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Anti-herpetic and anti-inflammatory activities of two new synthetic 22,23-dihydroxylated stigmastane derivatives

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ABSTRACT

Stromal keratitis resulting from ocular infection with Herpes simplex virus type 1 (HSV-1) is a common cause of blindness. This report investigates the antiviral and anti-inflammatory properties of two new synthetic stigmastane analogs in the experimental model of HSV-1-induced ocular disease in mice.

(*22S,23S*)-22,23-dihydroxystigmast-4-en-3-one (**1**) and (*22S,23S*)-22,23-dihydroxystigmasta-1,4-dien-3-one (**2**) exhibited anti-HSV-1 activity *in vitro* and ameliorated the signs of murine herpetic stromal keratitis (HSK), although none of the compounds showed antiviral activity *in vivo*. We discuss that the improvement of HSK could be due to an immunomodulatory effect of both compounds.

1. Introduction

Herpes simplex virus type 1 (HSV-1) induces an ocular disease in humans named herpetic stromal keratitis (HSK), which is a significant cause of ocular morbidity as a result of recurrent episodes of quiescent virus reappearing in the cornea from the innervating ganglion [1]. A chronic immunoinflammatory syndrome is established that can lead to vision impairment and blindness [2].

Murine corneal infection with HSV-1 has been extensively used as an experimental model of HSK. Within two weeks, HSV-1 infection of the eye leads to the development of a stromal necrotizing keratitis that resembles many histological characteristics of the recurrent disease in humans [3–5].

We have recently reported that some polyfunctionalized stigmastane derivatives have *in vitro* antiviral activity against several pathogen viruses [6,7]. Particularly, we have demonstrated that (*22S,23S*)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one prevents HSV-1 multiplication and viral spreading in a human conjunctival cell line, with no cytotoxicity. Despite the lack of anti-HSV activity of this compound *in vivo* conditions, it significantly decreases the incidence of HSK in the murine model of HSV-1 corneal infection, probably due to an immunomodulatory effect [8]. Furthermore, this stigmastane derivative inhibits the production of TNF-α in a LPS-stimulated murine macrophage cell line [9,10], and modulates the secretion of IL-6 and TNF-α in human epithelial cells derived from ocular tissues [9].

In order to improve the immunomodulatory activity of this kind of molecules, we designed new stigmastane analogs, keeping the (*22S,23S*)-22,23-dihydroxylated side chain of the steroidal structure and providing A and B rings, with structural features similar to those of the commercial anti-inflammatory drug Dexamethasone.

In the present work we describe the chemical synthesis of three new stigmastane analogs:

(*22S,23S*)-22,23-dihydroxystigmast-4-en-3-one (**1**), (*22S,23S*)-22,23-dihydroxystigmasta-1,4-dien-3-one (**2**) and (*22S,23S*)-4α,5,22,23-tetrahydroxy-5α-stigmastan-3-one (**3**) (Scheme 1), we

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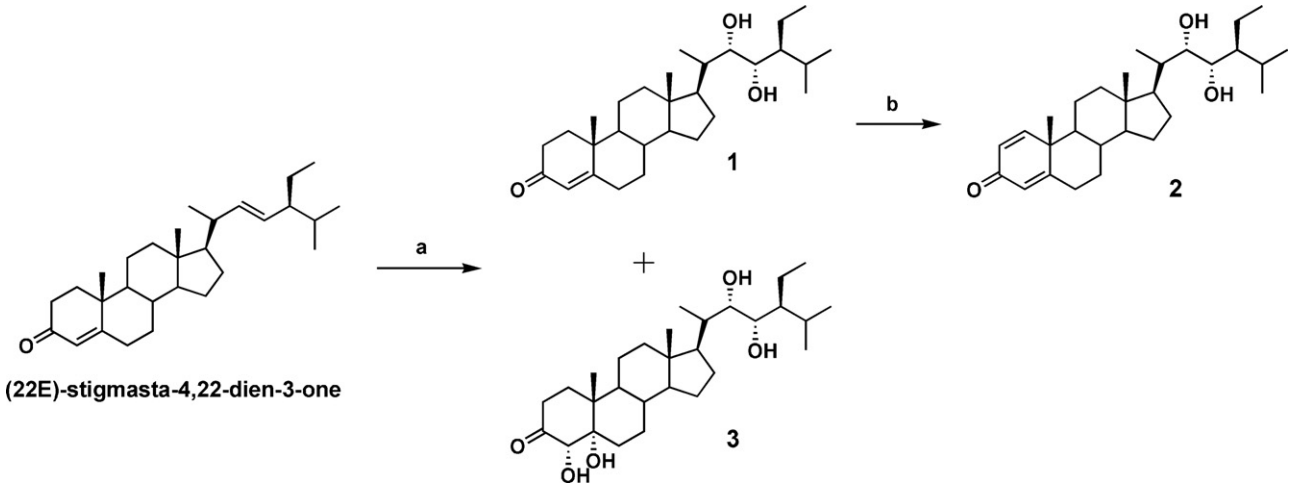

Scheme 1. Synthesis of compounds. *Reagents and conditions*: (a) *N*-Methylmorpholine *N*-oxide/OsO_4/$MeSO_2NH_2$/$NaHCO_3$/*t*-BuOH/THF/water, 50 °C, 24 h. (b) DDQ/dioxane, reflux, 24 h.

report their anti-HSV-1 activity *in vitro* and the healing effect displayed by compounds **1** and **2** on the development and evolution of HSV-1 induced-ocular disease in mice.

