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Synthetic stigmastanes with dual antiherpetic and immunomodulating activities inhibit ERK and Akt signaling pathways without binding to glucocorticoid receptors.

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ABSTRACT

Background

We have previously shown that some synthetic hydroxylated stigmastanes derived from plant sterols inhibit *in vitro* HSV-1 replication in ocular cell lines and decrease cytokine production in stimulated macrophages, suggesting that these steroids might combine antiviral and immunomodulating properties. In this paper we report the synthesis of some analogues fluorinated at C-6 in order to study the effect of this modification on bioactivity.

Methods

The following methods were used: organic synthesis of fluorinated analogs, cytotoxicity determination with MTT assays, cytokine production quantification with ELISAs, glucocorticoid activity determination by displacement assays, immunofluorescence and transcriptional activity assays, studies of the activation of signaling pathways by Western blot, antiviral activity evaluation through virus yield reduction assays.

Results

We report the chemical synthesis of new fluorinated stigmastanes and show that this family of steroidal compounds exerts its immunomodulating activity by inhibiting ERK and Akt signaling pathways, but do not act as glucocorticoids. We also demonstrate that fluorination enhances the antiviral activity.

Conclusions

Fluorination on C-6 did not enhance the anti-inflammatory effect, however, an increase in the *in vitro* antiviral activity was observed. Thus, our results suggest that it is possible to introduce chemical modifications on the parent steroids in order to selectively modulate one of the effects.

General significance

This family of steroids could allow the development of an alternative treatment for ocular immunopathologies triggered by HSV-1, without the undesirable side effects of the currently used drugs.

KEYWORDS

Synthetic steroids; fluorinated steroids; antiherpetic; cytokines; signaling pathways.

ABBREVIATIONS

ALDO: aldosterone

CC: cell control

CC50: Cytotoxic Concentration 50%

CORT: corticosterone

DEX: dexamethasone

EC50: Effective Concentration 50%

GFP: Green Fluorescent Protein

GR: glucocorticoid receptor

HCLE: Human corneal–limbal epithelial cell line

HSK: herpetic stromal keratitis

HSV-1: Herpes simplex virus type 1

IOBA-NHC: Normal human conjunctival cell line

m.o.i.: multiplicity of infection

MR: mineralocorticoid receptor

RSV: Rous sarcoma virus

SI: Selectivity index

25HC: 25-hydroxycholesterol

1. INTRODUCTION.

Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus with very high infective rates in humans [1]. Although the primary site of HSV-1 infection is the orolabial mucosa, it can also cause disease in all of the major ocular tissues, including the conjunctiva, cornea, uveal tract, and retina. 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 [2]. When reactivation occurs, HSV-1 is transported back to the primary site of infection where it causes recurrent ulcerations. The potent immune response to the viral proteins triggers the ingrowth of blood vessels, infiltration of leukocytes, and damage to the corneal stroma and endothelium that combine to promote corneal opacity and edema, leading to an immunopathology known as herpetic stromal keratitis (HSK) that may result in blindness and, in rare cases, brain infections [3].

Current HSK treatment involves the use of topical corticosteroids to reduce inflammation. But the immunosuppressive effect associated with corticosteroids usually induces viral reactivation that must eventually be mitigated with an antiviral drug such as acyclovir. A common outcome of this treatment is the emergence of acyclovir-resistant viral strains, essentially due to long-term antiviral therapy in combination with an impaired host response, which enables the less virulent viruses to continue to replicate [4]. Thus, the development of new antiherpetic compounds for HSK treatment is a crucial challenge.

We previously showed that synthetic steroids **1** and **2** (Figure 1) inhibit HSV-1 multiplication and viral spreading in a human conjunctival cell line (IOBA-NHC), with no cytotoxicity. Furthermore, these compounds significantly decrease the incidence of HSK in infected mice, even when administered during the inflammatory phase of the disease [5]. This fact led us to consider the possibility that these steroids might combine antiviral activity with immunomodulating properties. Indeed, in macrophages stimulated with lipopolysaccharide (LPS) and treated with compound **1**, the production of inflammatory cytokines such as TNF-α and IL-6 is significantly inhibited, comparable to the effects of dexamethasone (DEX), a widely used corticosteroid [6].

Compounds **1** and **2** belong to steroids of stigmastane series, which include sterols such as sitosterol and stigmasterol (commonly found in plants but absent in animals).. However, these compounds share some moieties with corticosteroids (a 3-keto-Δ^4 system in ring A and an hydroxylated side chain), which led us to hypothesize that their inmmunomodulating properties might be associated with a corticoid-like activity.

On the other hand, since the first report of fluorination of the glucocorticoid hormone cortisol in 1954 [7] it is known that the introduction of a 6α-fluorine into a glucocorticoid has a large and increasing effect on anti-inflammatory activity. Several topically active products containing this functionality, for example paramethasone, have been developed and are in clinical use [8].

Taking into account these precedents, and with the aim to study the effect of fluorination on the *in vitro* immunomodulating activity of compounds **1** and **2**, we decided to synthesize new analogs bearing a fluorine atom at C-6 (compounds **3a**, **3b**, **4a** and **4b**, Figure 1). Furthermore, the anti HSV-1 activity of the compounds was assessed in a cell line relevant to ocular infections.

2. MATERIALS AND METHODS.

2.1 Synthesis of the compounds.

All solvents and reagents were purchased from Sigma-Aldrich Chemical Co. and were of analytical grade. Satisfactory combustion analysis data were obtained for all new compounds. Structures of the new compounds were confirmed by 1-D and 2-D NMR spectroscopic analysis. Detailed synthetic procedures and full characterization of the new compounds are described in the Supplementary Data.

2.2 Cells, virus and treatment solutions.

Human corneal–limbal epithelial (HCLE) cells were kindly provided by Dr. Ilene K. Gipson and Dr. Pablo Argüeso (The Schepens Eye Research Institute, Harvard Medical School, Boston, USA), grown in GIBCO Keratinocyte Serum-Free Medium, supplemented with 25 µg/mL bovine pituitary extract 0.2 ng / mL epidermal growth factor and 0.4 mM $CaCl_2$, and maintained in low calcium DMEM/F12. Vero cells were grown in Eagle's minimal essential medium (MEM) supplemented with 5% inactivated fetal bovine serum (FBS) (MEM- FBS 5%) and 50 µg/mL gentamycin, and maintained in monolayer formation in MEM supplemented with 1.5% inactivated FBS (MEM-FBS 1.5%). The murine macrophage cell line J774A.1 (ATCC® Number TIB-67TM) was kindly provided by Dr. Osvaldo Zabal (INTA, Castelar, Buenos Aires) and grown in RPMI 1640 medium supplemented with 10% inactivated FBS (RPMI-FBS 10%) and maintained in RPMI supplemented with 2% inactivated FBS (RPMI-FBS 2%). Human embryonic kidney 293-T cells (HEK293T cells, ATCC® Number CRL-11268) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS previously stripped of endogenous steroids by adsorption on charcoal/dextran mixture, and re-

sterilized with 0.2 µm filters. The HSV-1 KOS strain was propagated at a low multiplicity of infection (m.o.i.) and used for in vitro experiments. [^{3}H]dexamethasone ([^{3}H]DEX) and [^{3}H]aldosterone ([^{3}H]ALDO) were purchased from New England Nuclear, Boston, MA, USA.

2.3 Reagents.

LPS (TLR4 ligand), a lipopolysaccharide from Escherichia coli serotype 055: B5 was obtained from Sigma (St. Louis, USA). Pam2CSK4 (TLR2 ligand) and ODN2395 (TLR9 ligand) were purchased from InvivoGen (California, USA). The rabbit polyclonal anti-IκBα antibodies, anti-ERK1 and anti-p-ERK1/2, and the peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies were obtained from Santa Cruz Biotechnology (Texas, USA). The anti-actin antibody was obtained from Calbiochem (Massachusetts, USA). Secondary goat anti-rabbit FluoroLinkTM CyTM2 and anti-mouse FluoroLinkTM CyTM3 antibodies were purchased from GE Healthcare Bio-Sciences (Buckinghamshire, England). Anti-Akt and anti-p-Akt Ser473 antibodies (Cell Signaling, Boston, USA) were kindly provided by Dr. A. Srebrow (IFBMC, FCEyN, UBA).

