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Research Paper

# Inhibition of herpes simplex-1 virus replication by 25-hydroxycholesterol and 27-hydroxycholesterol

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ABSTRACT

Oxysterols are known pleiotropic molecules whose antiviral action has been recently discovered. Here reported is the activity of a panel of oxysterols against HSV-1 with the identification of a new mechanism of action. A marked antiviral activity not only of 25HC but also of 27HC against HSV-1 was observed either if the oxysterols were added before or after infection, suggesting an activity unrelated to the viral entry inhibition as proposed by previous literature. Therefore, the relation between the pro-inflammatory activity of oxysterols and the activation of NF-kB and IL-6 induced by HSV-1 in the host cell was investigated. Indeed, cell pre-incubation with oxysterols further potentiated IL-6 production as induced by HSV-1 infection with a consequent boost of the interleukin's total cell secretion. Further, a direct antiviral effect of IL-6 administration to HSV-1 infected cells was demonstrated, disclosing an additional mechanism of antiviral action by both 25HC and 27HC.

## 1. Introduction

Oxysterols are a family of cholesterol oxidation derivatives containing an additional hydroxyl, epoxide or ketone group as to the parental compound. Due to their chemical structure, oxysterols have been consistently demonstrated as more reactive than unoxidized cholesterol, being easily diffusible through cell membranes, and possessing remarkable pro-inflammatory and cytotoxic effects in a number of cells and tissues [1].

The biological role of oxysterols has been reconsidered for their fundamental activity as physiological ligands of liver X receptors (LXRs), a feedback regulating system for cholesterol homeostasis, immune and inflammatory reactions. The oxysterols showing a high affinity for LXRs are essentially those originated enzymatically, mainly 25-hydroxycholesterol (25HC), 27-hydroxycholesterol (27HC) and 24-hydroxycholesterol (24HC) [2]. These oxysterols have been shown to activate either LXR-dependent or LXR-independent cell signaling pathways, suggesting their dual effects as anti- or pro-inflammatory molecules [3].

Of interest, oxysterols have recently been reported to exert antiviral activity against different enveloped and non-enveloped viruses [4–7]. Notably, the large majority of these studies were focused on 25HC, because the antiviral molecule interferon (IFN) proved to induce the transcription of cholesterol-25-hydroxylase (CH25H), the enzyme responsible for 25HC synthesis [4]. Indeed, also other oxysterols are endowed with antiviral activity, in particular side chain oxysterols. 27HC was shown to be active against murine cytomegalovirus [5], human papillomavirus, rotavirus and rhinovirus [6]. 24HC and 25-epoxycholesterol inhibited the growth of MCMV [5], while 22(S)-hydroxycholesterol and 20α-hydroxycholesterol were active against HBV [8].

If, on one hand, the broad antiviral activity of at least certain oxysterols is nowadays well demonstrated and supported by a still growing bulk of experimental evidence, on the other hand, the mechanisms underlying such an important property of different cholesterol oxidation products mostly remain to be elucidated (for an updated review see 7).

A suitable approach for understanding the antiviral effects of oxysterols, in our opinion, should be guided by the awareness that most viral infections induce in the host the activation of innate and acquired immune systems and trigger an inflammatory response. Of note, most of the oxysterols of pathophysiological interest are provided with a marked and wide pro-inflammatory effect [9–11]. In addition, at least with regard to 25HC, solid data are available that support its

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significant involvement in the regulation of immunologic response [12]. Hence, an oxysterol-mediated modulation of immune and flogistic reactions of the infected host appears very likely.

Out of the several viruses whose growth resulted to be inhibited by 25HC, herpes simplex-1 (HSV-1) appeared the most affected one, at least under the experimental conditions adopted [5]. Moreover, a number of reports have outlined an important role played by inflammation against HSV-1 infection. Indeed, an induction of pro-inflammatory cytokines, in particular IL-6, in response to HSV-1 infection was observed in different types of cells including leukocytes [13], EMT-6 epithelial cells, HaCat cells [14,15], cornea epithelial cells, fibroblasts [16], astrocytes [4]. HSV-1-induced up-regulation of IL-6 expression and synthesis was confirmed to be operated through the increase of Toll Like Receptor 3 (TLR3) and Nuclear Factor kappaB (NF-kB) protein levels [4]. The pro-inflammatory burst activated by HSV-1 infection was proved to have an antiviral effect, by the net enhancement of viral growth achieved every time the overproduction of IL-6 and other pro-inflammatory cytokines was quenched or inhibited [17,18]. In this regard, it is noteworthy the very strong enhancement of morbidity, symptoms severity and mortality following HSV-1 respiratory infection in knockout mice deficient for IL-6 [19].

