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In vitro inhibition of influenza A virus infection by marine microalga-derived sulfated polysaccharide p-KG03

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ABSTRACT

The sulfated polysaccharide, p-KG03, purified from the marine microalga, *Gyrodinium impudium*, is a unique compound comprising homogenous galactose units conjugated to uronic acid and sulfated groups. Although previous studies showed that p-KG03 suppresses tumor cell growth and infection by encephalomyocarditis virus, its effect against enveloped virus infection and the biological mechanism of action have not been elucidated. In this report, the inhibitory activity of p-KG03 against influenza virus was examined and compared with that of other sulfated polysaccharides (fucoidan and pentosan polysulfate) and antiviral agents (oseltamivir phosphate, oseltamivir carboxylate, amantadine, and ribavirin). The results of a cytopathic effect reduction assay using MDCK cells demonstrated that p-KG03 exhibited the 50% effective concentration (EC_{50}) values of 0.19–0.48 μg/ml against influenza type A virus infection (selectivity index >200) but not all influenza type B viruses. Mechanism studies showed that inhibition of influenza virus replication was maximized when p-KG03 was added during or within 6 h after viral infection, suggesting that mainly the viral adsorption and internalization steps are targeted by this compound. The results of influenza virus binding assay to p-KG03 and fluorescence microscopy indicate that the antiviral activity of p-KG03 is directly associated with its interaction with viral particles. The sulfated polysaccharide p-KG03 is a potent and specific influenza A viral entry inhibitor and may be a candidate for antiviral drug development.

1. Introduction

Several types of sulfated polysaccharides [such as dextran sulfate, heparin, and fucoidan (Fuc)] and sulfated polymers possess broad-spectrum antiviral activity against dengue virus, herpes simplex virus (HSV), human immunodeficiency virus (HIV), and influenza virus in cultured cells (Hidari et al., 2008; Hosoya et al., 1991; Lee et al., 2006; Talarico et al., 2005). Mechanism studies show that they target the viral attachment and/or internalization steps mediated by specific interactions between polysulfates and either viral particles or cellular surface molecules, resulting in virus (sero)type- or host cell type-dependent inhibitory activity. In particular, because negatively charged sulfated groups can be involved in antiviral efficacy, the size and degree of sulfation present in these compounds correlates relatively well with their ability to inhibit viral infection of cells (Ghosh et al., 2009). In addition, composition of the sugar units and diversity of the linkage chemistry are factors that determine not only the functional properties but also the target specificity of sulfated polysaccharides. Thus, when examining the potential of these compounds as antiviral drug candidates, it is desirable to assess their antiviral activity with as many viral types and isolates as possible.

The sulfated exopolysaccharide, p-KG03, is found in the marine dinoflagellate, *Gyrodinium impudium*. Its molecular weight ranges from 10^6 to 10^7 Da and the molecule comprises a homogenous mixture of galactose residues with uronic acid (2.9% w/w) and sulfate group (10.3% w/w) branches (Yim et al., 2003, 2007). One of the biological activities of p-KG03 is to activate nitric oxide production in a JNK-dependent manner and to stimulate the production of cytokines, such as interleukin-1 (IL-1), IL-6, and TNF-α in macrophages; thus, preventing tumor cell growth both *in vitro* and *in vivo* (Bae et al., 2006; Yim et al., 2005). Interestingly, p-KG03 also shows antiviral effects against encephalomyocarditis virus (EMCV), a non-enveloped, single-stranded, positive sense RNA virus belonging to the *Picornaviridae* family, but the molecular mechanisms involved have not been reported. Moreover, its inhibition of enveloped virus infection (such as HIV) is marginal *in vitro* (Yim et al., 2004).

Although antiviral agents and vaccines are available to cure or protect humans from influenza virus infection, other approaches solving the problems caused by rapidly emerging viral isolates,

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which are either drug-resistant or not neutralized by existing vaccines, are urgently needed. Thus, the present study was aimed to examine whether purified p-KG03 could be a potential anti-influenza virus agent by assessing its activity profile against type A (two H1N1 and one H3N2 isolates) and type B (two isolates) viruses. Notably, the 50% effective concentration (EC_{50}) values against influenza A virus for p-KG03 were comparable to those of oseltamivir carboxylate (the active form of a viral neuraminidase inhibitor, Tamiflu). p-KG03 was significantly more effective than Fuc, a well-studied sulfated polysaccharide with inhibitory effects against the entry of various DNA and RNA viruses (Andrei and De Clercq, 1990; Feldman et al., 1999; Garcia-Villalón and Gil-Fernández, 1991; Hidari et al., 2008). In this report, mechanism studies showed that p-KG03 not only inhibited the binding of influenza virus to host cells (virus–cell attachment) by capturing viral particles, but also prevented cellular internalization of the virus (virus–cell fusion) and early viral replication. Taken together, this study suggests that p-KG03 has strong and specific antiviral activity and could be a candidate compound for the development of an anti-influenza A virus agent to be used alone, or in combination with other licensed antiviral drugs.

