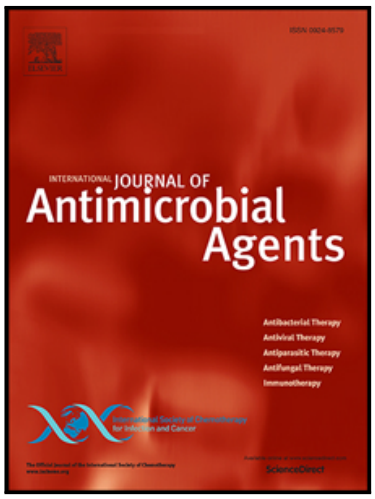

β-Carboline Derivatives as Novel Antivirals for Herpes Simplex Viruses

Gonzalez M. Micaela , Cabrerizo Franco M , Baiker Armin , Erra-Balsells Rosa , Osterman Andreas , Nitschko Hans , Vizoso-Pinto María G.

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Highlights Gonzalez et al.

- 11 out of 21 β-carbolines inhibited HSV-1 in a MTS-based antiviral screening.
- 6-methoxy-, 9-methyl-harmane, and 9-methyl-norharmane reduced HSV-1 &-2 virus yields.
- These compounds were not virucidal and did not inhibit adhesion of viral particles to cells.
- They worked at later stages of virus infection.
- The substances interfered with ICP0 localization and expression of late proteins.

β-CARBOLINE DERIVATIVES AS NOVEL ANTIVIRALS FOR HERPES SIMPLEX VIRUSES

Gonzalez, M. Micaela[1,2,*]; Cabrerizo, Franco M.[2]; Baiker, Armin[3]; Erra-Balsells, Rosa[4]; Osterman, Andreas[1,5]; Nitschko, Hans[1,5]; and Vizoso-Pinto, María G.[1,6,*]

[1]Max von Pettenkofer Institute, Virology, National Reference Center for Retroviruses, Faculty of Medicine, LMU

D-80336, Munich, Germany.

[2]IIB-INTECH-CONICET, Universidad Nacional de San Martín, 7130 Chascomús, Buenos Aires, Argentina.

[3]Bavarian Health and Food Safety Authority, Oberschleissheim, Germany.

[4]CONICET, Universidad de Buenos Aires. Centro de Investigación en Hidratos de Carbono (CIHIDECAR). Facultad de Ciencias Exactas y Naturales. Pabellón II, 3er P., Ciudad Universitaria, 1428 Buenos Aires, Argentina.

[5]German Center for Infection Research (DZIF), partner site Munich, Germany.

[6]INSIBIO-CONICET, Departamento Biomédico. Facultad de Medicina, Universidad Nacional de Tucumán, 4000, Argentina.

***Corresponding authors:**

Dr. M. G. Vizoso-Pinto

Permanent address: Instituto de Investigación en Medicina Molecular y Celular Aplicada (IMMCA) Dorrego 1080, T-4000, San Miguel de Tucumán & Dto. Biomédico, Facultad de Medicina, Universidad Nacional de Tucumán, Av. Kirchner 2100, T-4000, San Miguel de Tucumán, Tucumán, Argentina. Tel. +54 381 4369043 int.7960/7953, fax: +54 381 4248921 E-mail address: mgvizoso@fm.unt.edu.ar

Dr. M. M. Gonzalez

Permanent address: IIB-INTECH-UNSAM-CONICET (Chascomús). Intendente Marino Km 8,2. CC 164 (7130), Chascomús, Buenos Aires, Argentina. Tel: +54 02241 430323/424049 int. 123, fax: +54 02241 424048. E-mail: mgonzalez@intech.gov.ar

ABSTRACT

Several commercial and novel synthetic βCs were evaluated for their antiviral activity against HSV-1 using an adapted MTS assay. Of 21 drugs tested, although 11 exerted antiviral activity at non-cytotoxic concentrations, only three of them (9-methyl-norharmane, 9-methyl-harmane and 6-methoxy-harmane) completely avoided virus-driven cytopathic effects. Effective concentrations (EC50, 4.9 ± 0.4 μM, 5.9 ± 0.8 μM and 19.5 ± 0.3 μM, respectively) and selectivity indexes (SI, 88.8, 40.2 and 7.0, respectively) of the latter three βCs against HSV-1 were determined by MTS, flow cytometry and plaque reduction assays. The mode of action of these drugs was also evaluated. According to time-of-addition assays, the selected compounds were not virucidal and did not interfere with attachment or penetration of HSV-1, but with later events of virus infection. Western blot studies showed that early and late protein expression was significantly delayed or even suppressed. HSV-2 was also inhibited by the selected substances in a similar way. Interestingly, 6-methoxy-harmane, 9-methyl-harmane and 9-methyl-norharmane restricted HSV-1 ICP0 localization to the nucleus during later stages of infection, possibly affecting its functionality at the cytoplasm, where ICP0 normally inhibits antiviral signaling and promotes viral replication. *In silico* prediction of ADME (Absorption, Distribution, Metabolism and Excretion) properties showed that all compounds fulfilled Lipinski's rule and their calculated absorptions were over 95%.

Keywords: carbolines, antiviral, herpes simplex virus, HSV, ICP0, alkaloids, anti-herpetic

1. Introduction

Herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) are dsDNA human herpes viruses that belong to *Alphaherpesvirinae*. They are transmitted across epithelial mucosal cells as well as through skin lesions. These viruses migrate along axons towards neuronal ganglia, where they persist in a latent state and may reactivate several times during an individual's lifespan. HSV-1 predominantly causes orofacial lesions, whereas HSV-2 mainly infects the genital mucosa [1].

Aciclovir (ACV), a nucleoside analogue, represents the gold standard treatment for HSV infections [2]. However, its widespread use has led to viral resistance [3]. Lessons learned from strategies to control Human Immunodeficiency Virus (HIV) infections point towards the combined use of antiviral drugs for successful therapies. Therefore, there is a need to find new strategies against ACV-resistant HSV viruses, to prevent resistance and improve efficacy and pharmacokinetics of actual treatments [2, 4].

β-carbolines (βCs) alkaloids are widely distributed in nature; they can be found in mammals [5], plants [6] and other organisms. Depending on their intrinsic chemical structure, βCs show a broad pharmacological spectrum [6, 7] as promising anti-tumor [7], antimicrobial [8-11] and antiviral agents [12]. Regarding their antiviral properties, harmane derivatives inhibit the replication of HIV in H9 lymphocytic cells and the substitution of –H with 7-methoxy or an alkyl group at the indole nitrogen of the harmane backbone enhanced its anti-HIV activity [13]. Flazin, a βC isolated from *Suillus granulatus*, presented weak anti-HIV activity. However, its synthetic analogue, flazinamida, showed higher activity against HIV [14]. In addition, several 1,3-disubstituted βC derivatives bearing a substituted carbohydrazide group at C-3 were active against polio vaccine virus and HSV-1 [15]. Furthermore, 9-methylharmine inhibited dengue virus 2 *in vitro* [12].

The need to continue searching for novel antiviral agents, and the intrinsic chemical properties of βCs, lead us to evaluate 21 βCs (Scheme 1) for their activity against HSV-1. We also provide insight into the mechanism of action of selected active compounds against HSV-1. Lipophilicity, topological polar surface area (TPSA), absorption (% ABS) and simple molecular descriptors (using Lipinski's rule) of the compounds were predicted by computational analysis.

