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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 croup 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 antimicrobial 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.

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

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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 polycations 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 ciprofloxacin (Woo 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 (betacoronavirus; 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 (CH_3COOH, 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 Bruker IFS 48 spectrometer. NMR spectra were measured in a 1:1 mixture of deuterium oxide (D_2O) 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 NH_2 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 cyanohydroborate, $NaCNBH_4$, (1.2568 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% CD_3COOD solution in D_2O (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 24 h in the dark. Subsequently, FITC-HTCC was precipitated with acetone and isolated by centrifugation for 5 min at 4000 rpm. The precipitate was washed with acetone, dissolved in water, dialyzed against water for 2 days and further against acetone:water mixture (1:4 v/v) for 1 day and freeze dried. The degree of substitution, defined as the number of FITC groups per a glucose group of HTCC, was found to be 1.66% based on the UV–VIS spectra.

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% CO_2. 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% CO_2. LR7 cells (murine L cells stably transfected with the N-Ceacam 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% CO_2.

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/KB1/17/10 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 3×10^5 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 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 $TCID_{50}$ of 400.

Virus stocks for other viruses used in the study were generated by infecting HeLa cells (adenovirus type 4), RD cells (echovirus 9); LLC-MK2 cells (hMPV clade B2), and MDCK cells (influenza A H_3N_2). In all the cases, cells were lysed after appearance of the cytopathic effect by two freeze–thaw cycles. The virus-containing fluid was aliquoted and stored at −80 °C. A control cell lysate from mock-infected cells was prepared in the same manner as the virus stock. All experimental procedures were conducted as previously described (Pyrc et al., 2012).

Stock samples containing influenza A virus, human echovirus 9, and human adenovirus were kindly provided by Marcel Müller. Human metapneumovirus was kindly provided by Oliver Schildgen.

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 TCG ATG GCC ATT TAG GTG ACA CTA TAG ATG GCT AGT GTA AAT TGG-3′; reverse: 5′-TCC GGA TTT TTT TTT TTT TTT TTT TTT TTA ATG CAA AAC CTC GTT GAC-3′) and SP6MHV (forward 5′-ATG CAT TTA GGT GAC ACT ATA GAT GTC TTT TGT TCC TGG GCA AGA-3′; reverse 5′-TTA CAC ATT AGA GTC ATC TTC TA-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
Primers used for quantitative PCR.

Target species	Primer	Primer sequence (5′–3′)
HCoV-NL63	Sense primer [63NF2]	AAA CCT CGT TGG AAG CGT GT
	Antisense primer [63NR1]	CTG TGG AAA ACC TTT GGC ATC
	Probe [63NP]	FAM-ATG TTA TTC AGT GCT TTG GTC CTC GTG AT-TAMRA
MHV	Sense primer [MHV_NF]	TGG CCG AAG AAA TTG CTG CTC TTG
	Antisense primer [MHV_NR]	GCC TGA CTT CTT TGG CAC TTT 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 CD5 signal peptide and followed by sequences encoding a C-terminal artificial GCN4 leucine zipper trimerization motif and a Strep-tag for affinity purification (IBA GmbH) as described previously for the haemagglutinin (HA) ectodomain of influenza virus (Bosch et al., 2010). To express the NL63-Se, 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 Se protein was confirmed by Western blotting and SDS–PAGE analysis (data not shown).

2.13. Evaluation of interaction between S_E-NL63 and HTCC

To find out whether HTCC interacts with the Se-NL63 protein, the fluorescence spectra of HTCC labeled with FITC (at $c_{FITC\text{-}HTCC}$ = 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 spectrofluorimeter (λ_{ex} = 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

This paper reports the studies on the antiviral properties of modified natural polymers which are chitosan derivatives, i.e., *N*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HTCC, Fig. 1A) and HTCC hydrophobically modified with dodecyl groups, i.e., *N*-dodecyl-*N*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HM-HTCC, Fig. 1B). Both polymers were selected from a larger library of compounds (see Supplementary Fig. 1) and are polycations since they contain amine and ammonium groups. These polymers were selected for tests since cationic polymers, in particular those containing ammonium groups, may potentially show antimicrobial properties (Huang et al., 2008; Timofeeva et al., 2009).