On these bases, we deemed important to investigate the possible anti-HSV-1 effect of a panel of oxysterols, some of them already shown as provided with broad antiviral activity [6] and all being recognized to induce inflammatory cytokines, including IL-6 [20,21]. The results obtained, demonstrate that mainly 25HC and 27HC markedly inhibit HSV-1 replication and point to the oxysterol-dependent further induction of NF-kB nuclear translocation and IL-6 production in the infected cells as a contributing mechanism to their antiviral properties.

## 2. Materials and methods

### 2.1. Cell lines and virus

African green monkey kidney cells (Vero) (ATCC CCL-81) were cultured in Eagle's minimal essential medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% foetal calf serum (FCS) (Gibco/BRL) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany), at 37 °C in an atmosphere of 5% of $CO_2$. Human epithelial adenocarcinoma HeLa cells (ATCC® CCL-2™) were propagated in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Gibco-BRL) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany), at 37 °C in an atmosphere of 5% of $CO_2$. Clinical isolates of HSV-1 were kindly provided by Prof. M. Pistello, University of Pisa, Italy. Viral strains were propagated and titrated by plaque assay on Vero cells.

### 2.2. Cell viability assay

Vero and HeLa cells were seeded into 96-well plates at a density of $10^4$ cells/well, and incubated at 37 °C in a 5% $CO_2$ atmosphere for 24 h. Oxysterols dissolved in ethanol (25 hydroxycholesterol 25HC, 27 hydroxycholesterol 27-HC, 7α hydroxycholesterol 7αHC, 7β hydroxycholesterol 7βHC and 7Ketocholesterol 7KC) were added to the cells at different concentrations ranging between 150 μM and 0.1 μM, with a replicate number of three wells per concentration. 25-HC was purchased from Sigma Aldrich (Saint Louis, Missouri, USA); 7αHC, 7βHC, 7KC and 27-HC were purchased from Avanti Polar Lipid (Alabaster, Alabama). After 24 or 48 h incubation period, cell viability was measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay by the Cell Titer 96 Proliferation Assay Kit (Promega, Madison, WI,USA) according to the manufacturer's instructions. Absorbance was measured using a Microplate Reader (Model 680, BIORAD) at 490 nm. The percent of viability was calculated in comparison with cells treated with equal volumes of ethanol. The 50% cytotoxic concentrations ($CC_{50}$) and 95% confidence intervals (CI) were determined using GraphPad PRISM software (Graph-Pad Software, San Diego, CA).

### 2.3. HSV-1 antiviral assays

Inhibition of HSV-1 replication was evaluated with plaque reduction assay and virus yield reduction assay. Vero cells were seeded in 24-well plates at a density of $9\times10^4$ cells. The plaque reduction assay was performed pre-treating cells for 16 h with each oxysterol and subsequently infecting cell monolayers with HSV-1 at a multiplicity of infection (MOI) of 0.0004 pfu/cell for 2 h at 37 °C. The inocula were subsequently removed from the wells, and the cells were washed with medium twice and overlaid with a medium containing 1.2% methylcellulose (Sigma). Treatment of control samples with equal volumes of ethanol was performed in order to rule out the possibility of any cytotoxic or antiviral effect ascribable to the solvent. After further incubation for 48 h at 37 °C in 5% $CO_2$, the supernatant was removed, and the cells were fixed and stained with 0.1% crystal violet in 20% ethanol and viral plaques were counted. The plaques were visualized using a Leica inverted Microscope equipped with a Bresser MikroCam microscope camera and MikroCamLab software (Rhede, Germany). ImageJ software was used to quantify plaque sizes. Ethanol treated HSV-infected monolayers were used as internal control.

For the virus yield reduction assay, cells were pre-treated for 16 h with oxysterols and infected in duplicate with HSV-1 at a MOI of 0.01 pfu/cell. Following virus adsorption (2 h at 37 °C), the virus inoculum was removed and cells were incubated until control cultures displayed extensive cytopathology. Alternatively, cells were infected with HSV-1 at a MOI of 0.01 pfu/cell for 2 h and subsequently incubated with medium containing serial dilutions of oxysterols until control cultures displayed extensive cytopathology. Supernatants were pooled as appropriate 48 h after infection and cell-free virus infectivity titers were determined in duplicate by the plaque assay in Vero cell monolayers.

### 2.4. Oxysterols intracellular quantification

To a screw-capped vial sealed with a Teflon septum, cellular pellets resuspended in NaCl 0.9% were added together with 2500 ng of D6-25-hydroxycholesterol and 2500 ng of 27-hydroxycholesterol as internal standards, 50 μl of butylated hydroxytoluene (5 g/l) and 50 μl of K3-EDTA (10 g/l) to prevent auto-oxidation. Each vial was flushed with argon for 20 min to remove air.

