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Virucidal activity of polysaccharide extracts from four algal species against herpes simplex virus

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ABSTRACT

Herpes simplex virus types 1 and 2 (HSV-1, HSV-2) infections are common, but can cause serious infections in neonates and the immunocompromised. Drugs currently used to treat cutaneous or genital HSV infections are effective in limiting disease, but the emergence of drug resistant viruses in immunocompromised individuals can be problematic. While the prophylactic oral treatment with antiviral drugs can reduce virus shedding and transmission, there is a need for topical microbicides that have the potential to limit sexual transmission of the virus. Previous reports demonstrated the antiviral activity of complex sulfated polysaccharides extracted from various species of marine algae and suggested that they interfered with the attachment of virions to host cells. Here, we evaluated the antiviral activity of extracts from *Undaria pinnatifida*, *Splachnidium rugosum*, *Gigartina atropurpurea*, and *Plocamium cartilagineum* against HSV-1 and HSV-2. These extracts exhibited good activity when added during the first hour of viral infection, but were ineffective if added later. Plaque reduction assays, when the extracts were added prior to viral inoculation, yielded EC_{50} values that ranged from 2.5–3.6 μg/ml for HSV-1 and 0.7–6.6 μg/ml for HSV-2. None of the extracts exhibited significant toxicity in a neutral red uptake assay (IC_{50} >100 μg/ml). Subsequent assays showed that the compounds had potent virucidal activity and were active at very low concentrations. We conclude that these extracts are nontoxic and effective virucidal agents that warrant further investigation to examine their potential role in the prevention of HSV infections of humans.

1. Introduction

Many species of marine algae contain significant quantities of complex structural sulfated polysaccharides that have been shown to inhibit the replication of enveloped viruses including members of the flavivirus, togavirus, arenavirus, rhabdovirus, orthopoxvirus, and herpesvirus families (Witvrouw and De Clercq, 1997). The biological activity of these high molecular weight molecules is determined by their chemical structure including the degree of sulfation, molecular weight, constituent sugars, conformation and dynamic stereochemistry (Adhikari et al., 2006; Damonte et al., 2004; Lüscher-Mattli, 2000). Antiviral activity of the sulfated polysaccharides has been reported to result from the interference with early steps in the viral replication process including virus adsorption (Damonte et al., 2004). The mechanism of action can be attributed to an inhibitory effect on the initial attachment of the virus to the host cells, and is thought to be mediated by interactions of sulfated polysaccharides with positively charged domains of the viral envelope glycoproteins involved in the attachment of the virus to heparan sulfate proteoglycans on the surface of the host cells (Damonte et al., 2004; Duarte et al., 2001; Neyts et al., 1992; Witvrouw and De Clercq, 1997). Both the degree of sulfation and the distribution of sulfate groups on the constituent polysaccharides play an important role in the antiviral activity of these polysaccharides. Algal polysaccharides with low degrees of sulfation are generally inactive (Damonte et al., 2004), while more highly sulfated polysaccharides and other synthetically sulfated polymers including dextran sulfate and sulfated polyanionic dendrimers appear to inhibit virus adsorption (González et al., 1987; Witvrouw et al., 2000). Certain neutral polysaccharides have also been found to inhibit viral infection indicating that antiviral activity of polysaccharides is not only related to their negative charge but also to other characteristics of the molecules (Marchetti et al., 1995). Many sulfated algal polysaccharides have been shown to have antiviral activity *in vitro*, including fucans, galactans and xylomannans that presumably act by similar mechanisms (Baba et al., 1988; Carlucci et al., 1999; Hayashi et al., 2008; Kolender et al., 1997; Talarico et al., 2004; Thompson and Dragar, 2004; Witvrouw and De Clercq, 1997). A summary of the antiviral activity of extracts from red and brown algae against HSV is presented in Table 1.

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Table 1
A selection of polysaccharide extracts from red and brown algae with activity against HSV-1 and HSV-2.

