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In vitro susceptibility of six isolates of equine herpesvirus 1 to acyclovir, ganciclovir, cidofovir, adefovir, PMEDAP and foscarnet

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Abstract

Equine herpesvirus 1 (EHV-1) is an important equine pathogen that causes respiratory disease, abortion, neonatal death and paralysis. Although vaccines are available, they are not fully protective and outbreaks of disease may occur in vaccinated herds. Therefore, there is an urgent need for effective antiviral treatment. For three abortigenic (94P247, 97P70 and 99P96) and three neuropathogenic isolates (97P82, 99P136 and 03P37), the effect of acyclovir, ganciclovir, cidofovir, adefovir, 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP) and foscarnet on plaque number was studied. Additionally, for isolate 97P70, the effect on plaque size was investigated. Ganciclovir was most potent in reducing plaque number, followed by PMEDAP and acyclovir. Adefovir and cidofovir were less effective and foscarnet was the least effective compound. There were no differences detected for acyclovir, ganciclovir, adefovir and PMEDAP between the abortigenic and neuropathogenic isolates. One abortigenic isolate (99P96) was more susceptible to cidofovir and two neuropathogenic isolates (99P136 and 03P37) were less susceptible to foscarnet. For isolate 97P70, all compounds resulted in a significant reduction of plaque size. The most remarkable effect was observed for cidofovir. It was 40-fold more effective in reducing plaque size than in reducing plaque number. In conclusion, ganciclovir was the most potent compound and therefore, may be a valuable candidate for the treatment of EHV-1 infections in horses. The antiviral effect of foscarnet on plaque number was highly dependent on the viral isolate tested. Therefore, it is no valuable antiviral for the treatment of herpesvirus-infections. Cidofovir, although less effective in reducing plaque number, had a strong effect on plaque size.

Keywords: EHV-1; Antivirals; *In vitro*; Isolates

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1. Introduction

Equine herpesvirus 1 (EHV-1), a member of the Alphaherpesvirinae, is a major pathogen of horses. The virus is endemic worldwide and most horses become infected during their first year of life (Ostlund, 1993). After exposure, EHV-1 replicates in the upper respiratory tract. This can be associated with respiratory disorders, characterised by fever, anorexia, nasal discharge of varying severity and ocular discharge (Patel and Heldens, 2005), or the infection can be silent (Foote et al., 2006). Replication is followed by a leukocyte-associated viremia which enables EHV-1 to reach internal organs. There, its replication can result in abortion, neonatal death or nervous system disorders (Allen and Bryans, 1986; Bryans and Allen, 1989). Abortion can involve only one or two mares in a herd but also abortion storms can occur on a premise, associated with great economic losses (Allen and Bryans, 1986). Neurological disorders have been reported with increasing frequency (McMartan et al., 1995; Friday et al., 2000; van Maanen et al., 2001; Stierstorfer et al., 2002; van der Meulen et al., 2003a) and during an outbreak many cases may occur with devastating effects.

Although vaccines against EHV-1 are available, they are not fully protective (Kydd et al., 2006; Goodman et al., 2006; van der Meulen et al., 2006a) and outbreaks of disease may still occur (Buchner and Mostl, 1998; Friday et al., 2000; van der Meulen et al., 2000; Kohn et al., 2003; Goehring et al., 2006). Therefore, there is a need for effective antiviral chemotherapy. Antivirals need to meet several requirements. They have to reduce viral replication and spread in affected horses, and prevent viral replication in in-contact animals. The drug should be effective against isolates of EHV-1 associated with outbreaks of abortion as well as those associated with outbreaks of neurological disorders. Recently, it was postulated that a variation of a single amino acid of the DNA polymerase is strongly associated with neurological versus non-neurological disease outbreaks (Nugent et al., 2006). This may have an implication on the susceptibility of various isolates to anti-herpetic compounds as many of these compounds act on the DNA polymerase (De Clercq, 2004). Finally, the drug should be safe and devoid of adverse effects.