Alkaline hydrolysis was allowed to proceed at room temperature (22 °C) with magnetic stirring for 30 min in the presence of ethanolic 1 M potassium hydroxide solution. After hydrolysis, the sterols were extracted twice with 5 ml cyclohexane and oxysterols were eluted on SPE cartridge by isopropanol: hexane 30:70 v/v. The organic solvents were evaporated under a gentle stream of argon and converted into trimethylsilyl ethers with BSTFA.

Gas chromatography mass spectrometry (GC-MS) analysis was performed on a GC equipped with an Elite column (30 m × 0.32 mmid × 0.25 mm film; Perkin Elmer, USA) and injection was performed in splitless mode and using helium (1 ml/min) as a carrier gas. The temperature program was as follows: initial temperature of 180 °C was held for 1 min, followed by a linear ramp of 20 °C/min to 270 °C, and then a linear ramp of 5 °C/min to 290 °C, which was held for 10 min.

The mass spectrometer operated in the selected ion-monitoring mode. Peak integration was performed manually, and sterols were quantified from selected-ion monitoring analysis against internal standards using standard curves for the listed sterols. Additional qualifier (characteristic fragment ions) ions were used for structural identification. Interassay CV was 4.6% for of 25-hydroxycholesterol and 3.8% for 27-hydroxycholesterol. Recovery ranged from 98 up to 103%.

### 2.5. IL-6 quantification by Elisa

HeLa cells were plated in flasks at a density of $1.2 \times 10^6$ cells and the day after 25HC or 27HC were added to the incubation medium to reach 5 μM final concentration. In the control samples, the oxysterol was replaced by equal volumes of ethanol. The following day the medium containing oxysterols was removed, cells washed and infected with HSV-1 at MOI of 1pfu/cell. After 24 h, cell medium was harvested and used for ELISA analyses.

Levels of IL-6 were quantified using Human IL-6 ELISA kit (Peprotech, Rocky Hill, NJ, USA) following the manufacturer's instructions. Sample absorbance values were read at 450 nm with a wavelength correction of 550 nm in a microplate reader (Model 680 microplate reader Bio-Rad), and data analyzed using Slide Write Plus software (Advanced Graphics Software). Extracellular IL-6 levels were evaluated in triplicate and values were expressed in molarity.

### 2.6. Immunofluorescence

To test the localization of NF-kB, Vero cells were grown directly onto glass coverslips in 24-well plates at a density of $9 \times 10^4$ cells, treated with 25HC or 27HC for 16 h and infected with HSV-1 for 2 h at 37 °C. 24 hpi cell samples were fixed in 95% ethanol for 5 min, permeabilized with MSM-PIPES [5 mM PIPES, 18 mM MgSO4, 5 mM CaCl2, 40 mM KCl, 24 mM NaCl, 0.5% Triton X-100 (v:v), 0.5% Igepal CA-630 (v:v)] for 2 min at room temperature and incubated in a 100 mM sodium cyanoborohydride reducing agent for 10 min at 37 °C. In order to block nonspecific binding, coverslips were preincubated with 3% BSA in 0.01 M PBS (w:v) containing 5% normal goat serum (v:v) and 0.3% Tween-20 (v:v) for 30 min at room temperature. Further, slides were incubated in the presence of 1:150 dilution purified rabbit anti-p65 monoclonal primary antibody (NF-κB p65 (D14E12) XP® Rabbit mAb #8242, Cell Signaling Technology, Inc.). After 90 min of incubation at room temperature in a humidified chamber specimens were then incubated with a purified goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Alexa Fluor, Molecular Probes, Life Technologies) (1:300 dilution) at room temperature for 60 min.

Slides were sealed with glycerol after three additional washes with 0.1 M PBS for 5 min, and observed with a confocal microscope Zeiss LSM 510 (plan neofluar lens 20×/0.5). The instrument was set to 488 nm exciting laser band, with a 505–530 nm band pass emission filter, to detect green fluorescence. Exciting light intensity, black level, and photomultiplier gain were adjusted on control specimens; settings were maintained the same when scanning experimental samples. All images were processed using LSM 510 Image Examiner software (Zeiss S.p.A Arese, Milan, Italy).