	Polysaccharides	EC_{50} (μg/ml)[a]		References
		HSV-1	HSV-2	
Species of red algae				
Bostrychia montagnei	Sulfated galactan	13	11	Duarte et al. (2001)
Callophyllis variegata	Sulfated galactan	0.2	0.2	Rodríguez et al. (2005)
Cryptonemia crenulata	DL-Hybrid sulfated galactan	1.1	1.9	Talarico et al. (2004)
Gigartina skottsbergii	Lambda-type carrageenan	0.6	0.4	Carlucci et al. (1997)
	Kappa/iota-carrageenans	3.2	2.3	Carlucci et al. (1997)
	Mu/nu-carrageenans	0.7	0.5	Carlucci et al. (1997)
Grateloupia indica	Sulfated galactan	0.3	0.3	Chattopadhyay et al. (2007)
Gymnogongrus griffithsiae	Mixture of iota/nu/kappa-carrageenans	0.4	1	Talarico et al. (2004)
Gymnogongrus torulosus	DL-Hybrid sulfated galactan	NA	0.6	Pujol et al. (2002)
Meristiella gelidium	Mixture of iota/kappa/nu-carrageenans	NA	0.04	de S.F-Tischer et al. (2006)
Nothogenia fastigiata	Sulfated xylomannan	0.7	0.7	Kolender et al. (1997), Pujol et al. (1995)
Schizymenia binderi	DL-Hybrid sulfated galactan	0.8	0.6	Matsuhiro et al. (2005)
Scinaia hatei	Sulfated xylomannan	0.5	0.5	Mandal et al. (2008)
Stenogramme interrupta	Hybrid polysaccharides composed predominantly of iota-carrageenan	9.3	4.3	Cáceres et al. (2000)
	Hybrid polysaccharides composed predominantly of xi- and lambda-carrageenans	2.9	0.9	Cáceres et al. (2000)
Species of brown algae				
Adenocystis utricularis	Sulfated galactofucan	0.3	0.5	Ponce et al. (2003)
Cystoseira indica	Sulfated fucan	2.8	1.3	Mandal et al. (2007)
Leathesia difformis	Sulfated fucan	0.7	0.5	Feldman et al. (1999)
Sargassum horneri	Sulfated fucan	1	NA	Hoshino et al. (1998)
Sargassum patens	Sulfated galactofucan	5	1.3	Zhu et al. (2004, 2006)
Stoechospermum marginatum	Sulfated fucan	3.6	0.6	Adhikari et al. (2006)
Undaria pinnatifida	Galactofucan sulfate	32	0.5	Hayashi et al. (2008), Thompson and Dragar (2004)

[a] Values shown represent the concentration required to inhibit plaque formation by 50% and are given in units of μg/ml.

Here we report the isolation of sulfated polysaccharides from species of red and brown marine algae from New Zealand and the characterization of their antiviral activity against HSV. Of particular interest are polysaccharide extracts that have been found previously to have antiviral activity *in vitro*. These include carrageenans from the red alga *Gigartina atropurpurea* and galactofucans and fucans from the brown algae *Undaria pinnatifida* and *Splachnidium rugosum*, respectively. Sulfated carrageenans isolated from the gametophytic stage of the endemic New Zealand red alga *G. atropurpurea* contain a kappa-2 type carrageenan which comprises kappa- and iota-carrageenans together with their biochemical precursors, the mu- and nu-carrageenans. The tetrasporic form contains a lambda-type carrageenan, which is more highly sulfated than the kappa-2 carrageenans from the gametophytic plants (Falshaw et al., 2003). The lambda-type carrageenan from the tetrasporic form of *G. atropurpurea* is unusual in that it contains a significant amount (around 10%) of unsulfated 3,6-anhydro-galactosyl units that are not normally associated with lambda-carrageenans (Falshaw et al., 2003). The sporophylls and blades of *U. pinnatifida* contain a sulfated galactofucan with a structure that has been partially determined (Hemmingson et al., 2006). *S. rugosum* is found only in the Southern hemisphere and contains a sulfated fucan (Miller and Blunt, 2003). The structure of polysaccharides isolated from *Plocamium cartilagineum* have not been described to our knowledge. Extracts from each of these species exhibit antiviral activity and can directly inactivate HSV-2.

2. Materials and methods

2.1. Algal material

Samples of *G. atropurpurea* and *U. pinnatifida* were collected from the Marlborough Sounds, New Zealand, in September 2005 and April 2006, respectively. The sample of *P. cartilagineum* was collected from Marfells Beach, Marlborough, New Zealand in December 2005. *S. rugosum* was collected from Owhiro Bay, Wellington, New Zealand, in April 2006. All algal material was air-dried and ground prior to extraction. Samples were extracted from combined sporophyll and leaf tissues of *U. pinnatifida*; sporophytes of *S. rugosum*; female gametophytes of *G. atropurpurea*; tetrasporophytes of *G. atropurpurea*; and an unidentified life phase of *P. cartilagineum* (Table 2). A bulk sample of galactofucan sulfate (GFS) extracted from the sporophyll of *U. pinnatifida*, by New Zealand Pharmaceuticals Limited (Lot # 5403020), was received in October 2003. This sample of GFS was extracted from algal material sourced by New Zealand Pharmaceuticals Limited.

2.2. Isolation of algal extracts

U. pinnatifida and *S. rugosum*. Extracts from these species were prepared using three different extraction methods based on those described by Usov and colleagues (Usov et al., 1985, 2005) (Table 2). Three extracts from each species were prepared by extracting dry ground samples for 6 h with 1% (w/v) H_2SO_4 at 20 °C, 0.2 M HCl at 20 °C, or 2% $CaCl_2$ at 75 °C. Each extract was centrifuged (3000 × *g*, 20 min.), the supernatant was neutralized with 10% NaOH then dialyzed against several changes of distilled water (pH adjusted to 7.5 with 0.5 M sodium carbonate) to remove salts and lyophilized.

