Antiviral Research 215 (2023) 105634

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Identification of oxysterol synthetic analogs as a novel class of late-stage inhibitors of herpes simplex virus 2 replication

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ARTICLE INFO

Keywords:
Herpes simplex virus
Oxysterols
Synthetic derivatives
Glycoproteins

ABSTRACT

Genital herpes, most frequently caused by herpes simplex virus 2 (HSV-2) infection, is one of the most prevalent sexually transmitted infections. The current rationale for the treatment of HSV-2 infection involves nucleoside analogs (e.g. acyclovir) to suppress reactivation. Enzymatic oxysterols are endogenous 27-carbon atoms molecules produced by enzymatic cholesterol oxidation, and recently emerged as a broad-spectrum host targeting antivirals. In this study, we screened selected members of an in-house synthesized library of oxysterol analogs for their activity against HSV-2, identifying three compounds, named PFM064, PFM067, and PFM069, endowed with 50% effective concentrations (EC_{50}) in the micromolar range, without exerting any apparent cytotoxicity. Moreover, the results obtained showed the ability of the novel derivatives to inhibit both cell-to-cell fusion induced by HSV-2, and the production of an intracellular viral progeny. Further experiments performed with PFM067 (which was selected for more-in-depth studies as the most effective synthetic analog) showed that these molecules act in a late stage of HSV-2 replicative cycle, by sequestering viral glycoproteins in the Golgi compartment, and likely inhibiting the nuclear egress of neo-synthetized viral capsids.

Taken together, these results point to PFM067 as a promising chemical scaffold for the development of novel herpetic antivirals.

1. Introduction

Herpes simplex virus 2 (HSV-2) is one of the most prevalent sexually transmitted pathogens (James et al., 2016). Genital HSV-2 infections in the vaginal mucosa are frequently asymptomatic; nevertheless, non-symptomatic individuals can shed infective particles during reactivation periods (Koelle and Wald, 2000; Tronstein et al., 2011; Wald et al., 2000). Symptomatic patients are subject to frequent manifestations in the form of genital lesions (Johnston et al., 2012), largely due to damage to the genital epithelium caused by viral lytic replication (Schiffer and Corey, 2013). Unfortunately, HSV-2 is also a neurotropic virus, which can establish a latent infection in neuronal cells. Of note, neuronal infection can result in fatal herpes simplex encephalitis in newborns or immunocompromised individuals (Brown et al., 2005; Kimberlin et al., 2013).

Nucleoside analogs, such as acyclovir, valacyclovir, or famciclovir can reduce the frequency of HSV reactivation, but they cannot eliminate or completely prevent the viral shedding (Johnston et al., 2012).

Abbreviations: HSV-2, herpes simplex virus 2; HSV-1, herpes simplex virus 1; 25OHC, 25-hydroxycholesterol; 25*R*,26OHC, 25*R*,26-hydroxycholesterol; EC_{50}, 50% effective concentration; EC_{90}, 90% effective concentration; CC_{50}, 50% cytotoxic concentration; SI, selectivity index; CPE, cytopathic effect; EtOH, ethanol; DMSO, dimethyl sulfoxide; HIV-1, human immunodeficiency virus type 1; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; PFU, plaque-forming units; MOI, multiplicity of infection; ANOVA, analysis of variance; SEM, standard error of the mean.

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https://doi.org/10.1016/j.antiviral.2023.105634
Received 23 January 2023; Received in revised form 5 May 2023; Accepted 7 May 2023
Available online 8 May 2023

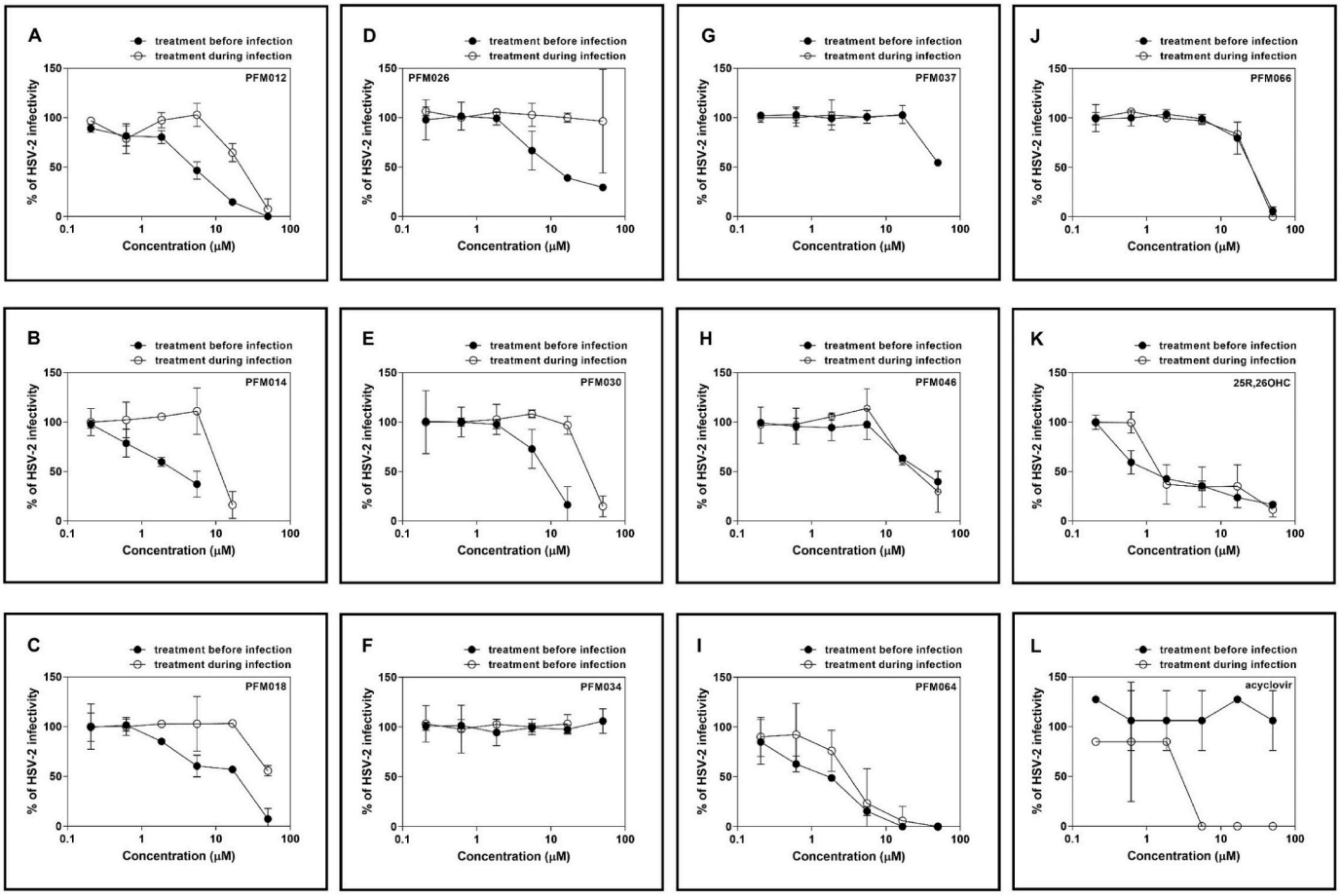

Fig. 1. Antiviral activity of the first series of synthetic oxysterols (namely PFM012, PFM014, PFM018, PFM026, PFM030, PFM034, PFM37, PFM046, PFM064, and PFM066), 25*R*,26OHC, and acyclovir on Vero cells. Cells were treated for 24 h with increasing concentrations of tested molecules and then infected with HSV-2; alternatively, cells were infected with HSV-2 in presence of increasing concentrations of tested molecules. Viral infections were detected 24 h after infection, by indirect immunoperoxidase staining. The percentage infection was calculated by comparing treated and untreated wells. The results are means and SD for two independent experiments, each one performed in triplicate.

Furthermore, resistance to antivirals such as acyclovir, particularly in immunocompromised individuals, has been observed (Jiang et al., 2016). More importantly, individuals with genital HSV-2 infection also hold a significantly increased risk of acquiring human immunodeficiency virus type 1 (HIV-1) infection (Looker et al., 2017; Van de Perre et al., 2008); thus, the inability to adequately address the HSV-2 epidemic has also been driving the HIV epidemic.

Oxysterols are 27-carbon atoms intermediates or end products of cholesterol metabolism, structurally characterized by the presence of extra oxygenated functions respect to the hydroxyl group at 3β-position. They are produced from cholesterol through oxidation reactions operated by reactive oxygen species or by specific endogenous enzymes. Among enzymatic oxysterols, 25-hydroxycholesterol (25OHC) and 25*R*,26OHC (more commonly referred to as 27-hydroxycholesterol) (Fakheri and Javitt, 2012) have raised particular interest as they are endowed with broad-spectrum host-targeting antiviral activity, against both enveloped and non-enveloped viruses (Blanc et al., 2013; Lembo et al., 2016; Liu et al., 2013).

The efficacy profile and antiviral mechanism of action of 25OHC have been investigated more thoroughly than that of 25*R*,26OHC. The reason is probably attributable to a more explicit involvement of 25OHC in innate immunity against viral infections: the enzyme responsible for the synthesis of 25OHC (i.e. the cholesterol-25-hydroxylase) is in fact an interferon-inducible protein (Blanc et al., 2013), with 25OHC acting as the actual downstream antiviral effector capable of blocking viral replication. As a matter of fact, the direct administration of 25OHC, to the cells inhibits viral replication by bypassing the trigger represented by interferon, even in interferon-deficient cells like Vero (Cagno et al., 2017). However, some recent evidence shows that 25*R*,26OHC has some particularly interesting features. While having an antiviral efficacy comparable to that of 25OHC towards several viral pathogens including HSV-1,[16–18] 25*R*,26OHC is present in various biological fluids (including blood and colostrum) at much higher concentrations than 25OHC (Civra et al., 2019). Furthermore, recent evidence shows that 25*R*,26OHC has a higher cytocompatibility than that of 25OHC (Civra et al., 2022). Moreover, its role in innate immunity against viral infections, although more nuanced than that of 25OHC, should be re-evaluated in the light of two recent articles, correlating low plasma levels of 25*R*,26OHC with the severity of two viral diseases, namely COVID-19 and chronic hepatitis B (Boglione et al., 2021; Marcello et al., 2020).