2. Materials and methods

2.1. Compounds, cells and viruses

Norharmane, harmane, harmine, harmaline, 6-methoxy-harmane and Acyclovir (Sigma-Aldrich Co.) were of the highest purity available (>98%). Non-commercial derivative synthesis was described previously (purity >98%) [16-19]. Stock solutions of βCs were prepared in pH 2 sterile ultrapure water [11, 20] and diluted (10%, vol/vol) to working concentrations in DMEM. The substances diluted in DMEM did not change the final pH of the medium.

Vero cells were grown in Dulbecco's Modified Eagle Medium (DMEM, 4.5 g/l glucose, (+)L-glutamine, (-)pyruvate) supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 1% non-essential aminoacids (NEAA), 1% L-glutamine (Q), 0.1% gentamycin (G) and 0.2% fungizone (F) (Life Technologies, Germany).

Two clinical isolates of HSV-1 and HSV-2 (Max-von-Pettenkofer Institute, LMU, Germany), previously identified by sequencing were propagated in Vero cells. Viral stocks were stored at –80°C and virus titers were determined by plaque assay as described elsewhere [21]. Briefly, Vero cell monolayers grown on 24-well plates were infected with serial dilutions of viral stocks, centrifuged at RT (500 rpm, 5 min) and incubated for 1h at 37°C in a 5% CO_2 humidified atmosphere. Supernatants were removed, and infected cells were incubated in DMEM added with 0.75% carboxymethylcellulose, (Sigma). After 72h, cell monolayers were fixed and stained with crystal violet (0.1% in ethanol 20%).

2.2. Computational study.

Simple molecular descriptors (using Lipinski's rule), topological surface area (TPSA) and absorption (%ABS) were calculated with the *Molinspiration online property calculation toolkit* (http://www.molinspiration.com).

2.3. Cytotoxicity assays

2.3.1. MTS assay. Vero cell monolayers, grown at 90% confluence in 96 well microplates, were exposed to different concentrations of the antiviral candidates or the vehicle as negative control diluted in DMEM (see 2.1.). After 21h, cell viability was examined by the MTS colorimetric assay [22] using CellTiter 96® AQueous One Solution Reagent (Promega), following manufacturer's

instructions. Absorbance ($A^{490\ nm}$) was measured in a microplate reader (Tecan, Sunrise™). The percentage of cell viability was calculated as:

$$\%\ cell\ viability = \frac{(A490\ sample - A490\ background)}{(A490\ cell\ control - A490\ background} \times 100 \quad (1)$$

where A^{490}_{sample} is the absorbance of each sample, and $A^{490}_{\text{cell control}}$ is the average of the absorbance measured from 3 wells containing cell controls, and $A^{490}_{\text{background}}$ is the average of the absorbance of the reagent without cells. Experiments were done in triplicates.

2.3.2. Propidium iodide (IP) exclusion assay. Monolayers of Vero cells were incubated with the compounds at different concentrations for 48h at 37°C. Then, supernatants, cell washes, and cells were harvested and centrifuged at 800 ×*g* for 10 min. Cells suspended in PBS were stained with IP (Sigma, 10 μg/mL) for 10 min and examined using a BD FACSCalibur flow cytometer. Data were analyzed using the FlowJo 7.6 software. The cells were excited at 488 nm and the IP fluorescence was read on FL3 in log scale (10,000 events). Two independent sets of experiments were done in triplicates. 50% cytotoxic concentration values (CC50) were calculated with GraphPad Prism 5 by non-linear regression analysis of %Cell Viability vs. Log [βC] (variable Hill's slope).

2.4. Screening for antiviral activity

The cytotoxicity protocol (section 2.3.1) was slightly adapted to screen for antiviral activity. Briefly, monolayers of Vero cells grown in 96 well microplates were incubated with different concentrations of the putative antiviral drugs and ACV. DMEM added with vehicle was used as control. The cells were infected with HSV-1 (100 PFU/well) and incubated until control cultures displayed extensive cytopathology. Then, we replaced the medium and conducted the MTS assay in triplicates. The percentages of antiviral activity were calculated using equation 2:

$$\%\ antiviral\ activity = \frac{(A490\ sample - A490\ virus\ control)}{(A490\ cell\ control - A490\ virus\ control)} \times 100 \quad (2)$$

where $A^{490}_{\text{virus control}}$ is the average of the absorbance measured from 3 wells containing virus controls (cells infected with viruses without drugs). A^{490}_{sample} and $A^{490}_{\text{cell control}}$ were defined in 2.3.1. We selected those compounds that both reduced viral load ≥60% and completely protected cells from HSV driven cytopathic effects at not cytotoxic concentrations.

2.5. Plaque reduction assays and EC50 determination

Vero cells were treated with medium containing the antiviral candidates 6-MeO-Ho, 9-Me-Ho, 9-Me-nHo at different concentrations or the vehicle as negative control prior to and during infection with HSV-1. After incubation for 48h at 37°C, cells were fixed and stained with crystal violet. Plaques were counted and the percentage of plaque reduction was calculated as follows: [1 - (V*d*/V*c*)]×100, where V*d* and V*c* refer to the number of plaques in the presence and absence of substances, respectively. To determine EC50, the percentage of plaque reduction was plotted against βCs concentrations, and non-linear regression analysis of plaque reduction (%) vs. Log [βC] (variable Hill's slope) was calculated with GraphPad Prism 5.

2.6. Experiments to assess the effect of selected drugs during different stages of infection. Time-of-addition experiments

2.6.1. Virucidal activity. HSV-1 and HSV-2 suspensions (2000 PFU/ml) were incubated with the selected βCs at the concentrations that resulted in a 2-log decrease (*i.e.* 30 μM, 50 μM and 60 μM for 6-MeO-Ho, 9-Me-Ho and 9-Me-nHo, respectively) or ACV (10 μM) or DMEM added with the vehicle for 1h at RT. Afterwards, they were added to Vero cell monolayers, centrifuged at 500 rpm for 5 min and incubated for 1h at 37°C to allow virus uptake. Then, medium was removed, cells were washed once with PBS and plates were incubated in fresh DMEM until control cultures displayed extensive cytopathology (48h). Cells were harvested, lysed by freeze-thaw cycling and infectious virus yield was titred on Vero cells.

2.6.2. Effect on viral attachment. To determine whether these drugs interfere in the absorption/penetration stage, Vero cell monolayers grown in 12-well plates were incubated for 1h, at 37°C, with DMEM supplemented with the selected drugs, ACV (10 μM) or the vehicle. Then, cells were washed and infected with HSV-1 or HSV-2 (2000 PFU/well). Plates were centrifuged at 500 rpm, for 5 min, and incubated at 37°C for 1h. The following procedures were as described in 2.6.1.

2.6.3. Treatment after infection. To test whether selected βCs interfere during replication, the cell monolayers were infected with HSV-1 or HSV-2. Plates were centrifuged at 500 rpm for 5 min and incubated for 1h at 37°C. Cells were washed once with PBS, and fresh DMEM supplemented with

the selected drugs, ACV (10 μM) or the vehicle was added. The following procedures were as described in 2.6.1.

