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Antiherpetic activity of a sulfated polysaccharide from *Agaricus brasiliensis* mycelia

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ABSTRACT

Sulfated polysaccharides are good candidates for drug discovery in the treatment of herpetic infections. *Agaricus brasiliensis* (syn *A. subrufescens*, *A. blazei*) is a Basidiomycete fungus native to the Atlantic forest region of Southeastern Brazil. Herein we report the chemical modification of a polysaccharide extracted from *A. brasiliensis* mycelia to obtain its sulfated derivative (MI-S), which presented a promising inhibitory activity against HSV-1 [KOS and 29R (acyclovir-resistant) strains] and HSV-2 strain 333, with selectivity indices (SI = CC_{50}/IC_{50}) higher than 439, 208, and 562, respectively. The mechanisms underlying this inhibitory activity were scrutinized by plaque assay with different methodological strategies. MI-S had no virucidal effects, but inhibited HSV-1 and HSV-2 attachment, penetration, and cell-to-cell spread, as well as reducing the expression of HSV-1 ICP27, UL42, gB, and gD proteins. MI-S also presented synergistic antiviral effect with acyclovir. These results suggest that MI-S presents multiple modes of anti-HSV action.

1. Introduction

Herpes Simplex Virus types 1 and 2 (HSV-1 and HSV-2) are human neurotropic viruses usually associated with infections of the skin and mucosae of different locations, most commonly the oral and genital regions. Although infections are often subclinical, HSV can cause mild to severe diseases, especially in neonates and immunocompromised individuals. Currently, there is no cure for the persistent infection, and prolonged therapy with the available antiherpes drugs has induced the emergence of drug-resistant virus strains. Moreover, HSV has been described as a risk factor for HIV infection (Roizman et al., 2007). This scenario has triggered the search for new antiherpetic agents, especially those with mechanisms of action different from that of nucleoside analogs, the major class of antiviral agents used for the management of HSV infections. Besides, a treatment based on the combination of different antiviral agents can be considered a promising approach to increase antiviral selectivity while simultaneously enabling the reduction of the active concentrations of the drugs (Chou, 2006).

Many synthetic or naturally occurring sulfated polysaccharides from different species of marine algae, bacteria, fungi, and animals have been previously shown to have antiviral activity against human and animal viruses (Ghosh et al., 2009). In the case of fungi, cell wall polysaccharides have been chemically modified to increase their solubility and enhance their biological activities (Liu et al., 2010), including their antiviral action (Zhang et al., 2004).

The pharmacological effects of *Agaricus brasiliensis*, a Basidiomycete fungus native to the Brazilian Atlantic forest region, have been mainly related to the presence of polysaccharides and protein–polysaccharide complexes (Firenzuoli et al., 2008). Concerning its previous antiviral evaluation, Sorimachi et al. (2001) showed that the ethanolic fractions of *A. brasiliensis* mycelium and fruiting bodies inhibited HSV, poliovirus, and Western equine encephalitis virus replication. The inhibition of HSV-1 and herpes bovine virus by an aqueous extract of *A. brasiliensis* fruiting bodies was also demonstrated by Bruggemann et al. (2006). Additionally, both aqueous and ethanolic fruiting bodies extracts and an isolated polysaccharide from this species displayed antiviral activity against poliovirus 1, as reported by Faccin et al. (2007).

Considering that fungal biomass is an important source of cell wall polysaccharides and that sulfated compounds are promising antiviral agents, this study attempted to produce a sulfated derivative of a polysaccharide obtained from *A. brasiliensis* mycelia. Since this compound presented a potential anti-HSV activity, its mechanism of action was also evaluated.

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2. Materials and methods

2.1. Fungal material

The fruiting bodies of *Agaricus brasiliensis* Wasser strain UFSC 51 (syn *A. subrufescens*, *A. blazei*) were collected in Biguaçu, Santa Catarina State, Southern Brazil. The characterization of the species was performed by Dr. Maria Alice Neves, and a voucher specimen (FLOR 11 797) was deposited in the FLOR Herbarium (Universidade Federal de Santa Catarina). The mycelium of *A. brasiliensis* was isolated and cultivated on potato dextrose agar (PDA) (Oxoid, UK) at 25 °C during 7 days. The liquid inoculum was produced by transference of mycelial disks to flasks containing Melin-Norkrans Modified medium (MNM) (Marx, 1969) and cultivated at 25 °C during 10 days. Mycelia were filtered and fragmented in 300 mL of NaCl 0.8%. The inoculum was then added to MNM in an airlift bioreactor (5 L) and cultivated during 7 days at 26 °C. The liquid culture was centrifuged and the mycelial biomass was dehydrated at 55 °C until constant weight.

