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Abstract

The genetic plasiticity of viruses is one of the main obstacles to the development of antivirals. The aim of this study has been to assess the ability of two physiologic oxysterols and host-targeting antivirals - namely 25- and 27-hydroxycholesterol (25OHC and 27OHC) - to select resistant strains, using human rhinovirus (HRV) as a challenging model of a viral quasispecies. Moreover, we selected 27OHC for further studies aimed at exploring its potential for the development of antiviral drugs. The results obtained with clonal or serial passage approaches show that 25OHC and 27OHC do not select HRV oxysterol-resistant variants. Moreover, we demonstrate the ability of 27OHC to inhibit the yield of HRV in 3D in vitro fully reconstituted human nasal and bronchial epithelia from cystic fibrosis patients and prevent virus-induced cilia damage. The promising antiviral activity of 27OHC and its competitive advantages over direct-acting antivirals, make this molecule a suitable candidate for further studies to explore its clinical potential.

Keywords: 25-hydroxycholesterol, 27-hydroxycholesterol, Rhinovirus, antiviral, resistance

1. Introduction

One of the main obstacles to the development of novel antiviral molecules is the genetic plasticity of viruses. The high mutation rate of certain viruses, when associated with the selective pressure exerted by a treatment with direct-acting antivirals (DAAs), can generate resistant strains, which often compromise both the development of otherwise promising active principles and the rationale for their use in vivo.

The frequency of this phenomenon is particularly acute when the viral population targeted by the DAA is a quasispecies, i.e. a group of genetically variegated, yet closely related viruses subjected to a constant process of genetic variation, competition and selection.

It is generally accepted that host-targeting antivirals (HTAs) are likely to generate a lower number of resistant variants than DAAs. Nevertheless, only a few studies have been aimed at empirically comparing the ability of DAAs and HTAs to "select" resistant variants from a viral quasispecies.

25-hydroxycholesterol (25OHC) and 27-hydroxycholesterol (27OHC) are physiological cholesterol-derived molecules that modulate several cellular pathways – some of which are involved in the innate immune response to viral infections –[1,2] and which have recently emerged as broad-spectrum HTAs.[3] In particular, 25OHC impairs the viral entry of at least two enveloped viruses endowed with quasispecies features (namely the human immunodeficiency virus [HIV] and the hepatitis C virus [HCV]), by inducing cellular membrane changes that alter the virus-cell fusion process.[4,5]

Interestingly, both 25OHC and 27OHC are also endowed with antiviral activity against a non-enveloped viral quasispecies, i.e. human rhinovirus (HRV).[6] 25OHC acts as an HTA against this virus (and a second *Picornaviridae* member, i.e. poliovirus), by inducing a delocalization of the oxysterol-binding protein (OSBP, a well-characterized cholesterol sensor and/or transporter) to the Golgi vesicles, thereby leading to a reduction in phosphatidylinositol 4-phosphate (PI4P) on the endoplasmic reticulum (ER) membrane, which is fundamental for the recruitment of viral RNA-dependent RNA polymerase.[7,8]

However, although HRV is one of the viral pathogens that has shown the highest in vitro sensitivity to both 25OHC and 27OHC, a further characterization of the anti-HRV activity of these molecules is lacking. Moreover, despite the importance of HRV as a pathogen in respiratory diseases, a specific treatment is also lacking: the massive serotypic diversity of HRV [9,10] precludes the generation of broad-spectrum vaccines, while the rapid emergence of resistance has been observed for previous inhibitors that targeted the virus itself – namely the capsid-binder pleconaril and the protease inhibitor rupintrivir – thanks to its fast replication and high mutation rate.[11,12]

The aim of this study has been to provide empirical proof of principle of the greater genetic barrier of 25OHC and 27OHC, using a highly challenging virus such as HRV as a quasispecies model, and comparing its ability to select resistant variants with that of two HRV-specific DAAs (pleconaril and rupintrivir). Consistently, we also demonstrate the antiviral activity of both oxysterols against a previously untested strain, i.e. HRV B48, which is intrinsically resistant to the capsid-binder pleconaril. Moreover, we disclose the improved cytocompatibility of 27OHC, with respect to 25OHC, and validate the antiviral activity of the former using fully reconstituted in vitro human nasal and bronchial epithelia.

2. Materials and methods

2.1 Antibodies and reagents

HRV A1- and HRV B48 VP2-specific antibodies were purchased from QED Bioscience Inc. (San Diego, CA) and Covalab (Villeurbanne, France), respectively. Anti-double strand RNA (dsRNA) monoclonal antibody J2 was purchased from SCICONS (Szirák, Hungary). Secondary antibody peroxidase-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG (H + L) was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). 25OHC and 27OHC (kindly provided by Panoxyvir Ltd, Turin, Italy; purity: > 95 % from HPLC and ^{1}H NMR) were dissolved in sterile ethanol at concentrations of 3mM or 30mM. Pleconaril and rupintrivir (Sigma Aldrich) were

prepared in DMSO at concentrations of 26mM and 16.7mM, respectively. All the compounds were stored at -20°C. Moreover, 2-hydroxypropyl β-cyclodextrin (2HPβCD) was used to prepare an hydrosoluble formulations of 27OHC (named 27OHC [2HPβCD]).

2.2 Cell lines and viruses

Cervix adenocarcinoma epithelial HeLa cells (ATCC® CCL-2™) were grown in Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich), supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma Aldrich). 3D in vitro fully reconstituted human nasal and bronchial epithelia (MucilAir™-CF, Ref # EP07MD) were purchased from Epithelix (Geneva, Switzerland). More in detail, these epithelia are reconstituted in vitro using primary cells from cystic fibrosis (CF) patients harboring the same mutation (ΔF508). Each tissue insert was allocated to a 24-well plate, filled basally with 700µL per well of proper medium (MucilAir™ culture medium, Ref # EP04MM), and provided with fresh medium every two days.

HRV group A and B, that is, HRV A1 (ATCC® VR-1559) and HRV B48 (Cat. N.: 0310051v; Culture Collections, Public Health England, The United Kingdom), were propagated in HeLa cells using DMEM supplemented with 2% (v/v) FBS at 34°C in a humidified 5% CO2 incubator. Supernatants were harvested when the full cytopathic effect (CPE) occurred; virus pools were clarified, aliquoted, and stored at -70°C, while titers were determined by infecting HeLa cells with serial dilutions of viral stocks. Any infected cells were detected 24 hours after viral inoculum by means of the indirect immunoperoxidase staining procedure, using specific monoclonal antibodies, as previously described.[13] HRV-positive cells were counted, and viral titers were expressed as focus-forming unit (FFU) per ml.

2.3 In vitro antiviral assays

The antiviral efficacy of 25OHC, 27OHC, and 27OHC (2HPβCD) was determined by means of a focus reduction assay on confluent HeLa cell monolayers seeded in 96-well plates. When the cells reached 80% of confluence, infection was performed with 200 FFU per well of HRV A1 or HRV B48

prepared in DMEM and 2% (v/v) FBS, in the presence of oxysterols, pleconaril, or rupintrivir at concentrations ranging from 0.002 to 50μM. Control samples (100% of infectivity) were prepared by treating the cells with the culture medium supplemented with equal volumes of ethanol or DMSO. The infected cells were incubated at 34°C for 24 hours, then fixed and subjected to indirect immunostaining to evaluate the viral titers as described above. Blockade of viral infectivity was expressed as the mean % of the untreated control ± standard error of the mean (SEM).

Yield reduction assays were performed on HeLa cells seeded in a 24-well plate to reach confluence on the same day as the assay. An aliquot of 100 FFU per well of HRV A1 or HRV B48 was inoculated into the cells in the presence of scalar dilutions of 25OHC, 27OHC, or 27OHC (2HPβCD), with concentrations ranging from 0.2 to 5.6μM. Control samples were obtained for the experiments with 25OHC and 27OHC by infecting cells in the presence of the culture medium and equal volumes of ethanol. Cells were incubated at 34°C for 3 days, and then the media were harvested along with scraped cell monolayers; cell lysates were then clarified by low-speed centrifugation for 10 minutes, and viral progeny was titrated as previously described.

