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Synthetic pregnenolone derivatives as antiviral agents against acyclovir-resistant isolates of Herpes Simplex Virus Type 1

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ABSTRACT

The conventional therapy for the management of Herpes Simplex Virus Type 1 (HSV-1) infections mainly comprises acyclovir (ACV) and other nucleoside analogues. A common outcome of this treatment is the emergence of resistant viral strains, principally when immunosuppressed patients are involved. Thus, the development of new antiherpetic compounds remains as a central challenge. In this work we describe the synthesis and the *in vitro* antiherpetic activity of a new family of steroidal compounds derived from the endogenous hormone pregnenolone. Some of these derivatives showed a remarkable inhibitory effect on HSV-1 spread both on wild type and ACV-resistant strains. The results also show that these compounds seem to interfere with the late steps of the viral cycle.

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1. Introduction

Herpes Simplex Virus Type 1 (HSV-1) is a double-stranded DNA virus with a very high infection rate in humans (Whitley and Roizman, 2001). The primary site of infection with HSV-1 is the orolabial mucosa. After this primary infection, the HSV-1 virus is transported to the trigeminal ganglion where it establishes a lifelong latent infection that can be reactivated by several stimuli (Egan et al., 2013). When reactivation occurs, HSV-1 is transported back to the primary site of infection and causes recurrent ulcerations. During recurrence, HSV-1 can eventually multiply in the eye triggering ocular keratitis (Rowe et al., 2013), that may result in blindness and, in rare cases, brain infections. The resulting HSV-1 encephalitis has mortality rates of up to 70% if left untreated (Hjalmarsson et al., 2007; Whitley, 2006). Conventional therapy for the management of HSV-1 infections mainly comprises acyclovir (ACV) (Hodge and Field, 2013). A common outcome of this treatment is the emergence of ACV-resistant viral strains, principally when used on immunosuppressed patients in the context of long-term prophylactic ACV therapy. This therapy, combined with an impaired host response, enables less virulent viruses to continue to replicate. ACV is a nucleoside analog that must be phosphorylated by the virus-encoded thymidine kinase (TK) prior to its incorporation into DNA by the viral DNA polymerase (DNA pol). Upon these two steps, ACV prevents chain elongation and therefore inhibits the viral DNA synthesis. HSV-1 can acquire resistance to ACV through several different mechanisms comprising alterations in TK and/or DNA pol genes. Current management of ACV-resistant HSV-1 infections includes the use of foscarnet (FOS), a direct inhibitor of DNA pol, but mutations in DNA pol associated with FOS resistance have also been reported (Andrei and Snoeck, 2013).

Thus, development of new antiherpetic compounds is a central challenge in Medicinal Chemistry. The search of new non-nucleoside drugs, which would avoid the development of the aforementioned mechanisms of resistance, is a promising approach. Recent publications have demonstrated the potential of this idea (Bag et al., 2014; Vilhelmova-Ilieva et al., 2014).

In this regard, steroids are an interesting source for alternative antiherpetic drugs, since they are known to have various biological properties and are often less prone to multi-drug resistance. The

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steroidal tetracyclic skeleton is often considered a privileged scaffold in drug discovery (Welsch et al., 2010).

We previously reported that some synthetic polyfunctionalized steroids derived from phytosterols display *in vitro* anti HSV-1 activity and are able to mitigate the severity of herpetic stromal keratitis in murine models (Michelini et al., 2008, 2004). Furthermore, we have recently synthesized a new family of azasterols with diamide side chains of general structure **1** (Fig. 1), some of which exerted antiherpetic activity (Dávola et al., 2012). Encouraged by these previous results, we decided to synthesize new families of steroids with diverse functionalities attached to the steroidal nucleus in the search for new antiviral steroids.

Pregnenolone (3β-hydroxypregn-5-en-20-one) is an important endogenous steroid in mammals, and a precursor of all major types of steroid hormones. Synthetic derivatives of pregnenolone with diverse biological activities have been described (Banday et al., 2010; Iqbal Choudhary et al., 2011; Porta et al., 2014), including some with antiviral action (Comin et al., 1999; Petrera et al., 2003). In this paper we describe a set of azasteroids of general structure **2** (Fig. 1), with pregnenolone as the core structural framework and amide moieties substitutions at C16. The new compounds were evaluated for their ability to inhibit HSV-1 yield in both wild-type and acyclovir-resistant strains.

2. Materials and methods

2.1. Chemical synthesis

Detailed synthetic procedures are included in the Supplementary data. Satisfactory combustion analyses (purity ⩾95%) were obtained for all new compounds using an Exeter CE 440 Elemental Analyzer. Their structures were confirmed by 1D and 2D NMR spectroscopic analysis. In some cases, the NMR spectra showed that the compounds were present in the solution as a mixture of two conformers; for simplicity, the chemical shifts and coupling constants were described only for the most populated conformer.

2.2. Treatment solutions

The synthetic compounds and ACV (Sigma–Aldrich) were dissolved in dimethylsulfoxide (DMSO) and diluted with Eagle's minimal essential medium supplemented with 1.5% inactivated fetal bovine serum (FBS) and 50 μg/ml gentamicin (MEM 1.5%). The maximum concentration of DMSO used (1% or 2%) exhibited no toxicity under *in vitro* conditions. FOS (Sigma–Aldrich) was dissolved in water and diluted with MEM 1.5%.

2.3. Cells and viruses

Vero cells were grown in MEM 5% and maintained after monolayer formation in MEM 1.5%.

The KOS strain was chosen as HSV-1 wild type reference. The B2006 strain, an HSV-1 TK-mutant, was a kind gift from Dr. E De Clercq (Rega Institute, Leuven, Belgium). Four new ACV-resistant HSV-1 clones (named 252, 503, 504 and 505) were obtained *in vitro* by one passage of KOS strain in the presence of a high concentration of ACV (25 or 50 μg/mL) following the procedure described by Andrei et al. (2005). All HSV-1 strains were propagated at low multiplicity of infection (moi) and plaque-assayed on Vero cells.

2.4. Cytotoxicity assay

Vero cells were seeded at a concentration of 10^4 cells/well in 96-well plates and grown at 37 °C for 1 day. Then, cells were treated with various concentrations of tested compounds in triplicates. DMSO was used as control. After 24 h of treatment, cell viability were determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, CellTiter 96 AQueous One Solution Reagent; Promega). The concentration required to reduce cell viability by 50% (CC_{50}) was obtained. Control with 1% DMSO showed no effect on cell viability. Cytotoxicity was also determined using confluent non-growing Vero cells after 72 h of treatment with the maximum concentration of the compounds employed.