Agaricus brasiliensis polysaccharide was isolated as previously described (Camelini et al., 2005), with minor modifications. Fifty grams of dried mycelial biomass were blended twice with five volumes of distilled water and refluxed at 100 °C for 3 h. The material was filtered under vacuum through a Whatman n°42 filter paper. Three volumes of ethanol were added to the filtrate. The mixture was maintained at 4 °C for 24 h and centrifuged (1100 *g*, 10 min). The mycelial polysaccharide was freeze-dried and designated as MI.

To produce the sulfated derivative, MI was sulfated using the pyridine-chlorosulfonic acid reagent as described by Zhang et al. (2003). After sulfation, resulting polysaccharides were dialyzed through a 5 kDa molecular weight cut-off membrane (Spectrum Laboratories, Rancho Dominguez, CA) against distilled water and freeze-dried yielding the sulfated derivative (MI-S).

MI and MI-S were characterized by spectroscopic methods [Fourier transform infrared (FTIR) and ^{13}C Nuclear magnetic resonance (^{13}C NMR)] and elemental analyses (C, H, O, S). Determination of homogeneity and molecular weight (M_w) was carried out by high-performance gel filtration chromatography (HPGFC) using a Perkin Elmer series 200 equipment coupled with a RI detector, using a gel filtration column (TSK-Gel 5000 PW 7.8 × 300 mm connected to a TSK PWH 5 × 7 mm guard column; Tosoh, Japan). Samples were eluted with 0.2 M NaCl mobile phase at a flow rate of 1 mL/min. Mean M_w was estimated by comparison with retention times of standard dextrans.

2.2. Cells and viruses

Vero cells (ATCC:CCL 81) were grown in minimum essential medium (MEM), supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (25 μg/mL). Cell cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere chamber. The virus strains used were: HSV-1 KOS and 29 R (Faculty of Pharmacy, University of Rennes, France), and HSV-2 333 (Department of Clinical Virology, Göteborg University, Sweden). Virus titers were determined by plaque assay and expressed as plaque forming units (PFU/mL) (Burleson et al., 1992).

2.3. Antiherpes evaluation

The cytotoxicity of samples was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, confluent Vero cells were exposed to different sample concentrations for 72 h. The medium was then substituted by the MTT solution and incubated for 4 h. After dissolution of formazan crystals, optical densities were read (540 nm) and the concentration of each sample that reduced cell viability by 50% (CC_{50}) was calculated based on untreated controls.

Subsequently, the potential antiherpetic activity was evaluated by the plaque reduction assay as previously described (Silva et al., 2010). Monolayers of Vero cells grown in 24-well plates were infected with 100 PFU per well of each virus for 1 h at 37 °C. Treatments were performed by adding samples either simultaneously with the virus (simultaneous treatment) or after the virus infection (post-infection treatment). Cells were subsequently covered with CMC medium (MEM containing 1.5% carboxymethylcellulose) and incubated for 72 h. Cells were then fixed and stained with naphthol blue black and viral plaques was counted. The concentration of each sample required to reduce the plaque number by 50% (IC_{50}) was calculated by standard method (Burleson et al., 1992). Acyclovir (ACV), dextran sulfate (DEX-S), and heparin (HEP) were purchased from Sigma (St. Louis, MO) and used as positive controls. IC_{50} and CC_{50} values were estimated by linear regression of concentration–response curves generated from the data. The selectivity index ($SI = CC_{50}/IC_{50}$) was calculated for each sample.

2.4. Evaluation of the mechanism of anti-HSV action

The virucidal assay was conducted as described by Ekblad et al. (2006), with minor modifications. Mixtures of equal sample volumes (20 μg/mL) and 4×10^5 PFU of HSV-1 (KOS and 29-R) or HSV-2 333 in serum-free MEM were co-incubated for 20 min at 4 or 37 °C. Samples were then diluted to non-inhibitory concentrations (1:1000) to determine the residual infectivity by plaque reduction assay as described above. Ethanol 70% (v/v) served as a positive control.

The attachment and penetration assays followed the procedures described by Silva et al. (2010). In the attachment assay, pre-chilled Vero cell monolayers were exposed to viruses (100 PFU per well), in the presence or absence of the samples. After incubation for 2 h at 4 °C, samples and unabsorbed viruses were removed by washing with cold phosphate-buffered saline (PBS) and cells were overlaid with CMC medium. Further procedures were the same as described above for the plaque reduction assay.