2.4 Cell viability and cytotoxicity assay

HeLa cells were seeded, at a density of 5×10^3 per well, in 96-well plates and treated the next day with 25OHC, 27OHC, 27OHC (2HPβCD), pleconaril, or rupintrivir at concentrations ranging from 0.07 to 1350μM. Control samples were prepared by treating the cells with the culture medium supplemented with equal volumes of ethanol, corresponding to 0.6% (v/v) or 0.0025% (v/v), or DMSO in the cell media. After 24 hours of incubation, cell viability was determined using a CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA), while cytotoxicity was assessed using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA), following the manufacturer's instructions. This timing was chosen to match the same treatment conditions used in focus-reduction assays (in which the treatment lasts 24 hours).

The absorbances of both the treated (Abs_T) and untreated (Abs_{NT}) samples were measured for the cell viability assays using a Microplate Reader (Model 680, BIORAD) at 490nm. The percentage of cell viability was calculated according to the following formula: (Abs_T X 100) / Abs_{NT}. A 100% toxicity control was prepared for the LDH-based cytotoxicity assays by adding 50µl of lysis buffer (provided by the supplier) to a set of non-treated samples. The absorbances of the 100% control samples, and of the treated and untreated samples (Abs_{Ctrl}, Abs_T, and Abs_{NT}, respectively) were measured at 490 nm; the effect of the treatment was expressed as the percentage of toxicity, according to the following formula: (Abs_T X 100) / Abs_{Ctrl}; (Abs_{NT} X 100) / Abs_{Ctrl}.

2.5 Selection of resistant HRV strains

The ability of 25OHC, 27OHC, pleconaril, or rupintrivir to generate resistant strains of HRV A1 was tested by exploiting clonal or serial passage approaches. In the former case, HeLa cells were seeded in 96-well plates; for each tested molecule, a total of 384 wells was infected with HRV A1 at a multiplicity of infection (MOI) of 0.1 FFU/cell. Infected monolayers were treated the next day with 25OHC, 27OHC, pleconaril, or rupintrivir at concentrations equal to the respective EC99s. Since this approach requires longer treatment times than those used in focus-reduction assays, uninfected treated controls were prepared in parallel with each culture passage, in order to exclude any cytotoxic effects due to the treatment. Non-treated control samples were prepared by incubating infected cells with ethanol or DMSO. The plate was incubated at 34°C and checked every day until a visible CPE occurred; CPE was observed in the control wells on day one of infection. In the few cultures in which CPE developed under drug pressure during the post-infection period, the supernatant was harvested and used independently to expand any of the contained infectious viruses.

For the serial passages approach, different 24-well-plate-seeded HeLa monolayers were inoculated at 34°C with HRV A1 (MOI=0.1 FFU/cell) in the presence of the selected compound at its EC_{50} dose. When full CPE occurred, supernatants from treated and non-treated wells were harvested, viral suspensions were clarified, aliquoted, titrated, and then tested for their sensitivity to the different

antiviral molecules, using antiviral assays as described above. This procedure was repeated several times, increasing the tested concentration of each compound at each passage. Cross-resistance of selected variants was assessed with in vitro antiviral assays, as previously described.

2.6 Experiments on 3D in vitro fully reconstituted human nasal and bronchial epithelia

Bronchial and nasal epithelia were infected apically with 150,000 FFU of HRV A1 and incubated at 34°C for 3 hours to allow viral entry. The inoculum was then removed, infected epithelia were washed three times, and an aliquot of 100µl of culture medium was added apically for 5 minutes to collect any residual viral particle. 24µl of culture medium, supplemented with 27OHC or 27OHC (2HPβCD) at 24µM or 72µM, were then added apically, and the infected epithelia were incubated at 34°C to allow HRV replication. Unless otherwise stated, the treatment with 27OHC or 27OHC (2HPβCD) was repeated every day. Viral progeny was harvested apically - by using the same procedure described above - every day for 3 or 4 days after inoculation (for nasal and bronchial epithelia, respectively). The titer of harvested infective particles produced each day was assessed by infecting HeLa cells with serial dilutions of viral stocks and performing an indirect immunoperoxidase staining at 24 hours post inoculum. HRV-positive cells were counted, and viral titers were expressed as FFU per ml.

Moreover, the toxicity of 27OHC or 27OHC (2HPβCD) on nasal and bronchial epithelia was evaluated using a cytotoxicity assay as follows: basal media of treated non-infected samples were harvested every 24 hours after treatment, for 5 or 6 days; toxicity was assessed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA), and measurements were performed according to the kit manufacturer's instructions, as described above.

Finally, epithelia were prepared at the end of each experiment to assess any eventual tissue morphology alterations, according to the different experimental conditions. Briefly, tissues were fixed in formaldehyde and embedded in paraffin prior to obtaining slices that were stained with haematoxylin/eosin dyes.

2.7 Statistical analyses

189 Half-maximal antiviral effective concentration (EC_{50}) and half-maximal cytotoxic concentration
190 (CC_{50}) values were calculated by means of regression analysis of the dose-response curves generated
191 from the experimental data using GraphPad PRISM 7 (GraphPad Software, San Diego, CA, USA).
192 A selectivity index (SI) was calculated by dividing the CC_{50} by the EC_{50} value. When necessary, EC_{50}
193 values were compared using the sum-of-squares F test. One-way ANOVA, followed by Bonferroni
194 test, was used to assess the statistical significance of the differences between the treated and untreated
195 samples, where appropriate. Significance was set at the 95% level.
196

197 # 3. Results

198 **3.1 25OHC and 27OHC showed in vitro antiviral efficacy against two HRV serotypes**
199 In this study, we have disclosed the efficacy of both 25OHC and 27OHC against HRV B48, and
200 confirmed it against HRV A1. To do this, focus reduction assays were performed on HeLa cells, and
201 two different markers of HRV replication (i.e. the capsid protein VP3 and the dsRNA) were detected.
202 Both oxysterols inhibited HRV infection in a dose-dependent manner up to a maximum of 100%
203 (Figure 1a-d), with EC50s being in the low micromolar range, and SIs above 100, and 27OHC being
204 characterized by the highest SI (Table 1). Control experimens were also performed with well
205 characterized anti-HRV DAAs, namely pleconaril and rupintrivir. As depicted in figure 1f and 1h,
206 rupintrivir inhibits as expected both HRV A1 and HRV B48 in a dose-response fashion up to a
207 maximum of 100%, with EC50s in the nanomolar range (Table 1); by contrast, pleconaril inhibits
208 only HRV A1 infectivity (with an EC_{50} in the low micromolar range), while it is totally ineffective
209 against HRV B48 (figure 1e and 1g).
210 The efficacy of both 25OHC and 27OHC was further investigated by means of yield reduction assays,
211 to assess the actual ability of these molecules to inhibit the production of viral progeny. In this
212 experimental setting, both oxysterols significantly ($0.0001<pANOVA<0.05$) inhibited the production

