JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Enhancement of Cyclophosphamide-Induced Antitumor Effect by a Novel Polysaccharide from *Ganoderma atrum* in Sarcoma 180-Bearing Mice

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ABSTRACT: The aim of this study was to investigate the enhancement of *Ganoderma atrum* polysaccharide (PSG-1) on cyclophosphamide (CTX)-induced antitumor effect in sarcoma 180 (S-180)-bearing mice. Results showed that both CTX and PSG-1 delayed tumor growth and resulted in tumor apoptosis. The combined regimen was superior to either modality alone. Moreover, the combined treatment-induced apoptosis was mediated via mitochondrial pathway, as evidenced by alterations of Bcl-2 family proteins, loss of mitochondrial membrane potential ($\Delta\psi_m$), cytochrome *c* release, and caspases activation. Our results also showed that thymus and spleen indexes, lymphocytes proliferation, and concentrations of cytokine in the CTX group were decreased, which were alleviated by PSG-1. Additionally, the combined treatment ameliorated oxidative stress as compared with CTX alone. Taken together, we conclude that PSG-1 improved the antitumor effect of CTX, possibly in part mediated by enhancing the induction of apoptosis via mitochondrial pathways, activating host immune function, and modifying the redox system in S-180-bearing mice.

KEYWORDS: Antitumor, combined treatment, cyclophosphamide, *Ganoderma atrum*, polysaccharide

INTRODUCTION

Ganoderma atrum, one of the most popular nutritional fungi, belongs to the polyporaceae family of Basidiomycota. Evidence for the protective effect of *G. atrum* in reduction of cancer risk has been reported. This protective effect is attributed to *G. atrum* polysaccharides, which have been shown to play an important role in the chemoprotection of the fungus. The major mechanism by which polysaccharides protect cells was traditionally thought to be through activating the host immune response. Moreover, different mechanisms seem to have some degree of interaction to synergistically afford chemoprevention.[1−3]

Cancer is a global health problem with high morbidity and mortality and poses both economic and psychological challenges. Conventional cancer therapies, including surgery, chemotherapy, and radiotherapy, as single modalities have limited roles in the overall treatment of most solid tumors. Thus, the strategies for cancer treatment using combined therapies or combined agents with distinct molecular mechanisms of action are considered to be more promising.[4−6] Chemotherapy-induced immunosuppression leads to serious morbidity and mortality, a major limiting factor in clinical chemotherapy without efficacious remedies. Cyclophosphamide (CTX), a traditional alkylating agent, is frequently used in cancer chemotherapy. The antitumor effect of CTX is in proportion to the dose of CTX administered, often resulting in immunosuppressive effects.[7] We have recently purified a novel polysaccharide (named PSG-1 with a purity of >99.8%). Its primary structural features and molecular weight were characterized. Briefly, PSG-1 is a homogeneous protein-bound polysaccharide, with a weight-average molecular mass of 1013 kDa based on the gel permeation chromatography (GPC) analysis. The predominant monosaccharides in PSG-1 are D-glucose, D-galactose, and D-mannose in a molar ratio of 1:1.28:4.91, detected by gas chromatography (GC). The β-elimination reaction demonstrated that the protein and carbohydrate are linked by an O-linkage.[8] We have further shown that PSG-1 possesses antioxidant activity and potent immunopotentiating activity against CTX-induced immunosuppression in vivo.[8,9]

Apoptosis, or programmed cell death, is a very important phenomenon in cytotoxicity induced by anticancer treatment.[10] The distinct mechanisms execute apoptosis according to various apoptotic stimuli and are classified into mitochondria-dependent apoptotic pathway and reticulum endoplasmic pathway.[11] The mitochondria-dependent apoptotic pathway is stimulated in various stresses including antitumor treatment. These stimuli induce loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) and release of cytochrome *c* from the impaired mitochondria to cytosol. The released cytochrome *c* forms a complex with apoptotic protease activating factor-1 in the presence of dATP, which recruits and activates caspase-9. Activated caspase-9 leads to the activation of caspase-3, which subsequently contributes to apoptotic cell death. The Bcl-2 family, which includes both antiapoptotic and proapoptotic proteins, acts as a checkpoint upstream of mitochondria dysfunction. Antiapoptotic proteins promote cell survival, whereas proapoptotic proteins mediate mitochondria-dependent apoptosis.[12]