The enzymatic oxysterols act as host-targeting antivirals by modifying the membrane composition of intracellular organelles, and their effect on viral replicative cycle differs depending on virus-host interactions; in particular, 25R,26OHC and/or 25OHC inhibit virus-cell fusion of several enveloped viruses (e.g. human immunodeficiency virus, and Ebola virus), the entry of human rotavirus, and the genome replication of human rhinovirus. Interestingly, both these enzymatic oxysterols are able to inhibit HSV replicative cycle determining a reduction of the titer of viral infective progeny (Cagno et al., 2017), although the effect on the replication cycle of this virus has not been investigated in depth.

The interesting antiviral activity showed by these two endogenous

Table 1
Antiviral activity of oxysterol analogs (first series).

ID	Treatment protocol	EC50[a] (μM) – 95% C.I.[b]	EC90[c] (μM) – 95% C.I.	CC50[d] (μM) – 95% C.I.	SI[e]
PFM012	*Before infection*	4.6 (3.3–6.3)	29.2 (14.3–59.4)	>250	>54.3
	During infection	20.5 (14.9–28.0)	41.9 (16.7–105.0)		>12.2
PFM014	*Before infection*	3.0 (1.9–4.8)	31.1 (7.6–126.3)	12.3 (10.1–15.0)	4.1
	During infection	n.a.	n.a.		n.a
PFM018	*Before infection*	12.2 (7.5–19.7)	100.4 (31.3–322.5)	>250	>20.5
	During infection	n.a.	n.a.		n.a.
PFM026	*Before infection*	13.8 (8.3–23.1)	113.8 (32.1–403.9)	>250	>18.1
	During infection	n.a.	n.a.		n.a.
PFM030	*Before infection*	8.4 (6.4–11.1)	21.0 (11.7–36.7)	>250	>29.8
	During infection	34.5 (19.7–60.3)	55.0 (36.4–82.9)		>7.2
PFM034	*Before infection*	n.a.	n.a.	36.7 (20.7–65.3)	n.a.
	During infection	n.a.	n.a.		n.a.
PFM037	*Before infection*	n.a.	n.a.	34.6 (25.1–47.8)	n.a.
	During infection	n.a.	n.a.		n.a.
PFM046	*Before infection*	32.6 (21.1–50.3)	201.5 (59.5–682.6)	1009 (214.7–4745)	31.0
	During infection	26.9 (16.9–42.7)	88.4 (31.0–251.5)		37.5
PFM064	*Before infection*	1.3 (0.9–1.9)	10.9 (4.6–25.7)	648.1 (197.1–2131)	498.5
	During infection	3.1 (2.3–4.2)	10.0 (5.6–17.7)		209.1
PFM066	*Before infection*	24.0 (20.0–29.4)	43.0 (27.8–66.4)	51.4 (34.6–76.3)	2.1
	During infection	20.6 (2.1–203.9)	n.a.		2.5
25*R*,26OHC	*Before infection*	2.0 (1.1–3.7)	56.5 (12.3–258.8)	>1350	>675
	During infection	2.4 (1.2–5.0)	17.4 (3.4–90.4)		>562.5
acyclovir	*Before infection*	n.a.	n.a.	>200	n.a.
	During infection	1.0 (0.7–1.4)	2.9 (1.3–6.4)	>200	>200.0

n.a. Not assessable.
[a] EC_{50} half-maximal effective concentration.
[b] CI confidence interval.
[c] EC_{90} 90% effective concentration.
[d] CC_{50} half-maximal cytotoxic concentration.
[e] SI selectivity index.

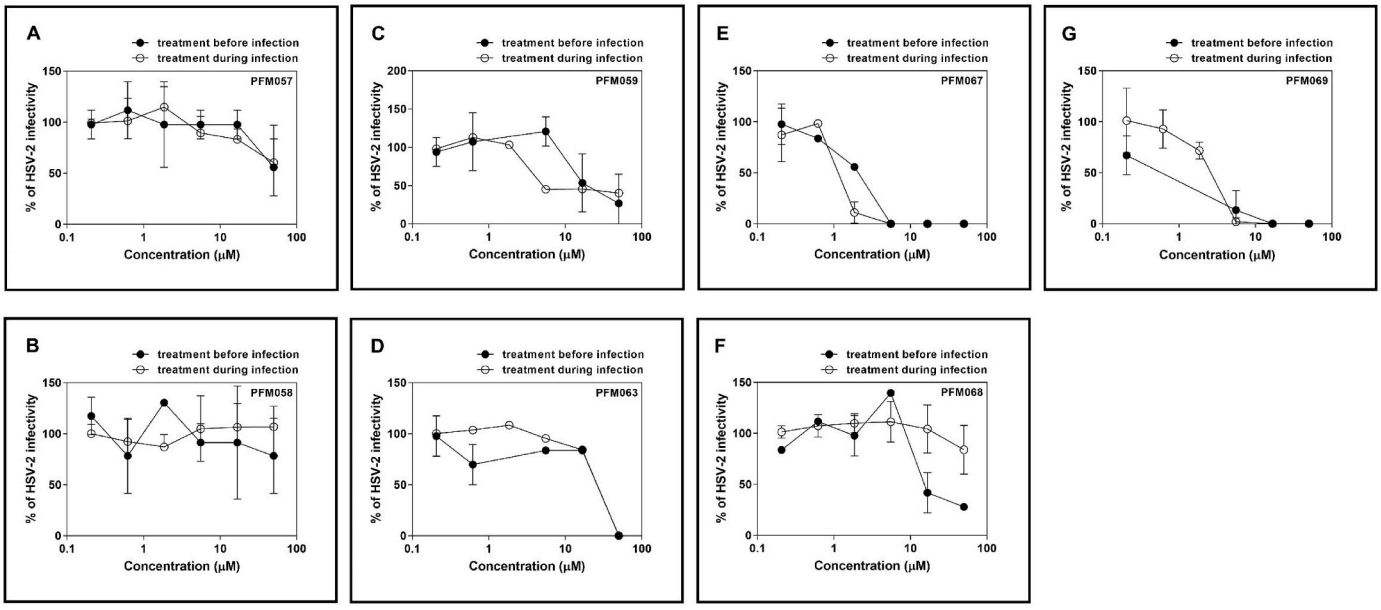

Fig. 2. Antiviral activity of the second series of synthetic oxysterols (namely PFM057, PFM058, PFM059, PFM063, PFM067, PFM68, and PFM069) on Vero cells. Cells were treated for 24 h with increasing concentrations of tested molecules and then infected with HSV-2; alternatively, cells were infected with HSV-2 in presence of increasing concentrations of tested molecules. Viral infections were detected 24 h after infection, by indirect immunoperoxidase staining. The percentage infection was calculated by comparing treated and untreated wells. The results are means and SD for two independent experiments, each one performed in triplicate.

oxysterols against HSV-1 (Schiffer and Corey, 2013), together with the medical need to develop a second class of anti-herpetic molecules to complement the nucleoside analogs, and with the paucity of published data about the evaluation as antiviral agents of non-endogenous, oxysterol analogs prompted us to screen an in-house synthesized, focused oxysterol library (Castro Navas et al., 2018; Marinozzi et al., 2017; Pontini et al., 2021). It should be noted that the items of such library are oxysterols in a broader sense: they all are characterized by the presence of side-chain oxygenated functions, although not all of them are 27-carbon derivatives. In this study, we will report the results of the screening of selected members of the library against HSV-2, culminating in the selection of two derivatives endowed with anti-HSV-2 activity. The results obtained also show that the selected molecules inhibit the latest stages of HSV-2 replication.

Table 2
Antiviral activity of oxysterol analogs (second series).

ID	Treatment protocol	EC50[a] (μM) – 95% C.I.[b]	EC90[c] (μM) – 95% C.I.	CC50[d] (μM) – 95% C.I.	SI[e]
PFM057	*Before infection*	n.a.	n.a.	>200	n.a.
	During infection	69.8 (25.3–192.7)	n.a.		>2.9
PFM058	*Before infection*	n.a.	n.a.	>150	n.a.
	During infection	n.a.	n.a.		n.a.
PFM059	*Before infection*	23.0 (9.4–56.1)	n.a.	>200	>8.7
	During infection	14.2 (6.9–28.9)	n.a.		>14.1
PFM063	*Before infection*	13.7 (5.2–35.6)	38.0 (4.4–326.6)	>200	>14.6
	During infection	20.9 (3.5–26.3)	28.2 (0.4–1786.0)		>9.6
PFM067	*Before infection*	1.9 (1.4–2.5)	4.3 (2.1–8.7)	>200	>105.3
	During infection	1.3 (0.8–2.0)	1.9 (1.6–2.3)		>153.8
PFM068	*Before infection*	18.5 (8.8–38.9)	n.a.	>200	>10.8
	During infection	n.a.	n.a.		n.a.
PFM069	*Before infection*	0.5 (0.2–1.2)	5.9 (0.7–45.9)	>200	>400
	During infection	2.3 (1.7–3.2)	4.0 (1.6–10.0)		>87.0

n.a. Not assessable.
[a] EC_{50} half-maximal effective concentration.
[b] CI confidence interval.
[c] EC_{90} 90% effective concentration.
[d] CC_{50} half-maximal cytotoxic concentration.
[e] SI selectivity index.

2. Materials and methods

2.1. Reagents

25*R*,26OHC (kindly provided by Panoxyvir Ltd, Turin, Italy; purity: >95% by HPLC and ^{1}H NMR) was dissolved in sterile ethanol (EtOH) to a concentration of 3 mM. PFM012, PFM014, PFM018, PFM026, PFM030, PFM034, PFM37, PFM046, PFM057, PFM063, PFM064, PFM066, PFM067, and PFM068, members of the in-house synthesized library, were dissolved in warm dimethyl sulfoxide (DMSO) at a concentration of 12.50 mM. PFM059 and PFM069 were dissolved with DMSO:EtOH (1:1) at a concentration of 8.33 mM. PFM058 was dissolved in DMSO:EtOH (2:1) at a concentration of 6.25 mM. The purity of all PFM compounds was >95% by microanalysis and ^{1}H NMR.

2.2. Cell line and virus

African green monkey kidney cells (Vero) and human lung fibroblast MRC-5 (ATCC® CCL-171) were grown in Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma Aldrich). Herpes simplex virus 1 (HSV-1) strain LV (Tognon et al., 1985) and herpes simplex virus 2 (HSV-2) strain MS (ATCC® VR-540) were propagated in Vero cells using DMEM supplemented with 2% (v/v) FBS at 37 °C in a humidified 5% CO_2 incubator. Supernatants were harvested when full cytopathic effect (CPE) occurred; virus pools were clarified, aliquoted, and stored at −70 °C and titers were determined by infecting Vero cells with serial dilutions of viral stocks. 24 h after viral inoculum, infected monolayers were washed with medium, fixed, and gD-positive syncytia were stained by indirect immunocytochemistry, as described in supplemental materials. HSV-1 plaques or HSV-2 syncytia were counted, and viral titers were expressed as plaque-forming unit (PFU) per ml.

