Pergamon

Biochemical Pharmacology, Vol. 47, No. 12, pp. 2187–2192, 1994.
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0006-2952/94 $7.00 + 0.00

0006-2952(94)E0082-W

ANTIVIRAL ACTIVITY OF A SULPHATED POLYSACCHARIDE FROM THE RED SEAWEED *NOTHOGENIA FASTIGIATA*

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(*Received* 28 *June* 1993; *accepted* 9 *February* 1994)

Abstract—The antiviral activity of polysaccharide fractions obtained from water extracts of the red seaweed *Nothogenia fastigiata* was investigated. Fraction F6, corresponding to a sulphated xylomannan, was found to inhibit efficiently the replication of herpes simplex virus type 1 (HSV-1). Furthermore, F6 selectively inhibited the replication of several other enveloped viruses including herpes simplex virus type 2, human cytomegalovirus (HCMV), respiratory syncytial virus, influenza A and B virus, Junin and Tacaribe virus and simian immunodeficiency virus. F6 was only weakly active against human immunodeficiency virus type 1 and 2. The mode of action of F6 against HSV-1 and HCMV could be ascribed to an inhibitory effect on virus adsorption.

Key words: sulphated polymers; antiviral; HIV; HCMV; HSV; influenza virus

Polysaccharides constitute a complex group of macromolecules known to possess a wide range of therapeutically important biological properties such as anticoagulant activity, antitumoral action, inhibition of DNA polymerase activity, interferon induction, immunomodulating and virus-inhibitory effects [1].

Although the antiviral properties of sulphated polysaccharides have been known for 30 years [2], interest in the antiviral potential of sulphated polysaccharides regained attention when more recent studies showed that several polyanionic substances were effective inhibitors of human immunodeficiency virus (HIV)§ replication [3]. Heparin, dextran sulphate, pentosan polysulphate, co-polymers of acrylic acid with vinyl alcohol sulphate (PAVAS), lentinan sulphate and sulphoevernan were shown to inhibit HIV replication in T4 cells [3–9]. Moreover, the antiretroviral activity of natural polysaccharides extracted from various sources has also been investigated [10, 11]. Herpes viruses, such as HSV types 1 (HSV-1) and 2 (HSV-2), and HCMV also proved susceptible to the antiviral action of naturally occurring and chemically synthesized polysaccharides [6, 12–15].

The present study deals with the antiviral activity of the water-soluble polysaccharide extracted from the red seaweed *Nothogenia fastigiata* [16], its antiviral activity spectrum and mechanism of antiviral action.

MATERIALS AND METHODS

Materials. The red seaweed *Nothogenia fastigiata* grows attached to the rocks, by means of a disc, in the middle littoral floor of the shores of Patagonia and Tierra del Fuego, Argentina. The sample was collected in water near the Estacion Algologica of Puerto Deseado in Southern Patagonia and was dried in the open air under strong winds. DS with a molecular weight of 5000 was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Separation and fractionation of the sulphated polysaccharides. Nothogenia fastigiata was extracted with boiling water as described by Matulewicz and Cerezo [16]. The water-soluble polysaccharides were fractionated with cetrimide. To a solution of the polysaccharides (4.95 g) in water (500 mL) a 10% (w/v) aqueous solution of cetrimide (50 mL) was added slowly with stirring. The complexed material was removed by centrifugation, suspended in water and subjected to fractional solubilization in sodium chloride solutions of increasing concentration. Finely divided sodium chloride was added with constant stirring so that its concentration was increased by 0.5–1.0 M each time. After each addition, stirring was continued overnight. Then the precipitate was centrifuged and discarded and the supernatant was extracted with 1-pentanol, dialysed, concentrated and freeze-dried. The upper limit of sodium chloride

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§ Abbreviations: CC_{50}, cytotoxic concentration 50%; $CCID_{50}$, cell culture infective dose 50%; CPE, cytopathic effect; DS, dextran sulphate; FCS, foetal calf serum; HCMV, human cytomegalovirus; HEL, human embryonic lung (cells); HIV, human immunodeficiency virus; HSV, herpes simplex virus; IC_{50}, inhibitory concentration 50%; MDCK, Madin Darby Canine Kidney (cells); MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline; RSV, respiratory syncytial virus; SIV, simian immunodeficiency virus.

