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B. Garré, A. Gryspeerdt, S. Croubels, P. de Backer, H. Nauwynck. Evaluation of orally administered valacyclovir in experimentally EHV1-infected ponies. *Veterinary Microbiology*, 2009, 135 (3-4), pp.214. 10.1016/j.vetmic.2008.09.062 . hal-00532506

HAL Id: hal-00532506

<https://hal.science/hal-00532506>

Submitted on 4 Nov 2010

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Accepted Manuscript

Title: Evaluation of orally administered valacyclovir in experimentally EHV1-infected ponies

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PII: S0378-1135(08)00439-2
DOI: doi:10.1016/j.vetmic.2008.09.062
Reference: VETMIC 4205

To appear in: *VETMIC*

Received date: 17-6-2008
Revised date: 1-9-2008
Accepted date: 15-9-2008

Please cite this article as: Garré, B., Gryspeerdt, A., Croubels, S., De Backer, P., Nauwynck, H., Evaluation of orally administered valacyclovir in experimentally EHV1-infected ponies, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2008.09.062

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Evaluation of orally administered valacyclovir in experimentally EHV1-infected ponies.

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Shortened running title: Valacyclovir in EHV1-infected ponies

Number of words in summary: 262

Number of words in text: 4011

Number of tables and figures combined: 6

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Abstract

The purpose of the current study was to investigate the therapeutic efficacy of valacyclovir against EHV1 in a controlled study. Eight naïve Shetland ponies were inoculated with $10^{6.5}$ TCID₅₀ of the neuropathogenic strain 03P37. Four ponies were treated with valacyclovir at a dosage of 40 mg/kg bodyweight, three times daily, for five (n=2) or seven (n=2) consecutive days, while the other 4 ponies served as untreated controls. The treatment regimen started 1 hour before inoculation. Ponies were monitored daily for clinical signs. At 0, 1, 2, 3, 4, 5, 7, 9, 11, 14, 17 and 21 days pi, a nasopharyngeal mucus sample was taken to determine viral shedding. At the same time points, blood was collected and peripheral blood mononuclear cells (PBMC) were isolated to determine viremia. During treatment, blood samples were collected 6 times daily, i.e. just before valacyclovir administration and 1 hour later, to determine the concentration of acyclovir in plasma. Also a nasopharyngeal swab was taken to measure the acyclovir concentration in nasal secretion. No differences could be noticed between valacyclovir treated and untreated ponies. The clinical signs, the viral shedding and the viremia were similar in both groups. Plasma acyclovir concentration could be maintained above the EC₅₀-value of EHV1 during 50% of the entire treatment period in valacyclovir-treated ponies. Acyclovir could be detected in nasal swabs at concentrations varying from 50-100% of the corresponding plasma concentration.

Although sufficiently high acyclovir levels could be reached in plasma and nasal mucus, no effect was seen of the treatment with valacyclovir on clinical signs, viral shedding and viremia of EHV1-infected ponies.

Keywords: EHV1, valacyclovir, experimental infection, therapy

1. Introduction

The use of antivirals in veterinary medicine is currently subject of increasing interest. Cytokines like interferons possess antiviral and anti-oncogenic properties and can therefore be used to treat certain cancers and viral infections. In cats, interferons have been administered for the treatment of feline infectious peritonitis (FIP) (Ritz *et al.*, 2007), feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV) (de Mari *et al.*, 2004) and feline herpes virus (FHV1) infections (Stiles, 2000; Haid *et al.*, 2007; Gutzwiller *et al.*, 2007).

