a primary infection involving the respiratory mucosa; this may be subclinical, but sometimes serious disease results. Respiratory signs are produced which are often accompanied by fever. Virus is present in respiratory secretions and a cell-associated viraemia can be detected (Scott et al., 1983). The infection can cross the placenta and produce abortion of an infected foetus (sometimes many weeks after the primary infection). This aspect is of great importance, since cases of abortion can result in closure of studs and interruption of breeding schedules (Allens and Bryans, 1986). Occasionally, neurological signs may also accompany infection in the horse (Bitsch and Dam, 1971).

To facilitate research into the natural disease, we have developed a murine model which mimics all the main features of the equine disease including the production of clinical signs, infection of the respiratory mucosa and a cell-associated viraemia during the acute disease (Awan et al., 1990). Recently, we have also confirmed that a latent infection is established in mice which can be reactivated upon appropriate stimuli (unpublished results). We have also proved that several antiviral agents including (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) are very effective in reducing the clinical signs as well as virus replication in the target tissues (Field and Awan, 1990). Compounds of the phosphonylmethoxyalkyl nucleoside series have activity against a variety of DNA and RNA viruses (De Clercq et al., 1986) and against TK-deficient varicella zoster virus and cytomegalovirus (De Clercq et al., 1987). HPMPA is also highly active against EHV-1, having an ED_{50} concentration of 0.1 $\pm$ 0.05 μg/ml tested in RK-13 cells (Field and Awan, 1990). The compound was shown to be efficacious in the murine model for EHV-1 infection and treatment ameliorated clinical signs, rapidly reduced virus replication in the lungs and nasal turbinate tissues, and cleared the viraemia (Field and Awan, 1990).

To date, the mechanism of action for the inhibition of EHV-1 by HPMPA has not been reported. The present communication describes the selection of viruses capable of growth at concentrations of HPMPA which inhibit the parental virus and confirmation of their resistant phenotype both in cell culture and in the murine model. Strong evidence is thus provided that the compound has a specific antiviral activity against EHV-1.

Materials and Methods

Virus and cell culture

The parental virus EHV-1 (AB4 strain) was a gift from Dr. Neil Edington, Royal Veterinary College, London, U.K. The provenance of this strain and the pathogenesis in mice following intranasal inoculation are described in detail in the paper of Awan et al. (1990). Virus stocks were prepared in rabbit kidney (RK-13) cells cultured in Eagle's minimal essential medium (EMEM)

supplemented with 10% new-born calf serum. RK-13 cells were also used for selection of the HPMPA-resistant mutants, for ED_{50} titration and for the isolation of virus from murine tissues. The thymidine kinase (TK) activity of the viruses was determined following growth in the TK-defective cell line M143(TK^-) from Upjohn, U.S.A., supplied by Dr. W.P.H. Duffus.

Antiviral compounds and determination of antiviral activity

HPMPA was a gift from Professor E. De Clercq, Rega Institute for Medical Research, Leuven, Belgium. The nucleoside analogue, Penciclovir, 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine (Boyd et al., 1987) was a gift from SmithKline Beecham Pharmaceuticals, Great Burgh, Epsom, Surrey, U.K.

The activity of HPMPA against EHV-1 was determined by plaque-reduction assay in RK-13 cells according to the technique described by Field and Awan (1990). In brief, approximately 100 PFU (plaque forming units) virus were inoculated onto preformed monolayers of RK-13 cells in multi-well dishes. After 1 h adsorption, medium containing various concentrations of HPMPA were added and incubation continued at 37°C for three days. The monolayers were then fixed, stained and virus plaques enumerated. The percentage reduction in plaques in the presence of drug was plotted against the $\log_{10}$ drug concentration, and the ED_{50} values determined directly from the curve.