3.2. Inhibition of HCoV-NL63 replication in vitro

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 ($TCID_{50}$) 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 IC_{50} 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 IC_{50} 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-

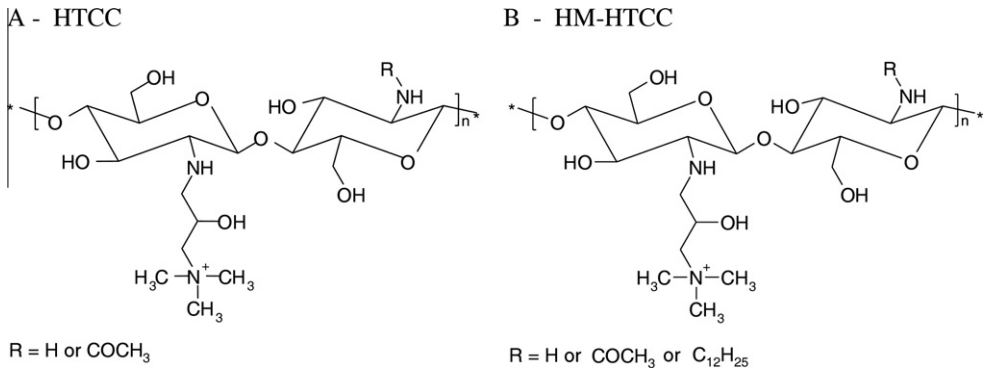

Fig. 1. Structures of the polymers showing anticoronaviral properties. In native chitosan the amino groups are partially deacetylated which means that the glucose groups may be substituted with both $-NH_2$ and CH_3CONH- groups in the same macromolecule. Since the distribution of acetylated and deacetylated NH_2- groups along the chitosan chain is random, this is often reflected by showing that R may be either H or $-COCH_3$ group. Consequently, since in HM-HTCC polymer some of the NH_2- groups are substituted with $-C_{12}H_{25}$ groups, some of R groups may be H, some $-COCH_3$ groups, and some $-C_{12}H_{25}$ groups in the same chitosan chain. Therefore, in HM-HTCC R may be H or $COCH_3$ or $C_{12}H_{25}$.

