Antiherpetic Mechanism of a Sulfated Derivative of *Agaricus brasiliensis* Fruiting Bodies Polysaccharide

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Key Words

Agaricus brasiliensis · Sulfated polysaccharide · Antiherpetic activity · Herpes simplex virus type 1 · Herpes simplex virus type 2 · Mechanism of action · Synergistic effect

Abstract

Objective: To study the anti-herpes simplex virus (HSV) activity of a (1→6)-(1→3)-β-D-glucan isolated from *Agaricus brasiliensis* fruiting bodies (FR) as well as its chemically sulfated derivative (FR-S). **Methods:** The antiherpetic activity and mechanism of action was studied by viral plaque assay applying different methodological strategies. **Results:** Although FR presented no in vitro antiherpetic action at 1 mg/ml, FR-S displayed promising anti-HSV-1 and anti-HSV-2 activities in both simultaneous and postinfection treatments, resulting in selectivity indices (CC₅₀/EC₅₀) higher than 393. FR-S had no virucidal effect, but significantly suppressed HSV-1 (EC₅₀ = 0.32 µg/ml) and HSV-2 (EC₅₀ = 0.10 µg/ml) adsorption. FR-S was less effective on adsorption inhibition of mutant virus strains devoid of gC (HSV-1 gC⁻39 and HSV-2

gCneg1), indicating a possible interaction with this glycoprotein. The reduction of viral adsorption upon cell pretreatment with FR-S also suggests its interaction with cellular components. FR-S inhibited HSV-1 (EC₅₀ = 8.39 µg/ml) and HSV-2 (EC₅₀ = 2.86 µg/ml) penetration more efficiently than heparin. FR-S reduced HSV-1 and HSV-2 cell-to-cell spread. A synergic effect between FR-S and acyclovir was also detected. **Conclusions:** FR-S displays an interesting mechanism of antiviral action and represents a promising candidate for the treatment and/or prevention of herpetic infections, to be used as a single therapeutic agent or in combination with acyclovir.

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Introduction

Herpes simplex virus (HSV) types 1 and 2 belong to the family Herpesviridae, subfamily Alphaherpesvirinae, and are responsible for a variety of mucocutaneous infections that are highly prevalent worldwide. Factors such as

the high morbidity in immunocompromised patients, the emergence of drug-resistant virus strains, and the undesirable side effects of the current antiviral chemotherapy have encouraged the search for new antiherpetic agents [1].

HSV attachment and entry into target cells, as well as virus cell-to-cell spread, entail the interaction of viral envelope glycoproteins with host cell surface receptors. These complex processes include a series of sequential steps and are essential for a productive infection in humans and, therefore, represent suitable targets for drug development. Three viral entry inhibitors have already been approved by the FDA: *n*-docosanol for orolabial herpes infections, and enfuvirtide and maraviroc for HIV-1 infections [2].

Agaricus brasiliensis Wasser et al. (syn. *A. subrufescens* Peck) is a Brazilian mushroom closely related to *A. blazei* Murrill (sensu Heinemann). In vitro and in vivo studies have shown mainly immunomodulatory [3–5], antitumoral [6], antigenotoxic [7], and antioxidant [3] activities for polysaccharides isolated from *A. brasiliensis* fruiting bodies and mycelia.

In the present study, we demonstrate the antiherpetic activity and mechanism of action of the sulfated derivative of a polysaccharide isolated from *A. brasiliensis* fruiting bodies chemically characterized as a sulfated (1→6)-(1→3)- β -D-glucan [8]. In previous studies, we demonstrated the in vitro and in vivo anti-HSV-1 and -HSV-2 activities of a sulfated derivative of a polysaccharide obtained from *A. brasiliensis* mycelia [9, 10].

Although some authors have reported the antiviral activity of *A. brasiliensis* extracts against Western equine encephalitis virus [11], HSV-1 [11, 12], bovine herpesvirus 1 [12, 13], and poliovirus 1 [14], as well as the anti-HSV activity of an *A. brasiliensis* polysaccharide and its sulfated derivative [15], the glucans described herein are structurally different and present diverse in vitro anti-HSV activity and modes of action.