Selection of resistant mutants

To select resistant mutants, RK-13 cells were inoculated with EHV-1 (AB4) at a multiplicity of infection of 0.1 PFU/cell in the presence of 0.1 μg/ml HPMPA. After three days, infected cells from single plaques were aspirated from the dish and inoculated onto RK-13 cells in the absence of drug. This virus preparation was then used to inoculate further cultures in the presence of successively higher concentrations of HPMPA as follows: 2, 4, 6, 10 and 15 μg/ml; single plaques were picked at each stage. Finally, four single plaque isolations were carried out in the absence of the drug and a working stock was prepared at this stage. The three EHV-1 mutants were thus isolated independently, and were termed HR1, HR2 and HR3. A TK-defective mutant of EHV-1 (PR3) was selected by two passages in RK-13 cells in the presence of first 10 and then 25 μg/ml Penciclovir followed by three rounds of single plaque purification in the absence of the inhibitor. Working stocks were then prepared in RK-13 cells. All mutants were confirmed to be EHV-1 by a virus neutralization test using an EHV-1-specific antiserum. For convenience the parental virus strain of EHV-1 will be referred to as wild type (EHV-1 w/t).

Determination of thymidine kinase activity

The method used was adapted from that described by Mittal and Field (1989) for the measurement of BHV-1 TK. This method was originally

described by Klemperer et al. (1967). In brief, M143(TK$^-$) cells were inoculated with EHV-1 at a multiplicity of 0.1–1.0 PFU/cell. The cells were harvested after 48 or 72 h incubation, and the enzyme activity determined in 25-μl aliquots of infected or uninfected cell extract using [^{3}H]thymidine (specific activity 20 μCi/mmol at a concentration of 1.6 μCi/ml) as substrate.

Inoculation of mice

Three-week-old female BALB/c mice were obtained from Bantin and Kingman Ltd, Hull, U.K. Mice at 4–5 weeks of age were inoculated intranasally under light general anaesthesia by instillation into both nares of 50 μl of virus suspension containing a total dose of 10^7 PFU of w/t or mutant virus.

Chemotherapy of mice

HPMPA was dissolved in sterile distilled water (1 mg/ml) and administered to mice for the duration of the experiment by s.c. injection starting from one day before virus inoculation. The drug was given twice a day to give a total dose of 50 mg kg^{-1} day^{-1}.

Isolation of virus from murine tissue and infectious centre assay

Previous study showed that the important target organs for virus replication are nasal mucosa, lungs and blood (Awan et al., 1990). Mice were killed at various times after inoculation and the lungs and turbinate bones were minced with scissors and homogenised in an electric blender in a small quantity of EMEM. The suspension was sonicated for 1 min and centrifuged at 500 $\times$ *g* for 10 min to remove cellular debris. Dilutions of the supernatant were made in EMEM and aliquots inoculated onto confluent RK-13 monolayers. After 45 min adsorption, EMEM containing 2% FCS and 1% carboxymethyl cellulose was added, and the cultures incubated at 37°C. Cultures were examined after two or three days and plaques stained with crystal violet for enumeration.

There was no evidence of drug carry-over interfering with virus isolation or titration. Viruses recovered from tissues of mice that had been inoculated with the resistant strains were found to retain the resistant phenotype of the inoculum.

To assess viraemia, blood was collected and mixed with anticoagulant (2 mg EDTA/ml). The blood was centrifuged in microfuge tubes and the buffy coat was mixed in distilled water for 1 min to lyse the erythrocytes. The osmotic balance was restored with 10 $\times$ strength phosphate-buffered saline (PBS). The cells were counted in a haemocytometer and a given number of cells added to confluent monolayers of RK-13 cells; the development of plaques was determined as above.

Histology

Mice were killed by lethal injection of pentabarbitone sodium. Small pieces of tissue were carefully excised and immediately fixed in 10% formal saline, and embedded in paraffin wax. Sections were stained by means of H and E, using standard methods. For immunohistochemistry of nasal epithelium, hard and soft palates of mice were removed with curved scissors. With the help of two 27-gauge needles and an inverted microscope, nasal epithelium was gently separated from the turbinate bones and spread on glass slides. These slides were fixed in cold acetone and treated with the indirect immunofluorescence method of staining using rabbit hyperimmune serum to EHV-1 and FITC goat anti-rabbit conjugate.

