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Iota-Carrageenan Inhibits Replication of the SARS-CoV-2 Variants of Concern Omicron BA.1, BA.2 and BA.5

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Abstract: Even with its endemic transition, the COVID-19 pandemic remains a public health threat, particularly in the light of emerging variants of concern (VoCs) and the need for pandemic preparedness in the future. In November 2021, the SARS-CoV-2 VoC Omicron emerged and its subvariants BA.1, BA.2 and BA.5 became predominant. Although the protease inhibitor Paxlovid® and the polymerase inhibitors Molnupiravir and Remdesivir were approved as specific antiviral treatment options for COVID-19 patients in the early stages after infection, effective prophylactically acting substances without adverse effects are not available yet. In a recent study, we demonstrated that iota-carrageenan, a sulfated polysaccharide extracted from red seaweed, efficiently inhibits the replication of the SARS-CoV-2 Wuhan Type and the VoCs Alpha, Beta, Gamma and Delta. Now, we extended this study by investigating the antiviral effects of iota-, lambda- and kappa-carrageenans on the VoC Omicron subvariants BA.1, BA.2 and BA.5. Using a VoC Omicron BA.1 spike pseudotyped murine leukemia virus (BA.1 MLV_{OM}VLP) as well as patient-derived SARS-CoV-2 Omicron isolates BA.1, BA.2 and BA.5 (SARS-CoV-$2_{OM\ BA.1}$, SARS-CoV-$2_{OM\ BA.2}$ and SARS-CoV-$2_{OM\ BA.5}$), we demonstrate that iota-carrageenan exhibits similar antiviral activity against all analyzed Omicron subvariants. As with other VoCs shown before, the biologically inert iota-carrageenan was more efficient than kappa- and lambda-carrageenan. Altogether, these results confirm that, independent of the current and potential future variants, the physical barrier provided by iota-carrageenan might be applicable for prophylaxis and early treatment of SARS-CoV-2 infections.

Keywords: COVID-19; SARS-CoV-2; coronavirus; variant of concern; omicron; pseudotyping; iota-carrageenan; kappa-carrageenan; lambda-carrageenan; sulfated polymer; virus variants; variants of concern; carrageenan types; omicron subvariants; BA.1; BA.2; BA.5

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1. Introduction

By now, coronavirus disease 2019 (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has resulted in around 576 million cases and 6.8 million deaths worldwide [1]. An ongoing major problem represents the emergence and spread of SARS-CoV-2 variants, particularly the alleged "Variants of Concern" (VoCs), which have the potential to escape vaccine- or infection-induced antiviral immune responses [2,3].

Since SARS-CoV-2 VoCs mainly carry mutations within the region of the spike glycoprotein, which might alter the interaction with the host receptors ACE-2 and TMPRSS2,

they change the infectivity, transmissibility or pathogenicity of the virus [4–7]. VoCs classified so far include SARS-CoV-2 Alpha [8], Beta [9], Gamma [10] and Delta [11], as well as SARS-CoV-2 Omicron [12]. Meanwhile, Omicron has been subdivided into the subvariants BA.1, BA.2, BA.3, BA.4 and BA.5 [13], with BA.5 still being predominant in most countries. In comparison to other VoCs, Omicron variants encompass a high number of deletions, insertions and mutations, particularly in the spike proteinn [13,14]. The subvariants BA.1, BA.2 and BA.5 have especially been spreading at an unprecedented rate [15–17], mainly caused by a higher transmissibility, viral affinity and antibody escape [14]. Moreover, these Omicron subvariants are highly resistant against clinically approved monoclonal antibodies [18,19]. Therefore, the development of broadly acting prophylactic and therapeutic counteractive measures remains of supreme importance.

In 2022, the first direct acting antiviral small molecule drugs were approved for high risk patients, predominantly for the application at early stages of infection with SARS-CoV-2 [20]. The first one is Paxlovid®, which is composed of Nirmatrelvir, an inhibitor of the 3-Chymotrypsin-like protease of SARS-CoV-2 and Ritonavir, an HIV-1-protease inhibitor [21,22]. Furthermore, Molnupiravir, which inhibits the RNA-dependent RNA-Polymerase of SARS-CoV-2, received approval for high-risk COVID-19 patients in the early stages after infection [23]. In addition to the side effects of these compounds, they are also directed against mutation-prone viral components that potentially might lead to the emergence of drug resistance.

Though numerous vaccines have been approved worldwide [24,25], herd immunity might be difficult to achieve, as it appears that the vaccines do not confer sterile immunity [26]. Therefore, there is still an unmet urgent necessity to develop prophylactical as well as safe therapeutic drugs, which should be widely obtainable and broadly effective against various viral strains of SARS-CoV-2. Taking into account the time- and cost-consuming path for the development of new therapeutics, assessing existing drugs and natural substances for their antiviral activity against the swiftly spreading SARS-CoV-2 VoCs is a fast and encouraging option.

For decades, natural substances have been known for their antiviral potential against a diversity of viruses. Since the outbreak of the SARS-CoV-2 pandemic, several natural substances were examined for their potential antiviral effects against SARS-CoV-2 [27–29]. Among them is carrageenan, a high molecular weight sulfated polymer originated from red seaweed (Rhodophyta) that has been widely used in food, cosmetic and pharmaceutical industry and is commonly acclaimed as safe by the FDA (GRAS). Three main forms of carrageenans are commercially used: iota, kappa and lambda. They vary from each other in the degree of sulfation, solubility and gelling properties [30]. In contrast to oligopeptide sized kappa- and lambda-carrageenan preparations, highly pure, high molecular weight iota-carrageenan (MW $\geq$ 1100 kDa) cannot enter cells and thus is known to be biologically inert in terms of adverse and immunomodulatory effects [31–34].

