Carbohydrate Polymers 90 (2012) 134–139

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Characterization of fungal sulfated polysaccharides and their synergistic anticancer effects with doxorubicin

Jing-Jy Cheng[a,b], Chia-Chuan Chang[a], Chi-Hsein Chao[a], Mei-Kuang Lu[a,*]

[a] National Research Institute of Chinese Medicine, 155-1 Li-Nung St., Sec. 2, Shipai, Peitou, Taipei 112, Taiwan
[b] Institute of Biophotonics, National Yang-Ming University, 155-1 Li-Nung St., Sec. 2, Shipai, Peitou, Taipei 112, Taiwan

ARTICLE INFO

Article history:
Received 16 March 2012
Received in revised form 6 April 2012
Accepted 1 May 2012
Available online 8 May 2012

Keywords:
Antrodia cinnamomea
Poria cocos
Sulfated polysaccharides
Doxorubicin
Anticancer
Angiogenesis

ABSTRACT

Sulfated polysaccharides (SPSs) from two edible fungal species, including two strains of *Antrodia cinnamomea* and *Poria cocos*, were isolated. Fucose, glucosamine, galactose, glucose, and mannose were the major sugars in the SPSs, and these SPSs had a high sulfate content. The area percentage of low-molecular-weight SPSs (1–100 kDa) covered almost half of the SPS mixture of the *A. cinnamomea* strains. In contrast, high-molecular-weight SPSs (>1000 kDa) of *P. cocos* covered a large proportion of the area at 30.06%. SPSs from *A. cinnamomea* B86 showed stronger inhibition of endothelial cell (EC) tube formation in an *in vitro* assay of angiogenesis, than did *A. cinnamomea* 35396 or *P. cocos*. The degree of sulfation paralleled their antiangiogenic activity. When tumor cells were concurrently exposed to doxorubicin (DOX) and fungal SPSs, SPSs synergistically increased the cytotoxicity of DOX to different degree up to 50-fold. Fungal SPSs may offer new applications for combinational-therapy drugs.

1. Introduction

For many years, interest has concentrated on sulfated polysaccharides (SPSs) as potentially useful, biologically active ingredients for pharmaceutical use. Drugs with a variety of biological activities, such as tumor suppression, anticoagulation, and antithrombosis, were developed (Miao et al., 2004; Roden et al., 1992; Veena, Josephine, Preetha, & Varalakshmi, 2007).

SPSs are defined as compounds with hemi-ester sulfate groups on a polysaccharide backbone. SPSs are considered an attractive class of compounds as drug candidates for anticancer therapies. There is now clear evidence that recognition of cell-surface heparan sulfate, with a structure similar to that of SPSs, is required for growth factor actions during angiogenic processes (Cohen et al., 1995; Vlodavsky, Miao, Medalion, Danagher, & Ron, 1996). Commercially available PI-88 is a mixture of highly sulfated, monophosphorylated mannose oligosaccharides, derived from the extracellular phosphomannan of the yeast *Pichia holstii* (Parish, Freeman, Brown, Francis, & Cowden, 1999), with potential antiangiogenic activity. Because SPSs can be easily commercially produced by submerged fermentation, it is worthwhile investigating this class of compounds for therapeutic purposes and to develop potential food supplements.

Physiologically active SPSs can be produced from commercially cultivated mushrooms. Due to the wide variety of biological effects elicited by bioactive SPSs, a current challenge is to investigate whether there are any differences and similarities in structural features of isolated SPSs which may account for certain biological effects of these compounds, including their ability to regulate inflammation, angiogenesis, apoptosis, and cell adhesion and to determine their anticarcinogenic efficacies. One of our previous studies was devoted to physiochemical characterization of SPSs from the fungus *Antrodia cinnamomea* (Polyporaceae) (Cheng, Huang, Lur, Kuo, & Lu, 2009). We also demonstrated that the SPSs from *A. cinnamomea* exhibit antiangiogenic and neuroprotective activities.