Results

Resistant phenotype of the mutants confirmed

The resistance of three mutants of EHV-1 independently isolated in the presence of inhibitory concentrations of HPMPA was confirmed by plaque reduction assay. HR1, HR2 and HR3 were shown to have ED_{50} concentrations of HPMPA of 3.4, 3.8 and 2.5 μg/ml, respectively (Table 1). The TK-defective mutant (PR3) was included for comparison, and was found to be equally sensitive compared with w/t virus.

The mutant viruses did not differ markedly from the parental virus in plaque morphology or growth characteristics at the normal incubation temperature 37°C. The thymidine kinase activity of the mutants selected in the presence of HPMPA was found to be similar to that obtained with the parental virus. The TK-defective virus (PR3) that had been selected for resistance to Penciclovir was also included for comparison (Table 1).

TABLE 1

The sensitivity of wild type and mutant viruses to HPMPA and thymidine kinase induction

Virus	ED_{50}[a]	Thymidine kinase induction[b]
EHV-1 AB4 (w/t)	0.1 ± 0.05	+
HR1	3.4 ± 0.1	+
HR2	3.8 ± 0.2	+
HR3	2.5 ± 0.1	+
PR3	0.1 ± 0.05	−

[a] Plaque reduction test performed in RK cells; results expressed as drug concentration in μg/ml.
[b] Thymidine kinase induced in M143(TK^-) cells infected at a multiplicity of 0.1–1 PFU/cell.
+: w/t level TK activity; −: TK activity < 1% w/t.