Table 1. Yields and analyses of the fractions obtained through redissolution in sodium chloride of the cetrimide salts of the sulphated polysaccharides from the red alga *Nothogenia fastigiata**

Fraction	Range of redissolution (M NaCl)	Yield† (%)	Sulphate (% SO_3Na)	Sugar composition						Mannose: sulphate
				Rha	Ara	Xyl	Man	Gal	Glc	molar ratio
F1	0–0.5	22.9	12.9	3.0	2.6	18.6	12.1	54.4	9.3	0.4
F2	0.5–1.0	11.8	19.2	2.6	2.5	23.7	57.1	11.9	2.2	1.5
F3	1.0–1.5	22.9	23.4	–	1.7	23.2	68.4	6.7	–	1.5
F4	1.5–2.0	11.6	27.6	–	1.9	10.6	77.8	9.8	–	1.3
F5	2.0–3.0	13.9	27.3	–	2.4	2.2	82.8	12.6	–	1.4
F6	3.0–4.0	6.6	24.7	–	–	2.1	97.9	–	–	1.7
F7	4.0‡	10.3	14.0	1.1	4.4	10.3	17.0	64.5	2.7	0.6

* Data taken from Matulewicz and Cerezo [16].

† Yields for fractions 1–7 are given as percentages of the recovered water-soluble polysaccharides (38% of the total polysaccharides).

‡ Insoluble in 4.0 M sodium chloride.

concentration was 4.0 M and the residual precipitate was suspended in water. The suspension was dialysed and freeze-dried. Range of the sodium chloride concentrations and analysis of the seven fractions obtained (F1–F7) are given in Table 1.

Viruses and cells. The virus strains used were as follows: HSV-1 (F and KOS strains); HSV-2 (G strain); HCMV (Davis and AD-169 strains); HIV-1 (HTLV-III_B strain); HIV-2 (LAV-2_{ROD} strain); SIV (MAC_{251} strain); influenza A virus (Ishikawa/7/82 H3N2 strain); influenza B virus (Singapore 222/79 strain); RSV (strain Long); parainfluenza virus (type 3) strain VR-93; Junin virus (XJC13 strain); Tacaribe virus (TRLV11573 strain); polio virus type 1 (Sabin strain). Vero, HeLa, E_6SM, HEL and MDCK cells were grown in MEM containing 5 or 10% FCS. MT-4 cells and MOLT-4 clone 8 cells were grown in RPMI medium (Gibco) containing 10% FCS.

Cytotoxicity assay. The concentration required to reduce cell viability by 50% was measured by: (1) the MTT method for MT-4 cells, or (2) inhibition of cell growth as determined by the Coulter counter for the other cell systems, after 5 days of incubation at 37° in the presence of the compounds.

Antiviral assays. For HSV-1 and HSV-2, Vero cell monolayers grown in 24-well plates (6×10^5 cells/well) were infected with 100 PFU/well in the absence or presence of varying concentrations of the test compound, and then overlaid with MEM containing 0.5% methylcellulose with or without compound. After 3 days of incubation, plaques were counted.

For influenza virus and RSV, respectively, confluent MDCK or HeLa cell cultures were inoculated with 50-fold the $CCID_{50}$ in the presence of various concentrations of the compound. For parainfluenza virus, polio, Junin and Tacaribe virus, confluent VERO cells were inoculated with 100 $CCID_{50}$ in the presence of various concentrations of the compound. The mean number of cells per well in the 96-well plates was 1.2×10^5 for HeLa cells, 8.4×10^4 for Vero cells and 7.0×10^4 for MDCK cells. Virus-induced cytopathicity was recorded at 5 days post infection. CPE was scored under an inverted microscope [score 0 = 0% CPE; score 1 = <20% CPE; score 2 = 20–40% (CPE); score 3 = 40–60% (CPE); score 4 = 60–80% (CPE); score 5 = 80–100% (CPE)]. The 50% effective concentrations were estimated from graphic plots.

The anti-HCMV activity was determined by a cytopathic effect reduction assay, as described by Snoeck *et al.* [18]. Briefly, HEL fibroblasts (2.0×10^5 cells/well) were grown in 96-well microtiter plates and infected with 100 PFU of virus per well in the presence of the compounds and incubated for 7 days. CPE was scored as was described for the RNA viruses.

For the antiretroviral assays, MT-4 cells were infected with HIV-1 or HIV-2 (100 $CCID_{50}$/well) and MOLT-4 clone 8 cells were infected with SIV (100 $CCID_{50}$/well). Cultures were incubated in the presence of varying concentrations of the test compounds, and the number of viable cells was recorded after 5 days by the MTT method as described before [17].

In all cases, the IC_{50} was calculated as the concentration required to reduce virus induced cytopathicity by 50%.