2.3. Plaque reduction assays

To assess the anti-HSV-2 efficacy of the compounds, plaque reduction assays were performed. Vero cells were infected with HSV-2 at a multiplicity of infection (MOI) of 0.001 PFU/cell. Serial dilutions of 25*R*,26OHC or PFMs were added on Vero cells at the same time of viral inoculum, 18 h after viral inoculum or 24 h before inoculum. Untreated control samples (100% of infection) were prepared by treating cells with culture medium supplemented with equal volumes of DMSO, DMSO:EtOH (1:1), DMSO:EtOH (2:1), or EtOH, ranging from 1.7% (v/v) to 0.0032% (v/v) in cell media. 24 h after the infection, the antiviral activity of the compounds was assessed by standard plaque method. Viral plaques were stained by an indirect immunoperoxidase staining procedure using the HSV-2/HSV-1 anti-gD monoclonal antibody, as described in supplementary materials. The same experimental design was used for the HSV-1 antiviral assays, except that cells were fixed 48 h post-infection. Blockade of viral infectivity is expressed as mean % ± standard deviation (SD).

2.4. Cell viability assay

To assess the eventual cytotoxicity of the analogs, cell viability assays were performed. Vero cells were treated in the same conditions used for the antiviral assays. After 24 or 48 h of incubation, cytotoxicity was determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI, USA), following the manufacturer's instructions. Viability of treated cells was expressed as a percentage relative to untreated cells.

2.5. Yield reduction assay

In this set of experiments, cells were infected with HSV-2 (at a MOI of 0.001 PFU/cells) in presence of a fixed concentration (16.7 μM) of 25*R*,26OHC or of PFMs. Alternatively, cells were treated 2 h, 4 h or 6 h post-infection. Untreated control samples were prepared for PFMs or 25*R*,26OHC itself, respectively by treating cells with culture medium supplemented with equal volumes of DMSO, DMSO:EtOH (1:1), or DMSO:EtOH (2:1) (ranging from 0.13% to 0.26% [v/v]) or EtOH (corresponding to 5.57% [v/v]) in cell media. When the full CPE developed, cells and supernatants were harvested, frozen and thawed three times, and clarified by low speed centrifugation. The viral progeny thus collected was titrated to the non-inhibitory concentration of the different antiviral molecules, and inoculated on freshly seeded Vero cells. In a second set of experiments, extracellular and intracellular infective viral progeny were harvested separately, at 24 h after inoculum. The titer of the viral progeny was determined by the standard plaque method – by staining viral plaques by indirect immunoperoxidase procedure as described above - and expressed as PFU/ml.

2.6. Single-cycle antiviral assessment

The effect of 25*R*,26OHC, PFM064, PFM067, PFM069, or acyclovir on HSV-2 syncytia formation was assessed by a single-cycle antiviral test. More in detail, in a first set of experiments Vero cells were infected for 12 h with HSV-2 in the presence of serial dilutions of the molecules, to allow only one single replicative cycle. Alternatively, cells were treated for 24 h with scalar dilutions of 25*R*,26OHC, PFM064, PFM067, PFM069, or acyclovir and then infected with HSV-2 for 12 h. The antiviral activity of the compounds was assessed by standard plaque method; viral plaques were stained by indirect immunoperoxidase procedure as described above.

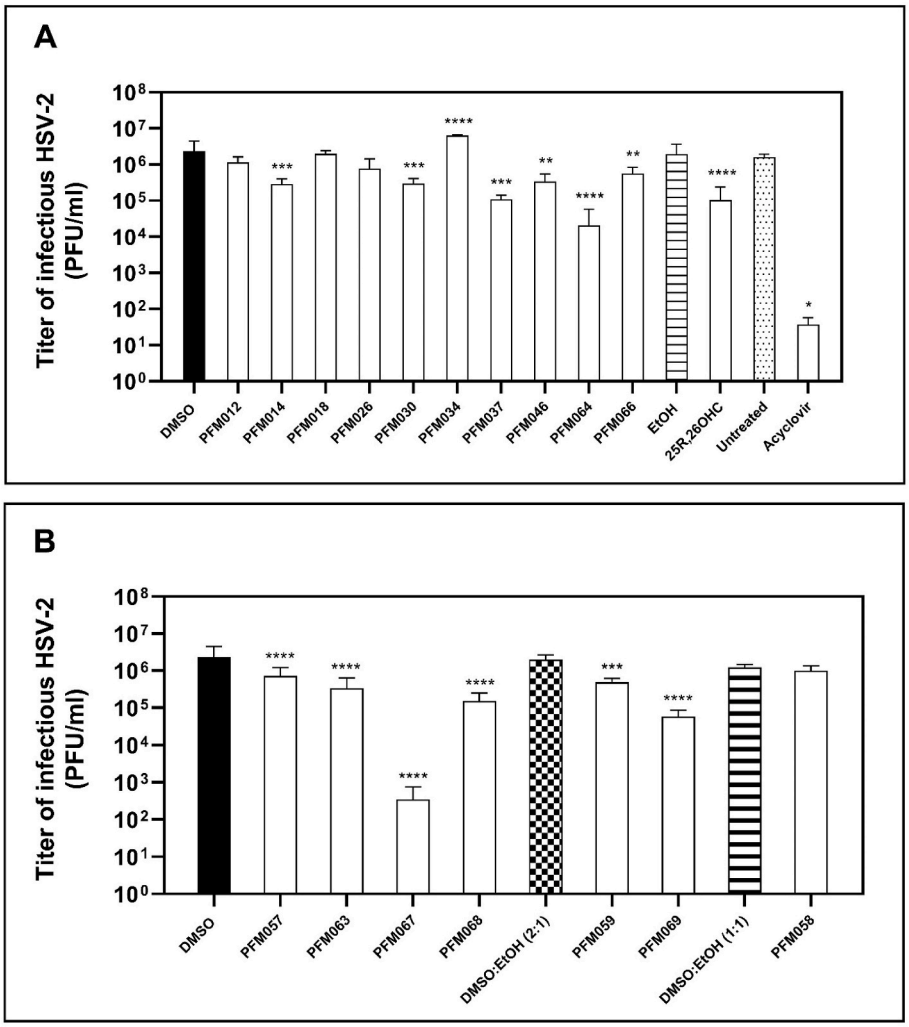

Fig. 3. Virus yield reduction assay with the synthetic oxysterols of the first library (namely PFM012, PFM014, PFM018, PFM026, PFM030, PFM034, PFM37, PFM046 PFM064, PFM066), 25*R*,26OHC, or acyclovir are depicted in panel A, while the ones of the second library (namely PFM057, PFM058, PFM059, PFM063, PFM067, PFM68, and PFM069) are represented in panel B. Cells were infected with HSV-2, in presence of a fixed concentration (16.7 μM) the different compounds. Untreated control samples were prepared for PFMs or 25*R*,26OHC, by treating cells with culture medium supplemented with equal volumes of DMSO, DMSO:EtOH (2:1), DMSO:EtOH (1:1), or EtOH, according to the solvent used to dissolve the molecules. When the cytophatic effect involved the whole monolayer of untreated samples, cells and supernatants were harvested and titrated. The results are means and SD for two independent experiments, each one performed in triplicate. $^{**}p_{ANOVA} < 0.01$; $^{***}p_{ANOVA} < 0.001$; $^{****}p_{ANOVA} < 0.0001$.

2.7. Electron microscopy experiments

To investigate the effect of 25*R*,26OHC and PFM067 on HSV-2 replicative cycle, transmission electron microscopy experiments were performed. Briefly, Vero cells were seeded on 60 mm plates. 24 h later, cells were infected at a MOI of 0.1 PFU/cell for 16 h and treated with 25*R*,26OHC or PFM067 at respective EC_{90}s. Pellet from both treated/infected and non-treated/infected Vero cells were harvested and manipulated as described in supplementary materials. Each sample was analyzed using a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a Mega-View-III digital camera and a Soft-Imaging-System (SIS, Münster, Germany) for the computerized acquisition of the images.

2.8. Immunofluorescence experiments

The intracellular localization of viral proteins was assessed by immunofluorescence and laser-scanning confocal microscopy. Vero cells were seeded in 24-well plate on glass coverslip at a density of 110,000 cells per well. 24 h later, cells were infected at a MOI of 0.1 PFU/cell for 11 h and treated with 25*R*,26OHC or PFM067 at respective EC_{90}s. Cells were then fixed with 4% paraformaldehyde, washed twice with sterile-filtered phosphate-buffered saline (PBS) and subjected to indirect immunofluorescence as described in supplemental materials. The coverslips were mounted and observed by a confocal fluorescence microscope (LSM800, Carl Zeiss, Jena, Germany) with a 63× objective.

2.9. Immunoblotting

The effect 25*R*,26OHC or PFM067 on HSV-2 early and late protein expression was tested by Western blot analysis. Briefly, cells were seeded in 6-well plate at a density of 150,000 cells per well. 24 h later, cells were infected at a MOI of 0.1 PFU/cell and treated with 25R,26OHC or PFM067 at respective EC90s. Control experiments were performed by treating cells with acyclovir.

24 h later, cells were lysed in denaturating conditions and a cytoplasmic proteins extraction was performed; more in detail, extracted proteins were denatured by boiling for 5 min, then separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-12% PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked overnight at 4 °C with PBS, 0.1% Tween 20, and milk powder 10%. Membranes were then incubated with primary antibodies for 1 h at 37 °C, washed 4 times with PBS, 0.1% Tween 20, and 15% milk powder, then incubated for 1 h at 37 °C with secondary antibodies coupled with HRP and washed extensively prior to developing by the enhanced chemiluminescence method.