Some antivirals have already been tested *in vitro* for their efficacy to inhibit EHV-1 replication by means of a plaque reduction assay, *i.e.* ganciclovir (Smith et al., 1983; Rollinson and White, 1983; Rollinson, 1987), three 2′-fluoropyrimidine nucleosides (Rollinson, 1987), (*S*)-9-[3-hydroxy-2-phosphonylmethoxypropyl]adenine or HPMPA (Field and Awan, 1990) and the HPMPA analogue cidofovir (Gibson et al., 1992). These compounds proved more effective than 9-[4-hydroxy-3-hydroxymethylbut-1-yl]guanine (Boyd et al., 1987) and penciclovir (de la Fuente et al., 1992). For acyclovir, contradictory data have been reported. Rollinson and White (1983) reported a 6-fold lower EC_{50} than Boyd et al. (1987). Further, based on comparison of EC_{50}-values reported in literature, adefovir would be the least active compound in this series (Field and Awan, 1990). However, data of these different studies are difficult to compare as different isolates, cell lines and assays have been employed.

The aim of the present study was to compare the efficacy of acyclovir, ganciclovir, cidofovir, adefovir, PMEDAP and foscarnet against EHV-1 *in vitro*. Four of these products have already been tested *in vitro* while foscarnet and PMEDAP are new in this study. Three abortigenic and three neuropathogenic isolates of EHV-1 were included and one cell line, EEL cells, was used. Additionally, the efficacy of the antivirals to reduce the size of EHV-1 induced plaques was investigated. Reduction in plaque size may be a potential parameter for the ability of an antiviral to inhibit cell to cell spread and, consequently to restrict the size of macroscopic lesions *in vivo*.

2. Materials and methods

2.1. Cells

All studies were conducted using equine embryonic lung (EEL) cells. EEL cells were cultured in growth medium (minimum essential medium (MEM) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 0.3 mg/ml glutamine and 5% foetal calf serum). Cells were passaged once a week.

2.2. Viruses

Six Belgian EHV-1 isolates were tested. The isolates 94P247, 97P70 and 99P96 were isolated

Table 1
Characteristics of the EHV-1 isolates

Viral isolate	Isolated from	ORF30 752[a] (D/N)	ORF68 strain[b] grouping	Origin
94P247	Abortion	N	3	Kluisbergen (50°46′ north–03°29′ east)
97P70	Abortion	N	3	Gouy-les-Pieton (50°25′ north–04° 25′ east)
99P96	Abortion	N	3	Hamme (51°05′ north–04°08′ east)
97P82	Neurological disorders	D	2	Nijvel (50°35′ north–04°19′ east)
99P136	Neurological disorders	D	3	Kampenhout (50°57′ north–04°34′ east)
03P37	Neurological disorders	D	3	Kozen (50°55′ north–05°20′ east)

[a] Nugent et al. (2006): ORF30 sequence variation is associated with pathogenic potential: 95% of the non-neurological isolates encoded A_{2254} (amino acid N_{752}), 86% of the neurological isolates encoded G_{2254} (amino acid D_{752}).
[b] Nugent et al. (2006): ORF68 as the primary strain grouping marker (geographical restriction of certain strain groups).

from lungs of an aborted fetus in 1994, 1997 and 1999, respectively. The isolates 97P82, 99P136 and 03P37 were isolated from the peripheral blood mononuclear cells (PBMC) of paralytic horses in 1997, 1999 and 2003, respectively. They have been sequenced across the ORF30 region associated with differences between neurological versus non-neurological isolates (Nugent et al., 2006). The abortigenic isolates were typed N_{752} and the neuropathogenic isolates were typed D_{752} (Table 1). They have also been sequenced across the ORF68 region. The ORF68 region is used as a marker system for distinguishing isolates into 6 common strain groups (Nugent et al., 2006). Five of the six isolates were located in strain group 3, while only the isolate 97P82 was located in strain group 2 (Table 1). Virus used for the experiments was between the fourth and sixth passage.

2.3. Antiviral compounds

The antiviral compounds used were acyclovir (GlaxoSmithKline, Genval, Belgium), ganciclovir (Roche Bioscience, Palo Alto, CA, USA), cidofovir and adefovir (Gilead Sciences, Foster City, CA, USA), 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP) and foscarnet (Astra Zeneca, Södertälje, Sweden). Compounds were dissolved in Roswell Park Memorial Institute (RPMI)-1640 medium at a concentration of 1 mg/ml and stored at 4 °C until use.