In the penetration assay, viruses (100 PFU per well) were adsorbed for 2 h at 4 °C on pre-chilled cells. After the removal of unbound viruses, the temperature was shifted to 37 °C to allow penetration. Then, the cells were treated with different concentrations of pre-warmed samples, and incubated for 1 h at 37 °C. Unpenetrated viruses were inactivated with citrate-buffer (pH 3.0). Cells were washed with PBS and covered with CMC medium. The percentage of inhibition was calculated based on the reduction of plaque number as mentioned previously.

Time-of-addition study was performed by virus yield reduction assay as reported by Carlucci et al. (1999), with some modifications. Briefly, monolayers of Vero cells were inoculated with HSV-1 at a MOI (multiplicity of infection) of 0.04, incubated for 60 min at 4 °C and 30 min at 37 °C to ensure synchronous viral replication. After removing virus inoculum, cells were maintained at 37 °C and treated with MI-S (20 μg/mL), DEX-S (20 μg/mL), or ACV (2 μg/mL) at 2, 4, 8, 12, 16, and 20 h post-infection (p.i.). After a 24 h period, cells were lysed by freeze-thawing three times and cellular debris were removed by centrifugation. Subsequently, virus titration was carried out by plaque assay. The percentage of viral inhibition of each sample treatment was calculated by comparing it with virus titers of untreated controls.

The effect of tested samples on HSV cell-to-cell spread was investigated as described by Ekblad et al. (2010). In brief, different concentrations of MI-S, ACV, or DEX-S were added to Vero cells 1 h after their infection with 100 PFU per well of HSV, and the plates were incubated throughout the entire period of plaque

development. Results were obtained by analyses of the images of 20 viral plaques formed in the absence (viral control) or the presence of different concentrations of each sample concentration. Images were captured using a cooled digital camera coupled to an Olympus BX41 microscope and the area of each plaque was determined using the Image J software (http://rsb.info.nih.gov/ij/).

2.5. Western blotting analysis

Western blotting analysis was performed as previously described (Kuo et al., 2001). Briefly, monolayers of Vero cells were inoculated or not with HSV-1 KOS at a MOI of 0.1. Plates were incubated for 60 min at 4 °C and 30 min at 37 °C to ensure synchronous viral replication. Then, infected cells were treated with MI-S (20 μg/mL), DEX-S (20 μg/mL) or ACV (2 μg/mL) at 1, 4, or 8 h p.i., and the plates were incubated for 18 h. Next, cells were lysed and protein quantification was carried out (Bradford, 1976). Each sample (5 μg of protein) was separated electrophoretically on a 12% SDS–PAGE gel and electroblotted onto polyvinylidene difluoride (PVDF) membranes. After blocking, membranes were incubated overnight with either anti-ICP27 (1:700, Millipore, Billerica, MA), or anti-UL42 (1:1000, Millipore), or anti-gB (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-gD (1:5000, Santa Cruz Biotechnology). After incubation with the corresponding secondary antibodies conjugated to horseradish peroxidase, protein bands were revealed by chemiluminescence on X-ray films (IBF, Brazil) using Pierce ECL substrate (Thermo Scientific, Waltham, MA) according to the Manufacturer's protocol. The relative amounts of protein in the detected bands were quantified by Image J software. The anti-β-actin antibody was used as a control for total protein loading.

2.6. Synergistic effect in combination with acyclovir

Potential synergistic effects of MI-S in combination with ACV was evaluated by plaque reduction assay, according to experimental design proposed by Chou (2006). Therefore, each drug alone or in combination was tested at an equipotency ratio, based on its corresponding IC_{50} value. The degree of interaction between MI-S and ACV was calculated through combination index (CI) equation, based on the median-effect principle of the mass-action law, 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.

