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# Immunomodulatory activity of an anti-HSV-1 synthetic stigmastane analog

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A B S T R A C T

Many viral infections are associated with the development of immunopathologies and autoimmune diseases, which are of difficult treatment and for which no vaccines are yet available. Obtaining compounds that conjugate both antiviral and immunomodulatory activities in the same molecule would be very useful for the prevention and/or treatment of these immunopathologies.

The compound (22*S*,23*S*)-22,23-dihydroxystigmast-4-en-3-one (compound **1**) displays anti-Herpes simplex virus type 1 activity in vitro and reduces the incidence of herpetic stromal keratitis (HSK) in mice, a chronic inflammatory syndrome induced by ocular HSV-1 infection.

In the present study, compound **1** showed opposite immunomodulatory properties in vitro. It induced the release of pro-inflammatory cytokines in HSV-1-infected epithelial cells of ocular origin, and significantly reduced the production of these cytokines in LPS-activated macrophages. RNA microarrays revealed various overexpressed and repressed genes in compound **1** treated infected epithelial cells and activated macrophages, many of which are associated with innate immune responses and inflammatory processes. These immunomodulatory properties of compound **1**, together with its previously reported antiviral activity, make it a potential drug for the treatment of HSK and many other immunopathologies of viral and non-viral origin.

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## 1. Introduction

Many viruses of public health significance may cause disease by triggering an immunopathology, in which the damage is produced by the host inflammatory response elicited by the virus, for example, the serious respiratory affections induced by human respiratory syncytial virus (HRSV) and influenza virus infections. In the case of Herpes simplex virus type 1 (HSV-1) corneal infection, a chronic inflammatory reaction occurs in the eye in response to viral reactivation, causing herpetic stromal keratitis (HSK), which may lead to vision impairment and even blindness.[1–3]

Previously, we have reported that polyfunctionalized stigmastane derivatives have in vitro antiviral activity and no cytotoxicity, preventing HSV-1 multiplication and spreading in human epithelial corneal and conjunctival cell lines.[4–7] Furthermore, preliminary results have shown that (22*S*,23*S*)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one (**6b**) and (22*S*,23*S*)-22,23-dihydroxystigmast-4-en-3-one (compound **1**) (Fig. 1) efficiently inhibit the multiplication of acyclovir-resistant strains in human epithelial corneal cells, a remarkable feature considering that the emergence of HSV-1 mutants resistant to antivirals commercially available is a frequent event.[6,7]

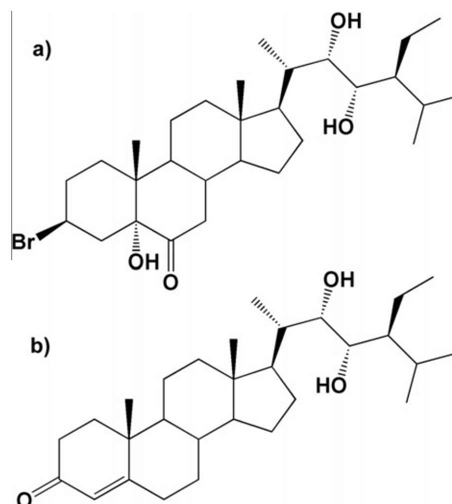


**Figure 1.** Structure of **6b** (a) and compound **1** (b).

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In addition, both compounds significantly decrease the incidence and severity of disease in the murine model of HSK. This effect may be attributed to their immunomodulatory activity rather than to an in vivo anti-HSV-1 action.[6] Alike **6b**, compound **1** decreases HSK incidence and severity when supplied both during virus replication and once the immune response has already been elicited.[8] Although compound **1** exhibits structural features similar to those of the commercial anti-inflammatory drug Dexamethasone (DEX), it seems not to act as a conventional steroidal drug because it significantly restrains HSK when administered during the first 3 days after HSV-1 infection, whereas DEX exacerbates ocular damage.[8,9]

When investigating an eventual immunomodulatory activity, we found that **6b** hinders TNF-α production in LPS-stimulated macrophages and modulates IL-6 and TNF-α secretion in human epithelial corneal and conjunctival cells.[7,10]

In the present work, we explored the immunomodulatory properties of compound **1** in order to evaluate its potential as a future drug for the treatment of HSK and other immunopathologies. For this purpose, we assessed the effect of compound **1** against inflammatory factors and mediators in cells subjected to viral and non-viral stimuli.

## 2. Materials and methods

### 2.1. Cell culture and viruses

The human IOBA-NHC cell line was grown in Dulbecco's modified medium and nutrient mixture F-12 (DMEM/F12, 1:1), supplemented with 10% inactivated fetal bovine serum (FBS) (DMEM/F12, 10%), and maintained in DMEM/F12 supplemented with 2% inactivated FBS (DMEM/F12, 2%).[11] Human corneal–limbal epithelial cells (HCLE) were kindly provided by Dr. Ilene K. Gipson and Dr. Pablo Argüeso (The Schepens Eye Research Institute, Harvard Medical School, Boston, USA) and grown in GIBCO Keratinocyte Serum Free Medium, supplemented with 25 μg/ml bovine pituitary extract (BPE), 0.2 ng/ml epidermal growth factor (EGF) and 0.4 mM $CaCl_2$, and maintained in low calcium DMEM/F12.[12] The murine macrophage cell line J774A.1 (ATCC® Number TIB-67™) was kindly provided by Dr. Osvaldo Zabal (INTA, Castelar, Buenos Aires, Argentina) and grown in RPMI 1640 medium supplemented with 10% inactivated FBS (RPMI 10%) and maintained in RPMI supplemented with 2% inactivated FBS (RPMI 2%). The HSV-1 KOS strain was propagated at low multiplicity of infection (m.o.i.) and used for in vitro experiments.

### 2.2. Cytotoxicity assay

To evaluate compound **1** cytotoxicity, cell viability was determined as previously reported.[13] For that purpose, compound **1** was dissolved in dimethylsulfoxide (DMSO) and diluted with culture medium. The maximum concentration of DMSO tested (1%) exhibited no cytotoxicity under experimental conditions. Dexamethasone 0.4% (DEX) was purchased from Sidus, Argentina.

