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In vitro inhibitory effect of carrageenan oligosaccharide on influenza A H1N1 virus

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ABSTRACT

Carrageenan polysaccharide has been reported to be able to inhibit the infection and replication of many different kinds of viruses. Here, we demonstrated that a 2 kDa κ-carrageenan oligosaccharide (CO-1) derived from the carrageenan polysaccharide, effectively inhibited influenza A (H1N1) virus replication in MDCK cells (selectivity index >25.0). Moreover, the 2 kDa CO-1 inhibited influenza A virus (IAV) replication better than that of 3 kDa and 5 kDa κ-carrageenan oligosaccharides (CO-2 and CO-3). IAV multiplication was suppressed by carrageenan oligosaccharide treatment in a dose-dependent manner. Carrageenan oligosaccharide CO-1 did not bind to the cell surface of MDCK cells but inactivated virus particles after pretreatment. Different to the actions of carrageenan polysaccharide, CO-1 could enter into MDCK cells and did not interfere with IAV adsorption. CO-1 also inhibited IAV mRNA and protein expression after its internalization into cells. Moreover, carrageenan oligosaccharide CO-1 had an antiviral effect on IAV replication subsequent to viral internalization but prior to virus release in one replication cycle. Therefore, inhibition of IAV intracellular replication by carrageenan oligosaccharide might be an alternative approach for anti-influenza A virus therapy.

1. Introduction

Influenza A virus (IAV) is a most formidable pathogen, which has been the cause of at least three pandemics in the last century. The most severe IAV pandemic caused more than 40 million deaths in the world during 1918–1919 (Lamb and Takeda, 2001; Yewdell and Garcia-Sastre, 2002). In late April 2009, a novel influenza A (H1N1) virus caused a pandemic in a short period of time, which attracted great attention all over the world. Current anti-IAV drugs are directed against the M2 protein (adamantanes) and neuraminidase (NA; zanamivir and oseltamivir) (Hay et al., 1985; Mendel et al., 1998). Despite these successes, the drug efficacy, resistance, toxicity, and cost remained to be unresolved issues (Hayden and Pavia, 2006). Recently, several other approaches including inhibitors of viral RNA transcription, small interfering RNA, inhibitors of virus-cell fusion or proteolytic processing of HA have been developed, but so far none of inhibitors have been developed into a drug (Lagoja and De Clercq, 2008). Hence, the development of novel antiviral agents that could be used alone or in combination with existing antiviral drugs is of high importance.

Carrageenan polysaccharide, extracted from red algae, shows different inhibitory effects on different viruses (Buck et al., 2006; Carlucci et al., 2004; Grassauer et al., 2008; Pujol et al., 2006; Talarico and Damonte, 2007). Previous studies have shown that carrageenan inhibits the replication of some enveloped viruses by interfering with virus adsorption and internalization into host cells (Buck et al., 2006; Grassauer et al., 2008; Talarico and Damonte, 2007). However, other reports have shown that carrageenan does not inhibit virus adsorption but inhibits some steps of virus life cycle in host cells (Gonzalez et al., 1987; Pujol et al., 2006). Recently, Leibbrandt et al. reported that ι-carrageenan polysaccharide could inhibit influenza A virus infection by directly binding to the virus particles (Leibbrandt et al., 2010), while Talarico et al. found that carrageenan could inhibit dengue virus infection in mosquito cells by targeting cellular proteins (Talarico et al., 2011). The inhibitory mechanism of carrageenans on virus replication seems to be dependent on the type of polysaccharide (Damonte et al., 2004) as well as the serotype of the virus and the host cells (Girond et al., 1991). Despite having good inhibitory effects on virus replication, the high molecular weight (MW) associated poor tissue-penetrating ability of sulfated polysaccharides limits their potential antiviral application in humans.

In recent years, marine oligosaccharides are attracting increasing interests in developing potential anti-viral drugs (Ji et al., 2011). Yamada et al. reported that *O*-acylated carrageenan oligosaccharides with different MW had increased anti-HIV activities after depolymerization and sulfation (Yamada et al., 1997, 2000). Ekblad et al. found that heparan sulfate (HS) mimetic PI-88, a sulfomannan oligosaccharide of low MW, efficiently reduced the

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cell-to-cell spread of herpes simplex virus (HSV) (Ekblad et al., 2010; Nyberg et al., 2004). The low-viscosity and high-solvency of marine oligosaccharides at neutral pH also suggest their potential use as anti-viral drugs. However, the molecular mechanisms underlying the anti-viral effects of oligosaccharides have not been systematically pursued.

In the present study, we investigated the capacity and molecular mechanisms of κ-carrageenan derived oligosaccharides in inhibiting IAV infection. Our results indicated that carrageenan oligosaccharide with low molecular weight (~2 kDa) could effectively inhibit IAV replication in vitro. IAV multiplication was suppressed by carrageenan oligosaccharide treatment in a dose-dependent manner. Carrageenan oligosaccharide CO-1 did not bind to the cell surface of MDCK cells but inactivated virus particles after pretreatment. CO-1 could enter into MDCK cells and did not interfere with IAV adsorption directly. Moreover, carrageenan oligosaccharide also inhibited IAV mRNA and protein expression after its internalization into cells.

