Novel polymeric inhibitors of HCoV-NL63

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ABSTRACT

The human coronavirus NL63 is generally classified as a common cold pathogen, though the infection may also result in severe lower respiratory tract diseases, especially in children, patients with underlying disease, and elderly. It has been previously shown that HCoV-NL63 is also one of the most important causes of group in children. In the current manuscript we developed a set of polymer-based compounds showing prominent anticoRonaviral activity. Polymers have been recently considered as promising alternatives to small molecule inhibitors, due to their intrinsic antimicrobial properties and ability to serve as matrices for anticoRonaviral compounds. Most of the antimicrobial polymers show antibacterial properties, while those with antiviral activity are much less frequent. A cationically modified chitosan derivative, N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HTCC), and hydrophobically-modified HTCC were shown to be potent inhibitors of HCoV-NL63 replication. Furthermore, both compounds showed prominent activity against murine hepatitis virus, suggesting broader anticoRonaviral activity.

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1. Introduction

Coronaviruses are positive-stranded RNA viruses with a genome of approximately 27–32 kb. The large coronaviral genome can be functionally divided into two regions. While 5’ two-thirds of the genome encodes a large polyprotein that contains all proteins necessary for RNA replication, the 3’ one-third encodes several structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N), which are incorporated into the virion and participate in numerous processes (e.g., virus budding). Nonstructural accessory protein genes are species-specific and are interspaced between the structural genes. The virus particle itself is enveloped and carries extruding S proteins on the membrane surface, providing the virion with the typical crown-like structure (Fields et al., 2007).

First human coronaviruses (HCoV-229E and HCoV-OC43) were described already in the mid-1960’s and for over forty years were believed to be the only representatives of the Coronaviridae family infecting humans, associated with relatively mild respiratory tract disease (Bradburne et al., 1967; Hamre and Procknow, 1966; McIntosh et al., 1967; Tyrrell and Bynoe, 1965). The change took place in 2003, when the SARS-CoV emerged in the Guandong province in China (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003). The epidemic spread was contained in early July of 2003, mostly by quarantine measures; however, there were also some sporadic cases in 2003–2004 season (Peiris et al., 2004). This sudden outbreak of a previously undescribed coronavirus brought the whole family of viruses back to the limelight, so in the consecutive two years two new human coronaviruses – HCoV-NL63 and HCoV-HKU1 – were identified. Both viruses have spread worldwide and are linked with the respiratory tract disease (van der Hoek et al., 2004; Woo et al., 2005).

The HCoV-NL63 is generally classified as a common cold virus, but severe lower respiratory tract infections are frequently observed in young children, patients with underlying disease, and elderly (Arden et al., 2005; Bastien et al., 2005a,b; Chiu et al., 2005; Ebihara et al., 2005; Kaiser et al., 2005; Perlman and Netland, 2009). The role of HCoV-NL63 infection in the development of the acute respiratory disease has been further emphasized by confirmation that HCoV-NL63 infection is associated with acute
respiratory disease and croup (Han et al., 2007; Sung et al., 2010; van der Hoek et al., 2005; Wu et al., 2008). Moreover, sporadic fatal cases were reported in patients tested positively for HCoV-NL63, where no other pathogen could be identified (Bastien et al., 2005a; Cabeca and Bellei, 2011; Oosterhof et al., 2010).

Considering the prevalence of the HCoV-NL63 and its indubitable association with diseases, an effective antiviral treatment is required for patients suffering from severe respiratory tract illness. The studies described in this paper indicate that such a treatment may be provided by polymeric substances. Polymers are intensively studied as potent antimicrobial systems both due to their intrinsic antimicrobial properties and as matrices for antimicrobial compounds (both organic and inorganic). The latter systems, however, require the diffusion of low-molecular-weight antimicrobials through a polymeric matrix, which may be toxic to the human body (Munoz-Bonilla and Fernandez-Garcia, 2012). Therefore, polymers which possess antimicrobial activity are the preferred solution. Moreover, polymers usually show a long-term activity. Of particular interest among antimicrobial polymers are polyacrylates containing phosphonium groups (Kenawy and Mahmoud, 2003) or those with quaternary nitrogen atoms such as pyridinium (Tiller et al., 2002), imidazolium (Anderson and Long, 2010), or ammonium groups (Huang et al., 2008; Timofeeva et al., 2009). Other antimicrobial polymers are those containing halogens, both fluorine (Caillier et al., 2009) and chlorine (Patel et al., 2004), polymers containing sulfo groups (Zaneveld et al., 2002), or those substituted with low-molecular-weight compounds such as antibiotics (Gibot et al., 2000) or norfloxacin (Yang and Santerre, 2001). Most of the antimicrobial polymers show antibacterial properties, while those with antiviral activity are much less frequent. It is still difficult to predict if a polymer shows antimicrobial properties just based on its structure, therefore its potential antimicrobial activity has to be verified experimentally.