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 a percentage of absorbance of treated cells with respect to untreated ones. The $CC_{50}$ was defined as the concentration of compound that caused 50% reduction in cell viability.

### 2.3. Indirect immunofluorescence

To analyze the immunomodulatory activity of compound **1**, we first assessed its effect on NF-κB translocation during HSV-1 infection and LPS stimulation. IOBA-NHC and HCLE monolayers grown on coverslips in 24-well plates were infected with 0.2 ml of HSV-1 KOS (m.o.i. = 1) and incubated for 1 h at 37 °C. Then, the inoculum was removed and cells were coated with DMEM/F12 without FBS in the presence or absence of compound **1** 40 μM or DEX 40 μM, for 24 h at 37 °C. J774A.1 cells were grown on coverslips in 24-well plates and stimulated with 100 ng/ml LPS (Sigma–Aldrich, USA) in RPMI without FBS in the presence or absence of compound **1** 40 μM or DEX 40 μM, for 8 h at 37 °C. After incubation, cells were fixed in methanol at −20 °C for 10 min, and processed for indirect immunofluorescence (IFI) with antibodies directed against the glycoprotein D (gD) of HSV-1 and/or the p65 subunit of NF-κB. After three washes with PBS, the coverslips were inverted on a drop of a 1/100 dilution of the primary antibody, mouse monoclonal anti-gD and/or rabbit polyclonal anti-p65 (Santa Cruz Biotechnology, USA) and incubated for 30 min at 37 °C, and then subjected to three additional washes with PBS. Then, the coverslips were incubated with a dilution 1/50 of the secondary antibodies, polyclonal goat anti-mouse IgG FluoroLinkTM CyTM3 and/or anti-goat polyclonal IgG rabbit conjugated to FITC (Sigma–Aldrich) for 30 min at 37 °C.

Finally, the coverslips were rinsed first with PBS and then with distilled water, mounted with buffered glycerin and observed under a Zeiss microscope with epifluorescence optics or a confocal microscope Olympus FB300.

### 2.4. Quantitative analysis of fluorescence

The images obtained with a 40× magnification were imported into the NIH ImageJ 1.34s program (designed by Wayne Rasband, NIMH, Bethesda). Immunofluorescence images were converted to an 8-bit grayscale from 0 (black) to 255 (white). We analyzed the total and nuclear fluorescence of the individual cells and obtained a mean fluorescence density for each. To compare the distribution of fluorescence within the cell, the results were analyzed in a spreadsheet (Excel ®). The total fluorescence intensity or nuclear density was calculated as the total or nuclear average per total or nuclear area, respectively. Then, we calculated the percentage of nuclear intensity with respect to the total intensity for each cell. Unstimulated cells were processed to establish a baseline of nuclear fluorescence (cut-off value).

### 2.5. Cytokine determination

Next, we analyzed the effect of compound **1** on pro-inflammatory cytokine production after infection with HSV-1 or stimulation with LPS. IOBA-NHC and HCLE monolayers grown in 24-well plates were infected with HSV-1 (m.o.i. = 1) in the presence or absence of compound **1** (40 μM) or DEX (40 μM), in duplicate. J774A.1 cells grown in 24-well plates were incubated at 37 °C with 100 ng/ml of LPS in the presence or absence of compound **1** (40 μM) or DEX (40 μM), in duplicate. After 24 h of incubation for the infected cells and 8 h for the LPS-stimulated cells, supernatants were harvested, centrifuged at 1000 rpm for 10 min, and cytokines were quantified by ELISA. Human or murine TNF-α, IL-6 and IL-8 were quantified by commercial ELISA sets (BD OptEIATM, Becton Dickinson, USA), according to the manufacturer's instructions.

### 2.6. Microarray procedures

HSV-1-infected cells and LPS-stimulated cells were transcriptionally analyzed by using the whole human genome microarray and whole mouse genome microarray (Agilent Technologies, Santa Clara, CA, USA), corresponding to 41,000 human or mice genes and transcripts.

Four biological replicates of HCLE cells infected with HSV-1 (m.o.i. = 1) and J774A.1 cells stimulated with LPS (100 ng/ml) were treated or not with 40 μM of compound **1**. Total RNA was extracted at 6 h p.i. and at 6 h post-LPS induction. In each case, two of the four biological replicates were dye swap to eliminate dye effects.

#### 2.6.1. Determination of gene expression profiles

Total RNA was isolated with phenol/chloroform as described by the manufacturer (Tri Reagent, Sigma-Aldrich, USA). Processed samples were quantified in a spectrophotometer (NanoDrop 1000 Thermo Scientific), exhibiting a high content of total RNA (1800 and 3100 ng/ml), and a good quality without degradation, according to the RIN (RNA Integrity Number) values obtained from the Bioanalyzer 2100 (Agilent Technologies), which were all above 8.

A 1-μg aliquot of total RNA was reverse-transcribed into cDNA, and this was transcribed into cRNA and labeled using the Quick Amp Labeling Kit, 2 Color (Agilent Technologies). The labeled cRNA was purified with Illustra RNAspin mini Isolation kit (GE Healthcare, USA). The quality of each cRNA sample was verified by total yield and specificity calculated based on NanoDrop ND-1000 spectrophotometer measurement (NanoDrop Technologies, USA).

After that, we proceeded with the hybridization, washing, assembling of the chips and scanning, according to the Agilent protocol. The glass slides were scanned using the Agilent microarray scanner G2565BA, using the default settings for all parameters.

The scanning software (Feature extraction) performed the scan quality control. The fluorescence intensity data obtained for each chip were analyzed using GeneSpring GX11 and Multi Experiment Viewer (MeV) programs.

The labeled samples were placed in human and mouse hybridizing chips for HCLE and J774A.1 cells, respectively, for 17 h at 60 °C and with a 10 rpm rotation. Successive washings were done with different washing, stabilization and drying solutions according to the Agilent protocol, and samples were scanned in the Agilent scanner G2565BA.

#### 2.6.2. Image analysis

We used Agilent Feature Extraction (version 9.5.1) for quality control, data filtering and data normalization. The software also converts the scanned images in quantitative data for further analysis.

