

HTCC as a highly effective polymeric inhibitor of SARS-CoV-2 and MERS-CoV

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www: <http://virogenetics.info/>.

38 **ABSTRACT**

39 The beginning of 2020 brought us information about the novel coronavirus emerging in China.
40 Rapid research resulted in the characterization of the pathogen, which appeared to be a member
41 of the SARS-like cluster, commonly seen in bats. Despite the global and local efforts, the virus
42 escaped the healthcare measures and rapidly spread in China and later globally, officially
43 causing a pandemic and global crisis in March 2020. At present, different scenarios are being
44 written to contain the virus, but the development of novel anticoronavirals for all highly
45 pathogenic coronaviruses remains the major challenge. Here, we describe the antiviral activity
46 of previously developed by us HTCC compound (N-(2-hydroxypropyl)-3-trimethylammonium
47 chitosan chloride), which may be used as potential inhibitor of currently circulating highly
48 pathogenic coronaviruses – SARS-CoV-2 and MERS-CoV.

49 **INTRODUCTION**

50 Coronaviruses mainly cause respiratory and enteric diseases in humans, other mammals,
51 and birds. However, some species can cause more severe conditions such as hepatitis,
52 peritonitis, or neurological disease. Seven coronaviruses infect humans, four of which (human
53 coronavirus [HCoV]-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1) cause relatively
54 mild upper and lower respiratory tract disease. Other three zoonotic coronaviruses - the severe
55 acute respiratory syndrome coronaviruses (SARS-CoV and SARS-CoV-2) and the Middle East
56 respiratory syndrome coronavirus (MERS-CoV) are associated with severe, life-threatening
57 respiratory infections and multiorgan failure ¹⁻⁷.

58 SARS-CoV-2 emerged in the Hubei province of China by the end of 2019 and caused
59 an epidemic that was partially contained in China by March 2020. However, the virus rapidly
60 spread globally and caused the pandemic ⁸. SARS-CoV-2 is a betacoronavirus and belongs to
61 a large cluster of SARS-like viruses in bats, classified in *Sarbecovirus* subgenus. While bats
62 are considered to be the original reservoir, it is believed that there is an intermediate host, and
63 pangolins were suggested as such ⁹. The virus is associated with a respiratory illness that, in a
64 proportion of cases, is severe. The mortality rate varies between locations, but at present, is
65 estimated to reach 3-4% globally. The virus infects primarily ciliated cells and type II
66 pneumocytes in human airways, hijacking the angiotensin-converting enzyme 2 (ACE2) to
67 enter the cell, similarly as SARS-CoV and HCoV-NL63.

68 MERS-CoV is related to SARS-CoV-2, but together with some bat viruses forms
69 a separate *Merbecovirus* subgenus. Bats are believed to serve as an original reservoir also in
70 this case ¹⁰, but camels were identified as the intermediate host ¹¹. The virus never fully crossed
71 the species border, as the human-to-human transmission is limited, and almost all the cases are
72 associated with animal-to-human transmission. The entry receptor for MERS-CoV is the
73 dipeptidyl peptidase 4 (DPP4) ^{12,13}. In humans, MERS-CoV causes a respiratory illness with

74 severity varying from asymptomatic to potentially fatal acute respiratory distress¹⁴⁻¹⁶. To date,
75 MERS-CoV infection was confirmed in 27 countries, with over 2,000 cases and a mortality rate
76 of ~35%.

77 Currently, there are no vaccines or drugs with proven efficacy to treat coronavirus
78 infection, and treatment is limited to supportive care. However, a range of therapeutics have
79 been experimentally used in clinic to treat SARS-CoV-2 and MERS-CoV-infected patients, and
80 their use is based on the knowledge obtained in previous years. The most promising drug
81 candidates include broad-spectrum polymerase inhibitors (remdesivir)¹⁷ and some re-purposed
82 drugs (e.g., HIV-1 protease inhibitors). However, until today none of these has proven effective
83 in randomized controlled trials. On the other hand, the antiviral potential of several small
84 molecules have been demonstrated in cell lines *in vitro*, but their effectiveness *in vivo* have not
85 been confirmed^{18,19}. For the ones that reached animal models, some promising drug candidates
86 have been shown to exacerbate the disease (ribavirin, mycophenolic acid)²⁰.

87 Drug development for SARS-CoV-2 and MERS-CoV is of great importance, but
88 considering the diversity of coronaviruses and the proven propensity to cross the species barrier,
89 the SARS-CoV-2 epidemic is probably not the last one. Warnings about the possibility of
90 another SARS-CoV epidemic have appeared in the scientific literature for a long time²¹.
91 Consequently, broad-spectrum antivirals are essential in long-term perspective.