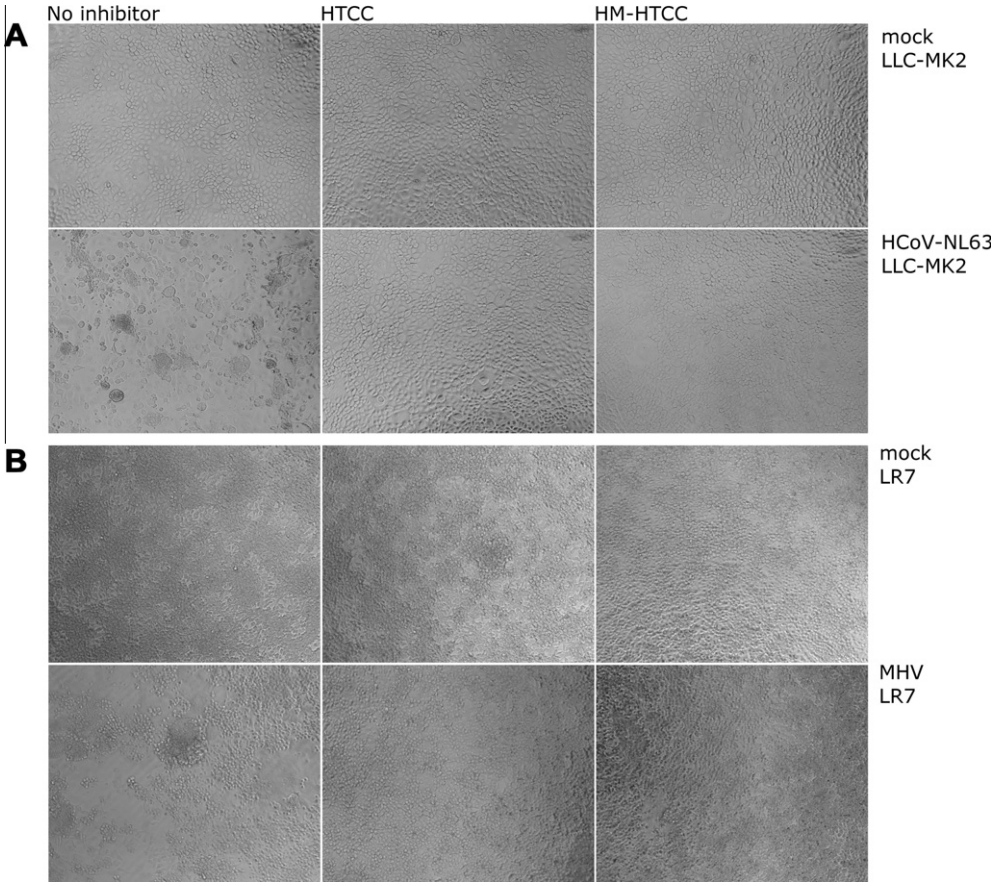

Fig. 2. Inhibition of HCoV-NL63 and MHV replication by HTCC and HM-HTCC compounds on LLC-MK2 (A) and LR7 cells (B), respectively. Infection was carried on in culture media, media supplemented with HTCC (100 μg/ml) or media supplemented with HM-HTCC (100 μg/ml). Cells were infected with HCoV-NL63 or MHV at $TCID_{50}$ 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.

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-

Table 2
Inhibition of HCoV-NL63 and MHV replication *in vitro*.

Polymer	HCoV-NL63 IC LLC-MK2 (CPE[a])	IC50 LLC-MK2 (qRT-PCR[b])	MHV IC50 LR7 (qRT-PCR[b])
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]

[a] Lowest compound concentration for which no CPE was noted.
[b] 50% inhibitory concentration as assessed by real-time RT-PCR analysis.

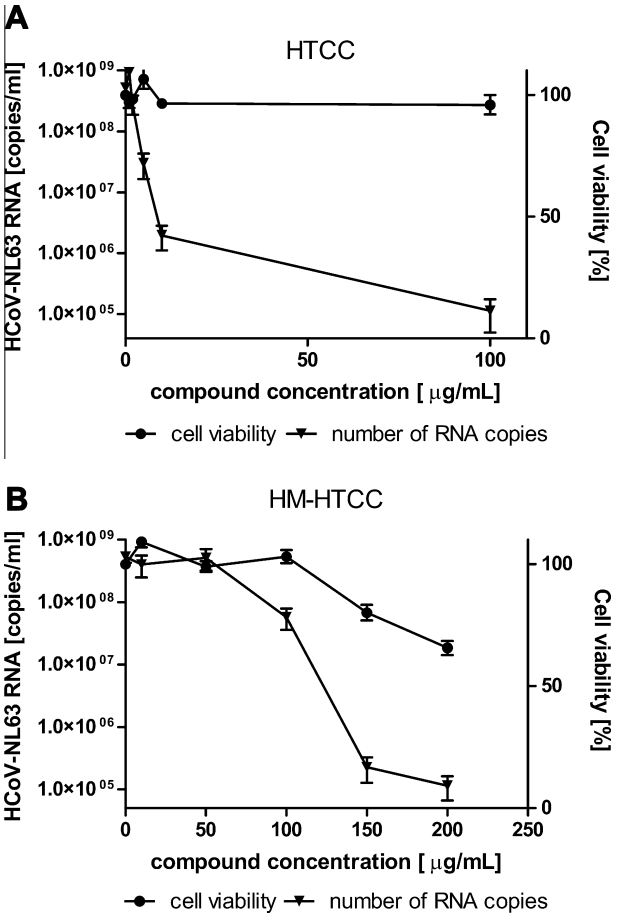

Fig. 3. Inhibition of HCoV-NL63 replication on LLC-MK2 cells by HTCC (A) and HM-HTCC (B). Data are presented as HCoV-NL63 RNA copies/ml (black triangles) and are reflected by values on the left *Y*-axis. The cell viability is presented on the right *Y*-axis (black circles). All assays were performed in triplicate and average values with standard errors (error bars) are presented.

