Archives of Virology (2020) 165:1385–1396
https://doi.org/10.1007/s00705-020-04562-1

ORIGINAL ARTICLE

Elucidation of the mechanism of anti-herpes action of two novel semisynthetic cardenolide derivatives

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Received: 6 November 2019 / Accepted: 22 January 2020 / Published online: 29 April 2020

Abstract

Human herpesviruses are among the most prevalent pathogens worldwide and have become an important public health issue. Recurrent infections and the emergence of resistant viral strains reinforce the need of searching new drugs to treat herpes virus infections. Cardiac glycosides are used clinically to treat cardiovascular disturbances, such as congestive heart failure and atrial arrhythmias. In recent years, they have sparked new interest in their potential anti-herpes action. It has been previously reported by our research group that two new semisynthetic cardenolides, namely **C10** (*3β-[(N-(2-hydroxyethyl)aminoacetyl] amino-3-deoxydigitoxigenin)* and **C11** (*3β-(hydroxyacetyl)amino-3-deoxydigitoxigenin*), exhibited potential anti-HSV-1 and anti-HSV-2 with selectivity index values > 1,000, comparable with those of acyclovir. This work reports the mechanism investigation of anti-herpes action of these derivatives. The results demonstrated that **C10** and **C11** interfere with the intermediate and final steps of HSV replication, but not with the early stages, since they completely abolished the expression of the UL42 (β) and gD (γ) proteins and partially reduced that of ICP27 (α). Additionally, they were not virucidal and had no prophylactic effects. Both compounds inhibited HSV replication at nanomolar concentrations, but cardenolide **C10** was more active than **C11** and can be considered as an anti-herpes drug candidate including against acyclovir-resistant HSV-1 strains.

Abbreviations

ACV	acyclovir
CC_{50}	50% cytotoxic concentration
CMC	carboxymethylcellulose
DEX-S	dextran sulfate
FBS	fetal bovine serum
HIV	human immunodeficiency virus
HSV-1	*herpes simplex virus* type 1
HSV-2	*herpes simplex virus* type 2
HPV	human papillomavirus
IC_{50}	concentration that inhibited 50% of viral replication
MEM	Eagle's minimum essential medium
MOI	multiplicity of infection
PBS	phosphate-buffered saline
PFU	plaque-forming units
SI	selectivity index

Handling Editor: Zhongjie Shi.

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Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are alphaherpesviruses that infect humans. They have an enveloped icosahedral capsid containing a proteinaceous tegument and a linear DNA genome. For replication, it is necessary that the viruses adsorb and penetrate host cells to express α genes (immediate early phase), which mainly regulate viral replication, such as ICP27; β genes (early phase), which are involved in synthesis and packaging of DNA, such as UL42; and γ genes (late phase), which synthesize structural components of the virion, such as gD [1].

HSV-1 and HSV-2 infections are common and ubiquitous, with a considerable social impact independently of

geographical region or population socioeconomic status, and are generally associated with ocular, orofacial and genital tract infections [2]. Most of the drugs used to treat herpesvirus infections are nucleoside analogs that share the same mechanism of action, affecting viral DNA synthesis by inhibiting viral DNA polymerase, such as acyclovir (ACV), the gold standard in the treatment of herpetic infections [3]. Such infections have become an important public health issue, mainly due to the HSV ability to cause acute and recurrent infections, as well as the emergence of resistant strains, which hinder the management of herpesvirus infections [4–6]. New anti-herpes drugs are therefore needed.

Cardenolides are cardiac glycosides mainly found in plant species, such as *Nerium oleander* L., *Asclepias curassavica* L. (Apocynaceae), *Digitalis lanata* Ehrh., and *Digitalis purpurea* L. (Plantaginaceae). These compounds have been used clinically for over 200 years to treat heart diseases [7] and are characterized by their powerful cardiotonic action [8]. The mechanism of their cardiotonic action occurs through the inhibition of Na^+/K^+-ATPase, involved in the Na^+/K^+ pump mechanism dependent on these ions, and promoting cardiac muscle contraction [9].

Despite the wide use of cardenolides as positive inotropic agents, the investigation of their effects on other pathologies has intensified in recent years, disclosing new potential therapeutic applications. Among them, their cytotoxic and antitumor effects recently reviewed by Cerella et al. [10], De et al. [11], Diederich et al. [12], Schneider et al. [13] and El-Seedi et al. [14], as well as their anti-inflammatory [15], antiprotozoal [16], anti-oxidant and anti-aging [17] activities can be cited. Another suggested possibility is their potential antiviral action, as reported by several authors against adenovirus [18], chikungunya virus [19], coronavirus [20, 21], cytomegalovirus [22–24], dengue virus [25], herpes virus [26–28], HIV [29–31], human papillomarivus (HPV) [32], influenza virus [33–35], and respiratory syncytial virus [36] replication. The effects of six well-known cardiac glycosides (digoxin, digitoxin, ouabain, convallatoxin, G-strophanthin and lanatoside C) on viral biology and the mechanisms by which they impair the replication of different RNA and DNA viruses were recently compiled [37]. Most of these studies only reported the antiviral activity of cardenolides, and it is required to understand the mechanistic aspects involved and how the compounds really act to further evaluate their potential therapeutic application.