2. Materials and methods

2.1. Chemical synthesis

2.1.1. General

Stigmasterol was purchased from Sigma–Aldrich Chemical Co. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker AM-500 at 500 MHz and at 125.1 MHz, respectively. Chemical shifts (δ) are given in ppm downfield from TMS as the internal standard. Coupling constant (J) values are in Hz. All solvents and reagents were of analytical grade. Reactions were monitored by TLC on precoated plates with silica gel F254 0.2 mm (Merck). Column chromatography was carried out on silica gel 60, 0.04–0.063 mm (Merck). Melting points (mp) were determined on a Fisher Johns apparatus and are uncorrected.

The preparations reported below are of compounds that were either new or synthesized using adaptations of literature methods. All new compounds gave satisfactory NMR and combustion analysis data.

*2.1.2. Synthesis of compound (22S,23S)-22,23-dihydroxystigmast-4-en-3-one (**1**)*

The known compound (*22E*)-stigmasta-4,22-dien-3-one [11] (1.2 g, 2.92 mmol) was dissolved in a mixture of 50 ml of tetrahydrofuran and 10 ml of water. To the resulting solution 0.15 g of $NaHCO_3$ (1.79 mmol), 1 ml of *t*-butanol, 0.28 g of methanesulfonamide (2.94 mmol), 0.25 g of *N*-methylmorpholine *N*-oxide (2.1 mmol) and 15 mg (0.06 mmol) of osmium tetroxide were added. The mixture was stirred at 50 °C for 24 h, cooled, and then extracted 3 times with 25 ml of EtOAc. The organic phase was dried over anhydrous sodium sulfate and evaporated under reduced pressure.

The crude product was purified by column chromatography over silica (hexane/EtOAc 1:1 as elution solvent) to give 0.81 g of (*22S,23S*)-22,23-dihydroxystigmast-4-en-3-one (**1**) (62% yield).

mp: 149 °C.

^{1}H NMR (500 MHz, $CDCl_3$): 5.72 (1H, s, H-4); 3.61 (2H, m, H-22 and H-23), 2.46 (1H, ddd, J = 14 Hz, 14 Hz, 5 Hz, H-7β); 1.19 (3H, s, H-19); 1.03 (3H, d, J = 6 Hz, H-21); 0.97 (3H, t, J = 7 Hz, H-28); 0.95 (3H, d, J = 6 Hz, H-27); 0.88 (3H, d, J = 6 Hz, H-28); 0.76 (3H, s, H-18).

^{13}C NMR (125 MHz, $CDCl_3$): 199.6 (C-6), 171.4 (C-4), 123.8 (C-5), 72.3 (C-22), 70.7 (C-23), 55.6 (C-14), 53.8 (C-9), 52.7 (C-17), 49.7 (C-24), 43.0 (C-13), 42.4 (C-20), 39.6 (C-12), 38.6 (C-10), 35.7 (C-1), 35.7 (C-8), 34.0 (C-2), 32.9 (C-6), 32.0 (C-7), 28.0 (C-16), 26.9 (C-25), 24.5 (C-15), 21.8 (C-27), 21.1 (C-11), 18.6 (C-28), 17.8 (C-26), 17.4 (C-19), 14.5 (C-29), 14.1 (C-21), 11.9 (C-18).

Anal. calculated for $C_{29}H_{48}O_3$: C 78.33%, H 10.88%. Found C 78.56%, H 10.71%.

Further elution gave 0.42 g of (*22S,23S*)-4α,5,22,23-tetrahydroxy-5α-stigmastan-3-one (**3**) (30% yield). mp: 184 °C.

^{1}H NMR (500 MHz, $CDCl_3$): 4.52 (1H, s, H-4β); 3.61 (2H, m, H-22 and H-23); 1.05 (3H, s, H-19); 1.03 (3H, d, J = 6 Hz, H-21); 0.97 (3H, t, J = 7 Hz, H-28); 0.95 (3H, d, J = 6 Hz, H-27); 0.88 (3H, d, J = 6 Hz, H-28); 0.74 (3H, s, H-18).