3. Results

3.1. Characterization of A. brasiliensis polysaccharides

Assignment of ^{13}C NMR spectrum (Fig. 1) was based on the previously published spectrum by Mizuno and colleagues (1999). Anomeric signals (C1) at δ 105.1 and 101.9 ppm were assigned to β-glucopyranosyl and β-mannopyranosyl residues, respectively. The signals at δ 98.1 and 94.3 ppm were assigned to the corresponding reducing end-groups. The characteristic resonances of C2, C3, C4, C5, and C6 of β-(1 → 2)-linked components were observed at δ 78.2, 73.7, 71.8, 77.9, and 62.9 ppm, respectively. The signals of β-(1 → 3)-linked components were assigned as C2 (75.0), C3 (86.6), C4 (71.9), C5 (76.3), and C6 (62.9). This result suggested that MI is a glucomannan with a main chain of β-1,2-linked D-mannopyranosyl residues and β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl residues as side chains. A symmetric single peak was obtained by gel permeation chromatography of MI-S, suggesting that the polymer is homogeneous. Based on calibration curves with standard dextrans, the apparent *M*w of MI-S was 86 kDa. In the MI-S spectrum, obtained by FTIR analyses, two new absorption bands

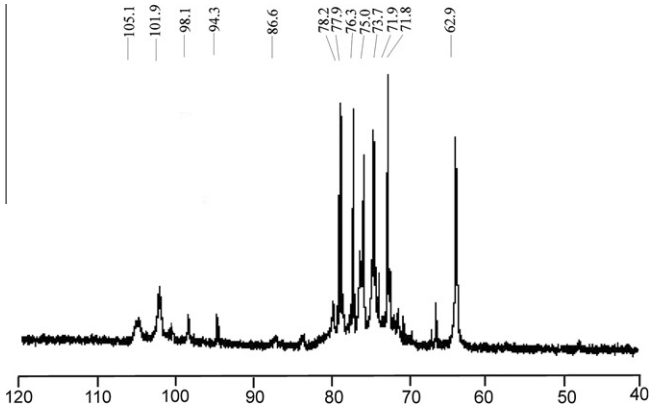

Fig. 1. ^{13}C-NMR spectrum of MI (*Agaricus brasiliensis* mycelial polysaccharide). ^{13}C NMR analysis of MI (60 mg, dissolved in D_2O) was performed at 25 °C with VARIAN Unity Plus spectrometer operating at 100,582 MHz (9.4 Tesla), using TSPA-d4 as external reference.

appeared at 1253 and 810 cm^{-1} (data not shown). These bands are related to S=O and C–S–O sulfate groups respectively, confirming that sulfation had actually occurred (Silverstein et al., 2005). In addition, the content of sulfur determined by elemental analyses was 14.77% and 10.72% for MI-S and DEX-S, respectively.

3.2. Antiherpetic activity

The cytotoxicity and antiviral activity results were used to calculate the selectivity index of each sample (SI = CC_{50}/IC_{50}) (Table 1). The data show that MI presented no antiviral activity, whereas MI-S inhibited both HSV-1 and HSV-2 replication, indicating that chemical sulfation was required for the antiviral activity. Since the simultaneous treatment was more efficient than the p.i. treatment, a direct inactivation of viral particles or inhibition of virus replication at the initial phases of the viral replication cycle could be involved.

Preincubation of virus suspensions with MI-S or controls (DEX-S and ACV), at 4 and 37 °C, had no significant inactivating effects (data not shown) on HSV-1 KOS and HSV-2 333 at the tested concentrations (20 μg/mL), which suppressed 100% of viral replication in the plaque reduction assay. These results indicate that the virucidal effect does not seem to be involved in the MI-S antiviral activity detected.

Along with the adsorption, the effect of MI-S on HSV penetration was also investigated (Table 2). The results demonstrated that MI-S, as well as DEX-S and HEP, strongly inhibited attachment of all viruses tested. Similarly to DEX-S, MI-S was also able to prevent penetration of all HSV strains into the cells, whereas HEP was much less effective for the HSV-2 strain.

To further clarify which steps of HSV infection are targeted by the samples, a time-of-addition study was performed (Fig. 2). The observed inhibition of HSV-1 KOS yield was higher than 50%, even when MI-S was added 16 h p.i. This might indicate that MI-S exerts some effect on virus cycle step(s), other than adsorption and penetration, as verified by the following results.

After penetration, HSV-1 expresses immediate early genes about 2–3 h p.i., early genes about 7 h p.i., and late genes after the viral DNA synthesis has begun. Western blotting analyses were carried out to evaluate if the MI-S antiviral mechanism was related to the inhibition of HSV-1 protein expression. To reduce the interference with any prior step of each protein expression stage in the viral replication cycle, samples were added at 1, 4, and 8 h p.i. for analysis of α, β, and γ proteins, respectively (Fig. 3). The results shown in Fig. 3B represent the quantification of each band in relation to the β-actin expression. As shown in Fig. 3, MI-S significantly

Table 1
Cytotoxicity and antiherpetic activity of *Agaricus brasiliensis* polysaccharides.