There is already considerable interest in the synergistic effects of SPS with antiviral and immunomodulatory agents. For example, SPSs from *Agaricus blazei* exhibited a synergistic antiviral effect with acyclovir for treating herpes infections and especially benefited patients who did not respond to acyclovir (de Sousa Cardozo et al., 2011). A higher sulfur content was correlated with a stronger inhibitory effect on the herpes simplex virus. In *Lucium barbarum*, a higher degree of sulfation of SPSs was reported to exhibit better immune activity, including generating antibodies earlier, accumulating them more quickly, and the effect lasting for a longer time. Also, its SPSs showed a synergistic effect with phytohemagglutinin in stimulating T lymphocyte proliferation (Wang et al., 2010). It is worthwhile investigating the synergistic effects of SPSs with anticancer agents.

* Corresponding author. Tel.: +886 2 2820 1999x7391; fax: +886 2 2826 4276.
E-mail address: mklu@nricm.edu.tw (M.-K. Lu).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.05.005

Angiogenesis is a multistep process that occurs early in tumor development and is rate limiting for tumor progression (Folkman, 2004). Angiogenesis and the development of metastases are intrinsically connected. The development of angiogenic inhibitors is an area that is ripe for expansion. In this study, the antiangiogenic activities of fungal SPSs were evaluated.

2. Materials and methods

2.1. Materials

Antrodia cinnamomea strains B86 and 35396 were a kind gift from Dr. Tun-Tschu Chang, Division of Forest Protection, Taiwan Forestry Research Institute, Taipei, Taiwan. *Poria cocos* (BCRC 36022) was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan).

2.2. Liquid culture

Fungi were maintained on potato dextrose agar (PDA) slants and transferred to fresh medium at 3-week intervals. In each pasteurized Petri dish, 25 ml of PDA medium (39 g/L) was used and incubated at 28 °C for 19 days. Fine mycelia on the media surface were transferred to 800-ml culture flasks containing 100 ml of 24 g/L potato-dextrose-broth (PDB), with 20 g/L glucose (pH 5.6) at 28 °C. SPSs were isolated from 49-day-old cultures. Following incubation, mycelia were rapidly washed with 1 L of 250 mM NaCl during aspiration to remove contaminating exopolysaccharides. Samples were then lyophilized, and stored at 4 °C, and the dry weight of mycelia was measured.

2.3. Isolation of SPSs

SPSs were isolated according to Albano and Mourio (1986) with the following modifications. Lyophilized mycelia (1 g) were extracted with 40 ml of 0.1 M sodium acetate (pH 5.5), containing 5 mM cysteine, 100 mg papain, and 5 mM EDTA at 60 °C for 24 h. Supernatants were collected after being centrifuged at 2000 × *g* for 10 min at 4 °C, and another 100 mg papain in 40 ml of the same buffer containing 5 mM cysteine and 5 mM EDTA was added to the precipitate for another 24 h at 60 °C. The supernatants of the two extractions were collected, and a 3.75-fold volume of 95% ethanol was added, precipitated at 4 °C overnight, and spun at 9000 × *g* for 10 min at 4 °C, and the pellets were collected. Pellets were dried and resuspended in 20 ml of distilled water, dialyzed (MW 12–14 kDa) against distilled water overnight at 4 °C, and centrifuged at 9000 × *g* for 10 min; then the supernatant was collected and lyophilized before use.

2.4. Size-exclusion chromatography (SEC) of SPSs

An SPS solution in milli-Q water was diluted to give a concentration of 1 mg/ml and was then filtered through a 0.22-μm filter (Millipore, Billerica, MA, USA) before injection onto the SEC column. The flow rate was 0.5 ml/min, with deionized water as the eluent. A calibration curve was constructed using an authentic standard, Sodex P-82 series (Showa Denko America, Mentor, OH, USA) containing polymaltotriose with molecular weights of 78.8×10^4, 40.4×10^4, 21.2×10^4, 4.73×10^4, and 1.18×10^4 Da. The TriSec software program was used to acquire and analyze the Viscotek data. SEC signal detection was performed using a ViscoTek model TDA-3-1 relative viscometer (ViscoTek, Houston, Texas, USA).

