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Evaluation of the antiviral activity of (1′S,2′R)-9-[[1′,2′-bis(hydroxymethyl) cycloprop-1′-yl]methyl]guanine (A-5021) against equine herpesvirus type 1 in cell monolayers and equine nasal mucosal explants

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ABSTRACT

Equine herpesvirus 1 (EHV1) is a ubiquitous equine alphaherpesvirus that causes respiratory disease, neurological symptoms and abortions. Current vaccines are not fully protective and effective therapeutics are lacking. A-5021 [(1′S,2′R)-9-[[1′,2′-bis(hydroxymethyl)cycloprop-1′-yl]methyl]guanine], previously shown to possess potent anti-herpetic activity against most human herpesviruses, was evaluated for its potential to inhibit EHV1 replication. In equine embryonic lung (EEL) cells, infected with either a non-neurovirulent (97P70) or a neurovirulent (03P37) EHV1 isolate, A-5021 proved to be about 15-fold more potent than acyclovir in inhibiting viral replication. Moreover, in equine nasal mucosal explants, A-5021 (at 8 and 32 μM) was able to completely inhibit viral plaque formation whereas acyclovir did not exert an antiviral effect at these concentrations. Our data demonstrate that A-5021 is a potent inhibitor of EHV1 replication and may have potential for the treatment and/or prophylaxis of infections with this virus.

1. Introduction

Equine herpesvirus 1 (EHV1), an equine alphaherpesvirus, is a major cause of abortion, neonatal foal death, respiratory disease and myeloencephalopathy in horses worldwide (Brosnahan and Osterrieder, 2009). The virus spreads via nasal secretions and establishes latency in neural and lymphoid tissue. As a consequence of increased occurrence of neurologic outbreaks and more frequently associated mortality, EHV1 has recently been classified as a potentially emerging disease by the US Department of Agriculture (USDA–APHIS, 2007). Prevention is difficult because available vaccines do not provide complete protection and treatment is limited to symptomatic care. Several antivirals have been shown to inhibit EHV1 replication *in vitro*, but efficacy in infected horses has not been proven (Kydd et al., 1994; Friday et al., 2000; Wong and Scarratt, 2006; Garré et al., 2007; Henninger et al., 2007; van der Meulen et al., 2007). The oral prodrug form of the anti-herpesvirus drug acyclovir, valacyclovir, has been evaluated in experimentally infected horses. The drug did not improve respiratory and other symptoms nor had it an effect on viral shedding and viremia (Garré et al., 2009). Thus, there is an urgent need for more efficacious EHV1 vaccines and potent and safe antiviral drugs for treatment or prophylaxis of EHV1-associated disease. In diseased animals, treatment might be appropriate, whereas prophylaxis of neighboring contact animals might be reasonable during an EHV1 outbreak.

A-5021 [(1′S,2′R)-9-[[1′,2′-bis(hydroxymethyl)cycloprop-1′-yl]methyl]guanine] has been previously shown to possess potent and selective anti-herpetic activity against herpes simplex virus type 1 (HSV1), herpes simplex virus type 2 (HSV2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), Epstein–Barr virus (EBV) and human herpes virus 6 (HHV-6A and HHV-6B). In mice experimentally infected with HSV1 or HSV2, A-5021 proved

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markedly more potent than acyclovir (Iwayama et al., 1998, 1999; Ono et al., 1998; Neyts et al., 2001).

In the present study, the effect of A-5021 on EHV1 replication was evaluated by studying the antiviral activity of this compound relative to acyclovir (and the acyclic nucleoside phosphonate PMEDAP [9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine]) in (i) an *in vitro* equine embryonic lung (EEL) cell model system and (ii) an *ex vivo* equine nasal respiratory explant model system.

2. Materials and methods

2.1. Viruses

Two different Belgian EHV1 isolates, 97P70 and 03P37 (Gryspeerdt et al., 2010), were used in this study. Isolate 97P70 was isolated from the lungs of an aborted fetus in 1997 and isolate 03P37 was isolated from the peripheral blood mononuclear cells of a paralytic horse in 2003. The isolates were genotyped by the Animal Health Trust, UK, in the ORF30 region which is associated with differences between neurovirulent and non-neurovirulent isolates (Nugent et al., 2006). Isolate 97P70 was typed as a non-neurovirulent isolate. Isolate 03P37 was typed as a neurovirulent isolate.