213 of HRV A1 and B48 infective progeny in a dose-response fashion to a maximum of 100% at 1.9μM
214 and 5.6μM, thus confirming their antiviral potential (Figure 2a and 2b).
215 Cytotoxicity assays were performed to assess the effect of both molecules on cellular integrity at even
216 higher concentrations than the ones tested for the antiviral assays. As shown in Figure 3, 25OHC
217 determined a significant ($0.001<pANOVA<0.01$) cytolysis at concentrations equal to or higher than
218 5.6μM, while 27OHC only determined a low-grade, albeit significant ($pANOVA<0.001$) cell death
219 at 450μM and 1350μM.
220
221 **3.2 25OHC and 27OHC did not select HRV oxysterol-resistant strains**
222 The tendency of 25OHC and 27OHC to select resistant HRV variants was explored through two
223 different approaches, and compared with that of pleconaril and rupintrivir.
224 The serial passages approach evaluated the variation in the antiviral efficacy of each tested molecule
225 against HRV (defined by their EC50 dose) after subsequent treatments with increasing concentrations
226 of 25OHC, 27OHC, pleconaril, or rupintrivir. Resistant variants to rupintrivir and pleconaril were
227 obtained after passages 7 and 6, respectively (Figure 4c and 4d, Tables 1S and 2S). The sensitivity of
228 these variants to each DAA was assessed by focus-reduction assays, as described in the paragraph
229 2.3; the pleconaril-resistant variant harvested at passage 6 was characterized by an EC50 >150μM
230 (approximately 375 times higher than the wild type strain; Table 2S), while the rupintrivir-resistant
231 variant harvested at passage 7 was characterized by an EC50 of 0.09μM (30 times higher than the
232 wild type strain; Table 1S). On the other hand, this approach did not result in the isolation of 25OHC-
233 or 27OHC-resistant variants (Figure 4a and 4b; Tables 3S and 4S) after 10 subsequent passages in
234 presence of increasing concentrations of these oxysterols. Importantly, for each passage, a set of
235 uninfected samples, treated under the same conditions, was prepared and observed daily under an
236 inverted microscope; no cytotoxicity ascribable to the treatment was detected on the treated cell
237 monolayers during any passage (data not shown).

In order to confirm these data, a clonal approach was used to assess the frequency of selection of the resistant variants in an entire assay-compiled plate for each tested molecule. Using this method, resistant variants were obtained in 23 out of 384 cultures for pleconaril, and in 43 out of 384 for rupintrivir (corresponding respectively to frequencies of 5.8% and 11.2%), but it was not possible to select resistant clones with 25OHC or 27OHC (Figure 4e). The variants that were obtained proved to be >4-fold less sensitive to the antiviral effect of pleconaril and rupintrivir than the wild-type virus (data not shown).

The susceptibility of the pleconaril- or rupintrivir-resistant mutants (obtained with the serial passages approach at passage 6 and 7, respectively) to the treatment with 25OHC and 27OHC, was tested by means of focus-reduction assays. The results showed that 25OHC and 27OHC inhibits the replication of both variants to a maximum of 100% (Figure 5a, 5b, and 5c), thus showing a comparable efficacy to that measured against the parental strain, with EC50s being in the sub-micromolar range (Table 2).

3.3 27OHC resulted histocompatible and exerted a protective effect on the infected epithelia from CF patients

The results described in the previous paragraphs showed that 27OHC and 25OHC were equivalent in terms of both antiviral potency and genetic barrier against the selection of resistant variants. However, the results of the cytotoxicity and cell viability assays demonstrated that 27OHC was less cytotoxic and cytostatic than 25OHC, thus suggesting that 27OHC should be selected for further studies aimed at exploring its putative potential of preclinical development in more detail.

To do so, we first tested the in vitro anti-HRV efficacy of a more hydrosoluble formulation of 27OHC, based on 2HPβCD. As shown in Figure 6a, 27OHC (2HPβCD) inhibited HRV A1 and B48 infectivity in a dose-dependent manner, to a maximum of 100%, with an EC50 in the low micromolar range (Table 3). The results of the viral yield assays confirmed that 27OHC (2HPβCD) significantly inhibited the production of viral progeny – up to a maximum of 100% - when tested at 0.6µM, 1.9µM, or 5.6µM (Figure 6b and 6c); by contrast, the corresponding vehicle (i.e. a “blank” 2HPβCD

formulation) was totally ineffective, thus confirming that the measured efficacy was totally ascribable to 27OHC.

More importantly, viral yield reduction assays were performed on 3D human nasal or bronchial airway epithelia, reconstituted in vitro using primary cells from CF patients (MucilAir™-CF). The results depicted in figure 7a show that both 27OHC and 27OHC (2HPβCD) totally inhibit HRV yield at 48 hours and 72 hours after infection when treatment of nasal epithelia is performed each day after infection respectively at 72µM and 24µM. Notably, also a single treatment performed with 27OHC or 27OHC (2HPβCD) at 72µM immediately after viral inoculum totally inhibited HRV yield at 48 hours post-infection. On bronchial epithelia, 27OHC inhibits viral yield of about 1 Log only 96 hours after inoculum, when treatment is repeated every day after infection at 24µM (Figure 7c). By contrast, when the treatment is performed in the same conditions with 27OHC (2HPβCD), viral yield is totally inhibited at 96 hours after viral inoculum (Figure 7c). A cytotoxicity assay was also performed, by sampling each day the basal medium of both nasal and bronchial epithelia treated apically every 24 hours for 6 or 5 days, respectively. Regarding nasal epithelia, cytotoxicity assays were performed also for samples treated only once at day one with 72µM of 27OHC or 27OHC (2HPβCD); also in this case, basal medium was sampled daily for 6 days. The results showed that neither the nasal nor the bronchial epithelia were damaged by 27OHC (2HPβCD) or 27OHC treatment at any tested condition, with cytotoxicity levels that were comparable with the untreated controls (Figure 7b-d).

As expected, histological preparations of untreated-infected bronchial epithelia showed a histological disruption characterized by the disappearance of cilia and a slenderer epithelial layer, both of which were consequences of viral replication and markers of tissue suffering (Figure 7e). However, when the epithelia were treated upon infection, the anatomic and functional features of the tissues were fully retained, and they appeared healthy, with intact cilia and an unaltered histologic structure, thus reflecting the protective role of 27OHC (2HPβCD) against the histologic damage induced by HRV infection (Figure 7e).

4. Discussion

In this study, we have demonstrated, through two different approaches, that 25OHC and 27OHC do not select HRV oxysterol-resistant variants. The results obtained by serially passaging HRV with increasing concentrations of 25OHC or 27OHC suggest that the tested oxysterols do not exert any remarkable selective pressure on the HRV quasispecies, and the emergence of oxysterol-resistant mutants therefore cannot be documented. On the other hand, the clonal approach suggests that the virus quasispecies of the inoculum contained few variants with a certain natural level of resistance to the tested oxysterols and/or that it is very difficult for the virus to acquire these mutations. The reason for these results requires more investigation: one intriguing hypothesis that could be explored is that, as 25OHC and 27OHC are well-demonstrated regulators of the lipid composition of intracellular membranes, and since these surfaces are involved in multiple steps of viral replication, these molecules could target multiple steps of the replicative cycle, thereby reducing the statistical risk of selecting a resistant variant.

In our experiments, the HRV-resistant "selective" potential of these physiologic HTAs was compared with that of pleconaril and rupintrivir, showing that both oxysterols are less prone to selecting resistant variants than the two DAAs.