2.5. Antiviral activity

The cytopathic effect (CPE) inhibition assay was used as a preliminary test to evaluate the antiviral activity. Vero cells were infected with HSV-1 at a moi of 0.1 and cultured for 1 h at 37 °C. Cells were then treated with the compound of interest or drug-free medium as a control virus, in triplicate. After 24 h the virus-induced CPE was recorded by analyzing cell morphology by inverted light microscope (Nikon Eclipse TS100). At that time, 100% cell death was observed in untreated infected control cells. Compounds were regarded protective when 25% or less rounding of Vero cells occurred.

The virus yield inhibition assay was performed as previously described (Dávola et al., 2012) and the concentration required to reduce the virus yield by 50% (EC_{50}) was obtained.

2.6. Virucidal assay

A virus suspension containing approximately 5×10^6 PFU/mL of HSV-1 strain KOS was mixed with 200 μM of the compound of interest in MEM 1.5% and incubated for 1 h at 37 °C. A virus control was performed by incubating the virus suspension with MEM 1.5%

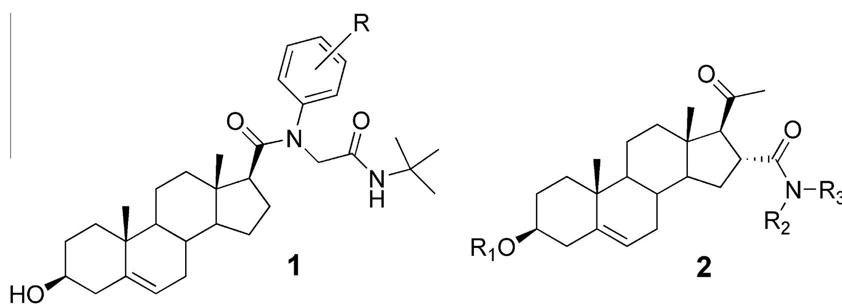


Fig. 1. General structure of synthetic azasteroids.

under the same conditions. Virus yields were subsequently titrated by plaque assay.

2.7. Indirect immunofluorescence (IFI) assay

Mouse monoclonal antibody to HSV-1 gD was obtained from Santa Cruz Biotechnology, USA. Secondary goat anti-mouse FluoroLinkTM CyTM3 antibody was purchased from GE Healthcare Bio-Sciences, Argentina. The procedure for IFI was performed as described previously (Dávola et al., 2012).

2.8. Time-of-addition and time-of-removal assays

Vero cells were exposed to the compound of interest before, during and after infection with HSV-1 KOS or B2006 strains (moi of 0.1 PFU/cell). For pre-infection assays, cells were treated with the compound during 14 h at 37 °C, washed with PBS and then infected with HSV-1. For co-infection, cells were simultaneously treated with HSV-1 and the compound of interest. After 1 h adsorption at 37 °C, the virus-drug mixture was removed and compound-free medium was added. For post-infection (p.i.) assays, cells were infected with HSV-1 for 1 h at 37 °C and then treated with the tested compound at 0 and 7 h p.i. For time-of-removal assay, cells were infected with HSV-1 and after 1 h of incubation at 37 °C, the inoculum was discarded and the compound was added. Drug was removed at 7 h p.i., then cells were washed with PBS and compound-free medium was added. A control culture that was infected but not treated (CV) was simultaneously performed. Total virus yields were always determined by plaque assay at 24 h p.i.

2.9. Extracellular and intracellular virus yields

Vero cells were infected with HSV-1 strain KOS or B2006 at moi of 10 PFU/cell for 1 h at 37 °C and then treated with 40 μM of the compound of interest at 0 h p.i. After 15 h of incubation, supernatants were harvested. Fresh medium was added and after cell disruption by three cycles of freezing and thawing. Supernatants were then pooled and intracellular virus was harvested. Extracellular and intracellular virus yields were determined by plaque assay.

2.10. Western blot analysis

Vero cells were infected with HSV-1 strain KOS at moi of 10 PFU/cell for 1 h at 37 °C and then treated with 40 μM of the compound of interest. After 14 h of incubation whole extracts were obtained and subjected to SDS–PAGE separation. Western blot analysis were performed employing mouse mAb to HSV-1 gD, mouse mAb anti-actin (JLA20, Calbiochem) and peroxidase-conjugated anti-mouse antibody (Santa Cruz Biotechnology), as described previously (Bueno et al., 2009).

2.11. Effect of **9c** on glucocorticoid receptor translocation

HEK 293T cells grown on coverslips pretreated with polylysine to promote cell adhesion were transfected with a chimeric murine glucocorticoid receptor GR associated with Green Fluorescent Protein (GFP). After 24 h of growth in a medium containing serum adsorbed with carbon/dextran (and thus steroid-free), cells were stimulated with 10 nM DEX, 50 μM of **9c**, or both compounds (at the aforementionated concentrations) simultaneously. After 30 min of incubation, the coverslips were washed with PBS, fixed with 3% p-formaldehyde, permeabilized with methanol at – 20 °C and the subcellular localization of GFP-GR observed with an Olympus IX71 epifluorescence microscope. Quantification was performed by counting cells and classifying them according to the location of the GR into five categories: (1) fully cytoplasmic, (2) mainly cytoplasmic, (3) homogeneous distribution, (4) majority nuclear, (5) completely nuclear. The results express the percentage of cells of categories 4 and 5 with respect to the total. In each experiment at least 50–100 cells were counted. The assay was carried out in triplicate.

2.12. Statistical analysis

CC_{50} and EC_{50} were calculated from dose–response curves using the software GraphPad Prism 4.0 (GraphPad Software; San Diego, CA, USA) as previously described (Alonso et al., 2014). All assays were carried out in triplicate. Statistically significant differences were evaluated by Student's *t*-test. *p*-Value <0.05 was considered significant.