^{13}C NMR (125 MHz, $CDCl_3$): 211.0 (C-3), 80.9 (C-5), 74.2 (C-4), 72.3 (C-22), 70.6 (C-2), 56.3 (C-14), 52.7 (C-17), 49.7 (C-24), 43.8 (C-9), 43.1 (C-13), 42.4 (C-20), 41.0 (C-10), 39.9 (C-12), 34.8 (C-1), 34.7 (C-8), 31.7 (C-7), 31.0 (C-2), 28.4 (C-6), 28.0 (C-16), 26.9 (C-25), 24.4 (C-15), 21.8 (C-27), 21.5 (C-11), 18.6 (C-28), 17.8 (C-26), 16.5 (C-19), 14.5 (C-29), 14.1 (C-21), 12.0 (C-18).

Anal. calculated for $C_{29}H_{50}O_5$: C 72.76%, H 10.53%. Found: C 72.48%, H 10.30%.

*2.1.3. Synthesis of compound (22S,23S)-22,23-dihydroxystigmasta-1,4-dien-3-one (**2**)*

120 mg of compound **1** were dissolved in 15 ml of dry dioxane. To this solution 180 mg of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) were added and the resulting mixture was refluxed, under an inert atmosphere, for 24 h. Formation of a solid was observed.

The precipitate was filtered and the liquid was evaporated under reduced pressure. The crude product was purified by column chromatography over silica (hexane/EtOAc 1:1 as elution solvent) to give 87 mg of (*22S,23S*)-22,23-dihydroxystigmasta-1,4-dien-3-one (**2**). mp: 142 °C.

^{1}H NMR (500 MHz, $CDCl_3$): 7.02 (1H, d, J = 10 Hz, H-1); 6.22 (1H, dd, J = 10 Hz, 2 Hz, H-2); 6.06 (1H, bs, H-4); 3.59 (2H, m, H-22 and H-23); 2.46 (1H, ddd, J = 14 Hz, 14 Hz, 5 Hz, H-7β); 1.22 (3H, s, H-19); 1.03 (3H, d, J = 6 Hz, H-21); 0.97 (3H, t, J = 7 Hz, H-28); 0.95 (3H, d, J = 6 Hz, H-27); 0.88 (3H, d, J = 6 Hz, H-28); 0.77 (3H, s, H-18).

^{13}C NMR (125 MHz, $CDCl_3$): 186.4 (C-3), 169.3 (C-5), 155.9 (C-1), 127.5 (C-2), 123.9 (C-4), 72.3 (C-22) 70.7 (C-23), 55.2 (C-14), 52.7 (C-17), 52.4 (C-9), 49.7 (C-24), 43.6 (C-13), 43.2 (C-10), 42.3 (C-20), 39.5 (C-12), 35.6 (C-8), 33.7 (C-6), 32.9 (C-7), 27.9 (C-16), 26.9 (C-

25), 24.6 (C-15), 22.9 (C-11), 21.8 (C-27), 18.7 (C-19), 18.6 (C-28), 17.8 (C-26), 14.5 (C-29), 14.1 (C-21), 12.0 (C-18).

Anal. calculated for $C_{29}H_{46}O_3$: C 78.68%, H 10.47%. Found: C 72.41%, H 10.22%.

2.2. Cells, viruses, and treatment solutions

Simian Vero cells were grown in Eagle's minimal essential medium supplemented with 5% inactivated fetal bovine serum (FBS) (MEM 5%) and 50 μg/ml gentamycin, and maintained in monolayer formation in MEM supplemented with 1.5% inactivated FBS (MEM 1.5%).

Human IOBA-NHC cell line (NHC) [12] was grown in Dulbecco's Modified Medium and Nutrient Mixture F-12 (DMEM/F12) 1:1 mixture, supplemented with 10% inactivated FBS (DMEM/F12 10%), and 50 μg/ml gentamycin, and maintained in DMEM/F12 supplemented with 2% inactivated FBS (DMEM/F12 2%).

HSV-1 $Cgal^+$ (HSV-1 Cgal) KOS strain was propagated at low multiplicity, plaque-assayed on Vero cell monolayers, and used for *in vitro* assays. This system contains the *Escherichia coli lacZ* reporter gene encoding β-galactosidase, which is under the control of a strong heterologous promoter from the human cytomegalovirus immediate-early (IE) regulatory region, and was kindly provided by Dr. Alberto Epstein (Université Claude Bernard, Lyon, France).

The HSV-1 KOS strain (wt) was also propagated at low multiplicity, and was used for *in vivo* experiments.

Compounds **1**, **2** and **3** were dissolved in ethanol and diluted with PBS or culture medium for testing. The maximum concentration of ethanol tested (1%) exhibited no toxicity under *in vitro* and *in vivo* conditions.

Dexamethasone 10 mM (DEX) and Acyclovir 133 mM (ACV) were purchased from Sidus and Elea, respectively.