Virus adsorption assays. The inhibitory effect of F6 on virus adsorption was measured by: (1) a virus plaque reduction assay for HSV-1 (strain F) in Vero cells upon different F6 treatment periods; (2) a HSV-1 (strain F) yield assay; (3) a binding assay of HCMV (strain Davis) to HEL cells, respectively.

1. Vero cells grown in 24-well plates were infected with 60 PFU of HSV-1 under different treatment conditions. In treatment A, the cells were exposed to HSV-1 in the presence of F6 (3.3 μg/mL) and after 1 hr virus adsorption at 4°, both compound and unadsorbed virus were removed, the cells were washed three times and were further incubated with MEM containing 0.5% methylcellulose. In treatment B, the cells were exposed to HSV-1 and after the virus adsorption period, unadsorbed virus was removed and the cells were further incubated with medium containing 3.3 μg/mL of F6. In treatment C, F6 was present both during and after the adsorption period. After 3 days of incubation, virus induced plaques were counted.

2. Vero cells were inoculated with HSV-1 at a MOI of 2 in the absence or presence of varying

concentrations of F6. After adsorption for 30 min at 4°, infected cells were washed three times with cold PBS and disrupted by freezing and thawing. The amount of infectious bound virus was then determined by titration.

3. The preparation of radiolabelled HCMV and the binding assay have been described previously [15]. Briefly, [methyl-^{3}H]dThd labelled HCMV was prepared from HCMV-infected HEL cells exhibiting 100% CPE. Confluent HEL cells in 96-well microtiter plates were incubated for 1–5 min at room temperature with 50 μL MEM or compounds dilution. Radiolabelled virus suspension (10 μL) (>10^8 pfu/mL and 5×10^5–10^6 cpm/mL) was then added for 30 min at 37°. Thereafter, cells were washed extensively with PBS and cell-associated radioactivity was determined.

Determination of HCMV-induced antigen in HEL cells. The method used was essentially the same as that described previously [15]. Briefly, HCMV-infected HEL cell cultures grown in 2-well tissue culture chamber slides were stained at 24 hr post infection with monoclonal antibody E_{13} (Biosoft, Paris, France) and were analysed by immunofluorescence microscopy.

HSV-1 internalization assay. Vero cells (1×10^5) grown in 35-mm Petri dishes plates were infected with 2×10^5 PFU of HSV-1 and the virus was allowed to adsorb to the cells for 60 min at 4°. Then, cells were incubated at 37° in MEM with or without F6 (25 μg/mL). At various times after infection internalized virus was determined by an infectious centre assay. To this end, cultures were washed with PBS and treated with proteinase K (0.5 mg/mL) in PBS for 45 min at 4° to remove external adsorbed virus. Protease treatment was stopped after adding 1 mM phenyl-methyl-sulphonyl-fluoride (PMSF) in PBS containing 3% bovine serum albumin; the cells were then pelleted by centrifugation and washed with PBS. The final pellet was resuspended in 1 mL of MEM and aliquots were layered on Vero cells. After 7 hr of incubation the cells were overlayed with MEM containing 0.5% methylcellulose. Plaques were counted at 3 days post infection.

RESULTS

Spectrum of antiviral activity of F6

All seven polysaccharide fractions were initially evaluated for their activity against HSV-1. Only F6 elicited marked anti-HSV-1 activity, its IC_{50} value being 0.6 μg/mL. F6 was then tested for its inhibitory effect on the replication of several RNA and DNA viruses. It also proved active against HSV-2, HCMV, influenza A virus, influenza B virus, RSV, Junin virus, Tacaribe virus, HIV-1, HIV-2 and SIV (Table 2). In comparison with other sulphated polysaccharides, such as DS, F6 showed a clearly different antiviral activity spectrum. The compound was only weakly active against HIV-1 and HIV-2, but, on the other hand, more active than DS against SIV. F6 was also more inhibitory than DS against influenza A and B viruses, although influenza B proved less sensitive to both compounds. F6 and DS showed similar activity against HSV-1 and HSV-2, but F6 was less active than DS against HCMV. F6 was only slightly less inhibitory than DS to the arena viruses Junin and Tacaribe, whereas both compounds were about equipotent against RSV. No activity was found with either F6 or DS against parainfluenza 3 and polio viruses.

The CC_{50} of F6 for the growth of Vero, HEL and HeLa cells was 100 μg/mL. The growth of MT-4 cells was reduced by 50% at a F6 concentration of 33 μg/mL.