The software automatically finds and places microarray grids, rejects outlier pixels, accurately determines feature intensities and calculates log ratios (Agilent's processed signal value), flags outlier pixels, and calculates statistical confidences. It also performs a dye normalization within arrays using lowess normalization.

#### 2.6.3. Statistical analysis

Microarray experiments were statistically compared using the Genespring software GX9 and the Multi Experiment Viewer (MeV) program (http://www.tm4.org/mev/). Genes significantly up- and down-regulated were identified by Student's *t*-test between subjects based on *t* distribution with a *p*-value of 0.01 and a Benjamini–Hochberg false discovery rate correction for multiple testing. The *t*-test consisted of a comparison between the infected cells against the infected treated ones.

#### 2.6.4. Real-time RT-PCR data validation

The RNA samples used in the microarray experiment were used to validate, by real-time PCR, some of the genes that were overexpressed expressed and some of those that were repressed in HSV-1-infected cells and LPS-stimulated cells. For each sample, cDNA was synthesized by reverse transcription using the SuperScript II Reverse Transcriptase (Invitrogen) with Random Primers and 2 μg of total RNA added as a template. Primers for the specific amplification of human glyceraldehyde 3-phosphate dehydrogenase (GADPH), interleukin 8 (IL-8), interleukin 1α (IL-1α), Chemokine (C-X-C motif) ligand 2 (CXCL2), interleukin 1 receptor-like 1 (IL1RL1), murine β-actin, activating transcription factor 3 (ATF3), dual specificity phosphatase 2 (DUSP2), suppressor of cytokine signaling 2 (SOCS2), interleukin 12-b (IL-12b), interleukin 1β (IL-1β), chemokine (C-X-C motif) ligand 10 (CXCL-10) and colony

**Table 1**
Primer sequences for real time RT-PCR

| Accession number[a] | Name of the gene | Primer sequence | Product size (bp) |
|---|---|---|---|
| NM_000584 | *Interleukin 8 (IL-8)* | FW: GAATGGGTTTGCTAGAATGTGATA<br>RV: CAGACTAGGGTTGCCAGATTTAAC | 128 |
| NM_000575 | *Interleukin 1, alpha (IL-1α)* | FW: TGACTGCCCAAGATGAAGACC<br>RV: TCCCAGAAGAAGAGGAGGTTG | 99 |
| NM_002089 | *Chemokine (C-X-C motif)ligand 2 (CXCL2)* | FW: TGCCAGTGCTTGCAGAC<br>RV: TCTTAACCATGGGCGATGC | 156 |
| NM_016232 | *Interleukin 1receptor like 1 (IL-1RL1)* | FW: TGATTTTGCCCTTCCTCTTG<br>RV: TGTTTCCAGTAATCGGAGCC | 192 |
| NM_008361 | *Interleukin 1 beta (IL-1b)* | FW: CAACCAACAAGTGATATTCTCCATG<br>RV: GATCCACACTCTCCAGCTGCA | 151 |
| NM_010090 | *Dual specificity phosphatase 2 (Dusp2)* | FW: GCAGTTCTCAATGTCTCT<br>RV: CTTCTACTGGAATGCTCTT | 74 |
| NM_007778 | *Colony stimulating factor 1 (macrophage) (Csf1)* | FW: ACTATGAGGAGCAGAACAAG<br>RV: ACCACATCTCGGCTAGAG | 88 |
| NM_007498 | *Activating transcription factor 3 (Atf3)* | FW:CAGCATTTGATATACATGCTCAACCT<br>RV: TCCGGTGTCCGTCCATTCT | 73 |
| NM_021274 | *Chemokine (C-X-C motif) ligand 10 (CXCL10)* | FW: TGAGATCATTGCCACGAT<br>RV: GCTTCACTCCAGTTAAGG | 129 |
| NM_008352 | *Interleukin 12b (IL-12b)* | FW: ACATCTGCTGCTCCACAAG<br>RV: GGTGCTTCACACTTCAGGAA | 92 |
| NM_007706 | *Suppressor of cytokine signaling 2 (Socs2)* | FW: GTTGCCGGAGGAACAGTCCC<br>RV: TCGGTCCAGCTGACGTCTTAAC | 205 |
| NM_002046 | *Glyceraldehyde 3-phosphate dehydrogenase (GADPH)* | FW: CACTCCTCCACCTTTGACG<br>RV: ACCACCCTGTTGCTGTAGC | 103 |
| NM_007393 | *β-Actin* | FW: TGCGTGACATCAAAGAGAAG<br>RV: GATGCCACAGGATTCCATA | 140 |

[a] Accession number in GenBank (NCBI).

stimulating factor 1 (Csf1) were designed on the basis of human and murine nucleotide sequences available from NCBI (http://www.ncbi.nlm.nih.gov/). The primer sequences and expected product lengths are listed in Table 1. Real-time reactions were performed using 5 μl Sybr Green (Brilliant II SybrGreen qPCR Master Mix, Agilent Technologies), 100 nM of forward and reverse primers and 1 μl of cDNA, in a final volume of 10 μl. Samples were analyzed in triplicate in a 72-disk Rotor-GeneTM 6000 (Corbett Life Sciences). Standard amplification conditions were 10 min at 95 °C and 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. After each PCR reaction, the corresponding dissociation curves were analyzed to ensure that the desired amplicon was being detected and to discard contaminating DNA or primer dimers.

The levels of expression of GADPH and β-actin were used as endogenous controls, following Pfaffl (2001).[14] These genes exhibited similar expression in all the samples analyzed. Each run of real-time PCR included gene expression measurement of GADPH or β-actin and the target gene in the corresponding samples.