92 Previously, we have demonstrated an antiviral potential of the HTCC polymer (N-(2-
93 hydroxypropyl)-3-trimethylammonium chitosan chloride), which efficiently hampered
94 infection of all low pathogenic human coronaviruses *in vitro* and *ex vivo*²² and several animal
95 coronaviruses (*unpublished data*). Furthermore, using several functional and structural assays,
96 we dissected the mechanism of the HTCC antiviral activity. We showed that the polymer
97 interacts with the coronaviral Spike (S) protein and blocks its interaction with the cellular
98 receptor²²⁻²⁴. Here, we analyzed the HTCC activity against SARS-CoV-2 and MERS-CoV *in*

99 *vitro* using permissive cell lines and *ex vivo*, using a model of human airway epithelium (HAE).
100 The study showed that the replication of both viruses was efficiently hampered. Overall, our
101 data show that HTCC polymers are potent broad-spectrum anticoronavirals and may be very
102 promising drug candidates for SARS-CoV-2 and MERS-CoV.

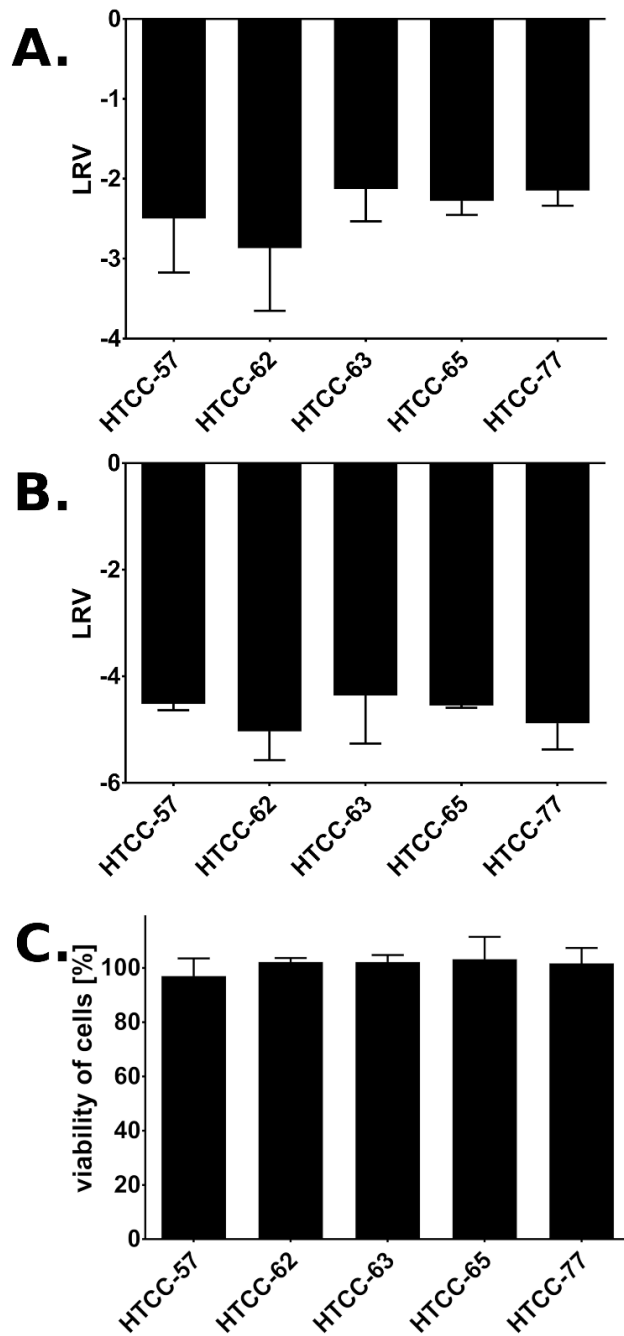
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104 **RESULTS AND DISCUSSION**

105 ***HTCCs hamper MERS-CoV and SARS-CoV-2 replication in cell lines***

106 Previously, we showed that HTCC with different degrees of substitution (DSs) is a
107 potent inhibitor of all four low pathogenic HCoVs²². DSs are expressed as the fraction of NH₂
108 groups of glucosamine units in chitosan substituted with glycidyltrimethylammonium chloride
109 (GTMAC). The DS of the studied HTCC polymers varied between 57% and 77%; thus, the
110 polymers were named HTCC-57, HTCC-62, HTCC-63, HTCC-65, and HTCC-77. The
111 synthesis and characterization of polymers are described elsewhere^{22,23}. The analysis showed
112 that HTCC-63 demonstrated the most significant inhibitory effect on HCoV-NL63, HCoV-
113 OC43, and HCoV-HKU1. On the other hand, HTCC-62 and HTCC-77 proved to be effective
114 inhibitors of HCoV-229E infection. Further, HTCC-65 effectively inhibited the replication of
115 HCoV-NL63 and HCoV-OC43, while HTCC-62 showed a potent antiviral effect on HCoV-
116 HKU1 infection.