During the past three decades, natural product-derived polysaccharides have been shown to exhibit potent immunotherapeutic properties with respect to the prevention and treatment of cancer. It has been well established that these compounds act as biological response modifiers that can amplify the antineoplastic effects of chemotherapy by enhancing the cytotoxic activities of effector immune cells and/or by antagonizing the immunosuppressive effects of radiation and anticancer drugs. Some of these

Received: December 25, 2010
Accepted: March 7, 2011
Revised: March 7, 2011
Published: March 07, 2011

polysaccharides have been used as adjuvants in the treatment of cancer.[13,14]

In this study, we show that the novel polysaccharide PSG-1 from *G. atrum* enhances CTX-induced antitumor effect in sarcoma 180 (S-180)-transplanted mice used as a human tumor model. Furthermore, the present study has for the first time demonstrated that PSG-1 could enhance the antitumor effect of CTX by triggering the mitochondrial pathway in S-180-bearing mice. Immunoenhancement and antioxidant activities of PSG-1 are related to the enhancement of PSG-1 on CTX-induced antitumor effect.

MATERIALS AND METHODS

Reagents. PSG-1 was extracted and purified by our group following our previously published method.[8] CTX (Shanghai Hualian Pharmaceutical Co.Ltd., Shanghai, China) was dissolved in 0.9% NaCl. Cell culture products were obtained from Life Technologies (Paisley, Scotland). Annexin V-FITC apoptosis detection kits were obtained from BD Biosciences (San Jose, SD). Anti-Bcl-2, anti-Bax, anticytochrome *c*, and anti-α-tubulin primary antibodies, as well as the HRP-linked secondary antibody, were from Santa Cruz Biotechnology (Santa Cruz, CA). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from SenXiong Biotech (Shanghai, China).

Cells and Animals. Murine S-180 cell line was purchased from Type Culture Collection (TCM) of the Chinese Academy of Sciences (Shanghai, China). S-180 cells were maintained as ascites in Kunming mice by weekly passage. S-180 cells, obtained from the peritoneal cavity of tumor-inoculated mice, were washed twice with phosphate-buffered saline (PBS) and adjusted to 1×10^7 cells/mL in PBS. Kunming mice, weighing 20.0 ± 2.0 g [Grade II, Certificate Number SCXK (gan) 2006-0001], were purchased from Jiangxi College of Traditional Chinese Medicine (Jiangxi, China). All animals used in this study were cared for in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH Publication No. 85-23, 1996), and all procedures were approved by the Animal Care Review Committee, Nanchang University (China).

Determination of Effect of Combined Therapy on Tumor Growth. S-180 ascites tumor cells were implanted subcutaneously into the left hind groin of the mice. The polysaccharide was dissolved in saline solution and given to the mice by orally (po) to the stomach (gavage) once daily in a volume of 0.2 mL for 10 consecutive days. At the beginning of the experiment, the mice were divided into five groups as follows. (1) Control group: after the day of inoculation (day 1), the mice were treated po with injection of vehicle saline. (2) PSG-1 group: After the day of inoculation (day 1), the mice were treated po with injection of PSG-1 (25 mg/kg body weight). (3) CTX group: After the day of inoculation (day 1), the mice were treated intraperitoneally (ip) with injection of CTX (20 mg/kg body weight). (4) PSG-1 + CTX group: After the day of inoculation (day 1), the mice were treated po with injection of PSG-1 (25 mg/kg body weight) and by ip injection of CTX (20 mg/kg body weight). (5) Normal group: The mice without inoculating received the same volume of vehicle saline injection. After 10 days of administration, mice were sacrificed by cervical dislocation. Tumor, spleen, and thymus weights of the mice were measured. The inhibition ratio was calculated by the following formula: inhibition ratio (%) = $[(A - B)/A] \times 100$, where A is the average tumor weight of control group, and B is that of the treat group. Thymus and spleen indexes were expressed as the thymus and spleen weight relative to body weight.

Determination of Survival in S-180-Bearing Mice. The effect of the polysaccharide on the survival of S-180 tumor-bearing animals was tested. The tumor was implanted subcutaneously into the left hind groin of the mice as described above. The treatments were started 1 day after tumor implant once daily for 10 days. The death pattern of animals due to tumor burden was recorded, and the percentage of increase in life span was calculated using the formula $[(T - C)/C] \times 100$, where "*T*" and "*C*" represent the number of days that treated and control animals survived, respectively.