Besides the use of these biological products, owners and veterinarians take much interest in chemotherapeutics for the treatment of viral infections.. Both in cats and horses, the therapeutic efficacy of several antivirals has been investigated. For FeLV and FIV, the molecules of interest are nucleoside reverse transcriptase inhibitors, e.g. zidovudine, abacavir, lamivudine and stampidine (Hartman, 1998; Arai *et al.*, 2002; Uckun *et al.*, 2003). Nucleoside analogues like acyclovir, valacyclovir, idoxuridine, trifluridine and vidarabine are the antiviral products mostly used to treat FHV1-infections (Owens *et al.*, 1996; Nasisse *et al.*, 1997; Galle, 2004). These analogues are structurally similar to the nucleosides of which DNA and RNA consist. They are incorporated into the viral DNA, thereby terminating the growing viral DNA chain. In horses, influenza- and herpesviruses are common viral pathogens. Both rimantidine (Rees *et al.*, 1999) and oseltamivir (Yamanaka *et al.*, 2006) could ameliorate the clinical signs of influenza virus infections. In contrast to the cat, there are no studies to evaluate the therapeutic efficacy of nucleoside analogues to treat herpesvirus infections.

EHV1 may result in several clinical syndromes in horses. The respiratory disease is usually silent (Foote *et al.*, 2006), but a new sporadic form of EHV1 results in respiratory distress and even death of the affected horses (Del Piero and Wilkins, 2001). EHV1 can also cause abortion or neonatal foal disease. Abortion can involve only 1 or 2 mares in a herd but also

73 abortion storms can occur on a premise, which are associated with great economic losses
74 (Allen and Bryans, 1986). Finally, neurological disorders due to EHV1 have been reported
75 with increasing frequency (McCartan *et al.*, 1995; Friday *et al.*, 2000; van Maanen *et al.*,
76 2001; Stierstorfer *et al.*, 2002; van der Meulen *et al.*, 2003; Henninger *et al.*, 2007). During an
77 outbreak many horses can exhibit neurological disorders with devastating consequences. The
78 use of the nucleoside analogue acyclovir has been described during outbreaks of neonatal foal
79 disease (Murray *et al.*, 1998) and neurological disorders (Friday *et al.*, 2000; van der Meulen
80 *et al.*, 2003; Wilkins, 2004; Henninger *et al.*, 2007). However, the benefit of these treatments
81 is difficult to evaluate since there were no untreated control animals included.

82 There are several reasons to use acyclovir for the treatment of EHV1-infected horses.
83 Acyclovir has proven to be effective against abortigenic and neuropathogenic strains of EHV1
84 *in vitro* (Garré *et al.*, 2007a). It is no longer protected by a patent and generic products are
85 available, so the expenses to treat horses during an outbreak are reasonable. Acyclovir has
86 been used in humans for almost 30 years and has proven to be safe, even in neonates (Hintz *et al.*,
87 1982) and pregnant women (Baker, 1999), which makes it attractive to use in pregnant
88 mares and foals. The pharmacokinetics of acyclovir have also been described in horses
89 (Wilkins *et al.*, 2005; Bentz *et al.*, 2006; Garré *et al.*, 2007b). However, the oral
90 bioavailability of acyclovir is low. Oral administration of the prodrug, valacyclovir, is
91 associated with higher bioavailability (Garré *et al.*, 2007b) resulting in higher plasma
92 concentrations. A recent study in horses and ponies demonstrated that multiple dosing of
93 valacyclovir every 8 h results in plasma concentrations exceeding the EC₅₀-value of EHV1
94 during the majority of the treatment period (Garré *et al.*, submitted).

95 The purpose of the current study was to investigate the therapeutic efficacy of valacyclovir
96 against EHV1 in a controlled infection experiment, by investigating clinical symptoms, viral
97 shedding and viremia of valacyclovir-treated and untreated ponies.

98

99 **2. Materials and methods**

100

101 **2.1 Virus**

102 The Belgian EHV1-strain 03P37 was used for the experimental inoculation. This strain
103 was isolated from the peripheral blood mononuclear cells (PBMC) of a paralytic horse in
104 2003 (van der Meulen *et al.*, 2003). The strain has been typed as neuropathogenic in
105 cooperation with the Animal Health Trust in the United Kingdom (Nugent *et al.*, 2006). The
106 virus used for inoculation was at the 6th passage; 2 passages were performed in rabbit kidney
107 cells (RK13) and the 4 subsequent passages in equine embryonic lung cells (EEL).