2.4 Cytotoxicity assay.

To evaluate the cytotoxicity of the compounds, cell viability was determined as previously reported [24]. Compounds were dissolved in dimethylsulfoxide (DMSO) and diluted with culture medium for testing. The maximum concentration of DMSO tested was between 0.1% and 1% v/v and exhibited no cytotoxicity under the experimental conditions. The absorbance of each well was measured on a Eurogenetics MPR-A 4i microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. Results are expressed as percent absorbance of treated cells with respect to untreated cells. The Cytotoxic Concentration 50% (CC50) was defined as the concentration of compound that caused a 50% reduction in cell viability.

2.5 Antiviral activity.

To evaluate antiviral activity, HCLE cells grown in 24-well plates were infected with HSV-1 (m.o.i. = 0.2) and then treated with different concentrations of the compounds or control media. After 24 h of incubation at 37 °C, free and cell-associated viruses 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 eliminated and replaced by maintenance medium with 0.7% of methylcellulose. After 72 h incubation at 37 °C,

cells were fixed with formaldehyde 10%, stained with Crystal Violet, and plaque forming units were counted.

The Effective Concentration 50% (EC_{50}) was defined as the concentration of compound that caused a 50% reduction in virus multiplication. Selectivity indices (SI) for each compound were obtained from CC_{50} / EC_{50} ratios.

2.6 Determination of cytokine profiles.

To determine cytokine responses, J774A.1 cells grown in 24-well plates were incubated at 37 ºC in the presence or absence of different concentrations of the Toll-like receptors 2, 4 and 9 (TLR2, TLR4 and TLR9) ligands, in duplicate. After 8 h, supernatants were harvested and murine TNF-α and IL-6 were quantified by commercial ELISA sets (BD OptEIATM, Becton Dickinson, USA) according to the manufacturer's instructions.

2.7 Western blot analysis.

Whole extracts of cells grown in 24-well plates for 24 h were run in a 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene fluoride membranes for 60 min at 75 mA. Membranes were blocked in PBS containing 5 % nonfat milk overnight and then incubated with diluted primary antibodies overnight at 4ºC. After washing, membranes were incubated with diluted peroxidase-conjugated antibodies for 1.5 h at 37ºC. The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, PerkinElmer).

2.8 Transfections.

Transfection assays with Lipofectamine 2000 reagent (Invitrogen) were performed according to the manufacturer's instructions. The NF-κB-LUC reporter vector and RSV-β-gal plasmid were kindly provided by Dr. Susana Silberstein (University of Buenos Aires). RSV-β-gal, which codes for the bacterial β-galactosidase gene under the control of the viral RSV promoter, was used as second control reporter plasmid.

2.9 Preparation of thymus cytosol.

Thymus cytosol was prepared following a previously described protocol [25]. Male Wistar rats weighing ~ 280 g were anesthetized intraperitoneally with a mixture of ketamine (80 mg/Kg) and xylazine (10 mg/Kg). The adrenal glands were removed by dorsal approach. Adrenalectomized animals were maintained with Purina rat chow, tap water and physiological solution ad libitum to prevent hyponatremia. This procedure

prevents the occupation of the glucocorticoid receptor (GR) by endogenous steroids and favors the physiological induction of GR, thereby increasing the sensitivity of the system. Thymuses were removed 48 h post-surgery, homogenized in HEM buffer (10 mM Hepes pH 7.4, 1 mM EDTA, 10% v/v glycerol, 20 mM sodium molybdate and supplemented with a cocktail of protease inhibitors) and centrifuged at 67,000 g for 30 min at 0°C. The supernatant hereafter referred to as cytosol was used immediately.

2.10 Preparation of fibroblast cytosol.

To prepare fibroblast cytosol, HEK293T cells were grown to ~ 50% confluence, at which point the culture medium was replaced by Opti-MEM. After 1 h, cells were transfected with either 2.5 µg of the plasmid pSV2rec-GR, which encodes for mouse GR, or with pCINeo-flagMR, which encodes for human MR, using a liposome (Transfast, Promega) vehicle, The cells were collected by trypsinization 48 h later, washed with PBS and homogenized in HEM buffer, as described for the preparation of thymus cytosol.

2.11 Displacement assays.

Cytosols were incubated with 20 nM [^{3}H]DEX or [^{3}H]ALDO and increasing amounts of unlabeled steroid (0.1-, 1-, 10-, or 100-fold excess) overnight at 0°C. Free hormone was separated from receptor-bound hormone by adsorption, with a volume of carbon/dextran in 10 mM Hepes buffer. The radioactivity associated with the supernatant was measured in a scintillation counter.

2.12 Immunofluorescence.

HEK293T cells grown on coverslips pretreated with polylysine to promote cell adhesion were transfected with a chimeric GR associated with Green Fluorescent Protein (GFP), as described in the previous section. After 24 h of growth in a medium containing serum adsorbed with carbon/dextran (and thus steroid-free), cells were stimulated with 100 nM DEX, 1 µM of the compound of interest, or both compounds 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 epifluorescence microscope.

2.13 Transcriptional activity assays.

HEK293T cells were transfected with 1 µg pCINeo-flag-MR or GFP-GR following a previously published protocol [26]. Concisely, 0.1 µg mouse mammary tumor virus-

luciferase (MMTV-Luc) and 50 ng of RSV-β-galactosidase were cotransfected with one of the receptors following the calcium phosphate precipitation standard method. After 36 h incubation in a medium containing charcoal-stripped serum, cells were stimulated with steroid for 12 h. Luciferase and β-galactosidase activities were measured and luciferase activity was normalized to β-galactosidase expression.

2.14 Statistical analysis.

One-way ANOVA analysis for paired samples was used to determine the significance of the differences between mean values in all control and tested samples using GraphPad Prism software. The analysis was complemented with a Tukey post-test to compare mean values.

3. RESULTS.

3.1 Synthesis of the fluorinated analogs.

The synthesis of the new fluorinated analogs is depicted in Scheme 1. Fluorination at C-6 was achieved via a well-established procedure that signifies the electrophilic fluorination of the corresponding steroidal 3,5-dienol acetate. Thus, the dienol acetate **6**, which was obtained from commercial stigmasterol in two steps, was subjected to fluorination with 1-(chloromethyl)-4-fluoro-1,4-diazabicyclo [2.2.2] octane bistetrafluoroborate (Selectfluor). This fluorinating agent was chosen because it is the most suitable reagent for the required fluorination [9].

The mixture of the 6α- and 6β-fluoroenones **7a** and **7b**, which was obtained in a 3:1 ratio, was separated by column chromatography. The configuration assignment at C-6 was established from the coupling patterns observed in the ^{1}H-NMR spectra of the corresponding H-6. In compound **7a**, besides the H-F coupling, the H-6 shows three additional H-H couplings, being the larger of 12.2 Hz. It suggests the presence of an axial-axial coupling for this proton, which is only compatible with a 6α configuration for the geminal fluorine. Furthemore, in compound **7b** not only this coupling pattern is absent, but a small long-range coupling between fluorine and the methyl-19 is observed, a typical feature of 6β-fluorosteroids [10,11].

Subsequently, the Δ^{22} double bond of the 6α-fluoro compound **7a** was selectively dihydroxylated under Sharpless´ conditions using $(DHQ)_2$-Phal as the catalyst and producing compound **3a** as the only product. The outcome of the reaction was the desired 22*S*,23*S* diol [12], a moiety that, as our previous studies suggest, is essential

for antiherpetic activity [13]. The configuration of the diol was established by comparison with the chemical shifts and coupling constants of known closely related structures. [14]

Finally, compound **3a** was treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to introduce an additional Δ^1 double bond, affording compound **4a** with 81% yield. Similarly, application of the dihydroxylation - dehydrogenation sequence on the 6β-fluoro isomer **7b** yielded compounds **3b** and **4b**.

3.2 Biological activity of the compounds.

3.2.1 Effect on cytokine production in stimulated macrophages.

It is well known that macrophages play a role in non-specific defense against viral infections and are a source of inflammatory mediators, such as TNF-α and IL-6. Specifically, in the HSK murine model, macrophages participate in the inflammatory response in the eye and are involved in the development of the immunopathology.