2.10. Statistical analysis

For plaque reduction assays, where possible, half-maximal antiviral effective concentration (EC50) and 90% effective concentration (EC90) values were calculated by regression analysis using the dose-response curves generated from the experimental data using GraphPad PRISM 7

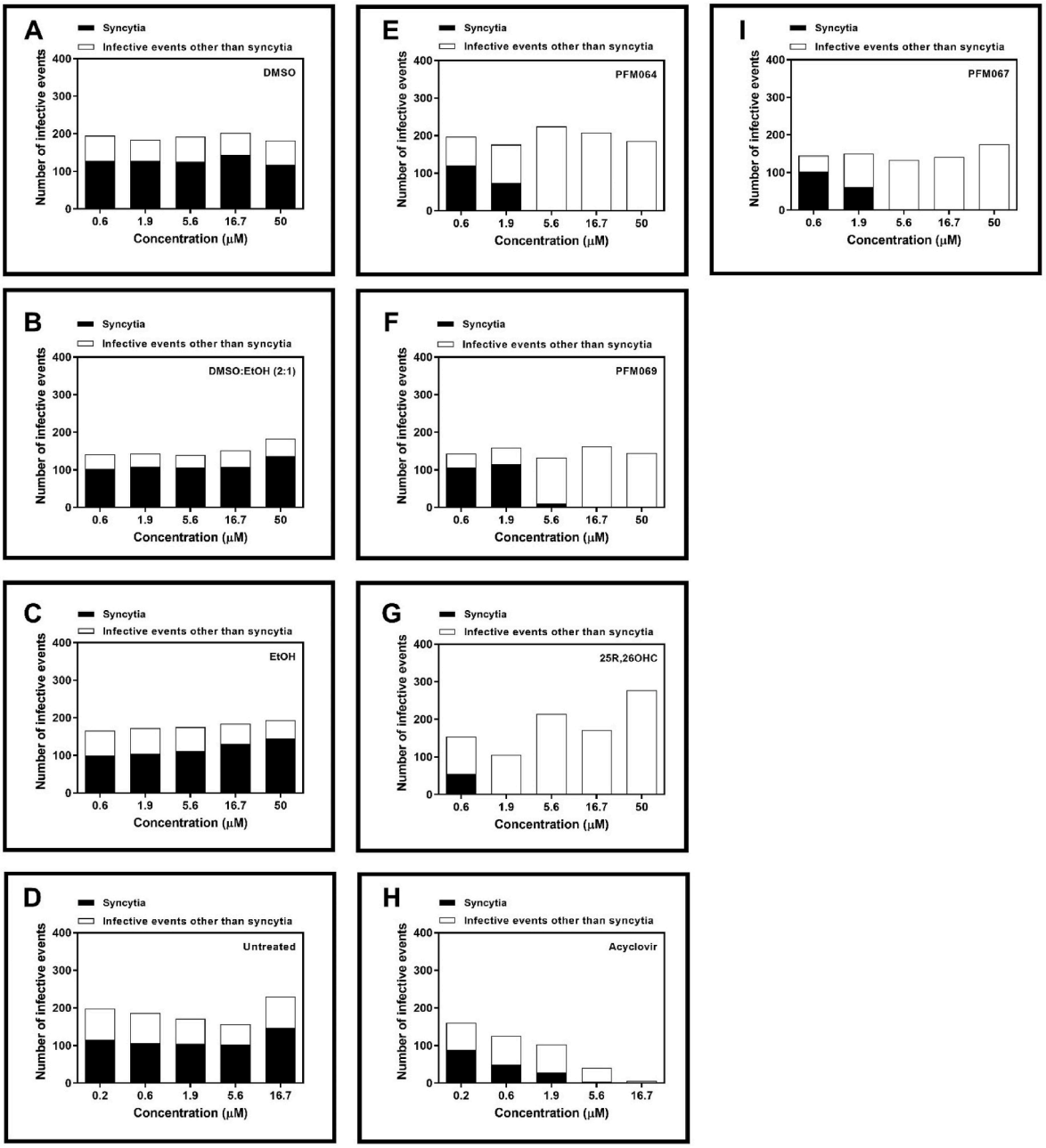

Fig. 4. Plaque reduction assays performed by fixing non-treated (A, B, C, and D) or treated (E, F, G, H, and I) infected cells at 12 h after infection. Each panel represents a contingency graph, in which the height of each bar represents the total number of infective events counted at each concentration. Each bar is furtherly divided in a black bar that indicates the number of syncytia, and in a white bar that indicates the number of non-complete infective events (named "infective events other than syncytia"). The results are mean values for triplicates from two independent experiments.

(GraphPad Software, San Diego, CA, USA). For cell viability assays, the 50% cytotoxic concentration (CC50) was determined using the same method and software. Where possible, a selectivity index (SI) was calculated by dividing the CC50 by the EC50 value. One-way analysis of variance (ANOVA), followed by Bonferroni test, was used to assess the statistical significance of the differences between treated and untreated samples, where appropriate. Significance was set at the 95% level.

3. Results

3.1. Effect of tested molecules on HSV-2 infectivity

When added on cells for 24 h before infection, both PFM014 and PFM066 inhibited viral infectivity (Fig. 1B and J), but with low SIs (Table 1). Both PFM012 and PFM064 exerted antiviral activity in a dose-response fashion against HSV-2 to maxima of inhibition of 100% (Fig. 1A and I), with favorable SIs, regardless of the timing of treatment. The other PFMs of this series showed limited or no antiviral activity (Fig. 1B-C-D-E-F-G-H-J), or unfavourable SIs (Table 1). 25*R*,26OHC inhibited HSV-2 infectivity in a dose-response fashion, with similar EC_{50}s whether incubated on cells 24 h before infection or added during viral inoculum (Fig. 1K). By contrast, acyclovir inhibited HSV-2 infectivity only when the treatment was performed during infection (Fig. 1L), consistently with its mechanism of action.

Among the derivatives belonging to the second series, PFM058 was the only one lacking antiviral activity at any tested concentration, regardless of the time of addition on the cells (Fig. 2B), while PFM057 showed only a weak efficacy at 50 μM (Fig. 2A). PFM063 inhibited viral

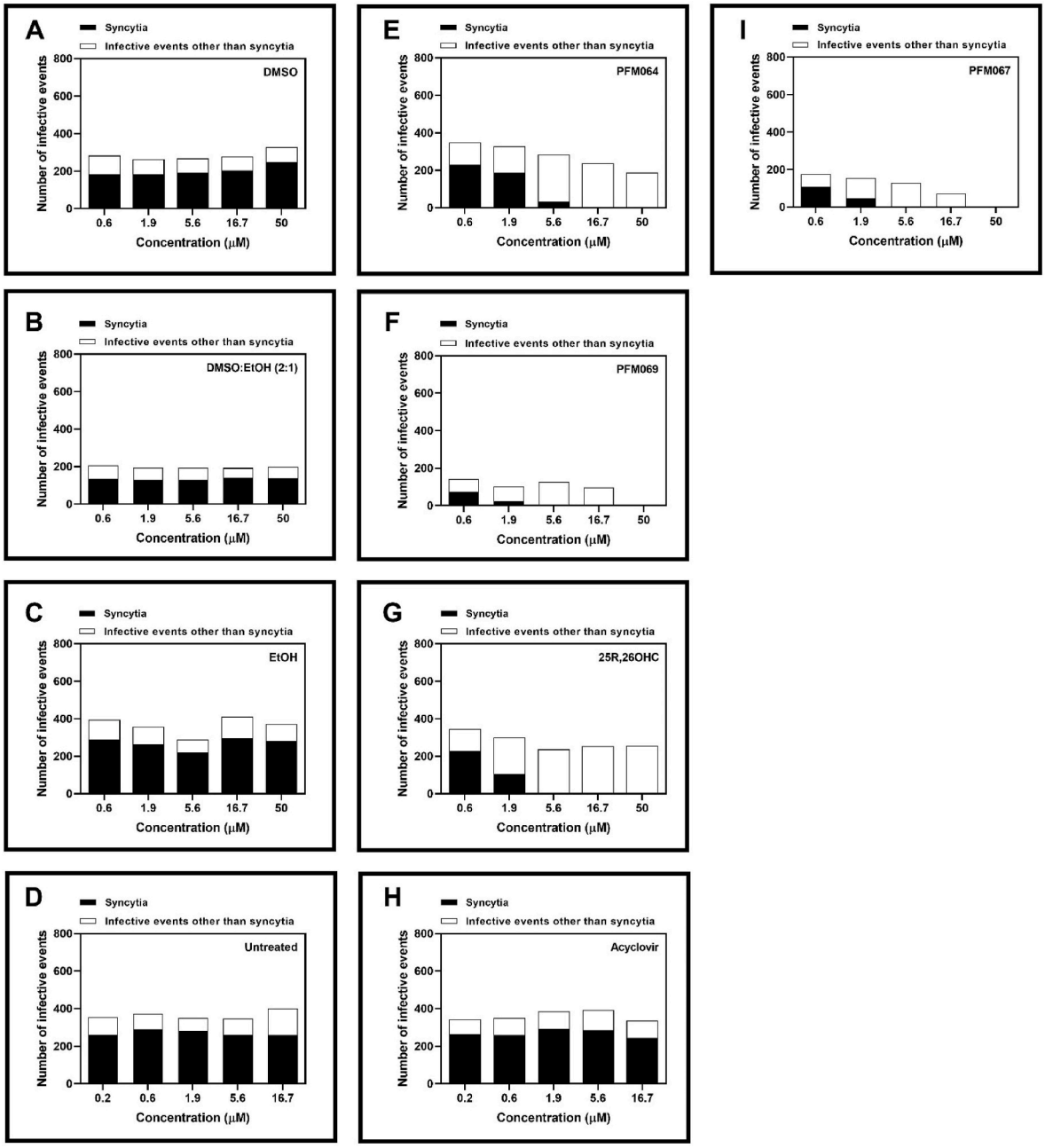

Fig. 5. Plaque reduction assay on Vero cells treated 24 h before HSV-2 inoculum. Non-treated (A, B, C, and D) or treated (E, F, G, H, and I) cells were fixed after 12 h of virus infection. Each panel represents a contingency graph, in which the height of each bar represents the total number of infective events counted at each concentration. Each bar is furtherly divided in a black bar that indicates the number of syncytia, and in a white bar that indicates the number of non-complete infective events (named "infective events other than syncytia"). The results are mean values for triplicates from two independent experiments.

infectivity to 100% only at 50 μM with both treatment protocols (Fig. 2D), but it was endowed with low SIs (Table 2). PFM059 and PFM068, while showing antiviral activity in a dose-dependent fashion, failed to inhibit viral infectivity to maxima of 100% in the range of concentrations tested (Fig. 2C and F). Notably only PFM067 and PFM069 exerted antiviral activity in a dose-response fashion against HSV-2 to a maximum of inhibition of 100%, with favorable EC_{50}s and SIs (Fig. 2E and G Table 2).

