Antiviral Sulfated Polysaccharide from *Navicula directa*, a Diatom Collected from Deep-Sea Water in Toyama Bay

Jung-Bum Lee,* Megumi Hirata, Eiko Kuroda, Eiko Suzuki, Yoshihiro Kubo, and Toshimitsu Hayashi

*Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan; and Toyama Prefectural Institute for Pharmaceutical Research; 17–1 Nakataikouyama, Imizu, Toyama 939–0363, Japan. Received April 24, 2006; accepted July 24, 2006; published online July 31, 2006

A sulfated polysaccharide named naviculan was isolated from a diatom, *Navicula directa* (W. Smith) Ralfs, collected in deep sea water from Toyama Bay. The polysaccharide consisted of fucose, xylose, galactose, mannose, rhamnose and sulfate with an apparent molecular weight of 220000. It showed antiviral activities against herpes simplex viruses type 1 and 2, and influenza A virus with selectivity indices (CC50/IC50) of 270, 510 and 32, respectively. Naviculan also showed an inhibitory effect on cell–cell fusion between CD4-expressing and human immunodeficiency virus (HIV) gp160-expressing cells that was used as a model system of infection with HIV.

Key words antiviral activity; polysaccharide; diatom; *Navicula directa*; deep sea water

There are several risks for emerging and re-emerging viral infectious diseases. Recently, a new avian influenza appeared in Southeast Asia and is becoming a serious problem worldwide. If an antigenic shift produces new influenza virus subtypes that can easily spread from human to human, these viruses could cause influenza pandemic outbreak. And now, it is predicted that this situation may result in high levels of illness, death, social disruption and economic loss. On the other hand, AIDS caused by human immunodeficiency virus (HIV) has been recognized as one of the most serious emerging infectious diseases worldwide. Numbers of HIV carriers and AIDS patients have been increasing not only in developing countries but also in Japan, and unprotected sexual contact is thought to be the most prominent cause of the spread of HIV. Furthermore, it is well known that infection with other sexually transmitted diseases including chlamydioidis, trichomoniasis and genital herpes increases the opportunity to transmit HIV during unprotected sex among infected and uninfected partners. There is substantial evidence that herpes simplex virus types 1 and 2 (HSV-1 and -2) are cofactors for HIV infection and progression in HIV-infected patients. Although various antiviral drugs have been developed for these viruses, drug-resistant mutations have often been observed, which reduces the efficiency of the available drugs. Therefore, new antiviral drugs are required and still more to be re-searched and developed.

Marine algae produce various metabolites and have been recognized as promising targets in the search for biologically active compounds. Our previous study revealed that marine algal extracts exerted antiviral activities against HSV-1. 21 A fucan sulfate, sodium horman (Na-HOR), was isolated from *Sargassum hornieri* (brown alga) as an antiviral ingredient.23 We have also isolated various sulfated polysaccharides showing antiviral activities.24 Therefore, marine algae can be important resources in the discovery of antiviral compounds. On the other hand, deep sea water has been suggested to be a promising resource to discover microorganisms producing bioactive compounds. In fact, antibiotics were isolated from actinomycete collected from deep sea water in Toyama Bay, Japan.25 *Navicula directa* is a diatom often collected at a sluice gate of deep sea water in Toyama Bay. This alga is unicellular and ubiquitously present in marine habitats. So far, hyaluronidase inhibitory effect was found in the water extract of *N. directa*, and its active substance was suggested to be a polysaccharide.25 However, there are no reports on polysaccharides from *Navicula* sp. In the present study, we report the results of bioactivity-guided fractionation of *N. directa* and antiviral properties of an isolated polysaccharide.

MATERIALS AND METHODS

**Materials** *N. directa* was collected at a sluice gate of deep sea water in Namerikawa, Toyama Prefecture, Japan. The diatom was cultured in deep sea water of Toyama Bay at 20 °C under 12 h photoperiod. Eagle’s minimal essential medium (MEM) was obtained from Nissui Pharmaceutical (Tokyo, Japan). DEAE 650M and GMPWXL columns were purchased from Tosoh (Tokyo, Japan). Sepharose 6B and Sephacryl HR-400 were from Amersham Biosciences (Picataway, NJ, U.S.A.). Dextran sulfate (MW 50000; DS) was from Sigma (St. Louis, MO, U.S.A.). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Preparation of Ethanol-Insoluble Fraction** Dried *N. directa* was suspended in distilled water, ultrasonicated for 30 min, and extracted at 90 °C for 30 min. Then, obtained extract was lyophilized to give a water extract. 80% EtOH was added to the extract to obtain insoluble part followed by lyophilization to give EtOH insoluble fraction (NDP).