Inhibition of virus adsorption

F6 was found to exert its anti-HSV activity by

Table 2. Inhibitory effect of F6 on the replication of several DNA and RNA viruses

Virus	Cell culture	IC_{50} (μg/mL)		CC_{50}* (μg/mL)	
		F6	DS	F6	DS
HSV-1 (F strain)	Vero	0.6	2.1	118	>200
HSV-1 (KOS strain)	Vero	1.5	1.9		
HSV-2	Vero	2.5	1.8		
HCMV (AD169 strain)	HEL	10	0.4	100	>200
HCMV (Davis strain)	HEL	2.8	0.1		
Poliovirus	Vero	>50	>200		
Influenza A virus	MDCK	0.2	8	ND†	ND†
Influenza B virus	MDCK	20	>200		
Parainfluenza 3 virus	Vero	>100	>200		
RSV	HeLa	0.9	1.6	100	>200
Junin virus	Vero	10	5		
Tacaribe virus	Vero	7.8	3.2		
HIV-1	MT-4	13.7	0.1	33	>500
HIV-2	MT-4	13.4	0.03		
SIV	MOLT-4	0.4	10		

* Determined by inhibition of cell growth for Vero, HEL and HeLa cells and the MTT assay for MT-4 cells.

† Not determined.

Table 3. Influence of various treatment periods on the anti-HSV-1 activity of F6

Treatment	F6 (μg/mL) present		Plaque number	Inhibition (%)
	During virus adsorption	After virus adsorption		
None	0	0	56	0
A	3.3	0	6	90
B	0	3.3	54	4
C	3.3	3.3	8	85

Vero cells were infected with 60 PFU of HSV-1. After 1 hr virus adsorption at 37° in MEM with or without F6 (3.3 μg/mL), unadsorbed virus and the compound were removed. The cells were overlaid with medium with or without F6, and further incubated for three days, whereafter the number of plaques was determined. Each value represents the mean of duplicate assays.

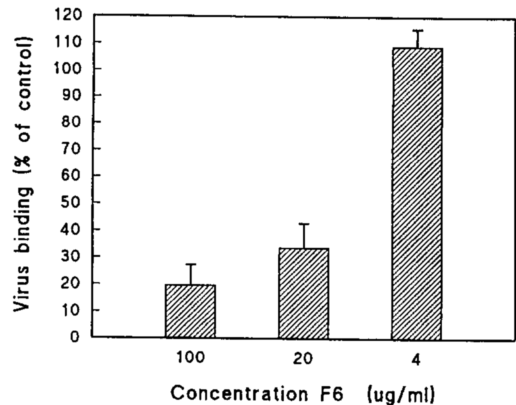

Fig. 2. Inhibitory effect of F6 on binding of radiolabelled HCMV (strain Davis) to HEL cells after a 30 min incubation period, as monitored by cell-associated radioactivity. Data are mean values for three separate experiments.

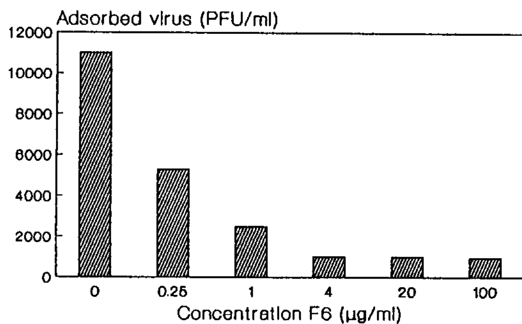

Fig. 1. Effect of F6 on HSV-1 adsorption. Vero cells were incubated for 30 min at 4° with HSV-1 in the presence of varying concentrations of F6. At the end of the incubation period, cell-bound infectivity was determined.

interference with a very early stage of the HSV-1 replication cycle, since replication was blocked only when the compound was present during the virus adsorption period. Further experiments were performed to determine whether F6 inhibited virus adsorption.

The compound was exposed to the cells either: (1) during the virus adsorption period only; (2) after virus adsorption only; or (3) both during and after virus adsorption. As can be concluded from the data presented in Table 3, F6 lost virtually all antiviral activity when not present during the virus adsorption period. The presence of only F6 during virus adsorption was as effective as the presence of the compound during the whole incubation period (pre and post adsorption).

The inhibitory effect of F6 on virus adsorption to the host cell was directly measured by monitoring the attachment of infectious HSV-1 to the cells in the presence of the compound. As shown in Fig. 1A, Vero cell associated infectivity was inhibited by F6 in a concentration-dependent manner with an IC_{50} of 0.25 μg/mL.