2.7. Viral replication in the presence of selected βCs.

To investigate the influence of selected compounds on viral replication, confluent Vero cells were treated with the selected drugs prior and simultaneously to infection with HSV-1 or HSV-2. At different time points, we collected the supernatants and cells independently and titred infectious viruses. Control wells were treated with ACV (10 μM) or the vehicle.

2.8. Quantitative Herpes simplex Virus PCR

Nucleic acid was extracted with the MagNA Pure LC 2.0 System (Roche Diagnostics GmbH, Penzberg, Germany). Specific HSV-1 sequences were amplified from 5 μl extracted DNA using a 7500 Fast Real-Time PCR System and TaqMan® Fast Universal PCR Master Mix (Thermo Fisher Scientific Inc., Waltham, USA). Optimized primers and a FAM-labeled probe amplified an 81-nt sequence of the polymerase. The thermocycler settings were: 20 sec, 95°C 45 cycles of (3 sec at 95°C and 30 sec at 60°C). Quantification was achieved by simultaneous amplification of four HSV-plasmid-DNA standards of 5×10^2, 5×10^3, 5×10^4 and 5×10^5 copies/ml.

2.9. Immunoblotting

Whole cell protein extracts derived from HSV-1 infected cells treated with βC or the respective controls (see 2.6) were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were blocked in 5% skim milk in 20 mM Tris, pH7.5, 150 mM NaCl, 0.5% Tween 20) rinsed and incubated with monoclonal anti-ICP8 (Sta. Cruz), anti-b-tubulin (Abcam), anti-ICP5 (Abcam) and anti-gB (Abcam), respectively for 3h at RT. Membranes were incubated for 1h at RT with HRP-labeled anti-mouse antibodies in 1X TBST and visualized using ECL Western blot detection kit (G&E).

2.10. Immunofluorescence staining and microscopy

HSV-1 (1000 PFU/well) and selected drugs, ACV (10 μM) or the vehicle were added to Vero cell monolayers grown in 24-well plates. After incubation (20h), medium was removed, cells were washed once with PBS and fixed for 30 min in 4% paraformaldehyde. Cells were permeabilized

with 0.5% Triton X-100 (Sigma-Aldrich). Non-specific binding was blocked with PBS added with goat serum (10% v/v) and Triton X-100 (0.3%). Cells were subsequently incubated for 3h at RT with monoclonal anti-ICP0 (Sta. Cruz) and anti-gB (Abcam), respectively.

All experiments described were performed at a MOI of 0.006. Tests presented in 2.5-2.10 were carried out in triplicates in two independent experiments.

2.11. Statistical analysis. One-way ANOVA with Dunnett's post-test was performed using GraphPad Prism 5 ($p < 0.05$).

3. Results and discussion

To identify novel substances capable of inhibiting HSV replication *in vitro*, we tested a library of 21 βCs consisting of norharmane, harmane and harmine, and 18 compounds derived from these molecules. The generic structure of βCs and substituents (methyl-, methoxy- or halogen-groups) at different C-positions are depicted in Scheme 1.

3.1. Computational Study

A high bioavailability and appropriate drug delivery are desired properties in the development of a drug candidate. Therefore, an *in silico* study for prediction of ADME properties of the 21 βCs investigated herein was performed by using Molinspiration toolkit. Simple molecular descriptors (using Lipinski's rule [23]) and topological surface area (TPSA) were determined. Percentages of absorption (%ABS) were estimated using the equation: %ABS = 109 – (0.345 × TPSA) [24]. According to Lipinski's rule, orally active drugs should follow these criteria: (1) no more than 5 hydrogen bond donors such as -OH or -NH groups (n-OHNH), (2) no more than 10 hydrogen acceptors such as -O or -N atoms (n-ON), (3) an octanol-water partition coefficient (Log P) not greater than 5 and (4) a molecular weight lower than 500. TPSA predicts drug transport properties: drugs with poor bioavailability and absorption have high TPSA values. Additionally, lipophilicity affects drug absorption, bioavailability, hydrophobic drug-receptor interactions, metabolism of molecules, and their toxicity.

According to these criteria, all studied drugs fulfilled Lipinski´s rule and have TPSA values ranged between 17 and 38, whereas %ABS were found to be higher than 95% (Table 1). Particularly, 9-methylated compounds showed the lowest TPSA values and, consequently, the

highest %ABS values. It is noteworthy that all compounds demonstrated better TPSA and %ABS than Acyclovir (67.9%). Thus, these derivatives should be active when orally administered. Further tests in animal models should confirm this prediction.

Although βCs are present in biological fluids, they may damage isolated DNA *in vitro* when exposed to UVA irradiation [25-27]. However, the level of DNA damage produced in whole cells was much lower [28] probably due to activation of DNA repair mechanisms. The use of light as adjuvant to anti-herpetic therapy βCs should be object of further studies, as well as the eventual side effects after light exposure if the substances are applied topically.

3.2. Cytotoxic and antiviral screening of novel compounds

Of 21 compounds tested using an adapted MTS assay, 11 displayed antiviral activity at least at one of the concentrations tested (Table 2). The cytotoxicity of βCs was tested on Vero cells to discard that the antiviral effect was due to a loss of cell viability. Several compounds, especially those substituted with halogen groups, precipitated at higher concentrations making impossible to calculate the CC50. Thus, we indicated in table 2 the highest concentration at which the compound was still soluble in culture medium and cells were viable as tested with the MTS or the PI exclusion assay.

Sako et al.[29] demonstrated that introducing a methyl group in γ-carbolines enhanced their antiviral activity. Later, Salim et al.[30] a stronger effect when introducing more methyl groups in the molecule. In our study, a methyl group at N9 enhanced antiviral activity in 9-Me-nHo, which showed a ~5-fold higher activity than its parental compound nHo (Table 2), whereas 9-Me-Ho almost doubled antiviral activity of Ho at 20 µM. At 40 µM, the measured antiviral activity of Ho was higher than 9-Me-Ho but it was not free of cytopathic effects as it was the case for 9-Me-Ho. We noticed that in contrast to Quintana et al.'s data [12], our assays showed only a discrete reduction of cytotoxicity in N9-methyl substituted βCs with respect to the unsubstituted βC-ring. Interestingly, N9-methyl and 6-methoxy substitutions appear to increase antiviral activity according to our screening (Table 2).

Bag et al.[31] reported the anti-HSV activity of harmaline, with a CC50=11.0-30.0 µg/ml (i.e. 51.3-140 µM) determined by an MTS assay, an EC50=1.1-3.1 µg/ml and a SI=27.27. In contrast,

harmaline was not inhibitory at 1.61-3.21 µg/ml in this study. This could be due to differences in methodology.

Later, Chen et al.[32] reported an EC50 value around 1.47 µM and a CC50 value at around 337.10 µM for harmine, a close related analog of harmaline, on HSV-2 in *in vitro* assays. In agreement with this, the two concentrations of harmine tested here (0.64 and 1.27 µg/ml) had a potent inhibitory activity of 39% and 71%, respectively. Nevertheless, cells incubated with harmine at 1.27 µg/ml showed signs of virus-driven cytopathic effects (several vacuoles in the cytoplasm and morphological changes such as cells rounding-up) indicating that the antiviral effect as determined by the screen was only partial (Table 2). Neither Bag et al.[31] nor Chen et al.[32] informed on cytopathic effects at these concentrations. In addition, we detected a similar cytopathology when checking infected cells treated with other βCs. Therefore, we rather used a more stringent criterion and, selected 6-MeO-Ho, 9-Me-Ho, and 9-Me-nHo (Scheme SI.1), which had both antiviral activity according to the modified MTS screen (>60% inhibition) and prevented signs of viral cytopathic effects (Table 2).