As mentioned above, new antiviral therapies are currently needed, mainly for treating drug-resistant infections. In this sense, our research group had therefore conducted studies with natural [26] and semisynthetic cardenolides [38]. In previous work, we reported the semisynthesis of 16 new derivatives based on the scaffold of digitoxigenin and demonstrated their anti-HSV-1 (KOS and 29-R strains) and anti-HSV-2 (333 strain) activities. Two derivatives emerged as the most promising compounds from this screening – **C10** (*3β-[(N-(2-hydroxyethyl)aminoacetyl]amino-3-deoxydigitoxigenin*) and **C11** (*3β-(hydroxyacetyl)amino-3-deoxydigitoxigenin*) – and they were selected in the present study for detailed investigation of the mechanism of their anti-HSV action.

Materials and methods

Compounds, viruses and cell line

C10 and **C11** (Fig.1) were synthesized, and their chemical structures and purity were confirmed as described previously [38], within an ongoing Brazil-Germany bilateral partnership.

All assays were performed on Vero cells (ATCC: CCL81) grown in Eagle's minimum essential medium (MEM; Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

HSV-1 (KOS and 29-R strains, which are ACV-sensitive and ACV-resistant, respectively; Faculty of Pharmacy, University of Rennes I, Rennes, France) and HSV-2 (333 strain; Department of Clinical Virology, Göteborg University,

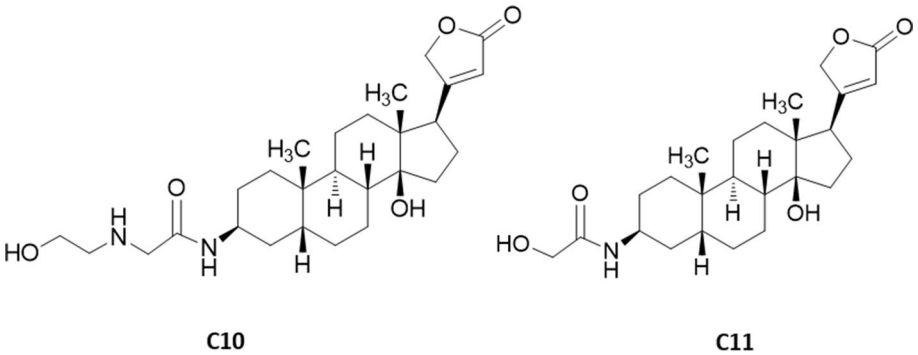

Fig. 1 Chemical structures of **C10** and **C11**

Göteborg, Sweden) viral stocks were propagated on Vero cells. They were titrated based on plaque-forming units (PFU), counted by plaque assay as described previously by Burleson et al. [39], and stored at -80 °C.

Evaluation of the anti-herpes mechanism of action

C10 and **C11** were selected for study based on their high selectivity indices ($SI = CC_{50} / IC_{50}$) obtained from anti-herpes screening as reported by Boff et al. [38]. The elucidation of their mechanisms of action was performed using the assays described below.

Plaque number reduction assay

This assay was performed as described by Boff et al. [40]. Briefly, confluent cell monolayers (2.5×10^5 cells per well) were infected with approximately 100 PFU of each virus strain [HSV-1 (KOS and 29-R strains) and HSV-2 (333 strain)] for 1 h at 37 °C. Treatments were performed by adding non-toxic concentrations (0.0625, 0.125, 0.25, 0.5 and 1.0 µM) of the samples after virus infection (post infection treatment). Cells were then washed with phosphate-buffered saline (PBS), overlaid with MEM containing 1.5% carboxymethylcellulose (CMC; Sigma-Aldrich, St. Louis, MO, USA) in the presence or absence of the samples, and incubated for 48 h. Cells were fixed and stained with naphthol blue-black (Sigma-Aldrich), and viral plaques were counted using a stereomicroscope. The concentration of each sample that reduced viral replication by 50% (IC_{50}) when compared to untreated controls was estimated. Acyclovir (ACV) was used as a positive control.

Virucidal assay

This assay followed the procedures described by Silva et al. [41]. Mixtures of equal volumes of **C10** and **C11** at ten different concentrations (1/5 to 100 × IC_{50}) and 4×10^4 PFU of HSV-1 or HSV-2 in serum-free MEM were co-incubated for 15 min at 4 °C or 37 °C. **C10** and **C11** were then diluted to non-inhibitory concentrations (1:100) to determine the residual infectivity by plaque number reduction assay as described above.

Pretreatment assay

This assay was conducted as described by Bertol et al. [26]. Confluent cell monolayers were pretreated with ten different concentrations (1/32 to 5 × IC_{50}) of the samples for 3 h at 37 °C. Then, cells were infected with 100 PFU of HSV-1 or HSV-2 per well and treated as described above for the plaque number reduction assay. ACV was used as an internal control.

Simultaneous treatment assay

This assay was executed as described by Argenta et al. [42]. Confluent cell monolayers were infected with HSV-1 or HSV-2 with simultaneous addition of the samples at ten different concentrations (1/32 to 5 × IC_{50}). Further procedures are described above for the plaque number reduction assay. ACV was used as an internal control.