3. Results

3.1. Synthesis

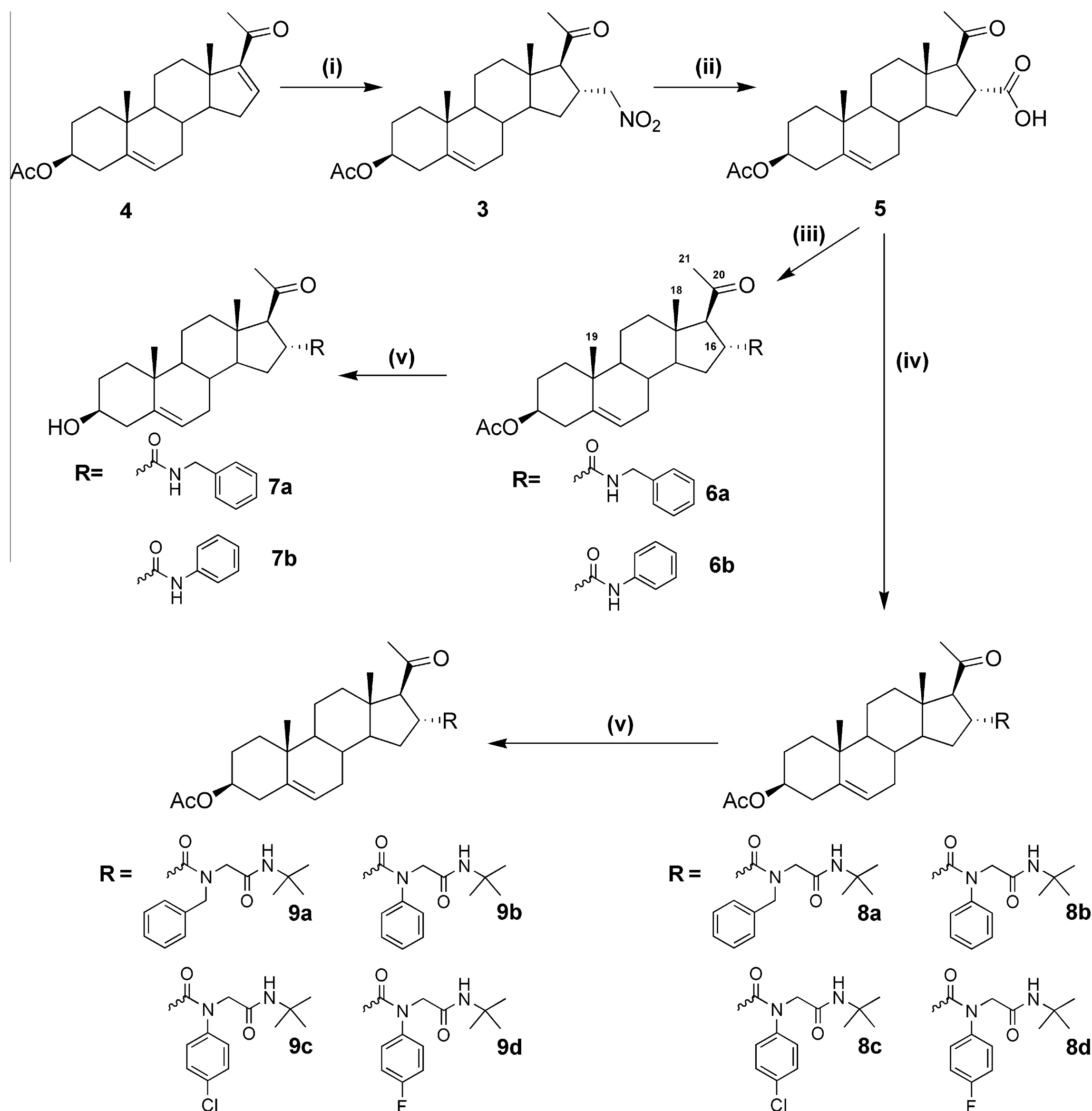
The new pregnenolone derivatives were designed to incorporate diverse functionalities at C16, a substitution rarely found in natural steroid. The synthetic route is shown in Scheme 1. The nitrosteroid **3** was obtained from 16-dehydropregnenolone (**4**) following an established procedure (Wankhede et al., 2008). Treatment of **3** with nitrite in acidic conditions yielded the steroidal acid **5** (Woroch, 1963). Then, the acid was condensed either with two different amines under standard conditions to give the amides **6a**–**b**, followed by the hydrolysis of the acetates at C-3. This provided the corresponding pregnenolone analogues **7a**–**b**.

In order to expand the structural diversity of the synthetic side chains, we employed the Ugi four-component reaction (U-4CR) (Dömling et al., 2012). This reaction introduces an α-aminoacylamide in one step, which allows the formation of pregnenolone derivatives with longer diamide side chains at C16. So, the steroidal acid **5** was treated with formaldehyde, t-butylisocyanide and a set of amines, to give the compounds **8a**–**d**, which were further hydrolyzed to give compounds **9a**–**d**. The amidation, the U-4CR and the subsequent hydrolyzes took place in good yields (>70%).

3.2. Antiviral activity screening

Once the steroid library was obtained, we performed a screening to assess the possible antiviral activity against HSV-1 (KOS strain). First, the cytotoxicity of the new analogs towards the uninfected Vero host cells was determined following 24 h of treatment using a colorimetric MTS assay. For comparison, we also included the endogenous steroidal hormone pregnenolone and all of the synthetic precursors shown in Scheme 1 (**3**–**5**).

CC_{50} was calculated and compounds showing values greater than 200 μM (Table 1) were selected to test their anti-HSV-1 activity by a CPE inhibition assay. This preliminary screening showed that compound **9c** most effectively prevented HSV-1-induced CPE (Fig. 2A); compounds **8d** and **9d** also had an interesting antiviral effect and pregnenolone elicited marginal inhibitory action (data not shown). Besides, MTS was also used to study the effect of these active compounds on Vero cells after a prolonged exposure time. The results obtained showed that **8d**, **9c** and **9d** were not toxic to Vero cells at a concentration of up to 200 μM even after 72 h of treatment. To confirm whether the new derivatives could inhibit the formation of infectious virions, EC_{50} values were calculated by the virus yield inhibition assay after 24 h of treatment. Fig. 2B shows that **8d**, **9c** and **9d** compounds exerted a dose-dependent



Scheme 1. Synthesis of compounds **6a–b**, **7a–b**, **8a–d** and **9a–d**. (i) DBU, CH_3NO_2, CH_2Cl_2; (ii) $NaNO_2$, AcOH, DMSO; (iii) R_2NH_2, EDC, DMPA, CH_2Cl_2; (iv) R_2NH_2, *t*-BuNC, H_2CO, MeOH; (v) K_2CO_3 (satd.).

Table 1
Cytotoxicity of compounds in Vero cells.

Compound	CC_{50} (μM)
Pregnenolone	>200
3	38.7 ± 1.0
4	63.7 ± 1.2
5	>200
6a	103.6 ± 1.1
6b	>200
7a	188.2 ± 1.1
7b	87.4 ± 1.1
8a	>200
8b	>200
8c	>200
8d	>200
9a	>200
9b	115.9 ± 1.1
9c	>200
9d	>200

inhibition of viral replication with selectivity indices ($SI = CC_{50}/CE_{50}$) of 26, 82 and 28, respectively. EC_{50}s were consistent with results found in the CPE inhibition assay, since HSV-1 yield was more susceptible to **9c** treatment than **8d** and **9d** treatments (Table 2).

Virucidal assays were performed to rule out the possibility that the antiviral action observed was caused by direct inactivation of the released virus. Suspensions of HSV-1 virus were incubated with hit compounds **8d**, **9c** and **9d** for 1 h at 37 °C followed by titration of the remaining infectivity. None of the three compounds significantly decreased the virus yield ($p < 0.05$), showing that these pregnenolone derivatives can be considered true antiviral agents that have the ability to interfere with some intracellular event during the replication cycle of HSV-1 in Vero cells (Fig. 2C).