**Fractionation and Isolation of an Antiviral Polysaccharide** NDP (8 g) was dissolved in H2O and centrifuged to remove the insoluble part. The soluble part was dialyzed against distilled H2O. Dialysate and non-dialysate were lyophilized to give NDP-L (1.15 g) and NDP-H (1.97 g), respectively. NDP-H (0.9 g) was then applied to a DEAE 650M column (5 i.d.×15 cm) which was subsequently eluted with each 1 l of H2O, 0.5 M NaCl, 1.0 M NaCl, 2 M NaCl, 4 M NaCl, and 0.2 M NaOH. The yield of each eluate was 131.5 mg (NDP-H1), 432.7 mg (-H2), 107.5 mg (-H3), 42.5 mg (-H4), 15.5 mg (-H5) and 58 mg (-H6), respectively. The fractionation with DEAE 650M was performed twice. NDP-H3 (200 mg) was subjected to a DEAE 650M column (2.5
i.d.×8 cm), which was eluted with linear gradient system prepared by H₂O (200 ml) and 1 M NaCl (200 ml), and finally eluted with 1 M NaCl (100 ml). Fractions of 10 ml were collected and monitored by phenol–H₂SO₄ method³ and UV absorbance at 258 nm. NDP-H3A (1.9 mg), NDP-H3B (48 mg) and NDP-H3C (49 mg) were obtained on the basis of its elution profile. NDP-H3B (30 mg) was applied to Sepharose 6B gel filtration (2.2 i.d.×96 cm) and eluted with 0.01 M citrate buffer (pH 7) containing 0.1 M NaCl. Fractions of 10 ml collected were monitored using a phenol–H₂SO₄ method and UV absorbance at 258 nm to give two fractions, NDP-H3B1 (21.5 mg) and -H3B2 (8.5 mg). NDP-H3B1 was applied to a Sephacryl S-400 HR column (2.2 i.d.×95 cm) and eluted with 0.1 M NaCl. Fractions of 10 ml were collected and monitored by phenol–H₂SO₄ method and UV absorbance at 258 nm. Carbohydrate-positive fractions were collected, dialyzed and lyophilized to give naviculan with white cotton-like appearance (12.7 mg).

Cellulose-Acetate Membrane Electrophoresis Naviculan was applied to a cellulose-acetate membrane (Separax) in pyridine–formic acid buffer (pH 3) and run at an 1 mA/cm². Naviculan was applied to a cellulose-acetate membrane (Separax) in pyridine–formic acid buffer (pH 3) and run at an 1 mA/cm². The apparent molecular weight of naviculan was estimated by HPLC analysis. The sample was applied on TSK GMPW XL gel filtration columns (7.6×300 mm×2) and eluted with 0.1 M NaCl at 0.5 ml/min. Commercially available pullulans (Shodex P-52, Showa Denko K.K., Tokyo, Japan) were used as standard molecular markers.

Chemical and Spectroscopic Analyses Naviculan was dissolved in MeOH (0.5 ml) and acetyl chloride (35 μl), and then heated at 80 °C for 16 h. The reaction mixture was dried with a N₂ stream at room temperature. The methyl glycosides were silylated using TMSI-H reagent (GL-Science Inc., Tokyo, Japan) at 60 °C for 1 h. The derivatives were analyzed by GC-MS using a DB-1 MS fused silica capillary column (J&W Scientific, Palo Alto, CA, U.S.A.) with the following oven temperature: initial temperature was set at 140 °C, and then immediately increased to 180 °C at a rate of 2 °C/min. Oven temperature: initial temperature was set at 140 °C, and then immediately increased to 180 °C at a rate of 2 °C/min. Sulfate content was analyzed using the rhodizonate method.³ Since NDP showed antiviral activities against glycogen (NDP-H) substances by dialysis. Since NDP showed antiviral activities against glycogen (NDP-H) substances by dialysis. The IR spectrum was measured on an FT/IR-460 spectrophotometer (JASCO, Kyoto, Japan) using the KBr method. NMR spectrum was run on Unity plus 500 spectrophotometer (Varian, Palo Alto, CA, U.S.A.). The sample was dissolved in D₂O and acetone was used as an internal standard (δH 2.229 ppm). Elemental analysis was performed by Perkin-Elmer 2400 Series II CHNS/O system (Wellesley, MA, U.S.A.). The protein content of the naviculan was calculated from the nitrogen content by multiplying the content by 6.25 (protein contains 16.5% nitrogen).

