Entry of human coronavirus NL63 to the cell.

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

Human coronavirus NL63 (HCoV-NL63) was discovered shortly after the emergence of the severe acute respiratory syndrome coronavirus (SARS-CoV) (1). Extensive studies on the pathogen’s biology and epidemiology revealed that it is prevalent worldwide, appearing with a seasonal distribution similar to that of other human coronaviruses. The clinical presentation may vary depending on the general health status of the patient. Usually, the virus causes a relatively mild respiratory tract disease, but fatal cases have been reported (2-5). Furthermore, broad studies on the association between infection and clinical symptoms reveal that HCoV-NL63 is associated with croup in young children (6-9).

Phylogenetically, HCoV-NL63 clusters within the genus alphacoronavirus, which also includes another human pathogen, HCoV-229E. Initially, these two viruses were considered distant relatives that diverged at some point due to an unknown reason. More recent research shows, however, that these two species most likely emerged in the human population during two separate zoonotic transmission events (10-12).

From the perspective of genome structure, HCoV-NL63 is similar to other alphacoronaviruses in that the 5’ terminal two-thirds of the genome encode a large polyprotein, which is cleaved to yield several non-structural proteins. Five genes (S-ORF3-E-M-N) are located at the 3’ terminus encode structural proteins. The spike protein (S) is a class I fusion protein comprising a rod-like domain anchored to the virion via its C-terminus, and a globular head responsible for the interaction with cellular entry receptors (13). It is generally assumed that alphacoronaviruses interact with and enter host cells using the CD13 (aminopeptidase N). However, HCoV-NL63 utilizes the ACE2 protein for this purpose, a characteristic shared only with SARS-CoV (14, 15). Virus tropism not only depends on the presence of a certain entry receptor but also may be modulated by other factors, e.g.,
attachment receptors, protease availability, and the activity of pathways responsible for internalization and trafficking of the virus particle (16, 17).

While binding to their cognate entry receptor provides sufficient stimulus for some viruses to initiate fusion between the viral and cellular membranes, most internalize via endocytosis; acidification and/or processing by cathepsins is then a pre-requisite for fusion (13). For a long time, endocytic entry of virions was classified as clathrin-dependent, clathrin-independent, or clathrin- and caveolin-independent. During recent years, a number of other pathways were identified and this complex machinery has become better understood. The occurrence, abundance, and mechanistic details of these pathways appear to vary between cell types, tissues, and species. Most often, the selection of a specific endocytic route is linked to cargo-directed trafficking and receptor-dependent trafficking. Nevertheless, many receptors/cargoes allow flexibility due to their capacity to enter a cell via multiple pathways.

The early stages of HCoV-NL63 infection have been described by us and others (18-20). Here, we made an effort to delineate events that occur early during HCoV-NL63 infection. First, the virus anchors to ciliated cells via heparan sulfate proteoglycans before interacting with the ACE2 entry receptor. Our results show that the virus-ACE2 interaction triggers recruitment of clathrin, followed by clathrin-mediated, dynamin-dependent endocytosis, which requires actin cortex remodeling. To ensure that our results were reliable, we used ex vivo cultured human airway epithelium (HAE), which mimics the microenvironment at the infection site.
Results

HCoV-NL63 enters the cell via endocytosis

We first determined whether entry of HCoV-NL63 requires endocytosis and acidification of endosomes. For this, we studied the effect of ammonium chloride (NH₄Cl) and bafilomycin A - lysosomotropic agents that inhibit acidification of endosomes (21-23) using two models of HCoV-NL63 infection: permissive LLC-Mk2 cells and HAE cultures. Cells were pre-incubated with NH₄Cl (50 mM), bafilomycin A (100 nM) or control DMSO for 1 h at 37°C, and subsequently incubated with the virus at TCID₅₀ of 100/ml (for LLC-Mk2 cells) or at TCID₅₀ of 400/ml (for HAE) for 2 h at 32°C in the presence of the inhibitor. Subsequently, supernatants were removed and cells were washed thrice with acidic buffer to inhibit the fusogenic activity of the virions retained on the surface (24). Next, LLC-Mk2 cells were washed with 1 × PBS (pH 7.4), overlaid with culture medium and incubated at 32°C for 4 days. Supernatant samples were collected for virus replication analysis. Simultaneously, HAE cultures were washed with 1 × PBS (pH 7.4) and further maintained at an air-liquid interphase at 32°C for 5 days. During this time HAE cultures were washed every 24 h with 1 × PBS supplemented with a given inhibitor for 10 min at 32°C, and apical washes were collected for virus replication analysis. Subsequently, viral RNA was isolated, reverse transcribed (RT), and HCoV-NL63 yield was determined using a quantitative real-time PCR (qPCR).

Bafilomycin A and NH₄Cl inhibited HCoV-NL63 infection in LLC-Mk2 cells, proving that acidification is a pre-requisite for the virus infection in vitro. No inhibition was observed in HAE cultures (Fig. 1A). No cytotoxic effect was observed in the presence of these inhibitors (Fig. 1B).