3.2. *Effect of tested molecules on HSV-2 yield*

When tested with viral yield reduction assays, PFM012 did not significantly inhibit ($p_{ANOVA} > 0.05$) the production of viral progeny (Fig. 3A). PFM064 confirmed as the most effective analog among those tested, being able to significantly ($p_{ANOVA} < 0.0001$) inhibit the production of viral progeny of about 100 times compared with untreated control (Fig. 3A). It is worth noting that, with the exception of PFM012, PFM018, PFM026, and PFM034, all the compounds tested were endowed with a slight but significant ($p_{ANOVA} < 0.001$) efficacy in inhibiting the yield of HSV-2 (Fig. 3A).

Of note, in this experimental setting 25R,26OHC inhibited significantly ($p_{ANOVA} < 0.0001$) viral yield by approximately 13 times (Fig. 3A). As expected, acyclovir significantly ($p_{ANOVA} < 0.05$) inhibited the production of HSV-2 infective progeny (Fig. 3A).

Consistently with the results of plaque reduction assays, PFM067 and PFM069 were able to significantly ($p_{ANOVA} < 0.0001$) inhibit the production of viral progeny of about 6000 and 34 times respectively, in comparison to untreated control (Fig. 3B). Of note, with the exception of

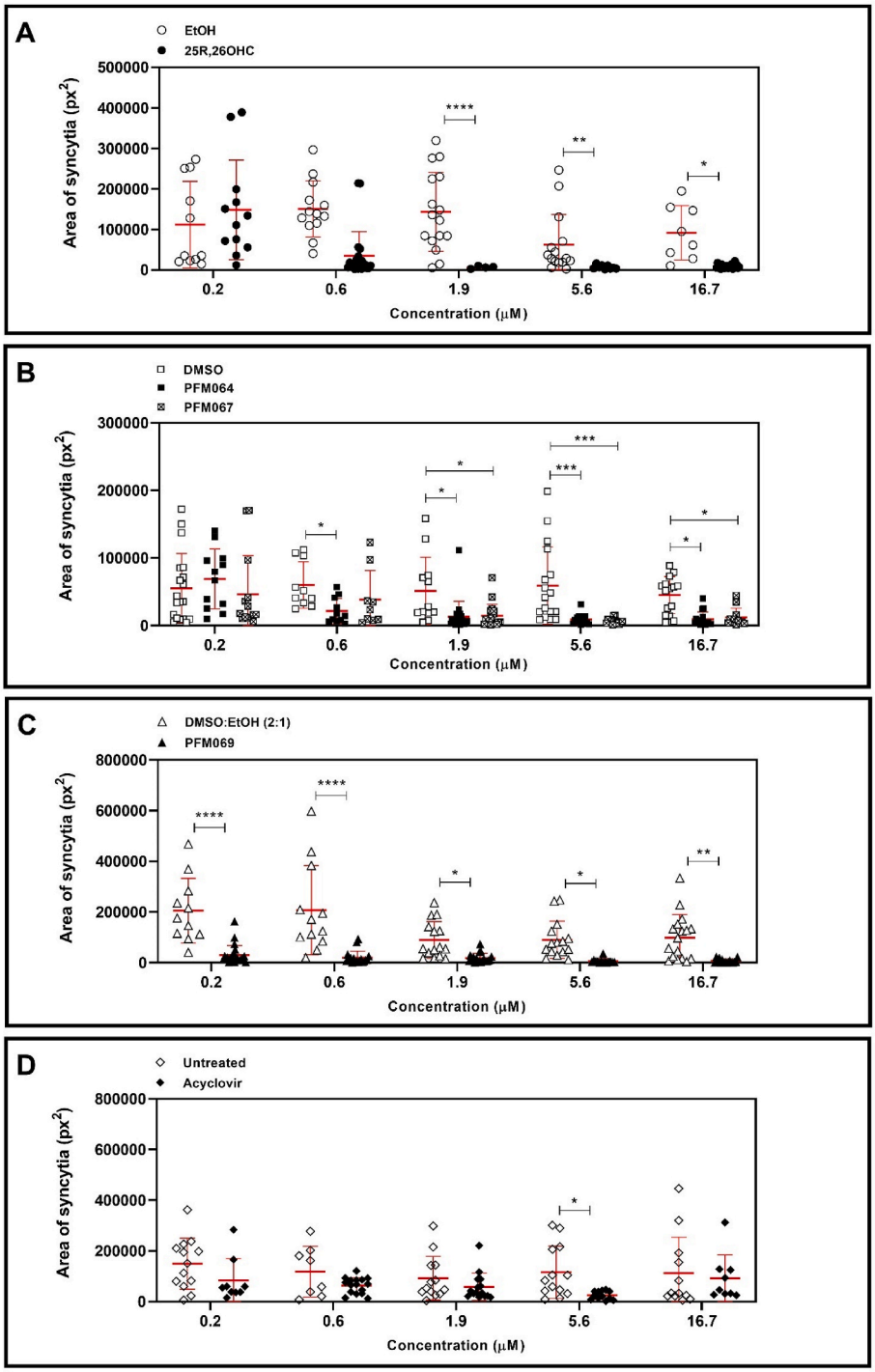

Fig. 6. Time-of-addition experiment. Cells were inoculated with HSV-2, then incubated for 18 h at 37 °C. After this time, cells were treated for additional 6 h with serial dilutions of PFM064, PFM067, PFM069, 25*R*,26OHC, or acyclovir. Untreated control samples were prepared for PFMs or 25*R*,26OHC, by treating cells with culture medium supplemented with equal volumes of DMSO, DMSO:EtOH (2:1), or EtOH, according to the solvent used to dissolve the molecules. Cells were then fixed with acetone:methanol (1:1) and gD positive syncytia were stained by indirect immunostaining. The area of syncytia was assessed by means of ImageJ software. On Y axis, the area of syncytia is expressed as squared pixels (px (Koelle and Wald, 2000)). Each point represents the area of one single syncytium. For each condition, a minimum of 8 to a maximum of 23 syncytia were counted. These result from two independent experiments, each one performed in duplicate. *$p_{ANOVA} < 0.05$; **$p_{ANOVA} < 0.01$; ***$p_{ANOVA} < 0.001$; ****$p_{ANOVA} < 0.0001$.

PFM058, all the tested analogs showed a significant ($0.0001 < p_{ANOVA} < 0.001$) efficacy in inhibiting the yield of HSV-2 (Fig. 3B).

Based on these data, PFM064, PFM067, and PFM069 were selected for further experiments. In particular, to further investigate their safety, the effect of the three molecules on cell viability was tested on uninfected cells by performing MTS assays at 24 or 48 h post treatment; as shown in Fig. 1S (panel A and B), a decrease in cell viability is detectable only at 200 μM. To exclude that the antiviral effect was determined by the ability of the molecules to selectively kill the infected cells, the viability assays were also performed on cells infected with HSV-2 under the same conditions used in the plaque assays; as shown in Fig. 1S (panel C) the results are superimposable to those obtained on uninfected cells, with a partial reduction of cell viability detected only at 200 μM. Representative images of the cell monolayers on which the viability assays were performed are collected in Figs. 2S, 3S and 4S).

3.3. Effect of 25R,26OHC and PFMs on HSV-2 replicative cycle

In a second of experiments, the cells were infected and contextually treated with 25*R*,26OHC, PFM064, PFM067, PFM069, or acyclovir, or treated for 24 h with the same compounds prior to virus infection; in each set, viral replication was blocked 12 h after infection, by fixing cell monolayers with cold acetone-methanol (50:50) upon the appearance of the first gD-positive syncytia. In this case, a count was performed not

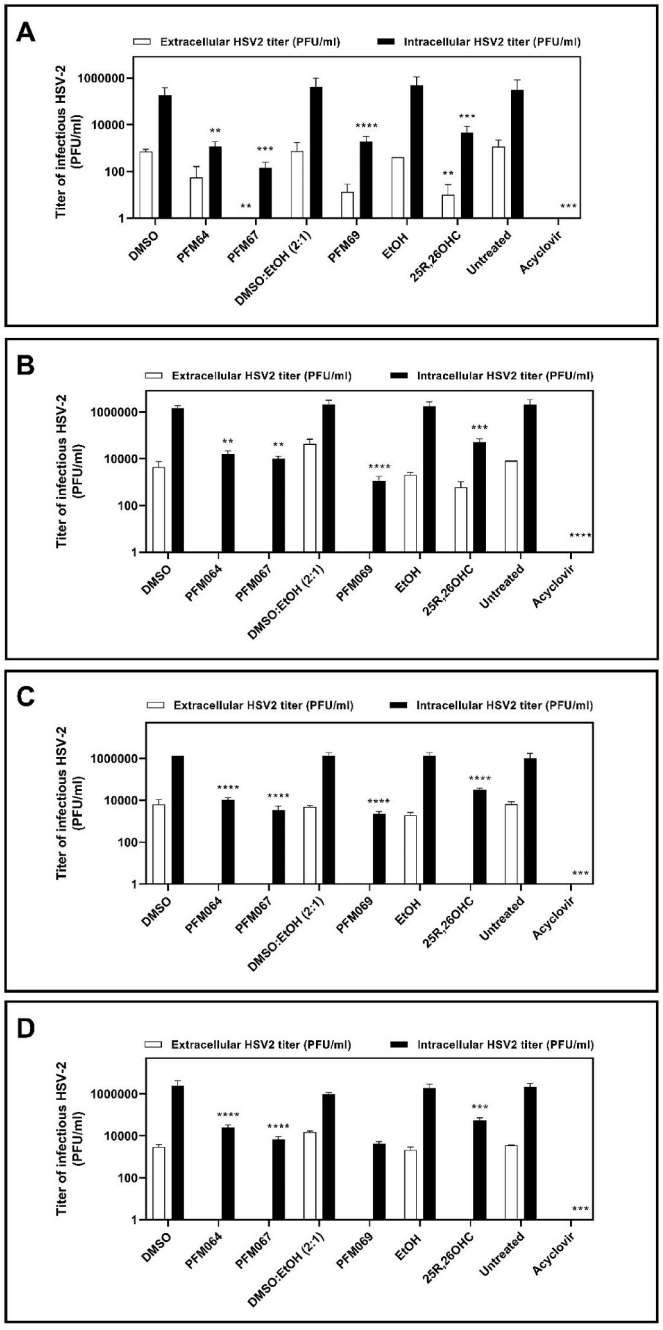

Fig. 7. Virus yield reduction assay. Cells were infected with HSV-2, in presence of a fixed concentration (5.6 μM) of PFM064, PFM067, PFM069, 25*R*,26OHC, acyclovir (panel A). Alternatively, cells were treated 2 h post-inoculum (panel B), 4 h post-inoculum (panel C), or 6 h post-inoculum (panel D). Untreated control samples were prepared for PFMs or 25*R*,26OHC, by treating cells with culture medium supplemented with equal volumes of DMSO, DMSO:EtOH (2:1), or EtOH, according to the solvent used to dissolve the molecules. 24 Hours post-inoculum, intracellular and extracellular infective virions were harvested and titrated separately. The results are means and SD for two independent experiments, each one performed in triplicate. $^{**}p_{ANOVA} < 0.01$; $^{***}p_{ANOVA} < 0.001$; $^{****}p_{ANOVA} < 0.0001$.