Adsorption, post-adsorption and penetration assays

These assays were performed following the general procedures described by Silva et al. [41]. Dextran sulfate (DEX-S; Sigma-Aldrich) was used as a positive control throughout the different assays. For the attachment assay, confluent cell monolayers were pre-chilled at 4 °C for 30 min, exposed to a mixture of 100 PFU of HSV-1 or HSV-2 per well in the absence (viral control) or presence of the samples at 10 different concentrations (1/32 to 5 × IC_{50}) and incubated at 4 °C for an additional 2 h. Unabsorbed viruses were removed by washing with cold PBS; cells were overlaid with CMC medium and treated as described above for the plaque number reduction assay.

For the post-attachment assay, confluent cell monolayers were pre-chilled at 4 °C for 30 min and incubated with 100 PFU of HSV-1 or HSV-2 per well at 4 °C for an additional 2 h to allow stable attachment of viruses without fusion with cell membranes. The samples were then added at 10 different concentrations (1/32 to 5 × IC_{50}), and the infected cells were incubated again at 4 °C for 2 h and treated as described above for the plaque number reduction assay.

For the penetration assay, 100 PFU of HSV-1 or HSV-2 per well were adsorbed for 2 h at 4 °C onto confluent cell monolayers that had been pre-chilled at 4 °C for 30 min (at this temperature the viruses can bind but cannot penetrate the cells). Then, the temperature was shifted to 37 °C for 5 min to allow virus penetration, and the cells were treated with 10 different concentrations of the samples (1/32 to 5 × IC_{50}) and incubated for 1 h at 37 °C. After incubation, unpenetrated viruses were inactivated with citrate buffer (pH 3.0) for 1 min. Cells were washed with PBS and treated as described above for the plaque number reduction assay.

Western blot analyses

To evaluate whether the compounds being tested interfere with HSV-1 (KOS strain) protein expression, experiments were performed following the procedures described by Argenta et al. [42]. Briefly, confluent cell monolayers were infected or not with HSV-1 at an MOI of 0.2 for 1 h

at 37 °C. Residual virus particles were then removed with PBS, and the cells were treated with five different concentrations of the samples (1/16, 1/8, 1/4, 1/2 and 1 × IC_{50}) or 5 μM of ACV (positive control) for 18 h (one viral replication cycle). The cells were then lysed, and protein quantification was carried out [43]. The protein content was separated electrophoretically in a 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After blocking, the membranes were incubated overnight with anti-ICP27 (1:1000, Millipore, Billerica, MA, USA), anti-UL42 (1:1000, Millipore), anti-gD (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin antibodies (1:5000, Millipore). The last of these was used as a control for total protein loading. After washing, the membranes were incubated for 1 h with the respective secondary antibodies conjugated to horseradish peroxidase. Protein bands were revealed using Pierce Enhanced Chemiluminescence ECL Western Blotting substrate (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Images were acquired using a Bio-Rad ChemiDocTM MP System and digitalized using the program Image Lab, version 4.1. Relative densitometry data for the blots were analyzed using ImageJ free software.

Viral release assay

This assay followed the procedures described by Bertol et al. [26], and the percentages of inhibition were calculated according to Su et al. [28]. Briefly, confluent cell monolayers were infected with HSV-1 or HSV-2 at MOI 0.4 for 1 h. Then, cell monolayers were washed, and different concentrations (1/2, 1 and 2 × IC_{50}) of the samples were added to the cells for 24 h at 37 °C, after which the supernatants and cell pellets were collected separately. Pellets were frozen and thawed three times before virus titration by plaque number reduction assay as described above. Digitoxin (Sigma-Aldrich) was used as a positive control.

Viral plaque size reduction assay

This assay was conducted as described by Argenta et al. [42]. Different concentrations (0.0625, 0.125, 0.25, 0.5 and 1 μM) of the samples were added to Vero cells after infection for 1 h with 100 PFU of HSV-1 or HSV-2. Further procedures were performed as described above for the plaque number reduction assay. After staining, images of 20 viral plaques formed in the presence or absence (viral control) of each concentration of the compounds were captured using a digital camera coupled to an Olympus IX71 inverted microscope (Center Valley, PA, USA). The area of each viral plaque was determined using the ImageJ free software.

Statistical analysis

The mean values ± standard deviations are representative of three independent experiments. Statistical analysis were performed by ANOVA followed by post-hoc tests as indicated.

Results

Anti-herpes mechanism of action

According to Boff et al. [38], the initial screening of 16 new cardenolide derivatives against HSV-1 (KOS and 29-R strains) and HSV-2 (333 strain) showed that **C10** and **C11** exhibited no relevant cytotoxic effects on Vero cells (>300 μM) and displayed the highest antiviral potential [IC_{50} values of **C10** and **C11**, respectively: 0.23 and 0.24 μM against HSV-1 (KOS strain); 0.18 and 0.19 μM against HSV-1 (29-R strain); and 0.27 and 0.30 μM against HSV-2 (333 strain)]. SI values were calculated based on their CC_{50} and IC_{50} values and found to be promising for both compounds. The SI values for **C10** and **C11** were as follows: HSV-1 (KOS strain) 1,304 and 1,250; HSV-1 (29-R strain) 1,667 and 1,579; HSV-2 (333 strain) 1,111 and 1,000, respectively. These values are similar to or even higher than those of acyclovir [HSV-1 (KOS strain) 1,449 and HSV-2 (333 strain) 619] [38].