Material and Methods

Fungal Material

A. brasiliensis fruiting bodies were collected in Biguaçu (Santa Catarina State, Brazil), and designated as strain UFSC 51. Species identification was performed by Professor Dr. Maria Alice Neves and a voucher specimen was deposited at the FLOR Herbarium (FLOR 11 797, UFSC) and at the Coleção Brasileira de Micro-organismos de Ambiente e Indústria – CBMAI/UNICAMP (<http://webdrm.cpqba.unicamp.br/catalogo/pycat/index.py>, code number: 1449). The dried fruiting bodies were ground, lyophilized, and stored at –80°.

Isolation of Fruiting Bodies Polysaccharide and Sulfation

A. brasiliensis cell-wall polysaccharide was obtained and sulfated as we reported previously [8]. Briefly, 50 g of lyophilized fruiting bodies were extracted with water at 100° (3 h), precipitated with ethanol, and centrifuged (1,100 g, 10 min). The obtained fruiting bodies polysaccharide (FR) was freeze-dried and chemically sulfated by the chlorosulfonic pyridine method. The highest molecular mass polysaccharides were separated by dialysis (membrane cutoff: 3.5 kDa) and freeze-dried generating the sulfated derivative (FR-S).

Cells and Viruses

Vero (ATCC: CCL 81, Rockville, Md., USA) and GMK-AH1 (Department of Clinical Virology, Göteborg University, Göteborg, Sweden) cells were grown in minimum essential medium (MEM, Cultilab, Brazil), supplemented with 10% fetal bovine serum (Cultilab), penicillin (100 U/ml), streptomycin (100 μ g/ml), and amphotericin B (25 μ g/ml; Cultilab), and maintained at 37° in a humidified 5% CO₂ atmosphere. The virus strains used were: HSV-1 KOS and HSV-1 29R (Faculty of Pharmacy, University of Rennes, France), HSV-1 gC-null variant gC-39 [16], HSV-2 strain 333 [17], and its gC-negative designated HSV-2 gCneg1 [18]. HSV-1 and HSV-2 were propagated in Vero and GMK-AH1 cells, respectively. Virus titers were determined by plaque assay and expressed as plaque-forming units (PFU/ml), as reported by Burleson et al. [19].

Cytotoxicity Evaluation

The cytotoxicity of samples on Vero and GMK-AH1 cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [20], with minor modifications. Briefly, 2×10^4 cells per well were cultivated in 96-well plates for 24 h. Then, the growth medium was replaced by 1:2 dilutions of each sample prepared in MEM. After 72 h of incubation, the medium was substituted by MTT solution (1 mg/ml) and the plates were further incubated for 4 h. MTT solution was removed and DMSO was added to dissolve formazan crystals. The optical densities were read at 540 nm and concentrations of the samples able to reduce cell viability by 50% (CC₅₀) were estimated by linear regression of concentration-response curves generated from the data. Dextran (DEX) 5 kDa, 410 kDa, and 670 kDa, acyclovir (ACV), and heparin (HEP) were purchased from Sigma (USA) and employed as controls. Stock solutions of tested compounds were prepared in MEM or in DMSO and maintained at –20° until use.

Evaluation of Antiherpes Activity (Plaque Number Reduction Assay)

For the plaque number reduction assay, confluent cells were infected with HSV-1 KOS or HSV-2 333 (100 PFU/well) at 37° for 1 h. Different concentrations of tested compounds were added either simultaneously with virus (simultaneous treatment) or after virus infection (postinfection treatment). After virus infection, plates were further incubated at 37° for 48 and 72 h for HSV-1 and HSV-2, respectively. Viral plaques were visualized by staining with naphthol blue-black (Sigma), and enumerated using a stereomicroscope. The concentrations of compound required to reduce plaque number by 50% (EC₅₀) and the respective selectivity indices (CC₅₀/EC₅₀) were calculated by standard methods [19].

Evaluation of Mechanism of Action

Virucidal Assay

The direct effect of FR-S, HEP, and ACV on HSV infectivity was assessed according to procedures described previously [10]. Equal volumes of tested compounds (20 µg/ml) and 4×10^4 PFU of HSV (KOS or 333) in serum-free MEM were coincubated for 20 min at 4, 22, or 37°. Ethanol (70% v/v) was used as a positive control. After incubation, mixtures were diluted 1,000 times in order to achieve noninhibitory concentrations. The residual infectivity of the tested compounds was then determined by plaque number reduction assay, as described above ('Evaluation of Antiherpes Activity').