Pfaffl relative quantification method was used to calculate the expression levels. A calibration curve was constructed with a pool of all the cDNA samples to determine the PCR efficiency. The efficiency was calculated from the slopes of the calibration curves, according to the equation ($E = 10\,[-1/\text{slope}]$), as described by Pfaffl (2001).[14]

We used the REST 2009 software (Pfaffl et al., 2002) to calculate the results.[15]

## 3. Results

### 3.1. Immunomodulatory activity of compound 1 in HSV-1-infected epithelial cells and activated macrophages

#### 3.1.1. NF-κB activation is inhibited by compound 1 after HSV-1 infection

Since HSV-1 is able to activate NF-κB, we investigated the effect of compound **1** on NF-κB p65 subunit intracellular localization.[16,17] For that purpose, IOBA-NHC and HCLE cells were infected with HSV-1 (m.o.i. = 1) and treated or not with compound **1** (40 μM). Double IFI staining was performed by adding anti-p65 and anti-HSV-1 gD antibodies to visualize gD as a marker of viral infection.

We found that the majority of infected IOBA-NHC and HCLE cells exhibited p65 fluorescence in the nuclei (85.9% and 72.5%, respectively) (Fig. 2A and D), most of them clustered in characteristic HSV-1 foci showing nuclear p65 fluorescence associated with gD staining. When compound **1** was added, the percentage of cells exhibiting NF-κB translocation to the nucleus dropped to 22.2 ± 1.2% in IOBA-NHC (Fig. 2B) and to 5.7% in HCLE cells (Fig. 2E), coincidentally with the limited appearance of fewer and scattered foci expressing gD. On the other hand, treatment with DEX did not prevent NF-κB nuclear translocation in any of HSV-1 infected cells (Fig. 2C and F).

Thus, compound **1** restrained NF-κB translocation to the nucleus of HSV-1-infected epithelial cells.

#### 3.1.2. NF-κB activation is not affected by compound 1 in stimulated J774A.1 cells

Since macrophages are essential for the onset of a pro-inflammatory response and are also involved in the development of several immunopathologies, we evaluated the effect of compound **1** on p65 subunit intracellular localization in macrophages. After 8 h of stimulation with LPS alone, p65 was detected in the nucleus of most of the cells, while the simultaneous addition of compound **1** did not prevent NF-κB translocation (Fig. 3). This observation was confirmed by means of a semiquantitative analysis of the images, which revealed that nuclear fluorescence was visualized in 79.4 ± 1.2% of LPS-stimulated non-treated cells and NF-κB activation was evidenced in 87.9 ± 0.2% of macrophages induced with LPS and treated with compound **1**. Treatment with DEX also failed to prevent NF-κB translocation, since 100% of the cells exhibited p65 fluorescence in the nucleus (Fig. 3F). Neither compound **1** nor DEX was able to block NF-κB activation.

#### 3.1.3. Modulation of cytokine production by compound 1 in infected epithelial cells

TNF-α, IL-8 and IL-6 are often released by corneal and conjunctival cells due to the presence of HSV-1 via NF-κB mechanisms.[18–20] To analyze the immunomodulatory activity of compound **1**, we investigated its effect on the secretion of these cytokines.

IOBA-NHC and HCLE cells infected with HSV-1 (m.o.i. = 1) were treated or not with 40 μM of compound **1** and DEX. After 24 h, cell supernatants were harvested and TNF-α, IL-6 and IL-8 were quantified by ELISA.

Compound **1** alone did not induce TNF-α and IL-6 release in uninfected cells, whereas it considerably increased IL-8 levels in both cellular types (data not shown). Besides, HCLE cells failed to produce TNF-α regardless of whether the supernatants were from HSV-1-infected or HSV-1-infected and treated cells, whereas IOBA-NHC cells were weak TNF-α producers. Uninfected cells yielded 40.9 pg/ml of the cytokine, whereas HSV-1 infection increased TNF-α yield to 77.1 pg/ml ($p$ <0.01) (Fig. 4A). DEX abrogated TNF-α production in HSV-1-infected IOBA-NHC cells completely, whereas compound **1** did not affect TNF-α production (Fig. 4A). The pattern of HSV-1-induced IL-6 expression varied depending on the cellular type. IL-6 production was 10-fold higher in IOBA-NHC infected cells than in HCLE cells (Fig. 4B and C). Noteworthy, compound **1** elicited no changes in IL-6 release from either infected cell type with respect to infected control cells, whereas DEX considerably reduced IL-6 levels.

IOBA-NHC and HCLE cells displayed high basal levels of IL-8 secretion (522.5 ± 23 pg/ml and 133.8 ± 0.6 pg/ml, respectively) ($p$ <0.01) (Fig. 4D and E). Whereas HSV-1 infection induced a reduction in IL-8 secretion to 252 ± 7.8 pg/ml ($p$ <0.001) in IOBA-NHC cells, infected HCLE cells showed a low but significant increase in IL-8 secretion, from 133.8 ± 0.6 pg/ml to 142.9 ± 0.5 pg/ml ($p$ <0.01). Treatment of infected cells with compound **1** induced a significant augment of IL-8 with respect to untreated infected IOBA-NHC and HCLE cells (471.8 ± 30 pg/ml, $p$ <0.001 and 163.6 ± 0.4 pg/ml, $p$ <0.00001, respectively). Unlike compound **1**, DEX slightly, but significantly, reduced IL-8 secretion both in uninfected and infected conjunctival and corneal cells ($p$ <0.001) (Fig. 4D and E).

In summary, compound **1** proved to elicit the release of pro-inflammatory cytokines in HSV-1-infected epithelial cells of ocular origin, while DEX produced an effect as an anti-inflammatory drug, revealing a different behavior of both structurally related compounds.

#### 3.1.4. Compound 1 abrogates cytokine secretion from activated J774A.1 cells

It is well known that macrophages act as a source of inflammatory mediators, such as TNF-α, IL-8 and IL-6. Thus, we evaluated the effect of compound **1** on the secretion of these cytokines. We found that compound **1** diminished TNF-α and IL-6 secretion in activated macrophages, in a dose-dependent manner (Fig. 5). The inhibitory concentration 50% ($IC_{50}$) values obtained were 7 μM for TNF-α and >40 μM for IL-6. Unlike IL-8 production in corneal and conjunctival cells, compound **1** (40 μM) reduced the secretion of IL-8 by 77.5% in activated macrophages.