Both of the oxysterols and DAAs were also tested, in focus reduction assays, to establish the correct concentration of molecule to be used in our experimental system in order to select eventual resistant mutants. The results confirm the efficacy of both 25OHC and 27OHC against HRV A1, as assessed in previous studies,[6,8] while pleconaril and rupintrivir showed comparable EC_{50}s with the ones documented in literature.[14,15] Moreover, our study discloses the antiviral activity of both of the oxysterols against HRV B48, with effective concentrations that are comparable with the ones obtained against HRV A1. Interestingly, while HRV A1 was found to be sensitive to both pleconaril and rupintrivir, HRV B48 was totally resistant to the former. This is not particularly surprising, since

312 the sensitivity of HRVs against pleconaril can be highly variable, and the existence of strains naturally
313 resistant to pleconaril has been well-documented.[16,14]
314 The lack of viral dsRNA production in both the 25OHC- and 27OHC-treated cells is consistent with
315 the previous findings of Roulin and colleagues, who demonstrated that 25OHC can inhibit HRV A1
316 RNA replication by acting as an HTA.[8] Nevertheless, this result does not exclude that the tested
317 oxysterols are able to block another (earlier) step of the replicative cycle, as hypothesized above.
318 Indeed, both of the oxysterols could have the potential to inhibit the entry process of an endocytosis-
319 dependent, non-enveloped virus such as HRV, through a similar mechanism to that previously
320 demonstrated for human rotavirus.[17]
321 The results of the cytotoxicity and cell viability assays exclude that the inability to isolate oxysterol-
322 resistant variants is trivially due to treatment-dependent cell damage or to the induction of a state of
323 senescence in the treated cells. Nevertheless, both assays indicated that 27OHC is remarkably less
324 cytotoxic than 25OHC. This result is particularly interesting, if associated with the greater
325 concentration of 27OHC in various biological fluids, including, for instance, blood and colostrum,
326 compared to that of 25OHC;[18] taken together, these data suggest a higher cytocompatibility of 27OHC
327 than of 25OHC. Intriguingly, previous findings correlated low plasma levels of 27OHC with the
328 severity of two viral diseases, namely COVID-19 and chronic hepatitis B,[19,20] thus suggesting that
329 the role played by this antiviral molecule deserves further studies.
330 The higher cytocompatibility of 27OHC, along with the latest evidences that suggest a putative role
331 in the progression of viral diseases, as well as the previous demonstration that 27OHC is effective
332 against SARS-CoV-2 and other endemic coronaviruses (i.e. OC43)[19], prompted us to validate its
333 efficacy on nasal and bronchial epithelia from CF patients. The ability of 27OHC to inhibit the yield
334 of HRV in this challenging and predictive model, and to prevent virus-induced cilia damage,
335 represents a particularly promising result, given the causal link between HRV infection and the
336 exacerbation of CF.[21–23] More importantly, these data disclose the higher anti-HRV efficacy of a
337 hydrophilic formulation of 27OHC (2HPβCD) than non-formulated 27OHC.

338 ## 4.1 Conclusions

339 The promising antiviral activity of 27OHC and its competitive advantages over direct-acting standard
340 antiviral drugs, make this molecule a suitable candidate for further studies to explore its clinical
341 potential to treat HRV infections in vulnerable patients affected by chronic respiratory diseases.

Glossary

Direct-acting antivirals (DAAs): antiviral drugs that exert their pharmacological activity by targeting viral proteins, hence inhibiting viral replication.

Host-targeting antivirals (HTAs): antiviral drugs that exert their pharmacological activity by targeting dispensable cell biochemical mechanisms essential for viral replication.

Oxysterols: a family of molecules derived from cholesterol by means of oxidation. They can be characterized by an additional hydroxyl, epoxide or ketone group in the sterol nucleus and/or a hydroxyl group in the side chain of the cholesterol molecule.

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415 .

Table 1. Antiviral activity of 25OHC and 27OHC

Tested antivirals	Virus	Viral marker	EC_{50}* (µM) – 95% C.I.**	EC_{90}*** (µM) – 95% C.I.	CC_{50}# (µM) – 95% C.I.	SI§
25OHC	HRV A1	VP3	0.22 (0.18-0.27)	0.94 (0.59-1.49)	64.48	293
		dsRNA	0.57 (0.47-0.69)	1.22 (0.70-2.12)	64.48	113
	HRV B48	VP3	0.17 (0.15-0.19)	0.28 (0.23-0.34)	64.48	379
		dsRNA	0.70 (0.53-0.92)	1.18 (0.59-2.35)	64.48	92
27OHC	HRV A1	VP3	0.61 (0.47-0.77)	2.02 (1.15-3.57)	> 1350	> 2213
		dsRNA	0.48 (0.31-0.76)	2.43 (0.90-6.58)	> 1350	> 2813
	HRV B48	VP3	0.19 (0.16-0.23)	0.67 (0.46-0.97)	> 1350	> 7105
		dsRNA	0.56 (0.38-0.83)	0.96 (0.72-1.29)	> 1350	> 2411
pleconaril	HRV A1	VP3	0.44 (0.43-0.44)	0.99 (0.97-1.01)	> 50	> 114
		dsRNA	0.42 (0.34-0.51)	1.59 (1.01-2.49)	> 50	> 119
	HRV B48	VP3	n.a.	n.a.	> 50	n.a.
		dsRNA	n.a.	n.a.	> 50	n.a.
rupintrivir	HRV A1	VP3	0.0028 (0.0027-0.0029)	0.0057 (0.0053-0.0062)	> 50	> 17857
		dsRNA	0.0011 (0.0008-0.0016)	0.0079 (0.0035-0.0176)	> 50	> 50000
	HRV B48	VP3	0.0032 (0.0028-0.0036)	0.0051 (0.0039-0.0066)	> 50	> 15625
		dsRNA	0.0040 (0.0030-0.0061)	0.0079 (0.0051-0.0122)	> 50	> 12500

* EC_{50}: half-maximal effective concentration
** CI: confidence interval
*** EC_{90}: 90% effective concentration
# CC_{50}: half maximal cytotoxic concentration
§ SI selectivity index
n.a. not assessable

Table 2. Antiviral activity of 25OHC and 27OHC against serially passaged pleconaril- or rupintrivir-resistant HRV A1.

Virus	Tested antiviral	EC_{50}* (µM) – 95% C.I.**	EC_{90}*** (µM) – 95% C.I.
Wild type HRV A1	25OHC	0.43 (0.40-0.47)	1.24 (1.07-1.45)
	27OHC	0.48 (0.46-0.51)	1.14 (1.03-1.27)
	pleconaril	0.52 (0.50-0.54)	1.14 (1.04-1.26)
	rupintrivir	0.0021 (0.0018-0.0024)	0.0072 (0.0055-0.0097)
pleconaril-resistant HRV A1#	25OHC	0.49 (0.43-0.55)	1.35 (1.09-1.71)
	27OHC	0.51 (0.45-0.58)	1.22 (0.94-1.61)
	pleconaril	n.a.	n.a.
	rupintrivir	0.0020 (0.0017-0.0023)	0.0068 (0.0052-0.0092)
rupintrivir-resistant HRV A1§	25OHC	0.47 (0.43-0.51)	1.30 (1.11-1.54)
	27OHC	0.50 (0.45-0.55	1.20 (1.00-1.46)
	pleconaril	0.49 (0.47-0.52)	1.10 (1.00-1.22)
	rupintrivir	n.a.	n.a.

* EC_{50}: half-maximal effective concentration

** CI: confidence interval

*** EC_{90}: 90% effective concentration

serially passaged HRV A1 (passage 6; see Table 2S)

§ serially passaged HRV A1 (passage 7; see Table 1S)

n.a. not assessable

Table 3. Antiviral activity of formulated 27OHC as assessed with the anti-VP3 antibody.

Tested antivirals	Virus	EC_{50}* (µM) – 95% C.I.**	EC_{90}*** (µM) – 95% C.I.	CC_{50}# (µM) – 95% C.I.	SI§
27OHC (2HPβCD)	HRV A1	0.56 (0.49-0.64)	0.88 (0.43-1.81)	415.9 (333.1-519.3)	743
	HRV B48	0.49 (0.39-0.62)	2.06 (1.24-3.44)	415.9 (333.1-519.3)	849
2HPβCD	HRV A1	n.a.	n.a.	677.6 (112.7-4072)	n.a.
	HRV B48	n.a.	n.a.	677.6 (112.7-4072)	n.a.