2. Materials and methods

2.1. Materials

From the marine microalga *G. impudium* KG03, p-KG03 was extracted, purified and characterized as described previously (Yim et al., 2007; Yim and Lee, 2004). Oseltamivir phosphate (OSV-P; the prodrug) was purchased from Hoffmann-La Roche Ltd. (Switzerland), oseltamivir carboxylate (OSV-C; the active form of OSV-P) from US biological (Swampscott, MA), and ribavirin (RBV), amantadine (AMT), Fuc (about 20 kDa), and pentosan polysulfate (PPS; about 5 kDa) from Sigma–Aldrich (St. Louis, MO).

2.2. Cells and viruses

Mardin–Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) were grown in minimum essential medium (MEM; Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen). Influenza virus A/Puerto Rico/8/34 (H1N1) (PR8; ATCC) was amplified by infection of 10-day-old chicken eggs at 37 °C for 3 days. Influenza viruses, A/Taiwan/1/86 (H1N1) (Tw), A/Seoul/11/88 (H3N2) (Se), and B/Panama/45/90 (Pan), were obtained from Korea National Institute of Health, South Korea. These viruses and influenza virus B/Lee/40 (Lee; ATCC) were propagated by infection of MDCK cells in the presence of 2 μg/ml TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)–trypsin (Sigma–Aldrich) at 33 °C (for Tw, Se, and Pan) or 35 °C (for Lee). Viral stocks were stored at −70 °C and titrated by plaque assay and hemagglutinin (HA) assay before use.

2.3. Cytopathic effect inhibition assay

MDCK cells were seeded in 96-well plates and cultured in MEM/10% FBS for 2 days at 37 °C to >90% confluence. Cells were then washed with PBS and infected with approximately 50 plaque-forming units (PFU) of influenza viruses suspended in 100 μl of serum-free MEM for 1 h at 37 °C (for PR8), 35 °C (for Lee) or 33 °C (for the other influenza viruses). Unadsorbed virus was removed and serial 3-fold dilutions of each compound in MEM containing 2 μg/ml TPCK–trypsin were added to the cell cultures. After 2 (for PR8) or 3 (for the other viruses) days of incubation at the corresponding temperatures, cell viability was measured using the fluorescein diacetate (FDA) method (Schols et al., 1988). In brief, cells were washed with PBS and treated with 100 μl of 30 μg/ml of FDA solution (Sigma–Aldrich) for 15 min at 37 °C. Fluorescence intensity was read at 485/538 nm. The 50% cytotoxic concentration (CC_{50}; the concentration of compound required to reduce cell viability by 50%) and the EC_{50} [the concentration of compound required to reduce the level of virus-induced cytopathic effects (CPE) by 50%] values were analyzed using Softmax Pro 5.3 software (Molecular Devices, Sunnyvale, CA).

2.4. Plaque assay

Confluent cultures of MDCK cells in 24-well plates were infected with 50 PFU/well of influenza virus PR8 at 37 °C for 2 h. Cells were washed with PBS and overlaid with serum-free MEM containing 0.5% carboxymethyl cellulose and 2 μg/ml TPCK–trypsin in the presence or absence of increasing amounts of the test compounds. After 2 days, the plaque number was counted by staining with 1% crystal violet solution.

2.5. Time of addition assay

Ten micrograms per ml of p-KG03 or Fuc were incubated with MDCK cells grown in 24-well plates for 2 h at 37 °C either before, during [with or without a 30-min pre-incubation of virus with p-KG03 at room temperature (RT)], or after a 2 h period of viral infection (50 PFU PR8/well). In each step, cells were washed with PBS. After removal of the supernatants and washing with PBS, the overlay medium with TPCK–trypsin was added and the viral plaque assay was performed as described in Section 2.4.

In another experiment, MDCK cells were infected with PR8 (100 PFU/well), either with 10 μg/ml of test compounds (time 0) or alone, at 4 °C for 1 h. Unadsorbed virus-containing supernatants were removed and the cells were washed with PBS. The cell monolayers were then treated immediately with MEM containing the same concentration of polysaccharides [0 and 1 h post-infection (p.i.)] or stepwise (2, 3, 4, 6, 8, and 10 h p.i.). Following incubation at 37 °C for 11 h, cells were washed with PBS and the plaque assay was performed as described in Section 2.4.

2.6. Solid-phase virus-binding assay

The binding of influenza viruses to p-KG03, Fuc, and PPS was examined using a solid-phase virus-binding assay as described previously (Totani et al., 2003). Briefly, each compound was coated onto 96-well plates (1 μg/well) using UV cross-linking followed by blocking with PBS/1% BSA. One hundred microliters of PR8 diluted 10-fold in PBS were added to the plates (5×10^1–5×10^3 PFU/well) and incubated at 4 °C overnight. After washing with PBS, they were incubated with house-made mouse anti-influenza A virus (Tw) antisera and HRP-conjugated goat anti-mouse immunoglobulin (Sigma–Aldrich). The absorbance was measured at 450/690 nm after incubation with a BM blue peroxidase substrate (Roche), according to the Manufacturer's instructions. For comparing the affinity of influenza type B viruses, Pan and Lee, to p-KG03, and Fuc were coated onto 96-well plates (1 μg/well) and then 100 μl of each virus (about 1×10^3 PFU/well) were incubated at 4 °C overnight. The amount of bound virus particles was measured with an anti-influenza B virus HA-specific antibody (Abcam, Cambridge, UK) and HRP-conjugated goat anti-mouse immunoglobulin (Sigma–Aldrich). The values were normalized by measuring the loading amount of type B isolates and their sensitivity to the primary antibody by ELISA using the same antibodies after coating of the virus on 96-well ELISA plates (Greiner bio-one, Kremsmunster, Austria).