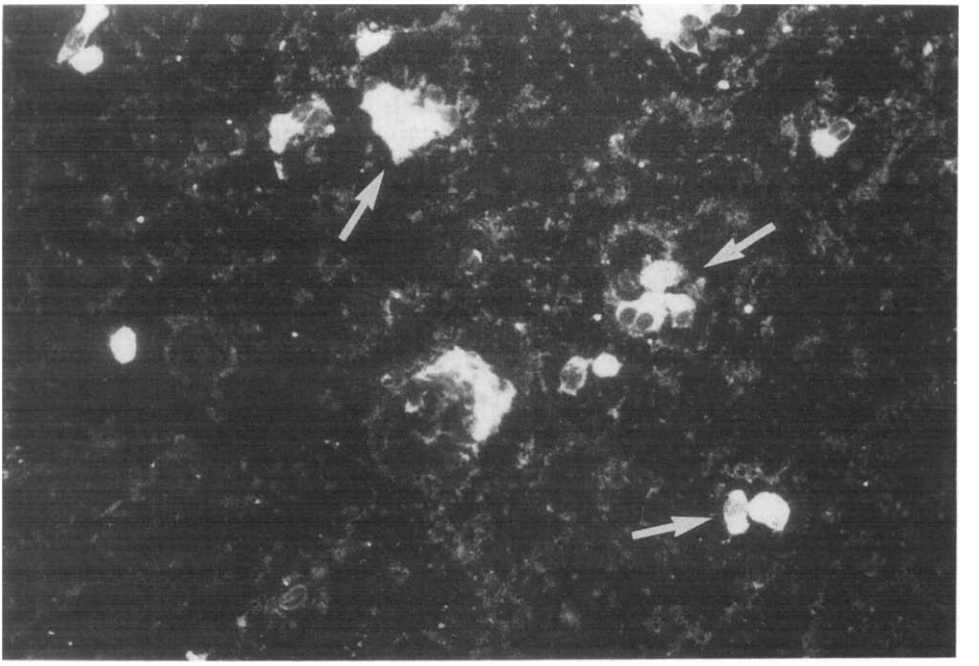
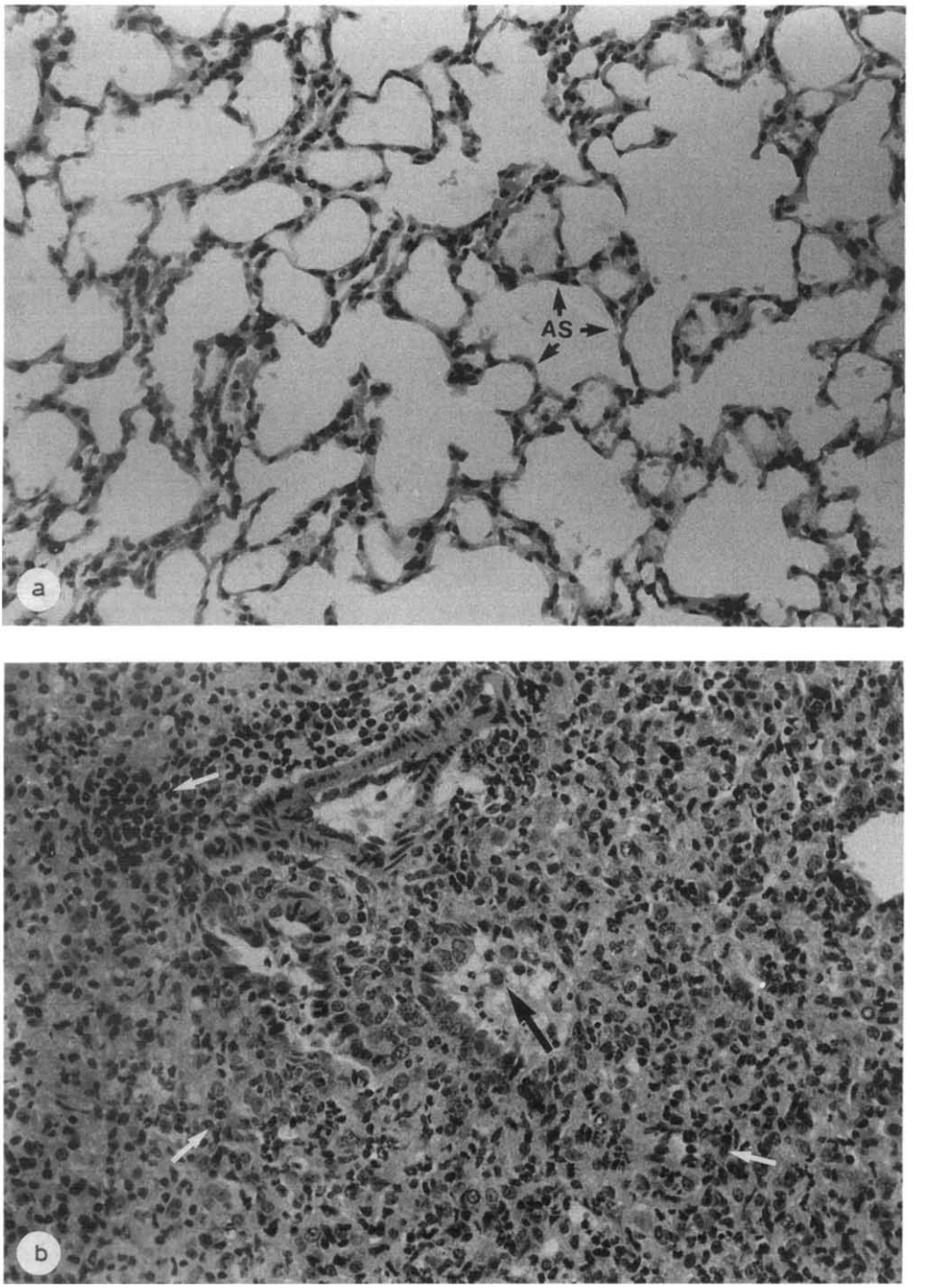


Fig. 1. Nasal epithelium of EHV-1 (w/t) infected mouse stained by the immunofluorescent technique three days after i.n. inoculation. Groups of positive staining cells (arrows) indicate primary virus lesion in the nasal epithelium. Magnification × 106.