117 The study on the MERS-CoV using the Vero cells revealed that all HTCC variants
118 inhibit virus replication to a similar extent (~100-1000-time decrease in viral yields), at non-
119 toxic concentration (**Figure 1A, C**). The inhibition of the SARS-CoV-2 infection in Vero E6
120 cells was even more pronounced, and all HTCC variants inhibited virus replication by ~10,000
121 times at non-toxic concentration (**Figure 1B, C**). In this case, the HTCC-63 was arbitrarily
122 selected for further studies on MERS-CoV, while HTCC-77 was selected for SARS-CoV-2.

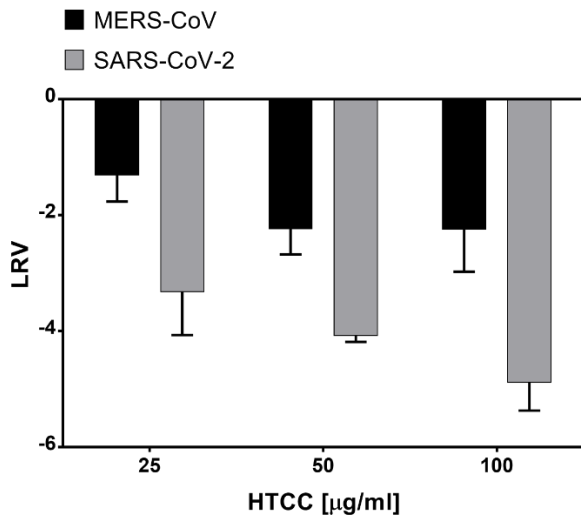


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124 **Figure 1. *In vitro* inhibition of MERS-CoV and SARS-CoV-2 by HTCC at non toxic concentration.** Vero cells were
125 infected with MERS-CoV (A), and Vero E6 cells were infected with SARS-CoV-2 (B). Briefly, cultures were
126 inoculated with a given virus in the presence of HTCC (100 $\mu\text{g}/\text{ml}$) or control PBS. Replication of viruses was
127 evaluated at 48 h post-inoculation using RT-qPCR. The data are presented as Log Removal Value (LRV) compared
128 to the untreated sample. The assay was performed in triplicate, and average values with standard errors are
129 shown. (C) Cytotoxicity of HTCCs with DS ranging from 57% to 77% *in vitro* at 100 $\mu\text{g}/\text{ml}$. Cell viability was assessed
130 with XTT assay. Data on the y-axis represent the percentage of values obtained for the untreated reference
131 samples. All assays were performed in triplicate, and average values with standard errors are presented. The
132 differences in cytotoxicity of HTCCs were not statistically significant.

133

134 Next, the dose-dependence was tested for the HTCCs. The inhibitory activity of selected
135 polymers was verified for three different concentrations, and obtained data are shown in **Figure**
136 **2**.



137 **Figure 2. Dose-dependent inhibition of MERS-CoV and SARS-CoV-2 replication.** Vero (MERS-CoV) or Vero E6 cells
138 (SARS-CoV-2) were inoculated with a given virus in the presence of different concentrations of HTCC. Replication
139 of viruses was evaluated at 48 h post-inoculation using RT-qPCR. The data are presented as Log Removal Value
140 (LRV) compared to the untreated sample. The assay was performed in triplicate, and average values with standard
141 errors are displayed.
142
143

144 Based on the data obtained, the basic parameters were calculated and are presented in **Table 1**.

145 **Table 1.** 50% Cytotoxic concentration (CC_{50}), 50% inhibitory concentration (IC_{50}), and the selectivity index (SI) of
146 two most effective HTCCs: HTCC-63 (for MERS-CoV) and HTCC-77 (for SARS-CoV-2).

	CC_{50} [µg/ml]	IC_{50} [µg/ml]	SI [CC_{50}/IC_{50}]
MERS-CoV	161.0	62.8	2.6
SARS-CoV-2	158.0	12.5	12.6

147
148 The parameters observed for the SARS-CoV-2 appear to be favorable with SI above 12. It is
149 also worth to note that HTCC was previously administered by inhalation in rats, and no adverse
150 reactions were observed ²⁵. In that study, HTCC was used as a carrier for the active substance,
151 and as such, was reported to be promising for local sustained inhalation therapy of pulmonary
152 diseases.

153 ***HTCCs hamper MERS-CoV and SARS-CoV-2 replication in human airway epithelium***

154 While the Vero cells constitute a convenient model for antiviral research, it is of utmost
155 importance to verify whether the results obtained are not biased due to the artificial system
156 used. This is especially important for compounds, which activity is based on electrostatic
157 interaction. To verify whether the natural microenvironment, which is rich in sugars and
158 charged molecules, does not abrogate the effectiveness of the inhibitors, we employed HAE
159 cultures that mirror the fully differentiated layer lining the conductive airways, as well as the
160 site of coronavirus replication. Briefly, fully differentiated HAE cultures were infected with a
161 given virus ²⁶ in the presence of previously selected HTCCs (200 µg/ml) or control PBS.
162 Following inoculation, apical lavage samples were collected daily, and replication kinetics for
163 each virus was investigated. The analysis revealed that the polymer efficiently hampered
164 SARS-CoV-2 and MERS-CoV also in this model. For MERS-CoV, the inhibitory effect was
165 the most evident at 72 h p.i., while for SARS-CoV-2 the most substantial decline of virus
166 progeny was observed at 24 h p.i. (**Figure 3**). Whether such kinetics will be reflected *in vivo* it
167 is to be investigated.