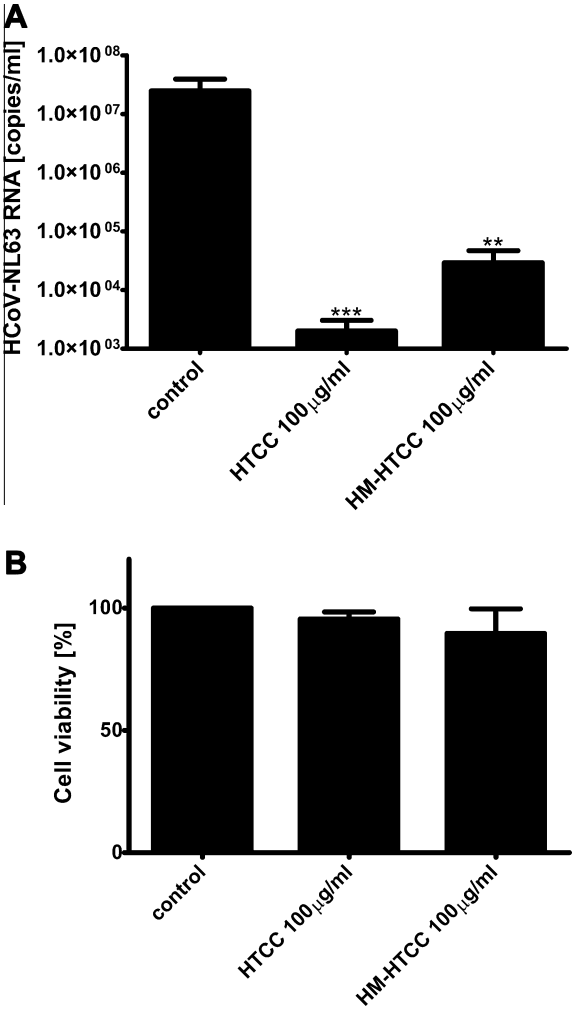

Fig. 4. Inhibition of HCoV-NL63 replication in HAE cultures. (A) Inhibition of virus replication in the cell culture, as determined with real-time PCR. Data on virus replication are presented as HCoV-NL63 RNA copies/ml. $^{***}p < 0.001$; $^{*}p < 0.01$; ns, not significant. (B) Cytotoxicity of HTCC and HM-HTCC on HAE cultures. Due to limited availability of HAE cultures, these experiments were conducted with limited number of concentrations. Cell viability was assessed with XTT assay and the data on the *Y*-axis represent the % of values obtained for control samples. All assays were performed in duplicate in at least two independent experiments and average values with standard errors (error bars) are presented.

tures with respective polymers did not result in a marked decrease in cell viability (Fig. 4B), which suggests these polymers may be potentially used in the treatment of coronaviral illnesses.

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 anticoronaviral 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 ($TCID_{50}$) 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