2.2. Antiviral compounds

The antiviral compounds used in this system were acyclovir, A-5021 [(1′S,2′R)-9-[[1′,2′-bis(hydroxymethyl)cycloprop-1′-yl] methyl] guanine] and PMEDAP [9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine]. A-5021 was synthesized as described previously (Sekiyama et al., 1998) and kindly provided by Dr. T. Tsuji from Ajinomoto Co. (Kawasaki, Japan). Acyclovir was obtained from GlaxoSmithKline (Genval, Belgium) and PMEDAP was kindly provided by Prof. A. Holý (IOCB, Prague, Czech Republic).

2.3. Antiviral assays

2.3.1. In vitro cell culture system

EEL cells were cultured in minimum essential medium (MEM) (Invitrogen, Paisley, UK) supplemented with 100 U/ml penicillin (Continental Pharma, Puurs, Belgium), 0.1 mg/ml streptomycin (Certa, Braine l'Alleud, Belgium), 0.1 mg/ml kanamycin (Sigma, St. Louis, MO), 0.3 mg/ml glutamin (VWR, Radnor, PA) and 5% fetal calf serum (Invitrogen). Cells were passaged once a week. Cells were seeded in 24-well culture plates (Nunc A/S, Roskilde, Denmark) at a density of 3.10^5 cells/ml and were cultured at 37 °C (5% CO_2). After 24 h, medium was removed and the cells were inoculated with 200 μl virus suspension containing 80 PFU/well. After 1 h incubation, cultures were rinsed twice with culture medium and overlaid with 0.94% carboxymethylcellulose (Sigma) medium containing 2-fold dilutions of each compound (0.5, 1, 2, 4, 8, 16 and 32 μM). Mock-treated EHV1-inoculated cells were used as a control. At 50 h post inoculation (pi), the medium was removed and the cultures were rinsed twice with phosphate-buffered saline (PBS). Subsequently, the cultures were fixed with 4% paraformaldehyde and methanol containing 1% H_2O_2. After fixation, an immunoperoxidase monolayer assay (IPMA) was performed. Therefore, cells were incubated (1 h, 37 °C) with polyclonal anti-EHV1 antibodies (van der Meulen et al., 2003), washed three times, incubated with goat anti-horse peroxidase-labeled antibodies (1 h, 37 °C) (Jackson, De Pinte, Belgium) and washed three times. Finally, the substrate 3-amino-9-ethylcarbazole (AEC) (Sigma) was added during 6 min (37 °C) and replaced by acetate buffer to block the enzymatic reaction. The number of EHV1 plaques was counted for mock-treated control samples and for each concentration of antiviral compound-treated samples using light microscopy (Olympus IX50, Olympus Corporation, Tokyo, Japan). The 50% effective concentration (EC_{50}) or concentration that reduces the plaque number by 50% was calculated.

The effect of the antiviral compound A-5021 on the viability of EEL cells was determined using an MTT-based method (Hansen et al., 1989). To this end, EEL cells were seeded in 96-well culture plates (Nunc A/S) at a density of 5.10^4 cells per well. After 24 h, growth medium was replaced by 100 μl serial 2-fold dilutions of the compound. Untreated cells were included as control. Additionally, untreated cells fixed with 4% paraformaldehyde at 69 h post incubation were included as background condition. At 69 h post incubation, cells were incubated overnight with 50 μl of a 3 mg/ml MTT solution (Sigma). After incubation with MTT, 100 μl medium was removed and 75 μl of detergent reagent (0.5% sodium dodecyl sulfate, 0.36% 10 M HCl in isopropanol) was added. The plates were placed for 5 min in the sonicater. Afterwards, 75 μl of detergent reagent was added and absorbance was measured on a Multiskan RC (Thermo Fisher Scientific, Waltham, MA) at a wavelength of 550 nm. The viability of the cells was calculated as follows: percentage of viable cells = $[(OD_t - OD_d)/(OD_c - OD_d)]^* 100\%$ where OD_t is the absorbance of cells incubated with compound, OD_d the absorbance of the background control and OD_c the absorbance of the untreated cells. For each concentration of antiviral compound and for each control sample, 3 wells were included per experiment; independent triplicate replicates of each experiment were performed.