2.3. Cytotoxicity assay

Cell viability in the presence of the compound was determined using the cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) by the mitochondrial enzyme succinate dehydrogenase to give a blue product (formazan) [13]. The absorbance of each well was measured on an Eurogenetics MPR-A 4i microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. Results were expressed as a percentage of absorbance of treated cell cultures with respect to untreated ones. The CC_{50} was defined as the concentration of the compound that caused a 50% reduction in absorbance.

2.4. Antiviral activity

NHC cells grown in 24-well plates were infected with HSV-1 KOS (m.o.i. = 0.2) and, after virus adsorption, they were treated with different concentrations of compounds **1**, **2** and **3** (from 0.1 to 20 μM) or control media. After 24 h of incubation at 37 °C, free and cell-associated virus were collected and titrated in Vero cells.

Vero cells grown in 24-well plates were infected with serial 10-fold dilutions of viral yields and incubated for 1 h at 37 °C. Residual inocula were replaced by maintenance medium with 0.7% of methylcellulose. After 72 h of incubation at 37 °C, cells were fixed with formaldehyde 10%, stained with Crystal Violet, and plaque forming units were counted.

2.5. Measurement of β-gal activity

NHC cell monolayers grown in coverslips inside 24-well plates were infected with HSV-1 Cgal (m.o.i. = 0.2). After incubation for 1 h at 37 °C, inocula were eliminated, and cells were covered with MEM 1.5%. At 24 h post-infection (p.i.), supernatants were harvested and stored at −70 °C for titration, and cells were stained *in situ* for β-gal. A solution containing 1% formaldehyde and 0.2% glutaraldehyde in PBS (200 μl per well) was added for 30 min at 4 °C. Subsequently, supernatants were removed, and cells were washed twice with PBS. A β-gal activity staining solution (50 mM potassium ferrocyanide, 50 mM potassium ferricyanide, 20 mM $MgCl_2$, and 1 mg/ml X-gal) was added to the cells for 3 h at 37 °C. Coverslips were mounted, and 'blue' cells were observed with an Olympus BX61 microscope, with a slider slot for Normarski DIC, and photographed.

2.6. Animals

Five to seven-week old male Balb/c mice were purchased from the I.N.T.A. (Castelar, Buenos Aires). All mice were handled according to the Animal Care Guidelines from the National Institute of Health (USA) and the Association for Research in Vision and Ophthalmology (ARVO, USA) resolution on the use of animals in research.

2.7. Corneal inoculation of HSV-1

Mice were anaesthetised with 2 mg intraperitoneal ketamine (Ketalar, Parke-Davis, Morris Plains, NJ) and xylazine (Rompun, Mobay, Shanee, KA). The right cornea of each mouse was scratched eight times in a criss-cross pattern with a 27 gauge needle, and 5 μl of an HSV-1 suspension containing 2.5×10^4 PFU of virus were instilled in the cul-de-sac. Mice were treated topically with **1** and **2** (40 μM), DEX (10 mM) and ACV (133 mM), supplied three times a day (volume = 7 μl), according to two schedules of treatment: at 1, 2 and 3 days p.i., and at 6, 7 and 8 days p.i. The control mice received PBS.

2.8. Clinical scoring

The signs of HSK were determined under a binocular microscope. The diagnosis of disease was based on the density of the inflammatory infiltrates, neovascularization, and the presence of superficial or deep ulcers. Thus, mice exhibiting puffy eyelids with moderate to severe crusting (blepharitis), vessel enlargement with corneal invasion (10% or more) (neovascularization), and slight to severe corneal turbidity/opacity, with or without signs of corneal necrosis or perforation, were considered as ill (stromal keratitis).

The percentages of animals with signs of disease were compared using the X^2-test, with 90–99% confidence intervals.

In the case of the severity of HSK, a scoring system based on the density of the inflammatory infiltrates, neovascularization, and the presence of superficial or deep ulcers was used as follows: 0, no significant lesion; 1, slight corneal turbidity, some iris detail visible; 2, moderate corneal opacity with iris detail obscured; 3, severe corneal opacity; 4, severe corneal opacity impeding the observation of the iris, with signs of corneal necrosis or perforation. Student's *t*-test was used for the statistical analysis of the data.