Sample	CC_{50}	IC_{50} **(SI)**					
		Simultaneous treatment			Post-infection treatment		
		HSV-1 (KOS)	HSV-1 (29R)	HSV-2 (333)	HSV-1 (KOS)	HSV-1(29R)	HSV-2 (333)
MI	3352.11 ± 206.64	NI	NI	NI	NI	NI	NI
MI-S	2415.29 ± 389.21	1.24 ± 0.05 **(1948)**	1.59 ± 0.25 **(1519)**	0.39 ± 0.17 **(6193)**	5.50 ± 0.58 **(439)**	11.62 ± 1.01 **(208)**	4.30 ± 0.36 **(562)**
HEP	>2,500	1.15 ± 0.30 **(>2174)**	2.01 ± 0.38 **(>1244)**	0.58 ± 0.01 **(>4310)**	12.68 ± 1.13 **(>197)**	NT	8.06 ± 1.46 **(>310)**
DEX-S	2465.53 ± 200.13	0.59 ± 0.01 **(4179)**	0.92 ± 0.08 **(2680)**	0.47 ± 0.16 **(5246)**	6.23 ± 0.96 **(396)**	11.92 ± 1.31 **(207)**	15.36 ± 2.79 **(160)**
ACV	>2500	NI	NI	NI	0.51 ± 0.05 **(>4902)**	760.00 ± 80 **(>3)**	3.91 ± 0.41 **(>639)**

CC_{50}, 50% cytotoxic concentration for Vero cells (μg/mL); IC_{50}, 50% inhibitory concentration (μg/mL); Selectivity index values ($SI = CC_{50}/IC_{50}$) are presented between parenthesis; NI, no inhibitory activity; NT, not tested; MI, *A. brasiliensis* mycelial polysaccharide; MI-S, sulfated *A. brasiliensis* mycelial polysaccharide; HEP, heparin; DEX-S, dextran sulfate; ACV, acyclovir. These values represent the mean ± SD of three independent experiments.

Table 2
Effect of *Agaricus brasiliensis* polysaccharides on HSV adsorption and penetration.

Sample	Inhibitory effect (IC_{50})					
	Attachment			Penetration		
	HSV-1 (KOS)	HSV-1 (29 R)	HSV-2 (333)	HSV-1 (KOS)	HSV-1 (29 R)	HSV-2 (333)
MI	NI	NI	NI	NI	NI	NI
MI-S	0.10 ± 0.01	0.26 ± 0.03	0.05 ± 0.01	1.04 ± 0.06	0.49 ± 0.10	1.21 ± 0.28
HEP	0.33 ± 0.04	0.62 ± 0.03	0.07 ± 0.01	13.34 ± 3.64	25.74 ± 2.64	17.99 ± 4.06
DEX-S	0.12 ± 0.04	0.11 ± 0.00	0.02 ± 0.00	0.85 ± 0.13	0.82 ± 0.11	0.69 ± 0.09
ACV	NI	NI	NI	NI	NI	NI

IC_{50}, 50% inhibitory concentration (μg/mL); NI, no inhibitory activity; MI, *A. brasiliensis* mycelial polysaccharide; MI-S, Sulfated *A. brasiliensis* mycelial polysaccharide; HEP, heparin; DEX-S, dextran sulfate; ACV, acyclovir; These values represent the mean ± SD of three independent experiments.

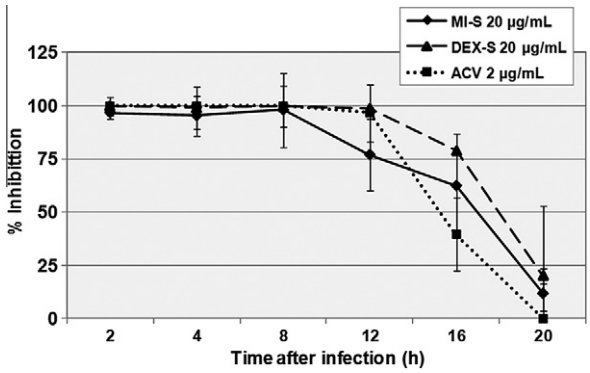

Fig. 2. Time-of-addition study. Percentage of inhibition of MI-S (sulfated *Agaricus brasiliensis* mycelial polysaccharide), ACV (acyclovir), and DEX-S (dextran sulfate) when added at different times after HSV-1 (KOS strain) infection. Each point represents the mean value ± SD of two independent experiments.

reduced the expression of ICP27, UL42, and gB. Moreover, the combination of MI-S and acyclovir (lane 4) reduced all the proteins expression more strongly than these compounds tested separately.