Virucidal activity

Even at concentrations 100 times higher than their IC_{50} values, as described above [38], the treatments were not able to inactivate all virus strains tested (data not shown).

Pretreatment effects

Treatment of Vero cells with both compounds at 1/32 to 5 × IC_{50} before virus inoculation did not affect HSV-1 or HSV-2 replication, suggesting that they did not have prophylactic effects (data not shown).

Effects on the early stages of viral replication

In the same way, simultaneous treatment did not inhibit HSV replication, suggesting that the next steps of viral replication (adsorption, post-adsorption and penetration) were not

affected. To confirme this finding, each of these steps was investigated individually, and the results confirmed that neither compound affected the early stages of viral replication (data not shown).

Effects on the expression of viral proteins

Since **C10** and **C11** did not interfere in the first steps of HSV-1 and HSV-2 replication, their effects on HSV-1 protein expression were evaluated by Western blot analyses. Fig. 2A shows a representative blot of the results obtained after 18 h of treatment (one HSV replication cycle) with **C10** and **C11** (1/16, 1/8, 1/4, 1/2 and 1 × IC_{50}) or ACV (5 μM), and Fig. 2B shows the ratio of the amount of each viral protein to the amount of β-actin protein.

The compounds **C10** (at concentrations of 1/4, 1/2 and 1 × IC_{50}) and **C11** (at concentrations of 1/2 and 1 × IC_{50}) completely abolished the expression of UL42 (β) and gD (γ) proteins, and partially reduced that of ICP27 (α) in a concentration-independent manner (Fig. 2A).

Effects on viral release

The ability of **C10** and **C11** to interfere with virus release was investigated by determining the intra- and extracellular HSV-1 and HSV-2 titers. Fig. 3 shows that both compounds, at all tested concentrations, significantly reduced the extra- and intracellular titers of HSV. The highest activity was against HSV-1 (29-R strain). At 1 × IC_{50}, virus release was inhibited by 97% (**C10**), 96% (**C11**) and 95% (digitoxin, positive control).

Effects on viral plaque size

The effects of different concentrations of **C10** and **C11** on the cell-to-cell spread of HSV-1 and HSV-2 were evaluated using a viral plaque size reduction assay. As shown in Fig. 4A and B, when compared to viral control, both compounds significantly reduced the areas of the viral plaques that were formed. The strongest reduction detected (**C10** from 79 to 100% and **C11** from 73 to 100%) was against HSV-1 (29-R strain), since the compounds almost completely closed the plaque area at the lowest concentration tested.

Discussion

The antiviral action is considered one of the new therapeutic possibilities of cardenolides besides their use as positive inotropic agents [44]. In the last decade, studies have highlighted these compounds as potent inhibitors of herpesvirus, as shown by Bertol et al. [26], Dodson et al. [27], and Su et al. [28]. Recently, our research group published the anti-herpes and cytotoxic screenings of 16 new semi-synthetic cardenolides [38]. The compounds with the best anti-HSV potential were **C10** and **C11**, and for this reason, several experiments were carried out to tentatively propose their mechanisms of action. In this study, both compounds were tested against HSV-1 (KOS and 29-R strains, which are ACV-sensitive and ACV-resistant, respectively) and HSV-2 (333 strain).

One approach that has great potential applicability in the therapy of sexually transmitted diseases, including those caused by HSV, is the topical application of drugs that can permanently inactivate viral particles [45]. However, in this study, **C10** and **C11** were not able to inactivate HSV-1 and HSV-2. This indicates that these compounds do not act directly on viral particles before they entered into the cells, which agrees with the findings of studies performed with other cardenolides [26, 28, 46, 47].

In order to detect possible preventive effects, **C10** and **C11** were evaluated directly on Vero cells in the absence of the viruses. This approach also did not affect HSV replication, attesting that both compounds did not present prophylactic effects. These findings are in accordance with the results obtained by Bertol et al. [26] for glucoevatromonoside, a natural cardenolide.

Viruses and compounds were then added simultaneously to investigate if the initial phases of the HSV-1 and HSV-2 replication cycles were affected by **C10** and **C11**. The results demonstrated that they did not act interfering with the early events of HSV infection, as it was confirmed posteriorly by the viral adsorption, post adsorption and penetration assays. These results are in line with the findings of the simultaneous treatment and corroborate the outcomes from Bertol et al. [26], Dodson et al. [27] and Su et al. [28].

It is well established that the HSV replication cycle is divided in six stages: adsorption, viral entry into host cells, expression of viral genes, DNA replication, assembly, and release of new viral particles [2, 48]. The results obtained so far demonstrate that **C10** and **C11** do not affect the initial phases of HSV-1 and HSV-2 replication, do not inactivate these viruses, and have no prophylactic effects.