2.3.2. Ex vivo explant system

Equine nasal respiratory explants were obtained from slaughter horses. Cultivation of equine nasal respiratory explants was performed as described previously (Vandekerckhove et al., 2009).

At 24 h after start of *in vitro* cultivation, explants were placed in a 24-well plate and washed twice with warm medium. The explants were then incubated with 1 ml inoculum containing $10^{6.5}$ $TCID_{50}$/ml EHV1 for 1 h at 37 °C and 5% CO_2. At 1 h pi, explants were washed twice with warm medium and were immersed for 1 h in medium with or without antiviral agent for antiviral-treated and mock-treated explants, respectively. In this antiviral assay, both acyclovir and A-5021 were used at concentrations of 2, 8 or 32 μM, respectively. Thereafter, explants were transferred back from the 24-well plate to their gauze with medium in the presence or absence of the antiviral agent for antiviral-treated and mock-treated explants, respectively. Ciliary beating of the explants was checked on a daily basis by examination of the ciliated surface of the explants for continuous beating of the cilia using an Olympus CK40 light microscope at magnification ×20.

At 48 h pi, explants were collected, embedded in methocel® (Sigma) and frozen at −70 °C. A total of 100 consecutive cryosections of 20 μm was made and fixed in methanol (100%, −20 °C) for 20 min. First, the cryosections were incubated with antibodies to visualize collagen VII, which is a component of the basement membrane. Therefore, cryosections were incubated (1 h, 37 °C) with monoclonal mouse anti-collagen VII antibodies (Sigma) (1:300 in PBS), followed by secondary TexasRed-labeled goat anti-mouse antibodies (Invitrogen) (1:50 in PBS, 1 h, 37 °C). EHV1-infected cells were detected by using biotinylated equine polyclonal anti-EHV1 IgG (1:10 in PBS, 1 h, 37 °C) (van der Meulen et al., 2003), followed by streptavidin-fluorescein isothiocyanate (FITC) (1:100 in PBS, 1 h, 37 °C) (Invitrogen). Finally, cryosections were washed three times in PBS and mounted with glycerin-DABCO (Janssen Chimica, Geel, Belgium). Immunofluorescence stained cryosections were analyzed using a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). A Gre/Ne 543 nm laser was used to excite TexasRed fluorochromes, an Argon 488 nm laser was used to excite FITC fluorochromes. The number of plaques and the presence of single EHV1-infected cells below

the basement membrane were determined in 10 mm^2 explant areas for each condition.

2.4. Data analysis

Data were statistically evaluated by SPSS software (SPSS Inc., Chicago, IL) for analysis of variance (ANOVA). Results with *P* values of $\leqslant 0.05$ were considered significant. Data shown represent means ± standard deviations (SD) of triplicate independent experiments.

3. Results

3.1. In vitro cell culture system

The effect of acyclovir, PMEDAP and A-5021 on viral replication of two different Belgian EHV1 isolates, 97P70, a non-neurovirulent isolate, and 03P37, a neurovirulent isolate, was evaluated in EEL cells. The number of EHV1-induced plaques was quantified for mock-treated and for drug-treated EHV1-infected cells. The mean number of plaques per 1.2×10^6 EEL cells ± SD of triplicate independent experiments are presented (Table 1). A-5021 inhibited viral replication with EC_{50}-values of 1.2 ± 0.4 μM and 1.6 ± 0.6 μM for 97P70 and 03P37, respectively. Acyclovir proved roughly 15-fold less effective (EC_{50} values of 23.5 ± 6.4 μM and 25.0 ± 8.6 μM for 97P70 and 03P37, respectively) whereas PMEDAP was about equipotent to acyclovir (EC_{50} values of 9.3 ± 3.0 μM and 15.6 ± 8.8 μM for 97P70 and 03P37, respectively).