In the current manuscript a cationically modified chitosan derivative, N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HTCC) and its hydrophobically-modified derivative (HM-HTCC), are shown to be potent inhibitors of HCoV-NL63 replication. Both polymers also showed a prominent activity against murine hepatitis virus (betacoronaviruses; MHV), suggesting that developed compounds may represent a novel class of antiviral compounds for the treatment of a wider spectrum of coronaviral diseases.

2. Materials and methods

2.1. Materials

Chitosan (CH, low molecular weight, 75–85% deacetylated, Sigma–Aldrich), glycidyltrimethylammonium chloride (GTMAC, Fluka, 90%), fluorescein thioisocyanate isomer 1 (FITC, Sigma–Aldrich), glacial acetic acid (CH3COOH, 99.5% pure p.a., CHEMPUR), acetone (POCH), DMSO (POCH), and methanol (POCH) were used as received. Water was distilled twice and deionized using the Millipore Simplicity system.

2.2. Apparatus

Fourier Transform infrared (FTIR) spectra were obtained on a BrukerIFS 48 spectrometer. NMR spectra were measured in a 1:1 mixture of deuterium oxide (D2O) and dimethyl sulfoxide-d6 (DMSO-d6) using a Bruker AMX 500 spectrometer. UV–Vis absorption spectra were recorded using an HP8452A diode-array spectrophotometer in 1-cm optical path quartz cuvettes. Elemental analysis was performed using a Vario Micro CHNS elemental analyzer (Elementar). GPC analyses were performed using a Waters GPC system equipped with a bank of three columns (PL Aquagel-Oh 30, 40, and 60) and tandem PDA/RI detectors. The eluent was 0.1 M NaCl, the flow rate was 0.6 ml/min, the sample volume was 150 µl, and the concentration of the polymers was 1.0 g/l.

2.3. Synthesis of N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HTCC)

Modification of the polymer was performed using the method previously described (Kaminski et al., 2010). Briefly, 2.5 g of chitosan was dispersed in 100 ml of distilled water supplemented with 10 ml of acetic acid (i.e., 0.5% v/v solution in water). The solution was stirred for 30 min and 6.9 ml of GTMAC was added to obtain the polymers with ~63% of substitution degree expressed as the fraction of NH2 groups substituted. The resulting mixture was heated and incubated at 55 °C for 18 h while stirring under reflux condenser. The suspension was subsequently centrifuged at 4000 rpm for 10 min to remove suspended unreacted chitosan. The product was extracted from the supernatant via precipitation in acetone supplemented with methanol and subsequent centrifugation at 4000 rpm for 20 min. The solution was decanted, and the resulting pellet was air dried and dissolved in distilled water. The purification process was repeated twice and the purified HTCC was dried in vacuum oven for 24 h. The GPC chromatograms revealed single peaks (data not shown). In the NMR spectra (data not shown) a signal appeared at 3.2 ppm (methyl protons of the trimethylammonium group), confirming the occurrence of the substitution reaction.

2.4. Synthesis of hydrophobically-modified HTCC (HM-HTCC)

HTCC (1 g, 3 mmol of glucose units) was dissolved in a 1:1 mixture of methanol and 1% acetic acid (pH 5.5). 0.3 mmol N-dodecyl aldehyde and sodium cyanohydrorate, NaCNBH4 (1,256 g, 20 mmol) were dissolved in 20 ml of methanol and added to the chitosan solution. The reaction mixture was then stirred for 36 h at 20 °C until a sol was formed. After the reaction was completed, the sol was precipitated by adding the methanol/diethyl ether (50:50 v/v) mixture. The white precipitate was washed using methanol and diethyl ether and vacuum-dried. The structure of the obtained product was verified by measuring 1H NMR spectra in 1% CD3COOD solution in D2O (data not shown).