Morphologic Assessment of Apoptosis. We selected four groups for morphologic assessment of apoptosis in S-180-bearing mice. Tumor tissue was cut into small pieces, homogenized into 1 mm × 2 mm, prefixed with 4% paraformaldehyde for 4 h, washed with PBS, postfixed with 1% osmic acid, dehydrated in graded ethanol−acetonum, embedded in an Epon 812 mixture, and cut into sections on an ultramicrotome. After they were stained with uranyl acetate and lead citrate, the sections were examined under a Hitachi-800 transmission electron microscope (Scotia, NY).

Flow Cytometric Analysis of Apoptosis. Fresh tumor tissue separated from S-180-bearing mice was chopped with a mechanical method. Single cell-suspended solution was obtained with 200 filter screens. The suspensions were washed twice and adjusted to a concentration of 1×10^6 cells/mL with PBS and stained with annexin V-FITC and propidium iodide (PI) in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$). Ten thousand events were collected for each sample. Stained cells were analyzed using a FACStar Plus flow cytometry in the FL1-H and FL2-H channels.

Determination of Mitochondrial Membrane Potential. The changes of mitochondrial membrane potential ($\Delta\psi_m$) were assayed by dye, 5,50′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (Beyotime Institute of Biotechnology, Shanghai, China). The $\Delta\psi_m$ was detected according to the changes of JC-1 fluorescence. The values of optical density (OD) at 590 and 530 nm were determined by a spectrofluorometry. As the $\Delta\psi_m$ is proportional to the ratio of OD590 to OD530, the $\Delta\psi_m$ was expressed as OD590/OD530.

Determination of Bcl-2, Bax, and Cytochrome *c* Proteins Expression. For Western blot analysis, fresh tumor tissue was collected after the mice were sacrificed and fractionated into mitochondrial and cytoplasmic compartments with a mitochondrial/cytosol fractionation kit (Biovision, Mountain View, CA). Samples were mixed with Laemmli sample buffer (Bio-Rad, Hercules, CA) and then boiled for 5 min and loaded onto 12 or 15% sodium dodecyl sulfate−polyacrylamide gels. After they were electroblotted onto PVDF membranes, the sample blots were blocked for 2 h with 5% nonfat milk powder in TBS (pH 7.6) containing 0.1% Tween-20 (TBST) [25 mM Tris-HCl, pH 7.6, 0.2 M NaCl, and 0.1% (v/v) Tween-20] at room temperature. Membranes were incubated with the antibodies overnight at 4 °C and then blotted with different antibodies in TBST. To ensure equivalent protein loading, the membranes were also incubated with anti-α-tubulin mouse monoclonal antibody (Cytosolic Loading Control) and anti-VDAC1 rabbit polyclonal antibody (Abcam, Cambridge) (Mitochondrial Loading Control) and subsequently with a corresponding horseradish peroxidase-conjugated second antibody IgG and developed using Chemiluminescence Reagent Plus (Waltham, Massachusetts). The densitometric analysis of bands was carried out using GDS-8000 UVP photo scanner (Upland, CA) and LAB WORK45 Image software.

Determination of Caspase Activities. Caspase activitives were evaluated by the use of caspase-3 and caspase-9 colorimetric assay kit (Biovision, CA). The assay is based on spectrophotometric detection of the chromophore ρ-nitroanilide (ρNA) after cleavage from the labeled substrate DEVD-ρNA. The absorbance at 405 nm of the released ρNA was monitored in a spectrophotometer.

Assay of Splenocyte Proliferation. Single-cell spleen suspensions were pooled in serum-free RPMI-1640 medium by filtering the suspension through sieve mesh with the aid of a glass homogenizer to exert gentle pressure on the spleen fragments. Samples were washed twice in PBS/0.1% (w/v) bovine serum albumin. After centrifugation

(200*g*, 5 min), the cells were placed into the 96-well flat-bottomed microplates in triplicate at 5×10^5 cells/well in RPMI 1640 medium supplemented with 10% fetal calf serum, and then, 2 μg/well of Con A or 20 μg/well of LPS was added to the wells. The cells were then incubated in a total volume of 200 μL/well. Serum-free RPMI-1640 medium was used as control. Cell proliferation was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. After incubation for 48 h, 20 μL of MTT (Sigma, St. Louis, MO) solutions (5 g/L) was added to each well, and the plate was incubated for another 4 h. The plate was then centrifuged at 200*g* for 10 min, and the supernatants were discarded. A total of 150 μL of DMSO was added to each well and shaken until crystals were dissolved. The absorbance (*A*) at 570 nm was detected on the Microplate Reader (Bio-Rad). The absorbance (*A*) was translated into lymphocyte proliferation ratio for comparison: lymphocyte proliferation ratio = $\text{test}_A/\text{normal control}_A \times 100\%$.