A-5021 had little or no effect on the viability of the uninfected EEL cells (CC_{50} value of 259 μM).

3.2. Ex vivo explant system

The effect of acyclovir and A-5021 on viral replication of the non-neurovirulent 97P70 EHV1 isolate and the neurovirulent 03P37 EHV1 isolate was evaluated in equine nasal respiratory mucosal explants. To this end, the number of EHV1 plaques was counted and the presence of single EHV1-infected cells below the basement membrane was determined for mock-treated control samples and for each concentration of antiviral compound-treated samples. The mean number of plaques per 10 mm^2 explant ± SD of triplicate independent experiments are presented in Table 2. Acyclovir did not reduce the number of plaques or the number of EHV1-infected single mononuclear cells in equine *ex vivo* explants. Acyclovir did have an effect on plaque size where EHV1 plaques showed a decreased plaque latitude when explants were treated with 32 μM acyclovir. A-5021 at concentrations of 8 and 32 μM reduced the total number of plaques by 100%. Representative confocal photomicrographs of EHV1 plaques in mock-, acyclovir- or A-5021-treated equine *ex vivo* explants are illustrated in Fig. 1. The EC_{50} value of A-5021 varied between 2.1 ± 2.1 μM for 03P37 and 4.4 ± 3.8 μM for 97P70. No EHV1-infected single mononuclear cells could be observed below the basement membrane when EHV1-infected explants were treated with 32 μM A-5021. EHV1-infected single cells were still present in EHV1-infected explants treated with A-5021 at concentrations of 8 and 2 μM (data not shown). Ciliary beating of the equine nasal respiratory explants remained intact and no histological or morphological alterations could be observed during antiviral treatment.

Table 1
Anti-EHV1 activity of acyclovir, PMEDAP and A-5021 in EEL cells.

Treatment		# plaques/1.2×10^6 cells	
		97P70	03P37
Mock		88 ± 22	86 ± 15
Acyclovir	0.5 μM	87 ± 21	88 ± 17
	1 μM	79 ± 11	84 ± 11
	2 μM	70 ± 18	65 ± 6.7
	4 μM	65 ± 4.2*	71 ± 9.5
	8 μM	65 ± 11	74 ± 11
	16 μM	54 ± 6.6*	53 ± 7.5*
	32 μM	31 ± 5.2*	39 ± 3.1*
PMEDAP	0.5 μM	68 ± 3.6	72 ± 9.0
	1 μM	72 ± 5.7	77 ± 9.1
	2 μM	58 ± 20	67 ± 16
	4 μM	50 ± 12*	59 ± 8.3*
	8 μM	51 ± 15*	53 ± 7.2*
	16 μM	29 ± 9.5*	37 ± 16*
	32 μM	13 ± 7.2*	20 ± 12*
A-5021	0.5 μM	51 ± 9.0*	58 ± 11*
	1 μM	47 ± 17*	48 ± 10*
	2 μM	37 ± 12*	38 ± 4.6*
	4 μM	18 ± 7.0*	34 ± 14*
	8 μM	4.7 ± 2.1*	17 ± 5.7*
	16 μM	1.3 ± 0.58*	4.3 ± 2.5*
	32 μM	0.33 ± 0.58*	1.3 ± 1.5*

Data represent mean values ± SD of triplicate independent experiments.
* Indicate statistically significant differences ($P \leqslant 0.05$) between the absolute number of plaques of antiviral-treated and mock-treated EHV1-infected cells.

Table 2
Anti-EHV1 activity of acyclovir and A-5021 in equine nasal mucosal explants.

Treatment		# plaques/10 mm^2	
		97P70	03P37
Mock		9.8 ± 10	21 ± 24
Acyclovir	2 μM	10 ± 7.1	26 ± 15
	8 μM	13 ± 5.9	42 ± 39
	32 μM	8.6 ± 4.4	22 ± 21
A-5021	2 μM	9.8 ± 7.9	9.5 ± 8.6
	8 μM	0.00 ± 0.00*	0.00 ± 0.00*
	32 μM	0.00 ± 0.00*	0.00 ± 0.00*

Data represent mean values ± SD of triplicate independent experiments.
* Indicate statistically significant differences ($P \leqslant 0.05$) between the absolute number of plaques of antiviral-treated and mock-treated EHV1-infected equine nasal mucosal explants.