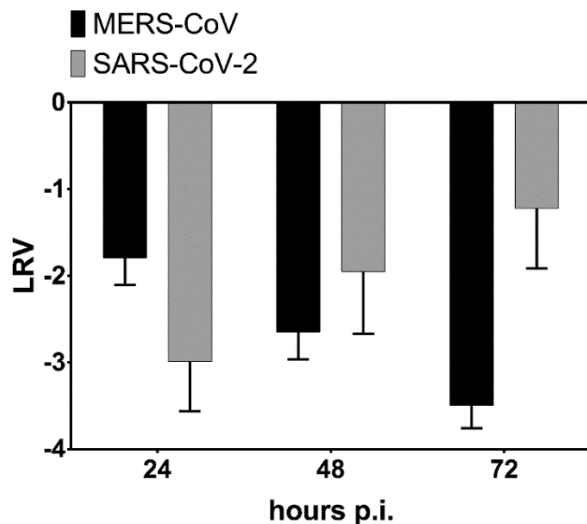
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174 **Figure 3. *Ex vivo* inhibition of MERS-CoV and SARS-CoV-2 by HTCC in human airway epithelium cultures.** HAE
175 cultures were exposed to MERS-CoV or SARS-CoV-2 in the presence of HTCC-63 (for MERS-CoV) or HTCC-77 (for
176 SARS-CoV-2) at 200 $\mu\text{g/ml}$ or control PBS. To analyze virus replication kinetics, each day post infection, 100 μl of
177 $1 \times \text{PBS}$ was applied to the apical surface of HAE cultures and collected after 10 min of incubation at 37°C .
178 Replication of viruses was evaluated using quantitative RT-qPCR. The data are presented as Log Removal Value
179 (LRV) compared to the untreated sample. The assay was performed in triplicate, and average values with
180 standard errors are shown.

181

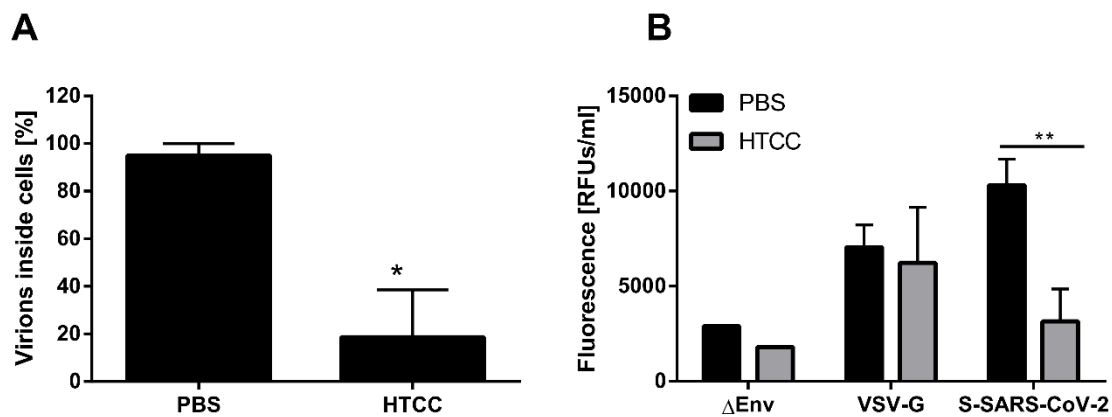
182 *HTCCs inhibits MERS-CoV and SARS-CoV-2 entry into susceptible cells*

183 Our previous research showed that the HTCC-mediated inhibition of coronaviral
184 replication results from the electrostatic interaction between the polymer and the Spike protein
185 of coronaviruses. We hypothesize that the selectivity of the inhibitors yields from the fitting
186 charge distributions on the polymer and on the S proteins on the viral surface. While the
187 interaction of a single charged moiety is relatively weak, the concatemeric nature of the virus
188 and the polymer stabilizes the binding. Such structure-based interaction may be an interesting
189 entry point for further fine-tuning of the polymeric inhibitors of viral replication.

190 To ensure that the observed effect was a result of coronavirus entry inhibition by HTCC,
191 two experiments were performed. First, HAE cultures were inoculated with MERS-CoV in the
192 presence of HTCC-63 (200 $\mu\text{g/ml}$) or control PBS and incubated for 2 h at 37°C . Next, cells
193 were fixed, immunostained for MERS-CoV N protein and actin. Virus entry was analyzed with
194 confocal microscopy. To visualize the effect, the signal attributed to intracellular MERS-CoV

195 was quantified, and the results show that the internalization of MERS-CoV was drastically
196 decreased (**Figure 4A**).