The reduction of HSV-1 and HSV-2 cell-to-cell spread was evaluated by comparing viral plaque areas between treated cells and untreated controls. Considering that significant differences in plaques sizes were only observed at concentrations higher than the IC_{50} values of all tested samples (data not shown), as well as the small number of plaques at this condition, an additional experiment was performed with samples at concentrations equivalent to their IC_{50} values. Mean plaque areas for each treatment and untreated controls are shown in Fig. 4. Regarding to HSV-1 (KOS strain), MI-S reduced the viral plaque size more extensively than did DEX-S and ACV. Although HSV-2 lateral diffusion was significantly reduced by all tested samples, MI-S resulted in the smallest mean plaque areas for both viruses. Even though the tested concentrations in this experiment were different, the reduction of viral plaque numbers was similar (~50%).

Given the fact that MI-S seemed to act in a different way than acyclovir, the potential synergistic effects between them were tested at different concentrations (Table 3). Since all combinations tested presented CI values less than 1, synergistic anti-HSV-1 and HSV-2 effects of MI-S with ACV were demonstrated.

4. Discussion

In order to evaluate the influence of the treatment period on the anti-HSV activity of MI-S, the plaque number reduction assay was performed under two different conditions. As shown in Table 1, MI-S was considerably more effective by simultaneous rather than post-infection treatment. The same result was observed for the other sulfated polysaccharides tested, HEP and DEX-S, as expected due to their similar nature. These results are in agreement with those of other authors who tested different sulfated polysaccharides, such as carrageenans (Carlucci et al., 1999), fucoidans (Karmakar et al., 2010), and sulfated β-glucans (Zhang et al., 2004), and found a stronger inhibition of HSV replication in the simultaneous treatment with these compounds than in post-infection treatments.

Although similar IC_{50} values were obtained for MI-S and HEP in the simultaneous treatment, we have not found an anticoagulant activity for MI-S at a 100% inhibitory concentration (data not shown), which represents an advantage for an antiherpes agent with these chemical features. Moreover, in the post-infection treatment, the inhibitory effect of MI-S was stronger than those of HEP for HSV-1 (KOS strain) and HSV-2, and of DEX-S for HSV-2. Differences among these results may be related to their structural diversity since, differently from MI-S and DEX-S, HEP is a linear polymer (Rabenstein, 2002), with a lower molecular mass (~18 kDa) than either MI-S (86 kDa) or DEX-S (500 kDa). Furthermore, the higher content of sulfur present in MI-S (14.77%) can be correlated to its stronger effect at inhibiting HSV-2 than DEX-S (10.79%). Indeed, the antiherpetic properties of sulfated polysaccharides are determined by a combination of structural features such as molecular