We previously found that compound **1** diminishes TNF-α and IL-6 secretion in a dose-dependent manner when murine macrophages are stimulated with LPS, a TLR4 agonist [6]. TLR-4 plays an important role in the innate immune system, and is proposed to be one of the TLRs involved in the recognition events following HSV-1 infection. Thus, we decided to extend this study to compound **2** and their fluorinated counterparts **3a**, **3b**, **4a** and **4b**. Compound **1** was also included in the assays for comparative purpose.

First, we determined the cytotoxicity of the compounds on J774A.1 cells. All of them exhibited CC50 values higher than 100 μM (Figure 2A).

We observed a basal production of 0.6 ± 0.1 ng/ml of TNF-α and 0.1 ± 0.1 ng/ml of IL-6 in the absence of LPS in J774A.1 cells. When macrophages were stimulated with 100 ng/ml of LPS, the secretion of TNF-α and IL-6 increased, 8 h after stimulation, to 152.3 ± 15.1 ng/ml and 18.8 ± 3.3 pg/ml, respectively (Figure 2B and C). When this experiment was performed in the presence of the steroidal compounds, a dose-response inhibition was observed (Figure 2B and C). In addition, none of the compounds were able to induce an increase in cytokine levels alone (shown in Supplementary data).

On the other hand, because TLR-2 and 9 receptors are also known to be involved both in the early recognition of HSV and cytokine secretion in the response to HSV infection

[15–17], the effect of the compounds on the production of TNF-α and IL-6 when macrophages were stimulated with the appropriate ligands was determined.

Figure 3 shows that when J774A.1 cells were treated with 100 ng/ml of a synthetic diacylated lipoprotein (Pam2CSK4, a TLR-2 agonist), or 5 µg/ml of ODN2395 (a TLR9 agonist), the production of TNF-α increased significantly (Figure 3A and C, respectively). When these experiments were preformed in the presence of 40 µM of the compounds, the cells activated with either stimulus reduced production of this cytokine. Stimulation with Pam2CSK4 induced an increase in IL-6 production (Figure 3B), while no secretion of IL-6 was observed in J774A.1 cells stimulated with ODN2395. All of the compounds tested were able to reduce IL-6 production. Figure 3 depicts the results for compounds **1**, **3a** and **4a** as representative examples of this behavior. Interestingly, although all of the compounds modulated both the TNF-α and the IL-6 production induced by the TLR ligands in a similar fashion to DEX [18], fluorination at C-6 does not seem, contrary to our expectations, to enhance the immunomodulatory effect.

3.2.2 Glucocorticoid activity of the synthetic steroids.

In order to confirm that the observed immunomodulatory effects were associated to glucocorticoid-like activity, we decided to directly evaluate the binding of the compounds to the glucocorticoid receptor (GR). Thus, we performed a displacement assay in which either native GR from murine thymus or mouse GR overexpressed in the human embryonic kidney cell line 293T (HEK293T) were used. As a displacement control we used unlabeled dexamethasone (DEX) or corticosterone (CORT).

To analyze possible cross reactions with the mineralocorticoid receptor (MR), which shares a very high homology with GR, displacement assays of the compounds were carried out with aldosterone (ALDO) as a control.

Figures 4A and B show the competition curves for GR extracts in thymus and HEK cells transfected with GR, respectively. As expected, both unlabeled CORT and DEX competed effectively with the tracer for GR. However, compound **1** did not show affinity for this receptor or cross reactivity to MR (Figure 4C). Furthermore, none of the other compounds (**2**, **3a**, **3b**, **4a** or **4b**) showed affinity for GR at a 100-fold excess of non-radioactive hormone (data not shown).

It is well known that steroid receptors are not statically confined to a given cell compartment, but move constantly between the cytoplasm and the nucleus. The

addition of steroids makes GR accumulate in the nucleus. Therefore, we studied the ability of the compounds to promote the nuclear accumulation of a chimeric GR associated with Green Fluorescent Protein (GFP-GR) in HEK cells. We also analyzed whether the presence of the compounds affects the normal hormone-dependent transit of GR.

None of the compounds induced GFP-GR nuclear localization *per se*, corroborating that they were not able to bind GR (Figure 5). In the presence of DEX, immunofluorescence images showed that GFP-GR localizes, as expected, in the nucleus.

To further confirm the failure of the compounds to trigger a glucocorticoid-like response, cells were transfected with a gene construction containing three glucocorticoid responsive elements that are able to recognize GR and command the induction of a reporter gene coding for luciferase. Cells were then stimulated with 10, 0.1 and 0.001 µM of the compounds for 12 h, or with the same concentrations of DEX as a positive control (Figure 6A). A similar scheme was used to study the transcriptional activity of MR (Figure 6B). Transfection efficiency was standardized to β-galactosidase activity.

Although 0.1 µM DEX led to 100% relative luciferase activity, none of the compounds assessed was able to activate the transcription via GR at any of the concentrations tested (Figure 6A). When transcriptional activity of MR was assayed, a similar result was obtained, since none of the compounds were able to induce the reporter gene via MR, and the positive control ALDO was (Figure 6B).

In conclusion, none of the synthetic stigmastanes were able to bind the glucocorticoid or mineralocorticoid receptors.

3.2.3 Activation of signaling pathways

Since the stigmastanes modulated both the TNF-α and the IL-6 production induced by TLR ligands, but by a different mechanism than glucocorticoids, we investigated whether NF-κB, Akt and ERK intracellular signaling pathways, which are involved in the regulation of cytokine production, were affected. As compound **1** was extensively studied in our previous work, it was chosen to perform the following experiments

NF-κB is a major signaling pathway activated by HSV-1 infection [19]. A series of phosphorylation / recruitment / activation events, such as degradation of IκB, lead to NF-κB nuclear translocation and transcription of inflammatory cytokine genes [20]. In

order to study the role of compound **1** in the NF-κB signaling cascade, we examined its potential effects on TLR-induced IκB degradation. We have previously shown that compound **1** is not able to block NF-κB nuclear translocation when J774A.1 cells are stimulated with LPS.[6] Hence, macrophages were stimulated with Pam2CSK4 (100 ng/ml), LPS (100 ng/ml) and ODN2395 (5 µg/ml), and treated with compound **1** (at 40 µM) for 1 h. Cells were then processed through Western blot using a rabbit polyclonal anti-IκB antibody.

Compound **1** did not degrade IκB in non-stimulated cells (Fig. 7A). TLR2, TLR4 and TLR9 stimulation caused IκB degradation, and the addition of **1** did not prevent IκB disappearance (Fig. 7A). Consequently, we hypothesized that compound **1** might inhibit a downstream step in IκB degradation and NF-κB translocation. Therefore, we used a NF-κB-LUC reporter plasmid to explore whether NF-κB-induced transcription was affected.

With this end, J774A.1 cells were transfected with the NF-κB-LUC reporter vector and β-galactosidase control plasmid and, 24 h later, were treated with **1** (40 µM). After 8 h, luciferase activity was measured in cell extracts and each value was normalized to β-galactosidase activity. All of the TLRs agonists caused significant NF-κB activation, but this activation was not hampered by compound **1** regardless of the TLR used (Fig. 7B).

Next, we determined if compound **1** could modulate ERK and/or Akt phosphorylation in cells stimulated with the TLR agonists. J774A.1 cells stimulated with Pam2CSK4 (100 ng/ml), LPS (100 ng/ml) and ODN2395 (5 µg/ml) were treated with compound **1** (at 40 µM) for 1 h. Cells were then processed in a Western blot using antibodies against pERK, ERK, pAkt and Akt.

Compound **1** was not able to activate either the ERK or the Akt pathways by itself, whereas the three TLR agonists induced, as expected, phosphorylation of ERK and Akt, (Figure 7C). Interestingly, compound **1** completely blocked both the ERK and the Akt phosphorylation triggered by the TLR agonists in macrophages (Fig 7C). Hence, the modulation of TNF-α and IL-6 secretion observed in compound **1** might be associated with the inhibition of the ERK and Akt signaling pathways.