*EC_{50}: half-maximal effective concentration

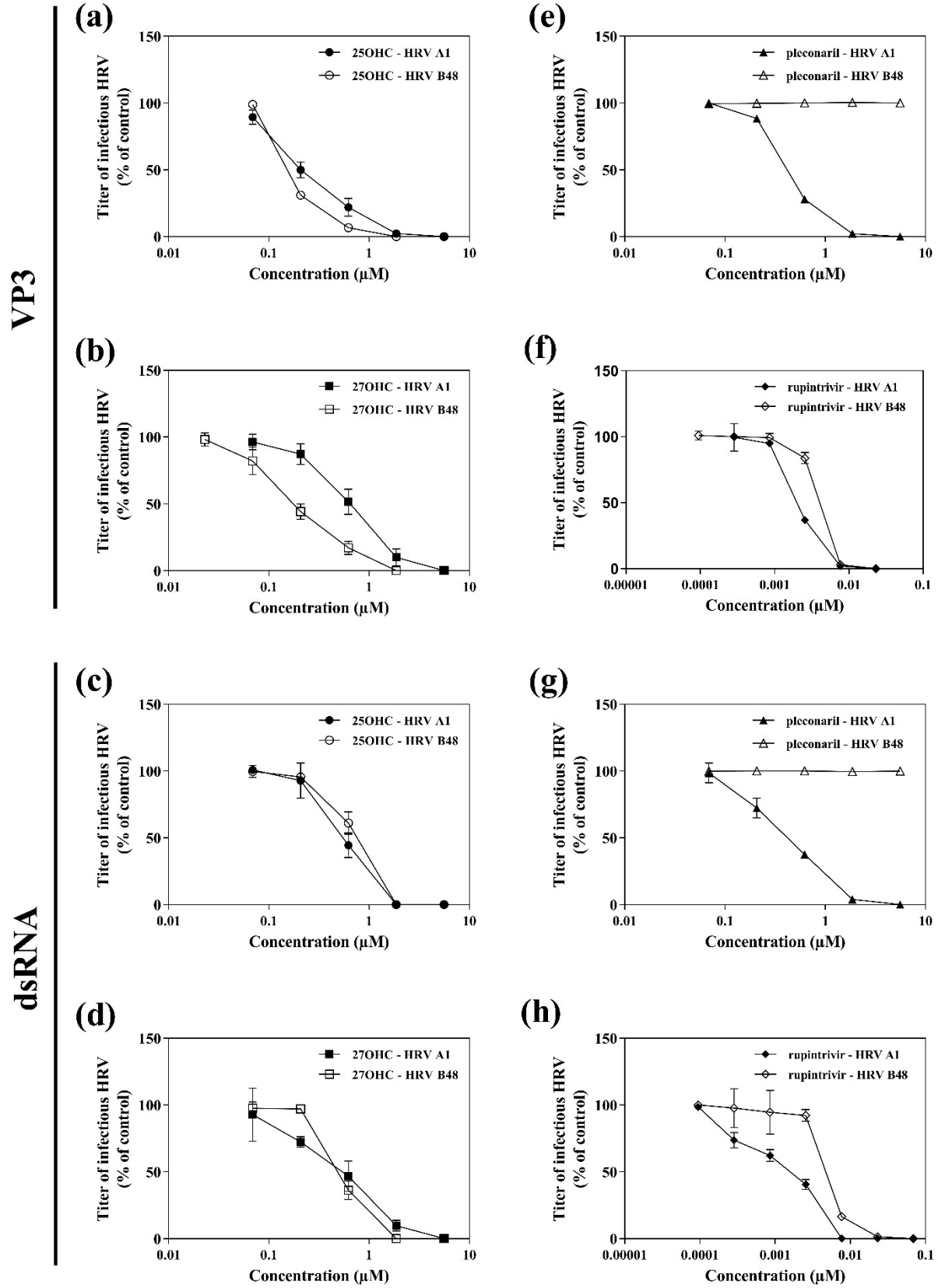
**CI: confidence interval

***EC_{90}: 90% effective concentration

CC_{50}: half maximal cytotoxic concentration

§ SI selectivity index

n.a. not assessable



438

439 **Figure 1.**

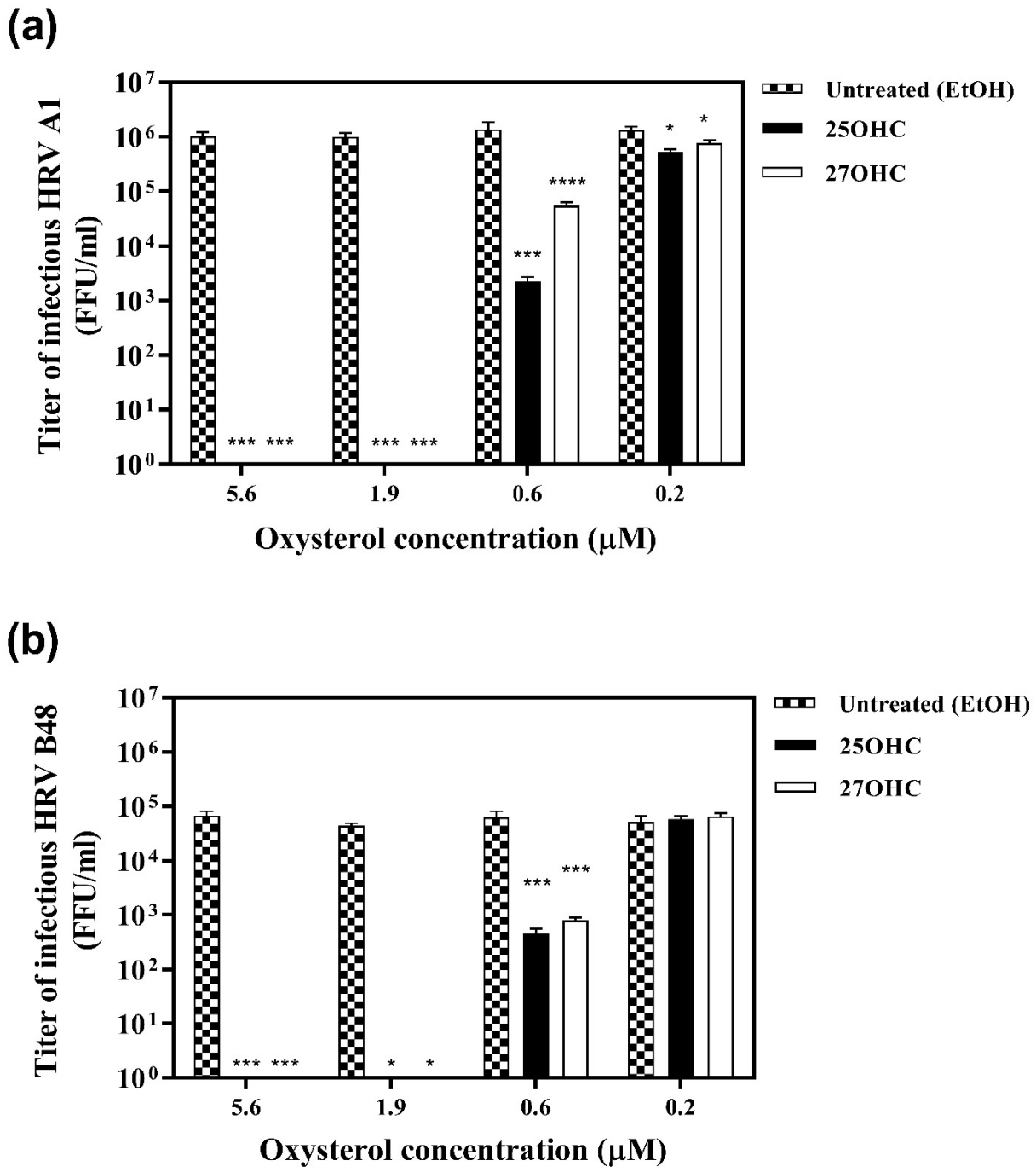
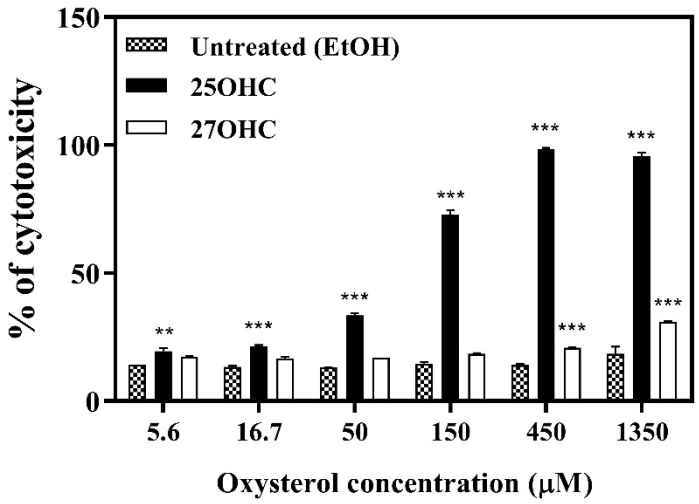