To identify the stages of the HSV replication cycle at which **C10** and **C11** could be acting, Western blot experiments were conducted. As already mentioned here, α genes regulate viral replication, β genes are responsible for DNA synthesis and packaging, and γ genes synthesize the structural components of the viruses [1]. Although both compounds gave promising results, **C10** significantly reduced UL42 (β) and gD (γ) protein expression at a lower concentration than **C11** and completely inhibited the expression of these proteins up to 1/4 × IC_{50}. This suggests that **C10** and **C11** could interfere with the synthesis of viral DNA and structural components. Additionally, both compounds

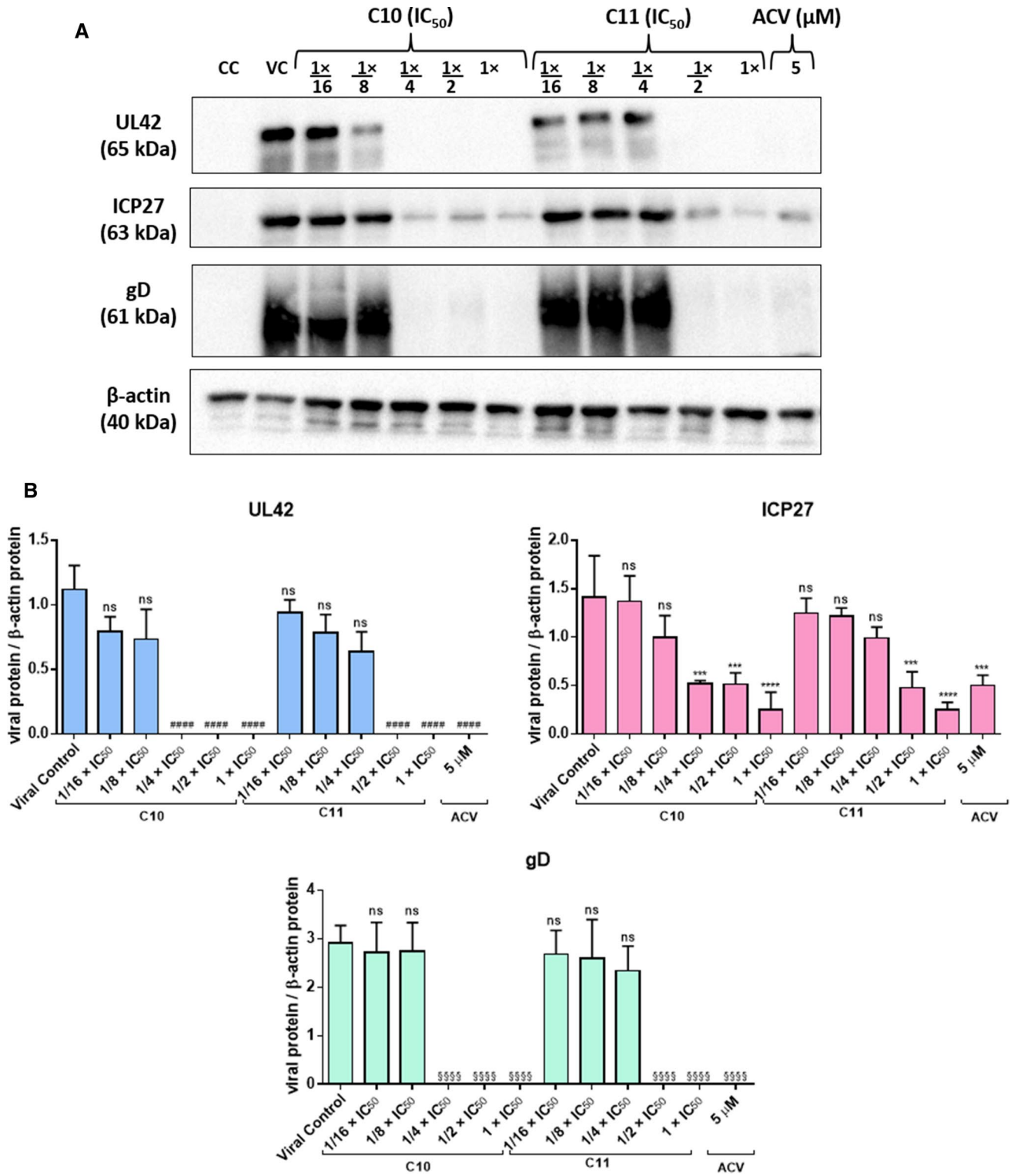

Fig. 2 Effects of **C10** and **C11** on HSV-1 (KOS strain) protein expression. **(A)** Confluent Vero cells were infected with HSV-1 (MOI 0.2) and treated with 1/16, 1/8, 1/4, 1/2 and 1 × IC_{50}, or 5 μM of ACV. Lysates were collected after 18 h of incubation, and the proteins were separated by SDS-10% PAGE and analyzed using antibodies specific for the viral ICP27, UL42, and gD proteins. CC (cell control), uninfected and untreated Vero cells; VC (viral control), infected but not treated Vero cells; ACV, acyclovir. Equal protein loading was confirmed by probing for β-actin. **(B)** The graph indicates the ratio of the amount of each viral protein to that of β-actin. ns, not significant; ####, $p < 0.0001$; ***, $p < 0.001$; ****, $p < 0.0001$; §§§§, $p < 0.0001$ *vs.* the respective viral controls (two-way ANOVA, Dunnett's post-hoc test)