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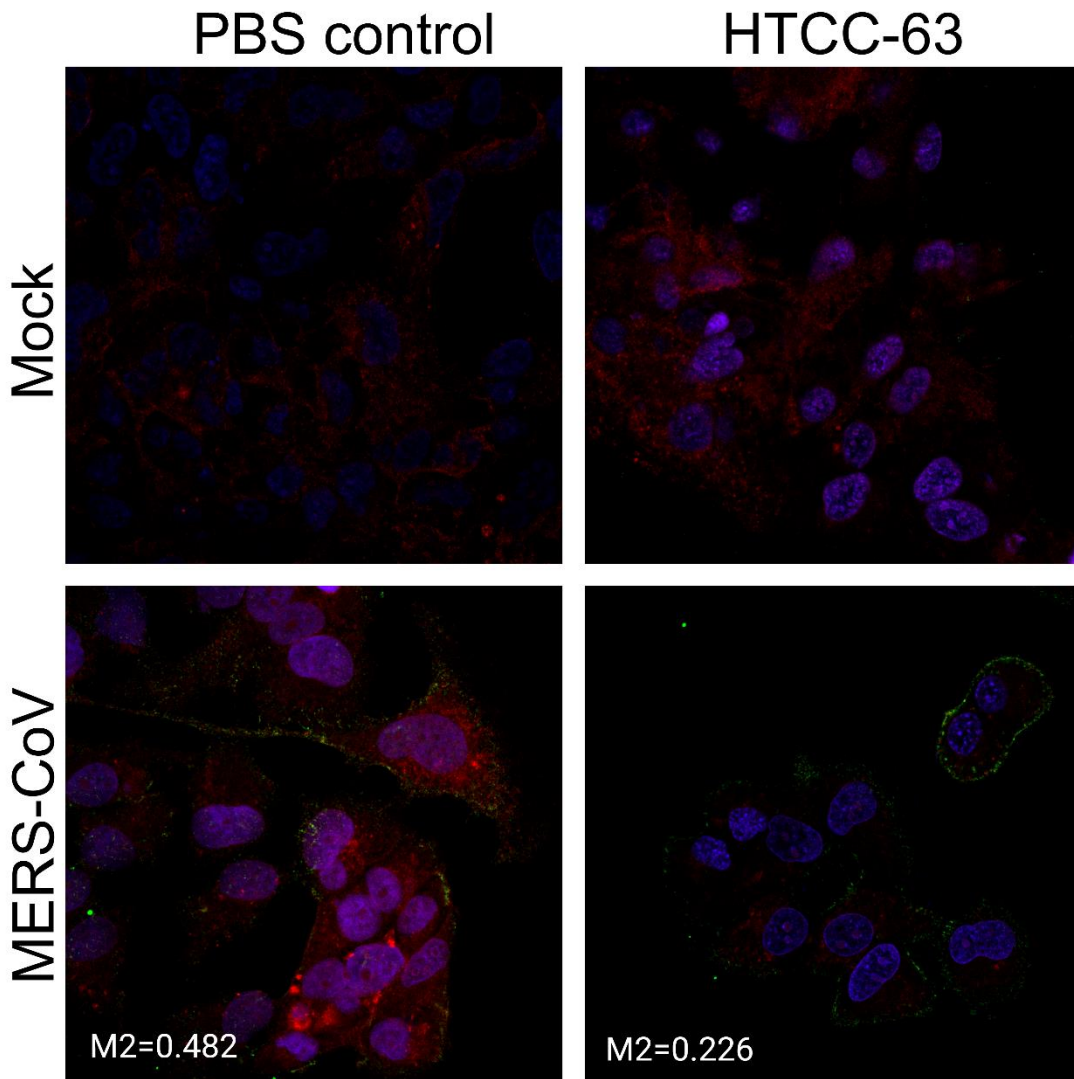
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199 **Figure 4. Coronavirus internalization into susceptible cells is hampered by HTCC.** (A) Pre-cooled HAE cultures
200 were incubated with ice-cold MERS-CoV suspension in the presence or absence of HTCC-63 (200 μ g/ml) for 2 h
201 at 37°C. Next, cells were fixed in PFA and immunostained for MERS-CoV N protein and actin. Virus entry was
202 analyzed with confocal microscopy. The data shown are representative of three independent experiments, each
203 performed in triplicate. * P < 0.05. (B) A549 cells overexpressing ACE2 were incubated with lentiviral particles
204 bearing GFP reporter gene, pseudotyped with SARS-CoV-2 Spike (S-SARS-CoV-2), VSV control G protein (VSV-G)
205 or particles without an envelope protein (Δ Env) in the presence of HTCC-77 or control PBS. After 2 h at 37°C cells
206 were washed with PBS and overlaid with fresh medium. Following 72 h incubation, GFP signal was measured
207 using fluorometer and pseudovirus entry is presented as Relative Fluorescence Units per ml. The assay was
208 performed in triplicate, and average values with standard errors are presented. ** P < 0.005.
209