2.4. Antiviral assays

EEL-cells were seeded in 24-well culture plates (Nunc A/S) at a density of 300,000 cells/well and were cultured at 37 °C for 24 h. Then, growth medium was removed and cultures were infected with 40 PFU of EHV-1/well. After 1 h, virus was removed. Cells were rinsed twice with culture medium and overlaid with carboxymethylcellulose 0.94% overlay medium (Sigma) containing serial dilutions of each compound. Infected, untreated cells served as control. Overlay medium was removed at 50 h post inoculation and cells were rinsed with PBS, after which the cell monolayer was fixed with 4% paraformaldehyde and methanol + 1% H_2O_2. Subsequently, an immunoperoxidase monolayer assay (IPMA) was performed. In brief, polyclonal antibodies against EHV-1 (van der Meulen et al., 2003b) were added for 1 h at 37 °C and, after three washing steps, goat anti-horse antibodies labeled with peroxidase (Jackson, De Pinte, Belgium) were added for 1 h at 37 °C. Again, three washing steps were performed, before adding the substrate 3-amino-9-ethylcarbazole (AEC) (Sigma). After 6 min of incubation at 37 °C, substrate was replaced by acetate buffer to block enzymatic reaction.

The number of EHV-1 induced plaques was counted for each concentration of antiviral compound and for untreated control samples using light microscopy (Olympus IX50). The inhibitory effect of the antiviral compounds on plaque number was calculated by following formula:

$$\text{Percentage inhibition} = \left[1 - \frac{(\text{number of plaques})\,\text{antiviral}}{(\text{number of plaques})\,\text{control}}\right] \times 100\%$$

The concentration of each compound required to reduce the plaque number by 50% (EC_{50}) was hand-calculated from the dose–response curves generated from the data.

For isolate 97P70, the effect of the antiviral compounds on virus-induced plaque size was

determined. Plaques were photographed using light microscopy (Olympus IX50) and a digital camera (Sony Progressive 3CCD), attached to a Macintosh computer. Plaques were randomly selected in each sample. At least three plaques were measured per well to allow statistical analysis. No more than 18 plaques were measured as this was the maximum number of well-isolated plaques per recipient (mean number measured per sample was 11.5 ± 3.3). Then, the size of the area of each plaque was determined in pixels using Scion 1.63 (National Institutes of Health, Bethesda, Maryland, USA) and finally, the mean plaque size was calculated for each concentration of antiviral.

For each experiment, three independent replicates were performed.

2.5. Cytotoxicity assay

The effect of the antiviral compounds on the viability of EEL cells was measured using an MTT-based method (Hansen et al., 1989). In brief, EEL-cells were seeded in 96-well culture plates (Nunc A/S) at a density of 50,000 cells/well. After 24 h, growth medium was replaced by serial dilutions of the compounds. Untreated cells served as control. Cells fixed with 4% paraformaldehyde were included as background condition. At 69 h post incubation, cells were incubated with 0.3% MTT (Sigma, Bornem, Belgium) and were further incubated for 3 h at 37 °C to allow the production of formazan. Then, MTT containing culture medium was removed and 150 μl of detergent reagent (0.5% sodium dodecyl sulphate in isopropanol) was added. Absorbance was measured on a Multiskan RC (Thermo Labsystems, Brussel, Belgium) at a wavelength of 550 nm. Viability of cells was calculated by following formula:

$$\text{Percentage of viable cells} = \frac{\text{ODt} - \text{ODd}}{\text{ODc} - \text{ODd}} \times 100\%$$

where ODt is the absorbency of cells incubated with antiviral compounds, ODd the absorbency of the background control and ODc the absorbency of the untreated cells.

For each concentration of antiviral compound and for control samples, eight wells were tested per experiment. Three independent replicates of each experiment were performed.

2.6. Data analysis

Statistical analysis was based on analysis of variance (ANOVA) using SPSS (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of the antiviral compounds on plaque number

The mean EC_{50}-values ± standard deviations are presented in Table 2. Ganciclovir proved most potent against all isolates and inhibited viral replication with a 50% effective concentration (EC_{50}) of 0.1–0.4 μg/ml. The efficacy of acyclovir (1.7–3.0 μg/ml) and the experimental molecule PMEDAP (1.3–2.9 μg/ml)