G. atropurpurea and *P. cartilagineum*. Extracts were prepared using a modification of the methods of Falshaw and Furneaux (1994) (Table 2). Samples of dry, ground algae (3 g) were placed in $NaHCO_3$ solution (0.05 M, 180 ml), left to swell (20 min) then heated (90 °C, 2 h). The hot extract was filtered through a Whatman GF/D filter (with a non-woven nylon filter above), the filtrate dialyzed against several changes of distilled water (pH adjusted to 7.5 with 0.5 M sodium carbonate) to remove salts and then lyophilized.

2.3. Partial hydrolysis of galactofucan sulfate and determination of the molecular weight

A 100 ml aqueous solution of GFS (24%, w/v) was hydrolyzed using $NaHSO_4$ (0.2 M, 44 ml) and stirred at 20 °C for 20 min, then

Table 2
Characteristics of algal extracts.

Sample number	Species	Life phase	Extraction medium	Polysaccharide type
1	*Undaria pinnatifida*	Sporophyte (sporophyll and leaf)	$CaCl_2$	Sulfated galactofucan
2	*Undaria pinnatifida*	Sporophyte (sporophyll and leaf)	HCl	Sulfated galactofucan
3	*Undaria pinnatifida*	Sporophyte (sporophyll and leaf)	H_2SO_4	Sulfated galactofucan
4	*Splachnidium rugosum*	Sporophyte (whole)	$CaCl_2$	Sulfated fucan
5	*Splachnidium rugosum*	Sporophyte (whole)	HCl	Sulfated fucan
6	*Splachnidium rugosum*	Sporophyte (whole)	H_2SO_4	Sulfated fucan
7	*Undaria pinnatifida*	Sporophyte (sporophyll)	H_2SO_4 then $NaHSO_4$	Depolymerized galactofucan sulfate (44 kDa)
7[a]	*Undaria pinnatifida*	Sporophyte (sporophyll)	H_2SO_4	Galactofucan sulfate (670 kDa)
8	*Gigartina atropurpurea*	Female gametophyte	$NaHCO_3$	Sulfated galactan (kappa-2 type carrageenan)
9	*Gigartina atropurpurea*	Tetrasporophyte	$NaHCO_3$	Sulfated galactan (unusual lambda-type carrageenan)
10	*Plocamium cartilagineum*	Unknown	$NaHCO_3$	Complex sulfated galactan

[a] Unhydrolyzed form of extract 7; GFS prepared by New Zealand Pharmaceuticals Limited.

heated to 47 °C and held at this temperature for 219 min with continuous stirring. After this time, the reaction mixture was cooled on ice, neutralized with 2.5 M NaOH and diluted to 50 mg/ml. The solution was then heated to 65 °C with continuous stirring. The solution was then removed from the heat source and left to cool to 20 °C. The solution was centrifuged at 7000 × *g* for 30 min and the supernatant was decanted, dialyzed and lyophilized.

The molecular weights of the GFS and the partially hydrolyzed material (Table 2) were determined by high performance size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS). The SEC-MALLS system consisted of a Waters 2690 Alliance separation module, a Waters 490E programmable multi-wavelength detector set at 280 nm, a DAWN-EOS multi-angle laser light scattering detector with a laser at 690 nm (Wyatt Technology Corp., Santa Barbara, CA) and a Waters 2410 refractive index monitor. Samples (2 mg/ml in 0.1 M $LiNO_3$) were heated (90 °C, 15 min or until dissolved) and then stirred at room temperature for 24 h. Samples were centrifuged before injection (100 μl) and eluted with 0.1 M $LiNO_3$ containing 0.02% NaN_3 (0.7 ml/min) from two columns (TSK-Gel G5000PWXL and G4000PWXL, 300 mm × 7.8 mm, Tosoh Corp., Tokyo, Japan) connected in series at 60 °C. Data for molecular weight determinations were analyzed using ASTRA software (Version 4.73.04, Wyatt Technology Corp.) and a dn/dc of 0.145 ml/g. The molecular weight of the partially hydrolyzed extract was also estimated by comparison of peak elution positions with pullulan standards (12.2, 23.7, 48, 100, 186 and 380 kDa).

2.4. Cells and viruses

Human foreskin fibroblast (HFF) cells were used in all assays and were grown in growth medium consisting of minimum essential medium (MEM) with Earle's salts supplemented with 10% fetal bovine serum (FBS) and standard concentrations of penicillin and gentamycin. The E-377 strain of HSV-1 and the MS strain of HSV-2 were used for cytopathic (CPE), and plaque assays. The MS strain of HSV-2 was used for the direct inactivation assays and adsorption, attachment and penetration assays were optimized and performed with the G strain of this virus.