Table 1. Effects of Combined Therapy on the Thymus and Spleen Indexes in S 180-Bearing Mice[a]

group	PSG-1 (mg/kg/day)	CTX (mg/kg/day)	spleen index (mg/g)	thymus index (mg/g)
control	vehicle	vehicle	4.10 ± 0.36	2.21 ± 0.11
PSG-1	25	vehicle	5.79 ± 0.43**	2.81 ± 0.16**
CTX	vehicle	20	3.16 ± 037**	1.92 ± 0.10*
PSG-1 + CTX	25	20	5.20 ± 0.40$^{\$\$}$	2.31 ± 0.17$^{\$\$}$

[a] S180-bearing mice were administered po with the test compounds for 10 days once daily. The spleen and thymus weights were measured on day 11. Thymus and spleen indexes were expressed as the thymus and spleen weight relative to body weight. Values are expressed as means ± SDs of eight mice for each group. *$P < 0.05$, and **$P < 0.01$ as compared to control group; $^{\$\$}P < 0.01$ as compared to CTX group.

Cytokines Assay. Serum was collected by retroorbital venous puncture 24 h after the last administration of the PSG-1. ELISA was performed to quantify interleukin (IL)-2 and tumor necrosis factor (TNF)-α in the serum according to the manufacturer's instruction. IL-2 and TNF-α concentrations were calculated from the absorbance values by plotting the values via IL-2 and TNF-α standard curves, which were performed for each assay.

Assays of Enzyme Activities and Malondialdehyde Contents. Serum was collected by retro-orbital venous puncture according to the above-described and immediately assayed for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA) contents using commercial kits (JianCheng Bioengineering Institute, Nanjing, China) with a spectrophotometer (UV-120-02, Shanghai, China), following the manufacturer's instruction.

Statistical Analyses. Data were expressed as means ± SDs. Statistical significance was analyzed using a linear mixed model (PASW Statistics software version 17.0, 2008, SPASS Inc., Chicago, IL), and a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of Combined Therapy on Thymus, Spleen Indexes, and Tumor Weights. As shown in Figure 1A,B, both PSG-1 and CTX showed a lower inhibition rate in S-180 tumor when they were used alone. However, when they were mixed, the inhibition rate was significantly raised. Meanwhile, CTX treatment resulted in a decrease of thymus and spleen indexes (Table 1). After the administration of PSG-1 or the mixture of it with CTX, the thymus and spleen indexes were increased