### 2.7. IL-6 antiviral assay

HeLa cells were plated in 24 wells plate at a density of $9 \times 10^4$ cells/well, the following day HSV-1 at MOI 1pfu/cell was added on cells for 2 h. Following virus adsorption, the viral inoculum was removed and cells were overlaid with medium containing IL-6 at final concentration of 100 nM, 50 nM and 20 nM. 24 h later, supernatants and cells were harvested and subsequently titrated on Vero cells. The percent infection was calculated comparing viral titers in treated samples with untreated ones. One way ANOVA was performed to reveal significant reduction of viral titers.

### 2.8. Statistics

The 50% effective concentrations ($EC_{50}$) and 95% confidence intervals (CI) were determined using GraphPad PRISM software (Graph-Pad Software, San Diego, CA).

Values of $p < 0.05$ were considered indicative of statistical differences. * $p < 0.05$ **$p < 0.01$ ***$p < 0.005$ Results represent the mean ± SEM of three independent experiments. With the exception of ELISA experiments, where data are means of four experiments ± SD.

**Table 1**
Anti-HSV-1 activity and cellular toxicity of oxysterols.

| Compound | $EC_{50}$ (μM) – 95% C.I. | $CC_{50}$ (μM) | SI |
|---|---|---|---|
| 25HC | 5.06 (3.47–7.38) | 77.59 | 15.3 |
| 27HC | 17.1 (10.2–30.1) | > 150 | > 8.78 |
| 7αHC | n.a. | 131 | n.a. |
| 7βHC | n.a. | 25.03 | n.a. |
| 7KC | n.a. | 79.06 | n.a. |
| Cholesterol | n.a. | > 150 | n.a. |

$EC_{50}$: half maximal effective concentration; C.I. confidence interval; $CC_{50}$: half maximal cytotoxic concentration; SI: selectivity index; n.a.: not assessable.

## 3. Results

### 3.1. Antiviral activity of oxysterols against HSV-1

In order to test the anti-HSV-1 activity of a panel of oxysterols of pathophysiological relevance, an inhibition assay was performed, in which the single 25HC, 27HC, 7αHC, 7βHC, 7KC or unoxidized cholesterol were added to Vero cells (see Methods), at different final concentrations, 16 h before viral infection. Experimental conditions were those previously adopted to test the effect of the same oxysterols on naked viruses [6], and the infection was evaluated by means of plaque assay (see Methods). To exclude the possibility that an antiviral activity might depend on oxysterols' cytotoxic effects, a cell viability assay was also performed on uninfected cells, challenged with the tested molecules under the same experimental conditions. As reported in Table 1, 25HC and 27HC were the only oxysterols affording a dose-dependent decrease in plaque number, thus an antiviral effect. The $CC_{50}$ and SI values indicate that the antiviral activity exerted by 25HC and 27HC was not due to cytotoxicity.

Of note, a significant reduction of plaque size was observed even with those oxysterols whose $EC_{50}$ was unmeasurable, when, according to Blanc et al. [5], a fixed dose of 5 μM of challenging compound was applied in a plaque reduction assay (Fig. 1, panel A). Since a reduced plaque area may result in a decreased production of virus progeny, even if the overall plaque number was not significantly reduced, oxysterols' inhibitory activity was analyzed by viral yield reduction assay. This is a more stringent test which allows multiple cycles of viral replication to occur before measuring the production of infectious viruses. To this aim, each oxysterol (5 μM) was added 16 h before viral inoculum (pre-treatment) (Fig. 1, panel B) or immediately after HSV-1 infection (treatment) (Fig. 1, panel C). 25HC and 27HC proved to be the most potent inhibitors of HSV-1 replication, but also 7αHC, 7βHC and 7KC significantly reduced viral yield in both the experimental conditions adopted.

The viral yield reduction ability of 25HC and 27HC in post treatment assays was further investigated in a dose-response manner and the relative $EC_{50}$ determined in two cell lines, namely Vero and HeLa cells. As shown in Table 2, the two compounds were active on both cell lines showing $EC_{50}$s much lower than those determined in the plaque assay.

### 3.2. Kinetics of the uptake of 25HC and 27HC by HeLa cells

Cell internalization of the two most powerful oxysterols, in terms of antiviral activity, was evaluated using HeLa cell cultures at various time points and different concentrations, namely from 0.2μM to 16 μM. Notably, the amount of the two oxysterols recovered within so treated cells, quantified by a mass spectrometry method, resulted to be consistently lower than that added and actually recovered in the total

