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24 **Abstract**

25 The genetic plasticity of viruses is one of the main obstacles to the development of antivirals. The
26 aim of this study has been to assess the ability of two physiologic oxysterols and host-targeting
27 antivirals - namely 25- and 27-hydroxycholesterol (25OHC and 27OHC) - to select resistant strains,
28 using human rhinovirus (HRV) as a challenging model of a viral quasispecies. Moreover, we selected
29 27OHC for further studies aimed at exploring its potential for the development of antiviral drugs. The
30 results obtained with clonal or serial passage approaches show that 25OHC and 27OHC do not select
31 HRV oxysterol-resistant variants. Moreover, we demonstrate the ability of 27OHC to inhibit the yield
32 of HRV in 3D in vitro fully reconstituted human nasal and bronchial epithelia from cystic fibrosis
33 patients and prevent virus-induced cilia damage. The promising antiviral activity of 27OHC and its
34 competitive advantages over direct-acting antivirals, make this molecule a suitable candidate for
35 further studies to explore its clinical potential.

36

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

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39

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}

65 However, although HRV is one of the viral pathogens that has shown the highest in vitro sensitivity
66 to both 25OHC and 27OHC, a further characterization of the anti-HRV activity of these molecules is
67 lacking. Moreover, despite the importance of HRV as a pathogen in respiratory diseases, a specific
68 treatment is also lacking: the massive serotypic diversity of HRV ^{9,10} precludes the generation of
69 broad-spectrum vaccines, while the rapid emergence of resistance has been observed for previous
70 inhibitors that targeted the virus itself – namely the capsid-binder pleconaril and the protease inhibitor
71 rupintrivir – thanks to its fast replication and high mutation rate.^{11,12}
72 The aim of this study has been to provide empirical proof of principle of the greater genetic barrier
73 of 25OHC and 27OHC, using a highly challenging virus such as HRV as a quasispecies model, and
74 comparing its ability to select resistant variants with that of two HRV-specific DAAs (pleconaril and
75 rupintrivir). Consistently, we also demonstrate the antiviral activity of both oxysterols against a
76 previously untested strain, i.e. HRV B48, which is intrinsically resistant to the capsid-binder
77 pleconaril. Moreover, we disclose the improved cytocompatibility of 27OHC, with respect to 25OHC,
78 and validate the antiviral activity of the former using fully reconstituted in vitro human nasal and
79 bronchial epithelia.

80

81 **2. Materials and methods**

82 **2.1 Antibodies and reagents**

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

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

93 **2.2 Cell lines and viruses**

94 Cervix adenocarcinoma epithelial HeLa cells (ATCC® CCL-2™) were grown in Dulbecco's
95 Modified Eagle Medium (DMEM; Sigma Aldrich), supplemented with 10% (v/v) fetal bovine serum
96 (FBS; Sigma Aldrich). 3D in vitro fully reconstituted human nasal and bronchial epithelia
97 (MucilAir™-CF, Ref # EP07MD) were purchased from Epithelix (Geneva, Switzerland). More in
98 detail, these epithelia are reconstituted in vitro using primary cells from cystic fibrosis (CF) patients
99 harboring the same mutation (Δ F508). Each tissue insert was allocated to a 24-well plate, filled
100 basally with 700 μ L per well of proper medium (MucilAir™ culture medium, Ref # EP04MM), and
101 provided with fresh medium every two days.

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

111 **2.3 In vitro antiviral assays**

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

115 prepared in DMEM and 2% (v/v) FBS, in the presence of oxysterols, pleconaril, or rupintrivir at
116 concentrations ranging from 0.002 to 50 μ M. Control samples (100% of infectivity) were prepared by
117 treating the cells with the culture medium supplemented with equal volumes of ethanol or DMSO.
118 The infected cells were incubated at 34°C for 24 hours, then fixed and subjected to indirect
119 immunostaining to evaluate the viral titers as described above. Blockade of viral infectivity was
120 expressed as the mean % of the untreated control \pm standard error of the mean (SEM).
121 Yield reduction assays were performed on HeLa cells seeded in a 24-well plate to reach confluence
122 on the same day as the assay. An aliquot of 100 FFU per well of HRV A1 or HRV B48 was inoculated
123 into the cells in the presence of scalar dilutions of 25OHC, 27OHC, or 27OHC (2HP β CD), with
124 concentrations ranging from 0.2 to 5.6 μ M. Control samples were obtained for the experiments with
125 25OHC and 27OHC by infecting cells in the presence of the culture medium and equal volumes of
126 ethanol. Cells were incubated at 34°C for 3 days, and then the media were harvested along with
127 scraped cell monolayers; cell lysates were then clarified by low-speed centrifugation for 10 minutes,
128 and viral progeny was titrated as previously described.

129 **2.4 Cell viability and cytotoxicity assay**

130 HeLa cells were seeded, at a density of 5×10^3 per well, in 96-well plates and treated the next day with
131 25OHC, 27OHC, 27OHC (2HP β CD), pleconaril, or rupintrivir at concentrations ranging from 0.07
132 to 1350 μ M. Control samples were prepared by treating the cells with the culture medium
133 supplemented with equal volumes of ethanol, corresponding to 0.6% (v/v) or 0.0025% (v/v), or
134 DMSO in the cell media. After 24 hours of incubation, cell viability was determined using a CellTiter
135 96 Proliferation Assay Kit (Promega, Madison, WI, USA), while cytotoxicity was assessed using a
136 CytoTox 96[®] Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA), following the
137 manufacturer's instructions. This timing was chosen to match the same treatment conditions used in
138 focus-reduction assays (in which the treatment lasts 24 hours).