3.2.4 Antiviral activity

The ocular surface consists of the corneal and conjunctival epithelia, which are closely linked, both histologically and functionally. Eye epithelial cells are the first cell types involved in the early development of HSK. These are the main sites of HSV-1

multiplication, a prerequisite for the induction of the immunopathology. It is of interest, then, to study the effect of compounds that are potentially useful in the treatment of HSK on the multiplication of HSV-1 in ocular tissue epithelial cells.

Since we have previously found that compounds **1** and **2** restrain HSV-1 propagation in infected conjunctival and corneal cells, we investigated the anti-HSV-1 activity of both compounds and their fluorinated counterparts in human corneal-limbal epithelial (HCLE) cells, another ocular cell line.

Firstly, the cytotoxic concentration 50% (CC_{50}) of each compound was determined to be higher than 200 µM (Fig 8A and B) in HCLE cells. Different concentrations ranging from 0.1 to 200 µM were then added to HSV-1-infected HCLE cells. Compounds **1**, **3a**, **4a** and **4b** inhibited the viral multiplication in a dose-dependent manner, with effective concentration 50% (EC_{50}) values of 10 µM or lower (Figures 8C and D). Interestingly, the most potent compound was **4b**, a surprising result considering that **2**, its non-fluorinated parent compound, showed no significant antiviral activity.

4. DISCUSSION

In previous studies we described a family of synthetic stigmastanes derived from stigmasterol, a major plant sterol, that share not only a wide spectrum of antiviral activity [13,21,22], but also anti-inflammatory properties [23,24]. The fact that the immunomodulation by these compounds resembled in part immunomodulation by glucocorticoids, led us to hypothesize that these two families of steroids might share a common mechanism of action. Nevertheless, the results shown in this work ruled out this possibility. We have demonstrated that these stigmastanes are not agonists of the glucocorticoid receptor; furthermore, they are not able to inhibit the NF-κB pathway when macrophages are stimulated with several TLRs ligands, as glucocorticoids do. Moreover, our results show that their immnunomodulating activity might be associated with the inhibition of the ERK and Akt signaling pathways.

On the other hand, although the antiviral activity of natural and synthetic steroids have been known for some decades [13,25–27], the understanding of their mechanism of action has remained elusive [28]. In recent years, however, a major breakthrough showed that endogenous oxysterols, especially 25-hydroxycholesterol (25HC), play an important role in both innate and adaptive immunity during viral infection and demonstrate antiviral activity, establishing a link between these two biological activities [29–32].

These findings may shed light on our results: it is possible that the stigmastanes exert their effects through a mechanism related to that of oxysterols. Firstly, there is a high structural similarity between the oxysterols and the stigmastanes described here. Furthermore, the hydroxylated side chain present on these stigmastanes is essential, as previously mentioned, for the antiviral and anti-inflammatory activity; interestingly, cholesterol is converted into 25HC by a Ch25h-mediated hydroxylation of the side chain. On the other hand, the stigmastanes inhibit both ERK and Akt signaling pathways: the activation of the ERK pathway is known to trigger the activation of the AP-1 family transcription factors [33], which is modulated by 25HC [32] during influenza infection. It is interesting to note that, although fluorination on C-6 did not enhance the anti-inflammatory effect, compound **4b** showed a five-fold increase in the *in vitro* antiviral activity. This result suggests that, even if both the antiviral and inmmunomodulating activities are linked by the same mechanism, it is possible to introduce chemical modifications on the parent steroids in order to selectively modulate one of the effects. Moreover, as these compounds do not act by the same mechanism as glucocorticoids, the work shown here could pave the way to the development of an alternative treatment for HSK without the undesirable side effects of the current treatments, which are based on glucocorticoids and nucleosides analogs. Further work in this direction, and in the elucidation of the molecular events involved in the activity is underway

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REFERENCES

[1] R.J. Whitley, B. Roizman, Herpes simplex virus infections, Lancet, 357 (2001) 1513–8.

[2] K.P. Egan, S. Wu, B. Wigdahl, S.R. Jennings, Immunological control of herpes simplex virus infections, J. Neurovirol., 19 (2013) 328–45.

[3] A.M. Rowe, A.J. St. Leger, S. Jeon, D.K. Dhaliwal, J.E. Knickelbein, R.L. Hendricks, Herpes keratitis, Prog. Retin. Eye Res., 32 (2013) 88–101.

[4] G. Andrei, R. Snoeck, Herpes simplex virus drug-resistance: new mutations and insights, Curr. Opin. Infect. Dis., 26 (2013) 551–60.

[5] F.M. Michelini, J.A. Ramírez, A. Berra, L.R. Galagovsky, L.E. Alché, Anti-herpetic and anti-inflammatory activities of two new synthetic 22,23-dihydroxylated stigmastane derivatives, J. Steroid Biochem. Mol. Biol., 111 (2008) 111–116.

[6] F.M. Michelini, P. Zorrilla, C. Robello, L.E. Alché, Immunomodulatory activity of an anti-HSV-1 synthetic stigmastane analog, Bioorganic Med. Chem., 21 (2013) 560–568.

[7] J. Fried, E.F. Sabo, 9α-Fluoro derivatives of cortisone and hydrocortisone, J. Am. Chem. Soc., 76 (1954) 1455–1456.

[8] J.-P. Bégué, D. Bonnet-Delpon, Biological impacts of fluorination: Pharmaceuticals based on natural products, in: A. Tressaud, G. Haufe (Eds.), Fluor. Heal. Mol. Imaging, Biomed. Mater. Pharm., Elsevier, 2008: pp. 553–622.

[9] V. Reydellet-Casey, D.J. Knoechel, P.M. Herrinton, Comparison of commercially available reagents for fluorination of steroid 3,5-dienol acetates, Org. Process Res. Dev., 1 (1997) 217–221.

[10] K. a M. Ampt, R.L.E.G. Aspers, M. Jaeger, P.E.T.J. Geutjes, M. Honing, S.S. Wijmenga, Application of fluorine NMR for structure identification of steroids, Magn. Reson. Chem., 49 (2011) 221–30.

[11] Y.S. Choe, J.A. Katzenellenbogen, Synthesis of C-6 fluoroandrogens: Evaluation of ligands for tumor receptor imaging, Steroids, 60 (1995) 414–422.

[12] M.J. Thompson, W.J. Meudt, N.B. Mandava, S.R. Dutky, W.R. Lusby, D.W. Spaulding, Synthesis of brassinosteroids and relationship of structure to plant growth-promoting effects, Steroids, 39 (1982) 89–105.

[13] M. Wachsman, J. Ramírez, L. Talarico, L. Galagovsky, C. Coto, Antiviral activity of natural and synthetic brassinosteroids, Curr. Med. Chem. -Anti-Infective Agents, 3 (2004) 163–179.

[14] A. Porzel, V. Marquardt, G. Adam, G. Massiot, D. Zeigan, ^{1}H and ^{13}C NMR analysis of brassinosteroids, Magn. Reson. Chem., 30 (1992) 651–657.

[15] N. Boivin, R. Menasria, J. Piret, G. Boivin, Modulation of TLR9 response in a mouse model of herpes simplex virus encephalitis, Antiviral Res., 96 (2012) 414–421.

[16] H. Li, J. Zhang, A. Kumar, M. Zheng, S.S. Atherton, F.S.X. Yu, Herpes simplex virus 1 infection induces the expression of proinflammatory cytokines, interferons and TLR7 in human corneal epithelial cells, Immunology, 117 (2006) 167–176.

[17] X. Jin, Q. Qin, W. Chen, J. Qu, Expression of toll-like receptors in the healthy and herpes simplex virus-infected cornea, Cornea, 26 (2007) 847–852.

[18] S. Bhattacharyya, C.K. Ratajczak, S.K. Vogt, C. Kelley, M. Colonna, R.D. Schreiber, L.J. Muglia, TAK1 targeting by glucocorticoids determines JNK and IκB regulation in toll-like receptor-stimulated macrophages, Blood, 115 (2010) 1921–1931.