(a)
Titer of infectious HRV A1 (FFU/ml)
Untreated (EtOH)
25OHC
27OHC
Oxysterol concentration (μM)
5.6
1.9
0.6
0.2
(b)
Titer of infectious HRV B48 (FFU/ml)
Untreated (EtOH)
25OHC
27OHC
Oxysterol concentration (μM)

Figure 2.



443

444 **Figure 3.**

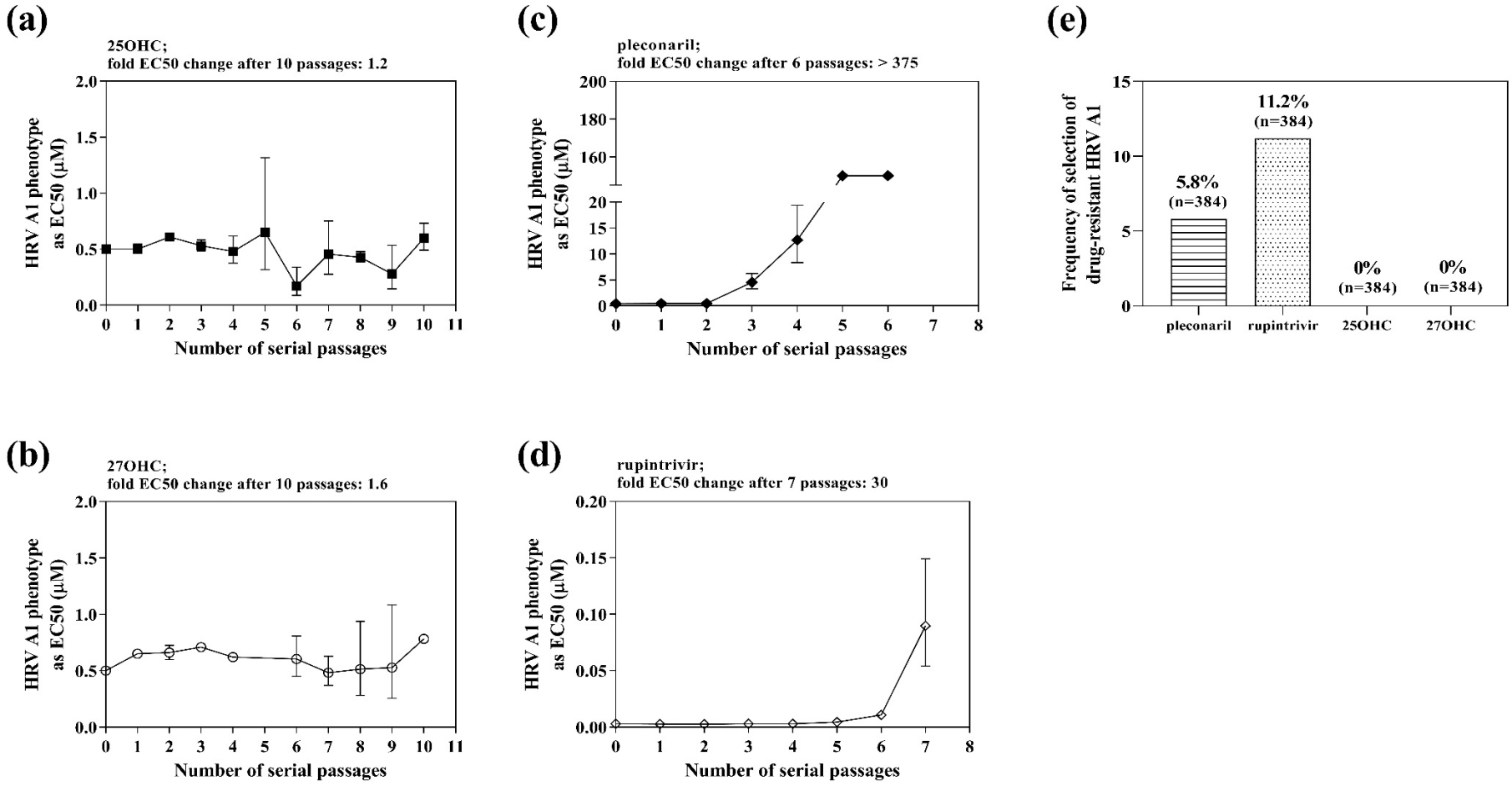
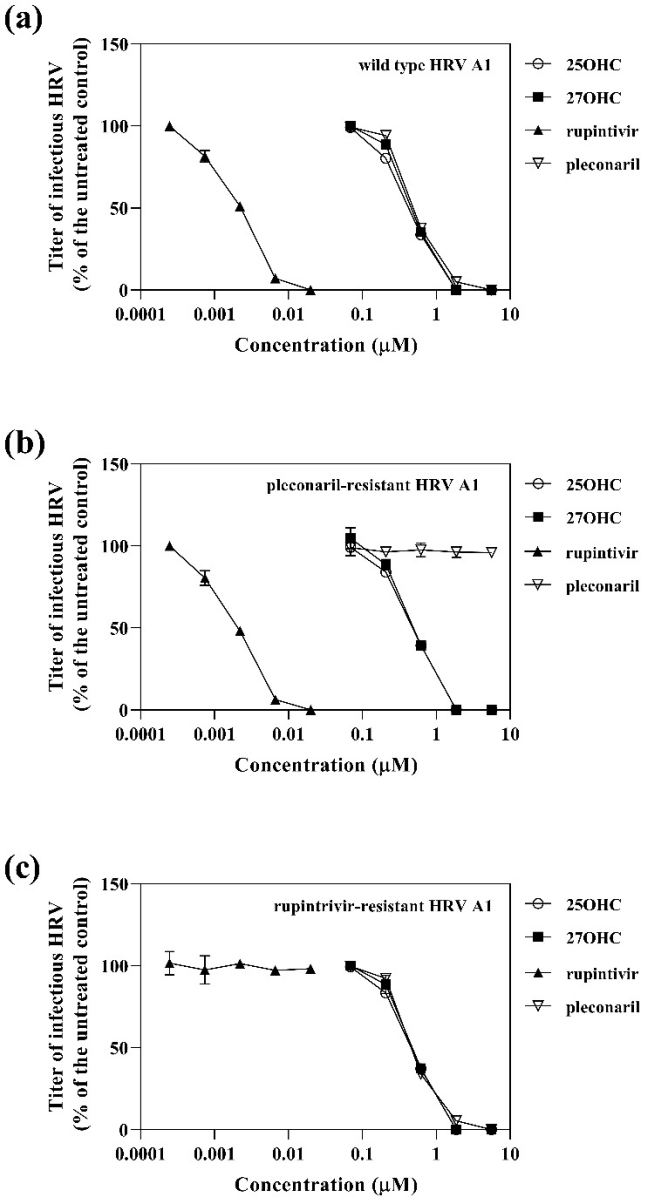

(a)
25OHC;
fold EC50 change after 10 passages: 1.2
HRV A1 phenotype as EC50 (μM)
Number of serial passages
(b)
27OHC;
fold EC50 change after 10 passages: 1.6
HRV A1 phenotype as EC50 (μM)
Number of serial passages
(c)
pleconaril;
fold EC50 change after 6 passages: > 375
HRV A1 phenotype as EC50 (μM)
Number of serial passages
(d)
rupintrivir;
fold EC50 change after 7 passages: 30
HRV A1 phenotype as EC50 (μM)
Number of serial passages
(e)
Frequency of selection of drug-resistant HRV A1
5.8% (n=384)
11.2% (n=384)
0% (n=384)
0% (n=384)
pleconaril
rupintrivir
25OHC
27OHC

Figure 4.