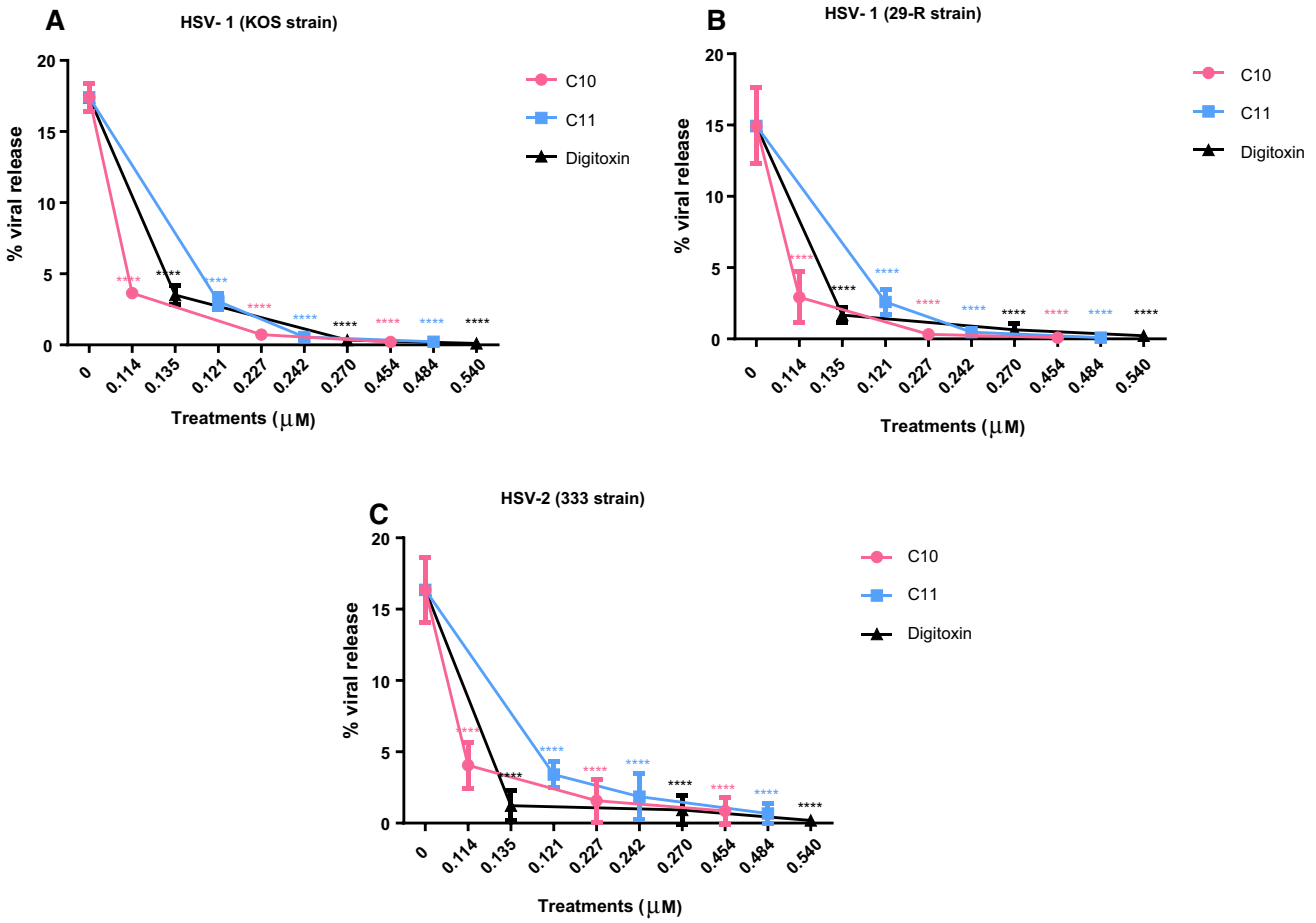

Fig. 3 Effects of **C10** and **C11** on virus release. Vero cells were infected with HSV-1 [(**A**) KOS and (**B**) 29-R strains] and (**C**) HSV-2 (333 strain) at MOI 0.4 for 1 h. Cell monolayers were washed and treated with different concentrations (1/2, 1 and 2 × IC_{50}) of **C10**, **C11** and digitoxin (positive control) for 24 h at 37 °C. The supernatants and cell pellets were then collected separately. Pellets were frozen and thawed three times before virus titration by plaque number reduction assay. ****, $p < 0.0001$ *vs.* the respective viral controls (two-way ANOVA, Dunnett's post-hoc test)

inhibited ICP27 (α) protein expression less than the other two proteins, but still significantly. At the molecular level, these results suggest that **C10** and **C11** interfere with the expression of all proteins evaluated, more intensively on the expression of early and late proteins in comparison with the immediate early proteins.