2.7. Inhibition of HA activity

Twenty-five microliters of 1/100 dilution of PR8 virus (HA titer of the stock, 2^8) were incubated with the same volume of PBS (mock) or serially diluted test compounds, p-KG03, Fuc, PPS and an H1N1 HA-specific monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), at RT for 20 min. Then each sample was mixed with 50 μl of 0.5% chicken red blood cells (RBC). After 30 min incubation at RT, the concentrations of the test compounds inhibiting HA activity were measured. The experiments were performed in triplicate.

2.8. Immunofluorescence microscopy

MDCK cells were grown to 80–90% confluence in 4-well chamber slides and infected with 400 μl of PR8 (5×10^5 PFU/ml) in the presence or absence of the test compounds for 2 or 5 h at 37 °C. After blocking with PBS containing 1% BSA (Sigma–Aldrich) and 10% goat serum (Santa Cruz), intracellular virus was detected with a mouse anti-influenza virus nucleoprotein (NP) monoclonal antibody (Santa Cruz) and FITC-conjugated goat anti-mouse immunoglobulin (Santa Cruz). Nuclei were counterstained with Vectashield mounting medium containing DAPI (Vector Laboratories, Peterborough, UK) for at least 4 h. Slides were visualized under a Ti-E fluorescence microscope (Nikon Instruments, Melville, NY) for viral protein detection.

3. Results

3.1. p-KG03 inhibits influenza A virus replication in MDCK

To study the *in vitro* anti-influenza activity of p-KG03, MDCK cells were infected with different strains of influenza A (H1N1: PR8 and Tw; H3N2: Se) and influenza B (Pan and Lee) followed by treatment with either sulfated polysaccharides (p-KG03, Fuc or PPS), or standard antiviral agents (OSV-P, OSV-C, AMT or RBV). Cell viability was measured using the FDA-based CPE assay to estimate the CC_{50} and EC_{50}. Although treatment with AMT reduced the viability of uninfected cells by about 30% at 100 μg/ml (data not shown), none of the agents used in this study caused severe cytotoxicity (CC_{50}, >100 μg/ml) (Table 1). The results from virus-infected cells showed that all the influenza A viruses were sensitive to p-KG03, with EC_{50} values of 0.48 ± 0.23, 0.19 ± 0.07, and 0.22 ± 0.15 μg/ml against PR8, Tw, and Se, respectively. Interestingly, one isolate (Lee) of influenza B virus was sensitive to p-KG03 (EC_{50}, 0.26 ± 0.05 μg/ml), while the other isolate (Pan) was not (EC_{50}, >100 μg/ml). This suggests that p-KG03 can inhibit influenza A viruses, but not all influenza B virus isolates. Compared with p-KG03, Fuc and PPS did not reduce the level of cytopathic damage suffered by virus infection. As expected, OSV-P, OSV-C and RBV effectively inhibited the CPE induced by all the influenza strains tested, while AMT was not effective against PR8 (H1N1), and Pan and Lee (type B), confirming the reliability of the assay system.

Several sulfated polysaccharides produced by naturally occurring organisms are thought to prevent influenza virus infection in cultured cells; however, they exhibit different levels of inhibitory activity depending on their molecular weight and degree of sulfation (Hasui et al., 1995; Hosoya et al., 1991; Ogura et al., 2010). To evaluate the putative therapeutic efficacy of p-KG03, the present study compared its antiviral potency with that of other sulfated polysaccharides in terms of their ability to inhibit influenza viral replication, *i.e.*, plaque formation. MDCK cells were infected with PR8, a representative influenza A virus (H1N1), and incubated with overlay medium supplemented with mock, or serially (10-fold) diluted test compounds (p-KG03, Fuc and PPS; 100–0.01 μg/ml) (Fig. 1). The plaque number of p-KG03-treated cells was markedly reduced on day 2 p.i. in a dose-dependent manner (at a dose of 0.01 μg/ml, plaque number was reduced by about 50% relative to that of PR8-infected, mock-treated control cells). Although Fuc was more active (the plaque number was reduced by 50% at a dose of 0.72 μg/ml) than PPS, it was still 70-fold less potent than p-KG03. The discrepancy of the antiviral activity of Fuc in Table 1 and Fig. 1 seems to be caused by the different sensitivity of the two assay systems: in the CPE assay, the results are read after complete cell lysis in 'virus only' wells, while in the plaque assay, the