only of syncytia (Fig. 5S, panels A-B-C), but also of single gD-positive cells (Fig. 5S, panels D and E). The aim of these experiments is to capture a picture of all the infectious events in each sample, both the complete ones (i.e. the syncytia), and the non-complete ones (i.e. the single gD-positive cells). In the untreated controls [i.e. DMSO, DMSO:EtOH (2:1), EtOH, and fresh medium; Fig. 4A–D and 5A-D] most part of infective events was represented by syncytia, while incomplete infectious events represented a smaller fraction. By contrast, the results showed that PFM064, PFM069, 25*R*,26OHC, acyclovir, and PFM067 significantly ($p_{ANOVA} < 0.001$) inhibited the formation of syncytia at concentrations ranging from 1.9 μM to 50 μM (Fig. 4, panels E, F, G, H and I and Fig. 5, panels E, F, G, H and I). Interestingly, when infection was run simultaneously to the treatment with 25*R*,26OHC or the selected PFMs, it was still possible to count a number of incomplete infectious events, at any tested concentration (Fig. 4, panels E, F, G, and I). By contrast, when cells were treated with the same molecules one day before infection, it could be observed a reduction of both syncytia and incomplete infective events, with PFM064, PFM069, and PFM067 showing a dose-response trend of such effect (Fig. 5, panels E, F, and I). The differences in the number of syncytia and non-complete infective events between treated and non-treated samples at each concentration ranging from 1.9 μM to 50 μM were significant ($p_{ANOVA} < 0.001$). As mentioned above, the treatment with acyclovir inhibited the total number of infective events (i.e. both the number of syncytia and non-complete infective events) at concentrations ranging from 16.7 μM to 1.9 μM (Fig. 4H) when cells are treated during viral replication, but resulted ineffective over a pre-treatment regimen (Fig. 5H). In a second set of experiments, 25R,26OHC, PFM064, PFM067, or PFM069 were added on cells 18 h after HSV-2 inoculum. In this case, the treatment did not reduce the number of syncytia (Fig. 6S), but their dimensions in treated wells were significantly ($0.0001 < p_{ANOVA} < 0.05$) reduced in a dose-response fashion, if compared to the untreated controls (Fig. 6A–C). More in detail, in the samples treated with 25R,26OHC or the selected PFMs, the syncytia appeared smaller and surrounded by a "crown" of single gD-positive cells; representative images of this phenomenon are depicted in Fig. 7S. This result suggests that the treatment was able to prevent cell-cell fusion phenomena essential for HSV-2 replication. Notably, the treatment with acyclovir did not reduce the dimensions of HSV-2-induced syncytia (Fig. 6D).

These results show that both 25R,26OHC and the selected PFMs inhibit the HSV-2-induced formation of syncytia, by targeting a step of the replicative cycle subsequent to the transcription and translation of the late genes (such as gD). For this reason, we evaluated the effects of the treatment on the processes that characterize the late stages of viral replication cycle.

Firstly, we tested the ability of the selected molecules to inhibit the assembly or release of the viral progeny. To do this, we performed viral yield reduction assays by separately collecting and titrating the progeny released in the cell supernatant (extracellular HSV-2) and infective virions within the cells (intracellular HSV-2). The results depicted in Fig. 7A-B-C-D demonstrate that 25*R*,26OHC, PFM064, PFM067, and PFM069 were able to significantly ($0.0001 < p_{ANOVA} < 0.01$) inhibit the viral progeny titer present in the host cells. PFM067 and 25*R*,26OHC were also able to significantly ($p_{ANOVA} < 0.01$) inhibit the viral titer present in cellular supernatant. As expected, acyclovir inhibited both the titer of extracellular and intracellular viral progeny.

Transmission electron microscopy images showed that HSV-2 capsids were present in the nuclei of both untreated and PFM067-or 25*R*,26OHC-treated cells (Fig. 8A-B-C), being able to interact with the nuclear membrane (Fig. 8D-E-F). By contrast, extra-nuclear viral particles were visible only in the non-treated/infected cells (Fig. 8G-H-I). Interestingly, immunofluorescence experiments showed that viral structural proteins (namely the pericapsidic glycoprotein gH/gL, gB, gD, and the capsidic protein ICP5) were expressed both in non-treated and in treated cells (Fig. 9); nevertheless, in PFM067-and 25*R*,26OHC-treated cells, viral glycoproteins mainly colocalized with the marker of cis-Golgi apparatus GM130 (Fig. 9); by contrast, in non-treated samples they were widely present in the cytoplasm of syncytia (in addition to being present in the Golgi apparatus; Fig. 9). Regarding ICP5, it was present only in the nuclea of PFM067-and 25*R*,26OHC-treated cells, while in the non-treated cells it was present in nuclea, cytoplasm and Golgi of the syncytia (Fig. 9). Finally, in the acyclovir-treated cells no structural protein

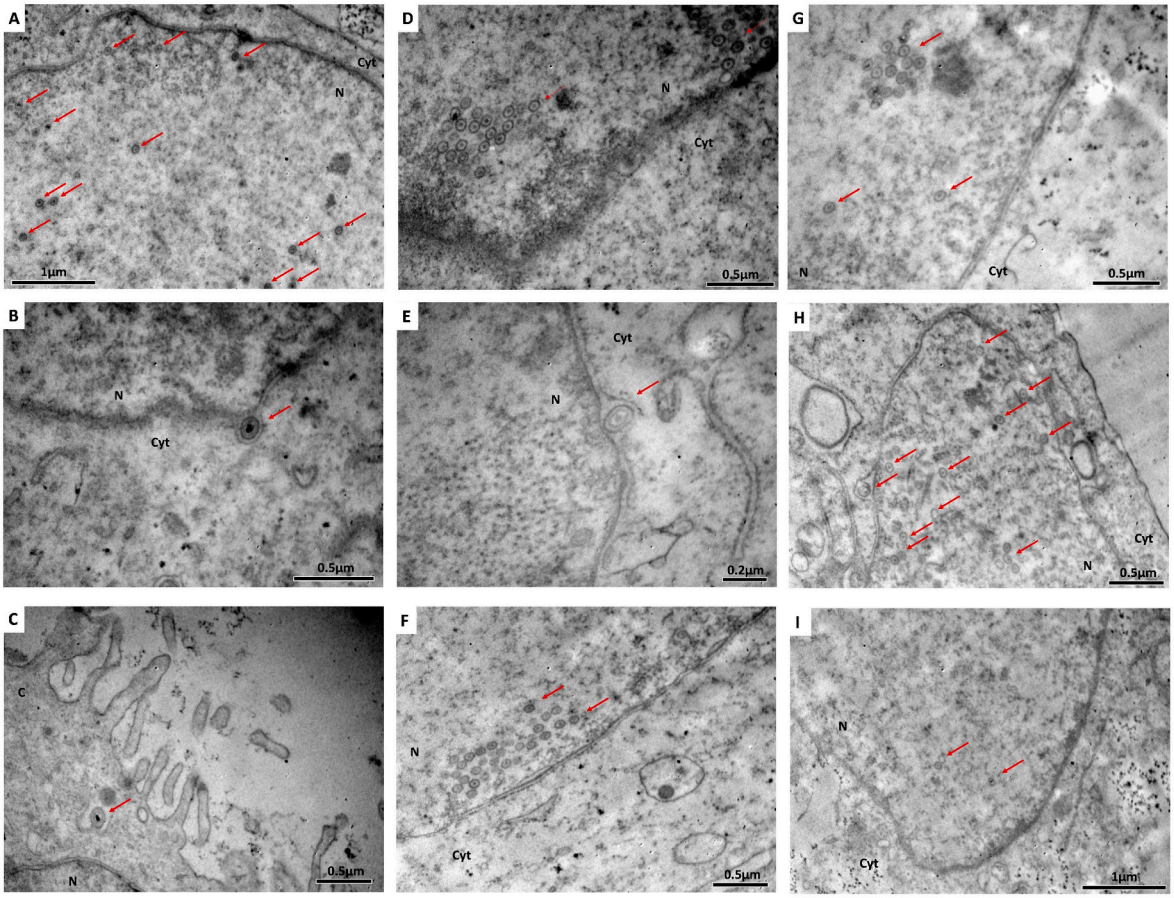

Fig. 8. Representative transmission electron microscopy images of HSV-2-infected Vero cells. Panels A, B, and C depict untreated-infected Vero cells at 16 h post-infection. Panels D, E, and F represent PFM067-treated and HSV-2-infected cells. Panels G, H, and I depict 25*R*,26OHC -treated and HSV-2-infected cells. N: nucleus; Cyt: cytoplasm. µm: micrometers. Red arrows indicate capsids and virions.

of the virus was detectable (Fig. 9).

These results are consistent with the ones of Western blot analysis; they show that in the cytoplasm of treated cells ICP5 is less represented than that present in untreated infected controls, while gB and gD are expressed comparable to untreated controls (Fig. 10). Notably, the early viral protein ICP8 is equally expressed in both treated and non-treated infected cells (Fig. 10), thus demonstrating that the virus is able to complete the early phases of its replicative cycle in the treated cells, and that, therefore, 27OHC or PFM067 do not inhibit the early phases of the replicative cycle (i.e. virus-cell entry) in host cells.

Taken together, these results suggest that 25*R*,26OHC and the selected PFMs inhibit late stages of viral replication, such as the assembly of newly formed virions and the fusogenic capacity of HSV-2.