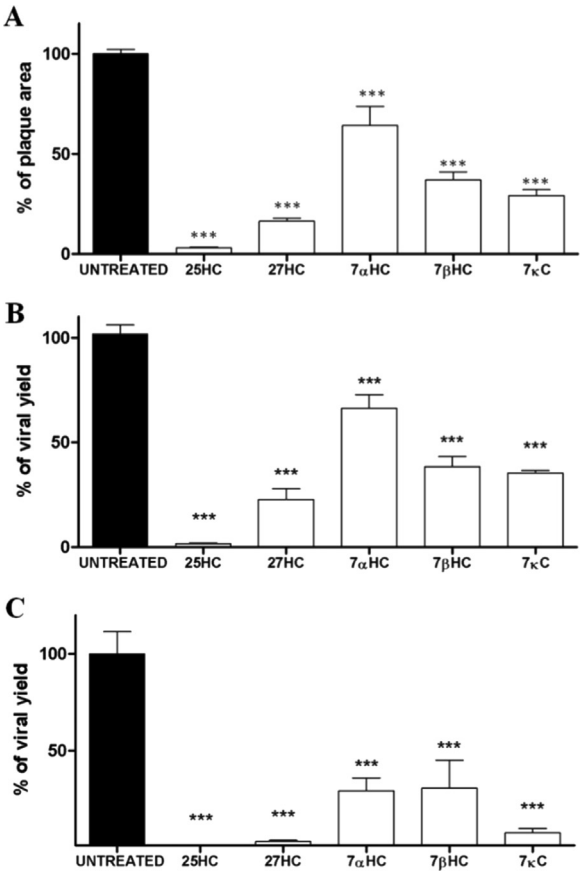


**Fig. 1. Plaque (A) and viral yield (B-C) reduction exerted by the different tested oxysterols on HSV-1 infected Vero cells.** The different oxysterols were added to cell monolayers at a final concentration of 5 μM, before viral inoculum (Panels A, B) or after viral inoculum (Panel C). See also methods for details. Panel A: percent of plaque areas measured in Vero cells 16 h pretreated with oxysterols then infected with HSV-1, taking the plaque area measured in infected but untreated cells as 100%. Values are means of three different experiments (15–20 measurements/experiment). Panel B: viral yield reduction in cells pretreated with oxysterols for 16 h and then infected. Panel C: viral yield reduction in cell culture infected with HSV-1 and then treated with the different oxysterols. * $p < 0.05$**, $p < 0.01$***, $p < 0.005$.

**Table 2**
Viral yield reduction by 25HC and 27HC.

| Cell line | Compound | $EC_{50}$ (μM) – 95% C.I. | $CC_{50}$ (μM) | SI |
|---|---|---|---|---|
| Vero | 25HC | 0.176 (0.124–0.250) | 77.59 | 440.8 |
| | 27HC | 0.220 (0.092–0.524) | > 150 | > 681.8 |
| HeLa | 25HC | 0.257 (0.206–0.321) | > 150 | > 583.6 |
| | 27HC | 0.984 (0.793–1.22) | > 150 | > 152.4 |

$EC_{50}$: half maximal effective concentration; C.I. confidence interval; $CC_{50}$: half maximal cytotoxic concentration; SI: selectivity index.

cell suspension (cell + incubation medium) at all the time points considered. Indeed, 25HC cell uptake, taken as % intracellular recovery, ranged from 13% to 24% of total added oxysterol, showing the higher percent values at 16.7 μM oxysterol concentration; notably, 25HC intracellular concentration tended to decrease with time, as evident after 72 and 96 h incubation in all but one experimental conditions (Fig. 2). With regard to the percent amount of 27HC recovered in the cells, values were steadily lower than those observed for 25HC, ranging from 9% to 15% of total added oxysterol. Also 27HC intracellular content showed to gradually decrease with time. In this case, however, the addition of the high oxysterol amount to cell suspension did not appear to efficiently accumulate 27HC within cells (Fig. 2).

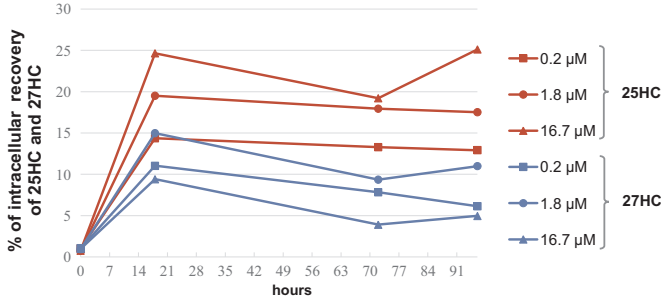


**Fig. 2. Intracellular recovery of 25HC and 27HC externally added to HeLa cell incubation medium.** Percent values were calculated as to the total amount added to the medium and were determined by mass-spectrometry at 18 h, 72 h and 96 h after oxysterols' addition.