3.3. Antiviral activity against ACV-resistant strains

One of the aims of this work was to discover new steroidal compounds that are able to inhibit the propagation of wild type and

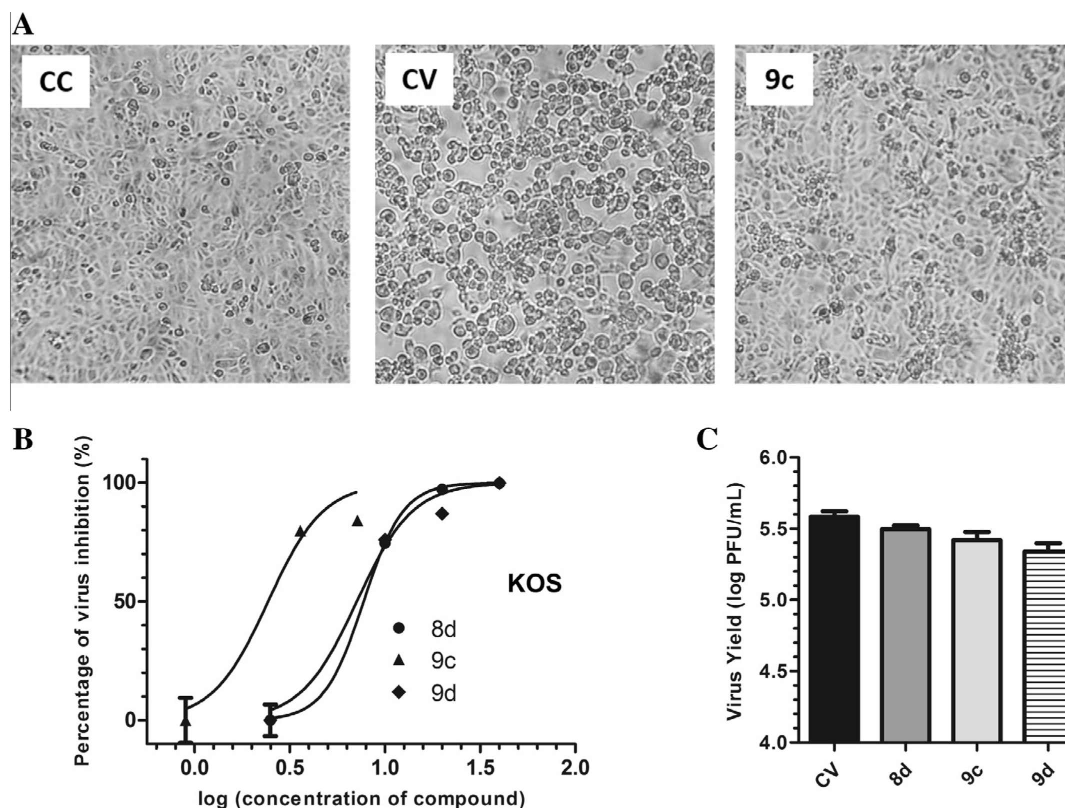


Fig. 2. Activity of hit compounds against HSV-1 strain KOS. (A) Vero cells infected with HSV-1 strain KOS at moi = 0.1 were treated or not (CV) with compound **9c** (25 μM). Images were obtained under inverted microscope. Non infected Vero cells were also analyzed (CC). Magnification ×100. (B) Vero cells infected with HSV-1 strain KOS at moi = 0.1 were treated with different concentrations of **8d**, **9c** or **9d** and EC_{50}s were calculated. (C) Suspensions of HSV-1 strain KOS were incubated with **8d**, **9c** or **9d** for 1 h at 37 °C and remaining infectivity was titrated. Incubation of virus suspension without drug was performed as control (CV).

Table 2
Antiviral activity of hit compounds against wild-type and ACV-resistant HSV-1 strains. EC_{50}s were calculated by nonlinear regression from data shown in Figs. 3 and 4.

Compound	EC_{50} (μM)					
	KOS	B2006	Clone 252	Clone 503	Clone 504	Clone 505
8d	7.73 ± 1.11	17.60 ± 2.33	8.29 ± 1.10	7.16 ± 1.39	7.20 ± 2.34	7.67 ± 1.38
9c	2.44 ± 1.22	16.84 ± 2.33	6.81 ± 1.07	5.10 ± 1.21	5.66 ± 1.53	3.23 ± 1.18
9d	7.12 ± 1.12	19.01 ± 1.06	12.37 ± 1.15	6.36 ± 1.32	7.22 ± 1.31	7.55 ± 1.15
ACV	0.11 ± 1.07	16.84 ± 1.20	12.50 ± 1.15	35.25 ± 2.34	28.74 ± 2.33	39.44 ± 1.28
FOS	49.07 ± 1.12	126.2 ± 1.0	100.8 ± 1.0	115.7 ± 1.0	134.2 ± 1.0	98.95 ± 1.35

ACV-resistant HSV-1 strains. Thus, we decided to obtain *in vitro* ACV-resistant HSV-1 clones from the KOS strain, the wild type reference used in this study. Their phenotypic features were compared to the previously described TK deficient HSV-1 B2006 strain isolated from an immunodeficient patient. The dose–response curves for ACV and FOS are shown in Fig. 3. Both the known strain and the new clones were highly resistant to ACV, since the increase in their EC_{50}s for ACV varied 113- to 358-fold with respect to that of the wild-type strain KOS (Fig. 3A). In contrast, FOS efficacy was similar to all ACV-resistant and wild-type strains (Table 2). The increase in EC_{50} values was low enough to be considered FOS- sensitive according to the commonly accepted criteria (Safrin et al., 1994).

The next step was to evaluate the ability of the hit compounds to inhibit the propagation of HSV-1 ACV-resistant strains. As shown in Fig. 4, the three compounds that were most active against HSV-1 strain KOS (**8d**, **9c** and **9d**) also protected *in vitro* against the resistant strains B2006 and the four isolated clones with remarkable EC_{50}s (Table 2).

3.4. Identification of antiviral effects by IFI assay

IFI staining was used to confirm the antiviral effects **9c**, the most active compound. As shown in Fig. 5, all cells were virus-positive in untreated HSV-1-infected cells (CV). As expected, ACV reduced the number of fluorescent cells only in HSV-1-KOS infected culture, while **9c** potently inhibited the propagation of all HSV-1 strains. Similar results were obtained for the other clones and hit compounds (data not shown).

*3.5. Influence of the duration of treatment with compound **9c** on HSV-1 infectivity*

To further characterize the inhibitory action of the most active compound, a time of-addition experiment was performed. Vero cells were exposed to compound **9c** pre-, during or post-infection with HSV-1 strains (KOS or B2006) and virus yields were determined at 24 h p.i. When **9c** was added before or during HSV-1 inoculation, no inhibition of infection was detected for either strain.