2.5. Cytopathic effect (CPE) reduction assay

The assessment of antiviral activity using CPE reduction assays was performed as described previously (Williams-Aziz et al., 2005). Briefly, HFF cells were seeded in 96-well plates at a concentration of 2.5×10^4 cells/well and incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h. After incubation, the medium was removed and 125 μl of the extracts were added to the first row in triplicate wells and all other wells contained 100 μl of growth media. Fivefold serial dilutions were then performed in the plate with a BioMek 2000 liquid handling machine. Cells were then infected with 100 μl of a virus suspension containing 1000 plaque forming units (PFU)/ml of either HSV-1 or HSV-2 and the plates were incubated at 37 °C for 4 days. Monolayers were stained with a 0.1% crystal violet solution and the optical densities were measured on a BioTek microplate reader at 630 nm.

2.6. Plaque reduction assay

Plaque reduction assays were performed as reported previously (Williams-Aziz et al., 2005). In brief, 6-well plates containing monolayers of HFF cells were infected with a virus suspension to yield 20–30 plaques per well. The virus was allowed to adsorb for 1 h, then the inocula were removed and dilutions of test extracts in growth media were added to the appropriate wells. After an incubation period of 3 days, the cells were stained with 0.1% crystal violet in 20% methanol and the plaques enumerated using a stereomicroscope. Concentrations of the extracts sufficient to reduce plaque number by 50% (EC_{50} values) were calculated by standard methods (Prichard and Shipman, 1990).

2.7. Plaque reduction assay with pretreatment

Plaque reduction assays using a pretreatment protocol were conducted by similar methods to the plaque reduction assays described above except that the test extract dilutions were incubated with the cell monolayers prior to infection. The medium was aspirated from the wells of 6-well plates containing monolayers of HFF cells and the extract dilutions in growth medium with 2% FBS were added to the appropriate wells. The plates were incubated for 1 h, and then the drug dilutions were aspirated from the wells and the monolayers were rinsed with phosphate-buffered saline (PBS) solution prior to infection with 20–30 PFU of the virus. The virus was allowed to adsorb for 1 h at 37 °C then extract dilutions were added again to each well and the infected monolayers incubated for 3 days. Plaque numbers were counted as described above and EC_{50} values were calculated.

2.8. Virus adsorption assay

Test extracts were serially diluted 1:5 to yield six concentrations in a final volume of 0.4 ml of growth medium. Equal volumes of the extract dilutions and a virus suspension at a concentration of 40–60 PFU/ml were placed in a tube and the mixtures were incubated at 37 °C for 1 h. The samples were then placed on monolayers of HFF cells and the virus was allowed to adsorb in the presence of the extracts. Adsorption efficiency was assessed by counting plaques, as described above for the plaque reduction assay. Pooled human immunoglobulin (IgG) (Polygam® S/D, Baxter Healthcare Corp. Hyland Immuno, Glendale, CA), which contains neutralizing antibody served as a positive control.

2.9. Virus attachment assay

Dilutions of the test extracts were added to each well of 6-well plates containing monolayers of HFF cells and the plates were incubated at 4 °C for 1 h. Extract solutions were then removed and virus suspensions containing virus to yield 20–30 plaques per well were added to each of the wells. Plates were incubated at 4 °C for 2 h to allow attachment, then monolayers were rinsed three times with cold PBS to remove the unbound virus. Growth medium was then added to each of the wells and the plates were incubated at 37 °C for 3 days. Plaques were counted and EC_{50} values were calculated, as described above. Dextran sulfate (Sigma Aldrich, St Louis, MO) served as a positive control.

2.10. Virus penetration assay

Virus suspensions were prepared on ice to yield 20–30 plaques per well on monolayers of HFF cells in 6-well plates. Suspensions were added to the plates, which were incubated at 4 °C for 2 h to allow attachment. Dilutions of each test extract were then added to the appropriate wells at room temperature and plates were incubated for 10 min at 37 °C to allow penetration. Dilutions of extracts were then aspirated and the monolayers were briefly washed with PBS at a pH of 3.0 to inactivate virions that had not penetrated the cells. Growth medium was then added to each of the wells and plates were incubated at 37 °C for 3 days. Plaques were enumerated and EC_{50} values were calculated as described above.

2.11. Virus inactivation assay

Dilutions of the test extracts were incubated with a concentrated virus suspension at 37 °C for 1 h. The samples were then placed on ice, the infectious virus remaining in the samples were diluted by 100-fold and the titer of infectious virus remaining in the suspension was quantified by plaque assay. This dilution essentially eliminated the potential effect of the remaining extract on subsequent binding events. In these studies, human IgG was used as a positive control.

2.12. Evaluation of cytotoxicity

The toxicity of each test extract to HFF cells was evaluated in a neutral red uptake assay by methods described previously (Williams-Aziz et al., 2005). Briefly, 2.5×10^4 cells were added to each well of a 96-well plate and incubated for 24 h. The growth medium was replaced and 1:5 serial dilutions of each extract were performed directly in the plate using a BioMek 2000 liquid handling machine. The plates were incubated for 7 days. The medium was then aspirated and neutral red stain in PBS was added and incubated for 1 h. Cell monolayers were washed and the dye contained in the cells was solubilized with a solution of 50% ethanol and 1% glacial acetic acid. The plates were mixed for 15 min on a rotating shaker and the optical densities were read at 550 nm. Concentrations of the extracts sufficient to reduce viability by 50% (CC_{50} values) were calculated.

