1   **Entry of human coronavirus NL63 to the cell.**
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3   *Running Head: Entry of HCoV-NL63.*
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Abstract

First steps of human coronavirus NL63 (HCoV-NL63) infection were previously described. The virus binds to target cells by heparan sulfate proteoglycans, and interacts with the ACE2 protein. Subsequent events, including virus internalization and trafficking, remain to be elucidated. In this study, we mapped the process of HCoV-NL63 entry into LLC-Mk2 cell line and *ex vivo* 3D tracheobronchial tissue.

Using a variety of techniques we have shown that HCoV-NL63 virions require endocytosis for successful entry to the LLC-MK2 cells, and interaction between the virus and the ACE2 molecule triggers recruitment of clathrin. Subsequent vesicle scission by dynamin results in virus internalization, and the newly formed vesicle passes the actin cortex, what requires active cytoskeleton rearrangement. Finally, acidification of the endosomal microenvironment is required for successful fusion and release of viral genome into the cytoplasm. Also for 3D tracheobronchial tissue cultures we observed that the virus enters the cell by clathrin-mediated endocytosis, but obtained results suggest that this pathway may be bypassed.

Importance

Available data on coronavirus' entry originate frequently from studies employing immortalized cell lines or undifferentiated cells. Here, using the most advanced 3D tissue culture system mimicking the epithelium of conductive airways, we systematically mapped HCoV-NL63 entry into susceptible cell. Obtained data allow for better understanding of the infection process and may support development of novel treatment strategies.

Introduction

49

50   Human coronavirus NL63 (HCoV-NL63) was discovered shortly after the emergence of

51   the severe acute respiratory syndrome coronavirus (SARS-CoV) (1). Extensive studies on the

52   pathogen's biology and epidemiology revealed that it is prevalent worldwide, appearing with

53   a seasonal distribution similar to that of other human coronaviruses. The clinical presentation

54   may vary depending on the general health status of the patient. Usually, the virus causes a

55   relatively mild respiratory tract disease, but fatal cases have been reported (2-5). Furthermore,

56   broad studies on the association between infection and clinical symptoms reveal that

57   HCoV-NL63 is associated with croup in young children (6-9).

58   Phylogenetically, HCoV-NL63 clusters within the genus alphacoronavirus, which also

59   includes another human pathogen, HCoV-229E. Initially, these two viruses were considered

60   distant relatives that diverged at some point due to an unknown reason. More recent research

61   shows, however, that these two species most likely emerged in the human population during

62   two separate zoonotic transmission events (10-12).

63   From the perspective of genome structure, HCoV-NL63 is similar to other

64   alphacoronaviruses in that the 5' terminal two-thirds of the genome encode a large

65   polyprotein, which is cleaved to yield several non-structural proteins. Five genes (S-ORF3-E-

66   M-N) are located at the 3' terminus encode structural proteins. The spike protein (S) is a class

67   I fusion protein comprising a rod-like domain anchored to the virion via its C-terminus, and a

68   globular head responsible for the interaction with cellular entry receptors (13). It is generally

69   assumed that alphacoronaviruses interact with and enter host cells using the CD13

70   (aminopeptidase N). However, HCoV-NL63 utilizes the ACE2 protein for this purpose, a

71   characteristic shared only with SARS-CoV (14, 15). Virus tropism not only depends on the

72   presence of a certain entry receptor but also may be modulated by other factors, e.g.,

73   attachment receptors, protease availability, and the activity of pathways responsible for

74   internalization and trafficking of the virus particle (16, 17).

75   While binding to their cognate entry receptor provides sufficient stimulus for some

76   viruses to initiate fusion between the viral and cellular membranes, most internalize *via*

77   endocytosis; acidification and/or processing by cathepsins is then a pre-requisite for fusion

78   (13). For a long time, endocytic entry of virions was classified as clathrin-dependent, clathrin-

79   independent, or clathrin- and caveolin-independent. During recent years, a number of other

80   pathways were identified and this complex machinery has become better understood. The

81   occurrence, abundance, and mechanistic details of these pathways appear to vary between cell

82   types, tissues, and species. Most often, the selection of a specific endocytic route is linked to

83   cargo-directed   trafficking   and   receptor-dependent   trafficking.   Nevertheless,   many

84   receptors/cargoes allow flexibility due to their capacity to enter a cell *via* multiple pathways.

85   The early stages of HCoV-NL63 infection have been described by us and others (18-

86   20). Here, we made an effort to delineate events that occur early during HCoV-NL63

87   infection. First, the virus anchors to ciliated cells *via* heparan sulfate proteoglycans before

88   interacting with the ACE2 entry receptor. Our results show that the virus-ACE2 interaction

89   triggers   recruitment   of   clathrin,   followed   by   clathrin-mediated,   dynamin-dependent

90   endocytosis, which requires actin cortex remodeling. To ensure that our results were reliable,

91   we used *ex vivo* cultured human airway epithelium (HAE), which mimics the

92   microenvironment at the infection site.

93   **Results**

94   **HCoV-NL63 enters the cell via endocytosis**

95   We first determined whether entry of HCoV-NL63 requires endocytosis and

96   acidification of endosomes. For this, we studied the effect of ammonium chloride (NH_4Cl)

97   and bafilomycin A - lysosomotropic agents that inhibit acidification of endosomes (21-23)

98   using two models of HCoV-NL63 infection: permissive LLC-Mk2 cells and HAE cultures.

99   Cells were pre-incubated with NH_4Cl (50 mM), bafilomycin A (100 nM) or control DMSO

100   for 1 h at 37°C, and subsequently incubated with the virus at $TCID_{50}$ of 100/ml (for LLC-Mk2

101   cells) or at $TCID_{50}$ of 400/ml (for HAE) for 2 h at 32°C in the presence of the inhibitor.