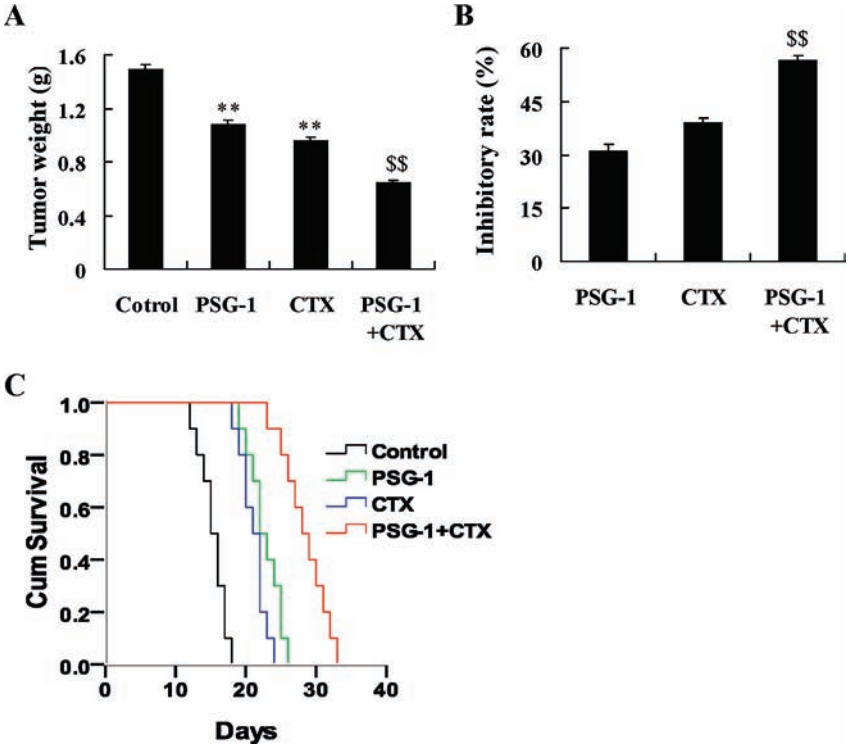

Figure 1. Effects of PSG-1 association with CTX on the antitumor activity in S-180-bearing mice. (A) The tumor weight in S-180-bearing mice. (B) The inhibition rate of PSG-1 on tumor. (C) Kaplan−Meier survival curves of groups of mice. The PSG-1 alone (25 mg/kg, po) or associated with CTX (20 mg/kg, ip) was administered starting 1 day after tumor implant. Control mice received vehicle (0.9% NaCl). Values are means ± SDs of eight mice. **$P <$ 0.01 vs control group.