In order to further validate the potential of PFM067 to be a suitable lead compound, we tested (a) the ability to inhibit the replication of another herpes virus, namely HSV-1 and (b) its ability to inhibit HSV-2 replication in a less surrogate context than Vero cells, i.e. MRC-5 diploid human fibroblasts. As depicted in Fig. 11A–B, PFM067 and 25R,26OHC inhibit HSV-1 infectivity in a dose-response fashion to a maximum of 100% of inhibition, either when added before or during infection, with EC50s in the micromolar range (Table 3). As expected, acyclovir is only effective when added during HSV-2 inoculation (Fig. 11C). Moreover, when tested on MRC-5, both molecules confirm their ability to inhibit HSV-2 replication (Fig. 11D–E), although, in this experimental setting, 25R,26OHC is less effective when added on cells during viral inoculum. Consistently with its mechanism of action, acyclovir is active only when added on MRC-5 cells during HSV-2 infection (Fig. 11F). Notably, the EC50 and EC90 of PFM067 assessed on MRC-5 cells are comparable with the ones obtained on Vero cells (Table 4).

4. Discussion

Whereas the antiviral activity of endogenous oxysterols is well documented, the same cannot be said for synthetic oxysterols. This lack of data along with the availability of an in-house synthesized, focused oxysterol library, consisting of about 100 items (named PFMs), prompted us to select some elements of such library to assay their activity against HSV-2. Within the library [originally designed to be a collection of potential Liver X Receptors (LXR) modulators], 10 derivatives (Fig. 12) were first picked following the criterion of the maximum heterogeneity sampling both in terms of structural diversity and biological profile towards LXRs. The results of the in vitro antiviral screening evidenced PFM064 as the most effective anti-HSV-2 derivative of this first series. To this point the library's items were re-examined according to a different criterion, that is the selection of the members as much as possible structurally similar to PFM064: 7 novel items were therefore identified (Fig. 13), screened, and two other effective antiviral derivatives, namely PFM067 and PFM069, were selected. Interestingly, we observed a very close structural similarity among the three active derivatives, all characterized by the presence of a dimethylamido moiety at the side-chain level. The previous data of the LXR-activity of the compounds evidenced a non-correspondence between the antiviral properties and the ability of modulating LXRs. Indeed, PFM064, PFM067, and PFM069 were not the best ones in terms of LXR activity. Moreover, PFM014, a very effective compound as LXR agonist, showed no antiviral efficacy, whereas PFM012, completely inactive at LXR, exerted antiviral activity even if it did not significantly inhibit the production of viral progeny.

25*R*,26OHC, PFM064, PFM067, and PFM069 were able to inhibit the

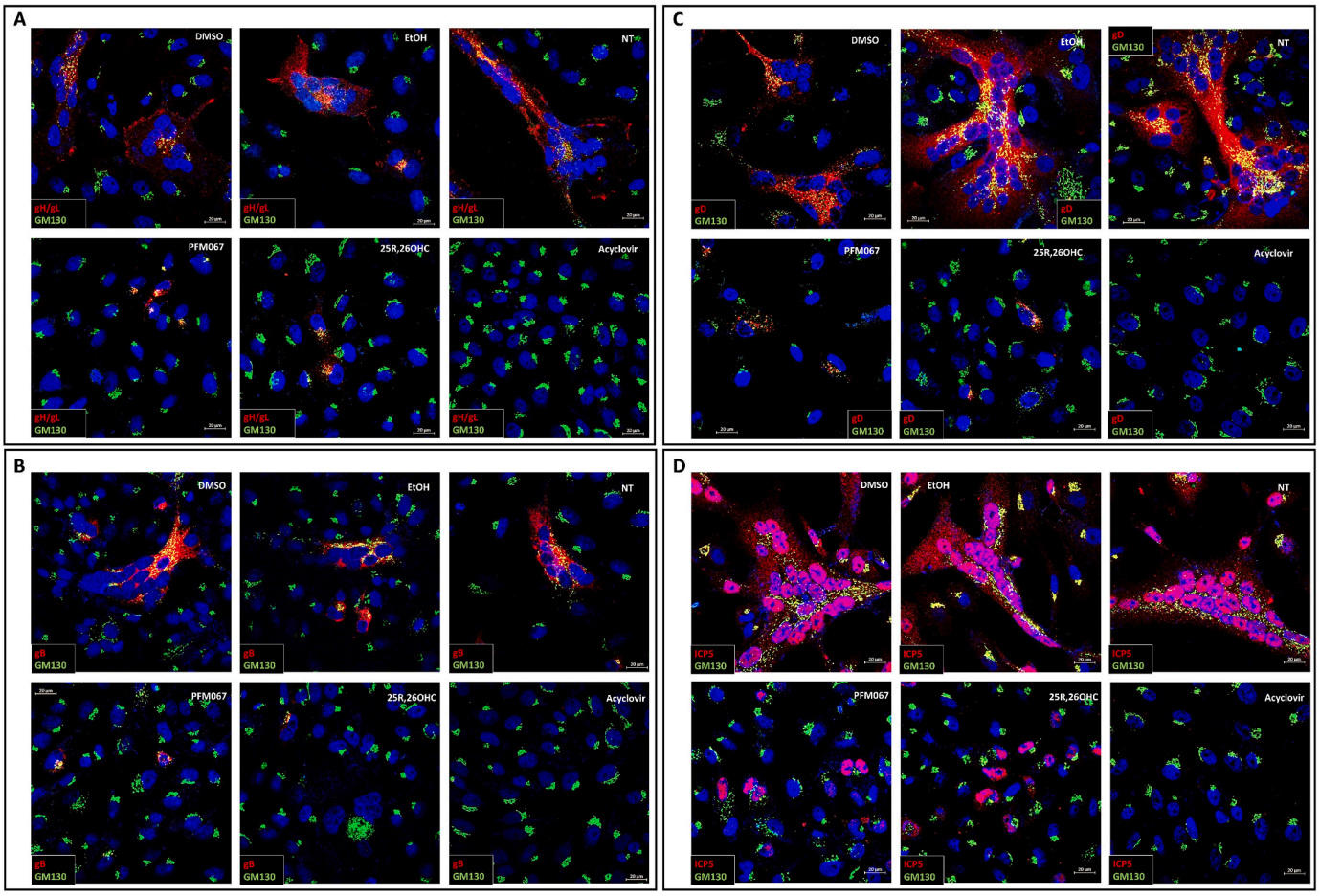

Fig. 9. Indirect immunofluorescence of HSV-2-infected Vero cells. Cells were inoculated and treated with PFM067, 25*R*,26OHC, or acyclovir for 11 h. Untreated control samples (100% of infection) were prepared by treating cells with culture medium supplemented with equal volumes of DMSO, EtOH, or sterile water (labeled as NT panels). All monolayers were stained with a GM130-specific antibody (targeting the cis-Golgi compartment; panels A–D), and counterstained with antibodies specific for HSV-2 structural proteins, namely gH/gL (panel A), gB (panel B), gD (panel C), or ICP5 (panel D). Nuclea were stained with DAPI.

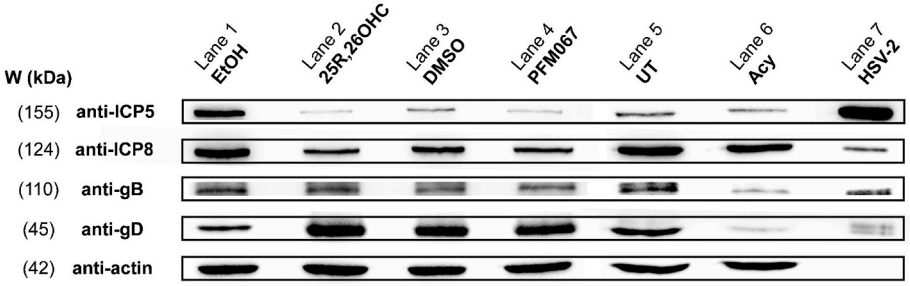

Fig. 10. Effect of 25*R*,26OHC, PFM067, or acyclovir treatment on the expression of HSV-2 early and late proteins, namely ICP5, ICP8, gB, and gD. Actin blotting was used as normalization. Cell-purified HSV-2 was also loaded on acrylamide gel (lane 7) to provide a molecular weight benchmark. EtOH: ethanol; DMSO: dimethyl sulfoxide; UT: untreated control; Acy: acyclovir.

production of HSV-2 progeny either the treatment was performed at the same time as the infection, or 24 h before performing the viral inoculation. These data are consistent with results obtained previously, which demonstrate that 25OHC and 25*R*,26OHC are effective against other viruses (namely rhinovirus, rotavirus, papillomavirus, and HSV-1) even when they are added on cells before viral inoculum, by targeting cellular proteins co-opted by viruses during replication, mainly involved in the regulation of membrane composition (Cagno et al., 2017; Civra et al., 2014, 2018, 2020; Liu et al., 2013; Pezacki et al., 2009). This feature is interesting in relation to the lower selective pressure exerted by host-targeting antivirals compared to direct-acting antivirals, and the consequent lower ability to select resistant variants (Civra et al., 2022).

Although further studies are needed to uniquely identify the mechanism of action of the tested molecules and their antiviral molecular target(s), in this study we have shown that 25*R*,26OHC, PFM064, PFM067, and PFM069 are able to target a different step of the replication cycle respective to acyclovir. The results have demonstrated the ability of these molecules to inhibit both HSV-2-induced cell-cell fusion and the production of the intracellular infective progeny. Interestingly, 25*R*,26OHC and PFM067 (which was selected for deeper studies as the most effective PFM) do not inhibit the production viral structural proteins, but rather sequester HSV-2 fusogenic glycoproteins in the cis-