108

109 **2.2 Animals**

110 Eight Shetland ponies were used in the study (A-H). Seven of the eight ponies were
111 younger than 8 months since the third incisor was not yet erupted. The other pony (A) was
112 approximately 2 years of age. Two females (D, F) and six males were included. None of the
113 ponies had been vaccinated prior to their arrival. Prior to the experiment, ponies were
114 monitored for 4 weeks. The rectal temperature was measured daily and complement-
115 dependent seroneutralization (SN)-tests and immunoperoxidase monolayer assays (IPMA)
116 were performed weekly to determine EHV-specific antibody titres. None of the ponies had a
117 raise in rectal temperature during this observation period which indicates that none of them
118 experienced an infection. None of the ponies had detectable EHV-specific antibody titres on
119 any occasion (SN < 2, IPMA < 10).

120 All ponies were housed in isolation stables. They were fed daily with a commercial,
121 complete feed. Drinking water and hay were supplied *ad libitum*.

122

2.3 Experimental inoculation

Each pony was inoculated oronasally with 20 ml of a virus suspension containing $10^{6.5}$ tissue culture infectious dose₅₀ (TCID₅₀). Ten ml were administered intranasally (5 ml per nostril) and 10 ml were inoculated orally. The virus titre was confirmed by titration of the inoculum. The ponies were inoculated at 8 am, i.e. 1 hour after the first valacyclovir administration.

2.4 Valacyclovir administration

Four of the eight ponies were treated with valacyclovir (VACV), while the other four ponies served as untreated controls. The treatment consisted of 40 mg of valacyclovir / kg body weight, administered three times daily, i.e. at 7 am, 3 pm and 11 pm for five (pony A, B) or seven (pony C, D) consecutive days. The doses were prepared identically for all horses by mixing the calculated amount of valacyclovir with apple sauce. The mixture was administered orally using a 60 ml syringe.

2.5 Clinical observation

Following virus inoculation, the ponies were monitored daily for three weeks for clinical signs via physical examination and measurement of rectal temperature. A rectal temperature of $\geq 38.5^{\circ}\text{C}$ was regarded as fever. During clinical examination, nasal discharge (serous, mucous or purulent), lymph node swelling, ocular discharge, tachypnoea, coughing and nervous system disorders were investigated. The breathing frequency was counted to evaluate breathing problems. Several tests were performed to evaluate nervous system disorders: walking the horse in a straight line, walking on and off a step, walking the horse in tight circles, pulling the horse's tail and backing the horse.

2.6 Virological examination

A nasopharyngeal mucus sample and 40 ml of heparinised blood were taken at 0, 1, 2, 3, 4, 5, 7, 9, 11, 14, 17 and 21 days post inoculation (d pi).

The nasopharyngeal samples were collected using a swab with a length of 40 cm that was inserted through the nostrils up to the nasopharynx. Immediately after collection, the swabs were immersed in transport medium containing phosphate buffered saline supplemented with 10% fetal bovine serum, 1000 U/ml penicillin, 1 mg/ml streptomycin and 0.5 mg/ml kanamycin. EHV1 was titrated in the nasopharyngeal secretions. Therefore, the nasopharyngeal swabs were vortexed in the transport medium, the medium was centrifuged and the supernatant was titrated on monolayers of RK13 cells.

Peripheral blood mononuclear cells (PBMC) were isolated from the heparinised blood by density centrifugation on Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). The cells were washed three times with phosphate buffered saline and resuspended in complete medium containing 2/1 MEM and RPMI 1640 supplemented with 10% fetal calf serum, 0.05 mM 2-mercapto-ethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 0.3 mg/ml glutamine, 1% non-essential amino-acids 100x (GibcoBRL, Life Technologies, Paisley, UK) and 1 mM sodium pyruvate. For co-cultivation, the cells were seeded on RK13 monolayers. The monolayers were placed at 37°C and 5% CO₂. After 36 hours of incubation, the number of plaques was counted. For pony C, D and E, 2 different co-cultures were made. Normal medium was added to the one co-culture, while acyclovir at a concentration of 4 µg/ml was added to the other co-culture to investigate whether acyclovir could exert an effect on the transmission of virus from infected PBMC to target cells.