dx.doi.org/10.1021/jf1049497 |*J. Agric. Food Chem.* 2011, 59, 3707–3716

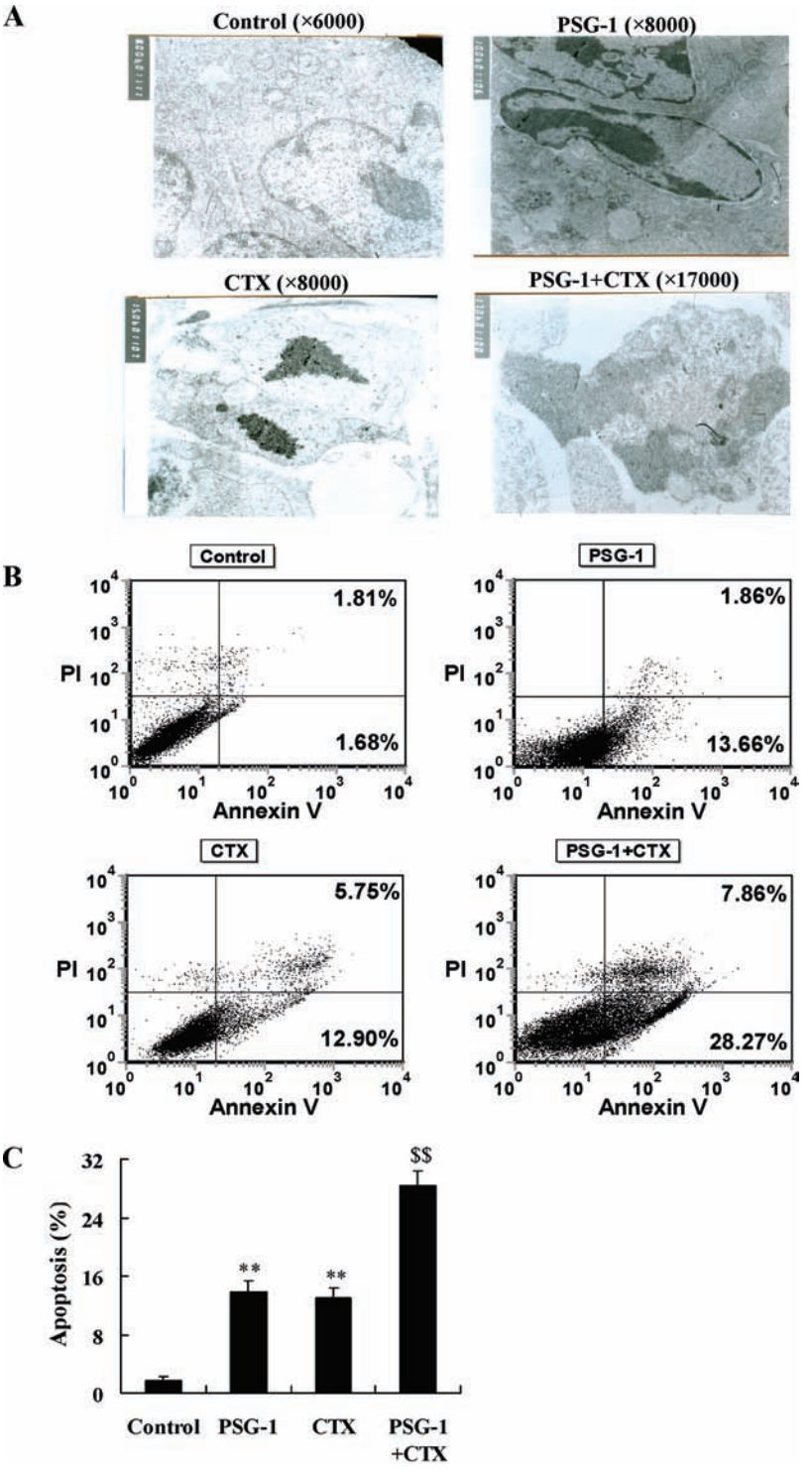

Figure 2. Effects of PSG-1 association with CTX on the apoptosis in the tumor of S-180-bearing mice. (A) Ultrastructure changes characteristic of apoptosis in the tumor of S-180-bearing mice were observed under transmission electron microscopy. (B and C) Flow cytometric analysis of apoptosis in the tumor tissue. (B) Detection of apoptotic cells by annexin V-FITC and PI double staining. Tumor cells stained with annexin V-FITC and PI labeling and analyzed by flow cytometry. (C) Column bar graph of apoptosis. Values were means ± SDs of eight mice. $^{**}P < 0.01$ vs control group, and $^{\$\$}P < 0.01$ as compared to CTX group.

significantly as compared with CTX group. These data suggested that PSG-1 can not only enhance the antitumor activity of CTX but also increase thymus and spleen indexes in S-180-bearing mice.

Effect of Combined Therapy on Survival of S-180-Bearing Mice. The effect of PSG-1 mixed with CTX on the survival of S-180 tumor-bearing animals was tested. In this experiment, the animals were treated (po) with PSG-1 alone or in combination

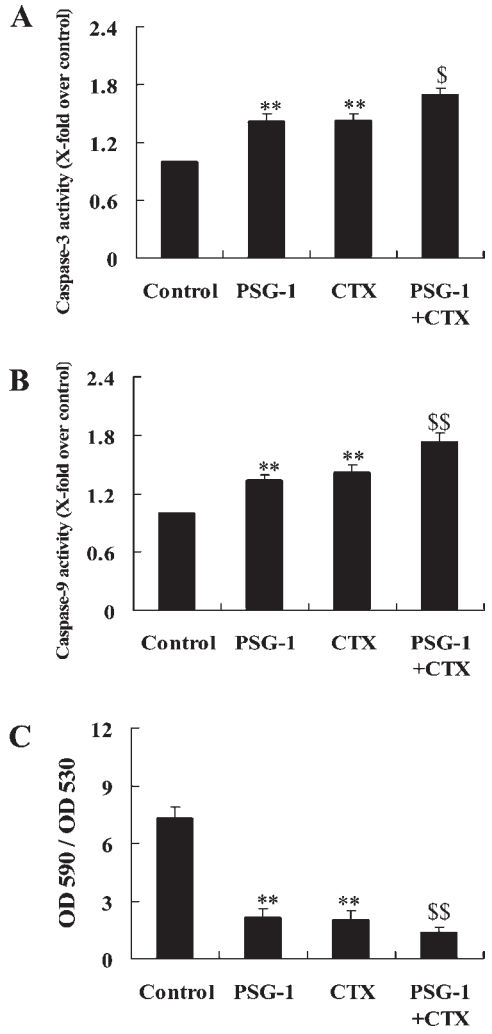

Figure 3. Effects of PSG-1 association with CTX on the activities of caspases and the loss of $\Delta\psi_m$ in the tumor of S-180-bearing mice. (A and B) Quantitative analysis of caspase-3 and caspase-9 activities. (C) Measurement of $\Delta\psi_m$ by JC-1 staining. The values of optical density (OD) at 590 and 530 nm were determined by a spectrofluorometry. The $\Delta\psi_m$ was expressed as the ratio of OD590/OD530. Values were means ± SDs of eight mice. $^{**}P < 0.01$ vs control group, $^{\$}P < 0.05$, and $^{\$\$}P < 0.01$ as compared to CTX group.

with CTX. The life span of solid tumor-bearing mice treated with the polysaccharide was found to be significantly increased (Figure 1C). The control animals survived only 15.3 ± 0.60 days after the tumor induction, while the CTX-treated animals survived 21.1 ± 0.59 days. PSG-1 alone and PSG-1 and CTX-treated animals survived 22.7 ± 0.73 and 28.4 ± 1.01 days, respectively.