4. Discussion

The currently available EHV1 vaccines are only capable of reducing, not preventing, viral shedding and clinical signs. An adequate vaccine should protect the horse against viremia and thus against severe EHV1-induced symptoms such as abortion and equine herpes myeloencephalopathy (EHM). Antiviral therapy against EHV1 is limited to experimental antiviral drugs and there exists no rigorous scientific evidence for their efficacy. Improved or new vaccines and/or antiviral drugs are urgently needed (Kydd et al., 1994; Friday et al., 2000; Wong and Scarratt, 2006; Henninger et al., 2007; van der Meulen et al., 2007).

A-5021 has been reported to be markedly superior to acyclovir against HSV1 and HSV2, VZV, CMV, EBV and HHV-6A and HHV-6B *in vitro* and against HSV1 and HSV2 infection in mouse models (Iwayama et al., 1998, 1999; Ono et al., 1998; Neyts et al., 2001). We therefore evaluated the antiviral activity of A-5021 against EHV1.

We demonstrated that A-5021 is roughly 15-fold more potent than acyclovir against EVH1 than *in vitro*. The potent activity of A-5021 against EHV1 was further corroborated in infected explants of the equine nasal respiratory mucosal explant model. This model was recently established at our laboratory (Vandekerckhove et al., 2009) and is a valuable and reproducible homologous model to study early events of EHV1 infection. Moreover, the model enables