2.5. Hydrolysis of SPSs

Acid hydrolysis of SPSs was carried out as follows. Lyophilized SPS (1 mg) was hydrolyzed with 4.95 N trifluoroacetic acid (TFA) at 80 °C in a heating block for 24 h. The mixture was cooled, evaporated, and then resuspended in milli-Q water.

2.6. High-performance anion-exchange chromatographic (HPAEC) analysis of SPSs

Hydrolysates of SPSs were separated by HPAEC (Dionex BioLC, Sunnyvale, CA, USA) equipped with a gradient pump, a pulsed amperometric detector (PAD-II) using a gold working electrode, and an anion-exchange column (Carbopac PA-10, 4.6 × 250 mm). Samples were applied with an autosampler (AS3500, SpectraSYSTEM) *via* a microinjection valve with a 200-μl sample loop. Monosaccharides were analyzed at an isocratic NaOH concentration of 18 mM at ambient temperature. Monosaccharides were identified and quantified by comparison to standards. Data were collected and integrated on a PeakNet system (Dionex). HPAEC standards of myo-inositol (99%), sorbitol (98%), fucose (99%), arabinose (99%), glucosamine (99%), galactose (99%), glucose (99.5%), mannose (99%), and fructose (99%) were purchased from Sigma (St. Louis, MO, USA).

2.7. Determination of sulfate ions (SO_4^{2-})

The method for estimating the liberated sulfate ions was based on Saito, Yamagata, & Suzuki (1968) with the following modifications. An aliquot (300 μl) of the acid hydrolysis of SPSs was pipetted into 700 μl of a $BaCl_2$-gelatin solution (see below). After mixing, the entire solution was allowed to stand for 10 min at room temperature. The absorbance of the test solution was measured against a blank at 360 nm. The $BaCl_2$-gelatin solution was prepared by dissolving 0.5 g gelatin in 100 ml of distilled water at 60 °C and allowing it to stand at 4 °C overnight. To this solution, 1.48 ml of 12.1 N HCl and 0.5 g $BaCl_2$ were added.

2.8. Cell culture

One endothelial cell line (human endothelial-like cells, Eahy926) and four cancer cell lines, MCF-7 (breast cancer), NCI-H460 (lung cancer), HT-29 (colon cancer), and CEM (leukemia), were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in DMEM or RPMI medium (Life Technologies, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies) under standard culture conditions. The cell viability and cell number were determined by the Trypan blue dye-exclusion method.

2.9. Determination of cell viability

To assess cell viability, the alamar blue (AB) assay (dye purchased from Biosource International, Nivelles, Belgium) was used. Doxorubicin (DOX) was used as a positive control. Cells were treated with different SPSs with or without DOX for 48 h, and the viability was determined. This involved aspirating medium at the end of each treatment period and adding 100 μl of fresh medium containing 10% (v/v) AB to the control and treated wells. Plates were incubated at 37 °C for 6 h prior to measuring the absorbance at 540 and 595 nm wavelengths using a spectrophotometric plate reader (DYNEX Technologies, Chantilly, VA, USA). Experimental data were normalized to control values. Serial dilutions were performed to

obtain values of the 50% inhibitory concentration (IC_{50}) of cell cytotoxicity.

2.10. Matrigel endothelial cell (EC) tube formation assays

Endothelial tube formation assay was modified from a previously described method (Jones et al., 1999). Matrigel (12.5 mg/ml) was thawed at 4 °C, and 50 μl was quickly added to each well of a 96-well plate and allowed to solidify for 10 min at 37 °C. Once the gel had solidified, wells were incubated for 30 min with ECs (3×10^4 cells/well). After adhesion of cells, the medium was removed and replaced by fresh medium supplemented with vascular endothelial growth factor (VEGF) with or without SPSs and incubated at 37 °C for 24 h. The growing tubes were visualized with an inverted Zeiss microscope (Oberkochen, Germany) at a magnification of 10×, and the length and total area of the capillary network were quantified with a map scale calculator (KURABO Angiogenesis Image Analysis Software, Osaka, Japan). The IC_{50} value was obtained after calculating 50% inhibition of the total tube area formation.