The antiviral activity of 6-MeO-Ho, 9-Me-Ho, and 9-Me-nHo was dose-dependent (Figure 1) below cytotoxic concentrations, suggesting that it was mediated by a specific mechanism and not by cytotoxicity. In our *in vitro* assays, 9-Me-nHo and 9-Me-Ho showed the same antiviral activity, within experimental error, whereas 6-MeO-Ho presented the lowest activity (see EC50 values in Figure 1 and Table 3). βCs are less effective than ACV (EC50 of 0.13±0.02 µM using the MTS assay). Similarly, a 7-methoxy group introduced at the indole nitrogen of the harmane backbone enhanced its anti-HIV activity [13] and a 9-methyl-substituted harmine was active against dengue virus [12].

To calculate SI of the 3 selected compounds, CC50 values were obtained by the IP exclusion assay after 48h. These values were comparable to CC50 previously determined with MTS, after a 21h-incubation (Table 1). Hence, SI for 9-Me-nHo, 9-Me-Ho and 6-MeO-Ho were 88.8, 40.2 and 7.0, respectively (Table 3).

3.3. Evaluation of possible antiviral mechanism.

To explore if selected βCs could deactivate virus particles directly, infectious particles of HSV-1 were incubated with selected compounds prior to monolayer infection. Figure 2A shows that 6-MeO-Ho, 9-Me-Ho nor 9-Me-nHo were not virucidal. Similarly, Nazari et al.[15] reported that other βCs lack virucidal activity against poliovirus and Quintana et al.[12] concluded the same for dengue virus 2.

Then, we evaluated if there was a prophylactic activity on host cells or interference on virus adsorption and/or penetration steps. A pre-treatment of cells with the selected βCs did not block viral adsorption/penetration nor induced activation of antiviral mechanisms in the cell (Figure 2B). Hence, these compounds do not interfere in viral entry. In agreement with this hypothesis, when compounds were added 1 hpi (Figure 2C), HSV-1 virus titers significantly decreased, showing that βCs are active after viral penetration in the host cell. Whether βCs act direct or indirectly remains to be further studied. We saw the same behavior for HSV-2 (Figure SI.1)

In virus yield reduction experiments, in the first 20 hpi virus yield did not change when comparing untreated vs. βC-treated wells (Figure 3A). After that time, virus yield decreases markedly for all compounds. Moreover, βCs' reduction of viral yield was comparable to ACV. The release of infective viral particles to the medium also significantly decreased in the presence of 9-Me-nHo (60 μM), 9-Me-Ho (50 μM), and 6-MeO-Ho (30 μM), at similar levels than observed with ACV (10 μM) (Figure 3B). Similar results were obtained for HSV-2 (Figure SI.2).

We found a significant reduction of HSV-1 genome equivalents/ml in the presence of βCs (Figure 4), though to a lesser extent (at least 1-log slighter) than ACV, which directly interferes in DNA replication. Hence, βCs may also reduce viral DNA replication in direct or indirect ways, which should be subject of further investigations.

After penetration, HSV replicates within the nucleus following three phases: immediate-early (IE), early (E) and late (L) [33]. To get a closer insight into the stage of the viral cycle influenced by βCs, we followed up the expression levels of HSV-1 E and L proteins by Western blot. In general, these assays revealed a down-modulation of protein expression when βCs were present. ICP8 expression was significantly reduced in the presence of βC, when compared to non-treated control or ACV-treated cells (Figures 5A and B). ICP8 acts as a nucleating factor responsible for formation

of pre-replicative sites containing the helicase-primase complex, appearing as punctate foci in the nucleus [34]. ICP0, a HSV IE protein, binds to several cellular proteins as well as viral promoters resulting in increased expression of viral E and L genes. Bag et al.[32] reported that harmine retarded IE events by blocking the binding of IE complex on the ICP0 promoter, leading to reduced expression of E proteins such as ICP4 and ICP7. Expression of IE, E and L proteins follow a cascade of events. Thus, the reduced level of ICP8 and gB expression observed in Western blot assays (Figures 5A and B) may be the consequence of a reduced activation of ICP0 transcription as it was the case for harmine. In contrast, ICP8 expression in the presence of ACV, a drug targeting later events, was not affected.

Several host defense mechanisms are triggered upon DNA virus entry, such as cytokine expression (especially type I IFNs) triggered by recognition of pathogen-associated molecular patterns by pattern recognition receptors. Type I IFNs act in an autocrine or paracrine way to activate the transcription of IFN-stimulated genes (ISG) whose protein products are responsible of mediating the antiviral state. This is a key step in immunity as IFNs bridge innate and adaptive immunity promoting immunological memory and virus clearance. Herpesviruses have several mechanisms to evade immunity. Their genome codes for proteins which may inhibit key activating pathways. ICP0 is an E3 ubiquitin ligase, which promotes the degradation of cellular proteins with antiviral effects (*e.g.*: PML, promyelocytic leukemia; IFIX, interferon-inducible protein X; and IFI16, interferon-inducible protein 16) enhancing virus replication and inhibiting host innate responses [35].

ICP0 is a multifunctional protein which has a nuclear localization signal (NLS) that initially causes it to translocate to the nucleus but, as the infection progresses, ICP0 moves to the cytoplasm [36]. There is accumulating evidence suggesting that ICP0 also has important functions in this compartment. ICP0 is recognized to act synergistically to promote viral gene expression and replication by modulating viral chromatin remodeling [37]. ICP0 can also simultaneously strike multiple host pathways by either degrading key restrictive factors or altering repressive complexes [36]. Interestingly, when βCs were present, the late ICP0 localization pattern changed. So, instead of localizing cytoplasmically as observed in the control or in cells treated with ACV, ICP0 remained

in the nucleus forming very small foci (Figure 5C). Bag et al.[31] revealed that harmaline interfered with the binding of the IE complex to the ICP0 promoter at very early stages of virus replication but to our knowledge, this is the first report of a mislocalization of ICP0 due to a drug treatment. Further experiments are still needed to evaluate the role of ICP0 in the antiviral mechanism of βCs.

Expression of late proteins like ICP5 and gD require the expression of E proteins such as ICP27 [38]. Chen et al.[32] showed that harmine downregulates IC27 expression resulting in an inhibition of gD and ICP5 expression. In our hands, L protein gB expression was significantly inhibited in the presence of 9-Me-nHo, 9-Me-Ho, and 6-MeO-Ho and slightly delayed by ACV treatment (Figures 5A-C). ICP5, could not be detected either when cells were treated with βCs or with ACV (Figures 5A and B). Accordingly, we show that βCs also act by inhibiting late protein expression.