2.5. Synthesis of fluorescein-labeled HTCC (FITC-HTCC)

150 mg of HTCC was dissolved in a mixture of 5.0 ml of distilled water and 15.0 ml DMSO. Five milligrams of fluorescein thioisocyanate (FITC) was dissolved in 1 ml of acetone, the solution was added to the sample containing HTCC, and vigorously mixed for 20 min. The precipitate was washed using methanol and diethyl ether and vacuum-dried. The structure of the obtained product was verified by measuring 1H NMR spectra in 1% CD3COOD solution in D2O (data not shown).

2.6. Cell culture

LLC-MK2 cells (ATCC: CCL-7; Macaca mulatta kidney epithelial cell line) were maintained in minimal essential medium (MEM), containing 2 parts of Hank’s MEM and 1 part of Earle’s MEM (PAA Laboratories) supplemented with 3% heat-inactivated fetal bovine serum (PAA Laboratories), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were cultured on T25 flasks (TPP) at 37 °C with 5% CO2. A549 cells (ATCC: CCL-185; human lung
carcinoma cell line) were maintained in Dulbecco-modified Eagle’s medium (DMEM; PAA Laboratories) supplemented with 3% heat-inactivated fetal bovine serum (PAA Laboratories), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were cultured on T25 flasks (TPP) at 37 °C with 5% CO₂, LR7 cells (murine L cells stably transfected with the N-Neacam receptor) were maintained in Dulbecco-modified Eagle’s medium (DMEM; PAA Laboratories) supplemented with 3% heat-inactivated fetal bovine serum (PAA Laboratories), penicillin (100 U/ml), streptomycin (100 µg/ml) and G418 (50 µg/ml; Sigma–Aldrich). Cells were cultured on T25 flasks (TPP) at 37 °C with 5% CO₂.

2.7. Human airway epithelium cultures

Human tracheobronchial epithelial cells were obtained from airway specimens resected from 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/KBI/17/10 dated on 16.02.2010). A written informed consent was obtained from all patients. Primary cells were expanded on plastic to generate passage 1 cells and plated at density of 3 x 10⁶ cells per well 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.

HAE cultures were infected with HCoV-NL63 by inoculation of the infectious material onto the apical surface. Following 2 h incubation at 32 °C, the unbound virus was removed by 1 × PBS washing (two times, 100 µl each) and HAE cultures were maintained at an air–liquid interface for the rest of the experiment. To analyze replication of HCoV-NL63, 72 h post infection, 100 µl of 1 × PBS was applied to the apical surface of HAE and collected following the 10 min incubation at 32 °C. All samples were stored at −80 °C.

2.8. Virus preparation, titration, and infection

HCoV-NL63 and murine hepatitis virus (MHV strain A59) stocks were generated by infecting LLC-MK2 and LR7 cells, respectively. Cells were lysed 6 days (HCoV-NL63) or 2 days (MHV) post-infection by two freeze–thaw cycles. The virus-containing fluid was aliquoted and stored at −80 °C. A control from mock infected cells (LLC-MK2 and LR7 cells) was prepared in the same manner as the virus stocks. Virus yield was assessed by virus titration on fully confluent LLC-MK2 cells or LR7 cells, according to Reed and Muench (1938). Cells on 96-well plates were incubated at 32 °C for 2 h (at 37 °C for 6 days (HCoV-NL63)) or at 37 °C for 2 days (MHV), and the cytopathic effect occurrence was scored using an inverted microscope. In subsequent experiments, fully confluent cells were exposed to HCoV-NL63 or MHV at TCID₅₀ of 400.