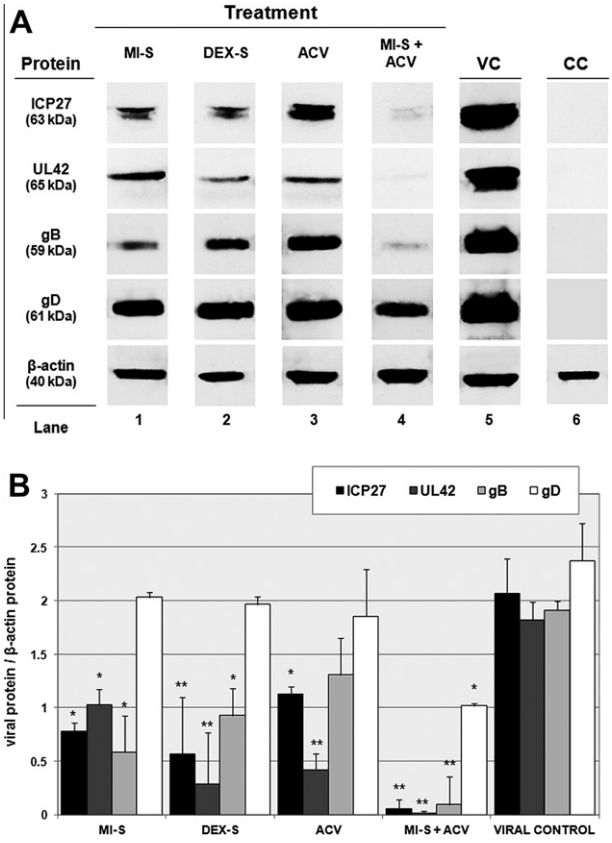

Fig. 3. Effect of MI-S (sulfated *Agaricus brasiliensis* mycelial polysaccharide) on HSV-1 (KOS strain) protein expression. Treated and untreated cells were analyzed by SDS–PAGE/Western blotting using specific antibodies for viral (ICP27, UL42, gB, or gD) and cellular (β-actin) proteins. (A) Lanes 1 to 4: HSV-1 infected cells treated with MI-S (20 μg/mL), dextran sulfate (DEX-S, 20 μg/mL), acyclovir (ACV, 2 μg/mL), and MI-S (20 μg/mL) plus ACV (2 μg/mL), respectively. Samples were added at 1, 4, and 8 h p.i. for analyses of α, β, and γ HSV proteins, respectively. Lane 5: untreated viral control; Lane 6: untreated cellular control. (B) The graph indicates the ratio of each viral protein to β-actin protein. Statistical analysis (one-way ANOVA followed by Newman-Keuls test) demonstrated significant differences in relation to viral controls with p values <0.05 (∗) and <0.01 (∗∗). The results are representative of two independent experiments.

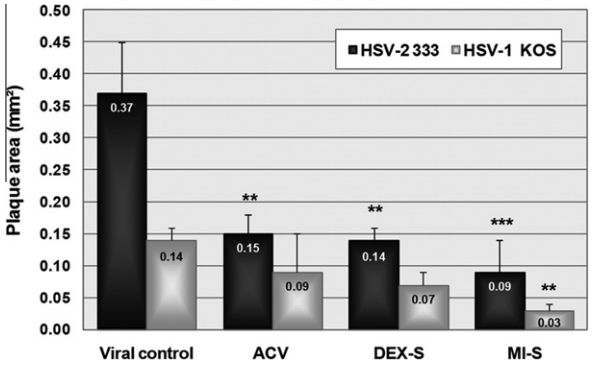

Fig. 4. Inhibition of HSV-1 and HSV-2 cell-to-cell spread by MI-S (sulfated *Agaricus brasiliensis* mycelial polysaccharide), DEX-S (dextran sulfate), and ACV (acyclovir) at their corresponding IC_{50} concentrations. The interference on cell-to-cell spread of MI-S, DEX-S, or ACV was evaluated at 5.50, 6.23, and 0.51 μg/mL for HSV-1 KOS, and at 4.30, 15.36, and 3.91 μg/mL for HSV-2 333, respectively. Results were expressed as average area of viral plaques developed in sample-treated cells and compared to untreated controls. Statistical analysis (one-way ANOVA followed by Dunnett's test) demonstrated significant differences in relation to the controls with p values <0.05 (∗), <0.01 (∗∗), and <0.005 (∗∗∗). Values shown represent means of three independent experiments.

mass, branching degree, charge density, and molecular composition of uncharged portions (Ghosh et al., 2009).

Sulfated polysaccharides may present an antiherpetic activity through different mechanisms, including virucidal effects. In this study, however, MI-S showed no virucidal effects, indicating that the antiherpes activity detected by the plaque reduction assay was due to the interference with some step(s) of the HSV replication cycle. By contrast, Bruggemann et al. (2006) have shown an HSV-1 virucidal activity for an aqueous extract of *A. brasiliensis*, but they used different methodologies for virucidal evaluation and extract preparation, which did not include the sulfation reaction. Still, other studies on the antiviral activity of sulfated polysaccharides have similarly reported no virucidal effects (Adhikari et al., 2006; Chattopadhyay et al., 2007, 2008; Karmakar et al., 2010; Matsuhiro et al., 2005; Zhu et al., 2006), or the virucidal action was detected only at concentrations higher than their IC_{50} values (Carlucci et al., 1999; Mazumder et al., 2002; Zhu et al., 2004).

Among the steps of HSV infection and replication, attachment and entry have been considered as potential targets. The findings presented in Table 2 are in agreement with those published by other authors, who stated that the mechanism underlying the antiherpes activity of polysaccharides, especially sulfated ones, may be related to the inhibition of HSV adsorption (Carlucci et al., 1999; Eo et al., 2000; Zhang et al., 2007). Since there was no detectable loss of HSV residual infectivity at 4 °C in the presence of MI-S, the virucidal mechanism in the adsorption assays was dismissed. Table 2 shows that MI-S and DEX-S displayed IC_{50} values lower than 1.21 μg/mL, whereas HEP showed values higher than 13.34 μg/mL. Since HEP was the only tested sulfated polysaccharide with a linear chain, it can be suggested that the presence of lateral branches could be important for the inhibition of the herpes virus penetration. The lack of inhibition of viral adsorption and penetration by the non-sulfated polysaccharide (MI) confirmed that the presence of sulfate groups is required for such activities.