3. Results

3.1. Synthesis of compounds *1* and *2*

The known steroid (*22E*)-stigmasta-4,22-dien-3-one [11] was hydroxylated using osmium tetroxide and *N*-methylmorpholine *N*-oxide as cooxidant to give compound **1** as the main product (62% yield), along with the tetrahydroxylated compound **3** (Scheme 1). Configurations at C-22 and C-23 in the new compounds were

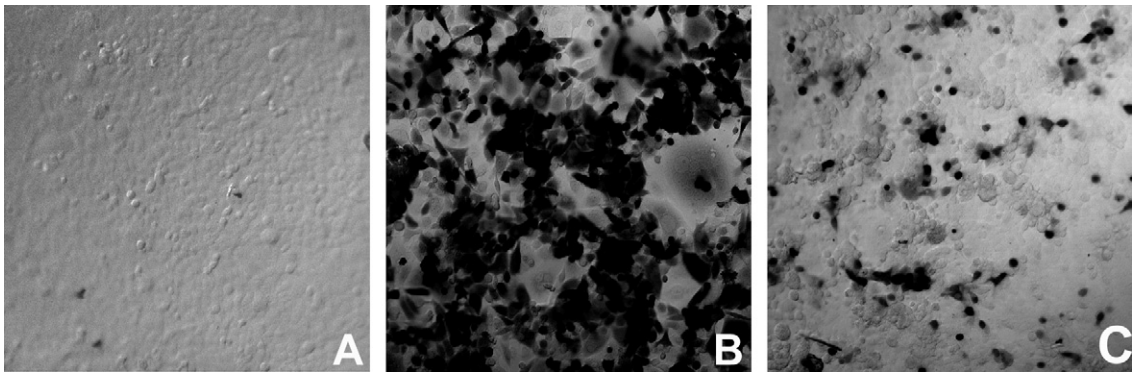

Fig. 1. Effect of **1** on HSV-1 propagation. NHC cells were infected with HSV-1 Cgal and treated with 20 μM of compound **1**. Cells were stained *in situ* for β-gal. (A) Uninfected control cells; (B) HSV-1 infected-untreated cells; (C) HSV-1 infected **1**-treated cells. Magnification: 40×.

established by comparison with NMR chemical shifts and coupling constants of known closely related structures [14].

The synthesis of compound **2** was achieved by treating compound **1** with DDQ in refluxing dioxane, in order to introduce the Δ^1 double bond.

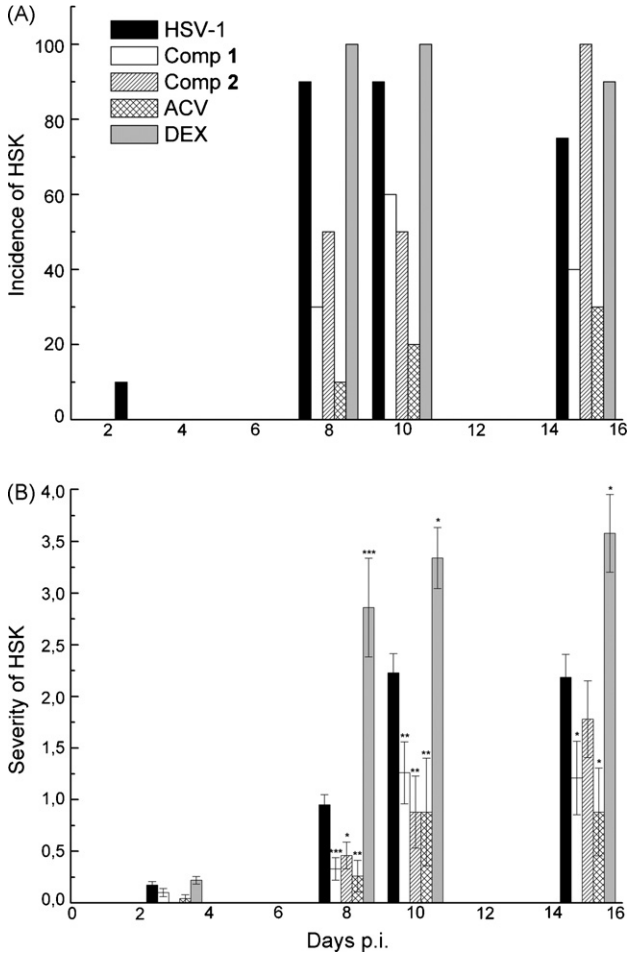

Fig. 2. Antiviral effect of **1** and **2** on the development of HSK. Mice were inoculated with HSV-1 KOS strain in their corneas. Five groups of 10 mice each received PBS (control group), compound **1** (40 μM), compound **2** (40 μM), ACV (133 mM) and DEX (10 mM), three times a day for three consecutive days, after infection. Signs of HSK (blepharitis, neovascularization, corneal clouding, edema, irritation and necrosis) were assessed for 15 days. Data shown are representative of two experiments. (A) Incidence of HSK; (B) Severity of HSK ($^*p < 0.1$; $^{**}p < 0.01$; $^{***}p < 0.001$).

3.2. Cytotoxicity and antiviral activity of the synthetic compounds

Anti-HSV-1 activity of compounds **1**, **2** and **3** was tested in human conjunctival cells. First, the 50% cytotoxic concentration (CC_{50}) for NHC cells was determined. The compounds were added to confluent non-growing cells in concentrations ranging from 1 to 340 μM and, after 24 h of incubation at 37 °C, a MTT colorimetric assay was performed. Compounds **1**, **2** and **3** exhibited CC_{50} values of 71.2, 70.8 and 42 μM, respectively.