2.7 Acyclovir quantification

In order to determine peak and trough concentrations in plasma, 5 ml of blood were collected into heparinized blood collection tubes (Venoject, Terumo, Leuven, Belgium) at 7 am, 3 pm and 11 pm just before valacyclovir administration (trough), and at 8 am, 4 pm and 12 pm (peak), during five (day 0-4 pi; pony A, B) or seven days (day 0-6 pi; pony C, D) of treatment. The next three days (day 5-7 pi for pony A, B; day 7-9 pi for pony C, D), blood samples were collected at 7 am, 3 pm and 11 pm to determine the elimination phase. Plasma was immediately harvested by centrifugation at 400 g for 10 minutes and was frozen at -70°C until assayed.

The nasopharyngeal mucus samples were taken daily at 4 pm during valacyclovir treatment. The nasopharyngeal samples were collected using a swab with a length of 40 cm inserted through the nostrils up to the nasopharynx. Immediately after collection, the cotton of the swab was cut off and put in an Eppendorf. Then, swabs were centrifuged for 10 minutes at 10.000 g to collect the nasal fluid which was transferred to another Eppendorf and stored at -70°C until assayed.

The acyclovir concentration was determined using high-performance liquid chromatography combined with tandem mass spectrometry (LC-MS/MS), based on Maes *et al.* (in press). The unbound concentration of acyclovir was obtained by a simple deproteinization step of the sample using a Microcon[®] filter. Ganciclovir was used as an internal standard.

2.8 Serological examination

The SN titre was determined at 0, 7, 14 and 21 d pi by means of a complement-dependent SN-test. Two-fold serum dilutions were prepared in duplicate rows of 96-well microtiter plates using MEM as diluent and mixed with 300 TCID₅₀ of the EHV1-isolate Arabica. After 23 hours of incubation at 37°C and 5% CO₂, 25 µl of unheated guinea pig serum was added to

each well as a source of complement. Plates were then incubated for an additional hour and the content of each well was subsequently transferred to a monolayer of RK13 cells. The monolayers were incubated at 37°C and 5% CO₂ and after 7 days examined for cytopathic effect. The SN titre was calculated as the reciprocal value of serum dilution that neutralized the cytopathic effect in 50% of the wells.

In case no detectable SN titre was observed, serum samples were tested using a more sensitive IPMA. RK13 cells were seeded in 96-well microtiter plates, grown to confluency and inoculated with 10³ TCID₅₀ of the EHV1-isolate 97P70. After 28 h, cells were washed, dried at 37°C for 1 h and stored at - 20°C until use. Plates were then thawed and subsequently fixed with 4% paraformaldehyde and a solution containing 1% hydrogen in methanol. Following extensive washing, serial 2-fold dilutions of the sera were added and cells were incubated for 1 h at 37°C. Then, cells were incubated with peroxidase-labeled goat anti-horse antibodies (Jackson ImmunoResearch Laboratories Inc., PA, USA). After 1 hour, a substrate solution of 3-amino-9-ethylcarbazole in 0.05 M acetate buffer with 0.05% hydrogen was added to each well. After 20 minutes of incubation at 37°C, the substrate solution was replaced with an acetate buffer to block the enzymatic staining reaction. The IPMA titre was calculated as the reciprocal value of highest serum dilution that induced visual staining of infected RK13 cells, as determined by light microscopy.

2.9 Statistical analysis

The Student's t-test was used to demonstrate possible differences in body temperature, duration of pyrexia, the area under the body temperature-time curve (AUC), duration of viral shedding, viral titres, duration of viremia and the number of infected PBMC in co-cultivation. Differences with P<0.05 were considered significant.