448

449 **Figure 5.**

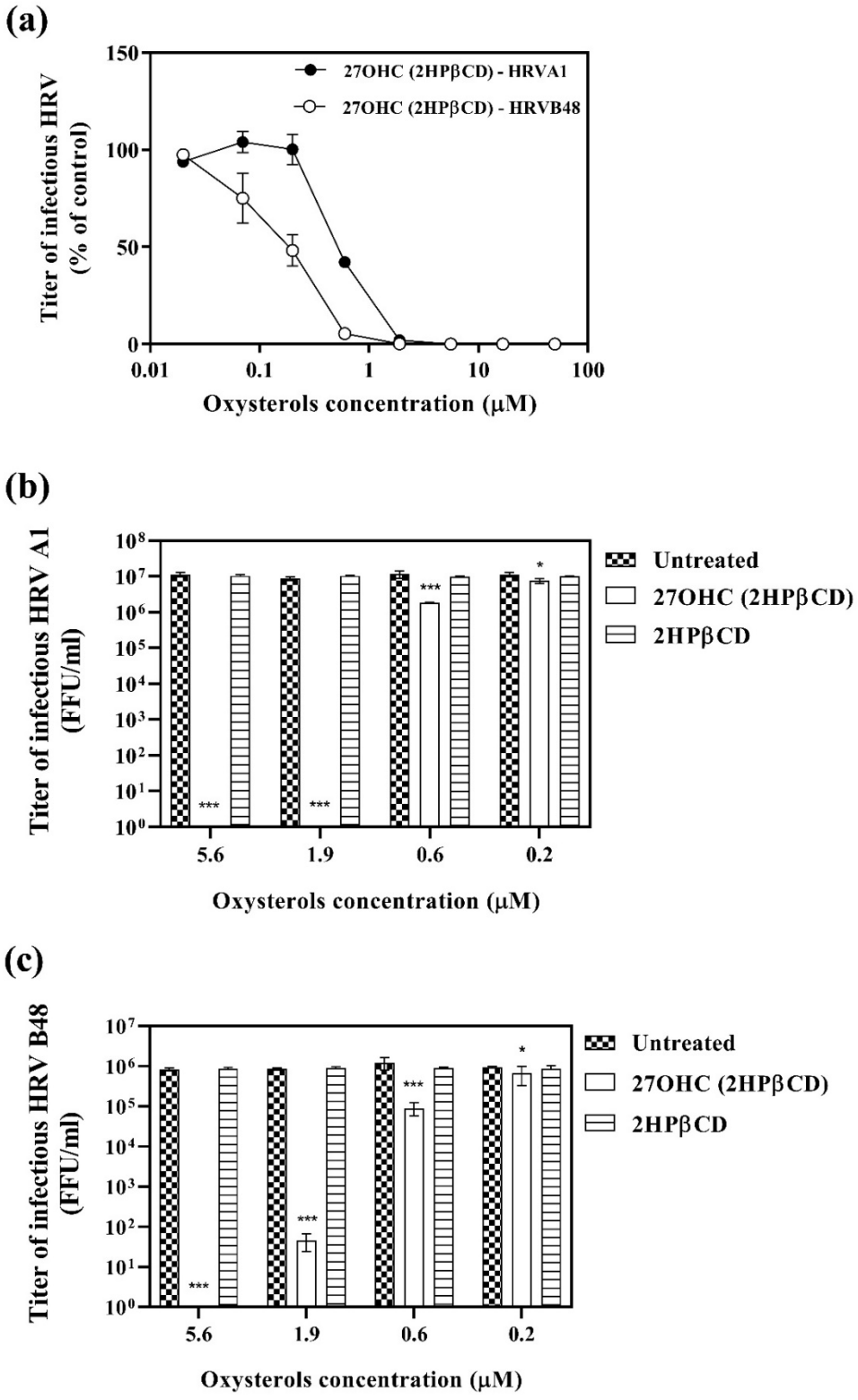

(a)
Titer of infectious HRV (% of control)
27OHC (2HPβCD) - HRVA1
27OHC (2HPβCD) - HRVB48
Oxysterols concentration (μM)
(b)
Titer of infectious HRV A1 (FFU/ml)
Untreated
27OHC (2HPβCD)
2HPβCD
Oxysterols concentration (μM)
(c)
Titer of infectious HRV B48 (FFU/ml)
Untreated
27OHC (2HPβCD)
2HPβCD
Oxysterols concentration (μM)

Figure 6.