Compounds **1** and **2** were able to prevent HSV-1 multiplication when added after infection in a dose-dependent manner, exhibiting 50% inhibition of virus yield (CE_{50}) at concentrations of 5.4 and 17.9 μM, and with selectivity indices ($SI = CC_{50}/CE_{50}$) of 13.2 and 3.9, respectively, whereas compound **3** did not restrain HSV-1 multiplication.

3.3. Compound *1* inhibits HSV-1 propagation in NHC cells

Considering that **1** exhibited the highest SI, we investigated its ability to restrain viral propagation in NHC cells infected with HSV-1 Cgal, treated with compound **1** and stained *in situ* for β-gal. In the absence of the compound, 'blue' cells clustered in characteristic HSV-1 foci were observed (Fig. 1B), whereas cell cultures treated with **1** exhibited only scattered 'blue' cells (Fig. 1C). Therefore, compound **1** impeded HSV-1 Cgal propagation as well as viral multiplication, since a decrease in 99.9% of viral titers obtained from supernatants belonging to **1**-treated cells with respect to untreated infected cells, was observed.

Similar results were observed after the treatment of infected cells with compound **2** (data not shown).

3.4. Antiviral effect of *1* and *2* on the development of HSK

Taking into account that replicating virus is only detectable in the cornea till 4–5 days p.i. and the anti-HSV-1 activity of **1**, we decided to evaluate its antiviral effect to heal HSK in an experimental model in mice. Although compound **2** did not exert a significant *in vitro* antiviral activity as compound **1** did, it was also administered to the ill corneas of infected mice because of its structural homology to DEX.

To evaluate the lack of toxicity of **1** and **2** in the eye of the mice, groups of 5 mice each received compounds **1** and **2** three times a day during three consecutive days. Daily observation of the animals confirmed no toxic effect at a concentration of 40 μM of none of the compounds.

To determine if **1** and **2** affected the clinical signs of murine HSK, groups of ten mice each were infected with HSV-1 KOS strain in the right eye. One group was mock-treated with PBS (control), whereas

the others were treated topically with compounds **1**, **2**, and ACV and DEX, three times a day, at 1, 2 and 3 days p.i. Signs of ocular disease that gradually developed from 6 to 7 days of infection onwards were observed until day 15 p.i. in all groups. The following results are the mean values obtained from two independent assays.

At the onset of disease (day 8 p.i.), 90% of untreated infected mice exhibited lesions of keratitis. By day 8 p.i., a healing effect of compounds **1** and **2** was evident, since both significantly restrained the incidence of HSK to only 30% ($p < 0.01$) and 50% ($p < 0.05$), respectively (Fig. 2A). In the case of DEX, the percentage of animals with ocular damage was higher than that observed in the control mice, while, as expected, ACV reduced the signs of keratitis to 10% ($p < 0.005$) (Fig. 2A).

At the end of the observation period, the percentage of sick animals treated with **1** remained unchanged ($p < 0.1$), but the incidence of HSK among animals treated with **2** raised to 100% (Fig. 2A).

The degree of corneal damage was also evaluated by following up the severity of disease. From day 8 to 15 p.i., typical lesions of HSK were observed in untreated infected mice, which showed a gradual increase in keratitis score values from 1 to 2.1–2.2 at the end of the assay (Fig. 2B). When compound **1** was administered, mice exhibited a significant decrease in the severity of disease, since values dropped to 0.33 ($p < 0.001$), 1.26 ($p < 0.01$) and 1.21 ($p < 0.1$) at 8, 10 and 15 days p.i., respectively (Fig. 2B). In the case of **2**, the scores of HSK were also significantly lower than those corresponding to untreated infected mice, but only at days 8 and 10 p.i., with values of 0.46 ($p < 0.1$) and 0.88 ($p < 0.01$), respectively (Fig. 2B). Similarly, ACV produced a diminishment in the severity of the lesion scores to 0.26 ($p < 0.01$) at day 8 p.i., and 0.88 ($p < 0.1$) after 10 days p.i. (Fig. 2B). When DEX was administered, ocular damage got worse, with keratitis score values of 2.86 ($p < 0.001$), 3.34 ($p < 0.1$) and 3.58 ($p < 0.1$), at 8, 10 and 15 days p.i., respectively (Fig. 2B).

