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Full Length Research Paper

Antiviral activities of ellagitannins against bovine herpesvirus-1, suid alphaherpesvirus-1 and caprine herpesvirus-1

Neli Vilhelmova-Ilieva[1], Ivo Sirakov[2], Remi Jacquet[3], Stephane Quideau[3] and Angel S. Galabov[1*]

[1]Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, 26 G. Bonchev Str., BG-1113 Sofia, Bulgaria.

[2]Department of Medical Microbiology, Medical Faculty, Medical University of Sofia, 2 Zdrave Street, BG-1431 Sofia, Bulgaria.

[3]Universitéde Bordeaux, Institut des Sciences Moléculaires (CNRS-UMR 5255), Institut Européen de Chimie et Biologie, 2 rue Robert Escarpit, 33607 Pessac Cedex, France. *Corresponding author. E-mail: galabov@microbio.bas.bg. Tel: +359 888 287 103. Fax: +359 2 870 0109.

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In vitro **antiviral activity of three ellagitannins - castalagin, vescalagin and grandinin was investigated against replication of three of the most common herpes viruses infecting animals, namely bovine herpesvirus-1 (BoHV-1), suid alphaherpesvirus-1 (SuHV-1), and caprine herpesvirus-1 (CapHV-1). The cytopathic effect inhibition test *via* the neutral red uptake assay in Madin-Darby bovine kidney (MDBK) cell culture was applied. The highest activity was demonstrated by castalagin followed by vescalagin against replication of SuHV-1, strain A2, with selectivity index values of 336.8 and 309, respectively, which are close to that of acyclovir (SI = 540). The activity of grandinin against the replication of SuHV-1 A2 strain (SI = 40.8) as well as the activity of all three substances against the replication of *BoHV-1* strain K22 was lower but still significant (castalagin SI = 45; vescalagin SI = 42.5; grandinin SI = 32.3). Against the E/CH strain of CapHV-1, the three ellagitannins showed a moderate to weak activity: Castalagin SI = 19.3, vescalagin SI = 18.8, grandinin SI = 11.8. The results obtained characterize compounds tested as perspective antivirals.**

Key words: bovine herpesvirus-1, suid alphaherpesvirus-1, caprine herpesvirus-1, ellagitannins, alpha acyclovir, antiviral activity.

INTRODUCTION

Among the viruses of the *Herpesviridae* family, some are common pathogens for humans and animals. They are easily transmitted from an infectious to a healthy individual and also they have the ability to form a life-long latent infection. In the herpesvirus species that attack animals, it is important that the animals are not only a reservoir for viruses, but also that the quality of the meat from the infected animals of the point of view of food

production is deteriorated and this leads to great economic losses.

Among the most important herpesviruses infecting animals are the members of the subfamily suid *Alphaherpesvirinae* bovine herpesvirus-1 (BoHV-1), alphaherpesvirus-1 (SuHV-1), and caprine herpesvirus-1 (CapHV-1). The economic importance of these viruses lies in the high economic losses incurred due to infections and the clinical symptoms caused by them. BoHV-1 infections lead to repeat breeding, rhinotracheitis in calves, vulvovaginitis-balanoposthitis in adult animals (Haralambiev, 2002), infertility, abortions (Yilmaz et al., 2016) and neonatal mortality (Wyler et al.,1989). CapHV-1 causes a similar clinical picture, with neonatal losses of up to 80%, repeat breeding 15%, reproductive disorders in adult animals - abortions 5% in affected flocks (Sirakov 2012), and death in kids (Saito et al., 1974).

Whereas BoHV-1 and CapHV-1 affect cattle and goats, SuHV-1 causes infections in a range of various domestic and wild animals. In swine, it is associated with abortions (~35%) and mortality (3–5%) (Yu et al., 2017), stillbirths and decrease in weight gain (Gerdts et al., 1997). It affects ruminants, horses, dogs (Quiroga et al., 1998), cats and panthers (Glass et al*.,* 1994). Although some cases have been reported in humans (Mravak et al*.,* 1987; Skinner et al*.,* 2001) and that this virus has limited zoonotic potential (Khan et al., 2013), SuHV-1 to some extent has social importance, as well.