Altogether, the mechanisms by which βCs inhibit virus replication may be at the transcriptional level, where they can bind to the viral genome and block transcription, as it was shown by Bag et al.[31] for harmine, which binds to ICP0 promoter. At the post-translational level βCs may interfere with ICP0 late location at the cytoplasm, where it participates in immune evasion. During infection, ICP0 inhibits type I IFN mediated antiviral effects, which are exerted through both nuclear and cytoplasmic events. ICP0 targets events which occur following the production of IFN-stimulated antiviral proteins [39]. Therefore, we speculate that mislocalization of ICP0 due to βCs treatment reduces ICP0 mediated evasion of IFN-stimulated antiviral proteins. Additionally, our results indicate that these substances also inhibit late protein expression, which warrants further investigation. It remains to be determined if there are other mechanisms involved in the antiviral activities of 9-Me-Ho, 6-MeO-Ho, and 9-Me-nHo and if there is a synergistic effect when co-administered with ACV.

Conclusions

In conclusion, 9-methyl-norharmane, 9-methyl-harmane and 6-methoxy-harmane were identified as novel antiviral agents against HSV-1 and HSV-2. These compounds are not virucidal and do not affect attachment. The mechanisms of antiviral action seem to be multiple and mainly

mediated by down-regulating expression of E and L proteins at early phases after penetration, by arresting DNA replication and by altering ICP0 cytoplasmic localization at later stages of infection.

9-methylated compounds presented lower SI, lower TPSA values, and higher %ABS. Therefore, they have potential as novel microbicides and should be further studied in murine models of acute infection to validate their efficacy and safety.

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Declarations

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Figure legends.

Fig. 1. Anti-HSV-1 activity and cytotoxicity of 9-methyl-norharmane (9-Me-nHo, A & D), 9-methyl-harmane (9-Me-Ho, B & E) and 6-methoxy-harmane (6-MeO-Ho, C & F) at different concentrations, obtained by plaque reduction assays and PI exclusion, respectively. Values represent the mean ± standard errors of two independent experiments in triplicates. To determine EC50, the percentage of plaque reduction was plotted against βCs concentrations, and non-linear regression analysis of plaque reduction (%) vs. Log [βC] (variable Hill's slope). 50% cytotoxic concentration values (CC50) were calculated by non-linear regression analysis of %Cell Viability vs. Log [βC] (variable Hill's slope).

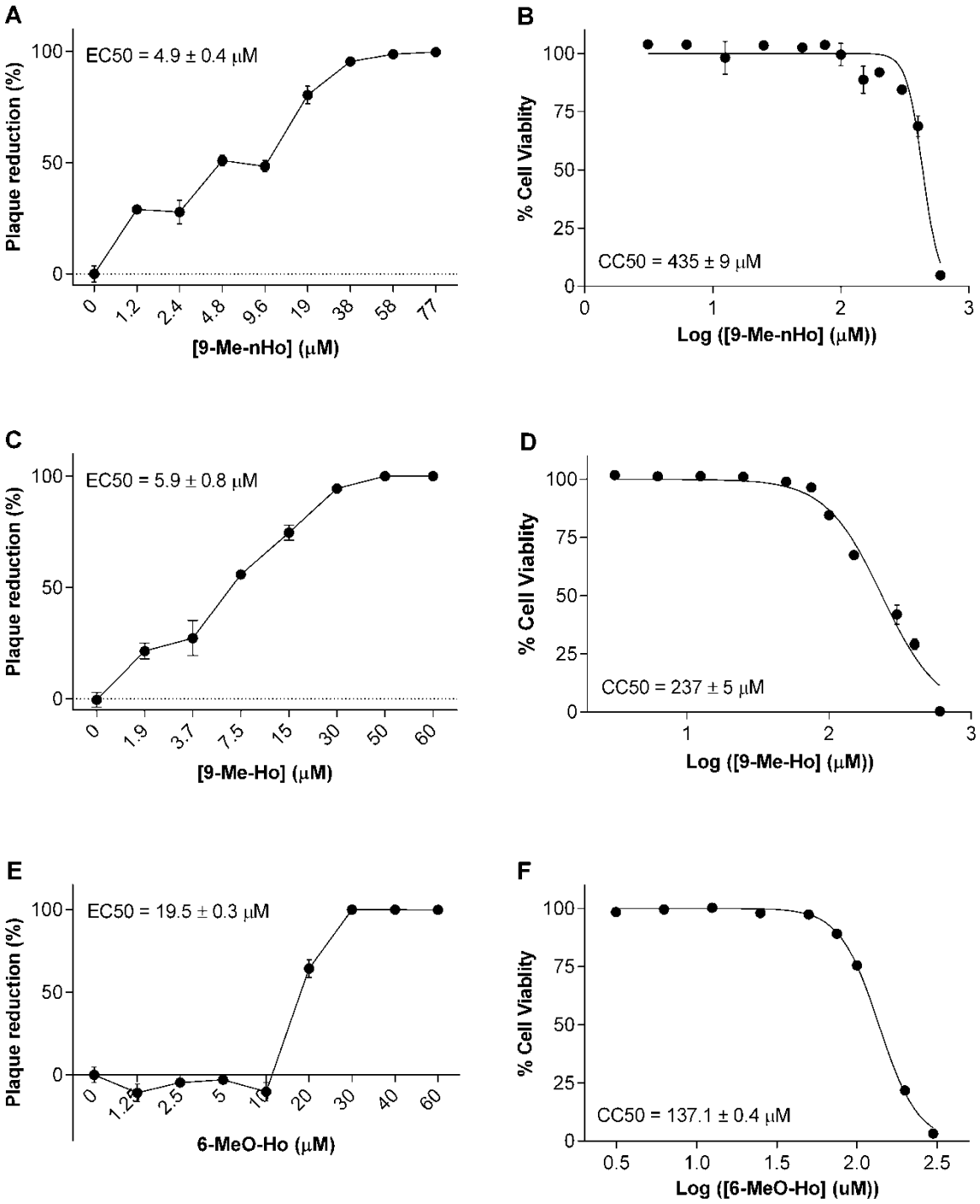
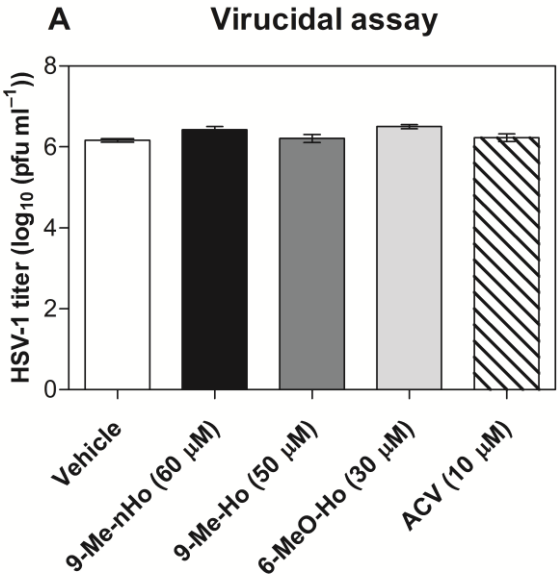
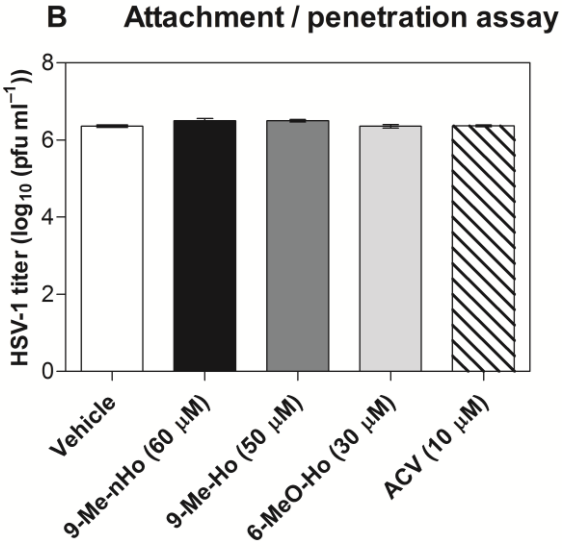