To determine whether compound **1** inhibited HSV-1 multiplication *in vivo*, the eyes of untreated and treated infected mice were gently washed with a 10 μl drop of MEM 1.5%, at 1, 2, 3, 7, and 9 days after infection. Each sample was 1:10 diluted and titrated in Vero cells. Student's *t*-test was used for the statistical analysis of the data. No significant differences in viral titers among samples from untreated and **1**-treated animals were detected, indicating that **1** did not exert an antiviral effect *in vivo* (Fig. 3). Similar results were observed with viral titers of eye washes from animals treated with

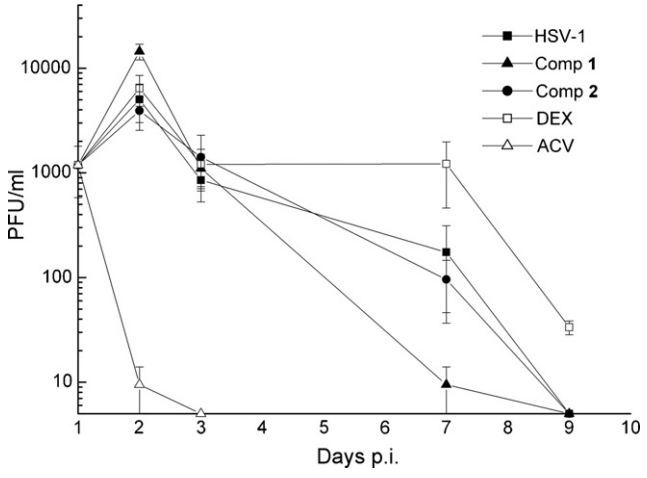

Fig. 3. Effect of **1** and **2** on HSV-1 multiplication in the eye. Viral infectivity was quantified in the eye washes of untreated infected mice (■), and treated with 40 μM of **1**(▲), 40 μM of **2** (●), ACV 133 mM (△), and DEX 10 mM (□) at 1, 2, 3, 7, and 9 days after infection.

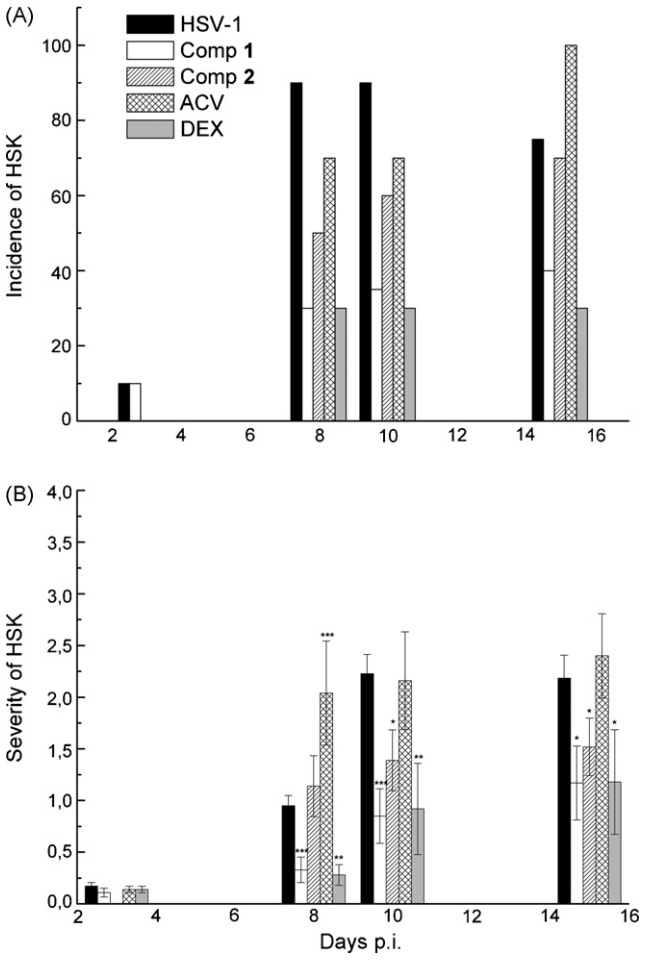

Fig. 4. Anti-inflammatory activity of **1** and **2** on the evolution of HSK. Mice were inoculated with HSV-1 KOS strain in their corneas. Five groups of 10 mice each received PBS (control group), compound **1** (40 μM), compound **2** (40 μM), ACV (133 mM) and DEX (10 mM), three times a day for three consecutive days, beginning at 6 day p.i. Signs of HSK (blepharitis, neovascularization, corneal clouding, edema, irritation and necrosis) were assessed for 15 days. Data shown are representative of two experiments. (A) Incidence of HSK; (B) Severity of disease (*$p < 0.1$; **$p < 0.01$; ***$p < 0.001$).

2 (Fig. 3). Likewise, ACV completely cleared viral infectivity by day 3 p.i., whereas DEX could not eliminate infectious virus up to 9 days p.i. (Fig. 3).