Table 2
Susceptibility of 6 isolates of EHV-1 to antiviral compounds

Viral isolate	EC_{50} ± S.D. (ng/ml)					
	Acyclovir	Ganciclovir	Cidofovir	Adefovir	PMEDAP	Foscarnet
94P247	2.7 ± 0.5	0.2 ± 0.2	5.8 ± 2.7	4.4 ± 1.4	1.5 ± 0.1	11.1 ± 2.5
97P70	2.0 ± 0.7	0.2 ± 0.1	4.4 ± 0.4	4.3 ± 0.7	2.1 ± 0.4	6.9 ± 0.8[+]
99P96	1.7 ± 0.5	0.2 ± 0.0	1.1 ± 0.6[*]	2.8 ± 1.8	1.3 ± 1.0	6.6 ± 1.1[+]
97P82	2.2 ± 1.2	0.1 ± 0.1	3.1 ± 1.5	4.2 ± 2.0	2.0 ± 0.7	7.9 ± 2.6[#]
99P136	2.5 ± 1.0	0.3 ± 0.1	4.8 ± 2.1	5.6 ± 1.2	2.3 ± 0.6	16.2 ± 2.6[+,#]
03P37	3.0 ± 0.4	0.4 ± 0.1	6.7 ± 1.2[*]	3.8 ± 1.8	2.9 ± 0.2	15.8 ± 3.6[+]

[*] Significant difference between isolate 99P96 and isolate 03P37.
[+] Significant difference between the isolates 97P70 and 99P96 on one hand, and the isolates 99P136 and 03P37 on the other hand.
[#] Significant difference between isolate 97P82 and isolate 99P136.