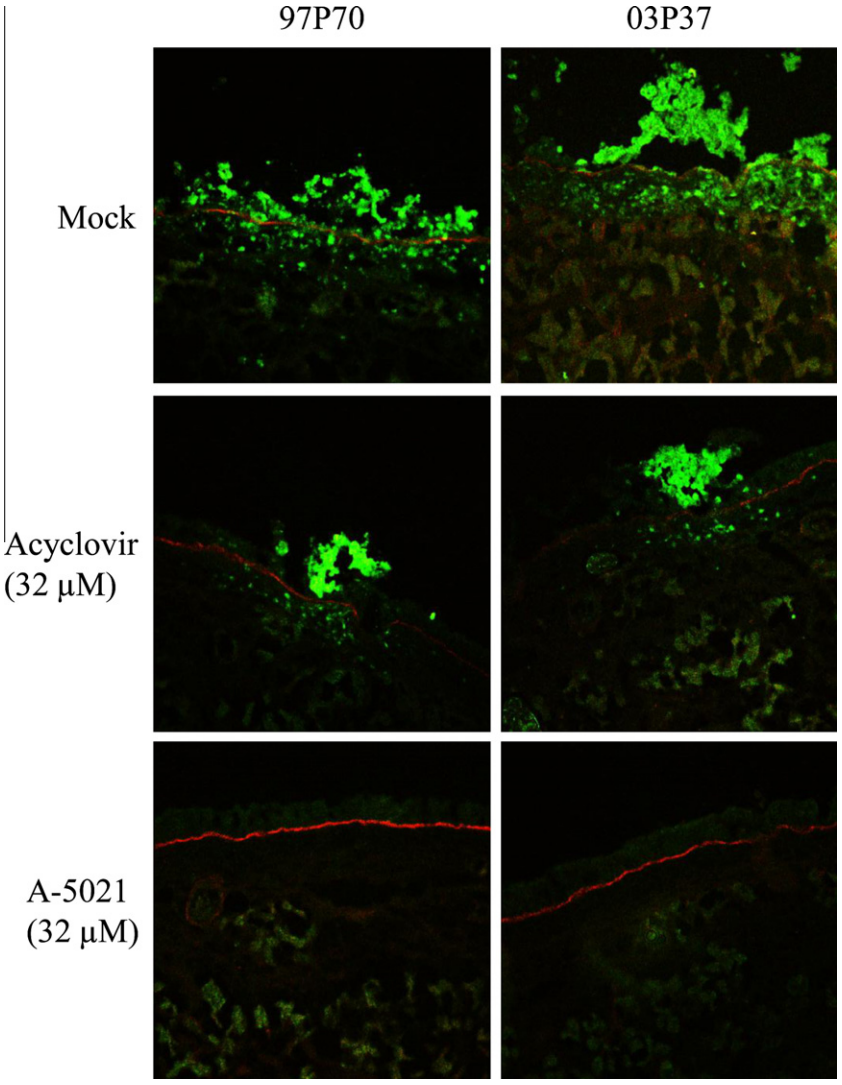

Fig. 1. Representative confocal photomicrographs of viral plaques and single viral-infected cells at 48 h pi in mock-treated, 32 μM acyclovir-treated or 32 μM A-5021-treated equine nasal respiratory *ex vivo* explants (objective 20×) for the EHV1-isolates 97P70 and 03P37. Collagen VII is visualized by red fluorescence. Green fluorescence visualizes EHV1 antigens. For mock- and acyclovir-treated explants, both viral plaques and single viral-infected cells could be detected for both isolates, whereas no viral plaques or single viral-infected cells were present in explants treated with 32 μM A-5021. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the study of differences between non-neurovirulent and neurovirulent strains (Vandekerckhove et al., 2010) and between EHV1 and EHV4 (Vandekerckhove et al., 2011). No EHV1-induced plaques were observed in the epithelium of explants infected with either the non-neurovirulent strain 97P70 or the neurovirulent strain 03P37 when treated with 8 or 32 μM of A-5021. Moreover, no single EHV1-infected mononuclear cells were observed below the basement membrane when EHV1-infected explants were treated with 32 μM of A-5021. EHV1 spreads plaque-wise in the epithelium (plaques remain confined to the epithelium), whereas the virus spreads in the lamina propria below the basement membrane via single EHV1-infected monocytic cells and T lymphocytes (Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). The observation that A-5021 can impede EHV1 infection at the level of both EHV1 plaques and single EHV1-infected cells, may have important implications for the outcome of A-5021-treatment of EHV1 infections in horses. Indeed, therapy with this compound could result in both reduced respiratory disease and, importantly, protect against viremia. However, the efficacy of A-5021 treatment needs to be evaluated in the target species before any firm conclusion can be made.

Acyclovir had no effect on either plaque number or presence of EHV1-infected single mononuclear cells below the basement membrane. Treatment of horses experimentally infected with EHV1 with valacyclovir, the oral prodrug of acyclovir, did not result in an effect on clinical signs, viral shedding and viremia (Garré et al., 2009). The lack of activity of acyclovir in the explant model is in line with the lack of a protective activity in the horse. Evaluation of the antiviral effect of (novel) inhibitors in the equine nasal explant model may thus provide more predictive information on the potential protective activity of novel anti-herpesvirus drugs in the horse than classical cell culture assays.

In summary, A-5021 efficiently inhibits EHV1 replication, and consequently reduces viral load, in an *in vitro* cell monolayer model and in an *ex vivo* explant model. It will be very interesting to evaluate the effect of this nucleoside analog for its potential to inhibit EHV1 replication in the natural host, the horse, as neither effective prophylactic nor therapeutic measures are currently available. As dosages cannot be extrapolated from dosages recommended in other species, an appropriate dosage regime should be designed by pharmacokinetic studies in horses. Moreover, taken the strong antiviral activity of A-5021 in the explant system into account,

diffusion of the drug into relevant tissues, the respiratory epithelium, should be studied. It may be worth testing whether intranasal administration would be an alternative to systemic administration of the drug. It may also be of interest to evaluate whether late start of treatment (i.e. a few days after challenge) is still efficacious in reducing EHV1 replication. Given the fact that drugs such as acyclovir, ganciclovir and cidofovir are effective against severe herpesvirus (HSV1, HSV2 and HCMV) infections in man, it is reasonable to expect that another nucleoside analog such as A-5021 (a proven potent inhibitor of EHV1 replication in equine tissues), might be effective in EHV1 infected horses.

Conflict of interest

Johan Neyts and Nesya Goris have a financial interest in Okapi Sciences N.V. None of the other authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

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