The antiviral action of iota-carrageenan is well established and has been demonstrated for a diversity of respiratory viruses [35–37] including SARS-CoV-2 [38–40]. Additionally, for lambda-carrageenan an antiviral activity against SARS-CoV-2 was reported [41]. In addition to that, in silico studies revealed several marine sulfated polysaccharides as promising antivirals against SARS-CoV-2. Generally, the potential mode of action is described as entry inhibitors [42–44]. Most importantly, a randomized, placebo-controlled, double-blinded, multicenter clinical study revealed that a nasal spray containing iota-carrageenan shows prophylactic efficacy in counteracting SARS-CoV-2 infection in healthcare workers caring for COVID-19 disease patients with a relative risk reduction of 79.8% [45]. A previous trial exploring a nasal spray comprising Ivermectin and iota-carrageenan revealed reduction in viral loads as well as COVID-19 disease severity [46]. Furthermore, clinical trials on COVID-19 cases running in the UK [47] and in Austria [48,49] investigate the prophylactic and therapeutic effect of iota-carrageenan containing nasal sprays and inhalants.

We have previously shown that iota-carrageenan exhibits antiviral activity against the SARS-CoV-2 Wuhan type and the VoCs Alpha, Beta, Gamma and Delta [38,39]. Here,

we show that carrageenans also exhibit antiviral activity against the most prevalent VoC Omicron subvariants BA.1, BA.2 and BA.5 with IC_{50} values comparable to the other VoCs and the Wuhan type of SARS-CoV-2. As before, this effect was shown in a human lung cell line (Calu-3) infected with SARS-CoV-2_{OM} isolates as well as with MLV_{OM}VLPs for the tested carrageenan types, with iota-carrageenan being the most effective.

2. Materials and Methods

2.1. Inhibitors

Iota- (Gelcarin PH 379), kappa- (Gelcarin PH 911) and lambda-carrageenan (Viscarin PH 109) were purchased from Dupont formerly FMC Biopolymers (both Philadelphia, PA, USA). Carboxymethylcelluslose (CMC) was purchased from Mare Austria GmbH and hydroxypropylmethylcellulose (HPMC) was from Fagron (Fagron BV, Rotterdam, The Netherlands). The dry polymer powders were dissolved in cell culture water (B Braun Melsungen AG, Melsungen, Germany) containing 0.5% NaCl (Merck KGA, Darmstadt, Germany) to a final concentration of 1.2 mg/mL. This stock solution was sterile filtered through a 0.22 µm filter (Sarstedt, Nuembrecht, Germany) and stored at 4 °C until use.

2.2. SARS-CoV-2 BA.1 Omicron Spike Pseudotyped Murine Leukemia Virus (BA.1 MLV_{OM}VLPs)

The pseudoviral particles are replication-deficient MLV pseudotyped with the SARS-CoV-2 Spike protein of the omicron subvariant BA.1 (Accession ID: EPI_ISL_6699757) and the firefly luciferase gene (eEnzyme, Catalog number SCV2-PsV-Omicron, Gaithersburg, MD, USA). This enables measuring the spike-protein-mediated cell entry via luciferase reporter activity.

2.3. Neutralization Test

An amount of 7.500 ACE2-HEK293 cells/well were seeded and cultured overnight. On the second day, BA.1 MLV_{OM}VLPs were incubated with buffer (controls) or the test substances for 30 min before infection. For infection, cell culture medium was removed, and cells were infected with around 55 µL of the BA.1 MLV_{OM}VLPs and spun at 700 rpm for 15 min at 4 °C. After 2 h at 37 °C, 50 µL DMEM containing 10% FCS was added to each well. After 42 h at 37 °C, plates were lysed by the freeze/thaw process before luciferase reagent (Bright Glow, Promega, Madison, WI, USA) was added to cells to measure the luciferase activity using a BMG Fluostar Microplate reader. Mock-infected cells and infected, mock-treated (0.5% NaCl) cells served as positive and negative controls. Cell toxicity was routinely checked by determining the metabolic activity of a parallel plate with an identical setup using Alamar blue.

2.4. Viruses

To obtain a clinical SARS-CoV-$2_{OM\ BA.1}$ isolate, 100 µL of an anonymized residual swap sample of a patient infected with the SARS-CoV-2 Omicron BA.1 subvariant was passaged on a confluent monolayer of Caco-2 cells. The integrity of the viral genome and the presence of mutations characteristic for the Omicron variant were confirmed by mutation-specific qRT-PCR (Novaplex™ SARS-CoV-2 Variants VII Assay, Seegene, Düsseldorf, Germany) and Illumina-based next generation sequencing using a MiSeq reagent kit v2 on a MiSeq™ instrument (Illumina, San Diego, CA, USA). Sequences were analyzed with CLC Genomics Workbench 21 (Qiagen Aarhus A/S, Aarhus, Denmark). The patient sample had, in addition to the usual Omicron mutations, an R346K mutation in the spike protein and an I4615V mutation in ORF1ab. Viral titers were determined by an endpoint titration assay.