102   Subsequently, supernatants were removed and cells were washed thrice with acidic buffer to

103   inhibit the fusogenic activity of the virions retained on the surface (24). Next, LLC-Mk2 cells

104   were washed with $1 \times$ PBS (pH 7.4), overlaid with culture medium and incubated at 32°C for

105   4 days. Supernatant samples were collected for virus replication analysis. Simultaneously,

106   HAE cultures were washed with $1 \times$ PBS (pH 7.4) and further maintained at an air-liquid

107   interphase at 32°C for 5 days. During this time HAE cultures were washed every 24 h with

108   $1 \times$ PBS supplemented with a given inhibitor for 10 min at 32°C, and apical washes were

109   collected for virus replication analysis. Subsequently, viral RNA was isolated, reverse

110   transcribed (RT), and HCoV-NL63 yield was determined using a quantitative real-time PCR

111   (qPCR).

112   Bafilomycin A and NH_4Cl inhibited HCoV-NL63 infection in LLC-Mk2 cells, proving

113   that acidification is a pre-requirement for the virus infection *in vitro*. No inhibition was

114   observed in HAE cultures (**Fig. 1A**). No cytotoxic effect was observed in the presence of

115   these inhibitors (**Fig. 1B**).

116   Next, we analyzed HCoV-NL63 co-localization with the early endosome antigen-1

117   (EEA1), a hydrophilic protein localizing exclusively to early endosomes (25). LLC-Mk2 cells

118   were fixed after 10, 20, 30 or 40 min post-inoculation (p.i.) with gradient-purified virus,

119   stained with antibodies specific to HCoV-NL63 N protein and EEA1, and analyzed under

120   confocal microscope. Measured co-localization expressed as Manders' coefficient increases

121   with time and reaches 0.68 forty min p.i. (n = 6 cells) (**Fig. 1C**).

122   We validated the obtained results using the HAE model. Briefly, HAE cultures were

123   inoculated with gradient-purified HCoV-NL63 and incubated at 32°C for 2 h. For this culture

124   model a longer incubation was required to observe virus attachment and entry, most likely due

125   to the requirement to cross the mucus layer. Subsequently, cells were fixed and labeled with

126   specific antibodies against HCoV-NL63 N protein and EEA1. Co-localization of HCoV-NL63

127   virus particles with EEA1 protein was analyzed using confocal microscope. Co-localization of

128   virus and EEA1 was observed in inoculated cells (**Fig. 1D**).

129

130   **Endocytosis of virus particles is induced by binding to the entry receptor**

131   HCoV-NL63 virus employs the ACE2 protein for cellular entry, while heparan sulfate

132   proteoglycans serve as attachment receptors (19). Here, we analyzed the consequence of

133   interaction between the virus particle and ACE2. First, we inoculated naturally permissive

134   LLC-Mk2 cells with HCoV-NL63 and incubated for 40 min at 4°C to enable virus adhesion

135   to a cell surface. Subsequently cells were fixed, the virus was labelled with specific antibodies

136   and its co-localization with the ACE2 and clathrin was studied. As shown in **Fig. 2A**,

137   HCoV-NL63 particles attach efficiently to the cell surface. However, only a proportion of

138   virions co-localize with the ACE2 (Manders'coeff. = 0.573; n = 5), suggesting that binding to

139   the HS precedes interaction with the entry receptor. At that point, there is no co-localization

140   of virus particles and clathrin-coated pits (Manders'coeff. = 0.140; n = 5) (**Fig. 2B**). Next, we

141   tested whether the virus binding to the adhesion or entry receptor triggers recruitment of

142   common cellular proteins responsible for pit formation by incubating cells for 5 min at 32°C.

143   Immunostaining showed that the virus particles bound to the ACE2 start to co-localize with

144   clathrin (Manders'coeff. = 0.849, n = 6) (**Fig. 2C**), while there is no co-localization between

145   non-ACE2-bound virions and clathrin (Manders'coeff. = 0.189, n = 6).

146

147   **HCoV-NL63 co-localizes with clathrin during entry**

148   To determine whether co-localization with clathrin following the ACE2 binding is

149   relevant, and indeed the virus enters the cell by clathrin-coated pits we analyzed

150   co-localization of intracellular virions with clathrin. Briefly, LLC-Mk2 cells were incubated at

151   32°C for 5-20 min with gradient-purified HCoV-NL63, fixed, immunostained and analyzed

152   with confocal microscopy. Results showed co-localization of virions entering the cell with

153   clathrin (Manders'coeff. = 0.584; n = 7) (**Fig. 3A**), whereas no co-localization with caveolin-1

154   was observed (Manders' coeff. = 0.053; n = 5) (**Fig. 3B**). HCoV-NL63 co-localization with

155   clathrin and caveolin was also studied in HAE model. For this, cultures were incubated with

156   gradient-purified HCoV-NL63 at 32°C for 2 h; the virus and the cellular proteins were

157   immunostained and analyzed with confocal microscopy. HCoV-NL63 virions also in this

158   model co-localized with clathrin, whereas no co-localization was observed for caveolin-1

159   (**Fig. 3**).

160

161   **Clathrin and dynamin are important for HCoV-NL63 entry**

162   As we already knew that HCoV-NL63 virions migrate to clathrin-coated pits, in the

163   subsequent step we aimed to determine whether the clathrin-mediated endocytosis is indeed

164   important for the virus entry. For this reason, we blocked the pathway using Pitstop 2 (N-[5-

165   [4-Bromobenzylidene]-4-oxo-4,5-dihydro-1,3-thiazol-2-yl] naphthalene-1-sulfonamide) – a

166   selective clathrin inhibitor targeting its amino terminal domain, and MitMAB

167   (tetradecyltrimethylammonium bromide) – a dynamin I and II GTPase inhibitor. Activity of

168   these compounds was verified with the positive control (fluorescently-labelled transferrin)

169   (26, 27). LLC-Mk2 cells were treated with Pitstop 2, MitMAB or control DMSO for 30 min

170   at 37°C, following transferrin uptake for 45 min at 37°C. Confocal images showed that both

171   inhibitors blocked transferrin endocytosis, as the protein was present only on the cell surface

172   (**Fig. 4A-D**).