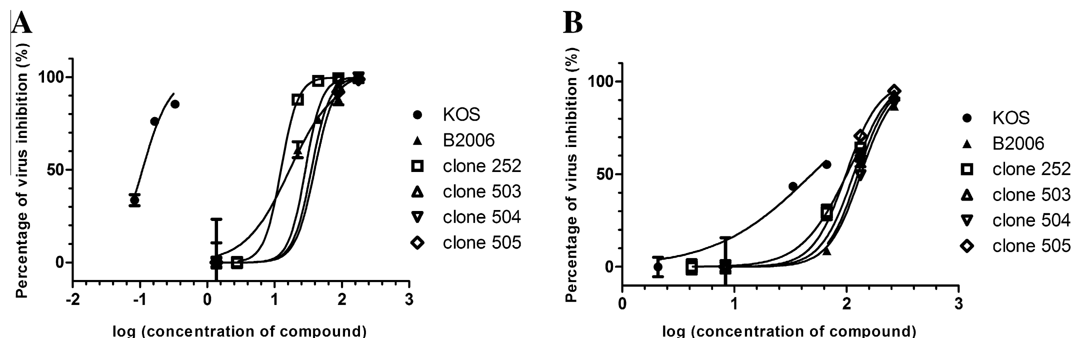


Fig. 3. Phenotypic characterization of ACV-resistant strains of HSV-1. Vero cells infected with HSV-1 strains KOS, B2006, 252, 503, 504 and 505 were treated with different concentrations of ACV (A) or FOS (B).

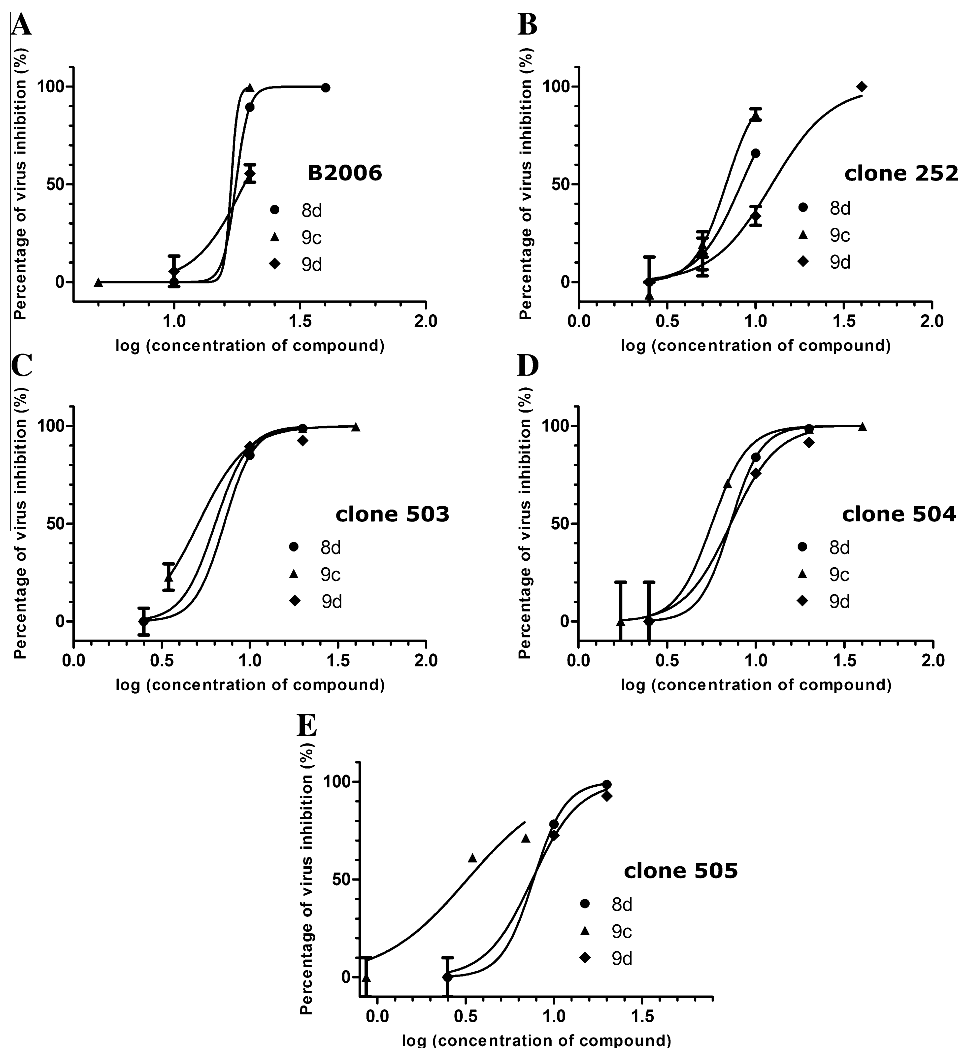


Fig. 4. Dose-dependent response of hit compounds on HSV-1 replication. (A), (B), (C), (D) and (E) show inhibitory effects of different concentrations of **8d**, **9c** and **9d** against HSV-1 strains B2006, 252, 503, 504 and 505, respectively.

However, the HSV-1 KOS and B2006 virus yields were significantly decreased when the steroid was supplemented post-infection (Fig. 6A and B). Then, we decided to make a time of addition/removal assay at 7 h p.i. to evaluate if compound **9c** exerts its anti-herpetic effect in the later stages of the virus replication cycle. The results show that **9c** was able to inhibit infectious particle formation, even when it was added at 7 h p.i., irrespective of HSV-1 strain. Furthermore, when compound **9c** was removed at 7 h p.i. inhibition decreased in both HSV-1 strains (Fig. 6C and D), suggesting that this pregnenolone derivative affects the later stages of the viral cycle.

To broaden this initial result, we determined the amount of cell-associated infectious particles as well as virus yield in the supernatants of treated cells. The formation of intracellular mature virus and extracellular enveloped virus were reduced to the same level in **9c** treated cells with respect to CV for both HSV-1 strains (KOS