210 Due to the limited availability of tools for the SARS-CoV-2 we were not able to replicate
211 the experiment for this virus. Here, we employed a surrogate system based on lentiviral vectors
212 pseudotyped with full-length Spike protein of SARS-CoV-2. A549 cells overexpressing the
213 ACE2 protein were incubated with pseudovirions harboring SARS-CoV-2 Spike or control
214 VSV-G protein in the presence of HTCC-77 (100 μ g/ml) or control PBS for 2 h at 37°C. After
215 72 h p.i. cells were lysed, and pseudovirus entry was quantified by measurement of the reporter
216 GFP protein. The analysis showed a significant reduction in SARS-CoV-2 Spike pseudoviruses
217 internalization in the presence of the polymer, while no inhibition was observed for the control
218 VSV-G (**Figure 4B**).

219 Next, to verify whether the mechanism of action for the highly pathogenic
220 betacoronaviruses is similar to that observed for alphacoronaviruses²², and is based on locking
221 the interaction between the virus and the entry receptor, we analyzed MERS-CoV
222 co-localization with its entry receptor, DPP4 in the presence or absence of the HTCC. For this,
223 human cell line Huh7 was inoculated with the virus or mock in the presence of HTCC-63
224 (100 µg/ml) or control PBS and incubated for 2 h at 4°C. Subsequently, cells were fixed and
225 immunostained for the DPP4 and MERS-CoV N using specific antibodies. Virus co-
226 localization with its receptor was examined using confocal microscopy. Obtained results
227 demonstrated that in the control samples, virions co-localize with the DPP4 protein, while in
228 the presence of the polymer, this interaction is blocked (**Figure 5**).

229



230

231 **Figure 5. HTCC blocks interaction between the virus and its entry receptor.** Pre-cooled Huh7 cells were
232 incubated for 3 h at 4°C with ice-cold MERS-CoV or mock in the presence or absence of HTCC-63 (100 µg/ml).
233 Next, cells were fixed with PFA and immunostained for MERS-CoV-N (green), DPP4 (red) and nuclear DNA (blue).
234 MERS-CoV interaction with the DPP4 protein was analyzed with confocal microscopy. Co-localisation of DPP4
235 with MERS-CoV-N was determined by confocal microscopy and is presented as Manders' M2 coefficient. The
236 decrease in colocalization was statistically significant ($P < 0.0005$). Each image represents maximum projection
237 of axial planes. Representative images are shown.

238

239 Taking together, we show here that the previously developed and described polymeric
240 HTCC anticoronaviral compounds based on chitosan are able to efficiently inhibit infection
241 with emerging coronaviruses. We believe that the HTCC can be fine-tuned to target any
242 coronavirus, and this interaction is specific to viruses that belong to the *Coronaviridae* family.
243 One may speculate that the inhibition results from the concatemeric nature of the virus surface
244 and the fact that the polymer with appropriate charge distribution can interact with multiple
245 sites on this surface. While the interaction of the monomer is relatively weak, and no inhibition
246 is observable for monomers, the sum of interactions stabilizes the binding and specific
247 inhibition is observed. Considering that the extended chain length for the HTCC used is
248 ~700 nm this scenario seems realistic ²⁷. If that would be true, HTCC would constitute a first
249 structure-specific inhibitor of viral replication. The major disadvantage of the HTCC is that, at
250 present, it is not registered for use in humans. However, previous experience with HTCC in
251 different laboratories shows that it may be delivered topically to the lungs, it is not associated
252 with toxicity, and it does not worsen the lung function ²⁵. We believe that HTCC is a promising
253 drug candidate that should be further studied, as it provides a ready-to-use solution for
254 SARS-CoV-2 and future emerging coronaviruses.

255

256 **MATERIALS AND METHODS**

257 **The active compound**

258 The HTCC was prepared in the same manner as previously described ^{22,23,28}.

259

260 **Plasmid constructs**

261 The codon-optimised full-length SARS-CoV-2 S gene was designed and purchased
262 from GeneArt (Thermo Fisher Scientific, Poland). The gene was cloned into pCAGGS vector

263 sequence verified that was a gift from Xingchuan Huang. psPAX (Addgene plasmid # 12260)
264 and pMD2G (Addgene plasmid # 12259) was a gift from Didier Trono. Lego-G2 vector
265 (Addgene plasmid #25917) was a gift from Boris Fehs.

266

267 **Cell culture**

268 Vero and Vero E6 (*Cercopithecus aethiops*; kidney epithelial; ATCC: CCL-81 and CRL-
269 1586), Huh7 (*Homo sapiens*; hepatocellular carcinoma; ECACC: 01042712) and A549 cells
270 with ACE2 overexpression (A549/ACE2)²⁹ were cultured in Dulbecco's MEM (Thermo Fisher
271 Scientific, Poland) supplemented with 3% fetal bovine serum (heat-inactivated; Thermo Fisher
272 Scientific, Poland) and antibiotics: penicillin (100 U/ml), streptomycin (100 µg/ml), and
273 ciprofloxacin (5 µg/ml). Cells were maintained at 37°C under 5% CO₂.