173   Subsequently, LLC-Mk2 cells were incubated with one of the inhibitors at 37°C for

174   30 min and inoculated with gradient-purified HCoV-NL63 at 32°C for 5 min. Following

175   immunostaining of the HCoV-NL63 N protein and actin, virus endocytosis was analyzed

176   using confocal microscopy. Results showed that virus internalization was hampered in cells

177   pre-treated with clathrin and dynamin inhibitors, as compared to the DMSO-treated cells

178   (**Fig. 4D-G**). Simultaneously, a cytotoxicity test of the entry inhibitors was performed, which

179   showed no toxic effect of the tested compounds to LLC-Mk2 cells (**Fig. 5**). In order to ensure

180   that our observations are not biased, statistical analysis of virus entry was performed. For this,

181   an algorithm was prepared for image analysis and 3D representation of the cell was prepared

182   and virus position in the cell was determined (**Fig. 6**).

183   Similar experiment was conducted using HAE cultures. For this, cultures were

184   incubated for 1 h at 37°C with inhibitors described above, following incubation with gradient-

185   purified HCoV-NL63 at 32º C for 2 h. A strong inhibition of virus internalization in cultures

186   pre-incubated with clathrin or dynamin inhibitors was observed, compared to control cells

187   (**Fig. 7**). No cytotoxicity to HAE was observed for the tested inhibitors after 3 h incubation at

188   37°C (**Fig. 8**).

189

190   **Clathrin mediated endocytosis is the main entry route for HCoV-NL63**

191   Even though certain cargo is usually internalized by a single route, frequently other

192   pathways may be used as alternatives. We therefore aimed to test whether inhibition of

193   clathrin-mediated entry with chemical inhibitors results in inhibition of virus replication. To

194   address this, we incubated LLC-Mk2 cells with a given inhibitor at 37º C for 1 h and infected

195   them with HCoV-NL63 (TCID$_{50}$ = 400 per ml) for 2 h at 32°C. Subsequently media were

196   removed and cells were washed thrice with acidic buffer, following washing with $1 \times$ PBS

197   (pH 7.4). Next, cells were overlaid with culture medium containing a given inhibitor and

198   incubated at 32°C for 4 days. Cells were fixed and immunostained for HCoV-NL63 N protein

199   to assess the number of infected cells. To assess the non-specific effect of entry inhibitors,

200   control cells were treated with these also 4 h p.i. Clearly, in the presence of clathrin-mediated

201   endocytosis inhibitors (Pitstop 2 and MitMAB) the number of HCoV-NL63-infected cells was

202   much lower compared to the control. However, MitMAB also inhibited virus replication at

203   later stages of the infection (**Fig. 9**). To ensure that entry inhibitors affected HCoV-NL63

204   infection in LLC-Mk2 cells, we analyzed by RT-qPCR virus replication at 120 h p.i. in the

205   presence of tested compounds. The analysis showed a ~2-log decrease in virus progeny

206   production in the presence of Pitstop 2 and MitMAB, compared to DMSO-treated cells and a

207   slight increase of RNA copy levels in the presence of nystatin (**Fig 10A**). Importantly, no

208   cytotoxic effect was observed for the tested inhibitors applied to LLC-Mk2 for 4 days at 32°C

209   (**Fig 10B**). The influence of tested inhibitors on HCoV-NL63 infection was analyzed also in

210   HAE cultures. For this, cultures were pre-incubated with a given inhibitor (Pitstop 2,

211   MitMAB, nystatin or control DMSO) for 1 h at 37°C and infected with HCoV-NL63 at

212   TCID$_{50}$ of 400 per ml for 2 h at 32°C. Subsequently, non-internalized virions were inactivated

213   by acid wash, cultures were washed with $1 \times$ PBS, and incubated with a given inhibitor for 10

214   min. After that time supernatants were discarded and cultures were incubated for 5 days at

215   32°C. During this period, cultures were incubated with a given inhibitor for 10 min at 32°C

216   every 24 h. Viral RNA from these samples was quantified by RT-qPCR. Virus replication in

217   HAE was not affected by any of the tested inhibitors (**Fig. 10A**).

218

TMPRSS2 is important during early stages of the infection

220   It was previously suggested that coronaviruses may bypass the endocytic entry route

221   employing TMPRSS2 protease, which primes the fusion protein and enable fusion of viral

222   and cellular membranes on the cell surface (31, 32). We have tested whether inhibition of the

223   TMPRSS2 proteases with camostat affects the HCoV-NL63 infection. We observed that

224   inhibition of TMPRSS2 protease hampers virus infection in HAE cultures, while it has no

225   effect on virus replication in LLC-MK2 cells (**Fig. 11A**). No inhibition of virus entry was

226   observed in any of the models, as tracked with confocal microscopy, visualizing the

227   nucleoprotein (**Fig. 11B**). As only single entry events per view were observed, several images

228   for camostat-treated and control cells are presented. In total, 500 entry events into HAE cells

229   were tracked and no difference between the camostat-treated sample and control sample was

230   noted.

231

HCoV-NL63 entry requires actin re-modelling

233   We studied trafficking of HCoV-NL63 inside the cell. As entry by endocytosis would

234   probably require re-modelling of the cytoskeleton, we evaluated virus internalization in the

235   presence of cytochalasin D, jasplankinolide or nocodazole. The first chemical inhibits actin

236   polymerization, whereas the second binds F-actin and stabilizes actin filaments (33, 34). The

237   last compound interferes with the microtubule formation. The analysis showed that actin

238   inhibitors prevented virus particles from penetrating the cell, with visible viral particles

239   accumulation on actin cortex or unstructured actin deposits. Microtubule inhibitor did not

240   affect virus entry (**Fig. 12**). No cytotoxicity was observed for the tested inhibitors (**Fig. 13**).