Cells and Viruses Vero and MDCK cells were grown in MEM supplemented with 5% fetal bovine serum (FBS). HSV-1 (HF strain) and HSV-2 (UW-268 strain) were grown on Vero cells. Influenza A virus (NWS strain, H1N1) (IFV) was grown on MDCK cells. After virus adsorption, the cells were maintained in the medium containing 2% FBS.

Antiviral Activity and Cytotoxicity Plaque yield reduction assay for antiviral activity has been described previouly.³ Bioactivity Guided Fractionation of Antiviral Polysaccharide Since NDP showed antiviral activities against HSV-1 and IFV (data not shown), we attempted further fractionation to obtain antiviral substances. First, NDP was fractionated to low molecular (NDP-L) and high molecular (NDP-H) substances by dialysis. Since NDP-H showed antiviral activity, it was applied to a DEAE 650M anion exchange chromatography and fractionated into six fractions. Among the fractions, NDP-H3 eluted with 1 M NaCl showed the most potent antiviral activity. This fraction was subjected to anion exchange column chromatography on DEAE 650M with linear gradient elution. The most abundant fraction (NDP-H3B) eluted with 0.6 M NaCl showed antiviral activity at 1 or 2 d incubation points in the medium containing 0.8% methylcellulose or 0.8% agar for HSV and IFV, respectively. The 50% inhibitory concentration (IC₅₀) was obtained from a concentration–response curve. For cell growth inhibition studies, cells were incubated in 48-well plates at an initial density of 1.2×10⁶ cells/well. After cells had been incubated for 8 h at 37 °C, naviculan was added to the cells and the incubation was continued for 3 d. Viable cell yield was determined by the trypsin blue exclusion test. The 50% cytotoxic concentration (CC₅₀) was obtained from concentration–response curves. Antiviral activities were estimated with selectivity indices (SIs) calculated from CC₅₀ and IC₅₀ values. HSV cell–cell fusion assay was performed with co-culture of HIV gp160-expressing HeLa (Env) and CD4-expressing HeLa (T4) cells. Briefly, Env and T4 cells were cocultured at a ratio of 1:20 in the absence or presence of sample in MEM supplemented with 5% FBS at 37 °C. After calculating the percentage of cell fusion value [(number of syncytium in the presence of sample/number of syncytium in the absence of sample)×100], the inhibitory effect was expressed as 50% inhibitory concentration.

Binding Inhibition Test by Infectious Center Assay Pre-cooled naviculan, HSV-1 (1 PFU/cell), and Vero cell suspension (4×10⁶ cells/ml) were mixed at 4 °C. After 1 h incubation at 4 °C, the cell suspensions were washed three times with ice-cold PBS to remove unbound viruses and free compound. The cell pellets were diluted serially with ice-cold PBS and immediately added to Vero cell monolayer, which were then overlaid with 0.5% methylcellulose to be plaque-assayed.

Penetration Inhibition Test Pre-cooled Vero cell monolayers were infected with HSV-1 (100 PFU/well) at 4 °C for 1 h. After washing three times with ice-cold PBS, cell monolayers were incubated at 37 °C in the medium containing naviculan. At 0, 0.5, 1, 2, 3, or 6 h after temperature shift to 37 °C, the cell monolayers were treated with 40 μM citrate buffer (pH 3.0) for 1 min and overlaid with 0.5% methylcellulose to be plaque-assayed.

Statistical Analysis All values were expressed as the mean±standard error of the mean (S.E.M.). Statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by Dunnett’s test. A probability value less than 0.001 was considered to be statistically significant.

RESULTS
activity. Then, the fraction was separated by gel filtration on Sepharose 6B. The first eluted fraction (NDP-H3B1) which exhibited antiviral activity was applied to a Sephacryl S-400HR column. The fraction eluted as a single peak was found to be antiviral. The antiviral polysaccharide thus obtained was named naviculan.

**Chemical Properties of Naviculan**

When naviculan was analyzed by cellulose acetate membrane electrophoresis, it was detected as a single band (Fig. 1) indicating its homogeneity. The apparent molecular weight of naviculan was estimated to be 2.22×10^6 by HPLC analysis. Its IR spectrum indicated the presence of sulfate groups (1252 cm⁻¹, S=O stretching). Colorimetric analysis with rhodizonate revealed that sulfate was contained 8.0% in naviculan. Sugar composition analyses indicated that it consisted mainly of fucose (26.6%), xylose (25.0%), galactose (20.7%), mannose (13.1%), and rhamnose (8.7%). In addition, small amounts of other sugar residues such as glucose and glucuronic acid were also detected. Elemental analysis revealed that naviculan contained nitrogen (2.41%), which was suggested to be originated from protein because both glucosamine and galactosamine were not detected. Thus, the protein content was estimated to be about 15.1%. The presence of fucose and rhamnose were further confirmed by methyl proton signals around 1.3 ppm were observed in its ¹H-NMR spectrum. Observation of signals due to numerous anomeric protons in ¹H-NMR spectrum suggested that several sugar residues were sulfated (data not shown).