[19] J. Melchjorsen, J. Sirén, I. Julkunen, S.R. Paludan, S. Matikainen, Induction of cytokine expression by herpes simplex virus in human monocyte-derived macrophages and dendritic cells is dependent on virus replication and is counteracted by ICP27 targeting NF-kappaB and IRF-3, J. Gen. Virol., 87 (2006) 1099–1108.

[20] M. Cai, M. Li, K. Wang, S. Wang, Q. Lu, J. Yan, K.L. Mossman, R. Lin, C. Zheng, The Herpes Simplex Virus 1-encoded envelope glycoprotein B activates NF-κB through the Toll-Like Receptor 2 and MyD88/TRAF6-dependent signaling pathway, PLoS One, 8 (2013).

[21] M.B. Wachsman, E.M.F. López, J.A. Ramirez, L.R. Galagovsky, C.E. Coto, Antiviral effect of brassinosteroids against herpes virus and arenaviruses, Antivir. Chem. Chemother., 11 (2000) 71–77.

[22] M.B. Wachsman, J.A. Ramirez, L.R. Galagovsky, C.E. Coto, Antiviral activity of brassinosteroids derivatives against measles virus in cell cultures, Antivir. Chem. Chemother., 13 (2002) 61–66.

[23] J.A. Ramírez, A.C. Bruttomesso, F.M. Michelini, S.L. Acebedo, L.E. Alché, L.R. Galagovsky, Syntheses of immunomodulating androstanes and stigmastanes: Comparison of their TNF-α inhibitory activity, Bioorganic Med. Chem., 15 (2007) 7538–7544.

[24] F.M. Michelini, A. Berra, L.E. Alché, The in vitro immunomodulatory activity of a synthetic brassinosteroid analogue would account for the improvement of herpetic stromal keratitis in mice, J. Steroid Biochem. Mol. Biol., 108 (2008) 164–170.

[25] J.-L. Giner, S.P. Gunasekera, S. a. Pomponi, Sterols of the marine sponge Petrosia weinbergi: implications for the absolute configurations of the antiviral orthoesterols and weinbersterols, Steroids, 64 (1999) 820–824.

[26] M.J. Comin, M.S. Maier, A.J. Roccatagliata, C.A. Pujol, E.B. Damonte, Evaluation of the antiviral activity of natural sulfated polyhydroxysteroids and their synthetic derivatives and analogs, Steroids, 64 (1999) 335–40.

[27] A.J. Roccatagliata, M.S. Maier, A.M. Seldes, C.A. Pujol, E.B. Damonte, Antiviral sulfated steroids from the ophiuroid Ophioplocus januarii, J. Nat. Prod., 59 (1996) 887–9.

[28] V. Castilla, J. Ramirez, C. E. Coto, Plant and animal steroids a new hope to search for antiviral agents, Curr. Med. Chem., 17 (2010) 1858–1873.

[29] M. Blanc, W.Y. Hsieh, K.A. Robertson, K.A. Kropp, T. Forster, G. Shui, P. Lacaze, S. Watterson, S.J. Griffiths, N.J. Spann, A. Meljon, S. Talbot, K. Krishnan, D.F. Covey, M.R. Wenk, M. Craigon, Z. Ruzsics, J. Haas, A. Angulo, W.J. Griffiths, et al., The transcription factor STAT-1 couples macrophage synthesis of 25-hydroxycholesterol to the interferon antiviral response, Immunity, 38 (2013) 106–118.

[30] S.Y. Liu, R. Aliyari, K. Chikere, G. Li, M.D. Marsden, J.K. Smith, O. Pernet, H. Guo, R. Nusbaum, J. a. Zack, A.N. Freiberg, L. Su, B. Lee, G. Cheng, Interferon-inducible cholesterol-25-hydroxylase broadly inhibits viral entry by production of 25-hydroxycholesterol, Immunity, 38 (2013) 92–105.

[31] C. Wilkins, M. Gale, Sterol-izing Innate Immunity, Immunity, 38 (2013) 3–5.

[32] E.S. Gold, A.H. Diercks, I. Podolsky, R.L. Podyminogin, P.S. Askovich, P.M. Treuting, A. Aderem, 25-Hydroxycholesterol acts as an amplifier of inflammatory signaling, Proc. Natl. Acad. Sci. U. S. A., (2014) 1–6.

[33] P. Monje, J. Hernández-Losa, R.J. Lyons, M.D. Castellone, J.S. Gutkind, Regulation of the transcriptional activity of c-Fos by ERK: A novel role for the prolyl isomerase Pin1, J. Biol. Chem., 280 (2005) 35081–35084.

Figure 1. Structures of the synthetic stigmastanes.

Figure 2. Effect of the compounds on cytokine production induced by TLR4 ligand. (A) Effect of the compounds on cell viability. J774A.1 cells were treated with serial two-fold dilutions of each compound at 37 ºC during 24 h. Cell viability was evaluated by the MTT assay and plotted as the percentage of cell viability with respect to untreated control. (B and C) J774A.1 cells were stimulated with TLR4 ligand (100 ng/ml) for 8 h and treated or not with different concentrations of the compounds (µM). TNF-α (B) and IL-6 (C) were determined by ELISA. Data are expressed as the mean ± S.D. of three separate experiments. CC: cell control (unstimulated cells); * $p < 0.001$, with respect to TLR4 ligand control.

Figure 3. Effect of the compounds on cytokine production induced by TLR2 and TLR9 ligands. J774A.1 cells were stimulated with TLR2 (100 ng/ml) and TLR9 (5 µg/ml) ligands for 8 h and treated or not with a different concentrations of the compounds. TNF-α (A and C) and IL-6 (B) were determined by ELISA. Data are expressed as the mean ± S.D. of three separate experiments. CC: cell control (unstimulated cells); * $p < 0.001$, with respect to TLR2 and TLR9 ligands controls.

Figure 4. GR and MR binding capacity of compound 1. Competition curves for GR in thymus extracts (A) and HEK cell extracts transfected with GR (B), and for MR (C) in HEK cell extracts transfected with MR. The cytosols were incubated with 20 nM [^{3}H]DEX or [^{3}H]ALDO and increasing amounts of unlabeled steroid (0.1-, 1-, 10-, or 100-fold excess). The free hormone was separated from the hormone bound to the receptor and the radioactivity associated with the supernatant was measured in a scintillation counter.

Figure 5. Capacity of the compounds to translocate GR to the nucleus (nuclear accumulation of GFP-GR in HEK cells). HEK cells grown on coverslips were transfected with a chimeric GR associated with GFP. After 24 h, cells were stimulated with 100 nM DEX, 1 µM of the compound under study, or both compounds at the same time. After 30 min of incubation, the coverslips were washed, fixed and permeabilized, and the subcellular localization of GFP-GR was observed with an epifluorescence microscope. Magnification: 60X.

Figure 6. Effect of the compounds on the transcriptional activity of GR and MR. HEK293T cells were cotransfected with 0.1 µg mouse mammary tumor virus-luciferase (MMTV-Luc) and 50 ng of Rous sarcoma virus (RSV)-β-galactosidase and with 1 µg of either receptor pCINeo-flag-MR or GFP-GR. After 36 h, cells were stimulated with steroid for 12 h. Both luciferase and β-galactosidase activities were measured, and the luciferase activity was normalized to β-galactosidase expression. GR (A) and MR (B) transcriptional activity in HEK cells.

Figure 7. Effect of compound 1 on the activation of intracelular signaling pathways. (A) Effect of the compound on IκBα degradation and NF-κB activation. J774A.1 cells were stimulated with TLR4 ligand (100 ng/ml), TLR2 ligand (100 ng/ml) and TLR9 ligand (5 mg/ml) and treated or not with compound 1 (40µM). After 60 min, macrophages were lysed and subjected to SDS-PAGE, followed by immunoblotting with antibodies against IκBα and actin. (B) J774A.1 cells were transfected with 0.5 µg of NF-κB-LUC reporter vector and 0.5 µg of β-galactosidase control plasmid. After 24 h, J774A.1 cells were stimulated with TLR2, TLR4 (100 ng/ml) and TLR9 (5 µg/ml) ligands for 8 h, in the

presence or not of compound 1 (40 μM). Luciferase activity was measured in cell extracts and each value was normalized to β-galactosidase activity in relative luciferase units (RLU). Data are expressed as the mean ± S.D. of three separate experiments. * indicates significant differences with respect to cell control (CC) ($p < 0.05$). (C) Effect of compound 1 on ERK and Akt activation. J774A.1 cells were stimulated with TLR2, TLR 4 or TLR 9 ligands for 60 min and treated or not with 40 μM of compound 1. Macrophages were lysed and subjected to SDS-PAGE, followed by immunoblotting with antibodies against pERK, ERK, pAkt and Akt.