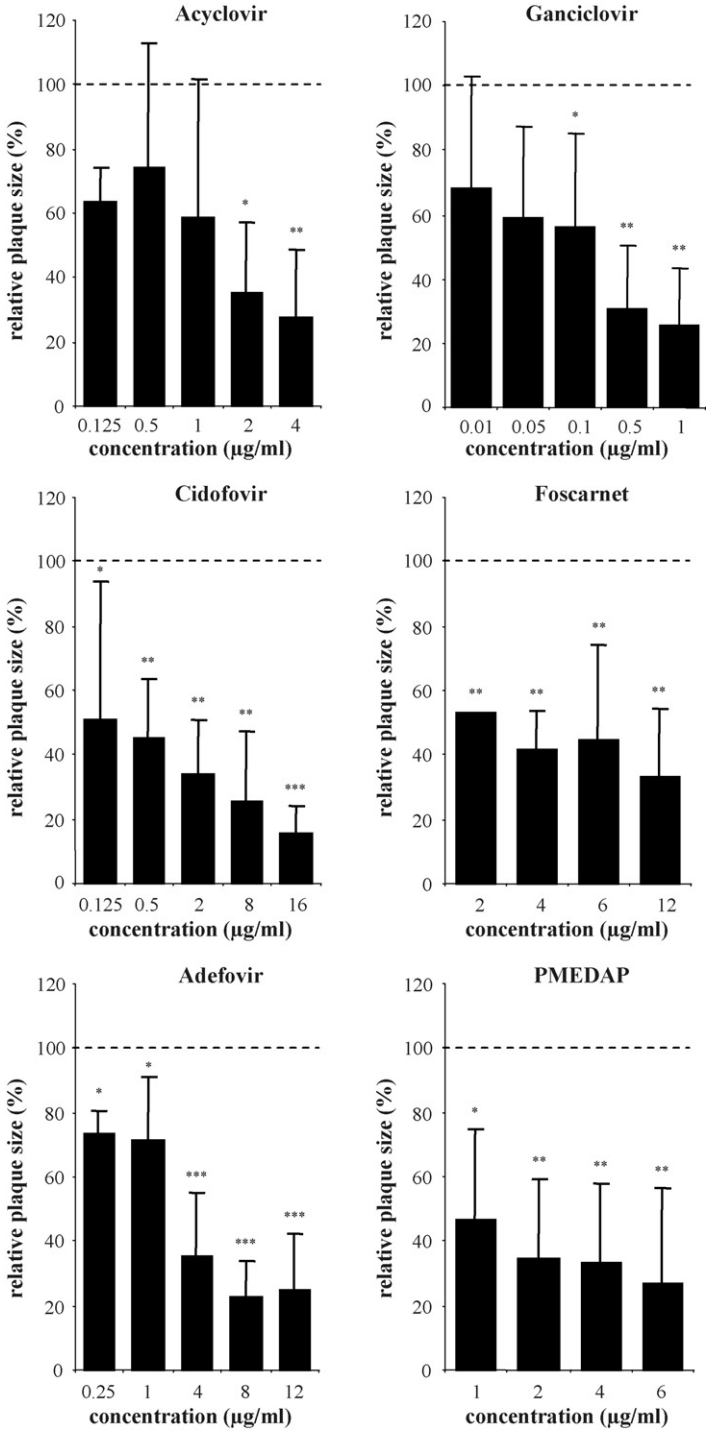

Fig. 1. The effect of antivirals on the size of virus-induced plaques. All products were able to reduce the plaque size significantly. The data represent the mean + S.D. of three independent experiments. Significant difference between test sample and untreated control (LSD, $^{*}P < 0.05$) ($0.01 < {}^{*}P < 0.05$; $0.001 < {}^{**}P < 0.01$; ${}^{***}P < 0.001$).

was comparable. Adefovir reduced plaque number at a concentration of 2.8–5.6 μg/ml. For all four compounds, there was no difference in susceptibility between the various isolates. The efficacy of cidofovir (3.1–6.7 μg/ml) was comparable with the efficacy of adefovir, except for isolate 99P96. This isolate displayed a clearly lower EC_{50} for cidofovir (1.1 μg/ml). Foscarnet was the least effective compound (6.6–11.1 μg/ml) and susceptibility showed a marked variation among isolates. The isolates 99P136 and 03P37 were clearly less susceptible to foscarnet than the other isolates (15.8 and 16.2 μg/ml).

3.2. Effect of the antiviral compounds on plaque size

As a complementary parameter of assessing the efficacy of the antiviral compounds, the mean plaque size (in pixels) of the antiviral treated relative to the size of plaques in untreated cultures was determined for isolate 97P70 (Fig. 1).

It is known that marked differences may occur in plaque size when EHV-1 is grown *in vitro*. Therefore, data obtained in the plaque size assay were subjected to an *F*-test to examine whether the variation in plaque size between samples was significantly different from the variation in plaque size within a sample (95% confidence interval). Or, in other words, the *F*-test was used to answer the question whether variation in plaque size was merely an artefact or whether it was related to the use of antiviral drugs. It was found that all compounds exhibited a significant effect on plaque size. Using a *post hoc* LSD-test it was demonstrated that the reduction in plaque size was significant for all antiviral compounds when compared to the untreated control (95% confidence interval).

Acyclovir reduced plaque size significantly at a concentration of 2 μg/ml or higher, ganciclovir at a concentration of 0.1 μg/ml or higher and PMEDAP at a concentration of 1 μg/ml or higher. Cidofovir, adefovir and foscarnet reduced plaque size significantly at respective concentrations of 0.125, 0.25 and 2 μg/ml or higher. The latter drugs are thus more effective (4-, 16- and 40-fold, respectively) in reducing plaque size than in reducing plaque number.

3.3. Effect of the antiviral compounds on the viability of EEL-cells

None of the compounds resulted in a significant decrease of viability of uninfected EEL cells at concentrations that were able to exert an antiviral effect. Acyclovir was toxic at a concentration of 320 μg/ml or more, ganciclovir at a concentration of more than 500 μg/ml, cidofovir at a concentration of 500 μg/ml or more, foscarnet at a concentration of 500 μg/ml or more, adefovir at a concentration of 80 μg/ml or more and PMEDAP at a concentration of 320 μg/ml or more (ANOVA, LSD, $P > 0.03$).

4. Discussion

The present study was conducted to compare the efficacy of a selection of anti-herpetic drugs against six isolates of EHV-1 *in vitro*. Based on the EC_{50}-value for inhibition of plaque formation, ganciclovir emerged as the most potent compound against all six isolates. Acyclovir and PMEDAP were approximately 10-fold less effective and adefovir was approximately 20-fold less effective than ganciclovir. For cidofovir and foscarnet, the efficacy was isolate-dependent. Isolate 99P96 was more susceptible to cidofovir as it had a lower EC_{50}-value than the other isolates. On the other hand, the isolates 03P37 and 99P136 were clearly less susceptible to foscarnet in comparison with the other isolates. There was no correlation between the susceptibility of an isolate to foscarnet and whether it was isolated from an outbreak of abortion or an outbreak of neurological disorders. Also, there was no correlation with the location or with the year of isolation. As foscarnet is the least potent compound against EHV-1 and as there is a great difference between various isolates in their susceptibility to this drug, foscarnet has no potential as a drug for the treatment of infected horses.