274

275 **Human airway epithelium (HAE) cultures**

276 Human airway epithelial cells were isolated from conductive airways resected from
277 transplant patients. The study was approved by the Bioethical Committee of the Medical
278 University of Silesia in Katowice, Poland (approval no: KNW/0022/KB1/17/10 dated
279 16.02.2010). Written consent was obtained from all patients. Cells were dislodged by protease
280 treatment, and later mechanically detached from the connective tissue. Further, cells were
281 trypsinized and transferred onto permeable Transwell insert supports ($\phi = 6.5$ mm). Cell
282 differentiation was stimulated by the media additives and removal of media from the apical side
283 after the cells reached confluence. Cells were cultured for 4-6 weeks to form well-differentiated,
284 pseudostratified mucociliary epithelium. All experiments were performed in accordance with
285 relevant guidelines and regulations.

286

287 **Cell viability assay**

288 HAE cultures were prepared as described above. Cell viability assay was performed by
289 using the XTT Cell Viability Assay (Biological Industries, Israel) according to the
290 manufacturer's instructions. On the day of the assay, 100 μ l of the 1 \times PBS with the 50 μ l of
291 the activated XTT solution was added to each well/culture insert. Following 2 h incubation at
292 37°C, the solution was transferred onto a 96-well plate, and the signal was measured at
293 $\lambda = 490$ nm using the colorimeter (Spectra MAX 250, Molecular Devices). The obtained results
294 were further normalized to the control sample, where cell viability was set to 100%.

295

296 **Virus preparation and titration**

297 MERS-CoV stock (isolate England 1, 1409231v, National Collection of Pathogenic
298 Viruses, Public Health England, United Kingdom) was generated by infecting monolayers of
299 Vero cells. SARS-CoV-2 stock (isolate 026V-03883; kindly granted by Christian Drosten,
300 Charité – Universitätsmedizin Berlin, Germany by the European Virus Archive - Global
301 (EVAg); <https://www.european-virus-archive.com/>) was generated by infecting monolayers of
302 Vero E6 cells. The virus-containing liquid was collected at day 3 post-infection (p.i.), aliquoted
303 and stored at -80°C . Control Vero or Vero E6 cell lysate from mock-infected cells was prepared
304 in the same manner. Virus yield was assessed by titration on fully confluent Vero or Vero E6
305 cells in 96-well plates, according to the method of Reed and Muench. Plates were incubated at
306 37°C for 3 days and the cytopathic effect (CPE) was scored by observation under an inverted
307 microscope.

308

309 **Virus infection**

310 In *in vitro* experiments, fully confluent Vero, Vero E6, or Huh7 cells in 96-well plates
311 (TPP) were exposed to MERS-CoV, SARS-CoV-2 or mock at a TCID₅₀ of 400 per ml in the

312 presence of tested polymer or control medium. Following a 2 h incubation at 37°C, unbound
313 virions were removed by washing with 100 µl of 1 × PBS and fresh medium containing
314 dissolved respective polymer was added to each well. Samples of cell culture supernatant were
315 collected at day 3 p.i. and analyzed using RT-qPCR.

316 For the *ex vivo* study, fully differentiated human airway epithelium (HAE) cultures were
317 exposed to the tested polymer or control PBS for 30 min at 37°C, following inoculation with
318 MERS-CoV or SARS-CoV-2 at a TCID₅₀ of 400 per ml in the presence of the polymer or
319 control PBS. Following 2 h incubation at 37°C, unbound virions were removed by washing
320 with 200 µl of 1 × PBS and HAE cultures were maintained at an air—liquid interphase for the
321 rest of the experiment. To analyze virus replication kinetics, each day p.i., 100 µl of 1 × PBS
322 was applied at the apical surface of HAE and collected following the 10 min incubation at 32°C.
323 All samples were stored at –80°C and analyzed using RT-qPCR.

324

325 **Isolation of nucleic acids and reverse transcription (RT)**

326 Viral DNA/RNA Kit (A&A Biotechnology, Poland) was used for nucleic acid isolation
327 from cell culture supernatants, according to the manufacturer's instructions. cDNA samples
328 were prepared with a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher
329 Scientific, Poland), according to the manufacturer's instructions.

330

331 **Quantitative PCR (qPCR)**

332 Viral RNA yield was assessed using real-time PCR (7500 Fast Real-Time PCR; Life
333 Technologies, Poland). cDNA was amplified in a reaction mixture containing 1 × qPCR Master
334 Mix (A&A Biotechnology, Poland), in the presence of probe (100 nM) and primers (450 nM
335 each).