LPS-induced cytokine secretion in J774A.1 cells was considerably decreased after treatment with compound **1**, reaching levels

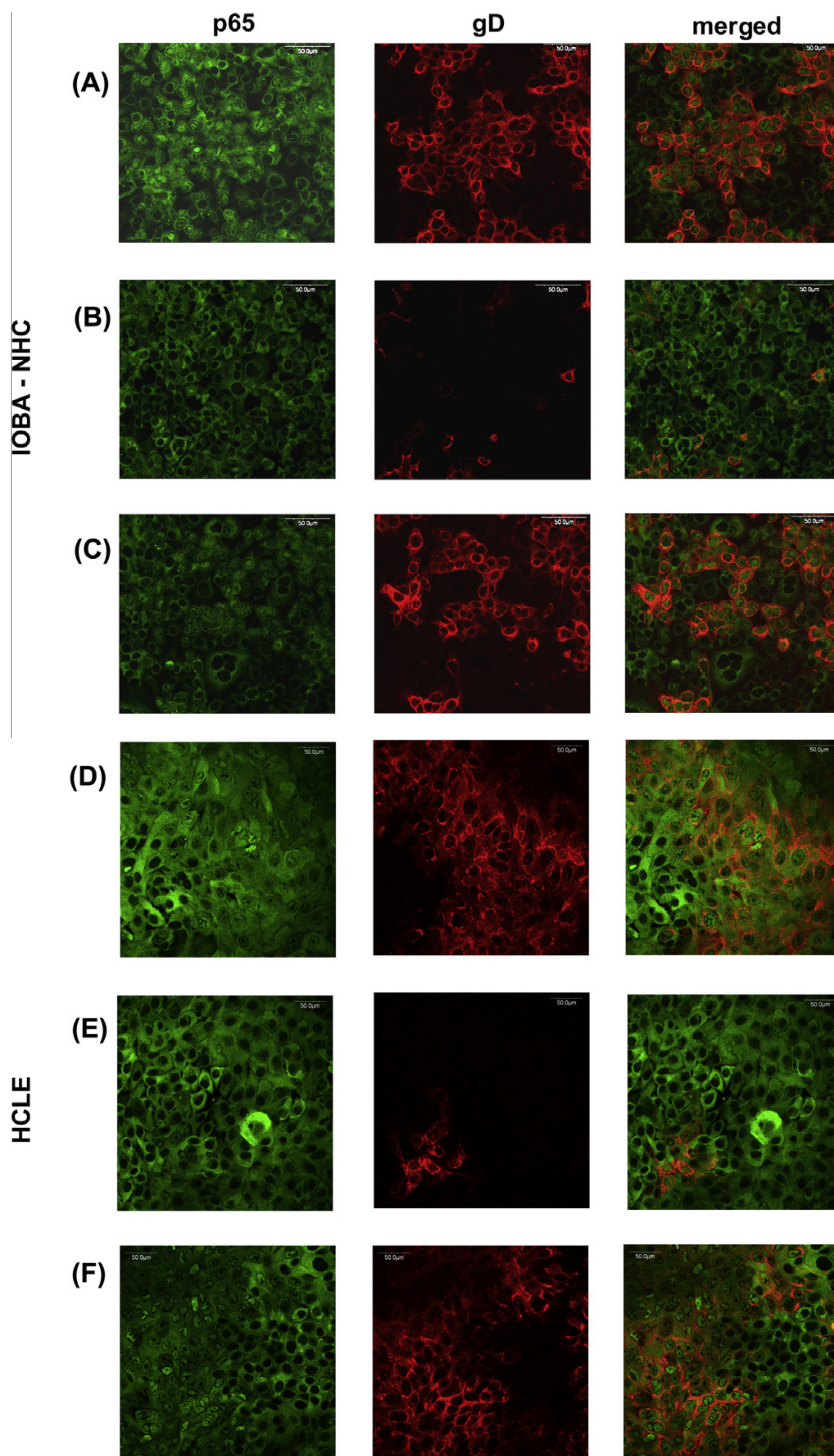


**Figure 2.** Effect of compound **1** and Dexamethasone (DEX) (40 μM) on NF-κB nuclear translocation in IOBA-NHC and HCLE cells infected by HSV-1. Double immunofluorescence staining was performed by adding anti-p65 and anti-HSV-1 gD antibodies. Untreated infected cells (negative controls) (A and D); infected cells treated with compound **1** (B and E) and DEX (C and F). Magnification: IOBA-NHC, 400× and HCLE, 600×.