Based on the results of sequencing across the ORF30 region, we were not able to detect differences in susceptibility between non-neurological and neurological isolates. However, there are other ways to distinguish isolates which may have an influence on the susceptibility to antivirals. For example, isolates can also be distinguished in six strain groups by using ORF68 as strain group marker (Nugent et al., 2006).

No conclusion could be drawn based on differences in ORF68, as all our isolates, except one, were assigned to one group. Also possible variations in ORF18 may be interesting to study as this gene encodes the processivity subunit of the DNA polymerase (Telford et al., 1998).

Antiviral compounds not only reduce the number of herpesvirus-induced plaques, they also contribute in the reduction of plaque size (Mikloska and Cunningham, 2001; Jenssen et al., 2004; van der Meulen et al., 2006b). We found that all compounds were able to significantly reduce EHV-1 induced plaque size. Thus, plaque size is a useful and complementary means of assessing antiviral efficacy. The lowest concentration that was able to significantly reduce plaque size approached the EC_{50} for acyclovir, ganciclovir and PMEDAP, while the other products were able to reduce plaque size at concentrations significantly lower than their EC_{50}. The most remarkable effect was seen for cidofovir. Cidofovir was 40-fold more effective in reducing plaque size than in reducing the plaque number. The relevance of this finding in view of treatment of horses against EHV-1 induced disease remains to be determined. However, as already speculated by Mikloska and Cunningham (2001), a significant reduction of plaque size *in vitro* may be a potential parameter for the ability of an antiviral agent to restrict the size of virus-induced lesions *in vivo*.

Although the acyclic nucleoside analogues acyclovir and ganciclovir share a similar requirement for enzymatic phosphorylation, the relative high efficacy of ganciclovir, compared to acyclovir, is noteworthy. The better activity of ganciclovir may be related to a more efficient phosphorylation of ganciclovir to its 5′-monophosphate form by the EHV-1 thymidine kinase, a more efficient activation of the 5′-monophosphate form to the triphosphate metabolite by cellular kinases (Smee et al., 1985; De Clercq, 1993) or a more efficient inhibition of the viral polymerase by ganciclovir 5′-triphosphate than by acyclovir 5′-triphosphate (Smee et al., 1985; Mar et al., 1985). Also for the acyclic nucleoside analogues adefovir and PMEDAP, the difference in efficacy is remarkable. The study of Kramata and Downey (1999) suggests that differences in inhibition of cellular DNA synthesis may be explained by different intracellular ratios of the analogue diphosphates to their corresponding deoxynucleoside triphosphates and different affinities of DNA polymerases for the nucleotide analogue diphosphates.

From our results, we can conclude that ganciclovir displays the best overall activity against EHV-1 infection *in vitro*, without affecting cell viability. However, due to the high cost price, there is no direct clinical application possible. Therefore, acyclovir seems a more valuable candidate for antiviral therapy against EHV-1 infection in horses. As acyclovir is from patent since 1997 and generic alternatives are available, it seems attractive to use this drug for the treatment of horses during an outbreak. Previous studies (Wilkins et al., 2005) indicated that twice daily IV infusions of acyclovir (10 mg/kg) results in plasma concentrations above EC_{50}-levels during the entire treatment interval. However, the EC_{50}-value reported in their study was 0.3 μg/ml, which is 10-fold lower than the one we found. Our EC_{50}-value implies that the infusion should be given much more frequently, *i.e.* 8 times per day, to exceed concentrations greater than 1.7–3.0 μg/ml for the entire treatment interval. This limits the clinical application due to the high costs and practical administration during an outbreak. Oral administration of acyclovir may be an alternative. However, the bioavailability of orally administered acyclovir is a restriction. Bentz et al. (2006) reported a bioavailability of only 2.8% resulting in low plasma concentrations, inadequate to expect any clinically relevant antiviral efficacy. The oral prodrug of acyclovir, valacyclovir, may be more effective due to its higher bioavailability (Ormrod and Goa, 2000). Also the experimental compound PMEDAP can be a useful antiviral in the future. Although cidofovir is not highly efficient in reducing plaque number, it is able to significantly reduce plaque size at very low concentrations. As foscarnet is the least potent compound against EHV-1 and as there is a great difference between various isolates in their susceptibility to this drug, foscarnet has no potential as a drug for the treatment of infected horses.

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