To date, there is no in general effective therapy of herpesvirus infections, nor vaccines created to avoid the formation of latent infection. Administration of anomalous nucleoside analogues is considered as the most potential by its efficacy chemotherapeutic agents used in the clinical practice, acyclovir been with largest application. A disadvantage of nucleoside analogues is the relatively faster development of drug-resistant mutants.

Limited scope of investigations was carried out on chemotherapeutic agents proved in humans against herpesvirus infections in domestic animals. Treatment with cidofovir has been shown to give good relief of clinical symptoms and a decrease in the viral shedding titers in CapHV-1 infection in goats (Tempesta et al*.,* 2008). Chervenkov et al*.* (2014) demonstrated that aqueous extracts of *Melissa officinalis* L. (*Lamiaceae*) have a marked antiviral effect against SuHV-1. Antiviral activity against this virus, as well as against BoHV-1, has also been shown by lambda-carrageenan prepared from red seaweed (Diogo et al., 2015). Moreover, Lisov et al*.* (2015) demonstrated that 2,5-dihydrobenzoic acid–gelatin conjugate has an anti-BoHV-1 effect and suggested that this agent would also be effective against other alphaherpesvirus subfamily members due to the similar mode of entry into the host cells. Some studies on the spread and replication of herpesviruses have been conducted using natural substances found in many foods that a person consumes daily.

Recently a special interest as anti-herpetic agents are the tannins which are a group of polyphenols, divided into two groups of condensed and hydrolysable compounds. Of the group of hydrolizable tannins are the ellagitannins. There is a lot of evidence in the literature that different types of ellagitannins show anti-herpesvirus activity (Kurokawa et al., 2001; Chattopadhyay et al*.*, 2010; Lin et al*.,* 2011).

In previous investigations we have also shown that three ellagitannins - castalagin, vescalagin and grandinin, possess remarkable activity against human herpes simplex virus (HSV)-1 and HSV-2, sensitive and resistant to acyclovir strains (Vilhelmova et al., 2011; Vilhelmova–Ilieva et al., 2014). In the present work we pay attention to the activity of these three substances on the replication of herpesvirus strains that are animal pathogens.

MATERIALS AND METHODS

Cells and viruses

Monolayer cultures of Madin-Darbey bovine kidney (MDBK) cells (CCLV 1992, RIE 261/ National Bank for Industrial Microorganisms and Cell Cultures, Sofia) were grown in Dulbeco minimal essential medium (DMEM) containing 10% bovine fetal serum (Gibco BRL, USA), supplemented with 10 mM HEPES buffer (Merck, Germany) and antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml), in CO_2 incubator (HERA cell 150, Heraeus, Germany) at 37°C/5% CO_2.

Suid alphaherpesvirus 1 (SuHV-1) strain A2, bovine herpesvirus-1 (BoHV-1), strain K 22, and caprine herpesvirus-1 (CapHV-1), strain E/CH (National Diagnostic and Research Veterinary Institute collection strains) were included in the study. The viruses were grown in MDBK cell cultivated in MEM-Eagle (Sigma–Aldrich, St. Louis, MO, USA) and MEM-Hanks (Sigma–Aldrich, St. Louis, MO, USA) supplemented with antibiotics (penicillin 100 IU/ml, streptomycin 100 IU/ml), essential amino acids and 2% fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA).

The virus titers were evaluated by the end-point dilution method, based on the cytopathic effect (CPE) inhibition and expressed as 50% cell culture infectious dose per ml ($CCID_{50}$/ml).

Compounds tested

Nonahydroxyterphenoyl-containing C-glucosidic ellagitannins: castalagin, vescalagin and grandinin, extracted from powdered pedunculate oak (that is, *Quercus robur*) heartwood and purified as previously described (Quideau and Feldman, 1996) were tested. The substances were dissolved in distilled water to a concentration of 0.01 M and then diluted in DMEM to the required concentrations. Acyclovir [9-(2-hydroxyethoxymethyl)-guanine] (ACV) was also dissolved in DMEM to the required concentration.

