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MICROBIOLOGY

In vitro activity of six antiviral drugs against equid alphaherpesvirus type 1 indicates ganciclovir as promising drug for *in vivo* studies

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ABSTRACT: *Equid alphaherpesvirus type 1 (EHV-1) is distributed worldwide and is a major agent of abortion, respiratory and neurological disease in horses. No specific treatment is available for EHV-1 infection, yet the potential of antiviral therapy has been explored. In this study we investigated the in vitro activity of Acyclovir, Ganciclovir, Foscarnet, Famciclovir, Vidarabina and Cidofovir against EHV-1. For this, the MTT test was performed, in which all the tested drugs showed no toxicity up to 200µg/mL. Subsequently, different drug concentrations were submitted to viral plaque reduction assays in cell culture. The selectivity index (SI) of the compounds was determined using the cytotoxic concentration for 50% of cells (CC_{50}), obtained by MTT, and effective drug concentration to inhibit by 50% the number of viral plaques (EC_{50}). Ganciclovir (SI: 490; EC_{50}: 1.9 µg/mL) was the most efficient and safest drug against EHV-1, followed by Cidofovir (SI: 150, EC_{50}: 5.7µg/mL), Acyclovir (SI: 37.4, EC_{50}: 22.2µg/mL), Famciclovir (SI: 25.1, EC_{50}: 24.5µg/mL), Vidarabine (SI: 12.2, EC_{50}: 40.9µg/mL) and Foscarnet (SI: 6.9, EC_{50}: 49.5 µg/mL), respectively. These results indicated that Ganciclovir (followed by Cidofovir), is a promising candidate for use in in vivo experiments.*
Key words: *EHV-1, plaque assay, Acyclovir, Ganciclovir, Foscarnet, Famciclovir, Vidarabine, Cidofovir.*

A atividade *in vitro* de seis drogas antivirais contra o alfaherpesvírus equino tipo 1 indica que o ganciclovir é uma droga promissora para estudos *in vivo*

RESUMO: *O alfaherpesvírus equino tipo 1 (EHV-1) está amplamente distribuído nos rebanhos equinos de todo o mundo e é um dos principais agentes causadores de abortos, doença respiratória e neurológica em equinos. Ainda não há tratamento específico para a infecção pelo EHV-1 em equinos, mas o potencial da terapia antiviral tem sido investigado. Neste trabalho, foi investigada a atividade anti-herpética in vitro dos fármacos Aciclovir, Ganciclovir, Foscanet, Famciclovir, Vidarabina e Cidofovir frente ao EHV-1. Para isso, foi realizado o teste de MTT, em que todas as drogas não apresentaram citotoxicidade até a dose de 200µg/mL. A seguir, diferentes concentrações dos fármacos foram submetidas ao teste de redução de placas virais em cultivo celular. O índice de seletividade (IS) dos compostos foi determinado usando a concentração citotóxica para 50% dos cultivos celulares (CC_{50}), obtida pelo MTT, e pela concentração dos fármacos efetiva para inibir em 50% o número de placas virais (EC_{50}). O Ganciclovir (IS: 490; EC_{50}: 1,9µg/mL) foi o mais eficiente e seguro frente ao EHV-1, seguido pelo Cidofovir (IS: 150; EC_{50}: 5,7 µg/mL), Aciclovir (IS: 37,4; EC_{50}: 22,2µg/mL), Famciclovir (IS: 25,1; EC_{50}: 24,5µg/mL), Vidarabina (IS: 12,2; EC_{50}: 40,9µg/mL) e Foscarnet (IS: 6,9; EC_{50}: 49,5µg/mL). Estes resultados indicam que o Ganciclovir constitui-se em um candidato para uso em experimentos in vivo.*
Palavras-chave: *EHV-1, ensaio de placa, Aciclovir, Ganciclovir, Foscarnet, Famciclovir, Vidarabina, Cidofovir.*

INTRODUCTION

Viruses belonging to the family *Herpesviridae* affect a wide range of mammals, and the subfamily *Alphaherpesvirinae* harbors the main herpesviruses of veterinary importance, including *Equid alphaherpesvirus 1* (EHV-1) (ICTV, 2016). This virus produces acute and latent infection in horses, whose reactivation and virus shedding may occur under immunosuppression and stressful situations (WALTER et al., 2013).