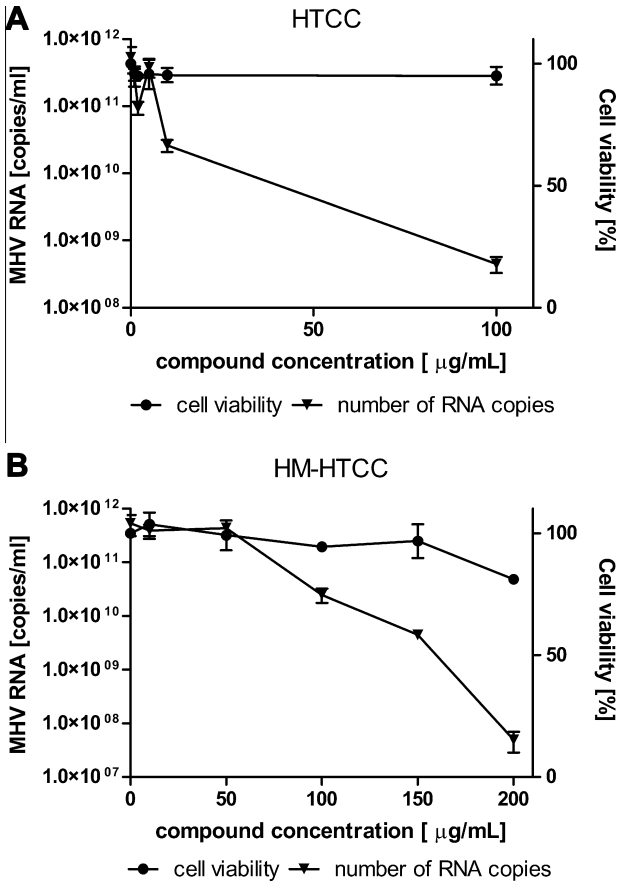

Fig. 5. Inhibition of MHV replication on LR7 cells by HTCC (A) and HM-HTCC (B). Data are presented as MHV RNA copies/ml (black triangles) and are reflected by values on the left *Y*-axis. The cell viability is presented on the right *Y*-axis (black circles). All assays were performed in triplicate and average values with standard errors (error bars) are presented.

confirm observations shown in Fig. 2 and prove that in the presence of HTCC or HM-HTCC MHV replication is limited by 3–4 logs, compared to the control sample (Fig. 5A and B). While for HM-HTCC some cytotoxicity may be observed at the highest concentration tested (200 μg/ml; ~20% toxicity), no marked toxicity was observed for the HTCC compound. The lower toxicity, compared to LLC-MK2 cells, most likely resulted from shorter incubation time for MHV (48 h instead of 144 h).

Subsequently, an effort was made to determine whether tested compounds are able to inhibit replication of other human viruses. To appropriately study the subject, a variety of viral species were tested, including human metapneumovirus, human adenovirus, human echovirus, influenza A virus, and human herpesvirus type 1. The analysis was conducted by CPE assessment and no inhibition of virus-mediated effect was observed (data not shown).

3.4. Cytotoxicity

The cytotoxicity of the tested polymers was assessed on LLC-MK2 cells which are susceptible to HCoV-NL63 infection, LR7 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 (IC_{50} > 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	CC_{50} LLC-MK2 (XTT[a]) (μg/ml)	CC_{50} LLC-MK2 (NR[b]) (μg/ml)	CC_{50} A549 (XTT[a]) (μg/ml)	CC_{50} A549 (NR[b]) (μg/ml)
HTCC	161.25	191.92	143.83	156.46
HM-HTCC	220.09	215.23	96.29	96.13

[a] Evaluated with XTT assay.
[b] Evaluated with neutral red assay.