139 The absorbances of both the treated (Ab_{ST}) and untreated (Ab_{SNT}) samples were measured for the cell
140 viability assays using a Microplate Reader (Model 680, BIORAD) at 490nm. The percentage of cell
141 viability was calculated according to the following formula: $(Ab_{ST} \times 100) / Ab_{SNT}$. A 100% toxicity
142 control was prepared for the LDH-based cytotoxicity assays by adding 50 μ l of lysis buffer (provided
143 by the supplier) to a set of non-treated samples. The absorbances of the 100% control samples, and
144 of the treated and untreated samples (Ab_{SCtrl} , Ab_{ST} , and Ab_{SNT} , respectively) were measured at 490
145 nm; the effect of the treatment was expressed as the percentage of toxicity, according to the following
146 formula: $(Ab_{ST} \times 100) / Ab_{SCtrl}$; $(Ab_{SNT} \times 100) / Ab_{SCtrl}$.

147 **2.5 Selection of resistant HRV strains**

148 The ability of 25OHC, 27OHC, pleconaril, or rupintrivir to generate resistant strains of HRV A1 was
149 tested by exploiting clonal or serial passage approaches. In the former case, HeLa cells were seeded
150 in 96-well plates; for each tested molecule, a total of 384 wells was infected with HRV A1 at a
151 multiplicity of infection (MOI) of 0.1 FFU/cell. Infected monolayers were treated the next day with
152 25OHC, 27OHC, pleconaril, or rupintrivir at concentrations equal to the respective EC99s. Since this
153 approach requires longer treatment times than those used in focus-reduction assays, uninfected treated
154 controls were prepared in parallel with each culture passage, in order to exclude any cytotoxic effects
155 due to the treatment. Non-treated control samples were prepared by incubating infected cells with
156 ethanol or DMSO. The plate was incubated at 34°C and checked every day until a visible CPE
157 occurred; CPE was observed in the control wells on day one of infection. In the few cultures in which
158 CPE developed under drug pressure during the post-infection period, the supernatant was harvested
159 and used independently to expand any of the contained infectious viruses.

160 For the serial passages approach, different 24-well-plate-seeded HeLa monolayers were inoculated at
161 34°C with HRV A1 (MOI=0.1 FFU/cell) in the presence of the selected compound at its EC₅₀ dose.
162 When full CPE occurred, supernatants from treated and non-treated wells were harvested, viral
163 suspensions were clarified, aliquoted, titrated, and then tested for their sensitivity to the different

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

167 **2.6 Experiments on 3D in vitro fully reconstituted human nasal and bronchial epithelia**

168 Bronchial and nasal epithelia were infected apically with 150,000 FFU of HRV A1 and incubated at
169 34°C for 3 hours to allow viral entry. The inoculum was then removed, infected epithelia were washed
170 three times, and an aliquot of 100µl of culture medium was added apically for 5 minutes to collect
171 any residual viral particle. 24µl of culture medium, supplemented with 27OHC or 27OHC (2HPβCD)
172 at 24µM or 72µM, were then added apically, and the infected epithelia were incubated at 34°C to
173 allow HRV replication. Unless otherwise stated, the treatment with 27OHC or 27OHC (2HPβCD)
174 was repeated every day. Viral progeny was harvested apically - by using the same procedure described
175 above - every day for 3 or 4 days after inoculation (for nasal and bronchial epithelia, respectively).
176 The titer of harvested infective particles produced each day was assessed by infecting HeLa cells with
177 serial dilutions of viral stocks and performing an indirect immunoperoxidase staining at 24 hours post
178 inoculum. HRV-positive cells were counted, and viral titers were expressed as FFU per ml.

179 Moreover, the toxicity of 27OHC or 27OHC (2HPβCD) on nasal and bronchial epithelia was
180 evaluated using a cytotoxicity assay as follows: basal media of treated non-infected samples were
181 harvested every 24 hours after treatment, for 5 or 6 days; toxicity was assessed using the CytoTox
182 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA), and measurements were
183 performed according to the kit manufacturer's instructions, as described above.

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

188 **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 EC_{50} s being in the low micromolar range, and SIs above 100, and 27OHC being
204 characterized by the highest SI (Table 1). Control experiments 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 EC_{50} s 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 ($0.0001 < p_{ANOVA} < 0.05$) inhibited the production

213 of HRV A1 and B48 infective progeny in a dose-response fashion to a maximum of 100% at 1.9 μ M
214 and 5.6 μ M, 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 ($0.001 < p_{ANOVA} < 0.01$) cytolysis at concentrations equal to or higher than
218 5.6 μ M, while 27OHC only determined a low-grade, albeit significant ($p_{ANOVA} < 0.001$) cell death
219 at 450 μ M and 1350 μ M.

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 >150 μ M
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 0.09 μ M (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).