3. Results

3.1 Clinical observation

The clinical signs as observed in the EHV1-inoculated ponies are summarized in Table 1. They were mild and consisted mostly of nasal discharge and swelling of the lymph nodes. Nasal discharge occurred for 14 days at most, varying from serous to purulent. Four ponies had ocular discharge for a short period of time. Swelling of the mandibular lymph nodes lasted for the entire observation period (21 d pi) in most ponies. The retropharyngeal lymph nodes could not be palpated but a painful reaction of the pony upon palpation indicated swelling. Tachypnoea and coughing occurred in 3 and 5 ponies, respectively. Also a short period of dullness could be noticed. None of the ponies developed nervous system disorders, such as ataxia or paresis. The temperature curves are presented in Figure 1. On 2 d pi, all ponies had fever, however valacyclovir treated ponies had a significantly lower body temperature (average = 38.8°C) than non-treated ponies (average = 39.9°C). Fever lasted for 5 or 6 days in most ponies, except for pony E (2-3 d pi) and G which was only pyretic for one day (2 d pi). There was no difference in duration of pyrexia between both groups. Also no difference in the AUC of body temperature could be noted.

3.2 Virus isolation from the upper respiratory tract

The results of virus isolation from the nasopharyngeal swabs are shown in Table 2. Five ponies started viral shedding on 1 d pi. All ponies shed virus on 2 d pi. Virus shedding lasted until 7 d pi in all untreated ponies. In valacyclovir treated ponies, viral shedding lasted until 9 or 11 d pi in 3 ponies, while pony C shed virus for 21 days. However, this difference in duration of viral shedding was not significant. Valacyclovir treated and untreated ponies had similar virus titres from 1 d pi up to 7 d pi.

3.3 Viremia

The numbers of infected PBMC as determined by co-cultivation are presented in Table 3. Infected PBMC were detected by co-cultivation between 3 and 9 d pi, 1 and 14 d pi, 1 and 17 d pi, 2 and 17 d pi in the valacyclovir-treated ponies A, B, C and D, respectively. In the untreated ponies, cell-associated viremia was detected between 2 and 11 d pi, 2 and 9 d pi, 7 and 9 d pi, 2 and 14 d pi in pony E, F, G and H, respectively. There was no difference in the duration of viremia between both groups. There were also no differences between treated and untreated ponies in the number of infected PBMC determined by co-cultivation.

There was no difference in the number of infected PBMC between normal co-cultures and co-cultures supplemented with acyclovir.

3.4 Serological response to experimental inoculation

The complement-dependent SN titres are shown in Table 4. A clear seroconversion was observed at 14 or 21 d pi, except for pony G which developed very low levels of antibodies even after 21 d pi.

3.5 Acyclovir concentration

The unbound plasma concentration versus time curve is presented in Figure 2. Plasma concentrations could be maintained above the EC₅₀-value of 1.7 µg/ml, i.e. EC₅₀ of an isolate highly susceptible to acyclovir, for 76% of the treatment period and above the EC₅₀-value of 3.0 µg/ml, i.e. EC₅₀ of an isolate less susceptible to acyclovir, for 46% of the treatment period.

Acyclovir could be detected in nasal mucus samples at concentrations varying from 0.5 µg/ml to 6.5 µg/ml. The concentrations in nasal mucus samples varied from 50 to 100% of the corresponding plasma concentrations.

4. Discussion

The purpose of the current study was to investigate whether valacyclovir, the oral prodrug of acyclovir, is effective in the treatment of EHV1-infected horses. Eight Shetland ponies were used in this study. Seven of them were 6 months of age. None of them had detectable EHV-specific antibody titres prior to the study. The use of weanling horses (five to nine months of age), free of EHV1 neutralizing antibodies, is a common tool in EHV1 research (Cornick *et al.*, 1990; Matsumura *et al.*, 1996; Heldens *et al.*, 2001). The foals were monitored for 4 weeks after arrival. During this observation period, no fever was recorded, no virus was isolated from nasopharyngeal swabs and no EHV-specific antibodies were detected on any occasion. This strongly indicated that all foals were EHV1 negative prior to the experiment. This was enforced by the absence of a quick serological booster reaction shortly after challenge. Working with naïve animals is important to minimise the effect of immune status on the clinical and virological outcome.