Effects on Viral Adsorption

Four distinct experimental protocols (treatments A–D) were employed to assess the inhibition of HSV-1 (strains KOS and gC-39) and HSV-2 (strains 333 and gCneg1) adsorption by FR-S, HEP, and ACV.

Treatment A (Attachment Assay). Cell monolayers grown in 24-well culture plates were prechilled at 4° for 1 h and exposed to viruses (100 PFU/well) in the absence or presence of serial dilutions of the sample (FR-S) and controls (HEP and ACV). After incubation for 2 h at 4° (adsorption period), cells were washed with ice-cold PBS to remove compounds and unbound viruses. As a control, wells in which viruses had been preattached to cells were treated with citrate buffer (pH 3.0) to verify if incubation at 4° allowed only viral attachment avoiding viral entry. Subsequently, cells were overlaid with CMC medium (MEM containing 1.5% carboxymethylcellulose) and the inhibitory activity of FR-S, HEP, and ACV was determined by the plaque number reduction assay, as described above ('Evaluation of Antiherpes Activity').

Treatment B (Pretreatment of Virions). Virus suspensions were incubated with FR-S, HEP, or ACV for 2 h at 4°. After that, the mixtures were added to prechilled cell monolayers to allow viral attachment. After the adsorption period (2 h, 4°), the same procedures described for treatment A were performed.

Treatment C (Pretreatment of Cells). Confluent cells grown in 24-well culture plates were pretreated for 1 h at 37° with different concentrations of FR-S, HEP, or ACV and then washed with PBS. Afterward, cells were chilled at 4° for 1 h, and exposed to viruses at 4° for another 2 h. The inhibitory effect was then determined as described in treatment A.

Treatment D (Postattachment Assay). Prechilled cells were firstly incubated with virus suspensions at 4° for 2 h to allow a stable attachment of HSV to cells. Unbound viruses were removed by aspiration and cells were washed with ice-cold PBS. FR-S, HEP, or ACV was then added and plates were further incubated at 4° for another 2 h. Cells were washed with ice-cold PBS or citrate buffer, overlaid with CMC medium and the plaque number reduction assay was conducted to determine the inhibitory activity of tested compounds, as described above ('Evaluation of Antiherpes Activity').

Effects on Viral Penetration

The inhibition of HSV-1 (KOS or gC-39) and HSV-2 (333 or gCneg1) penetration was assessed as described by Cardozo et al. [10]. Briefly, prechilled Vero or GMK-AH1 cells were incubated with viruses for 2 h at 4°. After the removal of unbound viruses, the temperature was shifted to 37° to allow penetration when the cells were treated with different concentrations of prewarmed

FR-S, HEP, or ACV. After the penetration period (1 h, 37°), non-penetrated viruses were inactivated with citrate buffer (pH 3.0). Cells were washed twice with PBS, and CMC overlay medium was added to allow plaque formation. The EC₅₀ values were calculated based on the reduction of plaque number as described in 'Evaluation of Antiherpes Activity'.

Plaque Size Reduction Assay

The effect of tested compounds on HSV cell-to-cell spread was investigated as previously described [10]. In brief, infected cells (100 PFU/well) were treated with FR-S, HEP, or ACV at concentrations equivalent to their corresponding EC₅₀ values and incubated throughout the entire period of viral plaque development. Results were obtained by analyses of the images of 20 plaques per each sample concentration, and compared with the viral controls (treated with medium only). The area of each plaque was determined using Image J software (<http://rsb.info.nih.gov/ij/>).

Combination of FR-S and ACV

The effects of FR-S in combination with ACV was evaluated by plaque reduction assay as described above and according to the experimental design proposed by Chou [21]. Briefly, each compound alone or in combination was tested at different ratios of its corresponding EC₅₀ concentration (i.e. at EC₅₀ × 0.25, × 0.5, × 1, × 2, and × 4). The interaction degree between FR-S and ACV was calculated through the combination index (CI) equation, using CalcuSyn software (version 2.1, Biosoft®). According to the CI theorem, CI values <1, = 1, and >1 indicate synergism, additive effect, and antagonism, respectively.