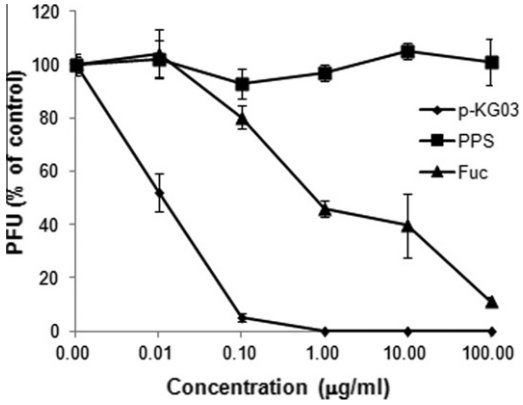

Fig. 1. Effect of sulfated polysaccharides on influenza A virus plaque formation. MDCK cells were infected with approximately 50 PFU of influenza A (PR8) virus for 2 h at 37 °C, and then overlaid with MEM containing 0.5% carboxymethyl cellulose, TPCK–trypsin (2 μg/ml) and increasing amounts of p-KG03, PPS or Fuc. The percentage of viral plaques formed by compound-treated cells relative to mock-treated cells was determined by staining with crystal violet on day 2 p.i. The results represent the mean ± SD of duplicate wells from three independent experiments.

Table 1
Cytopathic effect reduction assay using several sulfated polysaccharides and antiviral chemicals against influenza viruses.

Compound	CC_{50} (μg/ml)[a]	EC_{50} (μg/ml)[b]				
		PR8 (H1N1)	Tw (H1N1)	Se (H3N2)	Pan	Lee
p-KG03	>100	0.48 ± 0.23	0.19 ± 0.07	0.22 ± 0.15	>100	0.26 ± 0.05
Fuc	>100	>100	>100	>100	>100	>100
PPS	>100	>100	>100	>100	>100	>100
OSV-P	>100	8.75 ± 2.35	6.03 ± 0.49	0.12 ± 0.03	2.17 ± 0.59	7.64 ± 2.53
OSV-C	>100	0.10 ± 0.02	0.11 ± 0.09	<0.002	0.02 ± 0.01	0.07 ± 0.01
AMT	>100	>100	2.57 ± 0.34	21.77 ± 10.74	>100	>100
RBV	>100	14.25 ± 1.25	12.76 ± 0.40	11.17 ± 3.38	5.77 ± 2.03	5.60 ± 0.88

[a] CC_{50}, the concentration required to reduced normal, non-infected cell viability by 50%. Values represent the mean of duplicate samples from three independent experiments.
[b] EC_{50}, the concentration required to reduced inhibition of viral infection-induced cytopathogenicity by 50%. Values represent the mean of duplicate samples from three independent experiments.

plaques are counted at the time point of more than 50% cell viability to obtain separate plaques. Actually, in the plaque assay with 10-fold-higher virus titers (500 PFU/well) inducing complete cell lysis, Fuc was inactive (EC_{50}, >100 μg/ml), while p-KG03 was still active (EC_{50}, 0.1–1 μg/ml) (data not shown). In conclusion, Fig. 1 indicates that the recovery of virus-infected MDCK cell viability after treatment with p-KG03, as observed in Table, 1 is due to inhibition of virus-mediated cytopathicity.

3.2. Mechanism underlying the anti-influenza virus activity of p-KG03

There is one report showing the inhibitory activity of microalga (*Cochlodinium polykrikoides*)-derived sulfated polysaccharide against enveloped viruses, including respiratory syncytial virus, HSV-1, HIV-1, and influenza viruses (both type A and B), (Hasui et al., 1995). However, its antiviral mode of action and the stage of viral replication that it targets have not yet been clarified. In the present study, to elucidate the influenza virus replication steps influenced by p-KG03, cells were treated for 2 h at three different time points, *i.e.*, before, during and after viral infection. Fig. 2A shows that p-KG03 preferentially targets early viral entry steps by regulating viral infectivity, rather than modulating cell susceptibility to the virus. The control compound Fuc had a profile similar to that of p-KG03, but was much weaker, requiring pre-incubation with the virus for sufficient viral plaque reduction.