2.11. Statistical analysis

Data are presented as the mean ± standard error (SE), and *n* represents the number of experiments. In bar graphs, SE values are indicated by error bars. Statistical analyses were carried out using Student's unpaired *t*-tests when applicable. *p* values of <0.05 were considered significant.

3. Results and discussion

3.1. Molecular-mass determination

To elucidate the relationship between the structure and biological function of these SPSs, they were characterized according to their molecular size distributions and sugar compositions. The molecular-weight distribution of lyophilized SPS-containing preparations was chromatographed (Fig. 1). A calibration curve was constructed using a series of standards containing polymaltotriose with molecular weights of 788, 404, 212, 112, 47.3, 22.8, 11.8, and 5.9 kDa. The molecular-weight distribution of the lyophilized SPS-containing preparations was chromatographed and characterized as high- (>1000 kDa, denoted as peak 1), moderately high- (100–1000 kDa, denoted as peaks 2 and 3), low- (1–100 kDa, denoted as peaks 4 and 5), and very-low-molecular-weight SPSs (<1 kDa, denoted as peak 6). The results showed that the two strains of *A. cinnamomea* generated similar molecular-weight distributions of SPS polymers. SPSs of *A. cinnamomea* contained five fractions. The area percentage of low-molecular-weight SPSs (peaks 4 and 5) covered almost half of the SPS mixture. The area percentage of moderate-molecular-weight SPSs (peaks 2 and 3) was the second large group of the SPS mixture. The molecular-weight-distribution profile for *P. cocos* greatly differed from those of *A. cinnamomea* in that high-molecular-weight SPSs (peak 1) were the largest proportion of the SPS mixture with a value of 30.06%.

3.2. Composition of SPSs

To study the structure of SPSs, the sugar composition had to be analyzed. A compositional analysis was performed after SPS fractions were completely hydrolyzed. The chemical profile is shown in Fig. 2, and the carbohydrate components are presented in Table 1. Results showed that different fungal species contained different sugar compositions of SPSs. Fucose, glucosamine, galactose, glucose, and mannose were the neutral sugars in the SPSs. Comparisons were made among the three fungal strains. Sulfate contents