2.9. Virus detection by reverse transcription and quantitative PCR

Viral nucleic acids were isolated from cell culture supernatant or apical washes from HAE cultures by a total RNA mini kit (A&A Biotechnology), according to the manufacturer’s instructions. Reverse transcription was carried out with High Capacity CDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer’s instructions. HCoV-NL63 and MHV virus yields were determined using real-time PCR. In order to assess the copy number for HCoV-NL63 and MHV, RNA standards were prepared for both viruses. Briefly, HCoV-NL63 and MHV N genes were amplified and cloned into pTZ57R/T plasmids using InstAclone PCR cloning kit (Thermo Scientific). Subsequently, DNA fragments encoding N genes were ampliﬁed using primer sets SP6NL63 (forward 5′-TCG GCC TTC ATG ACC ATT TAG GTG ATG GCT AGT G/ TA TGG TGG-3′; reverse 5′-TCC GGA TTT TTT TTT TTC TTT TTT TTT TTT TTT TTT TTG GAA AGA-3′; reverse 5′-TTA CAC ATT AGA GTC ATC TTC TA ATG GCT TC-3′) for HCoV-NL63 and MHV, respectively (5′ primers contain the SP6 promoter). Ampliﬁed DNA was gel puriﬁed and used as a template for in vitro transcription using SP6 RNA polymerase (Thermo Scientific). Reaction was conducted according to the manufacturer’s instructions, and resulting reaction was DNase treated (DNase Turbo; Life Technologies). Resulting RNA was puriﬁed with total RNA mini kit (A&A Biotechnology) and its concentration was assessed using a spectrophotometer. Samples were serially diluted and used as an input for reverse transcription and real-time PCR reaction. Five microliters of cDNA was ampliﬁed in 10 μl reaction mixture, containing 1× TaqMan® Universal PCR Master Mix, No AmpErase®UNG (Applied Biosystems), speciﬁc probe labeled with FAM (6-carboxyﬂuorescein), and TAMRA (6-carboxytetramethylrhodamine) (200 nM) and primers (900 nM each). Rox was used as a reference dye. All primers and probes are listed in Table 1. The reaction was monitored on a 7500 fast real-time PCR machine (Applied Biosystems) with the following settings: 2 min 50 °C, 10 min at 92 °C, and 40 cycles of 15 s at 92 °C, and 1 min at 60 °C.

2.10. XTT assay

LLC-MK2, A549, and LR7 cells were cultured on a 96-well plate, as described above. Cell viability assay was done by using XTT Cell Viability Assay Kit (Biological Industries), according to the manufacturer’s instructions. Briefly, the medium was discarded and 100 µl of the culture medium with 30 µl of the activated XTT solution was added to each well. The plate was further incubated at 37 °C for 2 h. Following this step, the plate was transferred into the colorimeter (Spectra MAX 250; Molecular Devices) and a signal was measured at λ = 450 nm. The reference absorbance measure-

Table 1

| Target species | Primer | Primer sequence (5′–3′) |
|----------------|--------|------------------------|
| HCoV-NL63     | Sense primer [63NF2] | AAA CCT CGT TGG AAG GCT GT |
|               | Antisense primer [63NR1] | C TG TGG AAA ACC TTT GGC ATC |
|               | Probe [63NP] | FAM-ATG TTA TTC AGT GCT GT TGC CTC GTG AT-TAMRA |
| MHV           | Sense primer [MHV_NF] | TGG CGG AAG AAA TTA GTG CTC TTG |
|               | Antisense primer [MHV_NR] | GCC GTA CTT TTA TGC CAC TTC GCT |
|               | Probe [MHV_Np] | FAM-TTT GGC TAA GCT CGG TAA AGA TGC CG-TAMRA |
ment was done at λ = 630 nm. The obtained results were further normalized to the control sample, where cell viability was set to 100%.

For the assessment of the cell viability for HAE cultures, the apical surface was washed with 100 μl of 1× PBS and 100 μl of 1× PBS with 50 μl of the activated XTT solution was overlaid on the apical surface. Following the 2 h incubation, the apical solution was collected and transferred onto a new 96-well plate. The signal was evaluated as described above.

2.11. Neutral red uptake assay

LLC-MK2 or A549 cells were cultured on a 96-well plate, as described above. Cell viability assay was done by using neutral red dye (Sigma–Aldrich). Briefly, the medium was discarded and cells were washed twice with 100 μl of 1× PBS. One-hundred microliters of the neutral red dye working solution (50 μg/ml dye in 1× PBS) was added to each well. Cells were incubated at 37 °C for 2 h. Following this step, a supernatant was discarded, cells were washed twice with sterile 1× PBS, and lysed for 10 min with 100 μl of sample buffer (1% acetic acid, 50% ethanol in water). Resulting samples were analyzed with the colorimeter (Molecular Devices SpectraMax 250) and the signal was measured at λ = 540 nm. The obtained results were further normalized to the control sample, where cell viability was set to 100%.