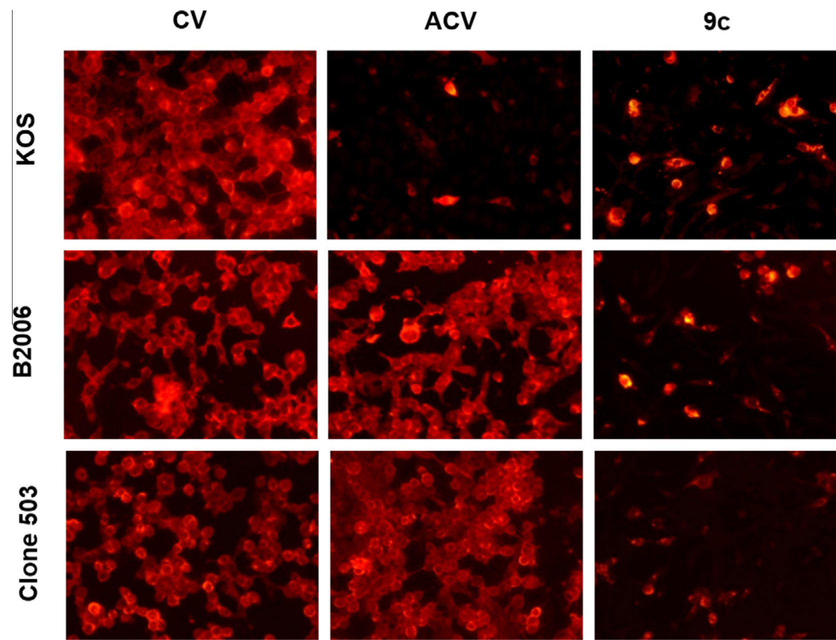


Fig. 5. Identification of antiviral effects by IFI assay. Vero cells infected with HSV-1 KOS or B2006 strains and clone 503 at moi = 0.1 were treated or not (CV) with ACV (10 μM) or **9c** (40 μM). IFI staining was performed by adding anti-gD antibodies to cells fixed with methanol at 24 h p.i. Magnification ×400.

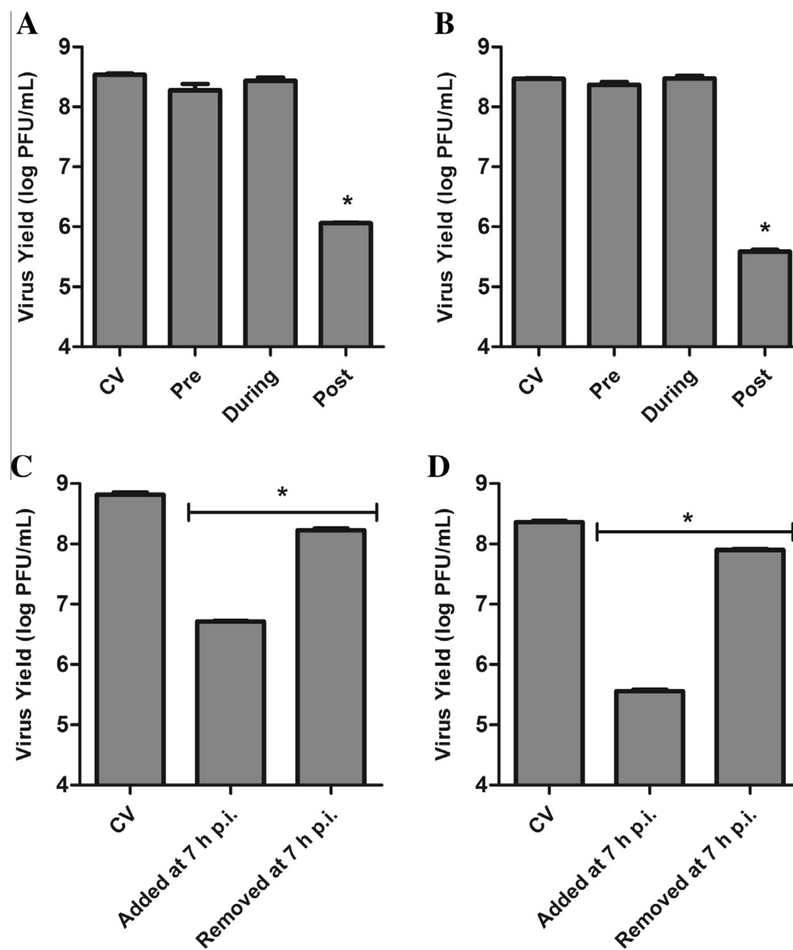


Fig. 6. Influence of time of treatment with hit compounds on HSV-1 infectivity. Vero cells were exposed or not (CV) to **9c** (40 μM) before, during and after infection with HSV-1 strains KOS (A and C) or B2006 (B and D). For time of addition/removal assays infected cells were treated with **9c** immediately after adsorption or at 7 h p.i. and the drug was removed at 7 h p.i. or at 24 h p.i. respectively. Total virus yields were always determined by plaque assay at 24 h p.i. *Significantly different from CV (p-value <0.05).

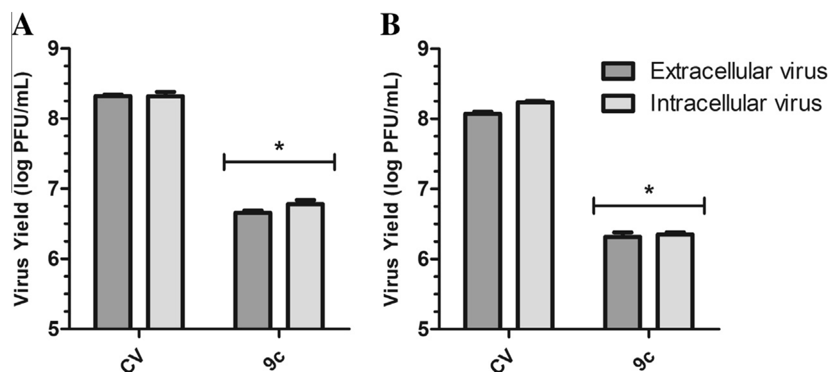


Fig. 7. Effect of **9c** on extracellular and intracellular virus yields. Vero cells were infected with HSV-1 strain KOS (A) or B2006 (B) (moi = 10) for 1 h at 37 °C and then treated or not (CV) with 40 μM of **9c**. After 24 h of incubation, extracellular and intracellular virus yields were determined by plaque assay. *Significantly different from CV (p-value <0.05).

or B2006) (Fig. 7A and B). Thus, the antiviral effect of **9c** would not be related to the inhibition of virus egress.

In a preliminary attempt to provide some insight into the mechanism of action, we evaluate the effect of **9c** on the synthesis of gD HSV-1 protein by immunoblotting. We found that compound **9c** did not significantly affect the intensity of viral or cellular bands, although the electrophoretic mobility of gD band was increased, suggesting an effect on oligosaccharide processing (Fig. 8A).

3.6. Glucocorticoid-like activity of the synthetic steroids

Glucocorticoids are prescripted in some cases during herpetic infections seeking to reduce the associated inflammation. Nevertheless, their immunosuppressive effect usually induces viral reactivation. Thus, we decided to assess whether these pregnenolone derivatives could mimic such a glucocorticoid-like activity. Firstly, we transfected HEK 293T cells with a GR-EGFP construct. When these cells were treated with dexamethasone at 10 nM, almost complete translocation of GR to the nucleus was observed. However, when cells were exposed to a high concentration of **9c** (50 μM) for one hour, GR signal remained almost completely in the cytoplasm, suggesting that **9c** was not able to induce the translocation of the receptor. Moreover, when cells were treated simultaneously with 10 nM of dexamethasone and 50 μM of **9c**, no competitive effect was observed (Fig. 8B).