3.5. Anti-inflammatory activity of *1* and *2* on the evolution of HSK

Even though viral replication occurred in the eyes of treated mice, compounds **1** and **2** improved the HSV-1-induced ocular disease, suggesting an eventual immunomodulating activity. To elucidate this possibility, we studied the effect of both compounds at the onset of the immuno-inflammatory syndrome, starting from day 6 p.i.

When animals received compound **1** at days 6, 7 and 8 p.i., the incidence of ocular disease remained low all along the observation period: 30% ($p < 0.01$), 35% ($p < 0.025$) and 40% ($p < 0.1$) at 8, 10 and 15 days p.i. A similar percentage was obtained after treatment with DEX (30%, $p < 0.025$) (Fig. 4A). With respect to the severity of disease, mice treated with compound **1** showed score values even lower than those observed in animals treated at 1, 2 and 3 days p.i., and very similar to those obtained after the administration of DEX: 0.33, 0.85 ($p < 0.001$) and 1.17 ($p < 0.1$), at 8, 10 and 15 days p.i., respectively (Fig. 4B). The healing effect of compound **2** was less

evident, and only at 8 days p.i., the difference between treated and untreated mice was significant (Fig. 4A and B). As expected, ACV had no effect in the development of HSK, because infective virus particles were no longer found in the eye at the beginning of the treatment (Fig. 4A and B). These results correspond to the mean values obtained from two independent assays.

4. Discussion

HSK initiates by infection of the cornea resulting in the infiltration of inflammatory cells necessary to clear viral infection. However, this inflammatory response can also be destructive to the host cornea, leading to scar formation and vision loss [15,16].

Progression of the disease in humans is not prevented by antiviral drugs, whereas symptoms of HSK can be alleviated by immunosuppresive reagents including systemic corticosteroids and cyclosporine A [17]. Nevertheless, treatment of HSK also includes ACV to mitigate viral reactivation due to the immunosupression provoked by these drugs.

The clinical importance of a drug potentially usable for the treatment of HSK relays on its capability to diminish the damage produced once the inflammatory response to the viral infection develops. We have previously reported that polyfunctionalized stigmastanes – brassinosteroid analogs – significantly improved the clinical signs of HSK in the murine model of corneal infection, when supplied from 1 to 3 days p.i. [8]. The administration of those compounds at the onset of disease did not restrain the signs of ocular damage (data not shown). In order to improve the immunomodulating activity, new compounds were synthesized by introducing a 3-keto group and a double bond in Δ^4 in the steroid A ring (compound **1**) and an additional double bond in Δ^1 (compound **2**) which provide the molecules with structural similarities to that of corticosteroids [18].

Only compound **1** exhibited a significant antiviral activity *in vitro* (Fig. 1), although this activity was not evident in the *in vivo* model (Fig. 3), as it occurs with (*22S*,*23S*)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one, previously reported [8].

Compound **1** ameliorated the signs of HSK when supplied under either of the two schedules of treatment, at days 1, 2 and 3 p.i. or 6, 7 and 8 p.i. (Figs. 2 and 4). Although compound **1** exerted an antiviral effect on HSV-1-infected NHC cells, it has not exhibited any antiviral activity in the murine experimental model of HSK, at the concentration tested (Fig. 3). Hence, its mode of action would not be related to that of ACV, which produced a complete viral clearance in the eye from 3 days p.i. onwards and led to the healing effect on the eyes of the animals (Fig. 3). Since the improvement of the disease observed when **1** was supplied immediately after infection cannot be ascribed to the antiviral effect reported *in vitro* conditions, we hypothesized that it would be playing a role as a modulator of the immune response, that could explain the favorable evolution of HSK. It has been demonstrated that Toll-like receptor 2 (TLR2) ligand activity of HSV may be a major mechanism by which the virus induces early events necessary for the development of the inflammatory disease [19]. We cannot discard that compound **1** would be interacting with TLR2 and possibly blocking the subsequent development of immunopathological lessions.

On the other hand, compound **1**, as well as DEX, could heal the inflammatory lesions when administered from 6 to 8 days p.i. (Fig. 4). However, compound **1** would not behave as a conventional steroidal anti-inflammatory drug like DEX, since **1** significantly restrained the signs of keratitis when administered during the first 3 days after infection, while DEX exacerbated ocular damage (Fig. 2). In fact, after DEX treatment, score values largely surpassed those of the control of disease (Fig. 2), probably due to the high viral loads that could not be completely eliminated until day 9 p.i. (Fig. 3).

In the case of **2**, the healing effect observed was only evident until day 10 p.i. At day 15 p.i., the incidence of keratitis in mice was similar or even higher than that of the control animals, depending on the schedule of administration.

In this work, we report, for the first time, the synthesis of two new molecules that, even when they lack *in vivo* antiviral activity at the doses tested, were effective to improve a pathology of viral origin, not only in the presence of replicative virus but also when the effect of viral replication has triggered the immune response.

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