The EHV-1 presents a worldwide distribution, and in Brazil, the first isolation occurred in 1966 in São Paulo State (NILSON & CORRÊA, 1966). Since then, several reports of virus isolation and antibody detection have been described,

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demonstrating the wide distribution of EHV-1 in the country (WEIBLEN et al., 1994; HEINEMANN et al., 2002; DIEL et al., 2006; LARA et al., 2010).

The EHV-1 is one of the main agents involved in outbreaks of abortion in horses, and it is also associated with respiratory and neurological disease, causing important economic losses in breeding horses, mainly in pregnant and young animals (LUNN et al., 2009). The EHV-1-associated myeloencephalopathy (EHM) is a neurological condition considered emergent by the United States Department of Agriculture (USDA, 2007). The disease may affect a large number of animals, similar to those that occurred in riding schools, racetracks and veterinary hospitals throughout North America and Europe (HENNINGER et al., 2007; WALTER et al., 2013). Cases of EHM and respiratory disease may occur even in vaccinated herds, in spite of vaccination since the efficacy of vaccines is questioned (PUSTERLA et al., 2009). Thus, in addition to vaccination, it is necessary to implement sanitary and management measures to susceptible and/or affected animals, aiming to reduce virus introduction and dissemination in equine herds, since there is no specific treatment for EHV-1 infection (LUNN et al., 2009).

Anti-herpetic therapy is a common practice in human medicine, yet is rarely used against animal herpesviruses. In this sense, Acyclovir has been the main drug used to treat animal viral infections, as BoHV-1, FeHV-1 and EHV-1 and EHV-4. This drug presented varied efficacy *in vivo* when used according to the required concentration to reduce 50% of viral plaques (EC_{50}) (VISSANI et al., 2016). Although, there are some investigations regarding to anti-EHV-1 activity *in vitro* using human anti-herpetics drugs (GARRÉ et al., 2007; AZAB et al., 2010), there is no defined guidelines on formulations available for treatment. Thus, more detailed *in vitro* studies are needed to verify the possibility of *in vivo* therapy using these drugs. Therefore, the aim of this research paper was to investigate, the susceptibility of EHV-1 *in vitro* to six antiviral drugs used in human medicine.

MATERIALS AND METHODS

Experimental design

The *in vitro* susceptibility of EHV-1 to antiviral drugs Acyclovir (ACV), Ganciclovir (GCV), Foscarnet (PFA), Famciclovir (FAM), Vidarabine (VID) and Cidofovir (CDV) was evaluated by plaque reduction assay (PRA). Initially, the cellular toxicity of the drugs was investigated by MTT. Then, the PRA was performed testing each drug against EHV-1. Lastly, the selectivity index (SI) of each compound was determined.

Cells and virus

Vero cells (*African Green Monkey kidney* – ATCC CCL-81, passage 65) was used for amplification and quantification of EHV-1 and herpesvirus simplex type 1 (HSV-1), cytotoxicity and PRA. Cells were cultured in RPMI medium (Roswell Park Memorial Institute), supplemented with 10% fetal bovine serum (FBS), antibiotics (streptomycin 0.4mg/mL; penicillin 1.6mg/mL) and antifungic (amphotericin B 0.0025mg/mL). The strain EHV-1 Kentucky D (p.10, Genbank: AB279610), kindly provided by Dr. Rodrigo Franco (Instituto Butantan, SP, Brasil) was tested against the six drugs. The HSV-1 KOS strain (Dr. Paulo Michel Roehe, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil) was used as control of drug efficacy.

Antiviral drugs

Six commercial anti-herpetic drugs used to treat human herpetic infections were used: ACV (molecular weight - MW 225.21; Dermapelle Farmácia de Manipulação, Santa Maria, RS, BR), GCV (MW 255.2; Sigma-Aldrich, St Louis, MO, USA), PFA (MW 300.04; Sigma-Aldrich, St Louis, MO, USA), FAM (MW 321.3; Sigma-Aldrich, St Louis, MO, USA), VID (MW 285.2; Sigma-Aldrich, St Louis, MO, USA) and CDV (MW 279.1; Sigma-Aldrich, St Louis, MO, USA). Drugs were diluted to 1mg/mL in saline solution (ACV); saline solution with 0.5% dimethyl sulfoxide (DMSO) (GCV); ultrapure water (PFA, CDV e VID); or only in dimethyl sulfoxide (DMSO) (FAM).