Evaluation of Anticoagulant Activity

The anticoagulant activity of FR-S was assayed using the activated partial thromboplastin time (APTT) kit (Biotécnica, Brazil). Briefly, 50 µl of FR-S or HEP solution, prepared at 20 µg/ml in PBS, was added to 100 µl of pooled human plasma and 100 µl of APTT reagent. PBS was used as negative control. The mixtures were incubated for 3 min at 37°. After that, preheated calcium chloride (100 µl, 20 mmol/l) was added and clot time was recorded.

Statistical Analysis

Data are presented as means ± SD of three independent experiments. The differences between treatments were analyzed by one-way ANOVA followed by Tukey's test. A p value less than 0.05 was considered to be statistically significant.

Results

Antiviral Activity

The native (FR) and sulfated (FR-S) *A. brasiliensis* polysaccharides were initially evaluated in terms of cytotoxicity by assessing their effects on Vero and GMK-AH1 cell viability. Antiviral activity was evaluated by plaque number reduction assay using simultaneous and postinfection treatments. The results presented in table 1 reveal that FR did not reduced viral plaque numbers in com-

Table 1. Cytotoxicity and antiherpetic activity of *A. brasiliensis* polysaccharides (FR and FR-S)

Sample	Cytotoxicity		Simultaneous treatment				Postinfection treatment			
	VERO CC ₅₀ ¹	GMK AH1 CC ₅₀ ¹	HSV-1 (KOS)		HSV-2 (233)		HSV-1 (KOS)		HSV-2 (333)	
			EC ₅₀ ²	SI ³	EC ₅₀ ²	SI ³	EC ₅₀ ²	SI ³	EC ₅₀ ²	SI ³
FR	3,062±167.55	>2,500	NI	–	NI	–	NI	–	NI	–
FR-S	2,650.09±190.32	2,520.25±460.11	1.43±0.06	1,853	0.62±0.08	4,065	6.74±0.01	393	4.62±0.38	546
DEX (5 kDa)	>2,500	>2,500	NI	–	NI	–	NI	–	NI	–
DEX (410 kDa)	>2,500	>2,500	NI	–	NI	–	NI	–	NI	–
DEX (670 kDa)	>2,500	>2,500	NI	–	NI	–	NI	–	NI	–
HEP	>2,500	>2,500	1.15±0.30	>2,174	0.58±0.01	>4,310	12.68±1.13	>197	8.06±1.46	>310
ACV	>2,500	>2,500	NI	–	NI	–	0.51±0.05	>4,902	3.91±0.41	>639

Values represent the means ± SD of three independent experiments. NI = No inhibitory activity at the maximum tested concentration (1,000 µg/ml); DEX = dextran. ¹ 50% cytotoxic concentration (µg/ml). ² 50% effective concentration (µg/ml). ³ Selectivity index (CC₅₀/EC₅₀).

Table 2. Effect of *A. brasiliensis* sulfated polysaccharide (FR-S) on HSV adsorption and penetration

Treatment protocol	Virus (strain)	EC ₅₀ ¹		
		FR-S	HEP	ACV
(A) Attachment assay	HSV-1 (KOS)	0.32±0.05 ^b	0.33±0.04 ^b	NI
	HSV-2 (333)	0.10±0.04 ^a	0.07±0.01 ^a	NI
	HSV-1 (gC [–] 39)	2.07±0.06 ^c	2.16±0.41 ^c	NI
	HSV-2 (gCneg1)	0.51±0.13 ^b	0.54±0.03 ^b	NI
(B) Pretreatment of virions	HSV-1 (KOS)	0.20±0.01 ^c	0.23±0.03 ^c	NI
	HSV-2 (333)	0.08±0.04 ^b	0.03±0.01 ^a	NI
	HSV-1 (gC [–] 39)	1.00±0.05 ^d	0.91±0.04 ^d	NI
	HSV-2 (gCneg1)	0.11±0.01 ^b	0.02±0.01 ^a	NI
(C) Pretreatment of cells	HSV-1 (KOS)	68.26±2.21 ^b	>100.00	NI
	HSV-2 (333)	6.09±1.81 ^a	>10.00	NI
	HSV-1 (gC [–] 39)	3.49±1.56 ^a	>10.00	NI
	HSV-2 (gCneg1)	2.43±1.07 ^a	>10.00	NI
(D) Post-attachment assay	HSV-1 (KOS)	3.00±0.01 ^a	>10.00	NI
	HSV-2 (333)	7.95±0.93 ^b	>10.00	NI
	HSV-1 (gC [–] 39)	8.76±0.83 ^b	>10.00	NI
	HSV-2 (gCneg1)	6.70±1.11 ^b	>10.00	NI
(E) Penetration assay	HSV-1 (KOS)	8.39±0.36 ^c	13.34±3.64 ^d	NI
	HSV-2 (333)	2.86±0.83 ^a	6.47±0.21 ^b	NI
	HSV-1 (gC [–] 39)	9.53±0.90 ^c	17.99±4.06 ^d	NI
	HSV-2 (gCneg1)	1.97±0.21 ^a	5.21±0.69 ^b	NI