3. Results

3.1. Antiviral activity of polysaccharide extracts

The antiviral activity of each of the polysaccharide extracts listed in Table 2 was evaluated initially in a standard CPE reduction assay where the extracts were added prior to virus infection. This initial analysis identified which among the many extracts tested exhibited antiviral activity against HSV-1 or HSV-2 and were nontoxic at the concentrations tested (Table 3). This apparent antiviral activity required confirmation by plaque reduction assays, which are a much better estimator of antiviral activity. Standard plaque reduction assays, in which the extracts were added following infection, were unable to detect any activity and suggested that the inhibition of virus replication may occur during the adsorption phase of the infection (Table 3). To test this hypothesis, extracts were incubated with the cell monolayers then removed prior to infection in the pretreatment plaque reduction assay. In this assay, antiviral activity was observed with all the extracts against either HSV-1 or HSV-2 and indicated that the polysaccharides needed to be present prior to the adsorption of the virus in order to exert their effect (Table 3). This was expected given the nature of the samples. These results were significant because the extracts had no antiviral activity per se, but inhibited viral adsorption even if they were removed prior to adsorption. The fact that the extracts retained activity under these conditions might indicate that the extracts are stably bound to virus receptors on the cell surface, but could also be a result of other inhibitory mechanisms.

Each of the three extracts from the sporophyll and leaf tissues of *U. pinnatifida* sporophytes inhibited both viruses in the plaque reduction assay when they were added prior to infection (Table 3), and was more effective against the MS strain of HSV-2 than the E-377 strain of HSV-1, which is consistent with results reported previously for multiple strains of these viruses (Thompson and Dragar, 2004). Extracts from *S. rugosum* also exhibited good antiviral activity and were also more effective against the MS strain of HSV-2 than the E-377 strain of HSV-1. However, the potency of the *S. rugosum* extracts was more modest than that of the *U. pinnatifida*

Table 3
Antiviral activity of algal extracts against HSV-1 and HSV-2.

Sample number	CPE assay[a]		Plaque reduction assay		Pretreatment plaque reduction assay[b]		Neutral red assay
	HSV-1	HSV-2	HSV-1	HSV-2	HSV-1	HSV-2	
1	2.3	3.1	>50	>50	7.2 ± 2.5	2.7 ± 1.3	>100
2	1.0	0.5	>50	>50	5.7 ± 2.9	1.7 ± 0.3	>100
3	7.1	1.1	>50	>50	5.9 ± 1.2	1.7 ± 0.3	>100
4	95	0.6	>50	>50	7.9 ± 0.7	6.6 ± 1.0	>100
5	44	7.2	>50	>50	29 ± 10	5.3 ± 1.8	>100
6	3.8	2.1	>50	>50	8.9 ± 0.5	5.5 ± 0.9	>100
7	42	0.5	>50	>50	33 ± 7.1	3.6 ± 2.9	>100
7[c]	0.4	0.5	–	–	8.0 ± 0.4	1.6 ± 0.3	>100
8	3.1	1.7	>50	>50	36 ± 15	2.4 ± 2.4	>100
9	15	8.3	>25	>25	1.5 ± 0.5	0.7 ± 0.1	>100
10	0.5	28	>50	>50	5.4 ± 1.3	2.4 ± 1.5	>100
ACV	0.2	0.3	0.3	0.7	0.3 ± 0.1	0.4 ± 0.1	>100

[a] Values shown from a single experiment represent the concentration required to inhibit plaque formation by 50% and are given in units of μg/ml.
[b] Each value represents the average of at least two experiments with standard deviation values shown and are given in units of μg/ml.
[c] Unhydrolyzed form of extract 7.

extracts tested. The partially hydrolyzed sample of GFS (44 kDa), commercially extracted from the sporophyll tissues of *U. pinnatifida* sporophytes and subsequently chemically degraded, also showed more modest antiviral activity, while the unhydrolyzed extract 7 (670 kDa) was more active. The polysaccharides from the red algae tested were also active, particularly the lambda-type carrageenan isolated from the tetrasporophyte form of *G. atropurpurea*, which had the best activity of all the samples tested. By comparison, the kappa-2 type carrageenan isolated from the gametophyte form of *G. atropurpurea* had more modest activity. Good activity was also observed with the extract from *P. cartilagineum* and was comparable to that observed with extracts from the brown algae. None of the extracts tested were toxic in a neutral red uptake assay, which confirmed that the antiviral effects observed were specific and were not the result of cytotoxicity.