Figure 8. Cytotoxicity and antiviral activity of the compounds in HCLE cells. (A and B) Effect of the compounds on cell viability. HCLE cells were treated with serial two-fold dilutions of each compound at 37 ºC during 24 h. Cell viability was evaluated by the MTT assay and plotted as the percentage of cell viability with respect to untreated control. (C and D) Effect of the compounds on viral yields. HCLE cells infected with HSV-1 KOS (m.o.i = 1) were treated or not (control) with serial two fold dilutions of the compounds. After 24 h of incubation at 37ºC, virus yields were determined by plaque assay in Vero cells and plotted as the percentage of inhibition with respect to untreated-infected control.

Scheme 1. Synthesis of fluorinated steroids. **a.** $HClO_4$ / Ac_2O / EtOAc / r.t, 1h. **b.** Selectfluor / AcCN / CH_2Cl_2 / -10°C → r.t, 10 h. **c.** K_2OsO_4 / $K_3Fe(CN)_6$ / $(DHQ)_2$-Phal / K_2CO_3 / t-BuOH / H_2O / $CH_3SO_3NH_2$, r.t, 4 days. **d.** DDQ / dioxane / reflux.

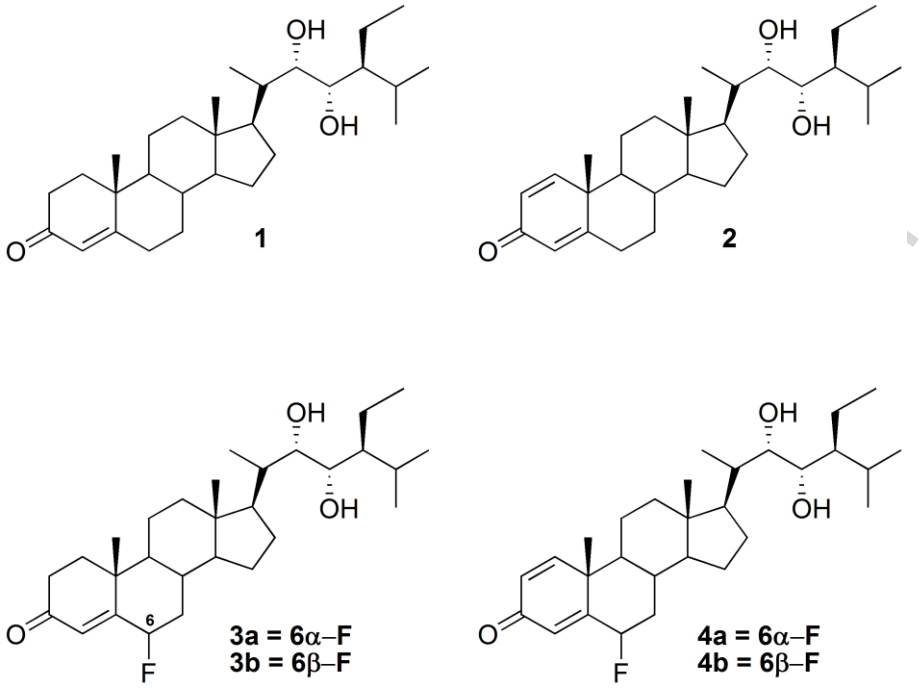


Figure 1

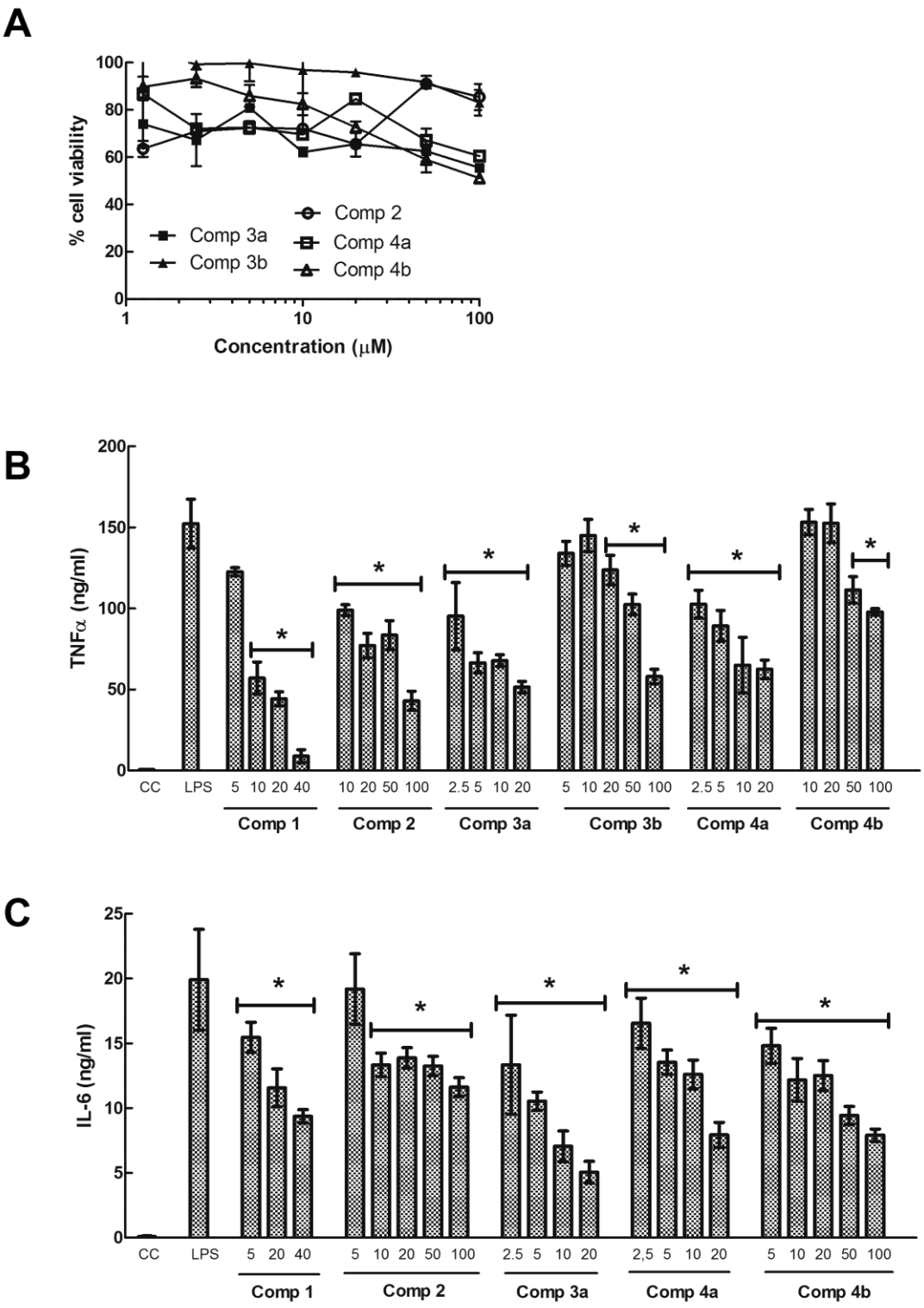


Figure 2

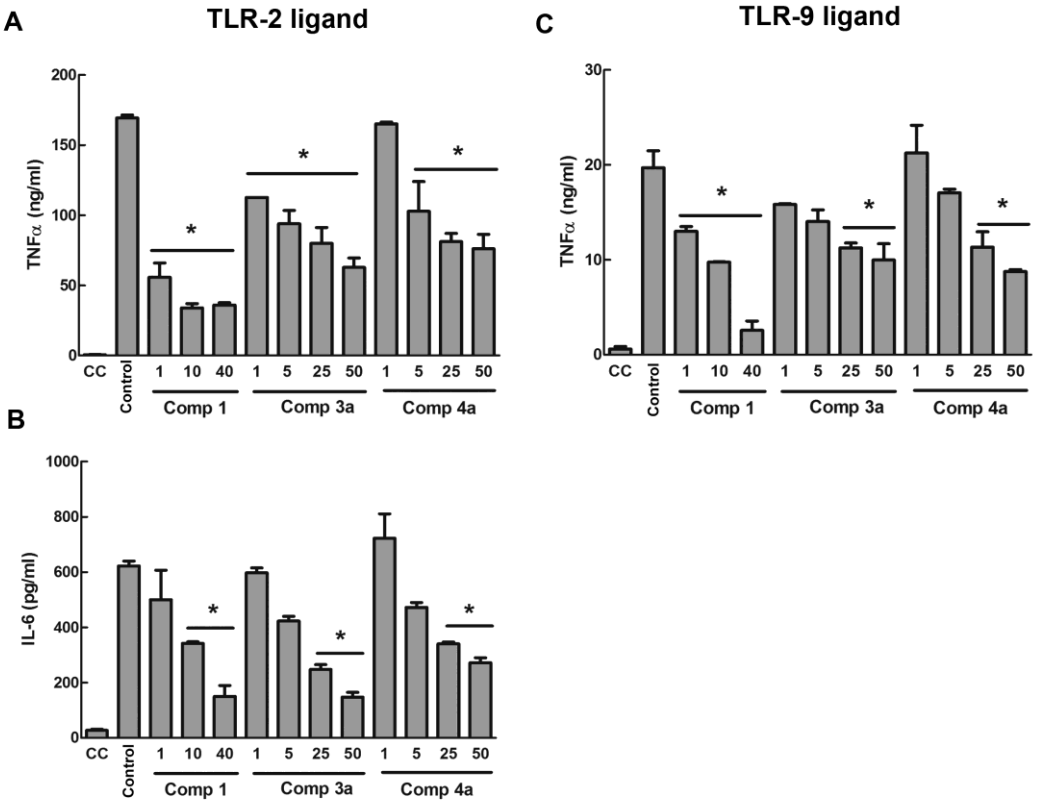


Figure 3

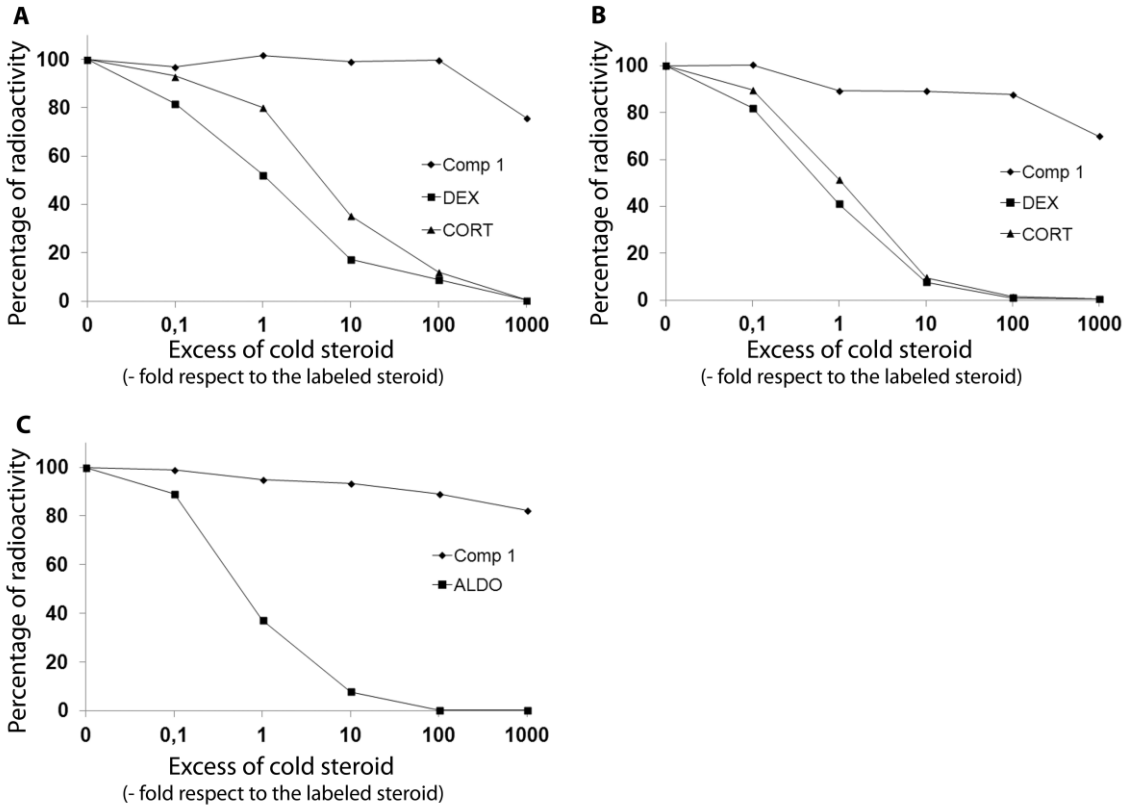