**Antiviral Activities of Naviculan**

Naviculan was treated for its inhibitory potency in the plaque reduction assay. Concentration-dependent inhibition of HSV-1, HSV-2, IFV-A infections and cell–cell fusion caused by HIV was observed as shown in Fig. 2. The resulting CC₅₀ and IC₅₀ values of naviculan were shown in Table 1, comparing with those of DS, one of the representative sulfated polysaccharides. It showed a low toxicity against cell growth, with CC₅₀ values being higher than 3800 μg/ml. On the other hand, IC₅₀ values were 14, 7.4, and 170 μg/ml for HSV-1, HSV-2 and IFV, respectively, when naviculan was added to the medium at the same time of virus adsorption (Table 1 (A)). The resulting selectivity indices (CC₅₀/IC₅₀) indicated that naviculan had potent anti-HSV-1, HSV-2, and IFV activities. When naviculan was added after virus adsorption (Table 1 (B)), its potency was less than that obtained in experiment A. These results suggest that naviculan might interfere with very early stages of viral replication such as virus binding to and penetration into host cells. Naviculan also interfered with the formation of cell–cell fusion between HIV gp160- and CD4-expressing HeLa cells. CD4-positive cells infected with HIV-1 contain the viral glycoprotein complex gp120/gp41 on their surface and merge healthy CD4 cells to produce multinuclear giant cells (syncytia). Thus, the assay system used in the present study mimics infection of cells with HIV-1 without handling...
DISCUSSION

So far, studies on polysaccharides derived from diatoms have been limited. For example, the extracellular polysaccharide produced by Chaetoceros curvisetus was reported to be partially sulfated and composed of galactose, rhamnose and fucose. In the report on the sugar composition of polysaccharide fractions from a marine diatom, Achnanthes longipes, cetylpyridinium chloride-insoluble fraction was found to contain an anionic polysaccharide consisting of fucose, galactose, xylose, glucuronic acid, glucose, mannose and rhamnose. Metabolism analyses of the fucoglucomannans revealed that the polysaccharides had a complex structure. In the present study, we isolated naviculan from N. directa as a novel antiviral sulfated polysaccharide, which consisted of fucose, xylose, galactose, mannose, rhamnose, and other trace amounts of sugar moieties.

In the present study, we showed that naviculan had a broad antiviral spectrum against enveloped viruses (Table 1). HSV and IFV infections are initiated by binding to carbohydrates, such as heparan sulfate or sialylate residues, on the host cell surface. As shown in Figs. 3 and 4, naviculan inhibited HSV-1 binding onto host cell and viral penetration into host cell around the concentration of its IC50. Thus, this polysaccharide is thought to be one of the inhibitors of virus–cell interaction. In general, sulfated polysaccharides are advantageous as antiviral candidates. That is, sulfated polysaccharides possess a broad antiviral spectrum against enveloped viruses such as HSV and HIV-1. HSV infection, particularly infection with HSV-2, is the most common cause of genital ulcer diseases worldwide, accounting for >60% of genital ulcer disease cases in developing countries. Genital ulceration is a risk factor for HIV infection as it provides a direct portal for HIV entry through mucosal disruption. Furthermore, HSV can activate HIV replication by the transactivation of provirus and expand HIV tropism by the pseudotype formation. These findings reveal that the discovery and development of agents carrying both anti-HSV and -HIV activities may be one answer to the prevention of HIV acquisition and transmission. There is an urgent need for microbicides that can protect women from infection with HIV. Since sulfated polysaccharides could be used in the form of a gel or support material, their application to the vagina before sexual contact may help prevent HIV infection. In regard to this, some sulfated polysaccharide derivatives, cellulose sulfate and carrageenans, are currently in phase III trials as vaginal microbicides in Africa. In future, it will be needed to estimate the in vivo antiviral activities of naviculan as an antiviral drug candidate.

Acknowledgement We would like to thank Ms. Kazuko Sawaya of the Life Science Research Center, University of Toyama, for the elemental analysis used in this study.

REFERENCES