Values represent the means ± SD of three independent experiments. Statistical analyses (one-way ANOVA/Tukey's test) were performed for each assay (A–E) separately. Different letters in superscript following values indicate statistically significant differences with *p* < 0.05. NI = No inhibitory activity at the maximum concentration tested (10 µg/ml). ¹ 50% effective concentration (µg/ml).

parison to the untreated controls, even when tested at 1 mg/ml. Similarly, nonsulfated dextrans with different molecular weights (5, 410, and 670 kDa) had no antiherpetic activity at 1 mg/ml. Contrarily, FR-S inhibited HSV-1 KOS and HSV-2 333 replication in both treatments, with EC₅₀ values lower than 6.74 µg/ml.

Virucidal Activity

Preincubation of virus suspensions with FR-S, HEP, or ACV at 4, 22, or 37° had no significant inactivating effects on HSV-1 KOS and HSV-2 333, at the tested concentrations (data not shown). These results indicate that virucidal effects do not seem to be involved in the detected antiviral activity of FR-S. Ethanol (70% v/v) was used as control and presented 100% inhibition of residual virus infectivity.

Inhibition of Viral Adsorption and Penetration

The EC₅₀ values obtained from different adsorption and penetration assays are summarized in table 2. Results show that FR-S strongly inhibited viral adsorption in all treatments, while HEP was only effective in treatments A and B. Results regarding penetration assay, reveal that FR-S inhibited HSV penetration more significantly than HEP for all virus strains tested. As expected, ACV did not inhibit viral adsorption or penetration at the maximum concentration tested (10 µg/ml).

Inhibition of Viral Cell-to-Cell Spread

Inhibition of HSV cell-to-cell spread was evaluated by measuring viral plaque areas of treated and untreated controls. Figure 1a displays the results of mean plaque areas of infected cells (HSV-1 KOS or HSV-2 333) treated

Fig. 1. a Inhibition of HSV cell-to-cell spread by FR-S, HEP, and ACV at their corresponding EC₅₀ concentrations. FR-S, HEP, and ACV were evaluated at 6.74, 12.68, and 0.51 µg/ml for HSV-1 (KOS) and at 4.62, 8.06, and 3.91 µg/ml for HSV-2 (333), respectively. Results are expressed as mean areas ± SD of viral plaques developed in cells sample-treated and untreated (controls). ANOVA/Tukey's test showed significant differences in relation to controls with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). **b** Viral plaques of Vero cells infected with HSV-1 KOS and treated with cell culture medium. **c** Viral plaques of Vero cells infected with HSV-1 KOS and treated with FR-S at 6.74 µg/ml.

with samples or culture medium (viral controls). Despite all tested compounds have significantly reduced plaque areas in comparison to untreated controls, infected cells treated with FR-S displayed the smallest plaque areas for both viruses. Figure 1b and c show cell monolayers with fixed and stained viral plaques.

Synergism between FR-S and ACV

Data of combined treatments of FR-S and ACV based on their respective EC₅₀ values were analyzed using CalcuSyn software in order to calculate the experimental CI values. The results compiled in table 3 show that the percentages of inhibition of the combinations increased in comparison to each compound alone, and since all combinations tested presented CI values lower than 1, FR-S was shown to have a synergistic anti-HSV effect with ACV for both tested viruses.