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

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

250 **3.3 27OHC resulted histocompatible and exerted a protective effect on the infected epithelia** 251 **from CF patients**

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

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

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

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

281 As expected, histological preparations of untreated-infected bronchial epithelia showed a histological
282 disruption characterized by the disappearance of cilia and a slenderer epithelial layer, both of which
283 were consequences of viral replication and markers of tissue suffering (Figure 7e). However, when
284 the epithelia were treated upon infection, the anatomic and functional features of the tissues were
285 fully retained, and they appeared healthy, with intact cilia and an unaltered histologic structure, thus
286 reflecting the protective role of 27OHC (2HPβCD) against the histologic damage induced by HRV
287 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 EC₅₀s 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

the sensitivity of HRVs against pleconaril can be highly variable, and the existence of strains naturally resistant to pleconaril has been well-documented.^{16,14}

The lack of viral dsRNA production in both the 25OHC- and 27OHC-treated cells is consistent with the previous findings of Roulin and colleagues, who demonstrated that 25OHC can inhibit HRV A1 RNA replication by acting as an HTA.⁸ Nevertheless, this result does not exclude that the tested oxysterols are able to block another (earlier) step of the replicative cycle, as hypothesized above. Indeed, both of the oxysterols could have the potential to inhibit the entry process of an endocytosis-dependent, non-enveloped virus such as HRV, through a similar mechanism to that previously demonstrated for human rotavirus.¹⁷

The results of the cytotoxicity and cell viability assays exclude that the inability to isolate oxysterol-resistant variants is trivially due to treatment-dependent cell damage or to the induction of a state of senescence in the treated cells. Nevertheless, both assays indicated that 27OHC is remarkably less cytotoxic than 25OHC. This result is particularly interesting, if associated with the greater concentration of 27OHC in various biological fluids, including, for instance, blood and colostrum, compared to that of 25OHC;¹⁸ taken together, these data suggest a higher cytocompatibility of 27OHC than of 25OHC. Intriguingly, previous findings correlated low plasma levels of 27OHC with the severity of two viral diseases, namely COVID-19 and chronic hepatitis B,^{19,20} thus suggesting that the role played by this antiviral molecule deserves further studies.

The higher cytocompatibility of 27OHC, along with the latest evidences that suggest a putative role in the progression of viral diseases, as well as the previous demonstration that 27OHC is effective against SARS-CoV-2 and other endemic coronaviruses (i.e. OC43)¹⁹, prompted us to validate its efficacy on nasal and bronchial epithelia from CF patients. The ability of 27OHC to inhibit the yield of HRV in this challenging and predictive model, and to prevent virus-induced cilia damage, represents a particularly promising result, given the causal link between HRV infection and the exacerbation of CF.^{21–23} More importantly, these data disclose the higher anti-HRV efficacy of a hydrophilic formulation of 27OHC (2HP β CD) 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.

342 **Glossary**

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

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

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

350

351

5. Acknowledgments

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415 .

416 **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

417 * EC₅₀: half-maximal effective concentration
418 ** CI: confidence interval
419 *** EC₉₀: 90% effective concentration
420 # CC₅₀: half maximal cytotoxic concentration
421 § SI selectivity index
422 n.a. not assessable

423 **Table 2. Antiviral activity of 25OHC and 27OHC against serially passaged pleconaril- or**
424 **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.

425 * EC₅₀: half-maximal effective concentration

426 ** CI: confidence interval

427 *** EC₉₀: 90% effective concentration

428 [#] serially passaged HRV A1 (passage 6; see Table 2S)

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

430 n.a. not assessable

431 **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.

432 *EC₅₀: half-maximal effective concentration

433 **CI: confidence interval

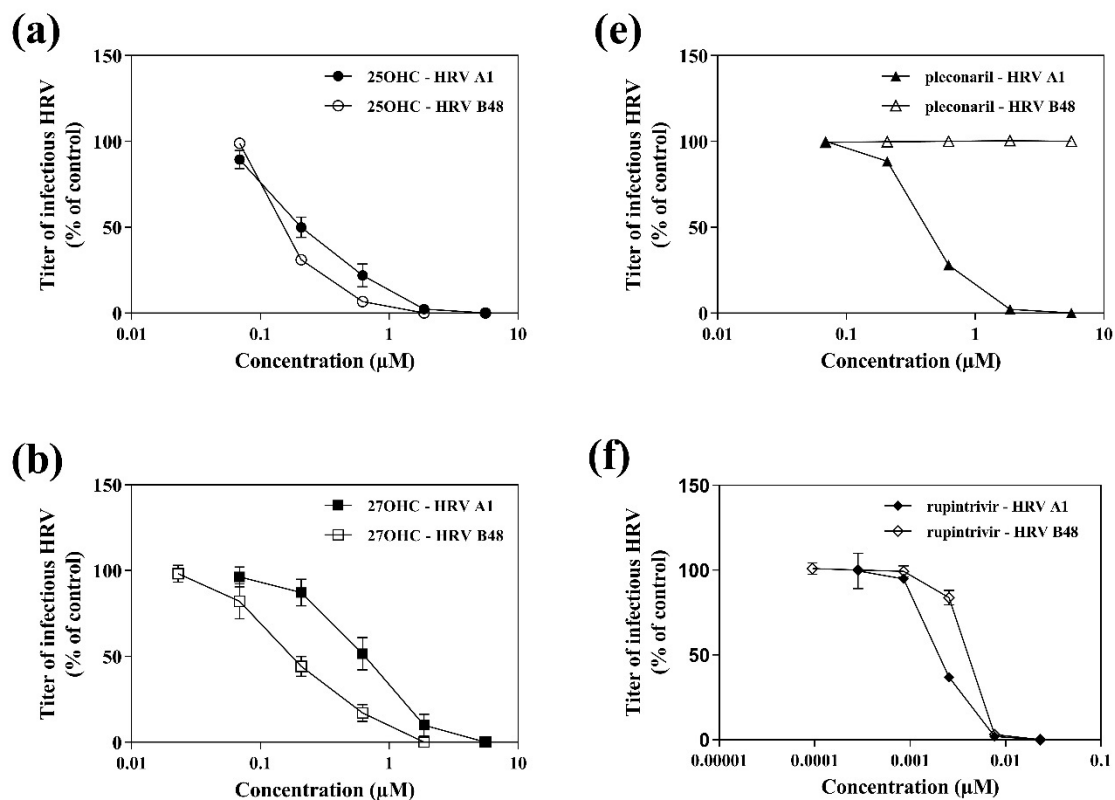
434 ***EC₉₀: 90% effective concentration