The foals were inoculated oronasally with the EHV1-isolate 03P37, which was isolated from PBMC of a paralytic horse during an outbreak in a Belgian riding school in 2003 (van der Meulen *et al.*, 2003). During this outbreak, neurological disorders were observed in 15% of all horses. The disorders consisted mainly of ataxia and paralysis of hind limb and tail, but also cerebral disorders, such as blindness, torticollis and severe apathy were observed. The mortality during the outbreak was 10%, so we can conclude that this EHV1-isolate is highly pathogenic. Despite the high virulence of the isolate, the clinical symptoms observed in our infection study were mild and consisted of pyrexia, nasal discharge and swelling of lymph nodes. Ocular discharge and respiratory symptoms such as tachypnoea and coughing were also observed. A short period of dullness could be noticed in six of the eight inoculated

ponies. Dullness was also noted by Kydd *et al.* (1994) who exposed Welsh Mountain ponies to the EHV1 strain Ab4, which was obtained from a quadriplegic mare affected during an outbreak of neurological disease in 1980 (Crowhurst *et al.*, 1981). The same strain was used by Smith *et al.* (1996) where all five infected mares showed dullness. Paralysis did not occur nor in one of these studies, nor in our study. In contrast, a study of Mumford *et al.* (1994) where mares were intranasally inoculated with the Ab4 strain, resulted in neurological disease in some animals. Development of neurological disorders appears to be dependent on several factors, including the animal's age and sex, and, in mares, the stage of their reproductive cycle (McCartan *et al.*, 1995). The severity of the disease may also be influenced by the physical condition of the host, the immune status of the host, and the pathogenic potential of the strain involved (Nugent *et al.*, 2006).

Four ponies were treated with valacyclovir, while the other four ponies served as untreated controls. The treatment was started 1 hour before inoculation as it has been shown that, after oral administration of valacyclovir, maximal plasma concentrations are reached after approximately 1 hour (Garré *et al.*, 2007b). The dosage regimen consisted of 40 mg/kg every 8 hours. We previously demonstrated that this dosage regimen was sufficient to maintain plasma concentrations in horses and ponies above the EC₅₀-value of EHV1 during the majority of the treatment period (Garré *et al.*, submitted). It has been suggested that maximal efficacy is reached when the length of time that the acyclovir concentration remains above the EC₅₀ is more than 50% of treatment period (Tod *et al.*, 2001). Also in this study, plasma concentrations could be maintained above the EC₅₀-value of 1.7 µg/ml for 76% of the treatment period and above the EC₅₀-value of 3.0 µg/ml for 46% of the treatment period. The isolate used in this study, 03P37, is susceptible to acyclovir *in vitro* at a concentration of 3.0 µg/ml (Garré *et al.*, 2007a), so this dosage regimen should have been effective to exert an antiviral effect. However, no differences could be noted between both groups of ponies in

clinical signs, viral shedding and viremia, except for the peak body temperature at 2 d pi, where valacyclovir treated ponies had a lower body temperature than untreated ponies ($P<0.05$).

Acyclovir could be detected in nasal mucus at concentrations varying from 50-100% of the corresponding plasma concentrations. This is in contrast with our earlier report where the acyclovir concentration in nasal mucus of healthy horses was only 7.5% of the corresponding plasma concentration (Garre *et al.*, submitted). This may be due to the fact that the ponies in the present study were infected with EHV1 and experienced nasal discharge. Infection of the respiratory tract causes hyperaemia (Allen and Bryans, 1986), which may influence the distribution of acyclovir into nasal mucus.