Inoculation of mice with parental and mutant virus

Mice were inoculated intranasally with 10^7 PFU of either the parental virus (EHV-1 (AB4)) or the HPMPA-resistant mutant HR2. Both parental and mutant viruses produced clinical signs in the mice, and there was no obvious difference in the severity of the disease produced by mutant or w/t virus. In both HR2- and w/t-inoculated mice without treatment, the mortality rate was similar. However, no mortality was observed in either group of mice treated with HPMPA. Mice were killed on days 1, 3 and 5 after inoculation. The respiratory disease was initiated with the production of infected foci of epithelial cells in the respiratory mucosa. Numerous discrete lesions were visible on day 3 by means of immunofluorescent staining (Fig. 1). By day 5, the lungs were heavily infiltrated and many bronchioles occluded with desqua-

Fig. 2. Histopathological changes in lungs of mice five days after i.n. inoculation with EHV-1 (w/t) or mock infection. (a) Lung of mock-infected mouse five days after i.n. inoculation with uninfected RK cell lysate showing normal architecture of lung (arrows); AS, alveolar septa. Magnification × 106. (b) Lung of a similar mouse to (a) five days after i.n. inoculation of 10^7 PFU EHV-1 (w/t). Note that there is a complete loss of normal alveolar architecture of the lung by inflammatory cells (white arrows). In some cases the bronchus (black arrow) is completely occluded by inflammatory exudate and desquamated epithelial cells. Magnification × 106. →


AS
a
b

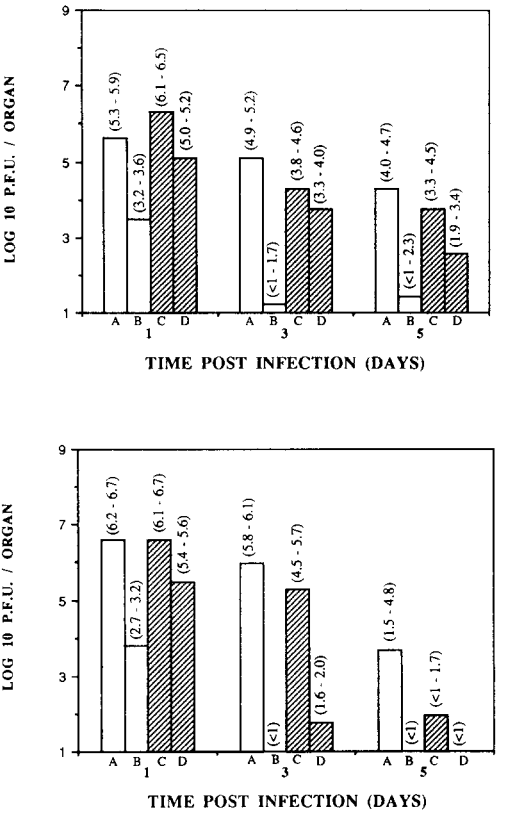


Fig. 3. Isolation of virus from tissues of mice with and without chemotherapy. Mice were inoculated i.n. with 10^7 PFU of either EHV-1 (w/t) or EHV-1 (HR2), the HPMPA-resistant mutant. The organs were tested for the presence of virus on days 1, 3, and 5 after inoculation. Groups A and C were untreated while groups B and D were treated with HPMPA 50 mg kg^{-1} day^{-1} commencing one day before virus inoculation for five days. Each bar represents the geometric mean titre with (range) of values for three or four mice processed individually at each time. Blank bars represent EHV-1 (w/t)-inoculated groups, while hash bars represent EHV-1 (HR2)-inoculated groups. Top panel: turbinate bones; bottom panel: lungs.