435 # CC₅₀: half maximal cytotoxic concentration

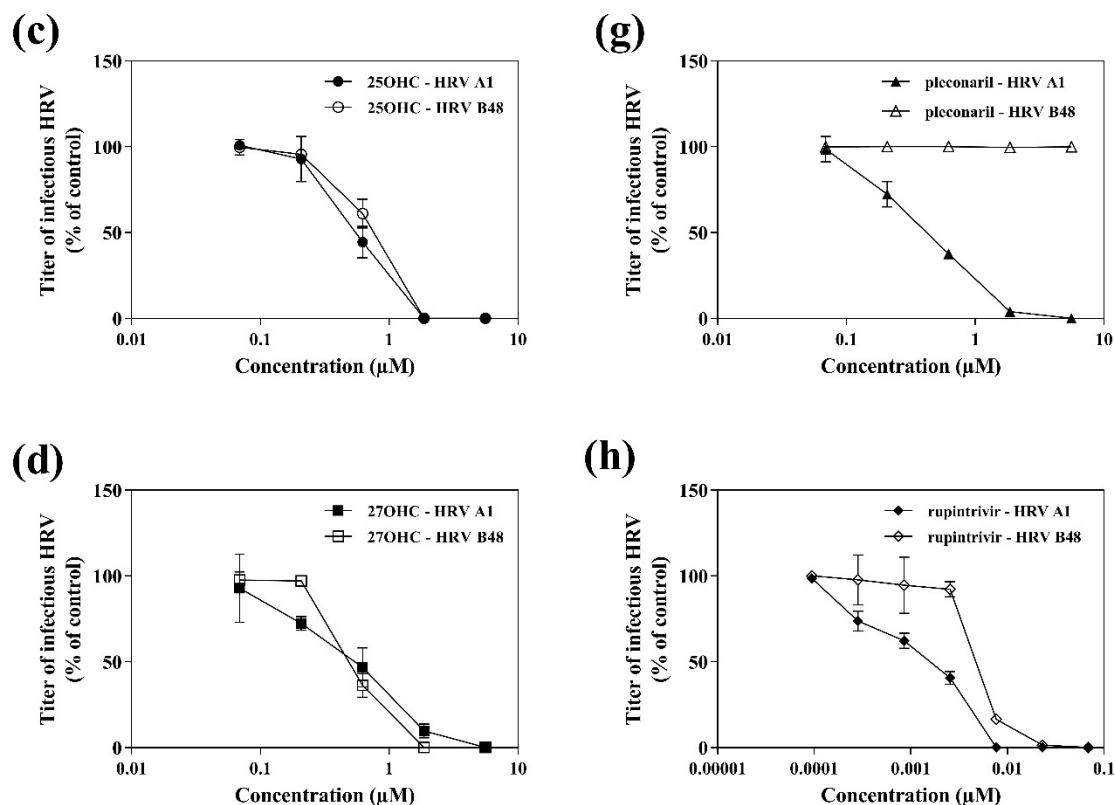
436 § SI selectivity index

437 n.a. not assessable

VP3