Next, we analyzed HCoV-NL63 co-localization with the early endosome antigen-1 (EEA1), a hydrophilic protein localizing exclusively to early endosomes (25). LLC-Mk2 cells...
were fixed after 10, 20, 30 or 40 min post-inoculation (p.i.) with gradient-purified virus, stained with antibodies specific to HCoV-NL63 N protein and EEA1, and analyzed under confocal microscope. Measured co-localization expressed as Manders’ coefficient increases with time and reaches 0.68 forty min p.i. (n = 6 cells) (Fig. 1C).

We validated the obtained results using the HAE model. Briefly, HAE cultures were inoculated with gradient-purified HCoV-NL63 and incubated at 32°C for 2 h. For this culture model a longer incubation was required to observe virus attachment and entry, most likely due to the requirement to cross the mucus layer. Subsequently, cells were fixed and labeled with specific antibodies against HCoV-NL63 N protein and EEA1. Co-localization of HCoV-NL63 virus particles with EEA1 protein was analyzed using confocal microscope. Co-localization of virus and EEA1 was observed in inoculated cells (Fig. 1D).

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

HCoV-NL63 virus employs the ACE2 protein for cellular entry, while heparan sulfate proteoglycans serve as attachment receptors (19). Here, we analyzed the consequence of interaction between the virus particle and ACE2. First, we inoculated naturally permissive LLC-Mk2 cells with HCoV-NL63 and incubated for 40 min at 4°C to enable virus adhesion to a cell surface. Subsequently cells were fixed, the virus was labelled with specific antibodies and its co-localization with the ACE2 and clathrin was studied. As shown in Fig. 2A, HCoV-NL63 particles attach efficiently to the cell surface. However, only a proportion of virions co-localize with the ACE2 (Manders’coeff. = 0.573; n = 5), suggesting that binding to the HS precedes interaction with the entry receptor. At that point, there is no co-localization of virus particles and clathrin-coated pits (Manders’coeff. = 0.140; n = 5) (Fig. 2B). Next, we tested whether the virus binding to the adhesion or entry receptor triggers recruitment of common cellular proteins responsible for pit formation by incubating cells for 5 min at 32°C.
Immunostaining showed that the virus particles bound to the ACE2 start to co-localize with clathrin (Manders’ coeff. = 0.849, n = 6) (Fig. 2C), while there is no co-localization between non-ACE2-bound virions and clathrin (Manders’ coeff. = 0.189, n = 6).

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

To determine whether co-localization with clathrin following the ACE2 binding is relevant, and indeed the virus enters the cell by clathrin-coated pits we analyzed co-localization of intracellular virions with clathrin. Briefly, LLC-Mk2 cells were incubated at 32°C for 5-20 min with gradient-purified HCoV-NL63, fixed, immunostained and analyzed with confocal microscopy. Results showed co-localization of virions entering the cell with clathrin (Manders’ coeff. = 0.584; n = 7) (Fig. 3A), whereas no co-localization with caveolin-1 was observed (Manders’ coeff. = 0.053; n = 5) (Fig. 3B). HCoV-NL63 co-localization with clathrin and caveolin was also studied in HAE model. For this, cultures were incubated with gradient-purified HCoV-NL63 at 32°C for 2 h; the virus and the cellular proteins were immunostained and analyzed with confocal microscopy. HCoV-NL63 virions also in this model co-localized with clathrin, whereas no co-localization was observed for caveolin-1 (Fig. 3).

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

As we already knew that HCoV-NL63 virions migrate to clathrin-coated pits, in the subsequent step we aimed to determine whether the clathrin-mediated endocytosis is indeed important for the virus entry. For this reason, we blocked the pathway using Pitstop 2 (N-[5-[4-Bromobenzylidene]-4-oxo-4,5-dihydro-1,3-thiazol-2-yl] naphthalene-1-sulfonamide) – a selective clathrin inhibitor targeting its amino terminal domain, and MitMAB (tetradecyltrimethylammonium bromide) – a dynamin I and II GTPase inhibitor. Activity of
these compounds was verified with the positive control (fluorescently-labelled transferrin) (26, 27). LLC-Mk2 cells were treated with Pitstop 2, MitMAB or control DMSO for 30 min at 37°C, following transferrin uptake for 45 min at 37°C. Confocal images showed that both inhibitors blocked transferrin endocytosis, as the protein was present only on the cell surface (Fig. 4A-D).

Subsequently, LLC-Mk2 cells were incubated with one of the inhibitors at 37°C for 30 min and inoculated with gradient-purified HCoV-NL63 at 32°C for 5 min. Following immunostaining of the HCoV-NL63 N protein and actin, virus endocytosis was analyzed using confocal microscopy. Results showed that virus internalization was hampered in cells pre-treated with clathrin and dynamin inhibitors, as compared to the DMSO-treated cells (Fig. 4D-G). Simultaneously, a cytotoxicity test of the entry inhibitors was performed, which showed no toxic effect of the tested compounds to LLC-Mk2 cells (Fig. 5). In order to ensure that our observations are not biased, statistical analysis of virus entry was performed. For this, an algorithm was prepared for image analysis and 3D representation of the cell was prepared and virus position in the cell was determined (Fig. 6).