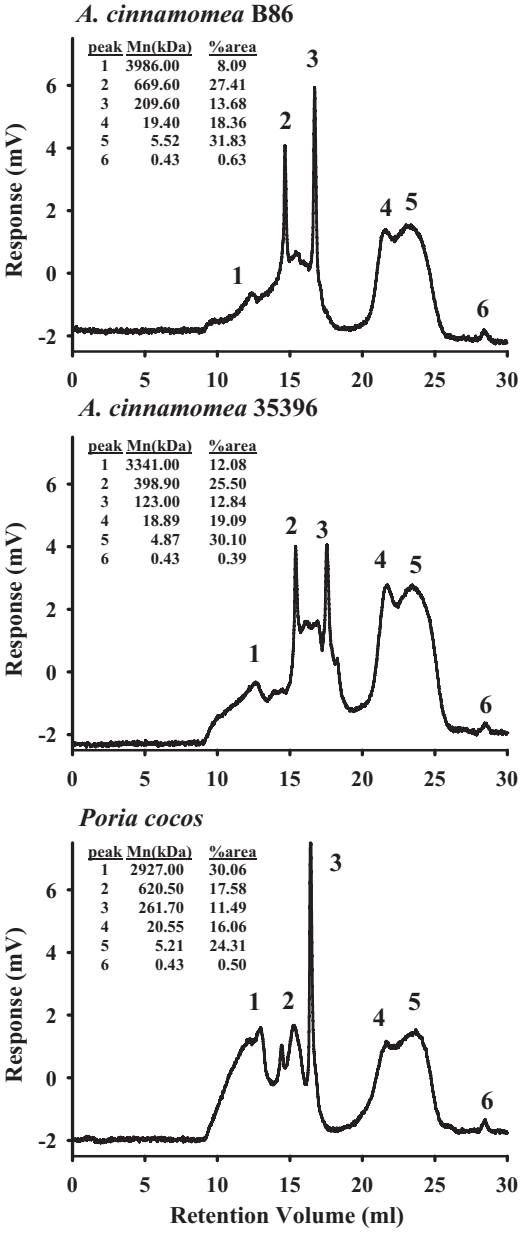


Fig. 1. Size-exclusion chromatographic (SEC) profile of fungal sulfated polysaccharides (SPSs). SPSs were isolated from 49-day cultured mycelia. SEC was performed using a ViscoTek model TDA-3-1 relative viscometer (Viscotek). An SPS solution in milli-Q water was diluted to give a final concentration of 1 mg/ml for the determination.

Table 1
Sugar compositions of sulfated polysaccharides (SPSs).

	Neutral sugars (μmol/g SPSs)		
	Antrodia cinnamomea B86	*A. cinnamomea* 35396	*Poria cocos*
Fucose	46.03 ± 3.40	34.29 ± 1.34	77.40 ± 0.15
Glucosamine	11.12 ± 0.09	24.39 ± 0.04	Trace
Galactose	375.49 ± 0.77	347.49 ± 0.53	289.85 ± 1.24
Glucose	796.60 ± 1.72	1278.01 ± 2.80	1347.08 ± 2.16
Mannose	41.90 ± 0.12	59.62 ± 1.36	158.87 ± 0.60
μμmol/g SPSs			
Sulfate[a]	607.20 ± 16.50	577.50 ± 6.60	496.70 ± 1.65

[a] Sulfate was determined by the $BaCl_2$-gelatin method (Saito et al., 1968).

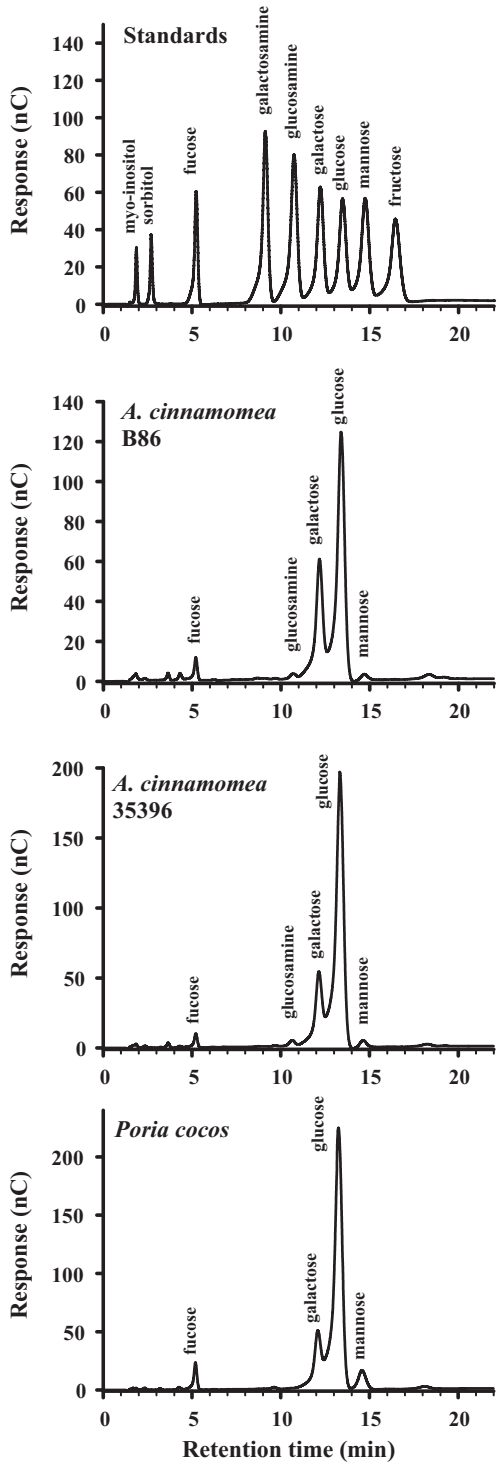


Fig. 2. High-performance anion-exchange chromatography (HPAEC) of fungal sulfated polysaccharide (SPS) hydrolysates. SPSs were completely hydrolyzed with 4.95 N trifluoroacetic acid (TFA) at 80 °C in a heating block for 24 h. The HPAEC analysis was carried out with 18 mM NaOH for 22 min at ambient temperature.

of strains B86, 35396, and *P. cocos* were determined to be 607, 578, and 497 μmol/g SPSs, respectively.