Discussion

241

242   Previously, we and others described the first steps of the HCoV-NL63 infection process,

243 showing that it begins with the virus binding to the cellular membrane *via* heparan sulfate

244 proteoglycans, which then enable/facilitate interaction with the entry receptor, ACE2 (14, 18,

245 19). Little is known about the subsequent virus internalization and its trafficking through the

246 cytoplasm, and some published data are contradictory. For example, the role played by

247 cathepsins and acidification of the microenvironment during transition of the HCoV-NL63 S

248 protein to its fusogenic form remains unclear.

249   We made an effort to systematically examine every step of the process. First, we tested

250 whether the virus requires endocytosis for successful entry. To do this, we carried out

251 experiments using chemical inhibitors of endosome acidification (ammonium chloride and

252 bafilomycin A). Both blocked virus infection in LLC-MK2 cells, suggesting a requirement for

253 transport of virions to endosomes, which then undergo acidification. Yet, such an approach

254 may have several disadvantages. First, we examined the role of endosome acidification based

255 on virus replication; thus we cannot rule out interference with virus infection at later stages

256 (as shown for MiTMAB). Second, the specificity and selectivity of chemical inhibitors are

257 questionable. An indirect proof for the pH dependence of HCoV-NL63 entry may be provided

258 by the fact that acidification of the environment (acid wash) results in inactivation of the

259 virus, suggesting the pH – directed structural switch in the S protein. To further confirm our

260 observations, we developed a method of visualizing single virions as they entered the cell.

261 Efforts to stain for virus surface proteins yielded poor results, most likely due to lack of

262 highly specific antibodies and post-translational modification of surface proteins and the best

263 results were obtained when antibodies specific to the N protein were used. Incubation of cells

264 with purified virions resulted in virus attachment, which was visualized by confocal

265 microscopy and co-staining for markers of the most commonly employed endocytic pathways

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266   allowed us to study the co-localization. If significant co-localization was detected, results

267   were confirmed with chemical inhibitors.

268   The results showed that HCoV-NL63 binding to the ACE2 initiates recruitment of

269   clathrin and subsequent formation of clathrin coated pits; no co-localization of the virus with

270   other markers (e.g., caveolin) was noted. Transferrin was used as a positive control for

271   clathrin-mediated endocytosis (35, 36). Importantly, chemical inhibitors of clathrin

272   completely blocked virus internalization and the virus remained on the cell surface. Analysis

273   of HAE cultures yielded identical results. The inhibitors of endocytosis also hampered virus

274   infection on LLC-Mk2 cells, highlighting that this pathway is relevant and the lack of an

275   equally effective alternative entry route in this culture model. Clathrin-mediated endocytosis

276   requires a number of other proteins, as dynamin, the GTPase responsible for scission of

277   clathrin-coated vesicles from the cell surface (37). Inhibiting dynamin also hampered virus

278   internalization into LLC-MK2 cells and HAE cultures, confirming our previous observations.

279   However, in this case the MitMAB compound blocked replication of HCoV-NL63 also during

280   subsequent stages of the infection.

281   It is noteworthy that we were not able to block virus infection of HAE cultures using

282   inhibitors of endocytosis. This may be related to the fact that the cultures were exposed to

283   inhibitors for a very short time during apical washes, which is not sufficient to permanently

284   block the infection. On the other side, it is also possible that in HAE HCoV-NL63 is able to

285   enter the cell by an alternative route. Recent reports on other coronaviruses (31, 32, 38)

286   suggested that these viruses may bypass the endocytic entry route using TMPRRS2 as the

287   priming protease, enabling the entry directly from the cell surface. Our experiments showed

288   that inhibition of this protease indeed inhibited virus infection. Interestingly, it did not hamper

289   virus internalization to the cell. Our data are consistent with the data presented by others (31,

290   32, 38), yet we believe that there is a different mechanistic explanation to the observed

291   phenomenon. We believe that indeed TMPRRS2 protease is required for the virus-cell fusion,

292   acting similarly to cathepsins, but it does not enable fusion on the cell surface and the

293   acidification of the microenvironment is required.

294   Our final research question was about virus trafficking. The endosome typically

295   translocates through the depolymerizing actin cortex and is subsequently sorted at the

296   endosomal hub and directed to different destinations. This sorting is highly dependent on the

297   cargo. Using two chemical inhibitors (jasplankinolide and cytochalasin B) (34, 39), we

298   showed that actin plays a vital role in virus entry. Stabilization of the actin cortex using

299   jasplankinolide similarly as inhibition of actin polymerization using cytochalasin D resulted in

300   immobilization of the virus at the cell surface. These two experiments suggest a scenario in

301   which virus-carrying endosomes pass along the actin cortex, which actively unwinds and

302   interacts with virions.

303   Summarizing, we show that HCoV-NL63 enters the cell by clathrin-mediated

304   endocytosis, but the pathway may be bypassed to some extent during the infection *ex vivo*.

305   HCoV-NL63 entry to the susceptible cell was summarized in **Fig 14**.

306 **Materials and methods**

307 **Cell culture**

308   LLC-Mk2 cells (ATCC: CCL-7; *Macaca mulatta* kidney epithelial) were maintained in

309 minimal essential medium (MEM; two parts Hanks' MEM and one part Earle's MEM;

310 Thermo Scientific, Poland) supplemented with 3% heat-inactivated fetal bovine serum

311 (Thermo Scientific, Poland), penicillin (100 U/ml), streptomycin (100 μg/ml), and

312 ciprofloxacin (5 μg/ml). Cells were cultured at 37°C under 5% CO_2.

313

314 **Ethics Statement**

315   Human tracheobronchial epithelial cells were obtained from airway specimens resected

316 from adult patients undergoing surgery under Silesian Center for Heart Diseases-approved

317 protocols. This study was approved by the Bioethical Committee of the Medical University of

318 Silesia in Katowice, Poland (approval no: KNW/0022/KB1/17/10 dated on 16.02.2010).

319 Participants provided their written informed consent to participate in the study, as approved

320 by the Bioethical Committee.