Similar experiment was conducted using HAE cultures. For this, cultures were incubated for 1 h at 37°C with inhibitors described above, following incubation with gradient-purified HCoV-NL63 at 32°C for 2 h. A strong inhibition of virus internalization in cultures pre-incubated with clathrin or dynamin inhibitors was observed, compared to control cells (Fig. 7). No cytotoxicity to HAE was observed for the tested inhibitors after 3 h incubation at 37°C (Fig. 8).

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

Even though certain cargo is usually internalized by a single route, frequently other pathways may be used as alternatives. We therefore aimed to test whether inhibition of
clathrin-mediated entry with chemical inhibitors results in inhibition of virus replication. To address this, we incubated LLC-Mk2 cells with a given inhibitor at 37º C for 1 h and infected them with HCoV-NL63 (TCID₅₀ = 400 per ml) for 2 h at 32°C. Subsequently media were removed and cells were washed thrice with acidic buffer, following washing with 1 × PBS (pH 7.4). Next, cells were overlaid with culture medium containing a given inhibitor and incubated at 32°C for 4 days. Cells were fixed and immunostained for HCoV-NL63 N protein to assess the number of infected cells. To assess the non-specific effect of entry inhibitors, control cells were treated with these also 4 h p.i. Clearly, in the presence of clathrin-mediated endocytosis inhibitors (Pitstop 2 and MitMAB) the number of HCoV-NL63-infected cells was much lower compared to the control. However, MitMAB also inhibited virus replication at later stages of the infection (Fig. 9). To ensure that entry inhibitors affected HCoV-NL63 infection in LLC-Mk2 cells, we analyzed by RT-qPCR virus replication at 120 h p.i. in the presence of tested compounds. The analysis showed a ~2-log decrease in virus progeny production in the presence of Pitstop 2 and MitMAB, compared to DMSO-treated cells and a slight increase of RNA copy levels in the presence of nystatin (Fig 10A). Importantly, no cytotoxic effect was observed for the tested inhibitors applied to LLC-Mk2 for 4 days at 32°C (Fig 10B). The influence of tested inhibitors on HCoV-NL63 infection was analyzed also in HAE cultures. For this, cultures were pre-incubated with a given inhibitor (Pitstop 2, MitMAB, nystatin or control DMSO) for 1 h at 37°C and infected with HCoV-NL63 at TCID₅₀ of 400 per ml for 2 h at 32°C. Subsequently, non-internalized virions were inactivated by acid wash, cultures were washed with 1 × PBS, and incubated with a given inhibitor for 10 min. After that time supernatants were discarded and cultures were incubated for 5 days at 32°C. During this period, cultures were incubated with a given inhibitor for 10 min at 32°C every 24 h. Viral RNA from these samples was quantified by RT-qPCR. Virus replication in HAE was not affected by any of the tested inhibitors (Fig. 10A).
TMPRSS2 is important during early stages of the infection

It was previously suggested that coronaviruses may bypass the endocytic entry route employing TMPRSS2 protease, which primes the fusion protein and enable fusion of viral and cellular membranes on the cell surface (31, 32). We have tested whether inhibition of the TMPRSS2 proteases with camostat affects the HCoV-NL63 infection. We observed that inhibition of TMPRSS2 protease hampers virus infection in HAE cultures, while it has no effect on virus replication in LLC-MK2 cells (Fig. 11A). No inhibition of virus entry was observed in any of the models, as tracked with confocal microscopy, visualizing the nucleoprotein (Fig. 11B). As only single entry events per view were observed, several images for camostat-treated and control cells are presented. In total, 500 entry events into HAE cells were tracked and no difference between the camostat-treated sample and control sample was noted.

HCoV-NL63 entry requires actin re-modelling

We studied trafficking of HCoV-NL63 inside the cell. As entry by endocytosis would probably require re-modelling of the cytoskeleton, we evaluated virus internalization in the presence of cytochalasin D, jasplakinolide or nocodazole. The first chemical inhibits actin polymerization, whereas the second binds F-actin and stabilizes actin filaments (33, 34). The last compound interferes with the microtubule formation. The analysis showed that actin inhibitors prevented virus particles from penetrating the cell, with visible viral particles accumulation on actin cortex or unstructured actin deposits. Microtubule inhibitor did not affect virus entry (Fig. 12). No cytotoxicity was observed for the tested inhibitors (Fig. 13).
Discussion

Previously, we and others described the first steps of the HCoV-NL63 infection process, showing that it begins with the virus binding to the cellular membrane via heparan sulfate proteoglycans, which then enable/facilitate interaction with the entry receptor, ACE2 (14, 18, 19). Little is known about the subsequent virus internalization and its trafficking through the cytoplasm, and some published data are contradictory. For example, the role played by cathepsins and acidification of the microenvironment during transition of the HCoV-NL63 S protein to its fusogenic form remains unclear.