F6 inhibited binding of radiolabelled HCMV to HEL cells with an IC_{50} of 16 μg/mL (Fig. 2). DS inhibited binding of radiolabelled HCMV to HEL cells with an IC_{50} of 0.8 μg/mL (data not shown). Also, when present at the time of infection at 100 μg/mL, F6 reduced the expression of HCMV immediate early antigens at 24 hr post infection by 96% as compared to the control cultures.

Effect of F6 on virus internalization

To rule out that F6 also interfered with a post-adsorption event, the effect of F6 on HSV-1 penetration was examined. To this end, cells were first infected with HSV-1 at 4° to allow virus adsorption but not virus internalization. The cells were then incubated with F6 and the temperature was immediately raised to 37° to initiate virus entry. Under these conditions, the amount of internalized virus, monitored by the number of infectious centres after incubation at 37°, was similar in F6-treated and untreated cells. Thus, F6 did not appear to inhibit virus penetration.

DISCUSSION

Several sulphated polysaccharides extracted from the red seaweed *Nothogenia fastigiata* inhibited the multiplication of HSV-1 and HSV-2 in Vero cells. The structural analysis of these polysaccharides revealed that they were composed of xylomannan (fractions F2–F6) and xylogalactan (fractions F1 and F7). The structure of the xylomannan consisted of an α-D-(1,3)-linked mannan backbone, 2- and 6-sulphated, having single stubs of β-(1,2)-linked D-xylose [16]. The analyses of the xylogalactan obtained so far indicated a sulphated α-(1,3)-, β-(1,4)-linked galactan chain with D-xylose and galactose side chains (unpublished results). Neither arabinose nor rhamnose was detected after the methylation analysis of the fractions. Minor amounts of 2,3,6-tri-*O*-

methylglucose were detected in permethylated fractions, which suggested the presence of cellulose-like glucan residues [19].

The xylomannan F6 proved active against a wide variety of enveloped viruses. HSV type 1 and type 2, influenza A virus, RSV and SIV proved most sensitive to F6 with IC_{50} values that were 2- to 30-fold lower than for DS (5000 kDa). These values were at least 100-fold lower than the cytotoxic concentrations (Table 2). Thus, F6 can be considered as a selective inhibitor of these viruses.

Full inhibitory activity was achieved only when the compound was present during the virus adsorption period. Binding of radiolabelled HCMV to the cells was blocked by F6 and little or no infectious virus (HSV-1) was found to be associated with cells that had been incubated with F6 during the virus adsorption period. In addition, F6 was shown to prevent the expression of immediate early antigens of HCMV, which were expressed shortly after infection. Furthermore, F6 was found not to interfere with the virus internalization process, an event which took place after virus binding to the cells. The mechanism of action of F6 could thus be ascribed to inhibition of virus adsorption.

The results reported here are in agreement with previous studies on the antiviral activity of sulphated polysaccharides, such as DS and heparin. The mechanism of antiviral action of this type of compound has been attributed to an interaction with virus attachment to the cells [4, 15, 20, 21]. An exception to this mode of action has been reported for the carrageenans by Gonzalez *et al.* [14]. According to these authors carrageenans would inhibit a step in the HSV cycle following viral internalization but preceding the onset of late viral protein synthesis.

Recent studies have shown that heparan sulphate proteoglycans present on the cell surface may be serving as the initial receptors for HSV types 1 and 2, pseudorabies virus, bovine herpes virus and HCMV [15, 22–26]. Heparin was found to interact with viral glycoproteins which were involved in the initial binding of the virus to the cells. It may be assumed that F6, akin to other sulphated polymers, interacts with positively charged domains on these glycoproteins. This will result in a shielding-off of these domains and thus prevent the virus from binding to the negatively charged heparan sulphate [15].

An interesting issue that remains to be elucidated is why F6 has an antiviral activity spectrum that is different from that of DS, namely why is it more active than DS against influenza viruses, RSV and SIV but less active than DS against HCMV and HIV. This differential activity may be related to the distribution of the sulphated groups on the polysaccharide backbone. F6 should be further pursued for its potential in the treatment of those virus infections for which it shows the greatest specificity (i.e. HSV-1, influenza A, RSV and SIV).

Acknowledgements—This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina), the Universidad de Buenos Aires, the AIDS Basic Research Programme of the European Community and by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (project 3.0026.91) and the Belgian Geconcerteerde Onderzoeksacties (project 90/94-2). We thank Silvia Coronato, Hyun-Jung Kim, Anita Camps, Anita Van Lierde and Frieda De Meyer for their excellent technical assistance. J. Neyts is a post-doctoral research assistant from the Belgian National Fund for Scientific Research (NFWO).

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