321

322 **Human airway epithelium cultures**

323   Primary human tracheobronchial epithelial cells were expanded on plastic to generate

324 passage 1 cells and plated on permeable Transwell inserts (6.5 mm-diameter) supports.

325 Human airway epithelium (HAE) cultures were generated by provision of an air-liquid

326 interface for 6-8 weeks to form well-differentiated, polarized cultures that

327 resemble *in vivo* pseudostratified mucociliary epithelium. Cultures were prepared and

328 maintained as previously described (24).

329

330 **Cell viability assay**

331   LLC-Mk2 cells were cultured on 96-well plates and HAE cultures were prepared as

332 described above. Cell viability assay was performed by using the XTT Cell Viability Assay

333 (Biological Industries, Israel), according to the manufacturer's instructions. Briefly, on the

334 day of the assay 100 μl of the culture medium (for LLC-Mk2) or $1 \times$ PBS (for HAE) with the

335 30 μl of the activated XTT solution was added to each well/culture insert. Following 2 h

336 incubation at 37°C, the solution was transferred onto a 96-well plate and signal was measured

337 at $\lambda = 490$ nm using the colorimeter (Spectra MAX 250, Molecular Devices). The obtained

338 results were further normalized to the control sample, where cell viability was set to 100%.

339

340 **Virus preparation and titration**

341   The HCoV-NL63 stock (isolate Amsterdam 1) was generated by infecting monolayers

342 of LLC-Mk2 cells. The virus-containing liquid was aliquoted and stored at −80°C. A control

343 LLC-Mk2 cell lysate from mock-infected cells was prepared in the same manner. The virus

344 yield was assessed by titration on fully confluent LLC-Mk2 cells in 96-well plates, according

345 to the method described by Reed and Muench (40).

346

347 **Purification of HCoV-NL63**

348   The virus stock was concentrated 25-fold using centrifugal protein concentrators

349 (Amicon Ultra, 10 kDa cut-off; Merck, Poland) and subsequently overlaid on 15% iodixanol

350 solution in $1 \times$ PBS (OptiPrep medium; Sigma-Aldrich, Poland). Following virus

351 concentration using iodixanol cushion (centrifugation at $175\,000 \times g$ for 3 h at 4°C) it was

352 overlaid on 10-20% iodixanol gradient in $1 \times$ PBS and centrifuged at $175\,000 \times g$ for 18 h at

353 4°C. Fractions (1 ml) collected from the gradient were analyzed on western blot, using

354 anti-HCoV-NL63 N IgGs (0.25 μg/ml; Ingenansa, Spain) and a secondary antibody coupled

355 with horseradish peroxidase (65 ng/ml, Dako, Denmark). The virus-containing fractions were

356 aliquoted and stored at −80°C. The control cell lysate (mock) was concentrated and prepared

357 in the same manner as the virus stock.

358

359 **Inhibition of virus entry**

360   LLC-Mk2 cells were seeded on coverslips in six-wells plates (TPP) and cultured for

361 2 days at 37°C. Subsequently, cells were incubated with a given inhibitor for 30 min at 37°C,

362 and later with 50 µl of purified HCoV-NL63 or mock sample for 1 h at 32°C. For the *ex vivo*

363 experiment, HAE cultures were exposed to the tested inhibitor or control PBS for 1 h at 37°C,

364 following inoculation with iodixanol-concentrated HCoV-NL63 or mock sample. Following

365 2 h incubation at 32°C, unbound virions were removed by washing with $1 \times$ PBS. Cells were

366 then washed with $1 \times$ PBS and fixed with 4% paraformaldehyde (PFA).

367   Transferrin and albumin were used as positive controls, as they were previously

368 described to serve as a cargo in the clathrin- and caveolin- dependent endocytosis,

369 respectively (41, 42). LLC-Mk2 cells were seeded on coverslips in six-wells plates (TPP,

370 Switzerland) and cultured for 2 days at 37°C. Subsequently, cells were incubated with a given

371 inhibitor for 30 min at 37°C, following incubation with Alexa Fluor 488-labeled transferrin

372 (100 µg/ml; Molecular Probes) or FITC labeled albumin (500 µg/ml; Sigma-Aldrich, Poland)

373 or control PBS for 45 min at 32°C. Cells were then washed with $1 \times$ PBS and fixed in

374 4% PFA.

375

376 **Immunostaining and confocal imaging**

377   Fixed cells were permeabilized with 0.1% Triton X-100 in $1 \times$ PBS and incubated

378 overnight at 4°C in $1 \times$ PBS supplemented with 5% BSA and 0.5% Tween 20. To visualize

379 HCoV-NL63 particles, cells were incubated for 2 h at room temperature with mouse

380   anti-HCoV-NL63 N IgGs (0.25 μg/ml; Ingenansa, Spain), followed by a 1 h incubation with

381   Alexa Fluor 488-labeled goat anti-mouse IgG (2.5 μg/ml; Thermo Scientific, Poland). The

382   following antibodies were used for endosomal markers: polyclonal goat anti-human

383   clathrin HC coupled with tetramethylrodamine (10 μg/ml; Santa Cruz Biotechnology) and

384   polyclonal rabbit anti-human early endosome antigen 1 (2 μg/ml; Santa Cruz Biotechnology);

385   polyclonal rabbit anti-human caveolin-1 (2 μg/ml; Sigma-Aldrich, Poland), and Alexa Fluor

386   633-labeled goat anti-rabbit (2.5 μg/ml; Thermo Scientific, Poland). Actin filaments was

387   stained using phalloidin coupled with Alexa Fluor 633 (0.2 U/ml; Thermo Scientific, Poland).