2.12. Expression and purification of the HCoV-NL63 S ectodomain

A human codon-optimized sequence encoding the S ectodomain (Se) of HCoV-NL63 (amino acids 16 to 1293) was cloned into a derivative of expression plasmid pS1-Ig (Li et al., 2003). The Se sequence was preceded by a sequence encoding an N-terminal tag of fusion to the haemagglutinin (HA) ectodomain of influenza virus (Bosch et al., 2010). To express the NL63-Sc, HEK293T cells were transfected with the Se expression plasmid using polyethyleneimine (PEI) in a 1:5 ratio (μg DNA to μg PEI). After 6 h of incubation the transfection medium was replaced by 293 SFM II expression medium (Invitrogen) supplemented with sodium bicarbonate (3.7 g/L), glucose (2.0 g/L), Primatone RL-UF (3.0 g/L), penicillin (100 U/ml), streptomycin (100 μg/ml), glutaMAX ( Gibco), and 1.5% dimethyl sulfoxide. Five to six days post-transfection, the Se protein was purified from the culture medium using Strep-Tactin affinity chromatography (IBA GmbH). Expression and purification of the HCoV-NL63 Sc protein was confirmed by Western blotting and SDS–PAGE analysis (data not shown).

2.13. Evaluation of interaction between Se-NL63 and HTCC

To find out whether HTCC interacts with the Se-NL63 protein, the fluorescence spectra of HTCC labeled with FITC (at C_{FITC-HTCC} = 0.5 μg/ml) in water were assessed in a control sample and in samples containing increasing concentrations of Se-NL63 or bovine serum albumin (Sigma–Aldrich). The measurement was conducted with SLM-AMINCO spectrophotometer (λ_{exc} = 494 nm). Further, the null hypothesis that the Se-NL63 protein or other content of the sample absorb electromagnetic wave at the excitation wavelength and interfere with the readout was rejected based on obtained results (data not shown).

2.14. Statistical analysis

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

3. Results

3.1. Inhibitory polymers

In order to appropriately evaluate the inhibitory activity of the tested polymers, two assays were employed. The HCoV-NL63 infection results in a considerable alteration of cell morphology and subsequent cell detachment. The cytopathic effect (CPE) typically occurs on days 4–6 post infection and it is associated with the production of infectious virions. Briefly, LLC-MK2 cells were seeded on a 96-well plate in media containing increasing concentrations of inhibitory polymers. Cells were infected with HCoV-NL63 at 50% tissue culture infectious dose (TCID₅₀) of 400 and the reduction of CPE in susceptible cells infected with HCoV-NL63 was investigated. Analysis revealed that HTCC and HM-HTCC hamper the appearance of morphological changes and cell death following the HCoV-NL63 infection (Fig. 2). For both tested polymers the minimal inhibitory concentration (IC) value was relatively low, amounting to 10 μg/ml (~50 nM) for HTCC and 50 μg/ml (~230 nM) for HM-HTCC (Table 2).

Reduction of the CPE during viral infection may result not only from an actual inhibition of virus entry or replication, but also from the cytoprotection (i.e., inhibited or delayed development of CPE not affecting the virus production). To rule out such a possibility, a quantitative RT-PCR – based assay was employed to assess the HCoV-NL63 yield in the cell culture supernatant. The assay was performed as previously described (Golda et al., 2011) and the number of RNA copies per milliliter of cell culture supernatant was assessed. Conducted analysis clearly shows that in the presence of the studied polymers there is a significant decrease in the number of viral copies produced during infection (Fig. 3). The best results were obtained for the HTCC polymer, for which the IC₅₀ value reaches 2.75 ± 1.18 μg/ml (13.41 nM) (Fig. 3A; Table 2), though pronounced inhibition was noted also for the HM-HTCC compound. The latter polymer appeared to be more toxic and the IC₅₀ value is markedly higher (68.52 ± 18.71 μg/ml; 308.65 nM) (Fig. 3B; Table 2).