Effects of Combined Therapy on Apoptosis. We used a transmission electron microscope to study apoptosis in the tumor tissue. Condensation of chromatin at margins of nuclei, disintegration of nucleolus, vacuoles in cytoplasm, and apoptotic bodies were observed in PSG-1 group, CTX group, and PSG-1 + CTX group (Figure 2A). In addition, flow cytometric analysis was used to quantify the rate of cell apoptosis using double staining of annexin V-FITC and PI. As compared with the S-180-bearing control group, a significant increase of apoptosis was observed in S-180-bearing mice treated with PSG-1 or CTX. After the treatment of PSG-1 mixed with CTX, the apoptosis increased significantly when compared with CTX group (Figure 2B,C). Therefore, PSG-1 increased the antitumor activity of CTX in association with the increase of apoptosis.

Effects of Combined Therapy on Caspase Activity. Caspase activation represents the "irreversible" or execution stage of apoptosis. We subsequently examined the activation of caspases in the tumor tissue of S-180-bearing mice undergoing combined therapy-induced apoptosis. In this work, the caspase-9 and caspase-3 activities were determined by using caspase-3 and caspase-9 colorimetric assay kits. Treatment with PSG-1, CTX, and combined therapy caused a significant increase of caspase-3 and caspase-9 activities (Figure 3A,B). These results suggested that combined therapy-induced apoptosis may be associated with the activation of caspase-3 and caspase-9.

Expression of Bcl-2 Family Proteins. Bcl-2 family proteins are key players in stress/toxicant-mediated apoptosis via their ability to regulate mitochondrial function. As shown in Figure 4, protein expression of mitochondrial Bcl-2 was decreased by the combined therapy. Western blot analysis also revealed that expression of mitochondrial Bax protein increased after the combined therapy, followed by a decreased expression of cytosolic Bax protein (Figure 4A–D). These data indicated that combined therapy induced the loss of Bcl-2 from mitochondria and Bax translocation.

Combined Therapy-Induced Death Signaling Is Mediated through Mitochondrial Pathway. Mitochondrial changes, including the collapse of the $\Delta\psi_m$, may contribute to the release of cytochrome *c* from mitochondria, initiating the cascade of events that result in caspase activation during stress-induced apoptosis. In this work, the changes of $\Delta\psi_m$ were determined by JC-1 staining (Figure 3C). The $\Delta\psi_m$ was expressed as the ratio of OD590/OD530 in accordance with literature.[15] Our results suggested that combined therapy was capable of inducing mitochondrial dysfunction.

The loss of $\Delta\psi_m$ appeared in parallel with the activation of caspase-3 and caspase-9, as well as with apoptosis. In addition, combined therapy induced a substantial release of mitochondrial apoptogenic protein (cytochrome *c*) from the mitochondria into the cytosol (Figure 4A,E,F). The loss of mitochondrial Bcl-2 and Bax translocation were also detected after the combined therapy (Figure 4A–D). These results suggested that mitochondrial dysfunction caused the release of cytochrome *c* into the cytosol and the activation of caspase-9 and caspase-3, thereby propagating the death signal. These data indicated that PSG-1 increased the antitumor activity of CTX via mitochondrial apoptotic pathways.

Effects of Combined Therapy on Lymphocytes Proliferation. The normal murine lymphocyte proliferation ratio induced by Con A or LPS and treated with RPMI 1640 medium was regarded as 100%. As shown in Figure 5A, the basal lymphocyte proliferation appeared to be decreased in the CTX group, as compared with control group. Meanwhile, proliferative responses of lymphocytes to both T cell and B cell mitogens (Con A and LPS, respectively) were also reduced markedly in CTX -treated mice as compared with control group (Figure 5B, C). This result indicated the side effect of CTX chemotherapy. When S-180-bearing mice were administrated with the mixture of PSG-1 and CTX, their lymphocyte proliferation activities were increased significantly as compared to CTX group.