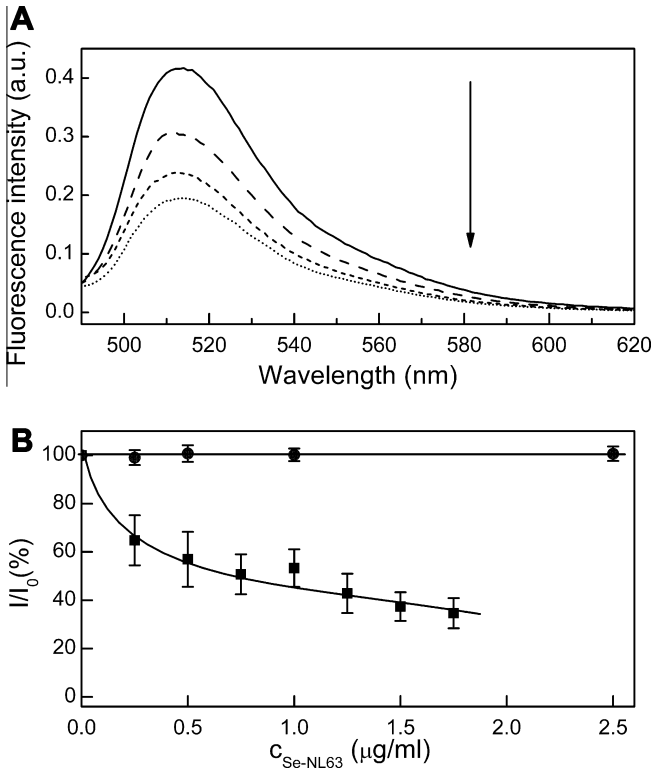

Fig. 6. Decreased fluorescence emission due to Se-NL63 and HTCC interaction. (A) Fluorescence spectra of FITC-HTCC in the presence of 0, 1.0, 2.0, and 2.5 μg/ml of Se-NL63 (the sequence of changes in the spectra for increasing concentration of Se-NL63 is given by an arrow; a.u., arbitrary units) and (B) The concentration-dependent decrease in relative fluorescence intensity (I/I_0) of FITC-HTCC at λ = 513 nm in the presence of Se-NL63 concentration (λ_{ex} = 494 nm, $c_{FITC\text{-}HTCC}$ = 0.5 μg/ml) (■). Analogical measurements of FITC-HTCC spectra in the presence of bovine serum albumin (BSA) were performed as a control (●). Both assays were performed in triplicate and the representative spectra are presented (Fig. 6A) or average values of fluorescence intensity at the band maximum with standard errors (error bars) (Fig. 6B).

a strong interaction between the tested biopolymers, as it results from the signal quenching during complex formation. No decrease in fluorescence intensity of FITC-HTCC was observed in the presence of bovine serum albumin (BSA) indicating that BSA does not interact with FITC-HTCC and, consequently, that there is some specificity in the interaction between Se-NL63 and HTCC.

4. Discussion

Human coronaviruses received relatively little attention as human 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 humans (Wise, 2012). As mentioned above, a clear link between HCoV-NL63 and the respiratory diseases was established in a prospective population-based study (PRI.DE) on lower respiratory tract infections in children less than three years of age in Germany. Of the children with HCoV-NL63 infection, 45% had laryngotracheitis (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 children (van der Hoek et al., 2005). Considering the observed pathogenicity 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 described for HCoV-NL63, interfering with different stages of HCoV-NL63 replication.

The unique mechanism of coronaviral replication, which includes 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 synthesized a set of specific M^{pro} inhibitors, repressing replication of multiple coronaviruses, including HCoV-NL63 (Yang et al., 2005). Another interesting approach was proposed by Pyrc et al., who used synthetic peptides interfering with S protein fusogenic activity 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 (Pyrc et al., 2006). An interesting and novel approach for inhibition of coronaviral infection was proposed initially by Pfefferle et al., who identified interaction between cyclophilins/immunophilins 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; Pfefferle 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(vinyl alcohol) chloride (HTPVA), poly(allylamine hydrochloride) (PAH), *N*-(2-hydroxypropyl)-3-trimethylammonium dextran chloride, low molecular weight chitosan, protamine sulfate, hydroxypropylcellulose grafted with poly(vinylamine), iota- and kappa-carrageenans, heparin, and copolymers of thymylethyl acrylate (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 replication of MHV in a cell culture. Evaluation of compounds using other human viruses (i.e., influenza A, hMPV, adenoviruses, enteroviruses, 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 compounds 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 ectodomain 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/ACE2 interaction, as the affinity of NL63-S to ACE2 is very low (Lin et al., 2008; Mathewson et al., 2008) and it was not possible to visualize this process with accessible methods. Careful analysis 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 fashion (data not shown).

Evaluated polymers can efficiently inhibit viral replication, thus one may consider these as potentially new class of anticoronaviral 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 anticoronaviral 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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