The clinical SARS-CoV-$2_{OM\ BA.2\ and\ BA.5}$ isolates were isolated from throat swabs collected at the Institute for Medical Virology and Epidemiology of Viral Diseases, University Hospital Tübingen, from PCR-positive patients, essentially as described previously [39].

In brief, throat swab material was centrifuged to remove any cellular debris and ~100 µL was diluted in 2 mL medium to directly inoculate 150,000 Caco-2 cells in six-well plates. At 48–72 h post-infection, supernatants were collected, centrifuged, sterile filtered

and stored at −80 °C. After two passages, RNA from the supernatant was prepared, and next generation sequencing (NGS) confirmed the authenticity of VoCs Omicron BA.2 and BA.5. Viral titers were determined by an endpoint titration assay.

To generate stocks, virus-containing cell culture supernatants were harvested at 72 h post-infection (hpi), sterile filtered and stored at −80 °C until further use.

2.5. Infection Experiments

For infection experiments, cells were inoculated with SARS-CoV-$2_{\text{OM BA.1, BA.2 and BA.5}}$ (multiplicity of infection (MOI): 2×10^{-2}) for 1 h, washed and further treated with interventions. At 72 hpi, virus-containing cell culture supernatants were incubated for 10 min at 95 °C and finally used for a qRT-PCR analysis. For titer determination of the SARS-CoV-$2_{\text{OM BA.1, BA.2 and BA.5}}$ virus stock, A549-ACE2/TMPRSS2 and Calu-3 cells were infected with serial dilutions of the virus stock over 72 h. Afterwards, cells were fixed (4% PFA), permeabilized (0.5 % Triton/PBS), blocked (1% BSA/PBS-T) and finally stained with a SARS-CoV-2 NP antibody (Biozol, Eching, Germany). The endpoint of virus infection was analyzed via fluorescence microscopy and a viral titer was calculated by the method of Reed and Muench [50].

2.6. Cell Culture

Calu-3 cells were maintained in Minimal Essential Medium (MEM) containing 20% (*v/v*) inactivated FCS, 1 mM l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 1 mM sodium pyruvate. A549-ACE2/TMPRSS2 cells were maintained in RPMI 1640 medium containing 10% (*v/v*) inactivated fetal calf serum (FCS), 1 mM l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.075% sodium bicarbonate, 1 µg/mL puromycin and 1 µg/mL blasticidin. A549 cells expressing ACE2 and TMPRSS2 were generated by retroviral transduction as described in [27] and cultivated in RPMI 1640 medium containing 10% (*v/v*) inactivated fetal calf serum (FCS), 2 mM l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 100 µg/mL blastomycin.

2.7. Determination of the Amount of Viral RNA Copies from Released Viruses by Quantitive Real-Time PCR (qRT-PCR)

The amount of viral RNA copies in the virus-containing samples was quantified by a Luna Universal Probe One-Step RT-PCR Kit from New England Biolabs (Cat: E3006L, Ipswich, MA, USA). This kit allows reverse transcription, cDNA synthesis and PCR amplification in a single step. Samples were analyzed by 7500 software v2.3 (Applied Biosystems, Waltham, MA, USA). PCR primers were designed and used as described previously in [51]. Thereby, the polynucleotide sequence contains parts of the SARS-CoV-2 Envelope (E) and RNA-dependent RNA-polymerase (RdRp) genes and was used as standard for the determination of viral RNA copies in the experiments. The sequences of the used primers were RdRp_forward (fwd): 5′-GTG-ARA-TGG-TCA-TGT-GTG-GCG-G-3′ and RdRp_reverse (rev): 5′-CAR-ATG-TTA-AAS-ACA-CTA-TTA-GCA-TA-C-3′. The probe was 5′-CAG-GTG-GAA-/ZEN/CCT-CAT-CAG-GAG-ATG-C-3′ (label: FAM/IBFQ Iowa Black FQ). A dsDNA polynucleotide sequence (Integrated DNA Technologies, Coralville, IA, USA) was used as a positive control: 5′-TAA-TAC-GAC-TCA-CTA-TAG-GGT-ATT-GAG-TGA-AAT-GGT-CAT-GTG-TGG-CGG-TTC-ACT-ATA-TGT-TAA-ACC-AGG-TGG-AAC-CTC-ATC-AGG-AGA-TGC-CAC-AAC-TGC-TTA-TGC-TAA-TAG-TGT-TTT-TAA-CAT-TTG-GAA-GAG-ACA-GGT-ACG-TTA-ATA-GTT-AAT-AGC-GTA-CTT-CTT-TTT-CTT-GCT-TTC-GTG-GTA-TTC-TTG-CTA-GTT-ACA-CTA-GCC-ATC-CTT-ACT-GCG-CTT-CGA-TTG-TGT-GCG-TAC-TGC-TGC-AAT-ATT-GTT-3′. By generating a series of dilutions (10^4, 10^5, 10^6 and 10^7 copies/mL) of this standard, the experiments were quantified using a standard curve to obtain absolute values of RNA copies in the sample.