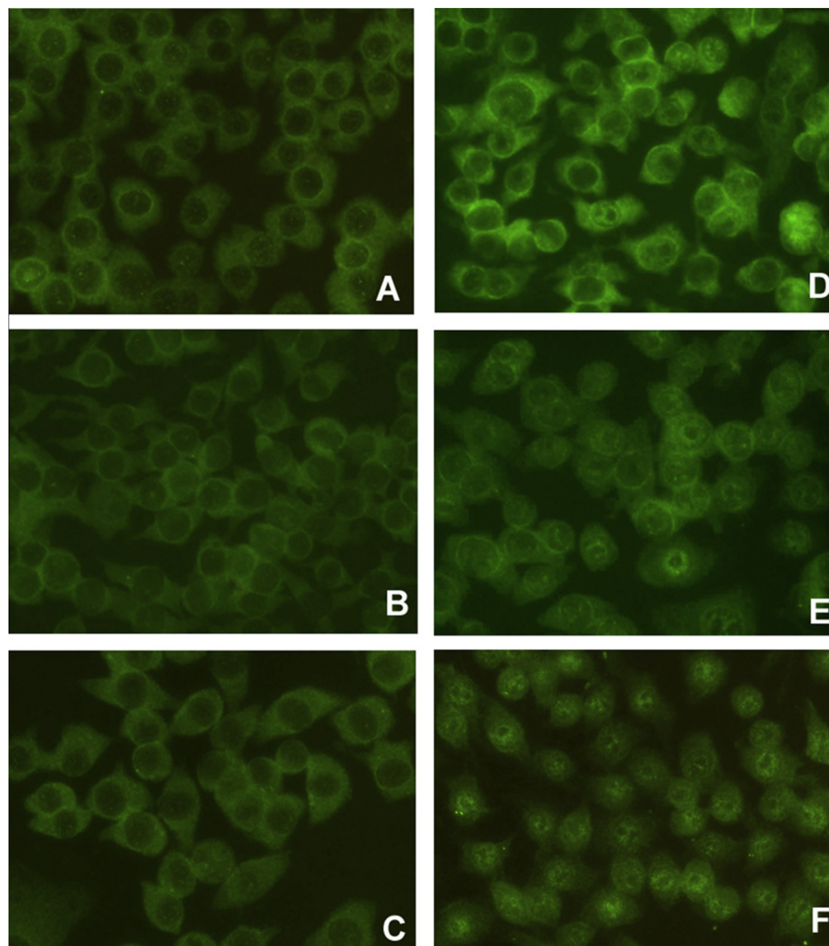


**Figure 3.** Effect of compound **1** on NF-κB nuclear translocation in macrophages. J774A.1 cells were stimulated with LPS for 8 h and treated or not with 40 μM of compound **1** or DEX. The localization of p65 was detected by IFI staining in methanol fixed cells. Magnification: 400×. (A) Control cells; (B) non-stimulated cells treated with compound **1**; (C) non-stimulated cells treated with DEX; (D) LPS-stimulated cells; (E) LPS-stimulated cells treated with compound **1**; (F) LPS-stimulated cells treated with DEX.

comparable to those obtained after treatment with DEX (data not shown).

## 3.2. Gene expression in epithelial and inflammatory cells is affected by compound 1

### 3.2.1. Microarrays

Since compound **1** proved to behave as an immunomodulator in vitro, we investigated if these findings correlated to the cell gene expression using viral and non-viral stimuli using microarray technology.

We found some overexpressed and some repressed genes in infected or stimulated and treated samples compared to infected or stimulated untreated ones. Genes differentially expressed in HCLE cells are shown in Table 2a, and genes differentially expressed in J774A.1 cells are shown in Table 2b.

IL-1α, IL-8, CXCL-2 and CSF-2 were overexpressed in HCLE cells infected with HSV-1 and treated with compound **1**, compared to infected and untreated controls. In LPS-stimulated macrophages treated with compound **1**, IL-10, IL-12b, IL-23α, CXCL10, Socs2, ATF3 and TLR4 genes were overexpressed. On the other hand, DUSP gene expression was repressed after treatment with compound **1**.

Therefore, we confirmed that compound **1** exerted an immunomodulatory effect in epithelial and inflammatory cells induced by a viral and non-viral stimuli, respectively.

### 3.2.2. Validation of microarray results by real-time PCR

Figures 6 and 7 show the results of gene expression calculated as relative expression ratio (R) of a target gene for HCLE and J774A.1 cells respectively:

$$\text{Ratio} = (\text{Etarget})\Delta\text{CPtarget}(\text{control} - \text{sample}) / (\text{Eref})\Delta\text{CPref}(\text{control} - \text{sample})$$

where Etarget is the real-time PCR efficiency of target gene transcript, Eref is the real-time efficiency of a reference gene transcript, ΔCPtarget is the CP deviation of control—sample of the target gene transcript and ΔCPref = CP deviation of control—sample of reference gene transcript. The data are shown as mean fold changes. Gene expression was estimated by the Pfaffl method using the GADPH and β-actin genes as the endogenous control for human and murine samples, respectively. Results were calculated with the REST 2009 (v2 0.13) software.

## 4. Discussion

In a previous work, we have shown that compound **1** significantly reduces the signs of corneal disease in the HSK murine model, probably due to an immunomodulatory activity. Given that the compound does not act as a conventional steroidal anti-inflammatory drug, we decided to further study its modulatory effects in epithelial and inflammatory cells in the presence of viral and non-viral stimuli.[9]