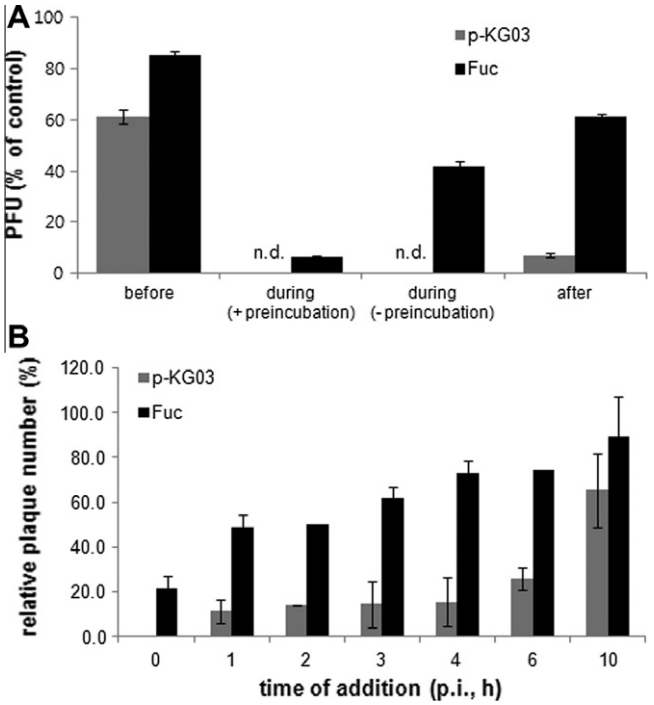

Fig. 2. Influence of time-of-addition on p-KG03 inhibitory activity against influenza A virus. (A) p-KG03 and Fuc (10 μg/ml) were added to MDCK cells for 2 h at 37 °C either before, during [with (+) or without (−) 30-min pre-incubation with virus], or after a 2-h period of viral infection (50 PFU/well in 24-well plates). The number of viral plaques of the compound-treated samples was expressed as a percentage relative to that of the mock-treated samples in each group. Values represent the mean ± SD of duplicate wells from three independent experiments. n.d., viral plaque not detected. (B) MDCK cells were infected with PR8 (100 PFU/well in 24-well plates), either with 10 μg/ml of test compounds (time 0) or alone, at 4 °C for 1 h. Unadsorbed virus-containing supernatants were discarded and the cells were washed with PBS. Cells were then treated immediately (0 and 1 h) or stepwise (2, 3, 4, 6, 8, and 10 h) with MEM containing the same concentration of polysaccharides. Following incubation at 37 °C for 11 h, cells were washed with PBS. On day 2, the viral plaques were counted and expressed as described in (A). The result is representative of three separate experiments.

To further investigate the mechanism, p-KG03 and Fuc were added to MDCK cells for an 11-h period for overall virus adsorption, internalization and early replication (time 0) or at different time points after virus adsorption (1–10 h p.i.) (Fig. 2B). Given that in the Fuc-treated samples, reduction of the viral plaque number was significant (∼80%) at time 0, but marginal at 1 h p.i. (<50%), it appears that Fuc controls influenza virus adsorption with a weak binding affinity to the virus. Interestingly, p-KG03 treatment resulted in complete viral clearance at 0 h and a significant prolonged reduction (∼75%) in the plaque number between 1 and 6 h. This suggests that p-KG03 can inhibit both virus attachment to the host cell surface and internalization, and also possibly early virus replication.

Next, we examined whether the antiviral activity of p-KG03 is derived from its ability to capture the virus particles. The binding affinity of PR8 for several sulfated polysaccharides was tested by a solid-phase virus-binding assay using a mouse anti-influenza virus antibody against the Tw (H1N1) strain. As expected, PR8 bound strongly to p-KG03 immobilized on plastic plates in a dose-dependent manner, but relatively weakly to Fuc and not at all to PPS (Fig. 3A). Furthermore, higher affinity of Lee to p-KG03 compared with Pan was confirmed by the same experimental method using an influenza B HA-specific antibody (Fig. 3B). In addition, to investigate if direct interaction between p-KG03 and virion can affect HA activity, a key protein essential for host cell receptor recognition, an HA inhibition assay in the presence of various polymers and an anti-HA monoclonal antibody (a positive control) was attempted. The results showed that PR8 HA activity disappeared by p-KG03 (5.6 μg/ml), Fuc (50 μg/ml) and anti-HA antibody (10 μg/ml), but not by PPS (Fig. 3B). Thus, it is likely that the strong and specific binding of p-KG03 to influenza A or some isolates of influenza B prevents free virus particles (or HA protein) from reaching host cell receptor(s) and, thus, from penetrating through the cell membrane.

3.3. p-KG03 protects cells against influenza virus infection

To visualize the effects of p-KG03 on influenza A infectivity, cells were inoculated with PR8 alone or with increasing concentrations of p-KG03 (0.1, 1, and 10 μg/ml). Intracellular localization of the viral NP protein was analyzed by fluorescence microscopy using an NP-specific primary antibody and an FITC-labeled secondary antibody. Accumulation of the NP protein within cells co-treated with p-KG03 (1 or 10 μg/ml) was markedly reduced 2 h after infection (Fig. 4). Interestingly, a lower dose (0.1 μg/ml) did not reduce the number of NP-positive cells compared with mock treatment, but rather stimulated migration of the influenza NP protein from the cytoplasm to the nucleus. This finding requires further investigation in the context of viral RNA replication in the nucleus and intracellular localization of viral proteins.

The inhibitory function of p-KG03 (10 μg/ml) during early virus replication was studied at a later time point, 5 h p.i. and compared with that of Fuc, OSV-P and AMT. Cells treated with virus alone showed increasing nuclear accumulation of the NP protein (compare the 'PR8 virus only' images in Figs. 4 and 5). Interestingly, in Fig. 5, incubation of the cells with virus plus p-KG03 significantly reduced the NP protein-positive cell populations but not fluorescence intensity in a single cell, indicating that viral RNA replication or protein expression efficiency is not affected by p-KG03 at early infection (5 h p.i.). As expected, Fuc and AMT, both inactive against PR8 in the CPE inhibition assay (Table 1), were unable to prevent viral entry into host cells. It is noteworthy that the NP amount was reduced by OSV-P. This event might be caused by OSV-P-mediated interruption of the NA activity as observed with OSV-C which inhibits infectious virus entry as well as the release of viral progeny as previously reported (Matrosovich et al., 2004; Ohuchi et al.,