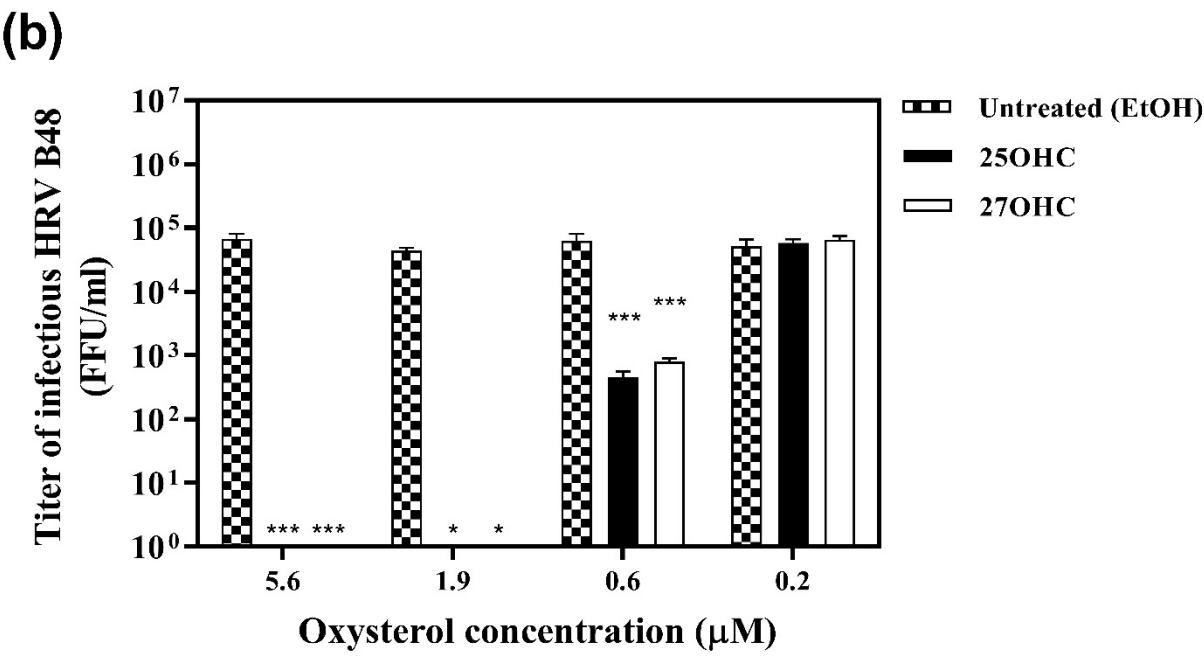
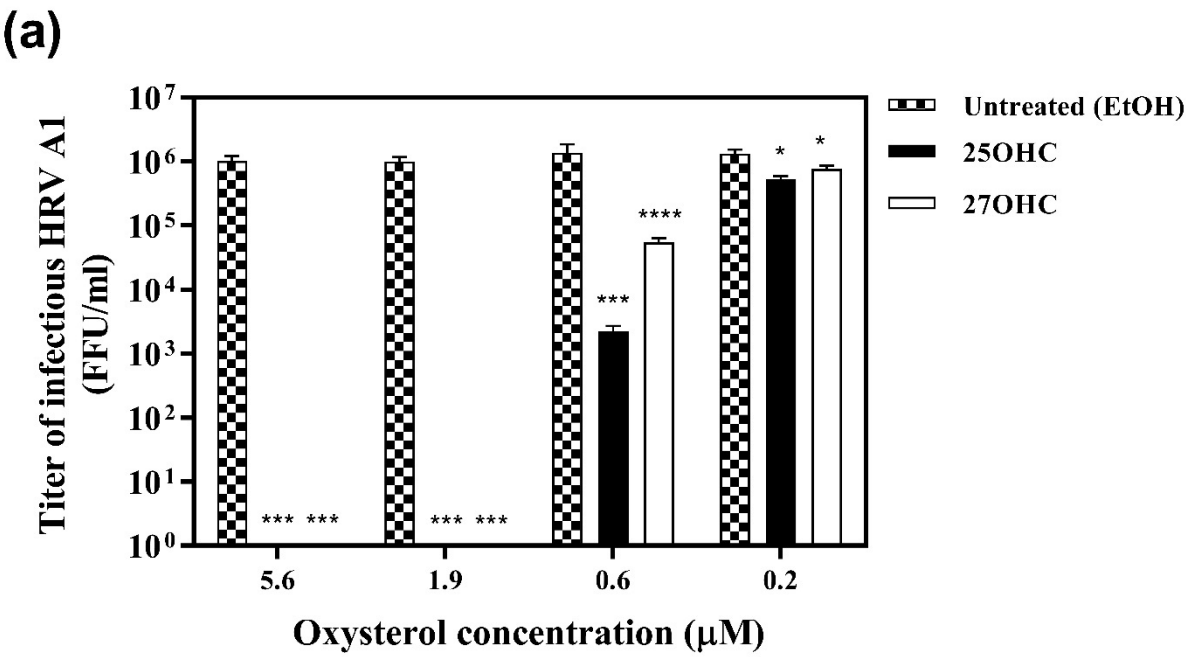
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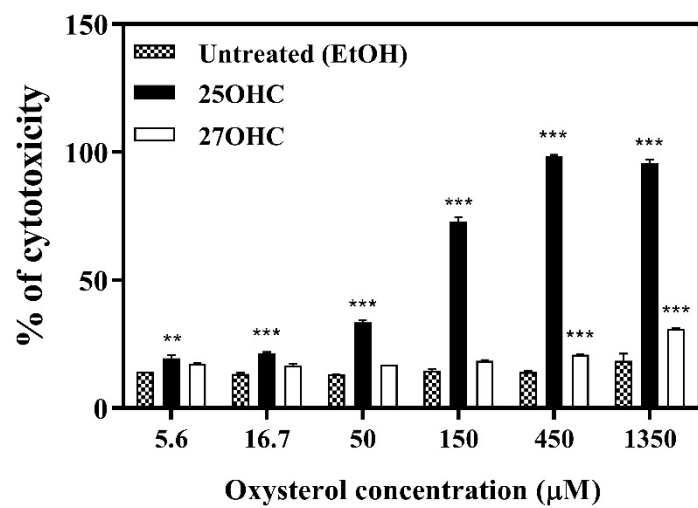
439 Figure 1.