3.2. Inhibition of viral adsorption

Studies described above showed that the pretreatment of cell monolayers with the extracts inhibited plaque formation even when they were removed prior to viral adsorption. Additional assays were performed with extracts added directly to virus suspensions both prior to and during adsorption to assess their effects. Under these conditions, the apparent efficacy of the polysaccharides was increased 10-fold (Table 4). These data indicated that the extracts were more effective if they were present both prior to and during the adsorption phase of infection and prompted additional studies to investigate specific effects on attachment, penetration and direct inactivation of virions.

3.3. Inhibition of virus attachment

The antiviral effects of the polysaccharides observed in the plaque reduction assay with pretreatment did not appear to be related to the virucidal activity of the extracts because they were not present during the adsorption phase of the infection. To further characterize the effects of the polysaccharides, a subset of the extracts was evaluated for their ability to inhibit attachment of HSV-2 to the host cells and was similar to methods reported previously, but the extracts were removed prior to infection (Wachsman et al., 2003). Data from plaque reduction assays with pretreatment showed that the extracts could be removed prior to infection without diminishing activity, and thus this assay could distinguish inhibition of attachment from direct virucidal effects. Each of the polysaccharides tested exhibited good activity in this assay and three of the samples were more effective than the dextran sulfate positive control (Table 4). The efficacy of dextran sulfate in this assay was also similar to that reported previously and confirmed that results from the assay were accurate (Baba et al., 1988). These results indicated that the extracts can interfere with the adsorption of the virus to the monolayers, and that the polysaccharides appeared to interact stably with the cell substrate.

Table 4
Effect of algal extracts on virus adsorption, attachment, penetration and inactivation of HSV-2.

Sample number	Adsorption[a]	Attachment[a]	Penetration[a]	Inactivation[a]
1	0.3 ± 0.05	0.95 ± 0.5	>26 ± 33	–
2	0.2 ± 0.11	–	–	–
3	0.3 ± 0.13	1.3 ± 0.07	>38 ± 17	2.2 ± 2.2
4	0.3 ± 0.16	–	–	–
5	0.3 ± 0.18	0.7 ± 0.4	>50 ± 0	0.4 ± 0.3
6	0.1 ± 0.02	–	–	–
7	0.4 ± 0.15	–	–	8.0 ± 1.6
7[b]	–	–	–	5.1 ± 1.8
8	0.1 ± 0.19	0.5 ± 0.5	45 ± 7.4	0.2 ± 0.1
9	0.2 ± 0.23	–	–	–
10	0.2 ± 0.19	0.55 ± 0.07	>30 ± 28	0.4 ± 0.3
Dextran sulfate	–	1.9 ± 1.3	>100 ± 0	–
IgG	0.06 ± 0.02	–	–	0.2 ± 0.2

[a] Values shown represent the concentration required to inhibit plaque formation by 50% and are given in units of μg/ml. Each value represents the average of at least two experiments with standard deviation values shown.
[b] Unhydrolyzed form of extract 7.

3.4. Inhibition of penetration

A set of experiments was also conducted to examine the effect of some of the polysaccharides on the penetration of the virus by methods described previously (Wachsman et al., 2003). In these experiments, the virus was incubated with cells at 4 °C for 2 h to allow attachment of the virus prior to the addition of the extracts. Polysaccharide dilutions were added during a 10 min penetration step at 37 °C, and then removed and replaced with growth media. None of the extracts inhibited virus penetration and indicated that polysaccharides must be present either before, or during, the virus adsorption phase to exhibit activity.

3.5. Virus inactivation

Some sulfated polysaccharides, such as lambda-carrageenans, exhibit virucidal activity *in vitro* and this property has been shown to be associated with increased activity in animal models (Carlucci et al., 2004). A subset of extracts was evaluated in a virus inactivation assay and all inactivated virions at concentrations within the range of 0.2–8 μg/ml (Table 4). Extracts from *S. rugosum*, *G. atropurpurea*, and *P. cartilagineum* exhibited levels of virucidal activity that were greater than those from *U. pinnatifida* and were comparable to those of the IgG controls. The unhydrolyzed and partially hydrolyzed samples of GFS exhibited the lowest levels of virucidal activity of the extracts tested, with the unhydrolyzed sample (670 kDa) having slightly better activity than the hydrolyzed sample (44 kDa). The EC_{50} values for these extracts were approximately 10-fold lower than those in the plaque reduction assay using pretreatment. This is also consistent with virucidal effects, since extracts were removed from the cell monolayers prior to the adsorption period, and were not replaced until 1 h following infection in the pretreatment assay.