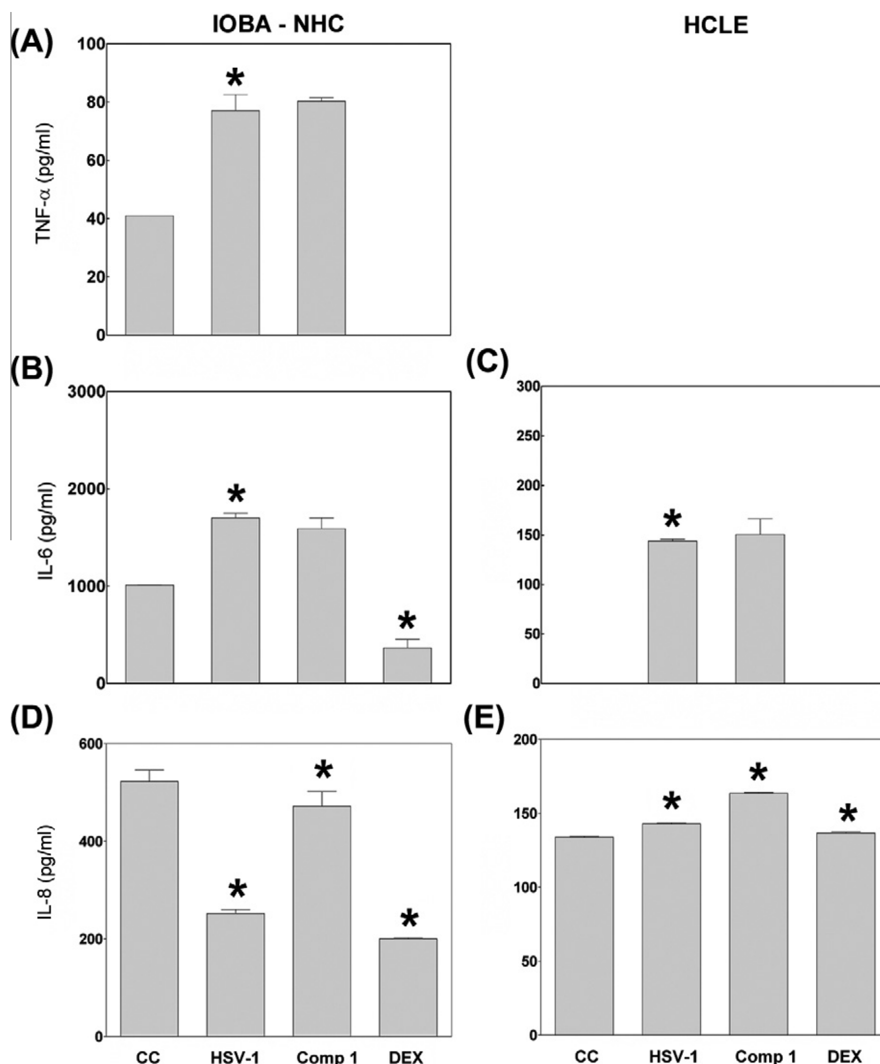


**Figure 4.** Effect of compound **1** and DEX on cytokine secretion by HSV-1 infected epithelial cells. (A, B and D) IOBA-NHC and (C and E) HCLE cells were treated or not with 40 μM of compound **1** or DEX. CC, untreated uninfected cells; HSV-1, untreated cells infected with HSV-1; Comp **1**, infected cells treated with compound **1** and DEX, infected cells treated with DEX. Data are representative of two independent experiments. * indicates statistical differences ($p < 0.01$).

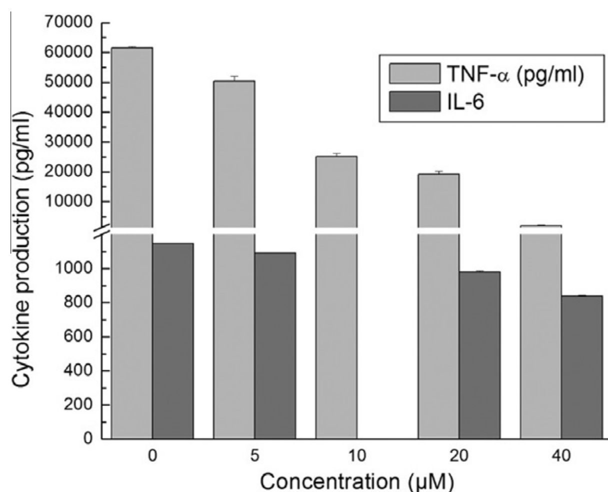


**Figure 5.** Concentration-dependence of TNF-α and IL-6 secretion in macrophages treated with compound **1**. J774A.1 cells were stimulated with LPS for 8 h, and then treated or not with different concentrations of compound **1**. TNF-α and IL-6 were determined by ELISA in the supernatants. Data shown are representative of two independent experiments and expressed as mean ± SEM.

HSV-1 infection induces NF-κB activation and the secretion of pro-inflammatory cytokines such as TNF-α and IL-6.[21,22] Compound **1** inhibited NF-κB translocation in epithelial infected cells, although this effect could be ascribed to its anti-HSV-1 activity. NF-κB activation occurs in two phases. The first one, which is rapid and transient, could be induced by the interaction of viral gD with receptors HVEA in the host cell and, thus, be independent of viral replication. A second phase of activation, which is sustained over time, requires active viral replication and viral protein synthesis.[23–26] It is important to mention that compound **1** was not present during the outbreak of the first wave of activation of NF-κB because it was added to the cells after the infection with HSV-1. Since compound **1** blocked NF-κB activation at 24 h p.i., it proved to inhibit the second phase of NF-κB activation. This would be the consequence of its inhibitory activity on HSV-1 replication in vitro. The compound did not exert a direct inhibition on NF-κB translocation, but reduced viral replication and thus the number of infected cells in which NF-κB nuclear translocation had occurred (Fig. 2).

Unlike DEX, which caused a significant inhibition of cytokine secretion, compound **1** did not induce a reduction in TNF-α, IL-6 and IL-8 levels in infected epithelial cells (Fig. 4B and C). The high

**Table 2**
Microarray results: Genes differentially expressed in HCLE cells infected with HSV-1 and J774A.1 stimulated with LPS and treated with compound **1**

| Accession number[a] | Gene | Name of the gene | *p* value[b] | Fold change[c] |
|---|---|---|---|---|
| (a) Differentially expressed genes in HCLE cells infected with HSV-1 and treated with compound **1** | | | | |
| *Overexpressed* | | | | |
| NM_000584 | IL-8* | *Interleukin 8* | *0.000152* | 4.4 |
| NM_000575 | IL-1α* | *Interleukin 1, alpha* | *0.000157* | 2.9 |
| NM_002089 | CXCL2* | *Chemokine (C-X-C motif) ligand 2* | *0.000647* | 2.9 |
| NM_016232 | IL-1RL1* | *Interleukin 1 receptor-like 1* | *0.001224* | 5.2 |
| NM_000758 | CSF2 (GM-CSF) | *Colony stimulating factor 2 (granulocyte-macrophage)* | *0.001908* | 2.2 |
| (b) Differentially expressed genes in J774A.1 cells activated with LPS and treated with compound **1** | | | | |
| *Repressed* | | | | |
| NM_008361 | Il1b * | *Interleukin 1 beta* | *0.0000379* | 2.58 |
| NM_010090 | Dusp2 * | *Dual specificity phosphatase 2* | *0.00304884* | 3.86 |
| *Overexpressed* | | | | |
| NM_007778 | Csf1 * | *Colony stimulating factor 1 (macrophage)* | *0.0000844* | 4.42 |
| NM_007498 | Atf3 * | *Activating transcription factor 3* | *0.000133* | 4.41 |
| NM_021274 | CXCL10 * | *chemokine (C-X-C motif) ligand 10* | *0.000353* | 11.41 |
| NM_008352 | Il12b * | *Interleukin 12b* | *0.009584472* | 4.19 |
| NM_007706 | Socs2* | *suppressor of cytokine signaling 2* | *0.042965613* | 2.29 |
| NM_011414 | Slpi | *Secretory leukocyte peptidase inhibitor* | *0.000149* | 1.53 |
| NM_021297 | TLR4 | *Toll-like receptor 4* | *0.002540414* | 1.95 |
| NM_010548 | IL-10 | *Interleukin 10* | *0.003137306* | 1.27 |
| NM_031168 | IL-6 | *Interleukin 6* | *0.004674967* | 1.97 |
| NM_008330 | Ifi47 | *Interferon gamma inducible protein 47* | *0.004765124* | 2.49 |
| NM_031252 | Il23a | *Interleukin 23, alpha subunit p19* | *0.004775975* | 3.19 |
| NM_019494 | CXCL11 | *Chemokine (C-X-C motif) ligand 11* | *0.008359252* | 6.01 |

[a] Accession number in the GenBank (NCBI).
[b] *t*-Test for paired samples, Benjamini–Hochberg false discovery rate correction for multiple testing.
[c] Relative expression treated/control.
* Genes analyzed for microarray data validation.