2.8. One-Dimensional and Two-Dimensional ^{1}H-NMR Analysis of Iota-, Kappa- and Lambda-Carrageenan

Samples of 10 mg of iota-, kappa- and lambda-carrageenan were sent to Spectral Services, Köln, for NMR measurements. In brief, 10 mg of substance was dissolved in 1 mL D_2O containing 3-(Trimethylsilyl) propionic acid-d4 sodium salt (0.01% as standard). Measurements of ^{1}H spectra were performed with an Avance III HD 500 MHz NMR spectrometer (Bruker, Billarica, MA, USA).

2.9. Size Exclusion Chromatography (SEC) of the Carrageenans

The molecular weight distribution of iota-carrageenan was determined by means of size exclusion chromatography (SEC) on an Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a TSKgel GMPWXL column (7.8 × 300 mm, 100–1000 Å, particle size 13 µm; Tosoh Bioscience, Griesheim, Germany) and a Shodex RI-101 refraction index detector (Thermo Scientific Dionex, Gemering, Germany). The column oven temperature was set to 30 °C and isocratic elution was performed at 0.6 mL/min using 100 mM $LiNO_3$. $LiNO_3$ was used as small monovalent cations, especially Li^+, which are reported to have non-gelling properties and form ordered structures with iota- and kappa-carrageenan, but no further aggregates [52–55]. An amount of 12 µL of a 1.2 mg/mL solution of carrageenan, i.e., 14.4 µg, was injected. The molecular weight distribution of the carrageenan samples was compared to dextran standards (1 mg/mL) with varying molecular weights (25–1000 kDa). Kappa- and lambda-carrageenan were analyzed analogously.

2.10. Field Flow Fractionation and Molecular Weight Determination of Iota-Carrageenan via Refractive Index (RI) and Light Scattering (LS)

Untreated, full-length iota-carrageenan was analyzed by Postnova Analytics GmbH (Landsberg am Lech, Germany) to determine the typical molecular weight distribution of iota-carrageenan dissolved in 0.5 % NaCl. A field flow fractionation (FFF) method for size fractionation was combined with a molecular weight determination via refractive index (RI) and light scattering (LS) using an FFF separation system AF2000 MT (Postnova Analytics GmbH, Landsberg am Lech, Germany), an RI detector PN 3140 or PN3150 and an LS detector PN3070, MALS (all Postnova Analytics GmbH, Landsberg am Lech, Germany). The solvent was 0.1 M $NaNO_3$ and 0.2 g/L NaN_3. A spacer of 350 µM and a NovaPES membrane (Postnova Analytics GmbH, Landsberg am Lech, Germany) with a 10 kDa cut-off were used. The injected volume of 100 µL was measured with the detector flow set at 1.0 mL/min. The molar mass was calculated based on the literature value for the refractive index increment of 0.148 mL/g.

2.11. Software and Statistics

Microsoft Word and Excel were used. GraphPad Prism 9.0 was used for statistical analyses and to generate graphs. Figures were generated with CorelDrawX7. The software 7500, v2.3, was used to evaluate the results obtained by qRT-PCR.

3. Results

3.1. Comparison of Iota-Carrageenan with Other Sulfated and Non-Sulfated Polymers in Their Antiviral Activity against SARS-CoV-2 Omicron BA.1

It was previously shown that iota-carrageenan exhibits antiviral activity against the SARS-CoV-2 Wuhan type and the VoCs Alpha, Beta, Gamma and Delta with a similar efficacy [38,39,41]. In addition to iota-carrageenan, the antiviral effects of lambda- and kappa-carrageenan was also analyzed, which differ from iota-carrageenan in their location and number of sulfate moieties on the hexose scaffold skeleton as well as in the polymerization grade and thus the molecular weight (iota-carrageenan > 1000 kDa, kappa- and lambda-carrageenan < 1000 kDa) [56,57]. As carrageenan homopolymers normally cannot be isolated in nature, the available preparations usually contain also

other subtypes of carrageenans. For this study, carrageenans were characterized by ^{1}H-NMR spectroscopy [38]. Thereby, we found relevant amounts of iota-carrageenan to be present in the lambda and kappa preparations (27.3 and 16.0%, respectively) [38]. Quality control by size exclusion chromatography, field flow fractionation and ^{1}H-NMR spectroscopy revealed that the iota-carrageenan used in this study was highly pure and homogenous, with at least 96% content (Supplementary Figures S1 and S2). Moreover, it has an average molecular weight of >1100 kDa, as shown by gel permeation HPLC and dynamic light scattering (Supplementary Figure S3). Different molecular structures between kappa- and iota-carrageenan were furthermore revealed by 2D ^{1}H NMR spectroscopy (Supplementary Figure S4). Thus, it was concluded that the iota-carrageenan preparation used in our experimental setup was homogeneous and as pure as possible by the current standard procedures.

We first wanted to determine whether iota-carrageenan and the other carrageenan types (kappa and lambda) interfere with the infection of cells with BA.1 MLV_{OM}VLPs. Therefore, ACE2-HEK293 cells were infected with BA.1 MLV_{OM}VLPs and spike driven infection was measured as described previously [38]. Carboxymethylcellulose (CMC) and hydroxypropylmethylcellulose (HPMC) were used as a control. These polymers contain no sulfate groups and, as shown previously, have no antiviral activity against SARS-CoV-2 [38].