Antiviral activity assay

CPE inhibition test was employed on confluent cell monolayer in 96-well micro plates infected with 100 $CCID_{50}$ in 0.1 ml (MOI = 0.0025). After 1 h of virus adsorption, compounds were added in various concentrations and cells were incubated for 48 h at 37 °C. Inhibition of cytopathic effect was determined using a neutral red

Table 1. Antiviral activity of ellagitannins castalagin, vescalagin and grandinin against replication of bovine herpesvirus-1 strain K 22, suid alphaherpesvirus 1 strain A2 and caprine herpesvirus-1 strain E/CH.

Compound	SuHV-1 A2		BoHV-1 K 22		CapHV-1 E/CH	
	IC_{50} µM/ml	SI	IC_{50} µM/ml	SI	IC_{50} µM/ml	SI
Castalagin	0.16 ± 0.073$^{***,\wedge\wedge\wedge}$	336.8	1.2 ± 0.12$^{***,<<<}$	45.0	2.8 ± 1.61$^{***,>>>}$	19.3
Vescalagin	0.22 ± 0.068$^{***,\wedge\wedge\wedge}$	309.0	1.6 ± 0.62$^{***,<<<}$	42.5	3.6 ± 2.11$^{***,>>>}$	18.8
Grandinin	0.87 ± 0.24$^{***,\#}$	40.8	1.1 ± 1.07$^{***,<<<}$	32.3	3.0 ± 3.2$^{***,>>>}$	11.8
ACV	2.4 ± 0.55$^{\wedge\wedge\wedge}$	540.0	3.8 ± 0.84$^{<<<}$	341.0	18.4,$^{>>>}$	70.4

*** p<0.0001, when comparing the values of each ellagitannin with that of ACV for the same strain. $^{\wedge\wedge\wedge}$ p<0.0001, when comparing the values against SuHV-1 (A2) and BoHV-1 (K22) strains of each substance. $^{<<<}$ p<0.0001, when comparing the values against BoHV-1 (K22) and CapHV-1 (E/CH) strains of each substance. $^{>>>}$ p<0.0001, when comparing the values against SuHV-1 (A2) and CapHV-1 (E/CH) strains of each substance. $^{\#}$ p>0.05, when comparing the values against SuHV-1 (A2) and BoHV-1 (K22) strains of each substance.

uptake assay (Borenfreund and Puerner, 1984). The IC_{50} values of ellagitannins were evaluated, that is, the concentrations that inhibited CPE development by 50%. The compounds cytotoxicity CC_{50} (50% cytotoxicity) values were used to determine the selective index (SI) values of the compounds tested. Cytotoxicity of ellagitannins and ACV was determined on MDBK cells in our previous study (Vilhelmova et al., 2011).The values of CC_{50} of the substances were as follows: castalagin - 53.9 µM/ml, vescalagin - 68.0 µM/ml, grandinin - 35.5 µM/ml and ACV - 1296.0 µM/ml.

Statistical analysis

Data on compounds antiviral effects were analyzed statistically. The values of IC_{50} are presented as means ± SD. The significant differences between the effects of each ellagitannin on each virus strain are compared with the corresponding acyclovir value; also those between the effects of each compound on the different strains were done through the One-Way ANOVA where p-values of <0.05 were considered as significant.

RESULTS

The IC_{50} values of ellagitannins tested against the three herpesviruses (SuHV-1, BoHV-1 and CapHV-1) are presented in Table 1. All three ellagitannins show the highest activity against SuHV, strain A2. Among them the antiviral effect of castalagin attained a pronounced value, SI = 336.8, followed by magnitude by the vescalagin's effect, SI = 309. The activity of both substances was close to that of ACV SI = 540. Grandinin manifested a marked activity but was substantially weaker.

The three ellagitannins demonstrasted a marked activity against BoHV-1, strain K22, with close SI values.