440



441

442 **Figure 2.**

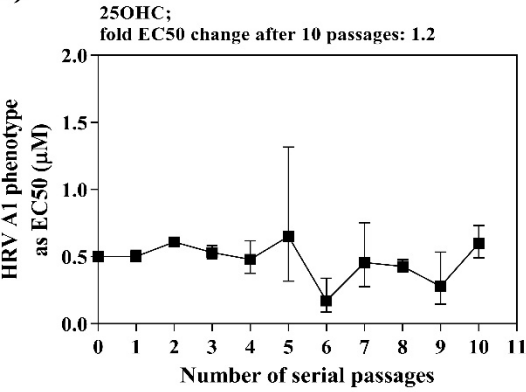


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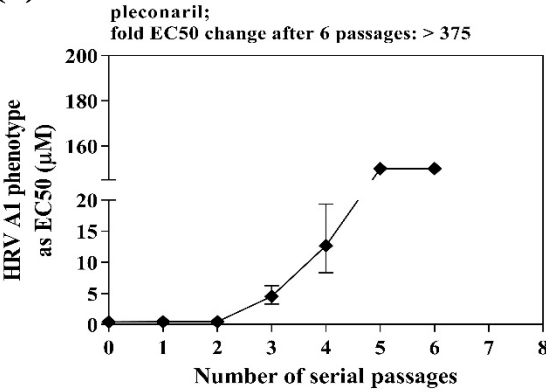
444 **Figure 3.**

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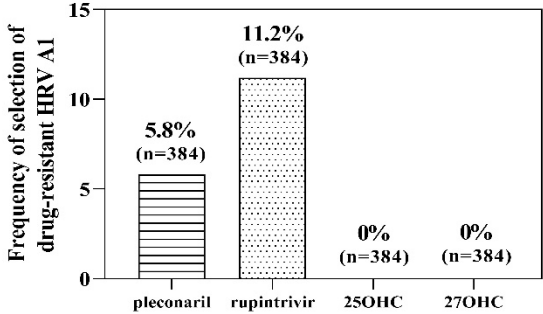
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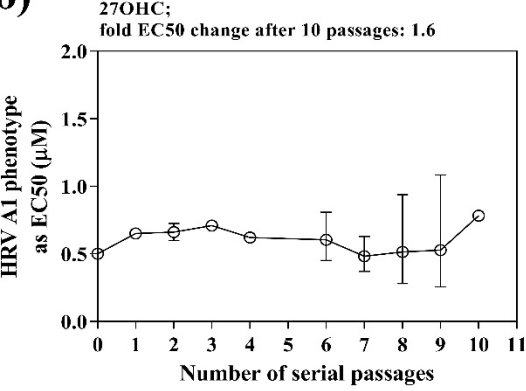
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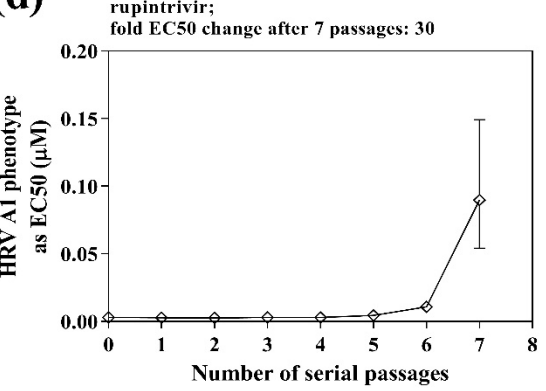
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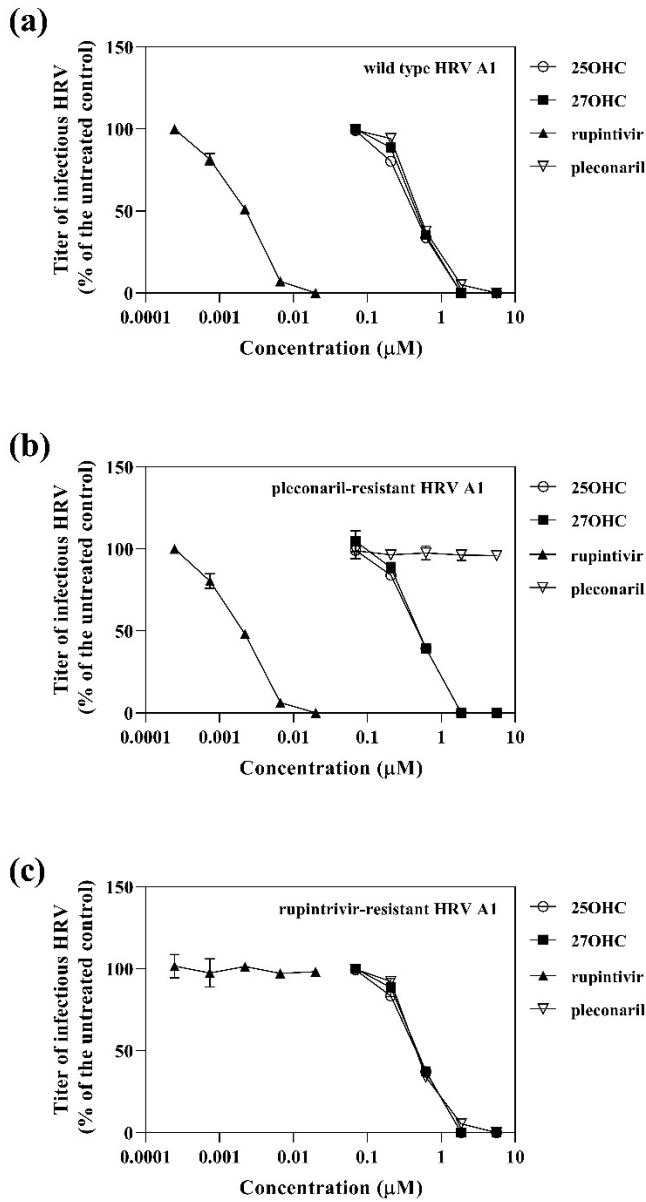