LLC-MK2 cells constitute the best described in vitro model that allows effective HCoV-NL63 replication and therefore these cells were used in the current study (Schildgen et al., 2006). To test whether the observed effect is not cell-specific, the inhibitory activity of the polymers in a more natural environment was also assessed; HAE cultures, mimicking natural conductive airway epithelium, were used for this purpose. HAE cultures are formed by the multi-layered, fully differentiated primary human airway epithelial cells growing on collagen-coated plastic supports on air/li-
quid interphase (Banach et al., 2009; Pyrc et al., 2010). Due to technical restrictions, it was not possible to use a standard assay with inhibitors present in the apical medium. HAE cultures were infected in the presence of tested inhibitors during 2 h incubation and subsequently the apical medium was removed. Further, the apical surfaces of HCoV-NL63-infected and mock-infected HAE cultures were rinsed every 24 h with 100 μl of the medium containing a given inhibitor. Samples of the apical fluid were concurrently collected. Real-time RT-PCR analysis shows the drastic decrease in the number of viral copies in the presence of the tested polymers, proving their effectiveness also in this semi-natural setting (Fig. 4A). It is also of note that incubation of HAE cul-

Fig. 1. Structures of the polymers showing anticonviral properties. In native chitosan the amino groups are partially deacetylated which means that the glucose groups may be substituted with both –NH2 and C6H12CONH– groups in the same macromolecule. Since the distribution of acetylated and deacetylated NH2– groups along the chitosan chain is random, this is often reflected by showing that R may be either H or –COCH3 group. Consequently, since in HM-HTCC polymer some of the NH2– groups are substituted with –C12H25 groups, some of R groups may be H, some –COCH3 groups, and some –C12H25 groups in the same chitosan chain. Therefore, in HM-HTCC R may be H or COCH3 or C12H25.

Fig. 2. Inhibition of HCoV-NL63 and MHV replication by HTCC and HM-HTCC compounds on LLC-MK2 (A) and LR7 cells (B), respectively. Infection was carried on in culture media, media supplemented with HTCC (100 μg/ml) or media supplemented with HM-HTCC (100 μg/ml). Cells were infected with HCoV-NL63 or MHV at TCID50 of 400. Images were taken on days 2 (MHV) or 6 (HCoV-NL63) post-infection with Nikon Eclipse Ti-S microscope. Magnification: 200×. The results shown are representative of at least three independent experiments.
3.3. Inhibition of MHV and other viruses in vitro

In order to test whether the investigated compounds selectively inhibit HCoV-NL63 replication, or show broader anticonviral activity, similar assays were performed for the MHV virus. Briefly, as MHV infection results in a massive cell death within 24–48 h post-infection, the CPE occurrence 48 h post-infection was scored on a 96-well plate in media containing inhibitory polymers. Cells were infected with MHV at 50% tissue culture infectious dose (TCID₅₀) of 400 and the reduction of CPE in susceptible cells infected with MHV was investigated. Analysis revealed that HTCC and HM-HTCC hamper the appearance of morphological changes and cell death following the infection, as presented in Fig. 2. Further, a quantitative RT-PCR – based assay was employed to assess the MHV yield in the cell culture supernatant. The assay was performed as described in Section 2 and the number of RNA copies per milliliter of cell culture supernatant was assessed. Obtained results

| Polymer | HCoV-NL63 (CPE) | IC₅₀ LLC-MK2 (qRT-PCR) | MHV | IC₅₀ LR7 (qRT-PCR) |
|---------|----------------|------------------------|-----|-------------------|
| HTCC    | 10 µg/ml       | 2.75 ± 1.18 µg/ml [13.41 nM] | 6.82 ± 1.16 µg/ml [33.27 nM] |
| HM-HTCC | 50 µg/ml       | 68.52 ± 18.71 µg/ml [308.65 nM] | 72.33 ± 18.24 µg/ml [325.81 nM] |

* Lowest compound concentration for which no CPE was noted.

* *50% inhibitory concentration as assessed by real-time RT-PCR analysis.
3.4. Cytotoxicity