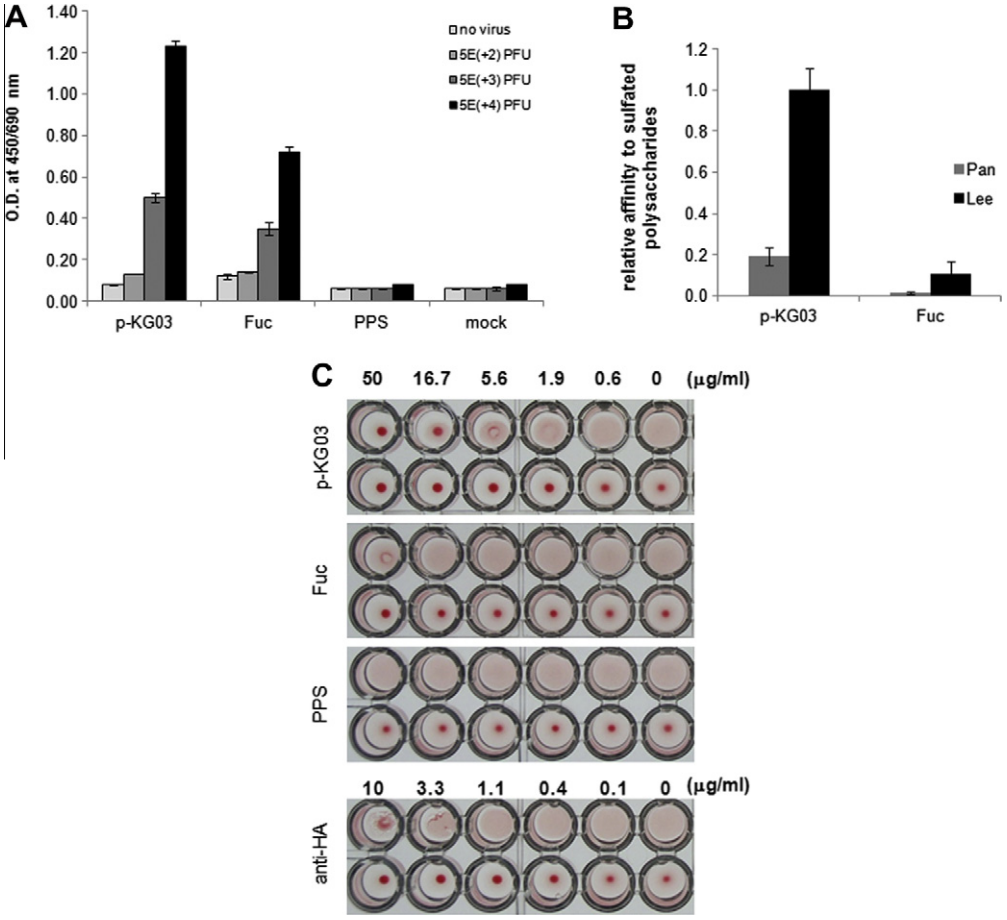

Fig. 3. Interaction between p-KG03 and influenza viral particles. (A) Different titers of the PR8 virus were incubated with either each sulfated polysaccharide (p-KG03, PPS, or Fuc; 1 μg/well) or mock-treated controls immobilized on 96-well plates. PR8 virus bound to the polysaccharides was quantitatively detected by incubation with an anti-influenza H1N1 (A/Taiwan/1/86) polyclonal antibody and an HRP-conjugated goat anti-mouse, secondary antibody. After addition of a peroxidase substrate and then 1N H_2SO_4 stop solution, the absorbance was measured at 450 nm against a reference wavelength of 690 nm. The results are expressed as the mean ± SD of triplicate samples. (B) Influenza B viruses, Pan (gray bar) and Lee (black bar) (100 μl of viral suspension about 1×10^4 PFU/ml), were incubated with either sulfated polysaccharide (p-KG03 or Fuc) or mock immobilized on 96-well plates (1 μg/well) at 4 °C overnight. Influenza type B virus bound to the polysaccharides was quantitatively detected by incubation with an anti-Flu B HA antibody and an HRP-conjugated goat anti-mouse. The viral titers bound to the polymers were measured as mentioned in (A). The viral loading amount and affinity to the primary antibody was normalized by measuring the absorbance from ELISA using the same antibodies, in which each virus was directly coated on an ELISA plate. The results are expressed as the mean ± SD of triplicate samples. The affinity of Lee to p-KG03 was defined as the normalized value '1'. (C) Twenty five microliters of PR8 virus (10^5 PFU/ml) in PBS (upper rows) or mock PBS (lower rows) were mixed with the same volume of 3-fold diluted compounds (p-KG03, Fuc, PPS and an anti-HA antibody; from 200 μg/ml for polymers and from 40 μg/ml for the antibody) at RT for 20 min. Then 50 μl of 0.5% chicken RBC was added to each well. RBC aggregation was monitored after 30 min incubation at RT. Final concentrations of test compounds are recorded on the top of panels.