388   Nuclear DNA was stained with DAPI (0.1 μg/ml; Sigma-Aldrich, Poland). Immunostained

389   cultures were mounted on glass slides in ProLong Gold antifade medium (Thermo Scientific,

390   Poland). Fluorescent images were acquired under a Leica TCS SP5 II confocal microscope

391   (Leica Microsystems GmbH, Mannheim, Germany) and a Zeiss LSM 710 confocal

392   microscope (Carl Zeiss Microscopy GmbH). Images were acquired using Leica Application

393   Suite Advanced Fluorescence LAS AF v. 2.2.1 (Leica Microsystems CMS GmbH) or ZEN

394   2012 SP1 software (Carl Zeiss Microscopy GmbH), respectively, deconvolved with Huygens

395   Essential package ver. 4.4 (Scientific Volume Imaging B.V.; The Netherlands) and processed

396   using ImageJ 1.47v (National Institutes of Health, Bethesda, Maryland, USA).

397

398   **Flow cytometry**

399   LLC-Mk2 cells were seeded on 6-wells plates (TPP), cultured for 2 days at 37°C with

400   5% CO_2. Cells in monolayer were incubated with each entry inhibitor for 1 h at 37°C,

401   following infection with HCoV-NL63 at $TCID_{50}$ of 100/ml or inoculation of the mock

402   sample. On day 4 p.i., cells were washed with sterile PBS, fixed with 3% PFA, permeabilized

403   with 0.1% Triton X-100 in $1 \times$ PBS and incubated for 1 h with 3% BSA in $1 \times$ PBS with

404   0.1% Tween 20. To quantify HCoV-NL63 infection, fixed cells were scraped from plastic and

405   incubated for 2 h at room temperature with mouse anti-HCoV-NL63 N IgG antibodies (1

406   µg/ml; Ingenansa), followed by 1 h incubation with Alexa Fluor 488-labeled goat anti-mouse

407   antibody (2.5 µg/ml; Molecular Probes). Cells were then washed, re-suspended in $1 \times$ PBS

408   and analyzed with FACS Calibur (Becton Dickinson) using Cell Quest software.

409

410   **Isolation of nucleic acids and reverse transcription**

411   Viral nucleic acids were isolated from cell culture supernatants (LLC-Mk2 cells) or

412   apical washes (HAE cultures) using the Viral RNA/DNA Isolation Kit (A&A Biotechnology,

413   Poland), according to the manufacturer's instructions. Reverse transcription was carried out

414   with a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Poland),

415   according to the manufacturer's instructions.

416

417   **Quantitative RT-PCR**

418   HCoV-NL63 yield was determined using an RT-qPCR (7500 Fast Real-Time PCR

419   machine, Life Technologies, Poland). Viral cDNA (2.5 µl per sample) was amplified in a

420   10 µl reaction mixture containing $1 \times$ Master Mix (RT Mix Probe, A&A Biotechnology,

421   Poland), specific probe labelled with 6-carboxyfluorescein (FAM) and 6-

422   carboxytetramethylrhodamine (TAMRA) (100 nM; 5'-ATG TTA TTC AGT GCT TTG GTC

423   CTC GTG AT- 3') and primers (450 nM each; sense: 5'-CTG TGG AAA ACC TTT GGC

424   ATC- 3'; antisense: 5' – CTG TGG AAA ACC TTT GGC ATC- 3'). Rox was used as the

425   reference dye. The reaction conditions were as follows: 2 min at 50°C and 10 min at 92°C,

426   followed by 40 cycles of 15 sec at 92°C and 1 min at 60°C. In order to assess the copy

427   number for the N gene, DNA standards were prepared. Briefly, N gene of HCoV-NL63 was

428   amplified and cloned into pTZ57R/T (Thermo Fisher Scientific, Poland) plasmid using the

429   InsTAclone PCR cloning kit (Thermo Scientific, Poland). Subsequently, DNA vectors were

430   amplified and linearized with *Eco*RI restriction enzyme. Linear nucleic acids were further

431   purified with the GeneJET™ PCR Purification Kit (Thermo Fisher Scientific, Poland),

432   according to the manufacturer's instructions and its concentration was assessed using a

433   spectrophotometer. The number of DNA copies/ml was assessed using Avogadro's constant

434   and molecular mass of RNA molecules. Samples were serially diluted and used as an input

435   real-time PCR reaction.

436   In this article, the data from quantitative PCR are presented as log removal values

437   (LRVs) in order to enable comparison of results obtained from different assays. LRV was

438   calculated according to the following formula: LRV = −log (c_i/c_0) where c_i is the number of

439   viral RNA copies per milliliter in the sample in the culture treated with a given polymer and

440   c_0 is the number of viral RNA copies per milliliter in control sample (untreated cells).

441

442   **Image analysis**

443   To evaluate the infection inhibition in the presence of various endocytosis inhibitors

444   image analysis was performed on 2 mm × 2 mm tile scan images. On each image, the number

445   of nuclei (expressed as a number of cells) and the mean pixel intensity for the virus were

446   calculated. For that, histograms of all images were adjusted to the min/max value, excluding

447   signal from the virus derived from images with no infected cells. Results are presented as

448   mean intensity of fluorescence per cell.

449   Co-localization analyses were performed under ImageJ using JACoP plugin (Bolte and

450   Cordelieres, 2006), where Manders' coefficient was calculated for 3D images of more than

451   5 cells.

452   Quantitative analysis of virus internalization in the presence of inhibitors was performed

453   with algorithm previously described by Berniak *et al*. with modifications (43). Cell surface

454   was estimated on each image slice manually using polygon selection tool in ImageJ and based

455   on this information 3D cell surface was modelled. Coordinates of virus particles were

456   determined using 3D Object Counter ImageJ plugin. Relative localization and distance

457   between virus particle and cell surface was calculated. Results are presented as a ratio

458   between virus particles inside a cell and the particles on the surface (up to 1.5 µm above).

459

460   **Statistical analysis**

461   All the experiments were performed in triplicate and the results are presented as mean

462   ± SD. To determine significance of the obtained results, a comparison between groups was

463   conducted using the Student's t-test. *P* values < 0.05 were considered significant.