We made an effort to systematically examine every step of the process. First, we tested whether the virus requires endocytosis for successful entry. To do this, we carried out experiments using chemical inhibitors of endosome acidification (ammonium chloride and bafilomycin A). Both blocked virus infection in LLC-MK2 cells, suggesting a requirement for transport of virions to endosomes, which then undergo acidification. Yet, such an approach may have several disadvantages. First, we examined the role of endosome acidification based on virus replication; thus we cannot rule out interference with virus infection at later stages (as shown for MiTMAB). Second, the specificity and selectivity of chemical inhibitors are questionable. An indirect proof for the pH dependence of HCoV-NL63 entry may be provided by the fact that acidification of the environment (acid wash) results in inactivation of the virus, suggesting the pH-directed structural switch in the S protein. To further confirm our observations, we developed a method of visualizing single virions as they entered the cell. Efforts to stain for virus surface proteins yielded poor results, most likely due to lack of highly specific antibodies and post-translational modification of surface proteins and the best results were obtained when antibodies specific to the N protein were used. Incubation of cells with purified virions resulted in virus attachment, which was visualized by confocal microscopy and co-staining for markers of the most commonly employed endocytic pathways.
allowed us to study the co-localization. If significant co-localization was detected, results were confirmed with chemical inhibitors.

The results showed that HCoV-NL63 binding to the ACE2 initiates recruitment of clathrin and subsequent formation of clathrin coated pits; no co-localization of the virus with other markers (e.g., caveolin) was noted. Transferrin was used as a positive control for clathrin-mediated endocytosis (35, 36). Importantly, chemical inhibitors of clathrin completely blocked virus internalization and the virus remained on the cell surface. Analysis of HAE cultures yielded identical results. The inhibitors of endocytosis also hampered virus infection on LLC-Mk2 cells, highlighting that this pathway is relevant and the lack of an equally effective alternative entry route in this culture model. Clathrin-mediated endocytosis requires a number of other proteins, as dynamin, the GTPase responsible for scission of clathrin-coated vesicles from the cell surface (37). Inhibiting dynamin also hampered virus internalization into LLC-MK2 cells and HAE cultures, confirming our previous observations. However, in this case the MitMAB compound blocked replication of HCoV-NL63 also during subsequent stages of the infection.

It is noteworthy that we were not able to block virus infection of HAE cultures using inhibitors of endocytosis. This may be related to the fact that the cultures were exposed to inhibitors for a very short time during apical washes, which is not sufficient to permanently block the infection. On the other side, it is also possible that in HAE HCoV-NL63 is able to enter the cell by an alternative route. Recent reports on other coronaviruses (31, 32, 38) suggested that these viruses may bypass the endocytic entry route using TMPRSS2 as the priming protease, enabling the entry directly from the cell surface. Our experiments showed that inhibition of this protease indeed inhibited virus infection. Interestingly, it did not hamper virus internalization to the cell. Our data are consistent with the data presented by others (31, 32, 38), yet we believe that there is a different mechanistic explanation to the observed
phenomenon. We believe that indeed TMPRSS2 protease is required for the virus-cell fusion, acting similarly to cathepsins, but it does not enable fusion on the cell surface and the acidification of the microenvironment is required.

Our final research question was about virus trafficking. The endosome typically translocates through the depolymerizing actin cortex and is subsequently sorted at the endosomal hub and directed to different destinations. This sorting is highly dependent on the cargo. Using two chemical inhibitors (jasplakinolide and cytochalasin B) (34, 39), we showed that actin plays a vital role in virus entry. Stabilization of the actin cortex using jasplakinolide similarly as inhibition of actin polymerization using cytochalasin D resulted in immobilization of the virus at the cell surface. These two experiments suggest a scenario in which virus-carrying endosomes pass along the actin cortex, which actively unwinds and interacts with virions.

Summarizing, we show that HCoV-NL63 enters the cell by clathrin-mediated endocytosis, but the pathway may be bypassed to some extent during the infection ex vivo. HCoV-NL63 entry to the susceptible cell was summarized in Fig 14.
Materials and methods

Cell culture

LLC-Mk2 cells (ATCC: CCL-7; Macaca mulatta kidney epithelial) were maintained in minimal essential medium (MEM; two parts Hanks’ MEM and one part Earle’s MEM; Thermo Scientific, Poland) supplemented with 3% heat-inactivated fetal bovine serum (Thermo Scientific, Poland), penicillin (100 U/ml), streptomycin (100 μg/ml), and ciprofloxacin (5 μg/ml). Cells were cultured at 37°C under 5% CO₂.