Anticoagulant Activity

The APTT values obtained for FR-S (20 µg/ml) and PBS were 82.01 ± 7.10 and 56.67 ± 1.53 s, respectively. Differently, no coagulation occurred for HEP at 1 µg/ml under the experimental conditions employed in this

study. These results indicate that, different from HEP, FR-S has only a slight anticoagulant activity at its 100% HSV inhibitory concentration.

Discussion

The chemical modification of carbohydrate polymers, especially sulfation, has often been applied to improve their intrinsic biological properties or provide new functional effects, including antiviral activities [22–25]. In this work, attempts were made to study the antiherpetic activity of a (1→6)-(1→3)-β-D-glucan isolated from *A. brasiliensis* fruiting bodies (FR) and its corresponding sulfated derivative (FR-S) [8].

Firstly, the viral plaque number reduction assay was performed under two distinct conditions: simultaneous treatment (samples were added to the cells at the same time of virus infection) and postinfection treatment (samples were added 1 h after virus infection). Results showed that the native polysaccharide FR did not reduce the number of viral plaques neither in simultaneous nor in postinfection treatment, even at the highest tested concentra-

Table 3. Synergistic anti-HSV effects of sulfated *A. brasiliensis* polysaccharide (FR-S) in combination with ACV

Compounds combination ratio	Sample concentration, µg/ml		Mean percentage of inhibition (%) FR-S + ACV	Experimental CI ¹ values	Description (graded symbols)
	FR-S	ACV			
<i>HSV-1 (KOS)</i>					
4 × EC ₅₀	26.96	2.04	100.00	0.662	synergism (+ + +)
2 × EC ₅₀	13.48	1.02	100.00	0.572	synergism (+ + +)
1 × EC ₅₀	6.74	0.51	99.22	0.286	strong synergism (+ + + +)
0.5 × EC ₅₀	3.37	0.26	69.15	0.239	strong synergism (+ + + +)
0.25 × EC ₅₀	1.68	0.13	55.19	0.443	synergism (+ + +)
<i>HSV-2 (333)</i>					
4 × EC ₅₀	18.88	15.64	100.00	0.550	synergism (+ + +)
2 × EC ₅₀	9.44	7.82	100.00	0.312	strong synergism (+ + + +)
1 × EC ₅₀	4.72	3.91	94.51	0.171	strong synergism (+ + + +)
0.5 × EC ₅₀	2.36	1.96	86.20	0.192	very strong synergism (+ + + + +)
0.25 × EC ₅₀	1.18	0.98	52.63	0.367	strong synergism (+ + + +)

In detail, the ranges of CI <0.1, 0.10–0.30, 0.31–0.70, 0.71–0.85, and 0.86–0.90 mean very strong synergism, strong synergism, synergism, moderate synergism, and slight synergism, respectively. Obtained values represent the mean of three independent experiments.

¹ Combination index, a quantitative index calculated by CalcuSyn software, which quantifies the interaction between the tested compounds, as described by Chou [21].

tion (1 mg/ml; table 1). We found the same negative results for the native polysaccharide isolated from the *A. brasiliensis* mycelia (MI), when tested at 1 mg/ml [10]. However, at 1 mg/ml under simultaneous treatment, FR reduced about 40% of plaque sizes in comparison to the viral controls, while at 0.5 mg/ml, no significant reduction in plaque areas was observed (data not shown). Since FR had no substantial antiviral effect, further investigations focused on determining the FR-S mechanism of action.

Seemingly in contradiction to our results, Yamamoto et al. [15] found an anti-HSV-1 activity under simultaneous (EC₅₀ = 454 µg/ml) and postinfection (83.6% of inhibition at 800 µg/ml) treatments with an *A. brasiliensis* native polysaccharide through viral plaque number reduction assay. However, our experiment tested the antiherpetic activity of a (1→6)-(1→3)-β-D-glucan, not a β-(1→6)-α-(1→4)-glucan-protein complex (PLS), as described by them [26]. They also found anti-HSV-1 activity for the PLS-sulfated derivative (SPLS), but unlike our findings there was not much improvement on antiviral activity after sulfation in the simultaneous treatment (EC₅₀ = 346 µg/ml), while no activity was detected for SPLS during postinfection treatment. These discrepant results are likely due not only to the disparity in chain configuration, but also to other chemical features, such as protein content [ours: FR = 1.76% (w/w), FR-S = not detected; Yamamoto et al.: PLS = 25.3%, SPLS = not

mentioned], molecular weight (ours: FR = 609 kDa, FR-S = 127 kDa; Yamamoto et al.: PLS = 97.7 kDa, SPLS = not mentioned), and probably the degree of substitution of sulfated groups (ours: FR-S = 1.88; Yamamoto et al.: SPLS = not mentioned) [8, 15, 26].