It is known that the genomic homology of HSV-2 is almost identical to that of HSV-1, and these viruses express the same proteins whose expression was evaluated in this work (gD, UL42 and ICP27) [49–51]. Earlier studies have shown that different samples, such as a cranberry extract [52] and a diterpene isolated from *Andrographis paniculata* Nees [53] were able to inhibit in a similar way the expression of these HSV-1 proteins as much as HSV-2 ones. Here, although the reduction of gD, UL42 and ICP27 expression by **C10** and **C11** was only tested using HSV-1 (KOS strain), all other experiments were performed against both strains of HSV-1 (KOS and 29-R) and strain 333 of HSV-2. The results showed similar inhibition profiles for all viral replication steps evaluated. Consequently, we presume that both compounds would act in the same way against HSV-1 (29-R strain) and HSV-2 replication by inhibiting the expression of the same proteins.

The capacity of **C10** and **C11** to block viral release was investigated by measuring the intra- and extracellular titers of HSV-1 and HSV-2. Both compounds reduced HSV release as much as the positive control (digitoxin), and for HSV-1 (29-R strain), this inhibition was greater than 80% at the lowest concentration tested. These results, together with those obtained by Western blot analyses, suggest that both compounds completely or partially inhibit the intermediate and final phases of HSV replication.

It is well established that the released viruses infect the adjacent cells of the infected tissues, replicate and continue to infect other cells through viral intercellular propagation (cell-to-cell spread), which is relevant to

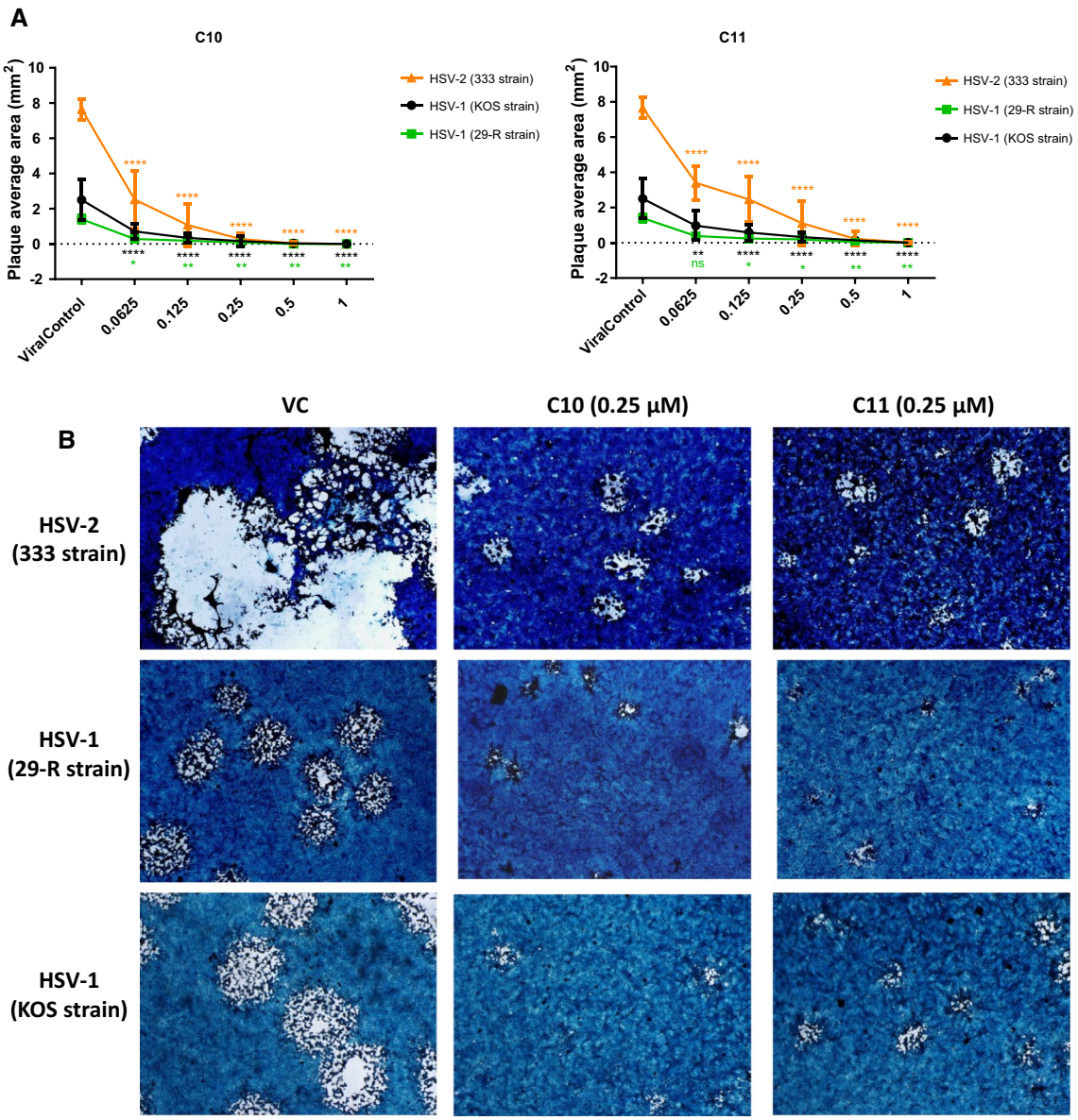

Fig. 4 Effects of **C10** and **C11** on viral plaque size of HSV-1 (KOS and 29-R strains) and HSV-2 (333 strain). **(A)** The areas of 20 lysis plates formed by the different viruses in the presence of the compounds (0.0625 to 1.0 µM) were measured, and **(B)** images formed in the presence and absence (viral control) of each concentration of **C10** and **C11** were captured. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; and ****, $p < 0.0001$ *vs.* the respective viral controls (two-way ANOVA, Dunnett's post-hoc test)

productive primary or recurrent infections establishment [54, 55]. **C10** and **C11** reduced significantly the areas of formed viral plaques when compared to viral controls, and these results could be a consequence of the inhibition of viral release.