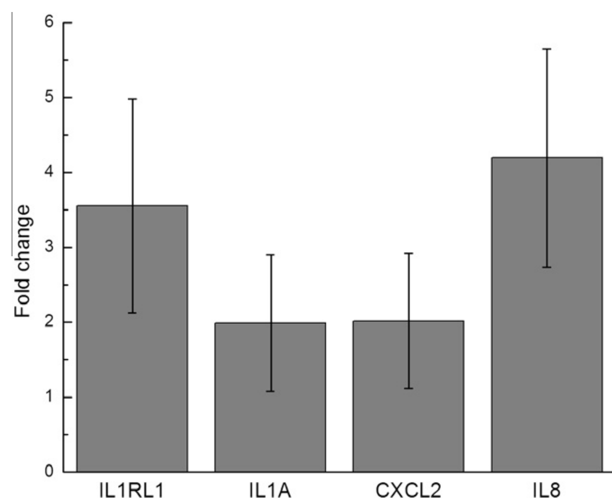


**Figure 6.** Relative expression in HCLE infected and treated cells versus non-infected cells (controls) of the following genes: IL1RL1, IL1A, CXCL2, IL8. Results are expressed as fold changes of each gene relative to the reference gene (β-actin). Gene expression was calculated with the Pfaffl method.

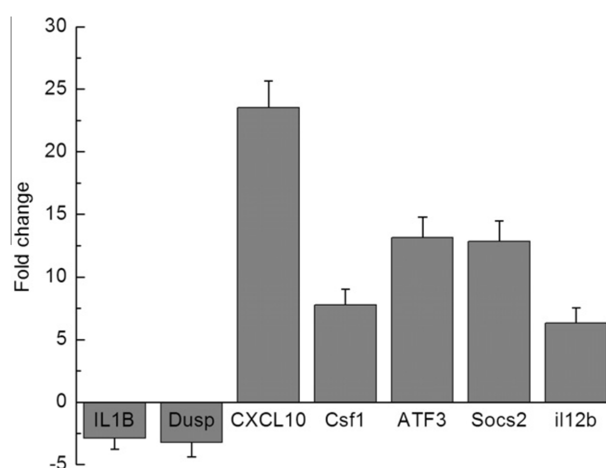


**Figure 7.** Relative expression in J774A.1 LPS-stimulated and treated cells versus non-stimulated untreated cells (controls) of the following genes: IL1B, Dusp2, CXCL10, Csf1, ATF3, Socs2, Il12b. Results are expressed as fold changes of each gene relative to the reference gene (β-actin). Gene expression was calculated with the Pfaffl method.

levels of these pro-inflammatory cytokines revealed a different behavior of both structurally related compounds (Fig. 4).

In LPS-stimulated macrophages, compound **1** behaved similarly to DEX, since it restrained the production of IL-6 (Fig. 5), as well as TNF-α and IL-8, and it did not alter p65 nuclear localization (Fig. 3).

Microarray experiments confirmed that compound **1** elicits a pro-inflammatory effect in epithelial infected cells, since it did not affect TNF-α and IL-6 RNA levels but induced an overexpression of IL-8 and other chemokines such as CXCL-2 and CSF-2. Both CXCL-2 and IL-8 are well known potent chemoattractants for polymorphonuclear cells. CSF-2 is one of the key regulators of the effector function of both mature neutrophils and macrophages. It delays apoptosis of these cells and induces the release of proteolytic enzymes and oxygen free radicals in them.[27–29] Therefore, the pro-inflammatory effect of compound **1** in epithelial infected cells could be helpful to elicit the innate immune response to eliminate the virus from the site of infection.

On the other hand, both IL-10 and Socs2 were overexpressed in LPS-stimulated cells in the presence of compound **1**, compared to the untreated control (Table 2). Socs2 protein is an anti-inflammatory mediator that blocks NF-κB and JAK-STAT pathways, thereby inhibiting the release of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8. IL-10 exhibits anti-inflammatory properties by itself, and one of its effects is the induction of Socs2 protein

expression.[30,31] Although Socs2 was overexpressed in cells treated with compound **1** for 6 h, the expression of TNF-$\alpha$ and IL-8 in control and treated-cells remained similar (Table 2). Moreover, both ATF3 and TLR4 were overexpressed in J774A.1 cells stimulated with LPS and treated with compound **1** (Table 2). ATF3, which displays an anti-inflammatory role, is activated via TLR4 and then inhibits gene induction stimulated by the same TLRs. ATF3 intervenes as a negative regulator of the inflammatory response in macrophages by antagonizing NF-κB induced responses.[32–34]

Dusp2 gene expression was restrained after the treatment of LPS-activated macrophages with compound **1** (Table 2). It is well known that the family of DUSP proteins regulates the activity of mitogen-activated protein kinases, critical in triggering the inflammatory response and cellular immune function. The absence of Dusp2 expression causes a decrease in the production of pro-inflammatory cytokines.[35]

Therefore, compound **1** seems to stimulate anti-inflammatory molecules and inhibit pro-inflammatory factors in macrophages activated with a non-viral stimulus, suggesting an immunosuppressive action over inflammatory cells.

We concluded that compound **1** exerts a dual immunomodulatory activity, depending upon the cell type involved, which makes it an interesting molecule to be further studied. It would be a promising drug for the treatment of immunopathologies and inflammatory processes of viral and non-viral origin.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.10.054. These data include MOL files and InChiKeys of the most important compounds described in this article.

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