A
EC50 = 4.9 ± 0.4 μM
Plaque reduction (%)
100
50
0
0
1.2
2.4
4.8
9.6
19
38
58
77
[9-Me-nHo] (μM)
B
% Cell Viablity
100
75
50
25
0
CC50 = 435 ± 9 μM
0
1
2
3
Log ([9-Me-nHo] (μM))
C
EC50 = 5.9 ± 0.8 μM
Plaque reduction (%)
100
50
0
0
1.9
3.7
7.5
15
30
50
60
[9-Me-Ho] (μM)
D
% Cell Viablity
100
75
50
25
0
CC50 = 237 ± 5 μM
1
2
3
Log ([9-Me-Ho] (μM))
E
EC50 = 19.5 ± 0.3 μM
Plaque reduction (%)
100
50
0
0
1.25
2.5
5
10
20
30
40
60
6-MeO-Ho (μM)
F
% Cell Viablity
100
75
50
25
0
CC50 = 137.1 ± 0.4 μM
0.5
1.0
1.5
2.0
2.5
Log ([6-MeO-Ho] (uM))

Fig. 2. Effects of 9-Me-nHo (60 µM), 9-Me-Ho (50 µM), 6-MeO-Ho (30 µM), and ACV (10 µM) at different stages of HSV-1: Virucidal assay (2A), Attachment / penetration assay (2B), and Post-infection assay (2C). All results were obtained by plaque assays of cell lysates. Data points represent the mean of three independent experiments in duplicate (±SE). Statistical differences ($p < 0.05$) are indicated as (*). ANOVA/Dunnett's t-tests were performed for each compound and compared to the respective controls. n.d.: not detected





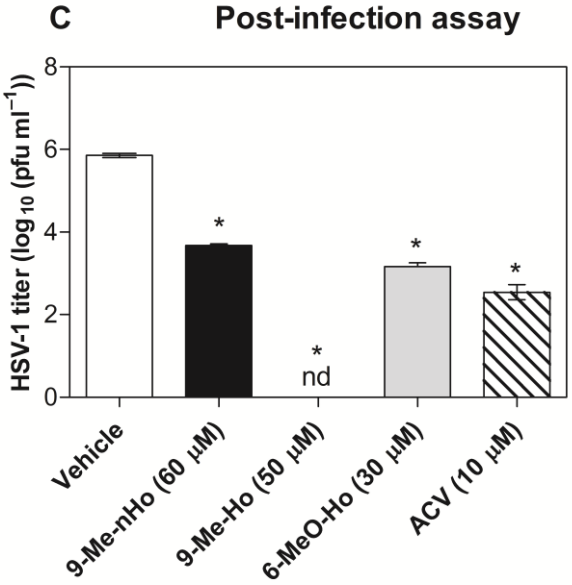


Fig. 3. Viral replication curves of HSV-1 in the presence of 9-Me-nHo (60 µM), 9-Me-Ho (50 µM), 6-MeO-Ho (30 µM) and ACV (10 µM). The values were obtained by virus yield titration of cell lysates (3A) or cell supernatants (3B) and represent the mean of three independent experiments in duplicate (±SE). (*) indicates significant statistical differences between the tested sample and virus control ($p < 0.05$). ANOVA/Dunnett's tests were carried out as appropriate.

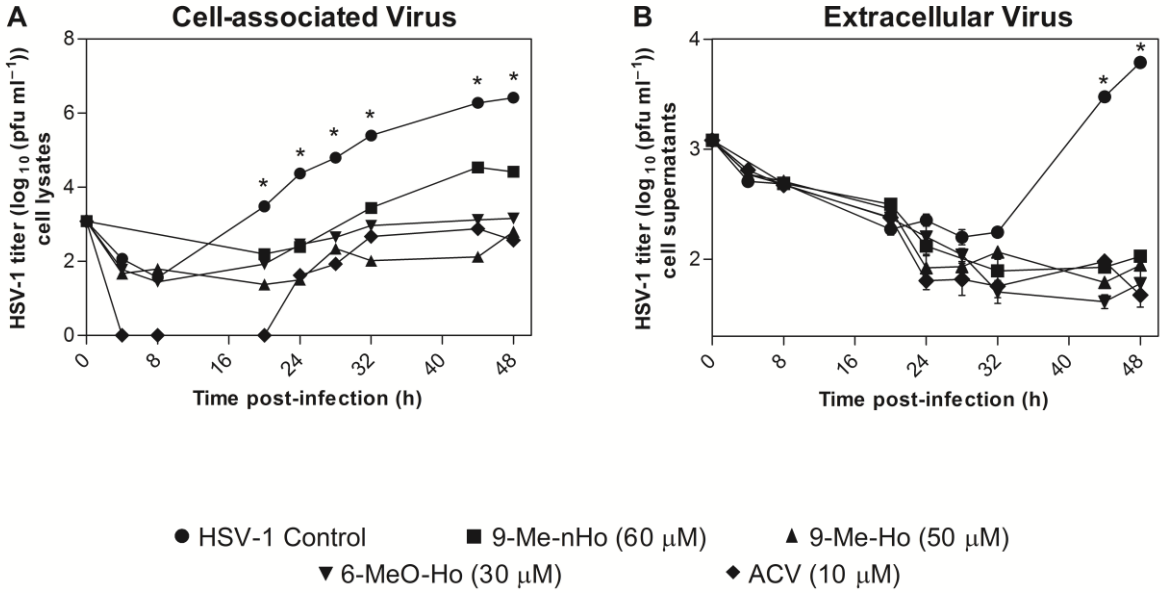


Fig. 4. Quantitative PCR was performed to assess the number of HSV-1 genome copies after treatment with 9-Me-nHo (60 µM), 9-Me-Ho (50 µM), 6-MeO-Ho (30 µM), and ACV (10 µM).