Although acyclovir was present in the nasal mucus, no effect could be demonstrated on nasal shedding of the virus. Valacyclovir-treated ponies did not show a shorter period of viral shedding or decreased viral titres in comparison with untreated ponies. It even seemed that valacyclovir-treated ponies experienced a longer period of viral shedding; however this difference was not significant. One pony, C, shed virus for 21 days. Normally, nasal shedding is observed from 1 day up till 7, 8 or 9 days after inoculation. However, nasal swabs may be virus positive up till 14 days after inoculation (Gibson *et al.*, 1992; Heldens *et al.*, 2001; van der Meulen *et al.*, 2006). Patel *et al.* (1982) also found virus in nasal swabs up to 21 d pi in a foal inoculated with a paresis isolate. Most researchers only sample the nasopharynx during the first 10 (Gleeson and Coggins, 1980; Chong and Duffus, 1992; Mumford *et al.*, 1994) or 14 days after inoculation (Heldens *et al.*, 2001). On these occasions, we can not draw any conclusion about the duration of nasal shedding as the latest collected sample is still virus positive at the end of the observation period (Chong and Duffus, 1992; Heldens *et al.*, 2001). This implies that three weeks of quarantine for horses arriving on a farm is the absolute minimum.

There was no difference between valacyclovir-treated ponies and untreated ponies in the duration of viremia or the number of infected PBMC. During cell-associated viremia, 98% of EHV1-infected PBMC do not show viral envelope proteins on their surface (van der Meulen *et al.*, 2006). Moreover, viral envelope proteins are also undetectable intracellular. Only the immediate-early protein and at least one early protein, ICP22 (regulatory protein), are present in the cytoplasm (van der Meulen *et al.*, 2006). This indicates that EHV1-infected PBMC circulating during cell-associated viremia are in an early phase of infection. It is possible that during cell-associated viremia, EHV1 replication is restricted to early events and that transcription of the remaining viral genome is postponed until a certain trigger occurs. Since acyclovir targets the DNA polymerase where it acts as a chain terminator, no effect on the number of infected PBMC is expected.

Earlier findings by Smith *et al.*, (2001, 2002) indicated that activation of adhesion molecules on both leukocytes and endothelial cells play a key role in the transfer of virus from infected PBMC to endothelial cells. This adhesion of infected PBMC to susceptible cells may provide the activation signal that triggers later events. Secondary replication in endothelial cells at the target sites results in vasculitis with secondary ischemia and haemorrhage resulting in the several symptoms (Edington *et al.*, 1986; 1991). Acyclovir may be able to exert an effect on the secondary virus replication and subsequently, be able to prevent abortion and neurological disorders. However, the results of the co-cultivation supplemented with acyclovir are in conflict to this theory. No difference was found in the number of infected PBMC in normal co-cultures and co-cultures supplemented with acyclovir. This indicates that acyclovir can not prevent the transmission of virus from infected PBMC to target cells and that acyclovir can not inhibit the secondary replication in experimentally EHV1-infected horses.

This study shows that acyclovir and its oral prodrug valacyclovir are not effective in EHV1-infected horses.

Acknowledgement

The authors thank C. Boone for titrations, C. Bracke for isolation of PBMC and performing SN-tests. We also thank N. Desmet for the chromatographic analysis of the samples.

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Figure legends:

Figure 1. Body temperature in valacyclovir-treated and untreated ponies following experimental inoculation with EHV1.

Figure 2. Unbound plasma acyclovir concentration versus time curve (mean + SD) during and after valacyclovir administration (40 mg/kg body weight, 3 times daily) in ponies treated for 5 (A, B) or 7 days (C, D)

◆: average of ponies A, B, C, D; ○: average of ponies A, B; □: average of ponies C, D

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Table 1. Clinical signs in valacyclovir-treated and untreated ponies following experimental inoculation with EHV1.