3.3. Effects of fungal SPSs on EC tube formation

To study the effects of fungal SPSs on angiogenesis, an *in vitro* assay was performed to evaluate EC tube formation in Matrigel. Serial dilutions of SPSs were evaluated for their effects on VEGF-induced angiogenesis revealed by tube formation on Matrigel after calculating the total area of the capillary network (Fig. 3). IC_{50} values of *A. cinnamomea* B86 and 35396, and *P. cocos* were 160.2, >200, and >200 μM, respectively. The ratio of the tube formation area, the ratio of tube length, and branches all indicated a dose-dependent effect of inhibiting angiogenesis with SPS pretreatment with *A. cinnamomea* B86. Among the three fungal SPSs, *A. cinnamomea* B86 showed stronger inhibition of tube formation than *A. cinnamomea* 35396 and *P. cocos*. This assay revealed that EC tube formation was attenuated by treatment with SPSs from *A. cinnamomea* B86 for 24 h.

Table 2
Cytotoxicity of fungal sulfated polysaccharides (SPSs) with doxorubicin (DOX) against four human tumor cell lines.

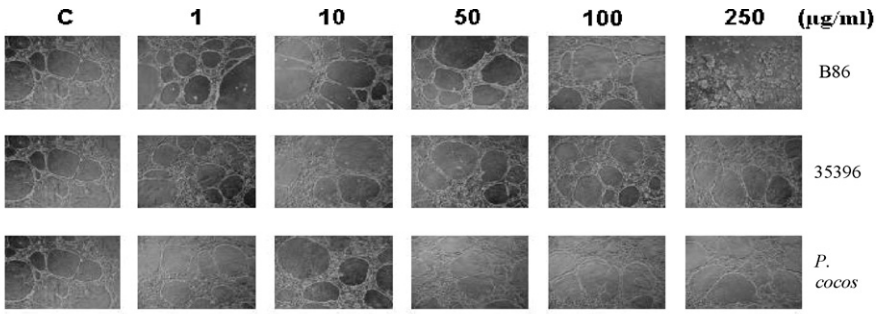
100 μg/ml SPSs	Cell line (IC_{50}, μM)[a]			
	MCF-7	NCI-H460	HT-29	CEM
Antrodia cinnamomea B86 + DOX	7.2	0.63	0.04	8.67
A. cinnamomea 35396 + DOX	46.43	22.03	49.49	15.42
Poria cocos + DOX	16.19	0.04	5.94	18.77
DOX	2.46	2.29	2.02	2.32

[a] IC_{50}, concentration that caused a 50% reduction in absorbance at 540 and 595 nm relative to untreated cells using the AB assay.

Heparan sulfate proteoglycans (HSPGs) modulate the binding of VEGF and fibroblast growth factors (FGFs) with their respective receptors (Presta et al., 2005). HSPGs also stabilize the FGF-2/FGF and VEGF/VEGF receptor complexes. Angiogenic inhibitors may possibly interfere with FGF-2 or VEGF activity in two ways: either directly by blocking interactions of VEGF or FGF with their receptors or by disrupting the bystander effect of HSPGs for efficient growth factor binding. Since fungal SPSs have similar structures as HSPGs, this property might be one reason that drives the antiangiogenesis activity of SPSs.