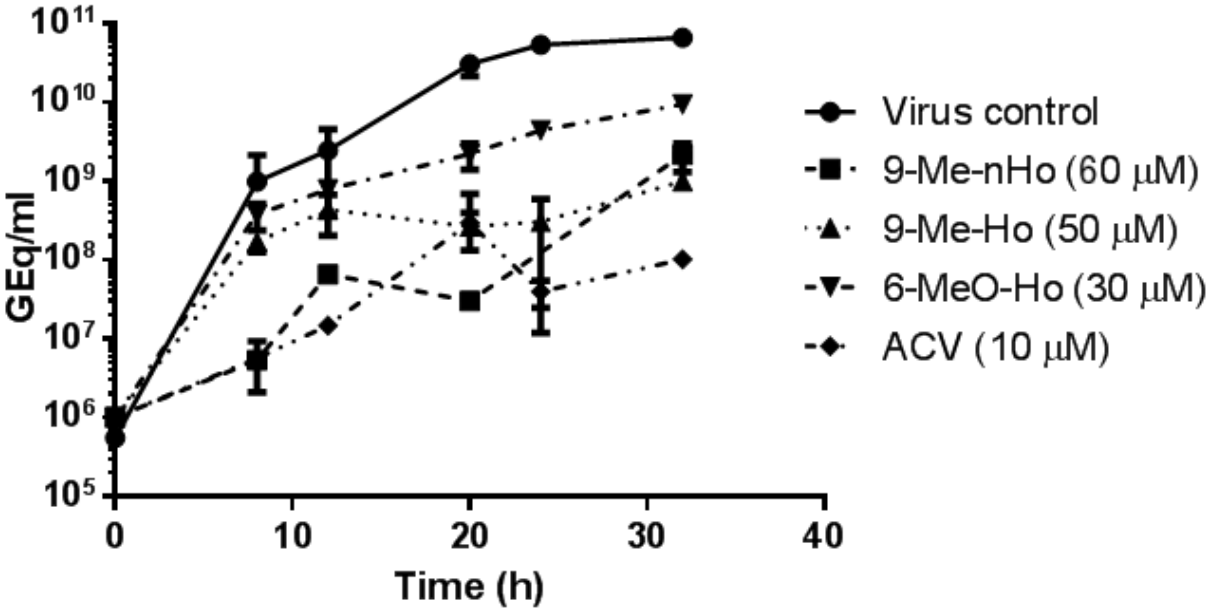
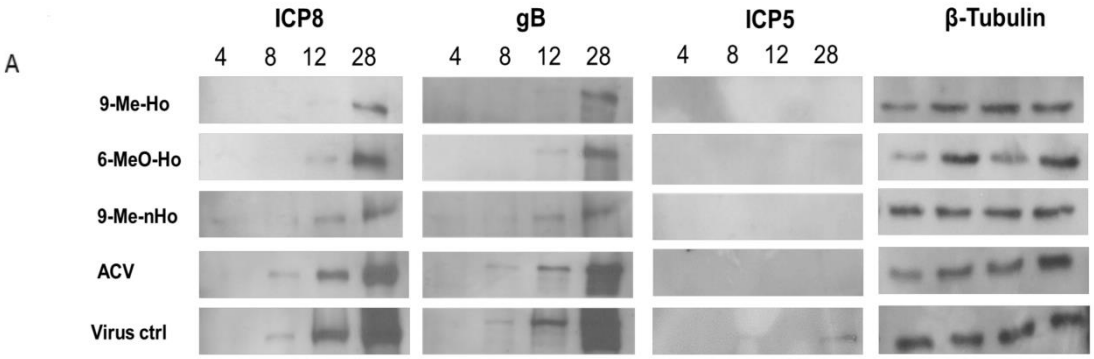
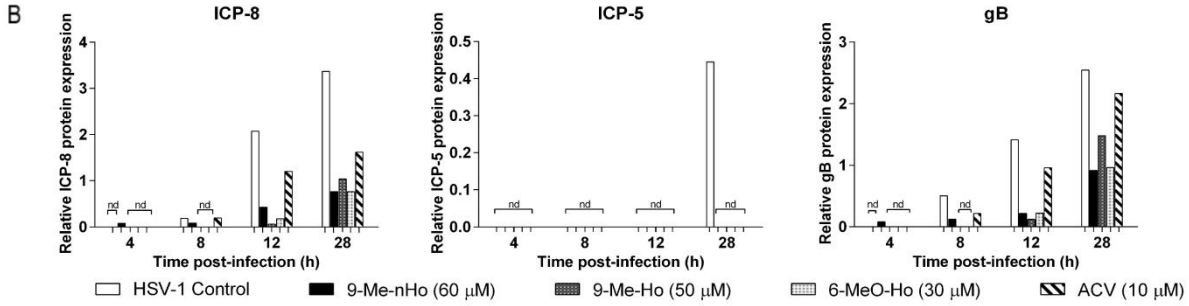
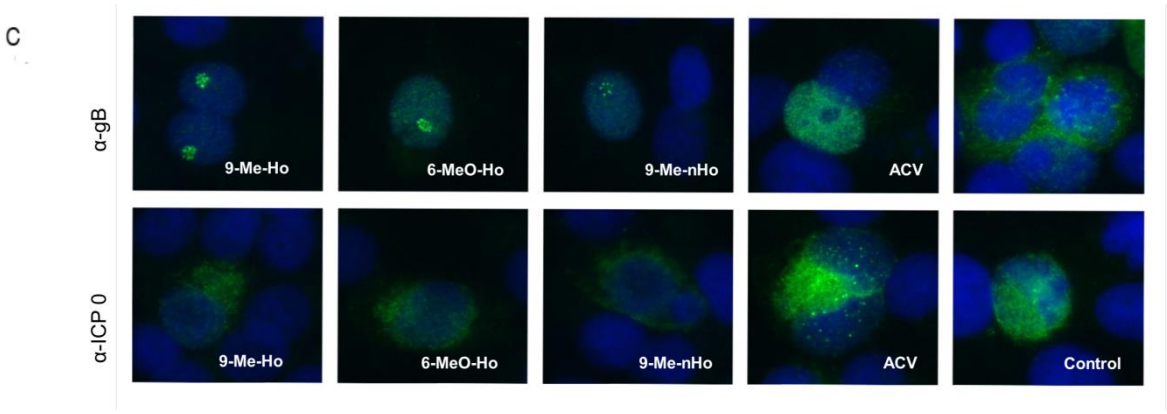


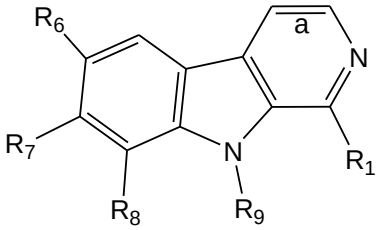
Fig. 5. Expression of HSV-1 immediately early, early and late proteins was evaluated by (A) Western blot at 4, 8, 12 and 28 h.p.i. Density measurements of Western blot bands as quantified using the Image J Software are depicted in (B). (C) Immunofluorescence microscopy examining ICP0 and gB subcellular localization after 20 h treatment with 9-Me-nHo (60 µM), 9-Me-Ho (50 µM), 6-MeO-Ho (30 µM), and ACV (10 µM). DAPI was used for nuclear staining.