4. Discussion

Sulfated polysaccharides isolated from many marine algae possess species specific structural variations that appear to impact their antiviral activity against HSV-1 and HSV-2 (Table 1). Most of the extracts have been reported to be effective against HSV-1 and HSV-2 at concentrations of about 1 μg/ml, but the efficacy varied depending on the species from which they were derived, and this is consistent with the notion that species specific structural variation impacts their efficacy. The number of species tested thus far is relatively small and there are likely many more polysaccharides with useful properties that remain uncharacterized (Schaeffer and Krylov, 2000). Antiviral activity was observed with each of the 10 samples examined in the present study. This was interesting given the range of structures (galactans, fucans and galactofucans), sulfation patterns (e.g. kappa-2 and lambda-carrageenans) and molecular weights (e.g. GFS with and without depolymerization) of the polysaccharides tested here. These results support the theory that the biological activity of sulfated algal polysaccharides is determined by a combination of structural features of which degree of sulfation, distribution of sulfate groups in the carbohydrate backbone and molecular weight are undoubtedly important

but other factors such as constituent sugars, molecular conformation and stereochemistry are also involved (Damonte et al., 2004; Lüscher-Mattli, 2000). Variation in the efficacy of the samples was observed, indicating that structural variations between the extracts from different species and, in the case of *G. atropurpurea*, between the life stages of a single species affect their antiviral activity. While the activity of *U. pinnatifida* extracts had been reported previously, the anti-viral activity of polysaccharide extracts from *S. rugosum*, *G. atropurpurea* and *P. cartilagineum* has to our knowledge not been reported previously.

Each of the extracts examined also exhibited potent virucidal activity, and was encouraging since this property has been associated with increased activity *in vivo* (Carlucci et al., 2004). The virucidal activity of sulfated polysaccharides is believed to be caused by the formation of a stable virion-sulfated polysaccharide complex where binding is not reversible and hence the sites on the viral envelope required for virus attachment to host cells are occupied by the sulfated polysaccharide (Damonte et al., 2004). The observation that all the extracts tested exhibited potent virucidal activity was unexpected given that there are few reports in the literature of sulfated polysaccharides possessing the ability to directly inactivate viruses. Additionally, the extracts tested in the present study all inactivated HSV-2 at lower concentrations than have been reported in the literature for other sulfated algal polysaccharides against herpes simplex viruses (Mandal et al., 2008; Pujol et al., 2002; Talarico et al., 2004; Zhu et al., 2004) with the exception of the lambda-type carrageenan from *Gigartina skottsbergii* which similarly inactivated HSV-2 at concentrations as low as 0.5 μg/ml (Carlucci et al., 1999). Of the extracts tested in the present study, the unhydrolyzed and partially hydrolyzed samples of GFS had the least virucidal activity, with the unhydrolyzed sample inactivating HSV-2 at slightly lower concentrations (5.1 μg/ml) than the partially hydrolyzed sample (8.0 μg/ml). The antiviral activity of sulfated polysaccharides is reported to increase with the molecular weight as well as with the sulfate content (Damonte et al., 2004; Duarte et al., 2001; Witvrouw and De Clercq, 1997). In the present study, the unhydrolyzed sample of GFS (670 kDa) was more active against HSV-1 and HSV-2 in the plaque reduction assay when added prior to infection compared with the hydrolyzed sample (44 kDa). However, results indicate that the considerable difference in the molecular weights of the hydrolyzed and unhydrolyzed GFS samples had relatively little effect on their virucidal activity. A possible explanation for this could be that the virucidal activity increases with the molecular weight up to a particular value, e.g. 100 kDa, after which the virucidal activity levels off, though further research would be needed to determine this.

The mechanism of virus inhibition was investigated for a subset of the extracts and each of them appeared to inhibit viral replication in a similar manner. Direct inactivation of virus clearly occurred, but the extracts also appeared to inhibit the attachment of virions to the cell surface. This effect was observed when HFF cells were pre-incubated with dilutions of the extracts and resulted in the inhibition of binding events even though the drug was removed prior to HSV infection. One interpretation of this result is that the sulfated polysaccharides interact with the cell monolayers in a stable manner and competitively inhibit binding sites typically utilized by the virus. This result was confirmed in a subset of the extracts by real-time PCR where the incubation of monolayers with dilutions of one extract, prevented the binding of virions as measured by the presence of viral DNA (data not shown).

Similar results have been reported for many polysaccharides isolated from other species of marine algae. Carrageenans (sulfated galactans) are the active constituents isolated from some species of red marine algae. The structures of these polysaccharides have been well characterized, and carrageenans are classified into different families based on their idealized disaccharide repeat units (Damonte et al., 2004). Variations in carrageenan structures occur among species of red algae and may also occur among the life stages of a single species, as is the case with Gigartinacean algae including *G. atropurpurea* (Falshaw et al., 2003). Lambda-carrageenans, such as 1T1 isolated from tetrasporic *G. skottsbergii*, exhibit good antiviral activity (Baba et al., 1988; Carlucci et al., 1997, 1999). This activity involves inhibition of virus adsorption and, as mentioned previously, 1T1 was also found to have virucidal activity against HSV-1 and HSV-2 (Carlucci et al., 1999). The kappa/iota- and mu/nu-carrageenans isolated from cystocarpic *G. skottsbergii* were also active against HSV-1 and HSV-2; the mu/nu-carrageenans were more active, but their activity appeared to be mediated exclusively by the inhibition of virus adsorption (Carlucci et al., 1999). Similarly, a carrageenan extract from tetrasporic *Stenogramme interrupta*, composed of predominantly lambda- and xi-carrageenans, was more highly active against HSV-1 and HSV-2 than the predominantly iota-carrageenan extract from cystocarpic plants; yet neither of these extracts had virucidal activity (Cáceres et al., 2000). The differences in activities of carrageenans are thought to be largely associated with differences in their primary structure including (i) the content of α-galactose 2,6-disulfate residues which is highest in lambda- and nu-carrageenans, (ii) the molar ratio of galactose to 3,6-anhydrogalactose which is highest in lambda- and mu/nu-carrageenans, and (iii) the position of the sulfate group in the β-galactose residues which is on C-2 in lambda-carrageenans and on C-4 in kappa/iota- and mu/nu-carrageenans (Carlucci et al., 1997, 1999).