Ethics Statement

Human tracheobronchial epithelial cells were obtained from airway specimens resected from adult patients undergoing surgery under Silesian Center for Heart Diseases-approved protocols. This study was approved by the Bioethical Committee of the Medical University of Silesia in Katowice, Poland (approval no: KNW/0022/KB1/17/10 dated on 16.02.2010). Participants provided their written informed consent to participate in the study, as approved by the Bioethical Committee.

Human airway epithelium cultures

Primary human tracheobronchial epithelial cells were expanded on plastic to generate passage 1 cells and plated on permeable Transwell inserts (6.5 mm-diameter) supports. Human airway epithelium (HAE) cultures were generated by provision of an air-liquid interface for 6-8 weeks to form well-differentiated, polarized cultures that resemble in vivo pseudostratified mucociliary epithelium. Cultures were prepared and maintained as previously described (24).
Cell viability assay

LLC-Mk2 cells were cultured on 96-well plates and HAE cultures were prepared as described above. Cell viability assay was performed by using the XTT Cell Viability Assay (Biological Industries, Israel), according to the manufacturer’s instructions. Briefly, on the day of the assay 100 μl of the culture medium (for LLC-Mk2) or 1 × PBS (for HAE) with the 30 μl of the activated XTT solution was added to each well/culture insert. Following 2 h incubation at 37°C, the solution was transferred onto a 96-well plate and signal was measured at λ = 490 nm using the colorimeter (Spectra MAX 250, Molecular Devices). The obtained results were further normalized to the control sample, where cell viability was set to 100%.

Virus preparation and titration

The HCoV-NL63 stock (isolate Amsterdam 1) was generated by infecting monolayers of LLC-Mk2 cells. The virus-containing liquid was aliquoted and stored at ~80°C. A control LLC-Mk2 cell lysate from mock-infected cells was prepared in the same manner. The virus yield was assessed by titration on fully confluent LLC-Mk2 cells in 96-well plates, according to the method described by Reed and Muench (40).

Purification of HCoV-NL63

The virus stock was concentrated 25-fold using centrifugal protein concentrators (Amicon Ultra, 10 kDa cut-off; Merck, Poland) and subsequently overlaid on 15% iodixanol solution in 1 × PBS (OptiPrep medium; Sigma-Aldrich, Poland). Following virus concentration using iodixanol cushion (centrifugation at 175 000 × g for 3 h at 4°C) it was overlaid on 10-20% iodixanol gradient in 1 × PBS and centrifuged at 175 000 × g for 18 h at 4°C. Fractions (1 ml) collected from the gradient were analyzed on western blot, using anti-HCoV-NL63 N IgGs (0.25 μg/ml; Ingenansa, Spain) and a secondary antibody coupled
with horseradish peroxidase (65 ng/ml, Dako, Denmark). The virus-containing fractions were aliquoted and stored at −80°C. The control cell lysate (mock) was concentrated and prepared in the same manner as the virus stock.

**Inhibition of virus entry**

LLC-Mk2 cells were seeded on coverslips in six-wells plates (TPP) and cultured for 2 days at 37°C. Subsequently, cells were incubated with a given inhibitor for 30 min at 37°C, and later with 50 μl of purified HCoV-NL63 or mock sample for 1 h at 32°C. For the ex vivo experiment, HAE cultures were exposed to the tested inhibitor or control PBS for 1 h at 37°C, following inoculation with iodixanol-concentrated HCoV-NL63 or mock sample. Following 2 h incubation at 32°C, unbound virions were removed by washing with 1 × PBS. Cells were then washed with 1 × PBS and fixed with 4% paraformaldehyde (PFA).

Transferrin and albumin were used as positive controls, as they were previously described to serve as a cargo in the clathrin- and caveolin-dependent endocytosis, respectively (41, 42). LLC-Mk2 cells were seeded on coverslips in six-wells plates (TPP, Switzerland) and cultured for 2 days at 37°C. Subsequently, cells were incubated with a given inhibitor for 30 min at 37°C, following incubation with Alexa Fluor 488-labeled transferrin (100 μg/ml; Molecular Probes) or FITC labeled albumin (500 μg/ml; Sigma-Aldrich, Poland) or control PBS for 45 min at 32°C. Cells were then washed with 1 × PBS and fixed in 4% PFA.