Several studies have demonstrated the anti-herpes activity of natural products, some of which interfere with early steps [41, 42, 56–64], while a few affect the last steps of HSV replication [65–68], as was demonstrated for **C10** and **C11**. For this reason, both cardenolides could be used in combination with other natural products to ensure that different stages of HSV replication would be inhibited, for example with trans-resveratrol, for which significant anti-HSV activity has also been described [69].

The mechanism by which cardenolides affect several steps of HSV replication could be related to the inhibition of Na^+/K^+-ATPase in host cells. Many studies have demonstrated the modulation of Na^+/K^+-ATPase functions in host cells by DNA viruses (adenoviruses [18], cytomegalovirus [23, 70–72], and HSV [27, 28]), and RNA viruses (chikungunya virus [19, 73], coronaviruses [20, 74, 75], respiratory syncytial virus [76, 77], Ebola virus [78, 79], influenza virus

[33, 80, 81], and HIV [30, 31, 82]). By activating signaling cascades or by altering the concentration of intracellular ions, the binding of cardenolides to Na^+/K^+-ATPase seems to create an unfavorable environment for viral replication. Bertol et al. [26] showed that the inhibition of Na^+/K^+-ATPase caused by glucoevatromonoside was correlated with virus release and viral protein synthesis due to a reduction in the K^+ concentration in the cell. Cohen et al. [23] found that the inhibitory activity of cytomegalovirus replication by convallatoxin was due to a decrease in immediate-early gene expression and that the antiviral potency depends on the structure of cardiac glycosides and their specific interactions with Na^+/K^+-ATPase. It was also shown that influenza virus replication was impaired by ouabain through the inhibition of viral protein translation and a decrease in the intracellular K^+ concentration [37]. Finally, Boff et al. [38] showed that **C10** and **C11** inhibited Na^+/K^+-ATPase, and a reduction in intracellular K^+ concentration could explain the inhibitory activity of those steps of viral infection. In summary, the main antiviral mechanisms suggested for both DNA and RNA viruses are decreased transcription of viral genes and impaired synthesis of viral proteins due to interference with the host translational machinery, such as the inhibition of Na^+/K^+-ATPase [37].

Structural differences among cardenolides, such as the substituents on the aglycone moiety and the number of sugars, may vary and alter their antiviral activity. Most of the cardenolides tested by Bertol et al. [26], Dodson et al. [27], and Su et al. [28] presented one or more sugar moieties bind to C3, unlike the cardenolides **C10** and **C11**. Both of which have substituted amine groups at C3 and are able to inhibit HSV replication at nanomolar concentrations, but not with the same potency as cardenolides bearing sugars at C3. Thus, the sugar moiety, although not essential for anti-herpes activity, nevertheless influences this activity positively. Moreover, **C10** was found to be more active than **C11** in all experiments, and therefore, we hypothesize that the secondary amine group located on the side chain might also contribute to the antiviral activity. Consistent with this, Fytas et al. [83] and Tataridis et al. [84] showed that the presence of additional amino groups in adamantane derivatives increased their antiviral activity.

Even though previous works have addressed the anti-herpes effects of different cardenolides, the investigation of the mechanism of anti-herpes action of **C10** and **C11** [HSV-1 (29-R strain) and HSV-2 (333 strain)] have not been explored so far, and we described it herein for the first time.

Conclusions

In conclusion, our findings suggest that **C10** and **C11** interfere mainly with the late steps of HSV replication, i.e., viral replication [HSV-1 (KOS strain)], assembling, release of new viruses and viral intercellular propagation [HSV-1 (KOS and 29-R strains) and HSV-2 (333 strain)]. In a minor extension, they also interfere with the intermediate stages of viral replication but not with the early steps. Additionally, they were not virucidal and have no prophylactic effects. These results encourage us to continue studying cardenolides as an alternative for the treatment of herpes simplex virus infections. Despite both compounds presented promising results, **C10** was more potent with a lower IC_{50} value and the best selectivity index. Wherefore, they can be considered new bioactive molecules with great potential as anti-herpes drug candidates mainly for HSV-1 (29-R strain), which is resistant to ACV treatment.

Acknowledgements The authors thank the Brazilian funding agencies CAPES (MEC), Fapemig (APQ-00538-17, RMP) and CNPq (MCTI) for research fellowships, as well as CNPq for financial support (grant numbers 305878/2016-6, CMOS, and 490057/2011-0, FCB). They are also grateful to BAYLAT (JM and RMP for mobility support) and Marie Curie Foundation/European Community (FP7 IRSES, grant 295251, WK).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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