In fact, the biological properties of sulfated polysaccharides have been shown not to be a simple consequence of the presence of sulfate groups, but a result of the combination of different structural features, such as sugar composition, chain configuration, molecular weight, sulfation degree, and specific positioning of sulfates [22]. Additionally, factors such as extraction procedure, period of collection, and species source also influence the chemical properties of native polysaccharides. Together, these aspects must be considered when comparing results for this class of compounds, although similar antiviral potencies might coincidentally be found for different polysaccharides.

The differences between FR-S and HEP results, particularly regarding their inhibitory capacity when added after viral entry, might also be explained by their structural diversity. The HEP used in this study is a linear glycosaminoglycan with a lower molecular mass (approx. 18 kDa) than FR-S (127 kDa), a branched (1→6)-(1→3)-β-D-glucan fully sulfated at C-6 and partially sulfated at C-4 [8]. When comparing the inhibition of HSV-1 and HSV-2 replication, it was observed that HSV-2 was more sensitive to FR-S and HEP, while HSV-1 was

more sensitive to ACV, in accordance with previous reports [27, 28].

Earlier reports have demonstrated HSV virucidal activity for isolated sulfated polysaccharides and polysaccharidic extracts obtained from marine algae [29, 30], while no virucidal effect was found for the sulfated derivative of a xylogalactofucan obtained from *Laminaria angustata* [31] or polysaccharide-rich fractions from *Lithothamnion muelleri* [32]. In order to investigate if FR-S exerts its antiherpes activity by direct virus particle inactivation, a virucidal assay was carried out. Since HSV-1 KOS and HSV-2 333 infectivity was not impaired by FR-S, we were led to believe that the antiherpetic activity detected by plaque assay (table 1) was due to the interference on the HSV replication cycle. Bruggemann et al. [12] reported HSV-1 virucidal activity for an aqueous extract of *A. brasiliensis*, but these researchers employed a different experimental design. The sample was extracted in water at room temperature and no sulfation derivatization was performed.

Attachment and entry are among the multiple steps of the HSV life cycle, which have attracted the attention of researchers as potential anti-infective targets. Adsorption and penetration are complex processes involving 5 (gB, gC, gD, gH/gL, gK) of the 12 known HSV surface glycoproteins. The glycoprotein C-1 (gC-1) has been reported to play a more important role in the attachment of wild-type HSV-1 than gB-1, while gB mediates the binding of gC-null mutants. Regarding gB, its contribution has been suggested to be higher for HSV-2 than for HSV-1 binding [18, 33]. Antiviral sulfated polysaccharides act by interfering with electrostatic interactions between viral glycoproteins and cell surface glycosaminoglycans [34]. Herein, mutant viruses devoid of gC were used to investigate the involvement of this glycoprotein in the antiherpetic activity of FR-S. Four different experimental strategies (A–D) were applied for the adsorption assays. Data shown in table 2 demonstrate that the HSV-1 gC-negative mutant virus was significantly less sensitive to FR-S than the parental strain (HSV-1 KOS) in protocols A (attachment assay) and B (pretreatment of virions). Similarly, EC_{50} values for HSV-2 333 in treatment A were significantly lower than for the HSV-2 gC-negative variant. These results indicate a possible interaction between gC and FR-S, as in the case of HEP [35]. These findings also suggest differences in FR-S binding properties between HSV-1 and HSV-2 or might simply be associated with the fact that gC plays a more important role in the attachment of HSV-1 than that of HSV-2. Furthermore, FR-S could bind to other membrane tar-

gets in addition to gC, similarly to HEP, which binds mostly to gC, but also to gB [33, 36]. Whether FR-S interacts with these HSV glycoproteins, and the way these interactions occur, remains to be studied.