Iota-carrageenan inhibited infection of ACE2-HEK293 cells with BA.1 MLV_{OM}VLPs with an IC_{50} of 2.67 µg/mL (1.83–3.69) (Figure 1) which was in a similar range to the IC_{50} values of the previously published SARS-CoV-2 Wuhan type and the VoCs Alpha, Beta, Gamma and Delta [39]. Similar to previous studies on VoCs, kappa- and lambda-carrageenan also showed some antiviral activity, albeit approximately 50-fold lower when compared to iota-carrageenan (Figure 1). CMC and HPMC showed no antiviral activity (Figure 1).

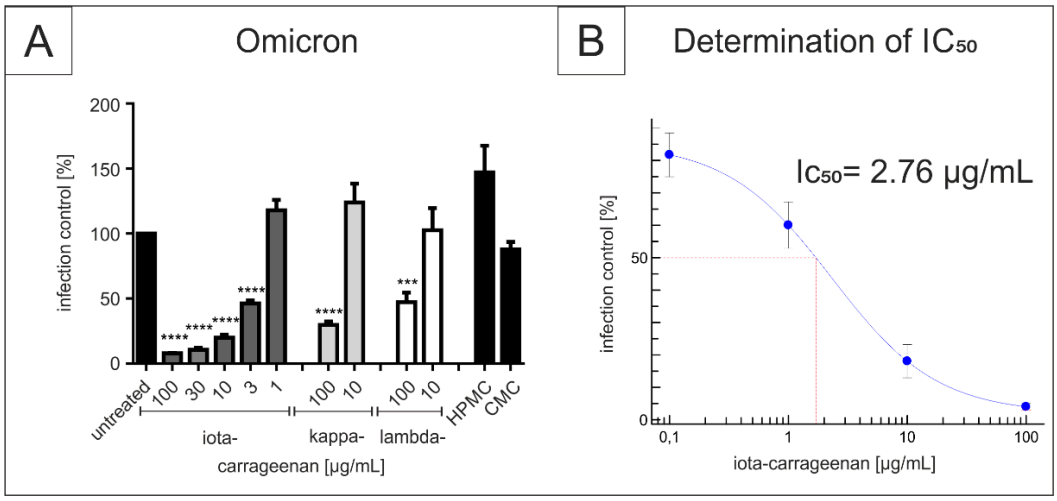

Figure 1. Effect of iota-, kappa- and lambda-carrageenan on SARS-CoV-2_{OM} BA.1 spike driven entry. (**A**) Luciferase activity following infection of ACE2-HEK293 cells with BA.1 MLV_{OM}VLPs. Untreated infected cells served as a positive control (100% infection control; y-axis). Amounts of 100, 30, 10, 3 and 1 µg/mL iota-carrageenan, 100 and 10 µg/mL kappa- and lambda-carrageenan and 100 µg/mL non-sulfated polymers (hydroxypropylmethylcellulose (HPMC) and carboxymethylcellulose (CMC)) were incubated with the MLV_{OM}VLPs for 30 min before infection. The efficiency of infection was determined in cell lysates by measuring the luciferase activity 48 h post-infection. (**B**) Determination of IC_{50} values using Excel XLfit 5.5.0.5. The data represent means of quadruplicates +/− standard deviation (*** $p < 0.001$, **** $p < 0.0001$) using a one sample *t* test, where each value is compared to the untreated control. Abbreviations: iota-carrageenan, kappa-carrageenan, lambda-carrageenan, CMC and HPMC.

3.2. Iota-Carrageenan Inhibits Replication of SARS-CoV-2$_{OM\ BA.1,\ BA.2\ and\ BA.5}$ in Calu-3 Human Lung Cells

With the aim of examining whether the results achieved with the BA.1 MLV$_{OM}$VLP system also apply to replication competent SARS-CoV-2, Calu-3 human lung cells, symbolizing the bona fide surrogate lung cell infection model expressing ACE2 and TMPRSS2 endogenously [58], were infected with the BA.1, BA.2 or BA.5 subvariants of the VoC SARS-CoV-2$_{OM}$ (Figure 2). One hour post-infection (hpi), the viral inoculum was removed and different concentrations of iota-carrageenan were added to the cells. Three days post-infection (dpi), cell culture supernatants were harvested and virus production was analyzed by quantitative RT-PCR (Figure 2).