Cytotoxicity assay

Cytotoxicity of the six drugs was determined by MTT test (*3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*) adapted from GARRÉ et al. (2007). For this, Vero cells ($2x10^6$ cells/ 96-well plates) were incubated at 37ºC with CO_2 at 5% and, after 24h, five different dilutions of each drugs were added onto cell monolayers (1; 10; 100; 200 and 500µg/mL). After 72h of incubation, the supernatant was removed and 50µL/well of MTT was added, followed by incubation for 2h at 37ºC in a CO_2 incubator at 5%. Then, the MTT was replaced by 150µL/well of DMSO, and absorbance (in optical density - OD) was measured at a wave-length of 550nm in microplate reader. Cell viability at each drug concentration was determined by the formula: *% viable cells = average OD (antiviral)*

x 100/average OD (controls). These results allowed determining the cytotoxic concentration to 50% of the cells (CC_{50}), by linear regression analysis. According to GARRÉ et al. (2007), in the MTT test, the evaluated drug concentration is considered cytotoxic when the cell viability is less than 80% when compared to controls.

Plaque reduction assays (PRA)

The antiviral activity of the drugs against EHV-1 and HSV-1 was determined by PRA, using Vero cells seeded in six-well polystyrene plates ($0.3x10^6$ cells/well). Initially, three wells of Vero cells monolayers received 1mL of RPMI containing the different drug concentrations (0.1; 1; 10; 25; 75 and 100µg/mL) and incubated for 1h at 37ºC in a CO_2 incubator at 5%. Two other wells were maintained as viral and cellular controls, receiving 1mL of RPMI. Next, the supernatant was removed, and 1mL of virus suspension containing 100 plaque forming units (PFU) of the respective virus was added. After 2h of adsorption, the inoculum was removed and cell monolayers were covered with 3mL of solid medium culture, containing RPMI, 10% fetal bovine serum and 1% low melting point agar, besides the different concentrations (µg/mL) of each drug. The plates were incubated at 37ºC in a CO_2 incubator at 5%. After 72h, the solid medium was removed and the infected monolayers were fixed and stained with 2ml/well of violet crystal solution and formalin (0.65g violet crystal; 62.5ml formalin and 500ml water q.s.p.). Finally, viral plaques were counted in both treated and control cells.

The inhibitory effect of antiviral compounds was determined by the formula: *% inhibition of viral replication = 1 - (mean number of viral plaques in the wells with antiviral/ mean number of viral plaques in the non-antiviral wells) x 100*. The concentration of each effective drug to inhibit 50% of the viral plaques (EC_{50}) and the selectivity index (SI) were calculated by linear regression analysis, considering line equation appropriate when the value R^2 was equal to or greater than 0.9. To EC_{50}, the line equation used was: $y = ax - b$ (where: y = EC_{50} e x = % inhibition of viral plaques). The SI is the relationship between drug CC_{50} (acquired by MTT) and EC_{50}. The value obtained in SI allowed estimating drug safety level for use in animals. The higher is the value (above 1), more significant will be the difference between the antiviral dose necessary to reduce in 50% the virus replication (EC_{50}) and cytotoxic dose (CC_{50}); therefore, safer the drug for use in animals (COEN & RICHMAN, 2007; DEZENGRINI et al., 2010).

Data analysis

Two independent tests were conducted, in triplicate, for each experiment. The data obtained was statistically analyzed by the analysis of variance (ANOVA) through the program GraphPad Prism 6.

RESULTS AND DISCUSSION

In the MTT tests performed with Vero cells, only the drugs Ganciclovir and Cidofovir did not present cytotoxicity at the concentration of 500µg/mL. These results are compatible with those obtained by GARRÉ et al. (2007). However, all evaluated drugs promoted cell viability greater than 80% when 200µg/mL was used. Then, all assays were performed using lower doses than 200µg/mL of each drug.