336

337 **Table 1. Primers and probes.**

	MERS-CoV	SARS-CoV-2
5' primer	GGG TGT ACC TCT TAA TGC CAA TTC	CAC ATT GGC ACC CGC AAT C
3' primer	TCT GTC CTG TCT CCG CCA AT	GAG GAA CGA GAA GAG GCT TG
probe	ACC CCT GCG CAA AAT GCT GGG (FAM / TAMRA)	ACT TCC TCA AGG AAC AAC ATT GCC A (FAM / BHQ1)

338

339 The reaction was carried out according to the scheme: 2 min at 50°C and 10 min at 92°C,
340 followed by 40 cycles of 15 s at 92°C and 1 min at 60°C. In order to assess the copy number
341 for N gene, DNA standards were prepared, as described before ²⁶.

342

343 **Immunostaining and confocal imaging**

344 Fixed cells were permeabilized with 0.1% Triton X-100 in 1 × PBS and incubated
345 overnight at 4°C in 1× PBS supplemented with 5% bovine serum albumin (BSA) and 0.5%
346 Tween 20. To visualize MERS-CoV particles, cells were incubated for 2 h at room temperature
347 with mouse anti-MERS-CoV N IgGs (1:000 dilution, Sino Biological, China), followed by 1 h
348 of incubation with Alexa Fluor 488-labeled goat anti-mouse IgG (2.5 µg/ml; Thermo Fisher
349 Scientific, Poland). Actin filaments was stained using phalloidin coupled with Alexa Fluor 633
350 (0.2 U/ml; Thermo Fisher Scientific, Poland). Nuclear DNA was stained with DAPI (4',6'-
351 diamidino-2-phenylindole) (0.1 µg/ml; Sigma-Aldrich, Poland). Immunostained cultures were
352 mounted on glass slides in ProLong Gold antifade medium (Thermo Fisher Scientific, Poland).
353 Fluorescent images were acquired under a Leica TCS SP5 II confocal microscope (Leica
354 Microsystems GmbH, Mannheim, Germany) and a Zeiss LSM 710 confocal microscope (Carl
355 Zeiss Microscopy GmbH). Images were acquired using Leica Application Suite Advanced
356 Fluorescence LAS AF v. 2.2.1 (Leica Microsystems CMS GmbH) or ZEN 2012 SP1 software
357 (Carl Zeiss Microscopy GmbH) deconvolved with Huygens Essential package version 4.4
358 (Scientific Volume Imaging B.V., The Netherlands) and processed using ImageJ 1.47v

359 (National Institutes of Health, Bethesda, MD, USA). At the time of the study, no antibodies
360 specific to SARS-CoV-2 were available to us.

361

362 **Pseudovirus production and transduction**

363 293T cells were seeded on 10 cm² dishes, cultured for 24 h at 37°C with 5% CO₂ and
364 transfected using polyethyleneimine (Sigma-Aldrich, Poland) with the lentiviral packaging
365 plasmid (psPAX), the VSV-G envelope plasmid (pMD2G) or SARS-CoV-2 S glycoprotein
366 (pCAGGS-SARS-CoV-2-S) and third plasmid encoding GFP protein (Lego-G2). Cells were
367 further cultured for 72 h at 37°C with 5% CO₂ and pseudoviruses were collected every 24 h and
368 stored at 4°C.

369 A549/ACE2 cells were seeded in 48-wells plates, cultured for 24 h at 37°C with 5%
370 CO₂ and transduced with pseudoviruses harboring VSV-G or S-SARS-CoV-2 proteins or
371 lacking the fusion protein (Δ Env) in the presence of polybrene (4 μ g/ml; Sigma-Aldrich,
372 Poland) and HTCC-77 (100 μ g/ml) or control PBS . After 4 h incubation at 37°C unbound
373 virions were removed by washing thrice with 1 \times PBS and cells were further cultured for 72 h
374 at 37°C with 5% CO₂. Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl,
375 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5) and transferred onto black
376 96-wells plates. Fluorescence levels were measured on a microplate reader Gemini EM
377 (Molecular Devices, UK).

378

379 **Statistical analysis.**

380 All the experiments were performed in triplicate, and the results are presented as mean
381 \pm standard deviation (SD). To determine the significance of the obtained results Student t test
382 was carried out. P values of < 0.05 were considered significant.

383

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392

393 **AUTHOR CONTRIBUTIONS STATEMENT**

394 A.M., Y.C., A.S., E. B. D., X.G., Y.G., J.L., L.C. conducted the experiments. M.O., M.U., and
395 S.R.M provided materials and methods for the study. A.M., K.P. designed the study and
396 experiments, analysed the data and wrote the manuscript. K.L., D.L., F.Z., M.N., K.S. analysed
397 the data. K.P. supervised the study. All authors reviewed the manuscript and approved the
398 submitted version. All authors agreed to be personally accountable for their own contributions
399 and to ensure that questions related the accuracy or integrity of any part of the work are
400 appropriately investigated, resolved, and the resolution documented in the literature.

401

402 **ADDITIONAL INFORMATION**

403 **Competing interests**

404 The authors declare no competing financial interests.

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