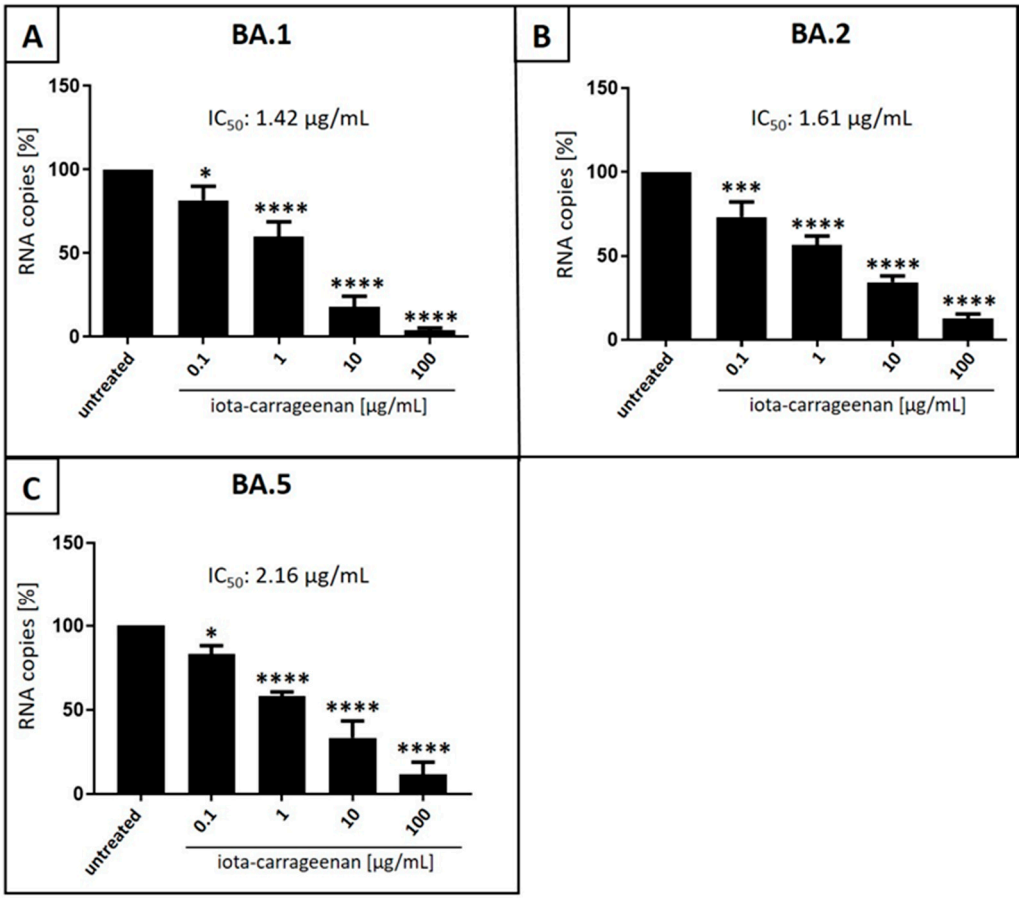

Figure 2. Iota-carrageenan inhibits replication of SARS-CoV-2$_{OM}$ BA.1 (**A**), BA.2 (**B**) and BA.5 (**C**). Calu-3 cells were infected with SARS-CoV-2$_{OM}$ BA.1, BA.2 or BA.5 at a MOI of 2×10^{-2}. One hpi and post-removal of input virus, cells were treated with indicated concentrations of iota-carrageenan. Cell culture supernatants were harvested at 3 dpi. The virions were purified and analyzed by qRT-PCR. Data represent means of three independent experiments ± standard deviation. A statistical analysis was performed using a multiple comparison Kruskal–Wallis test (Anova) followed by a Dunn's post hoc test (* $p < 0.02$; *** $p < 0.0003$; **** $p < 0.0001$ versus the untreated control).

As with the BA.1 MLV$_{OM}$VLP system, treatment with iota-carrageenan led to a strong reduction in virus replication, with an IC$_{50}$ of 1.42 µg/mL (confidence interval (CI): 0.14–2.7) following infection with SARS-CoV-2$_{OM\ BA.1}$ (Figure 2A). In addition, also following infection with SARS-CoV-2$_{OM\ BA.2\ or\ BA.5}$ and subsequent treatment with iota-carrageenan, virus replication was reduced with an IC$_{50}$ of 1.61 µg/mL (CI: 0.18–3.04) for BA.2 and 2.16 µg/mL (CI: 1.2–3.12) for BA.5 (Figure 2B,C). Thereby, the reduction was in a similar range as previously reported for SARS-CoV-2$_{PR-1}$ and the respective VoCs Alpha, Beta, Gamma and Delta [39].

Next, we wanted to confirm the data obtained by the BA.1 MLV_{OM}VLP system (Figure 1) regarding the side-by-side comparison of the antiviral activity of iota-, kappa- and lambda-carrageenan in a replication competent SARS-CoV-2 system. Therefore, Calu-3 cells were infected for one hour with the BA.1, BA.2 or BA.5 subvariants of the VoC SARS-CoV-2_{OM} and following treatment with the different carrageenans for three days, cell culture supernatants were harvested and virus production was analyzed by quantitative RT-PCR (Figure 3).