3.4. Effects of combination therapy utilizing fungal SPSs with DOX

The cytotoxic activity of fungal SPSs were evaluated against a panel of human tumor cell lines, including breast cancer (MCF-7), lung cancer (NCI-H460), colon cancer (HT-29), and leukemia (CEM) (Table 2). SPSs of *A. cinnamomea* B86 and 35396, and *P. cocos* alone had low cytotoxic effects on the four cancer cells with an IC_{50} value of 200 μg/ml for B86 against MCF-7; all the others had IC_{50} values of >250 μg/ml (data not shown). However, fungal SPSs at 100 μg/ml, which is non-cytotoxic to cancer cells, showed synergistic effects on *in vitro* cancer cell cytotoxicity when combined with DOX. The enhanced cytotoxicity was most evident in NCI-H460 and HT-29 cells where the IC_{50} value of 0.04 μM was much lower than that of DOX alone with respective IC_{50} values of 2.29 and 2.02 μM. Combination treatment with 100 μg/ml fungal SPSs generated a 50-fold reduction in the IC_{50} compared to DOX alone. Enhancing the sensitivity of chemotherapeutic agents in combination with other components from natural sources is a promising way to reduce their side effects. Tanshinones from *Salvia miltiorrhiza* provided synergism to DOX cytotoxicity in an *in vitro* DOX-resistant HCC model (Lee et al., 2010). In this study, it was obvious that SPSs differentially influenced the sensitivity of different kinds of cancer cell lines to DOX at nontoxic concentrations. The dramatic augmentation of sensitivity to SPSs and DOX observed in this study might facilitate overcoming the relatively high incidence of chemotherapy resistance. It was reported that polysaccharopeptide enhances the anticancer activity of doxorubicin on human breast cancer cells (Wan, Sit, & Louie, 2008). Further, *Phellinus linteus*, mostly consisted of polysaccharides, sensitized apoptosis induced by doxorubicin in prostate cancer (Collins, Zhu, Guo, Xiao, & Chen, 2006). Although in our study the synergistic mechanism was not determined.



Sulfated polysaccharides IC_{50} (μg/ml)	Conc. (μg/ml)	Percent area	Length	Branch
Antrodia cinnamomea B86 160.21	1	1.02	0.94	0.90
	10	0.97	1.01	0.97
	50	1.03	1.01	1.03
	100	0.99	0.99	0.94
	250	0.00	0.00	0.00
A. cinnamomea 35396 > 250	1	1.03	1.04	1.01
	10	1.02	1.07	1.07
	50	0.97	1.08	1.08
	100	1.00	1.04	0.98
	250	1.02	1.15	1.15
Poria cocos > 250	1	0.98	1.03	0.99
	10	1.03	1.06	1.08
	50	1.01	1.07	1.05
	100	1.02	1.07	1.05
	250	0.99	1.05	1.06

Fig. 3. Influences of sulfated polysaccharides (SPSs) of *Antrodia cinnamomea* and *Poria cocos* on endothelial cell (EC) tube formation. ECs were seeded onto Matrigel and cultured for 24 h under vascular endothelial growth factor (VEGF) stimulation with or without (control; C) pretreatment with a series of dilutions of SPSs for 1 h. Capillary tube formation on Matrigel was visualized with an inverted Zeiss microscope at a magnification of 10×. The ratio of the tube formation area and percentages of tube length, joints, and branches were calculated as a percentage of VEGF-treated ECs using KURABO Angiogenesis Image Analysis Software as indicated in the lower panel. IC_{50}, concentration that caused a 50% reduction in tube formation area relative to that of VEGF-treated cells.

However, SPSs in our study might augment Dox-mediated apoptotic signaling in the same way and requires further evaluation.

In conclusion, fungal SPSs in this study exhibited synergistic activity with DOX against some cancer cell lines. The fact that SPSs increased the sensitivity of DOX offers new applications for combinational-therapy drugs.

Acknowledgements

We thank Mr. D.P. Chamberlin for critically reading the manuscript. This work was supported in part by grants NRICM-99-HDNP-03 and NRICM-99-DBCMR-08 from the National Research Institute of Chinese Medicine, Taipei, Taiwan.

References

Albano, R., & Mourio, P. (1986). Isolation, fractionation, and preliminary characterization of a novel class of sulfated glycans from the tunic of *Styela plicata*. *Journal of Biological Chemistry*, *261*, 758–765.

Cheng, J., Huang, N., Lur, H., Kuo, C., & Lu, M. (2009). Characterization and biological functions of sulfated polysaccharides from sulfated-salt treatment of *Antrodia cinnamomea*. *Process Biochemistry*, *44*, 453–459.