The cytotoxicity of the tested polymers was assessed on LLC-MK2 cells which are susceptible to MHV infection, and on A549 cells. All the experiments aimed to determine the cytotoxicity of the tested compounds were conducted in identical conditions as those used for the assessment of the antiviral potential of the polymers (i.e., LLC-MK2 and A549 cells were incubated for 6 days, and LR7 cells were incubated for 48 h in the presence of the inhibitory compounds). Two different assays were used to appropriately evaluate the cytotoxic potential of the tested polymers. First, an XTT test was performed, based on the ability of eukaryotic mitochondrial enzymes to transform the substrate (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) to colored formazan salts. As there is a direct relationship between cell viability and the amount of the dye produced, it was possible to determine the relative viability of cells. The neutral red (NR) assay was used as the second method. The neutral red dye is able to penetrate living cells and it accumulates in endosomes, therefore it was possible to analyze and quantify the rate of this process, as described in Section 2. Following the 6-day incubation of LLC-MK2 or A549 cells in the presence of the tested polymers, the media were removed and the cell viability was evaluated. Both methods produced consistent estimation of the polymer toxicity and the results are presented in Table 3 and Fig. 3. The HTCC polymer is not toxic at effective concentrations (up to 100 µg/ml), showing highly specific inhibition of viral infection. At higher concentrations the cytotoxic effect becomes visible (IC₅₀ > 150 µg/ml), but one should remember that LLC-MK2 cells are incubated with polymer for six consecutive days due to low replication rate of the HCoV-NL63 in this model system. The second tested polymer – HM-HTCC – shows similar toxicity, though its inhibitory properties are inferior compared to HTCC. For LR7 cells, following the 2-day incubation in the presence of the tested polymers, the media were removed and the cell viability was evaluated with the XTT assay (Fig. 5B). Obtained results were consistent with those presented in Fig. 2. Also in this case no cytotoxicity was observed in an effective range for HTCC and only minor toxicity for HM-HTCC.

The cytotoxicity of the polymers was also tested on a fully differentiated human airway epithelium (HAE) (Fig. 4B). No significant cytotoxicity was observed with an XTT assay after 6 days of incubation and no visible alteration of HAE culture integrity and ciliation was observed. Lack of cytotoxicity may be easily explained in that case, as cells were not exposed to polymers throughout the whole time (apical surfaces of HAE cultures were rinsed every 24 h with medium containing a given polymer) and incubation time was shorter than for LLC-MK2 cells (72 h).

3.5. Interaction between HTCC polymer and Se-NL63 protein

One may assume that tested polymers inhibit HCoV-NL63 replication during early phases of infection, as no inhibition is noted if the compound is provided after the initial virion adherence phase (data not shown). In order to elucidate the mechanism of HTCC action, the interaction between HTCC and Se-NL63 protein was evaluated. For this purpose HTCC was substituted with FITC and fluorescence emission intensity in the presence of increasing concentrations of Se-NL63 was measured. As presented in Fig. 6, the fluorescence intensity diminished in the presence of Se-NL63 in a concentration-dependent manner. Such an observation indicates

Table 3  
Cytotoxicity of the polymers.

| Polymer  | CC₅₀ LLC-MK2 (XTT) (µg/ml) | CC₅₀ LLC-MK2 (NR) (µg/ml) | CC₅₀ A549 (XTT) (µg/ml) | CC₅₀ A549 (NR) (µg/ml) |
|----------|-----------------------------|---------------------------|-------------------------|------------------------|
| HTCC     | 161.25                      | 191.52                    | 143.83                  | 156.46                 |
| HM-HTCC  | 220.09                      | 215.23                    | 96.29                   | 96.13                  |

⁎ Evaluated with XTT assay.

⁎⁎ Evaluated with neutral red assay.
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et al., 2004; Woo et al., 2005, 2009). This is particularly important
studies on this family of pathogens revealed surprising variety of
man pathogens, as for over 40 years they have been considered to

4. Discussion
Human coronaviruses received relatively little attention as hu-
man pathogens, as for over 40 years they have been considered to
be common cold viruses only. SARS-CoV epidemic and subsequent
studies on this family of pathogens revealed surprising variety of
species infecting animals and humans, and a potential for future
coronavirus-related outbreaks (Poon et al., 2005; van der Hoek
et al., 2004; Woo et al., 2005, 2009). This is particularly important
considering recent identification of new coronavirus infecting hu-
mans (Wise, 2012). As mentioned above, a clear link between
HCoV-NL63 and the respiratory diseases was established in a pro-
spective population-based study (PRIME) on lower respiratory
tract infections in children less than three years of age in Germany.
Of the children with HCoV-NL63 infection, 45% had laryngotrache-
itis (croup) compared to only 6% in the control group. Multivariate
analysis demonstrated that the chance of croup is 6.6 times higher
in HCoV-NL63-positive children than in HCoV-NL63-negative chil-
dren (van der Hoek et al., 2005). Considering the observed patho-
genicity and worldwide distribution of the virus, in severe cases
(e.g., in children, immunocompromised and elder patients) the
application of a therapeutic drug may be required to hamper the
disease progression. Until present, some inhibitors have been de-
scribed for HCoV-NL63, interfering with different stages of HCoV-
NL63 replication.