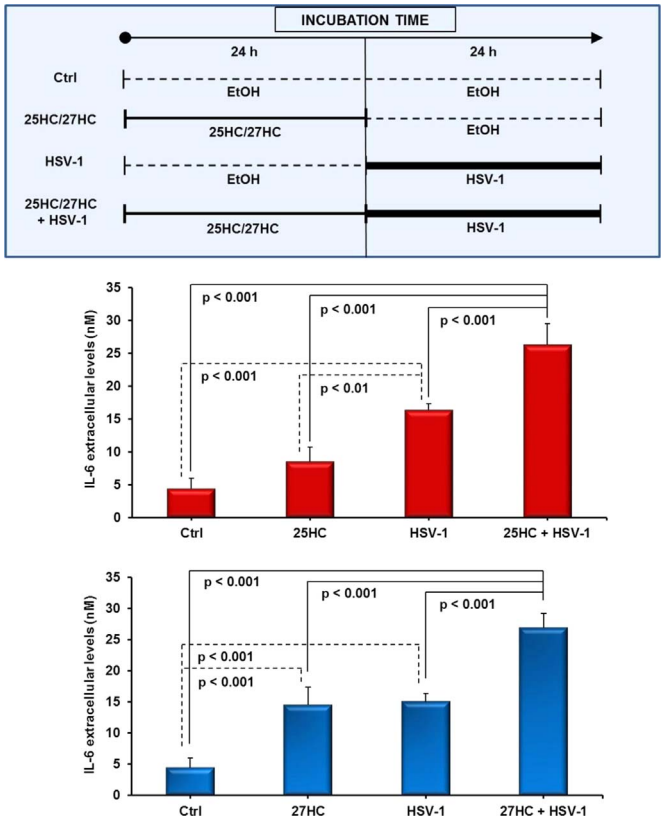


**Fig. 3. Effect of oxysterols on IL-6 secretion by HeLa cells infected with HSV-1.** The concentration of IL-6 was measured by ELISA in culture medium of HeLa cells treated or not with 5 μM 25HC or 5 μM 27HC (24 h), with or without HSV-1 cell infection (24 h). Data are means of four experiments ± SD. Statistical difference within experimental groups was calculated using ANOVA associated with the Bonferroni post-test.

### *3.3. Pretreatment with 25HC or 27HC additionally up-regulates IL-6 secretion by HeLa cells as induced by HSV-1 infection*

As reported in Fig. 3, HeLa cells infection with HSV-1 led *per se* to a significant stimulation of the cytokine IL-6 secretion into the incubation medium. A consistent increment of IL-6 extracellular levels was also achieved when cells were 24 h pretreated with 25HC and, in particular, with 27HC. Importantly, cell pre-incubation with oxysterols followed by 24 h HSV-1 infection led to a significant further increase of IL-6 total secretion, most likely because of an additive effect of the two agents.