At PRA, the most effective drug against EHV-1 was GCV ($EC_{50:}$ 1.9), followed by CDV ($EC_{50:}$ 5.7), ACV ($EC_{50:}$ 22.2), FAM ($EC_{50:}$ 24.5), VID ($EC_{50:}$ 40.9) and PFA ($EC_{50:}$ 49.5), respectively (Table 1, Figure 1). Most drugs completely inhibited HSV-1 replication at concentrations 75 and 100µg/mL, except FAM that did not show complete antiviral activity at the concentration of 75µg/mL. The HSV, prototype of the subfamily *Alphaherpesvirinae*, is widely used as control to development of antiviral chemotherapy/drugs, because it was the first virus effectively treated using an antiviral compound (Idoxuridine) (PRUSOFF, 1959); and, therefore, it was used as control of the antivirals tested.

The GCV presented lower EC_{50} resulting in the highest SI (Table 1). Then, it was the most effective and safest drug tested. The result obtained for this drug resembles that observed by MEULEN et al. (2006) and for GARRÉ et al. (2007), who evaluated the susceptibility of six antiviral drugs against FeHV-1 and EHV-1, using feline kidney cells (CRFK) and equine embryonic lung cells (EEL), respectively. The CDV also induced notable decrease in the number of viral plaques, differing from the results obtained by GARRÉ et al. (2007), where this drug was not highly efficient in inhibiting virus replication; although, it was able to significantly reduce the plaque size even when very low concentrations were used. The differences observed in both studies may be related to variable susceptibility of EHV-1 isolates used in viral inhibition assays.

MEULEN et al. (2006) and GARRÉ et al. (2007) showed that ACV had EC_{50} more than 20 times higher than GCV to FHV-1 and/or EHV-1, respectively. These results were similar to those obtained in our study, and indicated the higher efficacy of GCV compared to ACV against different virus species. Thus, GCV would

Table 1 - *In vitro* antiviral activity of Acyclovir, Ganciclovir, Foscarnet, Famciclovir, Vidarabine e Cidofovir against equine herpesvirus type 1.

Drug	CC_{50} [1]	EC_{50} [2]	SI[3]
Ganciclovir	931	1.9	490
Cidofovir	855.5	5.7	150
Acyclovir	830.7	22.2	37.4
Famciclovir	615.7	24.5	25.1
Vidarabine	500.4	40.9	12.2
Foscarnet	345.1	49.5	6.9

[1]CC_{50}: antiviral concentration in µg / mL cytotoxic to 50% of Vero cells. [2]EC_{50}: concentration of each antiviral required to inhibit the number of viral plaques in µg / mL by 50%. [3]SI: selectivity index; relationship between CC_{50} and EC_{50}.

be an attractive alternative to initiate *in vivo* experiments aiming to determine drug toxicity, bioavailability, pharmacokinetics and antiviral activity.

According to EC_{50} values, the activity of ACV and FAM against EHV-1 were similar as well as reported by MAXWELL et al. (2008). However, in horses, there is no pharmacokinetic study yet using FAM, possibly due to the high cost of the drug and the unavailability of parenteral products (MAXWELL, 2017). Though, in the study carried out by MALIK et al. (2009), FAM was a promising systemic drug for the treatment of ocular diseases attributed to FeHV-1, being more effective than ACV and its prodrug Valacyclovir. FAM resembles ACV in the structure, selectivity and mechanism of action. However, FAM has longer action, due to the longer half-life of its metabolites, formed after the uptake of the drug by the cells, thus decreasing the duration of the lesion (MAXWELL, 2017).

The drugs GCV and ACV replaced VID in the treatment of HSV infections, because they are metabolically less toxic and more stable (SHEN et al., 2009). In equine medicine, there are few reports of use of VID against EHV-1. In this study, this drug presented moderately satisfactory results, being more efficient that PFA. The PFA reduced the number of viral plaques, but presented the highest indexes of EC_{50} and lower SI for the EHV-1. These results differed from those obtained by DEZENGRINI et al. (2010), in which PFA presented the better results against BoHV-1, BoHV-2 and BoHV-5. Against EHV-1, *in vitro* antiviral activity and SI presented by PFA were lower than the other drugs tested, indicating that this drug should not be considered the main alternative for therapies and *in vivo* studies.