The unique mechanism of coronaviral replication, which in-
cludes post-translational processing of large 1a/1ab polyprotein
carried over by highly conserved and specific viral proteases seem
to be promising target for therapy. Yang et al. designed and synthe-
sized a set of specific Mpro inhibitors, repressing replication of mul-
tiple coronaviruses, including HCoV-NL63 (Yang et al., 2005).
Another interesting approach was proposed by Pyrce et al., who
used synthetic peptides interfering with S protein fusogenic activ-
ity by interacting with heptad repeat regions in the S2 domain.
The same group of authors suggested employment of RNAi technology
and small molecule inhibitors previously shown to inhibit other
viruses (Pyrce et al., 2006). An interesting and novel approach for
inhibition of coronavirus infection was proposed initially by Pfe-
ferle et al., who identified interaction between cyclophilins-immu-
nophilins and the coronaviral non-structural protein 1. Such an
interaction is maintained in all coronaviral species, though what
is even more important, commercially available peptide inhibitors
of cyclophilins/immunophilins are able to hamper coronaviral
infection (Carbajo-Lozoya et al., 2012; de Wilde et al., 2011; Pfe-
ferle et al., 2011; Tanaka et al., 2012).

In the current study a number of polymers were tested for
potential antiviral activity. They included N-(2-hydroxypropyl)-
3-trimethylammonium chitosan chloride (HTCC), N-dodecyl-N-
(2-hydroxypropyl)-3-trimethylammonium chitosan chloride
(HM-HTCC), O-(2-hydroxypropyl)-3-trimethylammonium poly
acrylate chloride, low molecular weight chitosan, proteamine sulfate,
hydroxypropylcellulose grafted with poly(vinyl alcohol), iota- and
kappa-carrageenans, heparin, and copolymers of thymylethyl acry-
late (TEA) with methacryloyl aminopropyltrimethylammonium
chloride (MAPTAC) (see Supplementary Fig. 1 for details). Only
polymers able to impede replication of the virus in vitro, namely
HTCC and its hydrophobic derivative, HM-HTCC, were selected
(HTPVA and PAH showed only very low inhibitory activity). Further
studies showed that both polymers were also able to inhibit repli-
cation of MHV in a cell culture. Evaluation of compounds using
other human viruses (i.e., influenza A, hMPV, adenoviruses, enter-
viruses, and human herpes virus type 1) showed no inhibitory
activity, suggesting that abolishment of coronaviral infection is a
highly specific process.

The exact mechanism of antiviral activity of the selected com-
pounds remains to be elucidated, though the size and charge of
polymers suggest that they are not actively transported into the
cell. Therefore, one may assume that the observed activity relies
on the direct interaction of the polymer with the virus (e.g., with
the S protein, essential for virus entry). Indeed, the analysis of
the interaction between HTCC polymer and the recombinant ecto-
domain of the S protein showed binding, resulting in the formation
of protein–polymer complexes. One may assume that such binding
will result in the efficient inactivation of the virus. Unfortunately, it
was not possible to determine whether HTCC polymer hampers
NL63-S/AEE2 interaction, as the affinity of NL63-S to AEE2 is very
low (Lin et al., 2008; Mathewson et al., 2008) and it was not possi-
ble to visualize this process with accessible methods. Careful anal-
ysis of polymers suggests that the combination of particular
polymeric chain and its proper substituent is indispensable for
its activity. For example, the non-modified oligochitosan did not
present any antiviral properties; furthermore, no inhibitory effect
was seen for other polymeric chains substituted in a similar fash-
ion (data not shown).
Evaluated polymers can efficiently inhibit viral replication, thus one may consider these as potentially new class of antiviral agents for future use in the treatment of acute respiratory illnesses in children and immunocompromised patients. One may also speculate that due to mucosal localization of the infection and mechanism of inhibition, polymers may be delivered in the form of an aerosol directly to the site of infection. Furthermore, the HTCC and HM-HTCC polymers showed anti-MHV activity, suggesting that the developed compounds may serve as antiviral drugs. Additional studies are required to synthesize derivatives of selected polymers, which would show better pharmacokinetic profiles and lower cytotoxicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.11.006.

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