The most active was castalagin with SI = 45, followed by vescalagin with SI = 42.5, and weakest activity was shown by grandinin - SI = 32.3. Here, it should be noted that, unlike the results obtained against SuHV-1, catalagin and vescalagin manifested significantly lower activity, while grandinin retained almost the same activity as that of ACV against SuHV-1. The weakest activity of the three ellagitannins was demonstrated against the replication of CapHV-1, E/CH strain. The values of the selective indices of castalagin, vescalagin and grandinin were SI = 19.3, SI = 18.8, and SI = 11.8, respectively. It could mark that the activity of acyclovir against this virus was lower than that established vs BoHV-1 and SuHV-1, although it was significantly superior (SI = 70.4) compared to the effect of ellagitannins.

DISCUSSION

Having in mind that the ellagitannins castalagin, vescalagin and grandinin, studied in the present work, were tested against viruses belonging to the subfamily *Alphaherpesvirinae* of the *Herpesviridae* family, it is not surprising that they manifested activities similar to that found against the subfamily members attacking humans (HSV-1 and HSV- 2) (Vilhelmova et al., 2011). When comparing the activity of ellagitannins on the replication of the SuHV-1 strain and their activity against human herpesvirus strains, it is noted that their activity is close to that observed with HSV-1 (Victoria strain) (Vilhelmova et al., 2011).

On the other hand, the activity of the tested ellagitannins on replication of BoHV-1 resembles their activity against HSV-2 (strain Bja) (Vilhelmova et al., 2011), although with lower values and that of castalagin and vescalagin was close to that of grandinin. Evidently, anti-BoHV-1 activity was considerably inferior when compared with the activity against SuHV-1. Similar dependence was observed in the ellagitannins activity against replication of CapHV-1 strain, where the activity was significantly lower and the activity of grandinin can be said to be insignificant.

Different authors have conducted various experiments to determine the viral replication stage inhibited by ellagitannins. Some ellagitannins have been shown to attack the extracellular virions (virucidal effect); others have an effect on the adsorption and entry of the virus into the host cell. There are ellagitannins that specifically inactivate viral DNA polymerase and inhibit replication of viral DNA or influence specific viral proteins necessary for viral replication (Tan et al., 2013). The results of our

study on the mode of anti-herpesvirus action of castalagin on the model of HSV-1 (Victoria strain) are in line with these data. The compound has been previously shown to have (i) a marked direct inactivating effect on extracellular virus; (ii) an inhibitory effect on virus adsorption; (iii) suppression of early steps in the viral replication cycle (Vilhelmova-Ilieva et al*.,* 2013). There are conflicting reports about the effect of acyclovir as the sole antiviral therapy against CapHV-1. Elia et al*.* (2015) observed no antiviral effect, whereas Camero et al. (2017) report good inhibitory activity. Camero et al.
(2017) explained these contradictory results to the different time of acyclovir application in the experimental setup. Both studies, however, show good antivirus activity of acyclovir in combination with mizoribine. It was established that there is a synergistic combined effect of acyclovir and mizoribine on the replication of strain CapHV-1 (Elia et al., 2015, Camero et al*.*, 2017) and on the human HSV-1 (Pancheva et al., 2002). In these previous experiments, acyclovir was used in sub-inactive concentrations, and with the addition of mizoribine a strong synergistic effect was obtained. This effect was mizoribine dose dependent.

Developing effective therapies to suppress herpes viral replication is important because the herpes infection of animals has not only economic significance for human infections but also for food production. In recent years, more and more attention has been paid to the ability of some herpes viruses to pass between species' barrier. In these cases, a virus that specifically infects a particular animal can also infect other species, and in some cases it can also be transmitted to humans (Reperant et al*.,* 2016). Data presented in this study enlarge the scope of ellagitannins' effects on replication of herpesviruses of *Alphaherpesvirinae* subfamily. It would be of interest to see the activity of these compounds against herpesviruses not belonging to this subfamily. Among the pathogens with economic importance are gallid herpesvirus 1 (laryngotracheitis virus), ovine herpesvirus 2 (malignant catarrhal fever virus), equine herpesviruses 1, 4 and 9 and cyprinid herpesvirus 3. So, experimental chemotherapeutic investigations including these viruses could be considered as a target of primary importance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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