Brown marine algae typically contain complex sulfated fucans with heterogeneous chemical structures that vary among species (Berteau and Mulloy, 2003). Due to the complexity of their structure and because most studies of biological activity are conducted using relatively crude fucan extracts, the relationship between structure and activity has not been firmly established for these compounds (Adhikari et al., 2006; Berteau and Mulloy, 2003). Sulfated fucans from various species of brown algae including *Sargassum horneri* (Hoshino et al., 1998), *Stoechospermum marginatum* (Adhikari et al., 2006), *Cystoseira indica* (Mandal et al., 2007), and sulfated galactofucans from *U. pinnatifida* (Thompson and Dragar, 2004) have been reported to inhibit *in vitro* HSV replication, presumably by inhibiting the adsorption and entry of the virus to the host cells. There are few reports of sulfated fucans from brown algae inactivating HSV as this property is either not studied or no direct inactivation is observed, although at high concentrations the polysaccharide from *Sargassum patens* (≥12.5 μg/ml) inactivated HSV-1 and HSV-2 (Zhu et al., 2004, 2006).

Typically, the antiviral activity of algal extracts observed *in vitro* has been predictive of results obtained in animal models, which have shown that they can reduce mortality when administered intraperitoneally immediately prior to infection with HSV-2 by the same route (Richards et al., 1978). Despite the inherent difficulties associated with the therapeutic use of sulfated polysaccharides that relate to their *in vivo* pharmacological properties (Lüscher-Mattli, 2000), there are numerous reports of the antiviral activity of sulfated algal polysaccharides in animal models. For example, a commercial lambda-carrageenan prepared from two species of *Gigartina*, *G. aciculaire* and *G. pistillata*, was shown to be effective in protecting mice from intra-vaginal infection with HSV-2 (Bourne et al., 1999). In a similar study, the lambda-carrageenan (1T1) from *G. skottsbergii* provided protection against HSV-2 when administered prior to infection and its activity was superior to a mu/nu-carrageenan suggesting that the virucidal activity of lambda-carrageenans may play an important role (Carlucci et al., 2004). Similarly, intraperitoneal administration of mu/nu-carrageenan from *G. skottsbergii* appeared to protect mice from HSV-1 infection when administered directly after HSV infection (Pujol et al., 2006). A iota/nu/kappa-carrageenan from

Gymnogongrus griffithsiae and a D-/L-hybrid galactan from *Cryptonemia crenulata* were found to inhibit the adsorption of HSV to cells and the D-/L-hybrid from *C. crenulata* protected mice from vaginal infection with HSV-2 when administered immediately prior to infection (Talarico et al., 2004). Oral administration of sulfated fucans from the edible brown alga *U. pinnatifida* also appeared to protect mice from HSV-1 challenge, and was hypothesized to be mediated by direct inhibition of viral replication and stimulation of innate or adaptive immune function (Hayashi et al., 2008).

One carrageenan-based topical microbicide under development, Carraguard, has been shown to be safe and well tolerated in both HIV-infected (van de Wijgert et al., 2007) and uninfected individuals (Kilmarx et al., 2008). Carrageenans appear to be very stable and do not affect the activity of nonoxynol-9, thus they might be capable of providing a measure of protection against infectious disease to existing spermicides (Maguire et al., 1998). However, results from a recent Phase 3 clinical trial of Carraguard did not show that it was effective in preventing HIV transmission during vaginal intercourse. Reasons for this are unclear, but it has been suggested that the overall low use of the microbicide during the trial likely impacted its efficacy (http://www.popcouncil.org/microbicides/). Nonetheless, the development of additional formulations is expected to continue (Turpin, 2002).

The addition of non-nucleoside reverse transcriptase inhibitor (NNRTI) MIV-150 to carrageenan appeared to improve its efficacy over Carraguard alone (Fernández-Romero et al., 2007). Conjugates of kappa-carrageenan and AZT also exhibited enhanced antiviral action against HIV and other such strategies should be feasible (Vlieghe et al., 2002). The identification of active polysaccharides from additional algal species, such as those described here, may also identify molecules with superior efficacy. Polysaccharides isolated from the four algal species described here should be considered as potential topical microbicides and each warrant additional studies to investigate their efficacy in animal models.

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