A
ICP8
gB
ICP5
β-Tubulin
4 8 12 28
9-Me-Ho
6-MeO-Ho
9-Me-nHo
ACV
Virus ctrl


B
ICP-8
ICP-5
gB
Relative ICP-8 protein expression
Relative ICP-5 protein expression
Relative gB protein expression
Time post-infection (h)
nd
HSV-1 Control
9-Me-nHo (60 μM)
9-Me-Ho (50 μM)
6-MeO-Ho (30 μM)
ACV (10 μM)


C
α-gB
α-ICP 0
9-Me-Ho
6-MeO-Ho
9-Me-nHo
ACV
Control

Scheme 1 Chemical structure of βCs and their derivatives studied in this work



Compound	R_1	R_6	R_7	R_8	R_9	a
norharmane	H	H	H	H	H	x
6-chloro-norharmane	H	Cl	H	H	H	x
8-chloro-norharmane	H	H	H	Cl	H	x
6-bromo-norharmane	H	Br	H	H	H	x
6,8-dibromo-norharmane	H	Br	H	Br	H	x
1-ethyl-norharmane	C_2H_5	H	H	H	H	x
9-methyl-norharmane	H	H	H	H	CH_3	x
harmane	CH_3	H	H	H	H	x
6-chloro-harmane	CH_3	Cl	H	H	H	x
8-chloro-harmane	CH_3	H	H	Cl	H	x
6,8-dichloro-harmane	CH_3	Cl	H	Cl	H	x
8-bromo-harmane	CH_3	H	H	Br	H	x
6,8-dibromo-harmane	CH_3	Br	H	Br	H	x
9-methyl-harmane	CH_3	H	H	H	CH_3	x
harmine	CH_3	H	OCH_3	H	H	x
6-chloro-harmine	CH_3	Cl	OCH_3	H	H	x
6,8-dichloro-harmine	CH_3	Cl	OCH_3	Cl	H	x
6-bromo-harmine	CH_3	Br	OCH_3	H	H	x
6,8-dibromo-harmine	CH_3	Br	OCH_3	Br	H	x
6-methoxy-harmane	CH_3	OCH_3	H	H	H	x
harmaline	CH_3	H	OCH_3	H	H	

Table 1: Lipinski parameters and molecular properties of all the investigated compounds**.**

	[a]*Lipinski´s rule*						
Compound	**[b]n-OHNH donors**	**[c]n-ON acceptors**	**[d]mi log P**	**[e]MW**	**n violations**	**TPSA**	**[f]%ABS**
Norharmane	1	2	2.37	168.2	0	28.68	99.1
6-chloro-norharmane	1	2	3.03	202.6	0	28.68	99.1
8-chloro-norharmane	1	2	3.00	202.6	0	28.68	99.1
6-bromo-norharmane	1	2	3.16	247.1	0	28.68	99.1
6,8-dibromo-norharmane	1	2	3.89	326.0	0	28.68	99.1
9-methyl-norharmane	0	2	2.44	182.2	0	17.83	102.8
1-ethyl-norharmane	1	2	3.17	196.2	0	28.68	99.1
Harmane	1	2	2.59	182.2	0	28.68	99.1
6-chloro-harmane	1	2	3.25	216.7	0	28.68	99.1
6,8-dichloro-harmane	1	2	3.85	251.1	0	28.68	99.1
8-chloro-harmane	1	2	3.22	216.7	0	28.68	99.1
8-bromo-harmane	1	2	3.35	261.1	0	28.68	99.1
6,8-dibromo-hamane	1	2	4.12	340.0	0	28.68	99.1
9-methyl-harmane	0	2	2.66	196.2	0	17.83	102,8
Harmine	1	3	2.63	212.2	0	37.92	95.9
6-chloro-harmine	1	3	3.23	246.7	0	37.92	95.9
6,8-dichloro-harmine	1	3	3.84	281.1	0	37.92	95.9
6-bromo-harmine	1	3	3.36	291.1	0	37.92	95.9
6,8-dibromo-harmine	1	3	4.10	370.0	0	37.92	95.9
Harmaline	1	3	2.68	214.3	0	37.39	96.1
6-methoxy-harmane	1	3	2.63	212.2	0	37.92	95.9
ACV	4	8	-1.61	225.2	0	119.06	67.9

[a]: www.molinspiration.com/cgi-bin/properties

[b] n-OHNH: number of hydrogen bond donors such as –OH or -NH groups

[c] n-ON: number of hydrogen acceptors such as -O or -N atoms

[d] mi log P: Method for log P prediction developed at Molinspiration online toolkit. P: octanol-water partition coefficient

[e] MW: molecular weight

[f] %ABS = 109 – 0.345 × TPSA

Table 2: Cytotoxicity and anti-HSV-1 activity of βC derivatives measured by the MTS assay.

			Antiviral Activity (% relative to the untreated control)	
Compounds	**CC_{50} (μM)**	**Cytopathic effects**	**Highest Concentration tested (6, 10, 15 or 40 μM)**	**Lowest Concentration tested (3, 5, 7.5 or 20 μM)**
norharmane [a]	>200		11 ± 11	13 ± 18
1-ethyl-norharmane [a]	>200		5 ± 8	1 ± 5
9-methyl-norharmane [a]	>300	–	**83 ± 2**	52 ± 9
6-chloro-norharmane [b]	>120		9 ± 13	32 ± 3
8-chloro-norharmane [b]	>120		27 ± 14	25 ± 12
6-bromo-norharmane [b]	>120		3 ± 1	8.0 ± 0.3
6,8-dibromo-norharmane [b]	>50		7 ± 4	23 ± 2
harmane [a]	226 ± 14	+	**94 ± 8**	33 ± 20
6-chloro-harmane [b]	122 ± 3		32 ± 15	30 ± 3
8-chloro-harmane [b]	246 ± 29	+	**104 ± 16**	11 ± 29
6,8-dichloro-harmane [b]	>120	+	**59 ± 5**	46 ± 3
8-bromo-harmane [b]	>120	+	**62 ± 13**	39 ± 7
6,8-dibromo-harmane [b]	>120	+	**63 ± 13**	40 ± 21
9-methyl-harmane [a]	268 ± 20	–	**69 ± 10**	54 ± 7
harmine [c]	73.0 ± 0.3	+	**71 ± 19**	39 ± 3
6-chloro-harmine [c]	83 ± 8	+	**108 ± 1**	**100 ± 5**
6,8-dichloro-harmine [c]	>120		15 ± 11	29 ± 22
6-bromo-harmine [c]	>120	+	**102 ± 5**	**59 ± 16**
6,8-dibromo-harmine [c]	>100		44 ± 4	22 ± 1
6-methoxy-harmane [a]	133 ± 5	–	**84 ± 11**	38 ± 3
harmaline [d]	187 ± 3		13 ± 12	0 ± 4

In this screening, the antiviral activity was tested for βC derivatives at two different concentrations, as indicated by [a]= 20 and 40 μM, [b]= 5 and 10 μM, [c]= 3 and 6 μM, and [d]= 7.5 and 15 μM, respectively.

Compounds with antiviral activity higher than 60% are **in bold**.

Values represent the mean ± standard errors of three independent experiments in duplicate.

CC_{50} = Due to the relatively low solubility of tested compounds in DMEM at high concentrations, the exact CC_{50} could not be determined in some cases (>highest concentration tested). In such cases, the given value corresponds to the highest soluble concentration tested in the MTS assay.

Table 3: HSV-1 antiviral activity (EC50), cytotoxicity (CC50) and selectivity indexes (SI) data for 9-Me-nHo, 9-Me-Ho and 6-MeO-Ho.

Compound	CC50 (μM)[a]	EC50 (μM)[b]	SI (CC50/EC50)
9-Me-nHo	435 ± 9	4.9 ± 0.4	88.8
9-Me-Ho	237 ± 5	5.9 ± 0.8	40.2
6-MeO-Ho	137 ± 0.4	19.5 ± 0.3	7.0

[a]: Values obtained by propidium iodide exclusion assay.
[b]. Values obtained by plaque reduction assays.