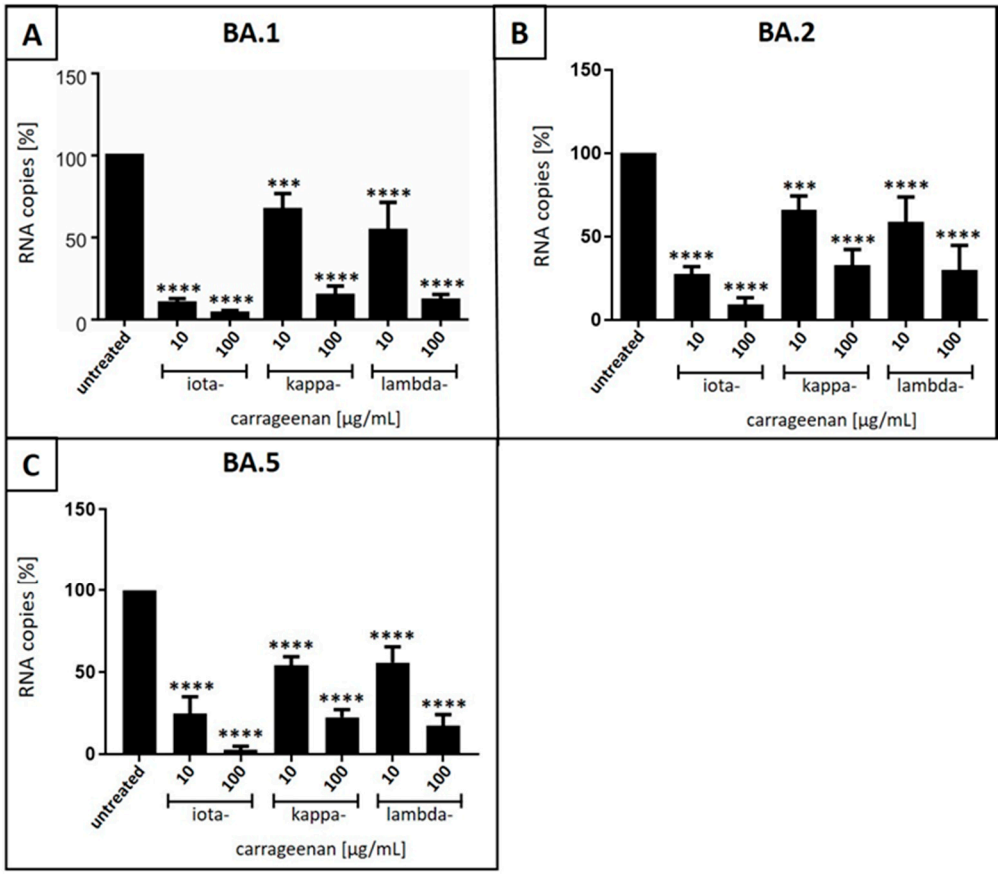

Figure 3. Comparison of the influence of iota-, kappa- and lambda-carrageenan on the replication of SARS-CoV-2_{OM} BA.1, BA.2 and BA.5. Calu-3 cells were infected with SARS-CoV-$2_{OM\ BA.1,\ BA.2\ or\ BA.5}$ at an MOI of 2×10^{-2}. One hpi, input virus was removed and cells were treated with the indicated concentrations of iota-, kappa- and lambda-carrageenan. Cell culture supernatants were harvested at 3 dpi. The virions were purified and analyzed by qRT-PCR. Data represent means of three ((**A**) + (**C**)) or four (**B**) independent experiments ± standard deviation. A statistical analysis was performed using a multiple comparison Kruskal–Wallis test (Anova) followed by a Dunn´s post hoc test (*** $p < 0.0009$; **** $p < 0.0001$ versus the untreated control).

Thereby, all used carrageenan types inhibit the replication of SARS-CoV-2_{OM} BA.1, BA.2 and BA.5 (Figure 3). However, iota-carrageenan was the most effective, with IC_{50} values of ~1 log-stage lower than that of kappa- and lambda-carrageenan (Figure 3A–C).

In summary, there are no differences in the antiviral effect of iota-carrageenan against SARS-CoV-$2_{OM\ BA.1,\ BA.2\ and\ BA.5}$ when compared to the other VoCs, further supporting the molecular mechanism by which carrageenans prevent virus entry by forming an extracellular physical barrier.

4. Discussion

Despite the fact that the COVID-19 pandemic has been phased out worldwide, infections with SARS-CoV-2 remain an important health and socioeconomic issue. Furthermore,

it is legitimate to assume that future coronaviruses, like previous SARS-CoV and MERS-CoV, could emerge via zoonotic transmission, potentially causing pandemic threats. This necessitates the development of novel broadly acting antiviral drugs to be part of the general pandemic preparedness. Thus, there is an ongoing vast need for the elaboration of new therapeutics that are broadly active, safe, cost-effective, biologically inert, chemically stable and thus easily distributable for a worldwide range of patients. Ideally, such a substance should work as a prophylactic or early therapeutic treatment by preventing infection or progression of infection with SARS-CoV-2, even before the virus reaches the target tissue cells, e.g., lung epithelial cells.

Due to their unspecific mode of action, most natural antivirals exhibit a broader activity spectrum than highly specific monoclonal antibodies or direct acting small molecule inhibitors [59]. For that reason, certain natural substances have proved effective against various viruses, including SARS-CoV, MERS-CoV and SARS-CoV-2 [27–29,60].

In this study, we demonstrate that the natural substance iota-carrageenan exerts antiviral activity with comparable efficacy not only against the originally emerged SARS-CoV-2 Wuhan type and the VoCs Alpha, Beta, Gamma and Delta, as shown previously [38–40], but also against various SARS-CoV-2 VoC Omicron subvariants. In addition, the antiviral effect of iota-carrageenan was previously shown in vitro for several other viruses, e.g., influenza A virus, human rhinovirus, endemic human coronaviruses and herpes simplex virus 2 [35,36,61,62].

Nasal sprays, throat sprays and lozenges containing iota-carrageenan have been approved as common cold preventions and treatment and have been sold in more than 30 countries on three continents [63] (for details, see Supplementary Figure S5). The clinical effectiveness of iota-carrageenan against respiratory cold viruses has been proven by an independent meta-analysis [64] and has been shown in several clinical trials [65–70]. A recent clinical study revealed that a nasal spray containing iota-carrageenan provided significant protection as a COVID-19 prophylaxis in health care workers caring for patients with COVID-19 [45]. Moreover, the German and the Austrian Society of Hospital Hygiene recommend the use of iota-carrageenan for the prevention of COVID-19 [71,72].