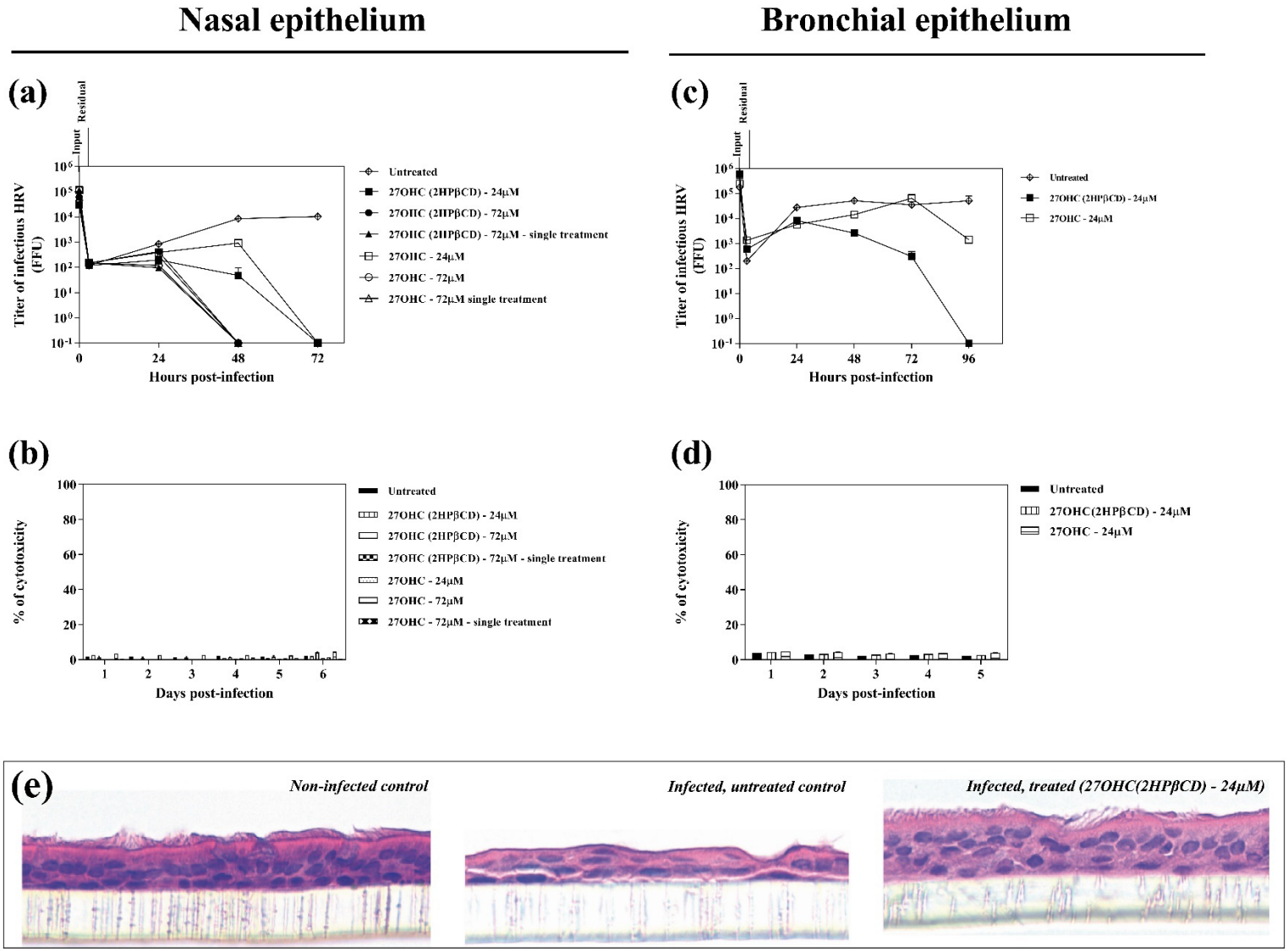

Nasal epithelium
Bronchial epithelium
(a)
(c)
(b)
(d)
(e)
Input
Residual
Titer of infectious HRV (FFU)
Hours post-infection
% of cytotoxicity
Days post-infection
Untreated
27OHC (2HPβCD) - 24μM
27OHC (2HPβCD) - 72μM
27OHC (2HPβCD) - 72μM - single treatment
27OHC - 24μM
27OHC - 72μM
27OHC - 72μM single treatment
Non-infected control
Infected, untreated control
Infected, treated (27OHC(2HPβCD) - 24μM)

Figure 7.

Legend

Figure 1. Antiviral activity of 25OHC (a, c), 27OHC (b, d), pleconaril (e, g), and rupintrivir (f, h) on HeLa cells, as assessed with the anti-VP3 antibody or the anti-dsRNA antibody. Cells were infected with HRV A1 or HRV B48 in the presence of increasing concentrations of tested molecules and then infected. Viral infections were detected 24 hours after infection, by means of indirect immunoperoxidase staining. On the y axis, blockade of viral infectivity is expressed as the mean percentage of the untreated control. In each panel, the black symbols represent the results of the antiviral assays performed with HRV A1, while the white symbols represent the results of the experiments with HRV B48.The results are means for duplicates; the error bars represent the standard error of the mean (SEM).

Figure 2. Virus yield reduction assays. Cells were infected with HRV A1 (panel a) or HRV B48 (panel b), in the presence of serial dilutions of 25OHC (black bars) or 27OHC (white bars), ranging from 0.2µM to 5.6µM. Untreated control samples were prepared by treating cells with a culture medium supplemented with equal volumes of ethanol (EtOH). When the cytopathic effect (CPE) involved the whole monolayer of the untreated samples, the cells and supernatants were harvested and titrated. On y axis, titers are expressed as focus-forming units per ml (FFU/ml). The results are means and SEM for triplicates. *p_{ANOVA}< 0.05; ***p_{ANOVA}< 0.001; ****p_{ANOVA}< 0.0001.

Figure 3. Cytotoxicity assays. Cells were treated for 24 hours with serial dilutions of 25OHC (black bars) or 27OHC (white bars), ranging from 5.6µM to 1350µM. Untreated control samples were prepared by treating cells with a culture medium supplemented with equal volumes of EtOH (checkered bars). Each bar represents the percentage of toxicity in the 25OHC-, 27OHC-treated, or untreated samples. The results are means and SEM for triplicates. **p_{ANOVA}< 0.01; ***p_{ANOVA}< 0.001.

Figure 4. Selection of resistant HRV strains by the serial passages approach (panels a-d) or by clonal approach (panel e). Regarding the former, experiments were performed by serially culturing HRV A1 in the presence of increasing concentrations of each antiviral molecule, namely 25OHC (panel a), 27OHC (panel b), pleconaril (panel c), or rupintrivir (panel d). The sensitivity of the viral progeny collected at each passage was tested by focus reduction assays, and is expressed on the y axis in terms of EC_{50}; error bars represent 95% confidence intervals of EC_{50}s. The results are means for duplicates. Panel (e) depicts the frequency of resistant HRV A1 variants in an assay-compiled plate clonal selection procedure. Briefly, a total of 384 samples were infected with HRV A1 for each tested antiviral and treated with the respective EC_{99}s of each compound. Each bar represents the percentage of cell monolayers with CPE calculated on the total number of infected samples.

Figure 5. Antiviral activity of 25OHC, 27OHC, pleconaril, and rupintrivir on HeLa cells against wild-type (panel a), pleconaril-resistant (panel b), or rupintrivir-resistant (panel c) HRV A1, as assessed by focus-reduction assay. Cells were infected in the presence of increasing concentrations of tested molecules and then infected. Viral infections were detected 24 hours after infection, by means of indirect immunoperoxidase staining with the anti-VP3 antibody. On the y axis, blockade of viral infectivity is expressed as the mean percentage of the untreated control. The results are means and SEM for duplicates.

Figure 6. In vitro antiviral activity assessment of formulated 27OHC (i.e. 27OHC [2HPβCD]), and blank formulation (2HPβCD). Panel (a) depicts the results of the focus reduction assays with HRV A1 and HRV B48; briefly, the cells were infected with HRV A1 or HRV B48 in the presence of increasing concentrations of 27OHC [2HPβCD] and then infected; viral replication was detected 24 hours after infection, by means of indirect immunoperoxidase staining with the anti-VP3 antibody. On the y axis, blockade of viral infectivity is expressed as the mean percentage of the untreated control. The results are means for duplicates; the error bars represent the standard error of the mean (SEM). Panels (b) and (c) show the results of the viral yield reduction assays for HRV A1 and HRV

503 B48, respectively. Cells were infected in the presence of serial dilutions of 27OHC (2HPβCD) (white
504 bars) or blank formulation (2HPβCD; striped bars), ranging from 0.2μM to 5.6μM. Untreated control
505 samples were prepared by treating cells with a culture medium supplemented with fresh medium
506 (checkered bars). When the CPE involved the whole monolayer of the untreated samples, the cells
507 and supernatants were harvested and titrated. On y axis, titers are expressed as FFU/ml. The results
508 are means and SEM for triplicates. *p_{ANOVA}< 0.05; ***p_{ANOVA}< 0.001.

509 **Figure 7.** Antiviral activity assessment of 27OHC and 27OHC (2HPβCD) against HRV A1, as
510 assessed on 3D in vitro fully reconstituted human nasal (panel a) and bronchial epithelia (panel c).
511 Briefly, the epithelia were infected with HRV A1, then treated with 27OHC or 27OHC (2HPβCD)
512 immediately after inoculum and each 24 hours after infection. Alternatively, nasal epithelia were only
513 treated after viral infection (this set of samples is named "single treatment" in panel a). Viral progeny
514 was harvested every 24 hours after infection for 72 hours or 96 hours; on y axis, titers are expressed
515 as FFU. Panels (b) and (d) represent cytotoxicity assays, performed respectively on nasal and
516 bronchial epithelia by sampling basal media from treated or non-treated samples every 24 hours after
517 treatment for 5 or 6 days. Briefly, in order to match the same treatment conditions used for the yield
518 reduction assays described above, the epithelia were treated each 24 hours for 5 or 6 days;
519 alternatively, nasal epithelia were only treated on day 1 (this set of samples is named "single
520 treatment" in panel b). Panel (e) depicts formaldehyde-fixed and haematoxylin/eosin-stained slices
521 of bronchial epithelia.