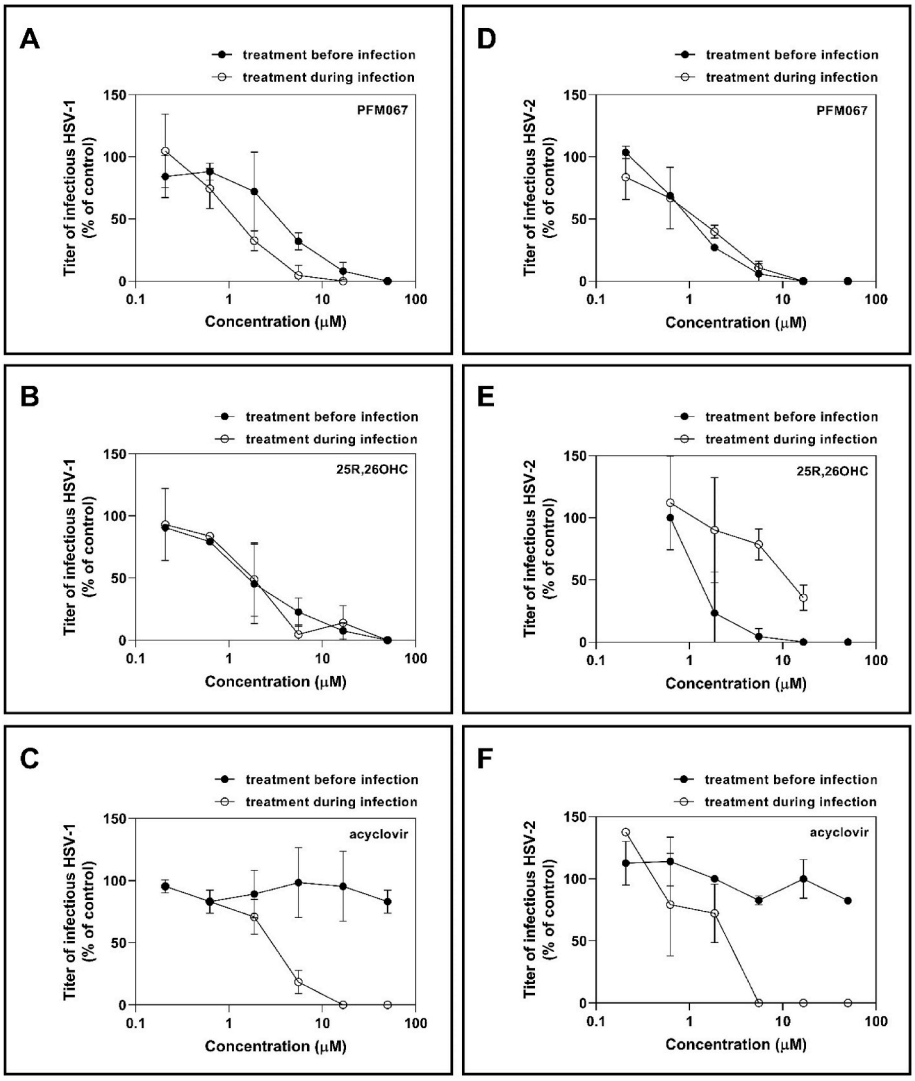

Fig. 11. Plaque reduction assays. Antiviral activity of PFM067, 25*R*,26OHC or acyclovir was tested against HSV-1 on Vero cells (panels A, B, and C respectively), and against HSV-2 on MRC5 cells (panels D, E, and F, respectively). Cells were treated for 24 h with increasing concentrations of tested molecules and then infected with HSV-1 or HSV-2; alternatively, cells were infected in presence of increasing concentrations of tested molecules. Viral infections were detected 48 h after infection for HSV-1 or 24 h after infection for HSV-2, by indirect immunoperoxidase staining. The percentage infection was calculated by comparing treated and untreated wells. The results are means and SD for two independent experiments, each one performed in triplicate.

Table 3
Antiviral activity of PFM067 and 25R,26OHC against HSV-1.

ID	Treatment protocol	EC50[a] (μM) – 95% C.I.[b]	EC90[c] (μM) – 95% C.I.	CC50[d] (μM) – 95% C.I.	SI[e]
PFM067	*Before infection*	3.3 (2.2–4.8)	16.4 (7.0–38.1)	>200	>60.6
	During infection	1.2 (0.9–1.6)	4.0 (2.3–6.9)		>166.7
25R,26OHC	*Before infection*	1.7 (1.1–2.6)	12.2 (5.5–26.9)	>1350	>794.1
	During infection	1.7 (1.0–2.8)	6.1 (2.2–17.1)		>794.1
acyclovir	*Before infection*	n.a.	n.a.	>200	n.a.
	During infection	2.7 (2.1–3.3)	9.1 (5.8–14.5)		>74.1

n.a. Not assessable.
[a] EC_{50} half-maximal effective concentration.
[b] CI confidence interval.
[c] EC_{90} 90% effective concentration.
[d] CC_{50} half-maximal cytotoxic concentration.
[e] SI selectivity index.

Table 4
Antiviral activity of PFM067 and 25R,26OHC against HSV-2 as assessed on MRC5 cells.

ID	Treatment protocol	EC50[a] (μM) – 95% C.I.[b]	EC90[c] (μM) – 95% C.I.	CC50[d] (μM) – 95% C.I.	SI[e]
PFM067	*Before infection*	1.0 (0.9–1.2)	3.5 (2.5–4.9)	>50	>50
	During infection	1.1 (0.7–1.8)	8.0 (3.1–28.8)		>50
25R,26OHC	*Before infection*	1.5 (0.4–5.6)	2.3 (0.7–7.0)	>1350	>900
	During infection	11.8 (4.9–28.3)	n.a.		>114.4
acyclovir	*Before infection*	n.a.	n.a.	>200	n.a.
	During infection	2.4 (1.2–4.7)	4.1 (0.5–31.4)		>83.3

n.a. Not assessable.
[a] EC_{50} half-maximal effective concentration.
[b] CI confidence interval.
[c] EC_{90} 90% effective concentration.
[d] CC_{50} half-maximal cytotoxic concentration.
[e] SI selectivity index.

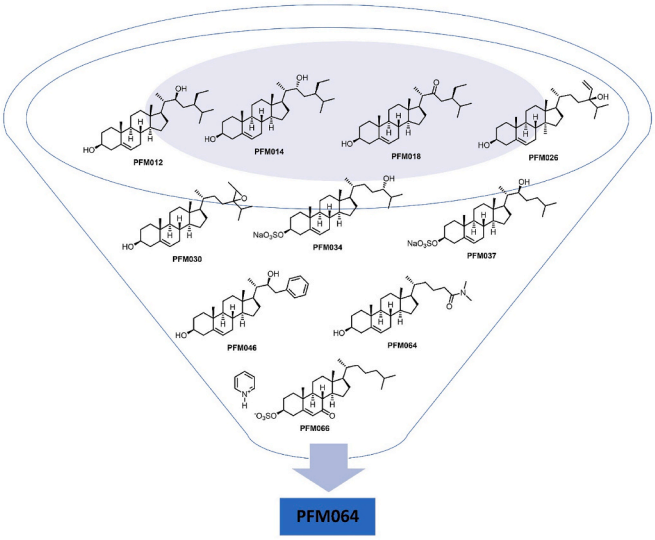

Fig. 12. Structure of the items picked from the library according to the criterion of diversity.

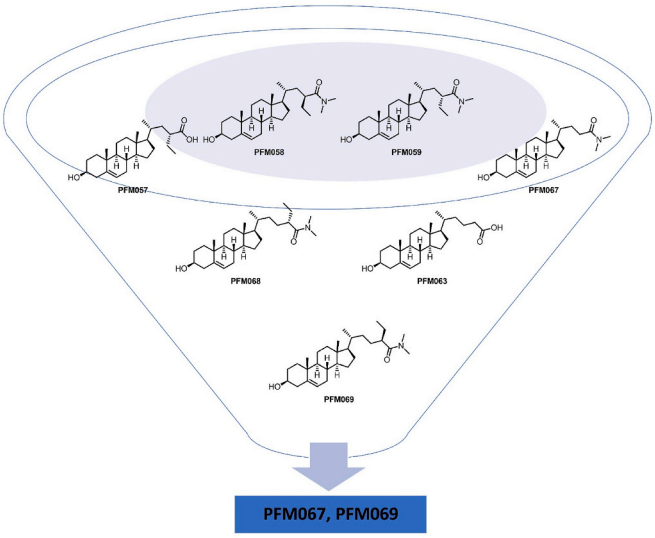

Fig. 13. Structure of the items selected from the library according to the criterion of similarity with PFM064.

Golgi compartment. This evidence explains the inhibition of HSV-2-induced CPE, the strictly nuclear localization of ICP5, and the consequent block of the maturation of the infective progeny, which represent three phenomena requiring the proper transport of viral glycoproteins out of the Golgi compartment.

Although further experiments are needed to shed light on the mechanism of action of oxysterol analogs, our data also suggest a block of capsids nuclear egress: indeed, contrary to what is visible in untreated cells, in PFM067-or 25*R*,26OHC-treated cells ICP5 does not appear to be present in the cis-Golgi. Furthermore, since the release of capsids from the nucleus to the endoplasmic reticulum and Golgi cisternae (Wild et al., 2017) is dependent on the presence of gD, gB, and gH/gL on nuclear membrane (Farnsworth et al., 2007; Johnson et al., 2011), this specific hypothesis is also consistent with the sequestration of viral glycoproteins in the Golgi apparatus detected by immunofluorescence.

5. Conclusions

Our data raise an intriguing question regarding the mechanism actually responsible for the observed accumulation/sequestration of viral glycoproteins in the Golgi. One hypothesis is that 25*R*,26OHC and PFM067 could impair the vesicular trafficking mediating the transport of viral glycoproteins from the Golgi to other cell districts (i.e. the cytoplasmic and nuclear membranes). This hypothesis is compatible with the ability of enzymatic oxysterols to alter the lipid composition of the membrane of various cellular compartments, including endosomes; this mechanism, relying on the ability of oxysterols to bind the cholesterol-shuttling oxysterol-binding protein (OSBP) has already been demonstrated for the human rotavirus (Civra et al., 2018).

More interestingly, the data obtained in this study show that PFM067 inhibits a different step of HSV-2 replication than acyclovir, providing a stimulus to investigate a combined use of PFM067 and nucleoside analogs; while it is likely to postulate at least an additive effect, one interesting question to investigate is the effect of a combined treatment with these two molecules with different mechanisms of action on the selection of resistant HSV variants.

The identification of the antiviral molecular target(s) of 25*R*,26OHC and PFM067 would also provide an efficient basis for a rational approach to design and synthetize a second generation of target-tailored analogs. In a more distant but fascinating perspective, our data suggest that PFM067 could represent the prototype of a novel class of wide-spectrum antivirals.

Glossary

Oxysterols: a family of molecules derived from cholesterol by means of oxidative processes at the level of the body as well as of the side-chain of the steroid. They are characterized by an oxygenated function, such as hydroxyl, epoxide or ketone moiety, additional to 3ß-hydroxyl group.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andrea Civra, David Lembo, Giuseppe Poli are the founders of Panoxyvir Ltd., a start-up developing the use of specific oxysterols as broad-spectrum antiviral agents. All other authors: none to declare.

Data availability

Data will be made available on request.

Acknowledgments

This research was supported by EU funding within the MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2023.105634.

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