The EFSA [73] and the FDA [74] approved iota-carrageenan as food safe for a quantum satis of 75 mg/kg b.w. per day, i.e., 4500 mg for a 60 kg person, while with the nasal spray, only 2.3 mg/day is applied [73]. Furthermore, it was shown that high-molecular weight iota-carrageenan exhibits no immunotoxicity and no local intolerance or toxicity upon intranasal application or inhalation [31]. In agreement with this, we previously demonstrated that iota-carrageenan has no toxic effect on Calu-3 and Vero B4 cells when treated with different concentrations of iota-carrageenan up to 100 µg/mL [39]. Most importantly, iota-carrageenan has been investigated in at least six clinical trials without any safety issues [45,46,66,70,74]. Hence, the side effects caused by iota-carrageenan are comparable to the application of saline solutions. Moreover, iota-, kappa- and lambda-carrageenan were intensively analyzed in numerous animal studies (for a review, see [75]). Thereby, it was administered by oral, dermal and inhalation routes in orders of magnitude above the concentration range in which iota-carrageenan is present in commercially available nasal and throat sprays or lozenges [75]. Histopathological as well as immunological and clinical chemistry investigations revealed that carrageenan had no adverse effects on organs and the general health of animals [75]. Even a lifelong administration of carrageenan to rodents and primates in high doses showed no pathologic effects in either species [75]. However, there are some studies showing that smaller, oligomeric fractions of kappa- and lambda-carrageenans can exhibit both anticancer and immunostimulatory effects [32–34].

To investigate whether different types of carrageenan have different antiviral properties, we compared iota-, kappa- and lambda-carrageenan. Although all carrageenan types showed antiviral effects against the subtypes BA.1, BA.2 and BA.5 of SARS-CoV-2_{OM}, iota-carrageenan clearly led to the strongest reduction, with IC_{50} values that are ~1 log-stage lower than those for kappa- or lambda-carrageenan. These results are in agreement with our previous work, where it was shown that iota-carrageenan exhibits the most po-

tent inhibition of the replication of SARS-CoV-2 Wuhan type and the VoCs Alpha, Beta, Gamma and Delta when compared to kappa- or lambda-carrageenan [39]. Moreover, it has been demonstrated that iota-carrageenan exhibits a superior antiviral activity against human rhinoviruses and Hepatitis A viruses when compared to kappa- and lambda-carrageenan [35,38,76]. Additionally, given the relatively high amount of iota-carrageenan in the kappa and lambda preparations, part of their observed antiviral activity might be attributed to the presence of iota-carrageenan [38].

It has been shown previously that the effectiveness of iota-carrageenan is not based on chemical, i.e., virucidal, activity [40]. Mechanistically, it is generally thought that the antiviral activity of iota-carrageenan is based on its ability to build a viscous barrier at the nasal mucosa, where inhaled virus particles become trapped when they first enter the nasal cavity. In the second step, newly synthesized virus particles that are released from infected cells are trapped as well. Finally, iota-carrageenan and trapped viruses are removed spontaneously from the nasopharynx by mucociliary clearance [77]. This results in a significant reduction in the viral load in the nasal cavity and hence a reduction in the duration of disease [66,68–70]. The underlying mechanism of the entrapment remains unknown. However, given the broad activity of iota-carrageenan against a variety of fundamentally different viruses, the trapping process is independent of specific cellular or viral receptors and thus unspecific and purely physical.

As iota-carrageenan non-specifically traps viruses, thereby preventing the interaction between virus and cellular surfaces, development of resistance due to the occurrence of escape mutants is unlikely. Meanwhile, there are also efforts to artificially imitate the effect of nasal sprays containing carrageenan [78]. Inhalation of a bioadhesive hydrogel is supposed to form a physical barrier on the mucosa of the nasopharynx and thus restrict SARS-CoV-2 from infecting host cells [78]. However, such chemical substances must undergo the standard preclinical and clinical development, a year-long and cost-intensive process, before the first in-person studies can be conducted.

The nonexistence of any adverse pharmacological activity and its lack of absorption or metabolism characterizes carrageenan as a safe and biologically inert antiviral that can be applied topically, e.g., as lozenges or a nasal/throat spray. The unspecific entrapment of virions mediated by iota-carrageenan supports its prophylactical and early therapeutic application against SARS-CoV-2, regardless of newly emerging variants.

In conclusion, our data strongly suggest that iota-carrageenan exhibits a broad and comparable antiviral activity against all VoCs, including the various Omicron subvariants. Thus, clinically approved nose/throat sprays and lozenges currently sold in more than 30 countries over the counter can be used as a first line defense without any concern about the nature of the infecting VoC (Supplementary Figure S5).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nutraceuticals3030025/s1, Figure S1: Identification and purity analysis of iota-, lambda and kappa-carrageenan by ^{1}H NR spectroscopy, Figure S2: Molecular weight (MW) distribution of iota-carraageenan using size exclusion chromatography (SEC), Figure S3: Molecular weight (MW) determination of iota-carrageenan by gel permeation HPLC and dynamic light scattering, Figure S4: Identification and purity analysis of iota-carrageenan by 2D ^{1}H NMR spectroscopy. Figure S5: World-wide approved iota-carrageenan containing products (nasal sprays, throat sprays and lozenges).

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