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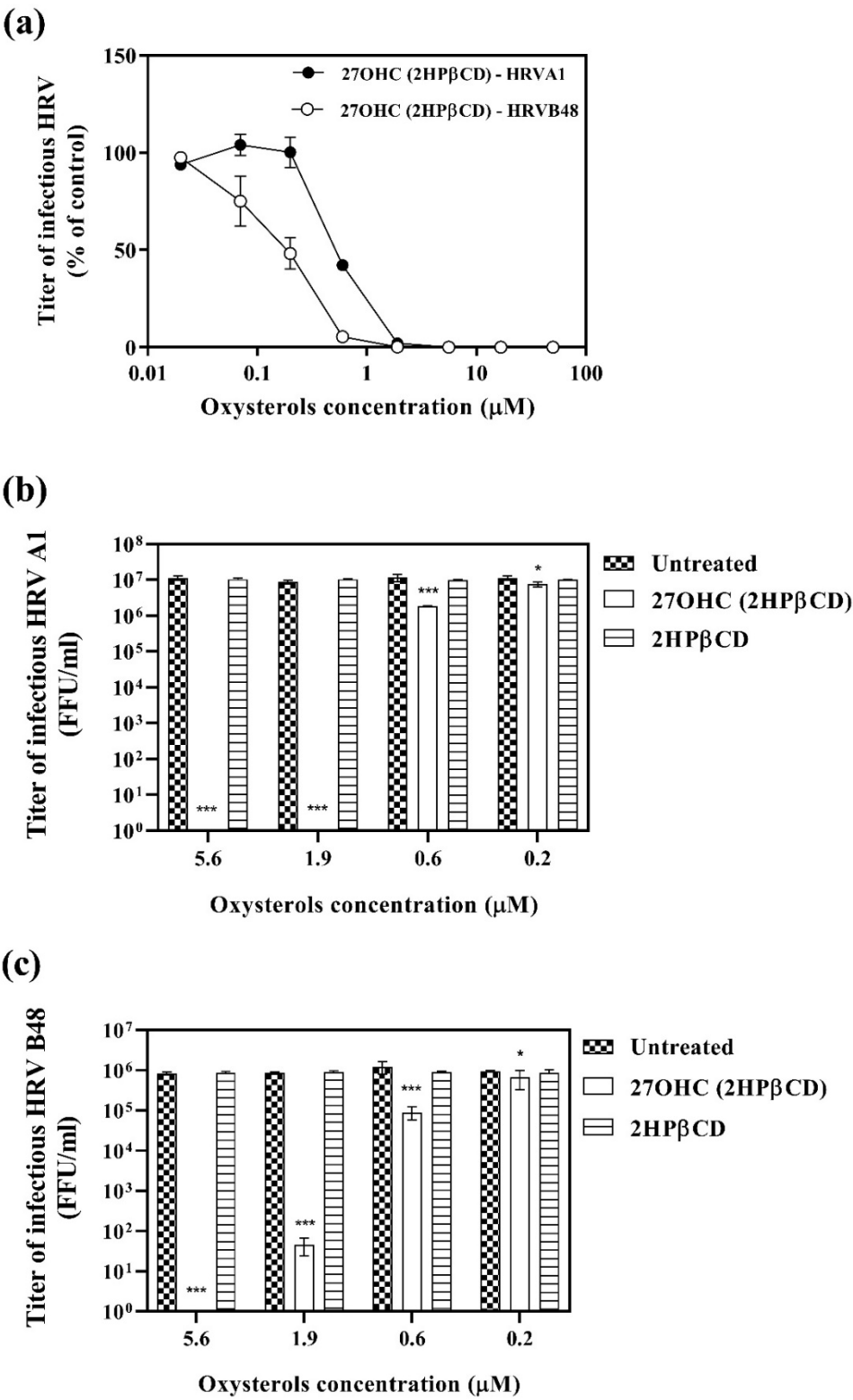
447 **Figure 4.**