Cohen, T., Gitay-Goren, H., Sharon, R., Shibuya, M., Halaban, R., Levi, B., et al. (1995). VEGF121, a vascular endothelial growth factor (VEGF) isoform lacking heparin binding ability, requires cell-surface heparan sulfates for efficient binding to the VEGF receptors of human melanoma cells. *Journal of Biological Chemistry*, *270*, 11322–11326.

Collins, L., Zhu, T., Guo, J., Xiao, Z., & Chen, C. (2006). *Phellinus linteus* sensitizes apoptosis induced by doxorubicin in prostate cancer. *British Journal of Cancer*, *95*, 282–288.

Folkman, J. (2004). Endogenous angiogenesis inhibitors. *Acta Pathologica Microbiologica et Immunologica*, *112*, 496–507.

Jones, M., Wang, H., Peskar, B., Levin, E., Itani, R., Sarfeh, I., et al. (1999). Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: Insight into mechanisms and implications for cancer growth and ulcer healing. *Nature Medicine*, *5*, 1418–1423.

Lee, W., Cheung, Y., Chartia, C., Liu, K., Fung, W., Wong, K., et al. (2010). Cytotoxic effects of tanshinones from *Salvia miltiorrhiza* on doxorubicin-resistant human liver cancer cells. *Journal of Natural Products*, *73*, 854–859.

Miao, B., Geng, M., Li, J., Li, F., Chen, H., Guan, H., et al. (2004). Sulfated polymannuroguluronate, a novel anti-acquired immune deficiency syndrome (AIDS) drug candidate, targeting CD4 in lymphocytes. *Biochemical Pharmacology*, *68*, 641–649.

Parish, C., Freeman, C., Brown, K., Francis, D., & Cowden, W. (1999). Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel in vitro assays for angiogenesis and heparanase activity. *Cancer Research*, *59*, 3433–3441.

Presta, M., Oreste, P., Zoppetti, G., Belleri, M., Tanghetti, E., Leali, D., et al. (2005). Antiangiogenic activity of semisynthetic biotechnological heparins. Low-molecular-weight-sulfated *Escherichia coli* K5 polysaccharide derivatives

as fibroblast growth factor antagonists. *Arteriosclerosis Thrombosis and Vascular Biology*, *25*, 71–76.

Roden, L., Ananth, S., Campbell, P., Curenton, T., Ekborg, G., Manzella, S., et al. (1992). Heparin: An introduction. *Advances in Experimental Medicine and Biology*, *313*, 1–20.

Saito, H., Yamagata, T., & Suzuki, S. (1968). Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. *Journal of Biological Chemistry*, *243*, 1536–1542.

de Sousa Cardozo, F., Camelini, C., Mascarello, A., Rossi, M., Nunes, R., Barardi, C., et al. (2011). Antiherpetic activity of a sulfated polysaccharide from *Agaricus brasiliensis* mycelia. *Antiviral Research*, *92*, 108–114.

Wan, J., Sit, W., & Louie, J. (2008). Polysaccharopeptide enhances the anticancer activity of doxorubicin and etoposide on human breast cancer cells ZR-75-30. *International Journal of Oncology*, *32*, 689–699.

Wang, J., Hu, Y., Wang, D., Liu, J., Zhang, J., Abula, S., et al. (2010). Sulfated modification can enhance the immune-enhancing activity of lyceum barbarum polysaccharides. *Cellular Immunology*, *263*, 219–223.

Veena, C., Josephine, A., Preetha, S., & Varalakshmi, P. (2007). Beneficial role of sulfated polysaccharides from edible seaweed fucus vesiculosus in experimental hyperoxaluria. *Food Chemistry*, *100*, 1552–1559.

Vlodavsky, I., Miao, H., Medalion, B., Danagher, P., & Ron, D. (1996). Involvement of heparan sulfate and related molecules in sequestration and growth promoting activity of fibroblast growth factor. *Cancer Metastasis Reviews*, *15*, 177–186.