**Immunostaining and confocal imaging**

Fixed cells were permeabilized with 0.1% Triton X-100 in 1 × PBS and incubated overnight at 4°C in 1 × PBS supplemented with 5% BSA and 0.5% Tween 20. To visualize HCoV-NL63 particles, cells were incubated for 2 h at room temperature with mouse
anti-HCoV-NL63 N IgGs (0.25 μg/ml; Ingenansa, Spain), followed by a 1 h incubation with Alexa Fluor 488-labeled goat anti-mouse IgG (2.5 μg/ml; Thermo Scientific, Poland). The following antibodies were used for endosomal markers: polyclonal goat anti-human clathrin HC coupled with tetramethylrodamine (10 μg/ml; Santa Cruz Biotechnology) and polyclonal rabbit anti-human early endosome antigen 1 (2 μg/ml; Santa Cruz Biotechnology); polyclonal rabbit anti-human caveolin-1 (2 μg/ml; Sigma-Aldrich, Poland), and Alexa Fluor 633-labeled goat anti-rabbit (2.5 μg/ml; Thermo Scientific, Poland). Actin filaments was stained using phallloidin coupled with Alexa Fluor 633 (0.2 U/ml; Thermo Scientific, Poland). Nuclear DNA was stained with DAPI (0.1 μg/ml; Sigma-Aldrich, Poland). Immunostained cultures were mounted on glass slides in ProLong Gold antifade medium (Thermo Scientific, Poland). Fluorescent images were acquired under a Leica TCS SP5 II confocal microscope (Leica Microsystems GmbH, Mannheim, Germany) and a Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy GmbH). Images were acquired using Leica Application Suite Advanced Fluorescence LAS AF v. 2.2.1 (Leica Microsystems CMS GmbH) or ZEN 2012 SP1 software (Carl Zeiss Microscopy GmbH), respectively, deconvolved with Huygens Essential package ver. 4.4 (Scientific Volume Imaging B.V.; The Netherlands) and processed using ImageJ 1.47v (National Institutes of Health, Bethesda, Maryland, USA).

**Flow cytometry**

LLC-Mk2 cells were seeded on 6-wells plates (TPP), cultured for 2 days at 37°C with 5% CO₂. Cells in monolayer were incubated with each entry inhibitor for 1 h at 37°C, following infection with HCoV-NL63 at TCID₅₀ of 100/ml or inoculation of the mock sample. On day 4 p.i., cells were washed with sterile PBS, fixed with 3% PFA, permeabilized with 0.1% Triton X-100 in 1×PBS and incubated for 1 h with 3% BSA in 1×PBS with 0.1% Tween 20. To quantify HCoV-NL63 infection, fixed cells were scraped from plastic and
incubated for 2 h at room temperature with mouse anti-HCoV-NL63 N IgG antibodies (1 μg/ml; Ingenansa), followed by 1 h incubation with Alexa Fluor 488-labeled goat anti-mouse antibody (2.5 μg/ml; Molecular Probes). Cells were then washed, re-suspended in 1 × PBS and analyzed with FACS Calibur (Becton Dickinson) using Cell Quest software.

**Isolation of nucleic acids and reverse transcription**

Viral nucleic acids were isolated from cell culture supernatants (LLC-Mk2 cells) or apical washes (HAE cultures) using the Viral RNA/DNA Isolation Kit (A&A Biotechnology, Poland), according to the manufacturer’s instructions. Reverse transcription was carried out with a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Poland), according to the manufacturer’s instructions.

**Quantitative RT-PCR**

HCoV-NL63 yield was determined using an RT-qPCR (7500 Fast Real-Time PCR machine, Life Technologies, Poland). Viral cDNA (2.5 μl per sample) was amplified in a 10 μl reaction mixture containing 1 × Master Mix (RT Mix Probe, A&A Biotechnology, Poland), specific probe labelled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) (100 nM; 5’-ATG TTA TTC AGT GCT TTG GTC CTC GTG AT- 3’) and primers (450 nM each; sense: 5’-CTG TGG AAA ACC TTT GGC ATC- 3’; antisense: 5’ – CTG TGG AAA ACC TTT GGC ATC- 3’). Rox was used as the reference dye. The reaction conditions were as follows: 2 min at 50°C and 10 min at 92°C, followed by 40 cycles of 15 sec at 92°C and 1 min at 60°C. In order to assess the copy number for the N gene, DNA standards were prepared. Briefly, N gene of HCoV-NL63 was amplified and cloned into pTZ57R/T (Thermo Fisher Scientific, Poland) plasmid using the InsTAclone PCR cloning kit (Thermo Scientific, Poland). Subsequently, DNA vectors were
amplified and linearized with EcoRI restriction enzyme. Linear nucleic acids were further purified with the GeneJET™ PCR Purification Kit (Thermo Fisher Scientific, Poland), according to the manufacturer’s instructions and its concentration was assessed using a spectrophotometer. The number of DNA copies/ml was assessed using Avogadro’s constant and molecular mass of RNA molecules. Samples were serially diluted and used as an input real-time PCR reaction.

In this article, the data from quantitative PCR are presented as log removal values (LRVs) in order to enable comparison of results obtained from different assays. LRV was calculated according to the following formula: LRV = −log (c_i/c_0) where c_i is the number of viral RNA copies per milliliter in the sample in the culture treated with a given polymer and c_0 is the number of viral RNA copies per milliliter in control sample (untreated cells).

**Image analysis**

To evaluate the infection inhibition in the presence of various endocytosis inhibitors image analysis was performed on 2 mm × 2 mm tile scan images. On each image, the number of nuclei (expressed as a number of cells) and the mean pixel intensity for the virus were calculated. For that, histograms of all images were adjusted to the min/max value, excluding signal from the virus derived from images with no infected cells. Results are presented as mean intensity of fluorescence per cell.