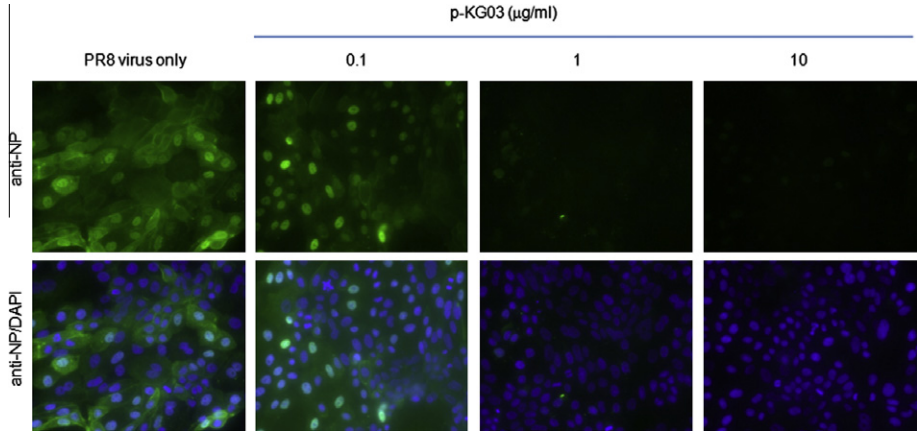

Fig. 4. Fluorescence microscopy to determine dose-dependent inhibition of influenza A virus entry to MDCK cells by pKG03. MDCK cells were seeded on 4-well chamber slides (2×10^5 cells per well) and infected with PR8 (2×10^5) in the presence or absence of p-KG03 (0.1–10 μg/ml) for 2 h at 37 °C. The viral NP protein was stained with an anti-NP antibody and a FITC-conjugated secondary antibody (Green). Nuclei were counterstained with DAPI (Blue). More than 10 images were captured for each sample. Magnification, 400×.

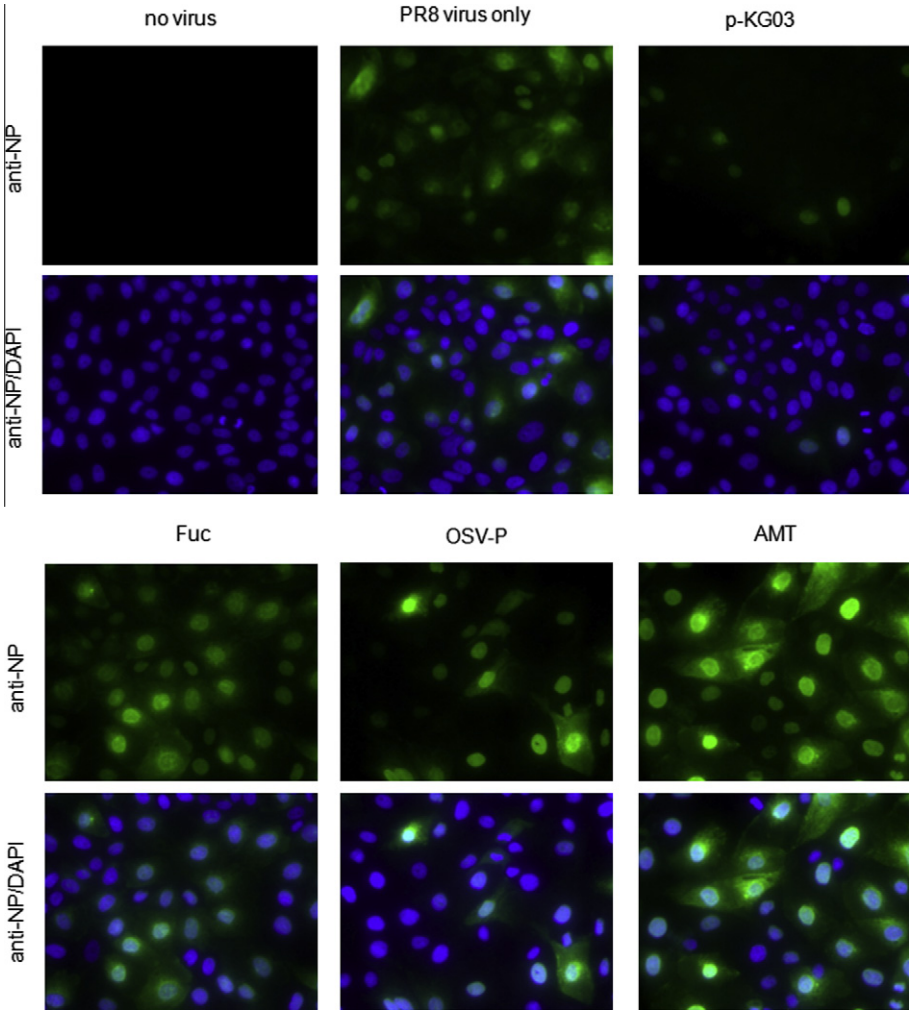

Fig. 5. Comparison of the inhibition of influenza virus entry by p-KG03 with that of other sulfated polysaccharides or antiviral therapeutics. MDCK cells were seeded on 4-well chamber slides (1.2×10^5×cells per well) and infected with PR8 (2×10^5) in the presence or absence of the indicated test compounds (10 μg/ml) for 5 h at 37 °C. The viral NP protein was stained with an anti-NP antibody and an FITC-conjugated secondary antibody (Green). Nuclei were counterstained with DAPI (Blue). Magnification, 400×.