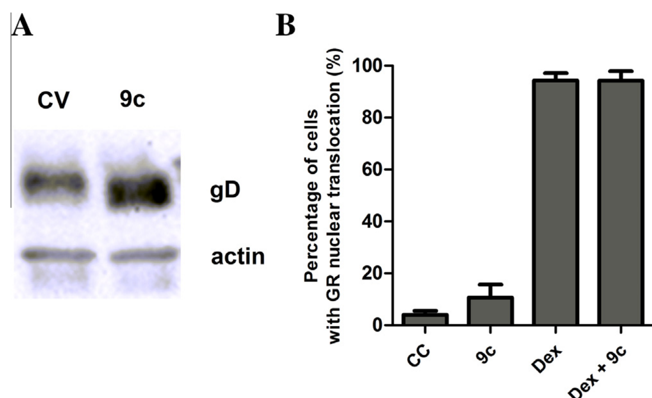


Fig. 8. (A) HSV-1 infected cells were treated or not (CV) with **9c**. After 14 h, cells were lysated and subjected to SDS–PAGE, followed by immunoblotting with antibodies against viral glycoprotein gD. Actin was used as a control cellular protein. (B) Upon addition of 10 nM dexamethasone to cells expressing pEGFP-GR, translocation of GR into the nucleus was observed, whereas GR-associated fluorescence appeared in the cytoplasm of untreated cells (CC). In cells exposed to 50 μM of **9c** no translocation was observed, suggesting that **9c** did not induce GR translocation. Co-treatment with both steroids did not show a competitive effect.

4. Discussion

In recent decades steroids have emerged as a new source of antiviral compounds (Castilla et al., 2010). In the current work, we show that three new pregnenolone derivatives with diamide side chains at C16 are able to reduce the HSV-1 viral yield without affecting cell viability (Table 1 and Fig. 2A and B). This antiviral activity could not be attributed to direct inactivation of virus particles (Fig. 2C). On the contrary, antiviral activity was demonstrated by a reduction in virus yield (Fig. 2B) and by the inability to detect intracellular viral antigen in IFI assay (Fig. 5).

Moreover, hit compounds were equally effective against different ACV-resistant HSV-1 strains (Figs. 4 and 5). The HSV-1 B2006 strain, isolated from an immunodeficient patient, was used as ACV-resistant reference strain. Furthermore, we characterized the phenotypic antiviral drug susceptibility of a panel of ACV-resistant clones isolated from HSV-1 KOS strain after a single round of selection. As a result, all selected clones were ACV-resistant and FOS-susceptible (Fig. 3), it is likely, therefore, that all clones have mutations at the TK gene. FOS does not require virus-specific intracellular phosphorylation, so does not show cross-resistance to ACV in TK affected cases. Nearly 95% of ACV-resistant clinical HSV isolates contain mutations in the viral TK and not in the viral DNA polymerase, therefore, FOS is recommended as alternative compound in ACV-resistant HSV infections (Andrei et al., 2005). Nevertheless, FOS has been associated with serious side effects, most commonly nephrotoxicity, which affects 30% of patients (Wang and Smith, 2014).

As previously mentioned, when the ACV therapy fails, alternative drugs, mainly nucleoside analogues, must be used. Unfortunately, the emergence of viral strains resistant to these drugs has also been reported (Andrei and Snoeck, 2013). The steroidal derivatives described in this work could represent an alternative starting point for the development of new antiherpetic drugs.

Although the mechanisms of action of these new pregnenolone derivatives are not known, it is highly probable that, because of their chemical structure, they interfere with different steps of the viral cycle than nucleoside analogues. The fact that some of the synthetic steroids described in this paper exert a remarkable antiviral effect against both wild type and ACV-resistant strains of HSV-1, supports this idea (Figs. 2, 4 and 5). Moreover, time-of-addition experiments revealed that these pregnenolone analogues do not affect virus adsorption or penetration into the cell (Fig. 6). Instead, the most active compound, **9c**, reduces infectious virion

formation even when it was added at 7 h p.i., thereby inhibiting late stages of the viral cycle (Fig. 7). This differs from the mechanism of action of ACV, which interferes with DNA replication, thus affecting earlier stages of the cycle.

A preliminary search aimed to find which step of the virus replication could be affected by **9c** revealed an alteration on the maturation of virus glycoproteins (Fig. 8B). This effect would explain the antiviral action of these synthetic steroidal compounds, because oligosaccharide processing of virus glycoproteins seems to be required for the proper virus assembly. (Ghosh-Choudhury et al., 1994).

On the other hand, the use of steroidal compounds for the treatment of HSV-1 infections may be questioned, since there is an important number of controversial results on the viral reactivation triggered by mammalian steroid hormones (Castilla et al., 2010). In particular, glucocorticoids that depress the innate immune inflammatory response are not recommended for HSV-1 treatment. Interestingly, our results suggest that, as **9c** was not able to significantly induce the nuclear GR translocation even at a concentration about 10-fold higher than its EC_{50}, this pregnenolone derivative would not have a glucocorticoid-like action. In conclusion, three synthetic pregnenolone derivatives showed remarkable inhibitory effects on the HSV-1 spread, both on wild type and ACV-resistant strains. Our observations also suggest that these compounds interfere with late steps in the viral replication cycle.

Additional studies to elucidate the molecular target and improve the antiviral activity are underway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2015.08.002.

References

Alonso, F., Cirigliano, A.M., Dávola, M.E., Cabrera, G.M., García Liñares, G.E., Labriola, C., Barquero, A.A., Ramírez, J.A., 2014. Multicomponent synthesis of 4,4-dimethyl sterol analogues and their effect on eukaryotic cells. Steroids 84, 1–6. http://dx.doi.org/10.1016/j.steroids.2014.03.002.

Andrei, G., Snoeck, R., 2013. Herpes simplex virus drug-resistance: new mutations and insights. Curr. Opin. Infect. Dis. 26, 551–560. http://dx.doi.org/10.1097/QCO.0000000000000015.

Andrei, G., Balzarini, J., Fiten, P., De Clercq, E., Opdenakker, G., Snoeck, R., 2005. Characterization of herpes simplex virus type 1 thymidine kinase mutants selected under a single round of high-dose brivudin. J. Virol. 79, 5863–5869. http://dx.doi.org/10.1128/JVI.79.9.5863-5869.2005.

Bag, P., Ojha, D., Mukherjee, H., Halder, U.C., Mondal, S., Biswas, A., Sharon, A., Van Kaer, L., Chakrabarty, S., Das, G., Mitra, D., Chattopadhyay, D., 2014. A dihydro-pyrido-indole potently inhibits HSV-1 infection by interfering the viral immediate early transcriptional events. Antiviral Res. 105, 126–134. http://dx.doi.org/10.1016/j.antiviral.2014.02.007.