Figure 4

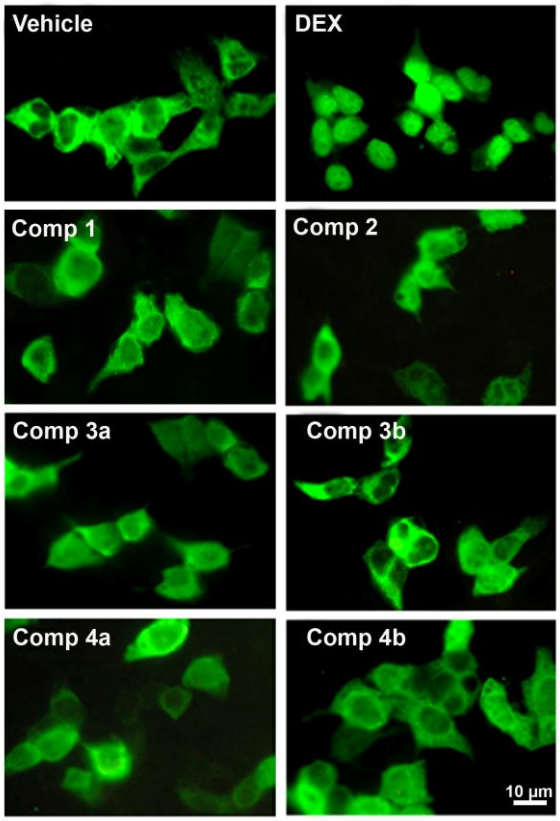


Figure 5

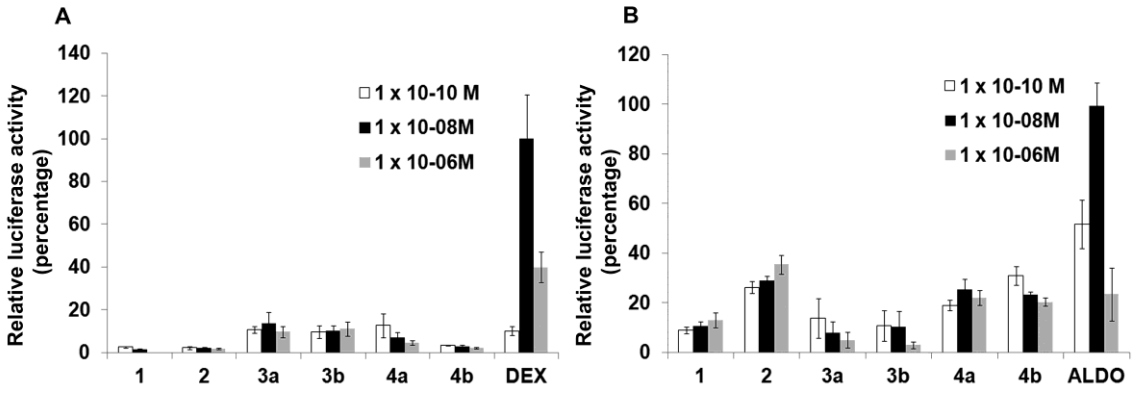


Figure 6

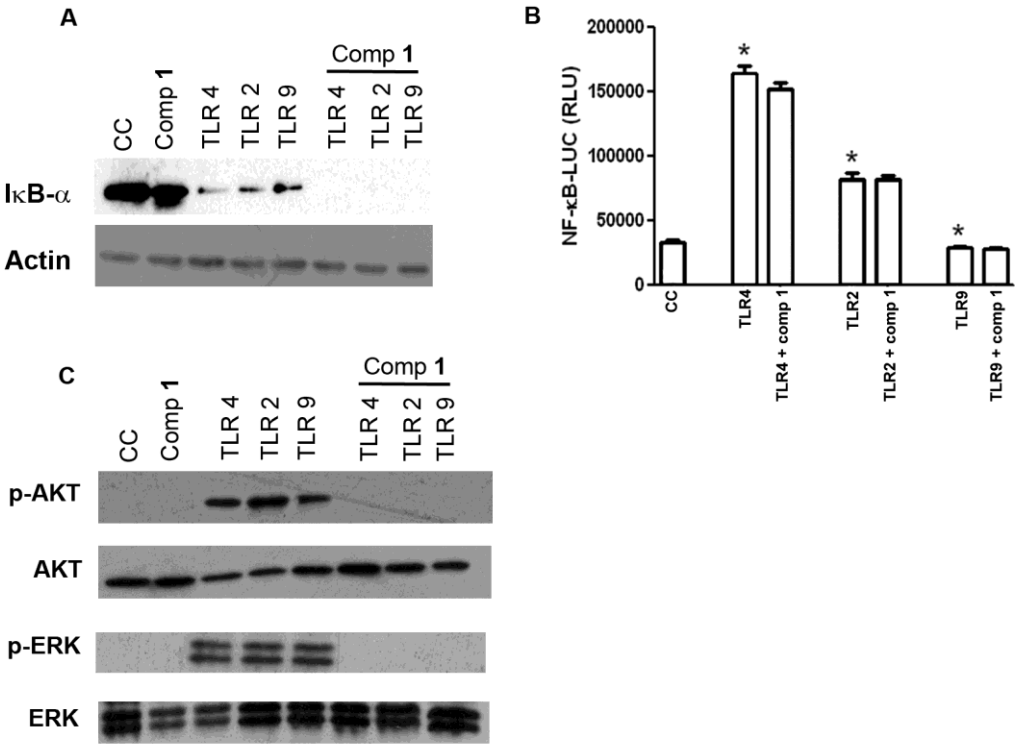


Figure 7

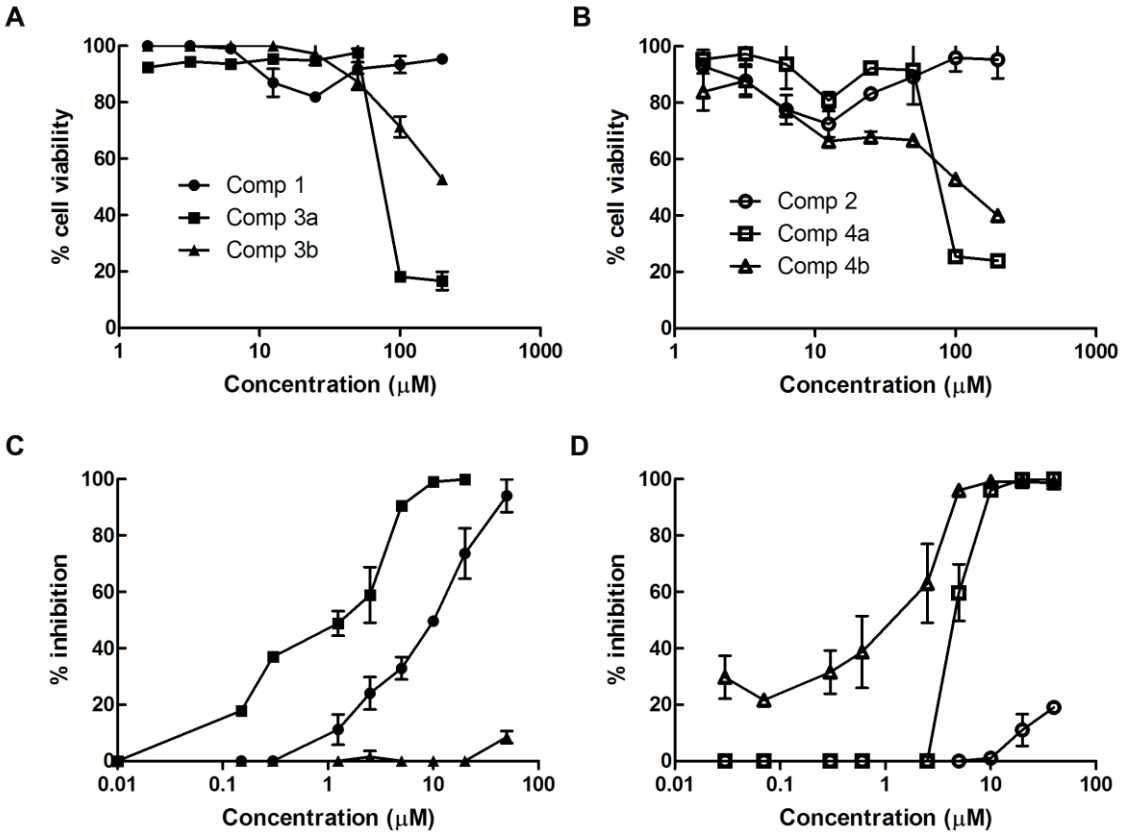
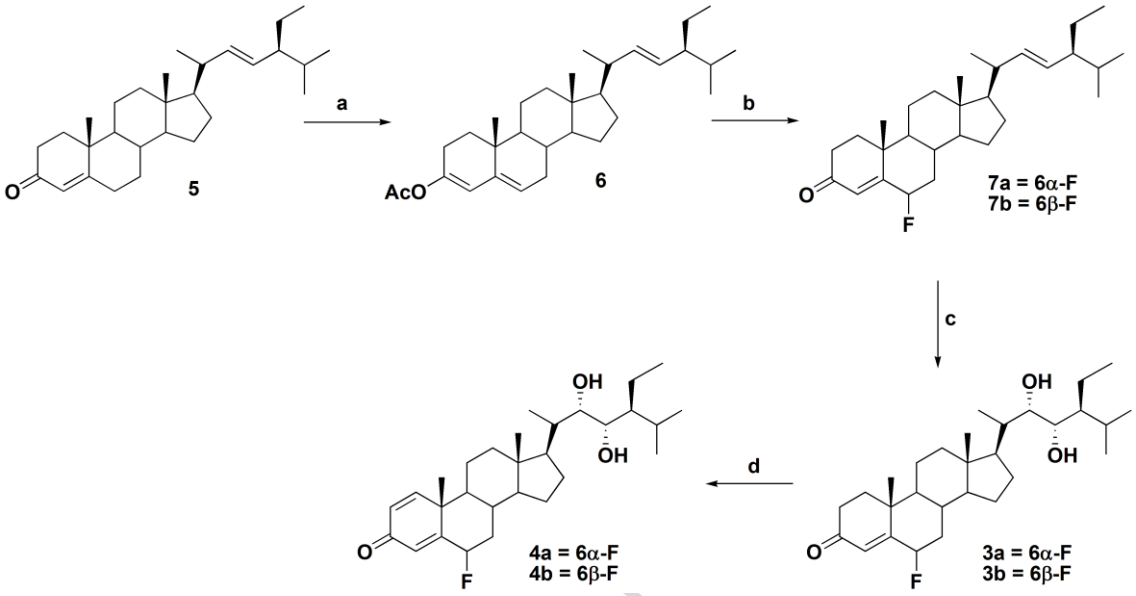


Figure 8



Scheme 1

HIGHLIGHTS

- The chemical synthesis of new stigmastanes fluorinated at C-6 is reported.
- These steroidal compounds exert both immunomodulating and antiviral activities.
- They inhibit ERK and Akt signaling pathways, but do not act as glucocorticoids.
- Fluorination enhances the antiviral activity.