Pony	Treatment	Duration of clinical signs (from ... to ... d pi)							
		Pyrexia	Nasal discharge	Ocular discharge	Swelling		Respiratory symptoms		Dullness
					mand.	retro.	tachypnoea	coughing	
A	VACV 5 days	2-6	2-11	5-6	3-21	-	-	-	-
B	VACV 5 days	2-6	1-11	-	1-21	2-11	-	-	3-6
C	VACV 7 days	2-5	1-14	1-4	1-19	2-5	-	3-6	-
D	VACV 7 days	2-6	2-12	3-7	1-21	1-5	-	3-6	4-7
E	None	2-3	1-13	-	3-17	2-14	4-6	-	3-7
F	None	2-7	1-14	-	1-21	4-10	4	2-7	8
G	None	2	1-11	-	4-11	-	-	2-5	2

H	None	2-8	2-11	1-5	2-18	-	3	2-10	3, 12
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EHV: equine herpesvirus; d pi: days post inoculation; VACV: valacyclovir; lnn: lymph nodes; mand: mandibular; retro: retropharyngeal

Table 2. Virus shedding in valacyclovir-treated and untreated ponies following experimental inoculation with EHV1.

Pony	Treatment	Virus titre ($\log_{10}$ TCID ₅₀ /g nasopharyngeal mucus)											
		0	1	2	3	4	5	7	9	11	14	17	21
A	VACV 5 days	-	3.2	2.9	3.9	6.2	5.9	5.9	4.0	-	-	-	-
B	VACV 5 days	-	2.6	4.8	4.4	6.0	7.2	5.8	3.6	5.0	-	-	-
C	VACV 7 days	-	3.1	5.7	5.1	6.0	6.1	5.6	2.5	5.0	3.7	5.0	4.2
D	VACV 7 days	-	-	6.4	5.1	4.8	5.8	5.0	5.6	3.5	-	-	-
E	none	-	4.8	6.0	5.7	5.0	5.8	2.8	-	-	-	-	-
F	none	-	3.5	5.3	4.9	4.7	5.9	4.5	-	-	-	-	-
G	none	-	-	4.5	3.2	4.9	5.4	5.6	-	-	-	-	-
H	none	-	-	5.5	2.9	4.0	5.1	4.5	-	-	-	-	-

EHV: equine herpesvirus; TCID: tissue culture infectious dose; VACV: valacyclovir; (-): $< 2.2 \log_{10}$ TCID₅₀/g nasopharyngeal mucus

Table 3. Cell-associated viremia in valacyclovir-treated and untreated ponies following experimental inoculation with EHV1.

Pony	Treatment	Number of infected cells able to transmit virus/ 10^7 PBMC (co-cultivation) at ... d pi											
		0	1	2	3	4	5	7	9	11	14	17	21
A	VACV 5 days	-	-	-	1	3	10	5	1	-	-	-	-
B	VACV 5 days	-	1	1	3	9	15	6	2	3	1	-	-
C	VACV 7 days	-	1	1	10	9	8	8	4	1	1	1	-
D	VACV 7 days	-	-	2	7	3	1	12	4	2	1	1	-
E	none	-	-	1	1	1	1	5	1	1	-	-	-
F	none	-	-	1	2	13	11	1	1	-	-	-	-
G	none	-	-	-	-	-	-	3	16	NS	-	-	-
H	none	-	-	1	1	1	1	6	2	NS	1	-	-

EHV: equine herpesvirus; PBMC: peripheral blood mononuclear cells; d pi: days post inoculation; VACV: valacyclovir; NS: no sample

Table 4. Serological response in valacyclovir-treated and untreated ponies following experimental inoculation with EHV1.

Pony	Treatment	SN titre at inoculation	Complement-dependent SN titres at ... d pi					
		0 d pi	7	9	11	14	17	21
A	VACV 5 d	< 2	< 2	12	32	192	320	512
B	VACV 5 d	< 2	< 2	< 2	4	16	128	640
C	VACV 7 d	< 2	< 2	3	8	64	96	96
D	VACV 7 d	< 2	< 2	3	8	24	48	64
E	none	< 2	< 2	< 2	6	32	64	160
F	none	< 2	< 2	< 2	2	32	96	256
G	none	< 2	< 2	< 2	NS	4	8	16
H	none	< 2	< 2	8	NS	12	64	192

NS: no sample