2. Materials and methods

2.1. Compounds and reagents

The κ-carrageenan-derived oligosaccharides named CO-1, CO-2, CO-3 and FITC-labeled oligosaccharide (CO-1-FITC) were provided by Glycoscience and Glycoengineering Laboratory, school of Medicine and Pharmacy, Ocean university of China. Mouse anti-influenza A virus nucleoprotein (NP) and anti-β-actin monoclonal antibodies were purchased from Santa Cruz Biotechnology (USA). Rabbit anti-influenza A virus neuraminase (NA) polyclonal antibody was purchased from Abcam (Hong Kong, China). FITC-labeled goat anti-mouse and goat anti-rabbit secondary antibodies were obtained from Boster (China). Alkaline phosphatase (AP)-labeled goat anti-mouse secondary antibody was purchased from Cell Signaling Technology (USA). Ribavirin injection (50 mg/mL) was obtained from LuKang Cisen (China).

2.2. Cell culture and virus infection

Madin-Darby canine kidney (MDCK) cells were grown in RPM1640 medium supplemented with 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin. Human lung epithelial cells (A549 cells) were cultivated in Ham's F-12 medium containing 10% FBS and 2 mM L-glutamine. Influenza virus (A/Puerto Rico/8/34 [H1N1]; PR8) was propagated in 10-day-old embryonated eggs for three days at 36.5 °C. For virus infection, virus propagation solution was diluted in PBS containing 0.2% bovine serum albumin and was added to cells at the indicated multiplicity of infection (MOI). Virus was allowed to adsorb 60 min at 4 °C. After removing the virus inoculum, cells were maintained in infecting media (RPMI1640, 4 μg/mL trypsin) at 37 °C in 5% CO_2.

2.3. Cytotoxicity assays

The cytotoxicity of compounds was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich, USA) assay. Confluent MDCK cell cultures in 96-well plates were exposed to different concentrations of compounds in triplicate, using incubation conditions equivalent to those used in the antiviral assays. Next, 10 μL of PBS containing MTT (final concentration: 0.5 mg/mL) was added to each well. After 4 h incubation at 37 °C, the supernatant was removed and 200 μL of DMSO was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance values were measured in a microplate reader (Bio-Rad, USA) at 570 nm. The CC_{50} was calculated as the compound concentration necessary to reduce cell viability by 50%.

2.4. Infectivity antiviral assays

For the determination of antiviral activity, a virus yield reduction assay was performed, as previously described (Basu et al., 2009; Leibbrandt et al., 2010). MDCK cell monolayers were firstly incubated with IAV at an MOI of 1.0 for 1 h at 4 °C, then after removed the virus, the infecting media containing different drugs were added to cells and incubated at 37 °C for 48 h. Then the 50% tissue culture infectious dose ($TCID_{50}$) was determined in MDCK cells with 10-fold serially diluted viruses incubated at 37 °C for 72 h. Virus titers in 50% tissue culture infectious doses ($TCID_{50}$)/ml were determined according to Reed and Muench (1938).

Furthermore, the antiviral activity was also evaluated by the CPE inhibition assay as described previously (Hung et al., 2009). MDCK or A549 cells in 96-well plates were firstly infected with IAV (MOI = 1.0), and then treated with different compounds in triplicate after removal of the virus inoculum. After 48 h incubation, the cells were fixed with 4% formaldehyde for 20 min at room temperature. After removal of the formaldehyde, the cells were stained with 0.1% crystal violet for 30 min. The plates were then washed and dried followed by solubilization of the dye with methanol, and the intensity of crystal violet staining for each well was measured at 570 nm. The virus inhibition (%) was calculated by the equation: Virus inhibition (%) = $[(A_{\text{sample } 570} - A_{\text{virus } 570})/(A_{\text{mock } 570} - A_{\text{virus } 570})] \times 100$; Where, $A_{\text{mock } 570}$ was the absorbance without virus infection, $A_{\text{sample } 570}$ was absorbance with virus infection and drug treatment, $A_{\text{virus } 570}$ was absorbance with virus infection but without drugs.

2.5. Influence of time of treatment on antiviral activity

Time course analysis was performed as previously reported (Buck et al., 2006; Talarico and Damonte, 2007). The MDCK cells were incubated with IAV (MOI = 3.0) for 1 h at 4 °C, washed to remove unbound virus, and then added with infection media. Various doses of carrageenan oligosaccharide CO-1 or ribavirin were added to the cultures at the indicated time points (0, 1, 2, 4, 8 h post-infection [p.i.]), respectively. For the 0 h time point, compounds were added to cultures simultaneously with the addition of infection media (after adsorption). Carrageenan oligosaccharide was also added separately to the cultures at 1, 2, 4, 6, and 8 h p.i. At 24 h p.i., the antiviral activity was evaluated by the CPE inhibition assay, as described above.

2.6. Hemagglutination (HA) assay

The hemagglutination (HA) assay was performed as previously reported (Wolkerstorfer et al., 2009). Standardized chicken red blood cell (cRBC) solutions were prepared according to the WHO manual 2002 (WHO, 2002). Virus propagation solutions (10^5 PFU/ml) were serially diluted 2-fold in round bottomed 96-well plate and 1% cRBCs were then added at an equal volume. After 60 min incubation at 4 °C, RBCs in negative wells sedimented and formed red buttons, whereas positive wells had an opaque appearance with no sedimentation. HA titers are given as hemagglutination units/50 μL (HAU/50 μL).

2.7. Flow cytometry analysis and confocal imaging

Flow cytometry analysis was performed as previously described (Miao et al., 2004) with some modifications. MDCK cells (5×10^5) were harvested by trypsination and resuspended in 1 mL PBS.