### *3.4. 25HC and 27HC further enhance NF-kB activation induced by HSV-1 infection*

On the basis of the unanimously recognized key role of the

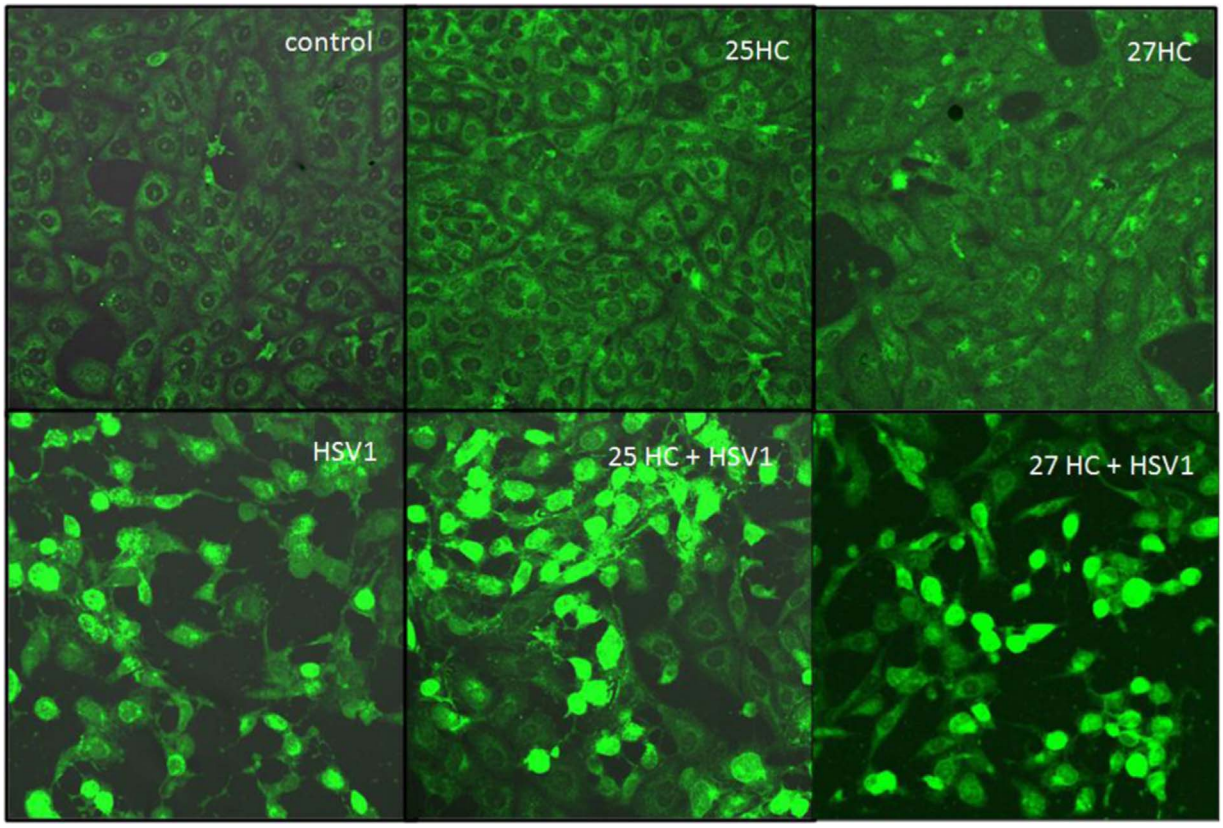


**Fig. 4. Effect of oxysterols on p65 expression and nuclear translocation in Vero cells treated with oxysterols and infected with HSV-1**. Vero cells were treated or not with 5 μM 25HC or 5 μM 27HC (16 h), subsequently infected or not with HSV-1 (24 h) and then subjected to immunofluorescence. Green Fluorescence was detected by using a confocal microscope Zeiss LSM 510 with a 20×/0.5 plan neofluar lens. Images were elaborated with Zeiss LSM 510 Image Examiner processing software.

transcription factor NF-kB in the expression of IL-6 and the already demonstrated ability of certain viruses to up-regulate, at least transiently, this transcription peptide's activity [22–24], the effect of the two oxysterols ± HSV-1 infection on the p65 component of NF-kB dimer was investigated using a confocal microscopy approach in a standard *in vitro* model system for HSV-1 infection, *i.e.* Vero cells.(Fig. 4).

While in untreated cells immunofluorescence detection showed p65 as essentially localized in the cytoplasm, a modest increase of fluorescence was observed in cells pretreated with 25HC or 27HC, but a much more evident trend to nuclear localization was evident in HSV-1 infected cells. Importantly, when cell infection was anticipated by oxysterol treatment, an even stronger fluorescence was detectable, especially when 25HC was employed.

### 3.5. Antiviral activity of IL-6

With the aim to verify if IL-6 up-regulation is actually one of the mechanisms by which 25HC and 27HC exert antiviral effect, HeLa cells were infected with HSV-1, then different cytokine concentrations were added to the cell incubation medium. At the end of the experiment, a viral yield reduction assay was performed. A dose-dependent inhibition of HSV-1 cell infection was observed, with a statistically significant reduction of viral titer at 100 nM and 50 nM IL-6 concentration (Fig. 5). Of note, cell viability assayed in IL-6 treated but not infected cells excluded that the observed IL-6 antiviral activity was a consequence of cytotoxic or antiproliferative effects (data not shown).

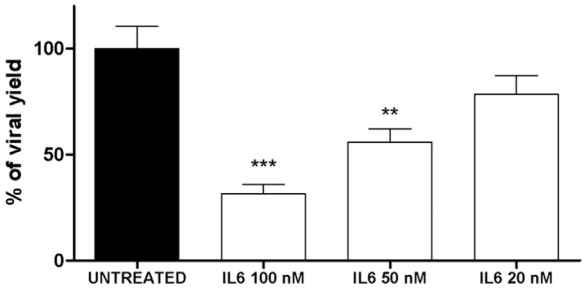


**Fig. 5. Antiviral activity of IL-6.** The graph shows the reduction of viral titer in presence of different concentration of IL-6. The cells were infected with HSV-1 (MOI 1) and then treated with different doses of IL-6; 24 h post infection, cells and supernatants were harvested and subsequently titrated. Bars are the % of infection calculated in comparison with untreated control. The experiment was performed three times in triplicated wells. ** $p < 0.01$, *** $p < 0.005$.