464

481

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482

483   **Figure legends**

484   **Fig 1. Importance of endosomal entry for HCoV-NL63 infection.**

485   (**A**) Inhibition of HCoV-NL63 infection in LLC-Mk2 cells and HAE cultures by

486   lysosomotropic agents: ammonium chloride (NH_4Cl, 50 mM) and bafilomycin A (Baf A,

487   100 nM), as determined with a RT-qPCR; values on the y axis are presented as LRV. The

488   assay was performed in triplicate, and average values with standard errors are presented. *P*

489   values < 0.05 were considered significant and are denoted with an asterisk (*). (**B**)

490   Cytotoxicity of the tested inhibitors was measured with an XTT assay. Data on the y-axis

491   represent viability of the treated cells compared to the untreated reference samples. The assay

492   was performed in triplicate and average values with standard errors are presented. (**C**, **D**)

493   Confocal images showing co-localization of HCoV-NL63 virions with early endosomal

494   marker EEA1 on LLC-Mk2 cells (**C**) and HAE cultures (**D**). Scale bar $= 5$ μm. Green:

495   HCoV-NL63, red: EEA1.

496

497   **Fig 2. HCoV-NL63 binding to the ACE2 triggers clathrin-mediated endocytosis.**

498   Pre-cooled LLC-Mk2 cells were incubated with gradient-purified HCoV-NL63 for 40 min at

499   4°C, following 0 min (**A**, **B**) or 5 min (**C**) incubation at 32°C. Co-localization of the virus

500   (green) and the ACE2 (red) was analyzed using confocal microscopy (**A**). No colocalisation

501   with clathrin was observed after 0 min incubation (**B**). Triple co-localization of virus with

502   ACE2 and clathrin (blue) is visible in panel (**C**). Images on the right side are zoomed-in

503   regions indicated by white rectangles on the left-side slides. A representative image is shown.

504   Scale bar $= 10$ μm.

505

506   **Fig 3. HCoV-NL63 co-localizes with clathrin, but not caveolin.**

507 LLC-Mk2 cells were incubated with gradient-purified HCoV-NL63 for 40 min at 4°C,

508 following 5 min (**A**) or 20 min (**B**) incubation at 32°C. HAE cultures were incubated with

509 gradient-purified HCoV-NL63 for 40 min at 4°C, following 120 min incubation at 32°C.

510 HCoV-NL63 co-localization with clathrin (**A**) or caveolin (**B**) was analyzed with confocal

511 microscopy (HCoV-NL63: green, clathrin and caveolin: red, nuclei: blue). Cells incubated

512 with mock and stained with isotypic antibodies were used as control (**C**). Scale bar = 5 μm.

513

514 **Fig 4. Clathrin and dynamin inhibitors hamper internalization of HCoV-NL63.**

515 In order to verify effectiveness of inhibitors, LLC-Mk2 cells were incubated with control

516 DMSO (**A**), 10 μM Pitstop 2 (**B**), or 10 μM MitMAB (**C**) for 30 min at 37°C, and inoculated

517 with Alexa Fluor-488-labelled transferrin. Following incubation (45 min, 37°C), cells were

518 fixed and stained for actin (red). Transferrin entry was evaluated with confocal microscopy.

519 Further, LLC-Mk2 cells were incubated with control DMSO (**E**), 10 μM Pitstop 2 (**F**), 10 μM

520 MitMAB (**G**) for 30 min at 37°C. Cells were inoculated with purified HCoV-NL63 and

521 incubated at 32°C for 1 h. Subsequently, cells were fixed and immunostained for HCoV-

522 NL63 particles (green) and actin (red). Mock-infected cells were used as control (**D**). Scale

523 bar = 10 μm.

524

525 **Fig. 5. Cytotoxicity of Pitstop 2 and MiTMAB on LLC-MK2 cells.**

526 Cytotoxicity of the tested endocytosis inhibitors was tested with an XTT assay. Cells were

527 incubated with control DMSO, 10 μM Pitstop 2, or 10 μM MitMAB for 2 h at 37°C Data on

528 the y-axis represent viability of the treated cells compared to the untreated reference samples.

529 The assay was performed in triplicate and average values with standard errors are presented.

530

531 **Fig 6. Numerical image analysis: clathrin and dynamin inhibitors block HCoV-NL63**

532 **entry.**

533 LLC-Mk2 cells were incubated with DMSO (**B**), 10 μM MitMAB (**C**), or 10 μM Pitstop 2

534 (**D**) for 30 min at 37°C, and subsequently inoculated with purified HCoV-NL63 and incubated

535 for 45 min at 32°C. Confocal images were digitalized, and localization of each virus particle

536 relative to the cellular membrane was assessed. In panel (**A**) a graph presenting the number of

537 internalized virus particles relative to number of virions on the cell surface (y axis) is

538 presented for cells treated with DMSO (control), Pitstop 2 or MitMAB. In panels (**B**), (**C**),

539 and (**D**) raw data for cells treated with DMSO, Pitstop 2, or MitMAB, respectively, are

540 presented. Histograms present an average number of virus particles (y axis) vs the distance

541 from cell surface (x axis). Values < 0 on the x axis indicate that the virus is inside the cell,

542 while for extracellular virions x value is ≥ 0.

543

544 **Fig 7. Clathrin and dynamin inhibitors prevent HCoV-NL63 from entering the cell in**

545 **the HAE model.**

546 HAE were incubated with control DMSO (**A**), 10 μM Pitstop 2 (**B**), or 10 μM MitMAB (**C**)

547 for 1 h at 37°C. Further, cells were inoculated with purified HCoV-NL63 and incubated at

548 32°C for 2 h. Subsequently, cells were fixed and immunostained for HCoV-NL63 particles

549 (green), actin (red) and nuclei (blue). Scale bar 5 μm.

550

551 **Fig. 8. Cytotoxicity of Pitstop 2 and MiTMAB on HAE cultures.**

552 Cytotoxicity of the tested endocytosis inhibitors was tested with an XTT assay. Cells were

553 incubated with control DMSO, 10 μM Pitstop 2, or 10 μM MitMAB for 2 h at 37°C. Data on

554 the y-axis represent viability of the treated cells compared to the untreated reference samples.