Co-localization analyses were performed under ImageJ using JACoP plugin (Bolte and Cordelieres, 2006), where Manders’ coefficient was calculated for 3D images of more than 5 cells.

Quantitative analysis of virus internalization in the presence of inhibitors was performed with algorithm previously described by Berniak et al. with modifications (43). Cell surface was estimated on each image slice manually using polygon selection tool in ImageJ and based
on this information 3D cell surface was modelled. Coordinates of virus particles were determined using 3D Object Counter ImageJ plugin. Relative localization and distance between virus particle and cell surface was calculated. Results are presented as a ratio between virus particles inside a cell and the particles on the surface (up to 1.5 µm above).

Statistical analysis

All the experiments were performed in triplicate and the results are presented as mean ± SD. To determine significance of the obtained results, a comparison between groups was conducted using the Student’s t-test. P values < 0.05 were considered significant.
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Figure legends

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

(A) Inhibition of HCoV-NL63 infection in LLC-Mk2 cells and HAE cultures by lysosomotropic agents: ammonium chloride (NH₄Cl, 50 mM) and bafilomycin A (Baf A, 100 nM), as determined with a RT-qPCR; values on the y axis are presented as LRV. The assay was performed in triplicate, and average values with standard errors are presented. *P values < 0.05 were considered significant and are denoted with an asterisk (*). (B) Cytotoxicity of the tested inhibitors was measured with an XTT assay. Data on the y-axis represent viability of the treated cells compared to the untreated reference samples. The assay was performed in triplicate and average values with standard errors are presented. (C, D) Confocal images showing co-localization of HCoV-NL63 virions with early endosomal marker EEA1 on LLC-Mk2 cells (C) and HAE cultures (D). Scale bar = 5 μm. Green: HCoV-NL63, red: EEA1.

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

Pre-cooled LLC-Mk2 cells were incubated with gradient-purified HCoV-NL63 for 40 min at 4°C, following 0 min (A, B) or 5 min (C) incubation at 32°C. Co-localization of the virus (green) and the ACE2 (red) was analyzed using confocal microscopy (A). No colocalisation with clathrin was observed after 0 min incubation (B). Triple co-localization of virus with ACE2 and clathrin (blue) is visible in panel (C). Images on the right side are zoomed-in regions indicated by white rectangles on the left-side slides. A representative image is shown. Scale bar = 10 μm.

Fig 3. HCoV-NL63 co-localizes with clathrin, but not caveolin.
LLC-Mk2 cells were incubated with gradient-purified HCoV-NL63 for 40 min at 4°C, following 5 min (A) or 20 min (B) incubation at 32°C. HAE cultures were incubated with gradient-purified HCoV-NL63 for 40 min at 4°C, following 120 min incubation at 32°C. HCoV-NL63 co-localization with clathrin (A) or caveolin (B) was analyzed with confocal microscopy (HCoV-NL63: green, clathrin and caveolin: red, nuclei: blue). Cells incubated with mock and stained with isotypic antibodies were used as control (C). Scale bar = 5 μm.

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

In order to verify effectiveness of inhibitors, LLC-Mk2 cells were incubated with control DMSO (A), 10 μM Pitstop 2 (B), or 10 μM MitMAB (C) for 30 min at 37°C, and inoculated with Alexa Fluor-488-labelled transferrin. Following incubation (45 min, 37°C), cells were fixed and stained for actin (red). Transferrin entry was evaluated with confocal microscopy.

Further, LLC-Mk2 cells were incubated with control DMSO (E), 10 μM Pitstop 2 (F), 10 μM MitMAB (G) for 30 min at 37°C. Cells were inoculated with purified HCoV-NL63 and incubated at 32°C for 1 h. Subsequently, cells were fixed and immunostained for HCoV-NL63 particles (green) and actin (red). Mock-infected cells were used as control (D). Scale bar = 10 μm.

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

Cytotoxicity of the tested endocytosis inhibitors was tested with an XTT assay. Cells were incubated with control DMSO, 10 μM Pitstop 2, or 10 μM MitMAB for 2 h at 37°C Data on the y-axis represent viability of the treated cells compared to the untreated reference samples. The assay was performed in triplicate and average values with standard errors are presented.
Fig 6. Numerical image analysis: clathrin and dynamin inhibitors block HCoV-NL63 entry.

LLC-Mk2 cells were incubated with DMSO (B), 10 μM MitMAB (C), or 10 μM Pitstop 2 (D) for 30 min at 37°C, and subsequently inoculated with purified HCoV-NL63 and incubated for 45 min at 32°C. Confocal images were digitalized, and localization of each virus particle relative to the cellular membrane was assessed. In panel (A) a graph presenting the number of internalized virus particles relative to number of virions on the cell surface (y axis) is presented for cells treated with DMSO (control), Pitstop 2 or MitMAB. In panels (B), (C), and (D) raw data for cells treated with DMSO, Pitstop 2, or MitMAB, respectively, are presented. Histograms present an average number of virus particles (y axis) vs the distance from cell surface (x axis). Values < 0 on the x axis indicate that the virus is inside the cell, while for extracellular virions x value is ≥ 0.