TABLE 2

Log_{10} reduction in virus titre in the organs of w/t or HR2-infected mice following treatment with HPMPA

Virus inoculated	Days post-inoculation					
	Day 1		Day 2		Day 3	
	Lung	Turbinate	Lung	Turbinate	Lung	Turbinate
EHV-1 (w/t)	2.7[a]	≥2.2	≥5.0	≥3.8	≥2.7	≥2.9
EHV-1 (HR2)	≥1.1	≥0.9	3.5	0.6	≥0.7	1.2

[a] Data are geometric mean log_{10} virus titre in organ without treatment minus geometric log_{10} titre in mice given HPMPA, 50 mg kg^{-1} day^{-1} from one day before virus inoculation.

mated epithelial and inflammatory cells (Fig. 2b). HPMPA was administered commencing from the day before virus inoculation. The levels of infectious virus were determined in the respiratory organs of treated and untreated mice. The treatment was effective and virus titres were reduced in HPMPA-treated mice in all tissues tested (Fig. 3). However, as shown in Table 2, at all times the mutant infected mice show less reduction with therapy in comparison with those inoculated with w/t. In w/t-inoculated mice the reductions ranged from approx. 5.0 to approx. 2.2 $\log_{10}$, while in the HR2-infected mice they were from 3.5 to 0.6 $\log_{10}$. However, as stated above, some reduction of virus titre was noted in all treated mice.

EHV-1 was detected in the blood of mice by means of infectious centre assay. Both mutant and w/t virus were found to cause transient viraemia in some mice (one of three mice tested on days 1 and 3, for w/t-inoculated mice, and two of three and one of three for mice inoculated with the mutant, HR2). The number of infected cells ranged from 6×10^6 to 9.6×10^5 leukocytes, and there was no significant difference between the mutant and w/t inoculated mice. Treatment with HPMPA ablated viraemia and no virus infected leukocytes were detected in the drug treated animals except for a low level (5×10^8 cells) in one of three w/t, HPMPA-treated mice on day 1 after inoculation.

Discussion

The phosphonylmethoxyalkyl derivative (HPMPA) was found to be extremely effective in clearing EHV-1 infection from the organs of mice, confirming the results of an earlier publication concerning the same compound (Field and Awan, 1990). The mechanism by which HPMPA inhibits EHV-1 has not been reported; however, it is likely that this is similar to other alphaherpesviruses and that the virus-specific DNA polymerase is an important target for the antiviral effect. Direct evidence for the role of DNA polymerase in the mode of action of HPMPA has been obtained for HSV (Votruba et al., 1988) and for BHV-1 in this laboratory (Field et al., unpublished results). The isolation of a series of mutants which have acquired resistance to HPMPA is consistent with this hypothesis. It is generally held that it is more difficult to select herpesvirus mutants with resistance mutations in the DNA-polymerase locus than for TK-mediated resistance to nucleoside analogues. EHV-1 is known to induce a virus-specific thymidine kinase (Robertson and Whalley, 1988). We observed that TK-defective mutants were readily generated by selection with a nucleoside analogue and that these mutants, as expected, retained sensitivity to HPMPA, confirming that an active EHV-1 TK is not required to activate the compound in infected cells. The isolation of HPMPA-resistant mutants proved to be more difficult, and several passages in the presence of the drug were required to obtain evidence for reduced sensitivity. However, the resistance of the mutants, once obtained, was reproducible when tested in a plaque reduction assay, although the degree of

resistance (approx. 25-fold) was relatively small compared with the resistance acquired by the TK-defective mutant to the compound used for its selection (>65-fold).

The HPMPA mutant tested in mice was found to be fully pathogenic in this infection model. Not only were clinical signs and mortality produced in the mutant-infected mice, but the multiplication of virus in respiratory tissue and the production of viraemia were shown to be similar to the w/t inoculated controls. However, treatment of mutant-infected mice with HPMPA prevented mortality and showed some reduction in virus titres, suggesting that the tissue levels of compound achieved in vivo were sufficiently high to effect the growth of a mutant showing only moderate resistance. This serves to emphasise the efficacy of this compound at least in the murine model; however, the reduction of virus titre was consistently less than that observed in w/t-treated mice. This therefore provides firm evidence for a specific antiviral mode of action in vivo, and suggests that the amelioration of disease signs was not caused by a non-specific mechanism such as an anti-inflammatory or immunomodulatory effect of the compound. In due course, the biochemical characterization of the resistant mutants described above should enable more precise location of the antiviral target for HPMPA in EHV-1.

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