555 The assay was performed in triplicate and average values with standard errors are presented.

556

557 **Fig 9. Clathrin and dynamin inhibitors limit the number of LLC-MK2 infected cells.**

558 LLC-Mk2 cells were incubated with control DMSO (**A**), 5 ug/ml nystatin (**B**), 10 μM

559 MitMAB (**C**), 10 μM Pitstop 2 (**D**) for 1 h at 37°C and inoculated with HCoV-NL63

560 ($TCID_{50} = 100$/ml). After 2 h incubation at 32°C, virions that were not internalized were

561 inactivated with acidic buffer (pH = 3) and cells were incubated for 4 days at 32°C in the

562 presence of tested inhibitors or control DMSO. Identical procedure was applied to cells

563 presented in panels **E** and **F**, yet in these MitMAB and Pitstop 2 were applied, respectively,

564 after the acid wash. Fixed cells were immunostained with anti-NL63 nucleocapsid protein

565 (green) and nuclei (blue) and confocal images were collected. Scale bar: 200 μm.

566

567 **Fig. 10. Clathrin and dynamin inhibitors hamper replication of HCoV-NL63 in LLC-**

568 **MK2 cells.**

569 (**A**) HCoV-NL63 replication in LLC-Mk2 cells and HAE cultures in the presence of entry

570 inhibitors or control DMSO was analyzed using RT-qPCR. Cultures were incubated with

571 10 μM Pitstop 2, 10 μM MitMAB, 5 ug/ml nystatin or DMSO for 1 h at 37°C and inoculated

572 with HCoV-NL63 ($TCID_{50} = 400$/ml). After 2 h incubation at 32°C, virions that were not

573 internalized were inactivated with acidic buffer (pH = 3) and cells were incubated for 5 days

574 at 32°C. The data are presented as Log Reduction Value (LRV), compared to the control

575 sample. The assay was performed in triplicate, and average values with standard errors are

576 presented. *P* values < 0.05 were considered significant and are denoted with an asterisk (*).

577 (**B**) Cytotoxicity of the tested inhibitors was tested with an XTT assay. Cells were incubated

578 with 10 μM Pitstop 2, 10 μM MitMAB, 5 ug/ml nystatin or DMSO for 5 days at 32°C. Data

579 on the y-axis represent viability of the treated cells compared to the untreated reference

580   samples. The assay was performed in triplicate and average values with standard errors are

581   presented.

582

583   **Fig 11. TMPRSS2 is required for entry to HAE cells, but not enables virus – cell fusion**

584   **on the cell surface.**

585   (**A**) HCoV-NL63 replication in LLC-Mk2 cells and HAE cultures in the presence of camostat

586   or control DMSO was analyzed using RT-qPCR. Cultures were incubated with 100 μM

587   camostat or DMSO for 1 h at 37°C and inoculated with HCoV-NL63 (TCID$_{50}$ = 400/ml).

588   After 2 h incubation at 32°C, virions that were not internalized were inactivated with acidic

589   buffer (pH = 3) and cells were incubated for 5 days at 32°C. The data are presented as Log

590   Reduction Value (LRV), compared to the control sample. The assay was performed in

591   triplicate, and average values with standard errors are presented. *P* values < 0.05 were

592   considered significant and are denoted with an asterisk (*). (**B**) HAE were incubated with

593   control DMSO or 100 μM camostat for 1 h at 37°C. Further, cells were inoculated with

594   purified HCoV-NL63 and incubated at 32°C for 2 h. Subsequently, cells were fixed and

595   immunostained for HCoV-NL63 particles (green), actin (red) and nuclei (blue). Scale bar =

596   5 μm.

597

598   **Fig 12. Actin is important for HCoV-NL63 entry.**

599   LLC-MK2 cells were incubated with DMSO (**A**), 10 μM cytochalasin D (**B** and **E**), 1.5 μM

600   jasplakinolide (**C** and **F**), or 400 nM nocodazole (**D** and **G**) for 1 hour at 37°C and inoculated

601   with purified HCoV-NL63 and incubated at 32°C for 1 h. Actin and virus localization was

602   verified with confocal microscopy; fixed cells were immunostained for HCoV-NL63 particles

603   (green), actin (red) and nuclei (blue). Scale bar = 10 μm.

604

605 **Fig. 13. Cytotoxicity of the cytoskeleton modifying compounds.**

606 Cytotoxicity of the tested endocytosis inhibitors was tested with an XTT assay. Cells were

607 incubated with DMSO, 10 μM cytochalasin D, 1.5 μM jasplakinolide, or 400 nM nocodazole

608 for 2 h at 37°C. Data on the y-axis represent viability of the treated cells compared to the

609 untreated reference samples. The assay was performed in triplicate and average values with

610 standard errors are presented.

611

612 **Fig 14. Early events during HCoV-NL63 infection.**

613

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736

LLC-MK2   HAE

A   Clathrin

B   Caveolin

C   mock

Transferrin

A DMSO

B Pitstop

C MiTMAB

D Control

HCoV-NL63

E DMSO

F Pitstop

G MiTMAB

A — surface/internalized virus ratio for control, Pitstop, MiTMAB

B, C, D — Number vs Distance [μm] histograms

A   Control

B   Pitstop

C   MiTMAB

A   C   E

B   D   F

A.

LRV

LLC-Mk2   HAE

2

0

-2

*   *

DMSO   Pitstop   MitMAB   Nystatin   Pitstop   MiTMAB   Nystatin

B.

Cell viability [%]

LLC-Mk2   HAE

100

50

0

DMSO   Pitstop   MitMAB   Nystatin   Pitstop   MiTMAB   Nystatin

A.

B.

1. Binding to HS proteoglycans

2. Receptor binding

ACE2

4. Dynamin-mediated vesicle scission

5. Crossing the actin cortex

3. Formation of the clathrin-coated pit and virus internalization

ACE2

6. pH dependent fusion

ACE2