In addition to the inhibition of HSV replication at 1 h p.i. treatment, MI-S presented inhibitory activity even when added at longer times after infection (Fig. 2), suggesting an action in post-entry events. This hypothesis was investigated by Western blotting analyses, in which a considerable reduction of α (ICP27), β (UL42), and γ (gB) HSV-1 proteins expression was found when MI-S was added at 1, 4, and 8 h p.i., respectively. Differently, infected cells treated with MI-S resulted in a slight reduction of gD expression. As for now, considering the performed experiments, the authors are unable to point out the reason for differences observed in reduction of expression of the late proteins gB and gD. Furthermore, the detected general reduction of protein production by MI-S could be associated with a secondary effect on another step of the viral cycle, as observed for ACV, for which inhibition of protein expression was due to an indirect effect on suppression of viral DNA replication. Although we are not aware of previous reports indicating the inhibition of HSV protein expression by sulfated polysaccharides, one study described the reduction of HIV protein expression by a sulfated oligosaccharide as well as by dextran sulfate (Artan et al., 2010).

Since an efficient dissemination of virus has an important role in its infectivity, the inhibition of viral intercellular diffusion is an attractive target for new antiviral drugs. In the plaque size reduction assay, MI-S significantly reduced plaque areas. Recently, Ekblad and colleagues (2010) have shown the inhibition of HSV cell-to-cell spread by a sulfated tetrasaccharide.

Here, a synergistic effect of MI-S with ACV was also found, supporting the results of their combination by Western blotting assay. These findings suggest that MI-S can be used either alone or in combination with ACV for the treatment of herpes infections, espe-

Table 3
Synergistic effects of combination of sulfated *Agaricus brasiliensis* polysaccharide (MI-S) with acyclovir (ACV) on anti-HSV activity.

Compounds combination ratio	Sample concentration (μg/mL)		Mean percentage of inhibition (%) MI-S + ACV	Experimental CI values	Description (graded symbols)
	MI-S	ACV			
			HSV-1 (KOS)		
$4 \times IC_{50}$	22.00	2.04	100.00	0.398	Synergism (+ + +)
$2 \times IC_{50}$	11.00	1.02	100.00	0.199	Strong synergism (+ + + +)
$1 \times IC_{50}$	5.50	0.51	100.00	0.099	Strong synergism (+ + + +)
$0.5 \times IC_{50}$	2.75	0.26	64.00	0.431	Synergism (+ + +)
$0.25 \times IC_{50}$	1.38	0.13	39.00	0.383	Synergism (+ + +)
			HSV-2 (333)		
$4 \times IC_{50}$	17.20	15.64	100.00	0.372	Synergism (+ + +)
$2 \times IC_{50}$	8.60	7.82	100.00	0.186	Strong synergism (+ + + +)
$1 \times IC_{50}$	4.30	3.91	86.86	0.472	Synergism (+ + +)
$0.5 \times IC_{50}$	2.15	1.96	59.79	0.646	Synergism (+ + +)
$0.25 \times IC_{50}$	1.08	0.98	19.59	0.816	Slight synergism (+)

CI, combination index, a quantitative measure calculated by Calcusyn Software. This index quantifies the interaction between the tested compounds as described by Chou (2006). In detail, CI from 0.10 to 0.30 means strong synergism, 0.30–0.70 means synergism, 0.70–0.85 means moderate synergism, and 0.85–0.90 means slight synergism. Obtained values represent the mean of two independent experiments.

cially those in which patients do not respond to ACV. Some other HSV entry inhibitors have already been reported to present synergistic effects with ACV. For example, a complex polysaccharide–protein from *Ganoderma lucidum* (Eo et al., 2000), docosanol (Marcelletti, 2002), and oxyresveratrol (Chuanasa et al., 2008).

In summary, our findings indicate that MI-S interferes with various steps of the HSV replication cycle, mainly adsorption and penetration, but also viral protein expression, as well as with HSV cell-to-cell spread. Taking into account the prospect of an economically feasible biotechnological production of this polysaccharide and its promising antiherpetic activity herein reported, further investigation is needed to clarify the potential of such compound for clinical application.

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