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449 **Figure 5.**

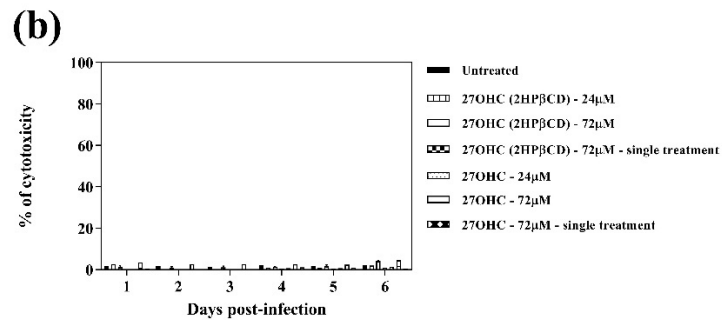
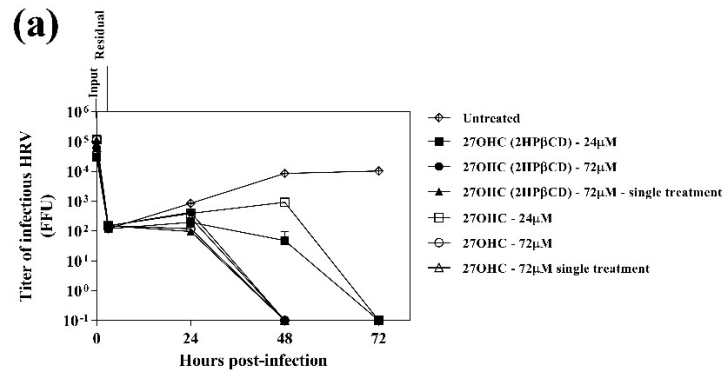
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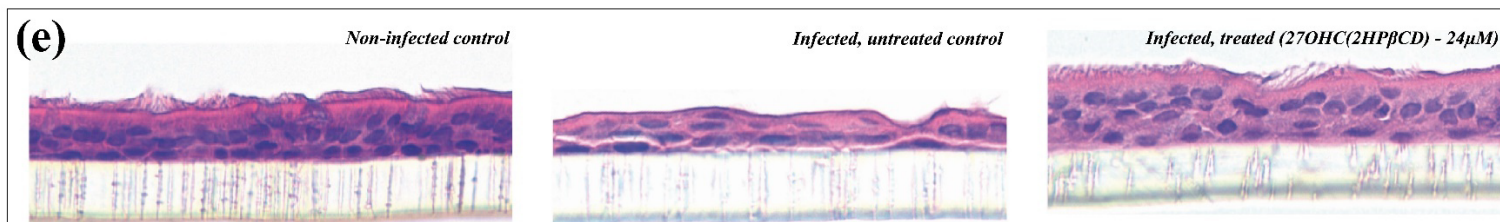
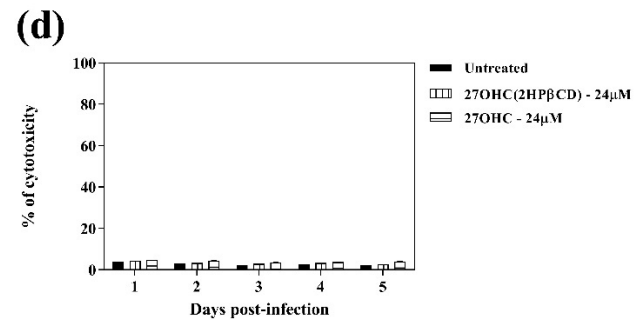
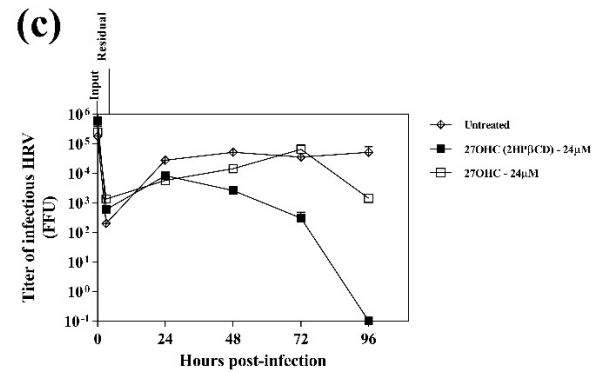
451

452 Figure 6.

Nasal epithelium



Bronchial epithelium



453

454 **Figure 7.**

455 Legend

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

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

472 **Figure 3.** Cytotoxicity assays. Cells were treated for 24 hours with serial dilutions of 25OHC (black
473 bars) or 27OHC (white bars), ranging from 5.6 μ M to 1350 μ M. Untreated control samples were
474 prepared by treating cells with a culture medium supplemented with equal volumes of EtOH
475 (checkered bars). Each bar represents the percentage of toxicity in the 25OHC-, 27OHC-treated, or
476 untreated samples. The results are means and SEM for triplicates. ** $p_{ANOVA} < 0.01$; *** $p_{ANOVA} <$
477 0.001.

478 **Figure 4.** Selection of resistant HRV strains by the serial passages approach (panels a-d) or by clonal
479 approach (panel e). Regarding the former, experiments were performed by serially culturing HRV
480 A1 in the presence of increasing concentrations of each antiviral molecule, namely 25OHC (panel a),
481 27OHC (panel b), pleconaril (panel c), or rupintrivir (panel d). The sensitivity of the viral progeny
482 collected at each passage was tested by focus reduction assays, and is expressed on the y axis in terms
483 of EC₅₀; error bars represent 95% confidence intervals of EC₅₀s. The results are means for duplicates.
484 Panel (e) depicts the frequency of resistant HRV A1 variants in an assay-compiled plate clonal
485 selection procedure. Briefly, a total of 384 samples were infected with HRV A1 for each tested
486 antiviral and treated with the respective EC₉₉s of each compound. Each bar represents the percentage
487 of cell monolayers with CPE calculated on the total number of infected samples.

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

495 **Figure 6.** In vitro antiviral activity assessment of formulated 27OHC (i.e. 27OHC [2HP β CD]), and
496 blank formulation (2HP β CD). Panel (a) depicts the results of the focus reduction assays with HRV
497 A1 and HRV B48; briefly, the cells were infected with HRV A1 or HRV B48 in the presence of
498 increasing concentrations of 27OHC [2HP β CD] and then infected; viral replication was detected 24
499 hours after infection, by means of indirect immunoperoxidase staining with the anti-VP3 antibody.
500 On the y axis, blockade of viral infectivity is expressed as the mean percentage of the untreated
501 control. The results are means for duplicates; the error bars represent the standard error of the mean
502 (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 (2HP β CD) (white
504 bars) or blank formulation (2HP β CD; striped bars), ranging from 0.2 μ M to 5.6 μ M. 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 (2HP β CD) 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 (2HP β CD)
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.