## 4. Discussion

Oxysterols are known pleiotropic molecules, and over the last years, antiviral properties have been attributed to this class of compounds. However, analyzing the work done so far in this relation, there are two main limitations. First, mechanistic studies of their potential antiviral effects mainly focused on 25 HC due to the induction of the synthetizing enzyme (CH25H) by IFN, while oxysterols'family is broad and variegate. Second, although the pro-inflammatory activity of several oxysterols is well known, to our knowledge, the possible interplay between pro-inflammatory and antiviral mechanisms of action has not been investigated yet.

To contribute to overcome these restraints, HSV-1 infection was here chosen as a model because of the demonstrated antiviral cytokine reaction triggered in targeted cells by the virus itself [4,13–16].

As reported in Table 1 and Fig. 1, 25HC and 27HC were the only oxysterols, among the tested, able to dose-dependently reduce the plaque formation, while all of them reduced plaque size and the viral yield both in pre-treatment and post-treatment conditions, although to different extents These findings point to possible additional mechan-

isms of action by side chain oxysterols, beside the entry inhibition mechanism suggested by Blanc et al. [5]. 25HC was reported to have the property to modify the composition of membranes, therefore it was suggested to broadly inhibit enveloped viruses by alteration of fusion, and adenoviruses were used as non enveloped control viruses to support this hypothesis. However, some reports suggest that 25HC is effective also against non enveloped viruses, such as poliovirus and rhinovirus through an inhibition of the assembly of viral replication machinery [7], supporting the possibility that the antiviral action of 25HC is linked to different mechanisms of action.

This is most likely the first report showing an anti-HSV-1 effect of oxysterols other than 25HC, all being of pathophysiological relevance, and certainly the first one emphasizing the remarkable antiviral action of 27HC in both preventing and stopping HSV-1 infection, at least in *in vitro* standard tests.

Since 25HC and 27HC were shown to be by far the most active oxysterols in inhibiting HSV-1 also in post infection tests, and in different cell lines (Fig. 1C and Table 2), deeper investigation of possible interrelationship between their inflammatory and antiviral features was then performed.

But before that, it is important to remark that the concentrations of 25HC and 27HC recovered within cells were comparable (27HC) or close (25HC) to those physiologically recovered in human plasma [22,23]. Cell supplementation with 27HC resulted to be less efficient than that with 25HC (Fig. 2), due to reduced entry and/or persistence within cell, any way a likely explanation of the 27HC slightly minor antiviral efficacy observed in comparison with 25HC. Further, the intracellular levels of both oxysterols were quite maintained at 96 h post treatment, suggesting long-term effects of the two molecules.

With regard to NF-kB activation, a number of viruses were shown to trigger and up-regulate the NF-kB pathway in the infected cells, most often at the onset of the infection process and in a transient way [24–26]. Although such an activation might be exploited by some viruses to enhance specific steps of their replicative cycles, it certainly represents an alarm signaling priming a cascade of defense reactions by the invaded cells. In this relation, a large bulk of literature points to NF-kB dependent cellular overproduction and secretion of inflammatory cytokines, in particular IL-6, as able to antagonize viral replication [17–19]. However, in the long run, viruses tend to build up a variety of immune based avoidance strategies able to by-pass or even down-regulate the previously enhanced NF-kB transcriptional pathway [27,28]. Still, a suitable boosting of the initial inflammatory reaction by cells under viral attack does represent a valid tool to more efficiently fight the infection itself.

Indeed, oxysterols of enzymatic origin, like 25HC and 27HC, whose pro-inflammatory properties are well recognized [1,29] would appear as potential physiological modulators of innate and acquired immune system in fighting viral infections. The here reported additive interaction between 25HC/27HC and HSV-1 in up-regulating IL-6 cellular levels (Fig. 3) is an interesting and new example of fine interplay between cholesterol metabolism and immune/inflammatory response to xenobiotics.

Such an additive interaction between HSV-1 virus and 25HC or 27HC in boostering IL-6 production and secretion by the infected cells could contribute to the observed antiviral effect exerted by the two side chain oxysterols. This hypothesis is supported by the dose-dependent inhibition of HSV-1replication induced by IL-6 concentrations compatible with those detected in HeLa cells pretreated with 25HC or 27HC then infected with the virus (Fig. 5).

In conclusion, 25HC and 27HC, the two oxysterols most considered for their potential involvement in the pathophysiological tuning of innate and acquired immune reactions, including inflammation, were for the first time demonstrated, in the *in vitro* standard model system, to be provided with strong inhibiting but also preventing effect against HSV-1 infection. Several are most likely the mechanisms underlying such a striking effect, being certainly included the reinforcing of IL-6 mediated restraining of viral replication and spreading.

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