2006). The results of the present study indicate that p-KG03 is a potent and specific inhibitor of influenza A virus entry into host cells.

4. Discussion

The marine microalga-derived sulfated polysaccharide p-KG03 is a novel compound with therapeutic potential for inhibiting tumor cell growth or interfering with infection by non-enveloped viruses such as EMCV (Bae et al., 2006; Yim et al., 2004, 2005). This antiviral activity against EMCV is a distinctive feature that discriminates it from other sulfated oligo- or polysaccharides that control infection primarily by enveloped viruses such as HIV, HSV or dengue virus (Ekblad et al., 2010; Hosoya et al., 1991; Lee et al., 2006; Talarico et al., 2005). In this study, the antiviral activity of p-KG03 against different (sub)types of enveloped influenza virus was tested to evaluate its inhibitory activity and potential as a new anti-influenza agent. Its activity was compared with that of existing drugs (OSV-P, OSV-C, AMT, and RBV) and with commercially available sulfated polysaccharides [Fuc (comprising fucose units) and PPS (comprising β-D-xylopyranose units)]. An *in vitro* cell-culture based CPE assay showed that it predominantly reduced infectivity of influenza A (but not all influenza B) as efficiently as OSV-P or OSV-C (Table 1). This antiviral activity was also observed in a plaque reduction assay (Fig. 1). However, the control sulfated polysaccharides, Fuc and PPS were ineffective in the CPE assay. In the plaque assay, PPS was also inactive, although Fuc did reduce influenza virus infectivity, but only marginally relative to p-KG03 (Table 1 and Fig. 1). This suggests that there might be a threshold binding affinity between the virus and sulfated polysaccharides for inhibiting virus entry.

The mechanism underlying the activity of antiviral sulfated polysaccharides derived either from synthetic products or from natural plants or seaweeds appears to involve targeting viral entry steps, such as virus particle attachment to host cell surface receptors or internalization via virus–cell fusion, or both (Ekblad et al., 2010; Hosoya et al., 1991; Talarico et al., 2005). However, studies investigating the antiviral activity and mechanism of action of sulfated polysaccharides purified from marine microalgae have not been performed. Previously, a sulfated polysaccharide from the marine microalga, *C. polykrikoides*, was suggested to have inhibitory activity against RSV, HSV-1, and HIV-1 infection, as well as infection by two different types of influenza virus [EC_{50}, 0.45–1.1 μg/ml against type A (H3N2) and 7.1–8.3 μg/ml against type B] in a CPE assay (Hasui et al., 1995). In spite of the similar source (marine microalgae), its chemical structure and broad-spectrum coverage against enveloped viruses are totally different from those of p-KG03; *i.e.*, sulfated polysaccharides from *C. polykrikoides* are

composed of a mixture of mannose, galactose and glucose and are active against various enveloped viruses mentioned above, whereas p-KG03 is a homopolysaccharide composed of galactose and is active against influenza virus but not against HIV at subtoxic concentration of the compound (Hasui et al., 1995; Yim et al., 2004). These similar, but not identical, characteristics emphasize the need for a more systematic understanding of how p-KG03 suppresses influenza virus replication. The time-of-addition experiments, in which its treatment was given at different time points relative to influenza H1N1 virus infection, showed that it was active during the early stages of the virus replication cycle, particularly between 0 and 6 h p.i. (Fig. 2). This strongly suggests that p-KG03 inhibits virus attachment, penetration into cells and, possibly, early viral replication. Inhibition of viral entry mediated by direct interaction between p-KG03 and influenza virus was proved by solid-phase binding and HA inhibition assays as well as fluorescence microscopy (Figs. 3–5). However, it did not affect viral RNA polymerase activity in a mammalian cell line when applied with a reverse genetics system-based polymerases/NP assay (Lutz et al., 2005) (data not shown). Currently, it is assumed that p-KG03 could down-regulate the early virus life cycle via stimulating the innate immune system suppressed by viral NS1 protein as observed in the case of chlorite-oxidized oxyamylose (COAM) (Li et al., 2008; Newby et al., 2007).

In addition to its significant antiviral potential, p-KG03 has several benefits points in terms of its future commercial application. First, suspension culture of the microalga strain KG03 is feasible, allowing high yield productivity and quality-controlled culture and, second, it is thermodynamically stable, permitting long-term storage of stock preparations. However, it has limited solubility in hydrophilic buffers (up to about 10 mg/ml) and thus is viscous. Therefore, to improve solubility and treatment dose range, its molecular size is currently being modified and optimized by artificial fragmentation of the full-length p-KG03 (MW; 10^6–10^7 Da), together with over-sulfation of the fragmented derivatives. To date, most sulfated polysaccharides that showed antiviral activity in cultured cells or animal models have proved ineffective in clinical trials (in patients infected with HIV for example), due to shielding of the anionic groups by positively-charged serum compounds or cervical secretions (Cohen, 2008). Given that p-KG03, with its lower EC_{50} than other sulfated polysaccharides, is highly effective *in vitro*, and that the same route used for influenza virus infection, via the respiratory tract, could be followed for treatment, it would seem advisable to test the antiviral activity against of mouse-adapted influenza viruses via simple intranasal administration of p-KG03.

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