Results obtained by *in vitro* assays are essential for the choice of drug to be used for in *in*

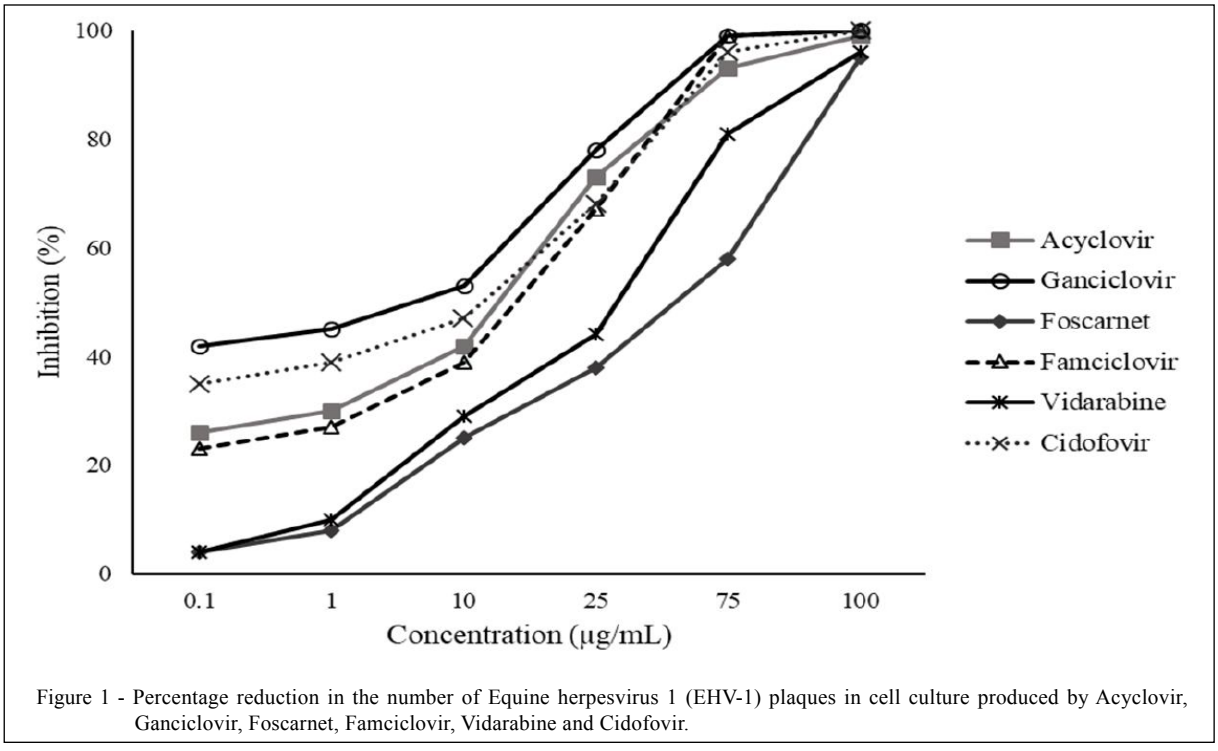

Figure 1 - Percentage reduction in the number of Equine herpesvirus 1 (EHV-1) plaques in cell culture produced by Acyclovir, Ganciclovir, Foscarnet, Famciclovir, Vidarabine and Cidofovir.

vivo studies. The use of animal models is an attractive alternative for *in vivo* studies of antiviral drugs, mainly to determine doses, administration routes, time of treatment, toxicity and drug efficacy. Experimental infection of rabbits with EHV-1 (KANITZ et al., 2015) resulted in infection and development of respiratory and neurological signs. In this sense, the initial evaluation *in vivo* of the antiviral activity of drugs such as GCV, may be performed in this animal model, as they developed respiratory disease similar to naturally infected horses.

CONCLUSION

The obtained results in plaque reduction assay indicate that the drug Ganciclovir is the safest and most effective against EHV-1. Thus, the compound has the potential to be used in *in vivo* experiments.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

RW, EFF, ALO and JFC conceived and designed experiments. ALO, APGM and JFC performed the experiments and carried out the lab analyses. ALO and JFC prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

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