When virions were pretreated with samples (treatment B), FR-S and HEP were more active than in treatment A. These results support a possible interaction of FR-S with viral particles, avoiding virus adsorption into host cells, which has already been described for HEP [27].

Interestingly, when the cells were treated with samples prior to viral attachment (treatment C), FR-S also reduced the viral infectivity, even though this effect occurred at higher concentrations, suggesting that FR-S may further interact with cell surface components, blocking virus-cell interactions during entry events. Differently, HEP had no inhibitory effects at the tested concentrations.

When added to cell cultures after the initial binding period (treatment D), FR-S was also able to inhibit virus infectivity, whereas HEP was not active at the tested concentrations. Taken together, these findings support that, contrary to HEP, FR-S may act by interacting with both viral and cellular structures, inhibiting viral adsorption and post-binding events more efficiently.

After the initial viral attachment to cellular glycosaminoglycans, further interaction of viral glycoproteins, especially gB, gD, and gH, with cell receptors is required for the entry process [1]. As shown in table 2 (treatment E), FR-S, a branched polymer, inhibited the penetration of all HSV-tested strains more efficiently than HEP, a linear polymer. These results bring forth the hypothesis that branching affects positively the ability of polysaccharides to inhibit viral adsorption and penetration. There were no statistical differences between the EC_{50} values of wild-type and gC-null strains for FR-S and HEP during the penetration assay, probably due to the minor role of gC in HSV cell penetration [33].

Viral glycoproteins interfering with HSV entry (gB, gD, and gH/gL) are also involved in HSV cell-to-cell spread, as well as the heterodimer gE/gI. Since the rapid spread between adjacent cells allows virus escape from the host's immune response, the inhibition of intercellular diffusion is a potential target for therapeutic intervention [1]. The reduction of HSV-1 and HSV-2 lateral spread was evaluated by measuring 20 viral plaque areas in infected cells treated with FR-S, HEP, or ACV at their corresponding EC_{50} postinfection values, i.e. at concentrations in which the reduction of the viral plaque number was almost identical (50%).

In our previous study, we also found the HSV cell-to-cell spread inhibition by the sulfated derivative of the

(1→3)- β -gluco-(1→2)-D-mannan (MI-S) isolated from *A. brasiliensis* mycelia. Despite the different chemical structures of FR-S and MI-S, they presented similar antiherpetic mechanism and effectiveness [10], showing the potential for drug development of polysaccharides from this species.

The HSV-1 cell-to-cell spread inhibition by suramin, a polysulfonated naphthylamine, was reported by Aguilar et al. [37]. Other sulfated compounds that inhibited HSV-1 and HSV-2 intercellular diffusion were a capsular sulfated polysaccharide from *Escherichia coli* [38], disulfated cyclitols [39], and a sulfonated oligosaccharide (PI-88) [40] and its glycoside derivative [41]. A study performed by Cheshenko et al. [42] showed that some sulfated/sulfonated compounds (PRO 2000, polystyrene sulfonate, cellulose sulfate, and polymethylenehydroquinone sulfonate) inhibited HSV-2 attachment, penetration, and cell-to-cell spread. However, Bergefall et al. [34] did not find any inhibition of HSV-1 and HSV-2 cell-to-cell spread by the chondroitin sulfate polysaccharide.

Since sulfated polysaccharides might present anticoagulant activity, we also performed the APTT assay. HEP, whose in vitro anti-HSV activity was originally published by Nahmias and Kibrick [43], is a sulfated

polysaccharide analogous to heparan sulfate clinically used as anticoagulant [44]. Different from HEP, FR-S showed only a slight anticoagulant activity at its 100% anti-HSV concentration, which represents an advantage for an antiherpetic drug candidate, minimizing possible side effects and drug-drug interactions. However, in vivo studies are needed to further assess the toxicological profile and clinical applicability of FR-S for human use.

In summary, this study shows that FR-S, a sulfated (1→6)-(1→3)- β -D-glucan [8], presents an interesting mechanism of antiherpetic activity by inhibiting viral adsorption, penetration, and cell-to-cell spread. FR-S was also found to have a synergistic anti-HSV effect with ACV, indicating that FR-S is a promising drug candidate for the prevention and/or treatment of HSV infections, to be used as a single therapeutic agent or in combination with ACV.

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