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

HAE were incubated with control DMSO (A), 10 μM Pitstop 2 (B), or 10 μM MitMAB (C) for 1 h at 37°C. Further, cells were inoculated with purified HCoV-NL63 and incubated at 32°C for 2 h. Subsequently, cells were fixed and immunostained for HCoV-NL63 particles (green), actin (red) and nuclei (blue). Scale bar 5 μm.

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

Cytotoxicity of the tested endocytosis inhibitors was tested with an XTT assay. Cells were incubated with control DMSO, 10 μM Pitstop 2, or 10 μM MitMAB for 2 h at 37°C. Data on the y-axis represent viability of the treated cells compared to the untreated reference samples. The assay was performed in triplicate and average values with standard errors are presented.
LLC-Mk2 cells were incubated with control DMSO (A), 5 μg/ml nystatin (B), 10 μM MitMAB (C), 10 μM Pitstop 2 (D) for 1 h at 37°C and inoculated with HCoV-NL63 (TCID₅₀ = 100/ml). After 2 h incubation at 32°C, virions that were not internalized were inactivated with acidic buffer (pH = 3) and cells were incubated for 4 days at 32°C in the presence of tested inhibitors or control DMSO. Identical procedure was applied to cells presented in panels E and F, yet in these MitMAB and Pitstop 2 were applied, respectively, after the acid wash. Fixed cells were immunostained with anti-NL63 nucleocapsid protein (green) and nuclei (blue) and confocal images were collected. Scale bar: 200 μm.

(A) HCoV-NL63 replication in LLC-Mk2 cells and HAE cultures in the presence of entry inhibitors or control DMSO was analyzed using RT-qPCR. Cultures were incubated with 10 μM Pitstop 2, 10 μM MitMAB, 5 μg/ml nystatin or DMSO for 1 h at 37°C and inoculated with HCoV-NL63 (TCID₅₀ = 400/ml). After 2 h incubation at 32°C, virions that were not internalized were inactivated with acidic buffer (pH = 3) and cells were incubated for 5 days at 32°C. The data are presented as Log Reduction Value (LRV), compared to the control sample. The assay was performed in triplicate, and average values with standard errors are presented. P values < 0.05 were considered significant and are denoted with an asterisk (*).

(B) Cytotoxicity of the tested inhibitors was tested with an XTT assay. Cells were incubated with 10 μM Pitstop 2, 10 μM MitMAB, 5 μg/ml nystatin or DMSO for 5 days at 32°C. Data on the y-axis represent viability of the treated cells compared to the untreated reference.
samples. The assay was performed in triplicate and average values with standard errors are presented.

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

(A) HCoV-NL63 replication in LLC-Mk2 cells and HAE cultures in the presence of camostat or control DMSO was analyzed using RT-qPCR. Cultures were incubated with 100 μM camostat or DMSO for 1 h at 37°C and inoculated with HCoV-NL63 (TCID₅₀ = 400/ml). After 2 h incubation at 32°C, virions that were not internalized were inactivated with acidic buffer (pH = 3) and cells were incubated for 5 days at 32°C. The data are presented as Log Reduction Value (LRV), compared to the control sample. The assay was performed in triplicate, and average values with standard errors are presented. *P* values < 0.05 were considered significant and are denoted with an asterisk (*). 

(B) HAE were incubated with control DMSO or 100 μM camostat for 1 h at 37°C. Further, cells were inoculated with purified HCoV-NL63 and incubated at 32°C for 2 h. Subsequently, cells were fixed and immunostained for HCoV-NL63 particles (green), actin (red) and nuclei (blue). Scale bar = 5 μm.

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

LLC-MK2 cells were incubated with DMSO (A), 10 μM cytochalasin D (B and E), 1.5 μM jasplakinolide (C and F), or 400 nM nocodazole (D and G) for 1 hour at 37°C and inoculated with purified HCoV-NL63 and incubated at 32°C for 1 h. Actin and virus localization was verified with confocal microscopy; fixed cells were immunostained for HCoV-NL63 particles (green), actin (red) and nuclei (blue). Scale bar = 10 μm.
Fig. 13. Cytotoxicity of the cytoskeleton modifying compounds.

Cytotoxicity of the tested endocytosis inhibitors was tested with an XTT assay. Cells were incubated with DMSO, 10 μM cytochalasin D, 1.5 μM jasplakinolide, or 400 nM nocodazole for 2 h at 37°C. Data on the y-axis represent viability of the treated cells compared to the untreated reference samples. The assay was performed in triplicate and average values with standard errors are presented.

Fig 14. Early events during HCoV-NL63 infection.

Reference list