Banday, A.H., Shameem, S.a., Gupta, B.D., Kumar, H.M.S., 2010. D-ring substituted 1,2,3-triazolyl 20-keto pregnenanes as potential anticancer agents: synthesis and biological evaluation. Steroids 75, 801–804. http://dx.doi.org/10.1016/j.steroids.2010.02.015.

Bueno, C.A., Barquero, A.A., Di Cónsoli, H., Maier, M.S., Alché, L.E., 2009. A natural tetranortriterpenoid with immunomodulating properties as a potential anti-HSV agent. Virus Res. 141, 47–54. http://dx.doi.org/10.1016/j.virusres.2008.12.013.

Castilla, V., Ramírez, J.A., Coto, C.E., 2010. Plant and animal steroids a new hope to search for antiviral agents. Curr. Med. Chem. 17, 1858–1873.

Comin, M.J., Maier, M.S., Roccatagliata, A.J., Pujol, C.A., Damonte, E.B., 1999. Evaluation of the antiviral activity of natural sulfated polyhydroxysteroids and their synthetic derivatives and analogs. Steroids 64, 335–340.

Dávola, M.E., Alonso, F., Cabrera, G.M., Ramírez, J.A., Barquero, A.A., 2012. Sterol analogues with diamide side chains interfere with the intracellular localization of viral glycoproteins. Biochem. Biophys. Res. Commun. 427, 107–112. http://dx.doi.org/10.1016/j.bbrc.2012.09.019.

Dömling, A., Wang, W., Wang, K., 2012. Chemistry and biology of multicomponent reactions. Chem. Rev. http://dx.doi.org/10.1021/cr100233r.

Egan, K.P., Wu, S., Wigdahl, B., Jennings, S.R., 2013. Immunological control of herpes simplex virus infections. J. Neurovirol. 19, 328–345. http://dx.doi.org/10.1007/s13365-013-0189-3.

Ghosh-Choudhury, N., Butcher, M., Reid, E., Ghosh, H.P., 1994. Effect of tunicamycin and monensin on the transport to the cell surface and secretion of a viral membrane glycoprotein containing both N- and O-linked sugars. Biochem. Cell Biol. 72, 20–25. http://dx.doi.org/10.1139/o94-004.

Hjalmarsson, A., Blomqvist, P., Sköldenberg, B., 2007. Herpes simplex encephalitis in Sweden, 1990–2001: incidence, morbidity, and mortality. Clin. Infect. Dis. 45, 875–880. http://dx.doi.org/10.1086/521262.

Hodge, R.A.V., Field, H.J., 2013. Chapter one – antiviral agents for herpes simplex virus. In: De Clercq, E. (Ed.), Antiviral Agents, Advances in Pharmacology. Academic Press, pp. 1–38. http://dx.doi.org/10.1016/B978-0-12-405880-4.00001-9.

Iqbal Choudhary, M., Shahab Alam, M., Atta-Ur-Rahman, Yousuf, S., Wu, Y.-C., Lin, A.-S., Shaheen, F., 2011. Pregnenolone derivatives as potential anticancer agents. Steroids 76, 1554–1559. http://dx.doi.org/10.1016/j.steroids.2011.09.006.

Michelini, F.M., Ramírez, J.A., Berra, A., Galagovsky, L.R., Alché, L.E., 2004. In vitro and in vivo antiherpetic activity of three new synthetic brassinosteroid analogues. Steroids 69, 713–720. http://dx.doi.org/10.1016/j.steroids.2004.04.011.

Michelini, F.M., Ramírez, J.A., Berra, A., Galagovsky, L.R., Alché, L.E., 2008. Anti-herpetic and anti-inflammatory activities of two new synthetic 22,23-dihydroxylated stigmastane derivatives. J. Steroid Biochem. Mol. Biol. 111, 111–116. http://dx.doi.org/10.1016/j.jsbmb.2008.05.005.

Petrera, E., Joselevich, M., Ghini, A., Burton, G., Coto, C.E., 2003. Antiherpes virus activities of new 6–19 carbon-bridged steroids and some synthetic precursors. Antivir. Chem. Chemother. 14, 243–248.

Porta, E.O.J., Carvalho, P.B., Avery, M.a., Tekwani, B.L., Labadie, G.R., 2014. Click chemistry decoration of amino sterols as promising strategy to developed new leishmanicidal drugs. Steroids 79, 28–36. http://dx.doi.org/10.1016/j.steroids.2013.10.010.

Rowe, A.M., St. Leger, A.J., Jeon, S., Dhaliwal, D.K., Knickelbein, J.E., Hendricks, R.L., 2013. Herpes keratitis. Prog. Retin. Eye Res. 32, 88–101. http://dx.doi.org/10.1016/j.preteyeres.2012.08.002.

Safrin, S., Elbeik, T., Mills, J., 1994. A rapid screen test for in vitro susceptibility of clinical herpes simplex virus isolates. J. Infect. Dis. 169, 879–882. http://dx.doi.org/10.1093/infdis/169.4.879.

Vilhelmova-Ilieva, N., Jacquet, R., Quideau, S., Galabov, A.S., 2014. Ellagitannins as synergists of ACV on the replication of ACV-resistant strains of HSV 1 and 2. Antiviral Res. 110, 104–114. http://dx.doi.org/10.1016/j.antiviral.2014.07.017.

Wang, Y., Smith, K.P., 2014. Safety of alternative antiviral agents for neonatal herpes simplex virus encephalitis and disseminated infection. J. Pediatr. Pharmacol. Ther. 19, 72–82. http://dx.doi.org/10.5863/1551-6776-19.2.72.

Wankhede, K.S., Vaidya, V.V., Sarang, P.S., Salunkhe, M.M., Trivedi, G.K., 2008. Synthesis of novel isoxazole-linked steroidal glycoconjugates-an application of a novel steroidal nitrile oxide. Tetrahedron Lett. 49, 2069–2073. http://dx.doi.org/10.1016/j.tetlet.2008.01.130.

Welsch, M.E., Snyder, S.a., Stockwell, B.R., 2010. Privileged scaffolds for library design and drug discovery. Curr. Opin. Chem. Biol. 14, 347–361. http://dx.doi.org/10.1016/j.cbpa.2010.02.018.

Whitley, R.J., 2006. Herpes simplex encephalitis: adolescents and adults. Antiviral Res. 71, 141–148. http://dx.doi.org/10.1016/j.antiviral.2006.04.002.

Whitley, R.J., Roizman, B., 2001. Herpes simplex virus infections. Lancet 357, 1513–1518. http://dx.doi.org/10.1016/S0140-6736(00)04638-9.

Woroch, E.L., 1963. The structure proof of 16α-carboxypregnenolone. J. Org. Chem. 28, 855–857. http://dx.doi.org/10.1021/jo01038a507.