Effects of Combined Therapy on Cytokine Production. Effects of test groups on IL-2 and TNF-α level in murine serum were determined by ELISA. As shown in Figure 6, IL-2 and

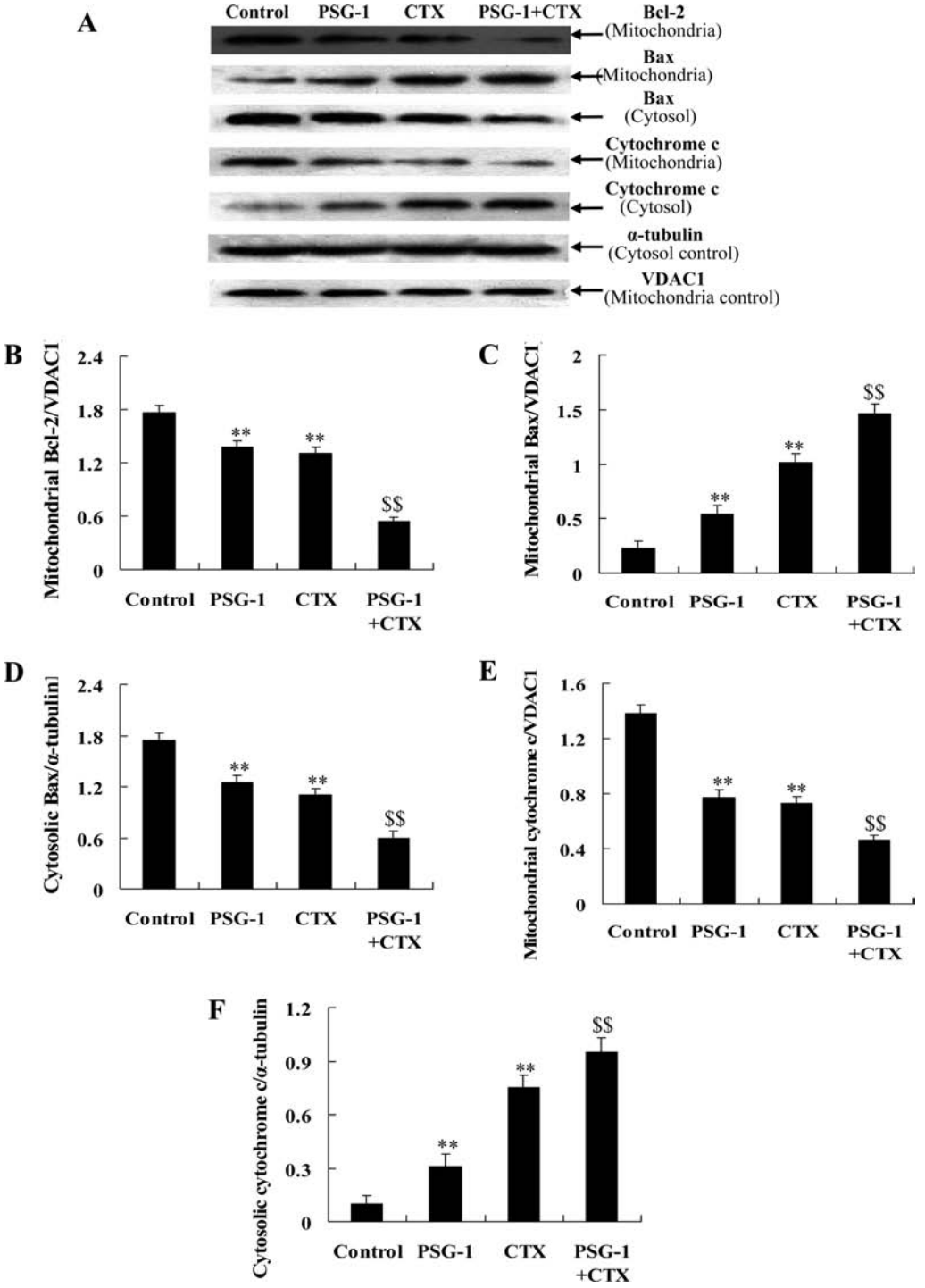

Figure 4. Effects of a combination of PSG-1 with CTX on expression of the apoptosis-associated proteins in the tumor of S-180-bearing mice. (A) Expression of the various apoptosis-associated proteins in the tumor tissue. The cytosolic and mitochondrial proteins in the tumor were analyzed by Western blotting with anti-α-tubulin, VDAC1, cytochrome *c*, Bax, and Bcl-2 antibodies. (B−F) Blots were scanned, and expression of Bcl-2, Bax, cytochrome *c*, α-tubulin, and VDAC1 was quantified by densitometric analysis. The ratios for these proteins are shown. Values are means ± SDs of eight mice. $^{**}P < 0.01$ vs control group, and $^{\$\$}P < 0.05$ as compared to CTX group.

TNF-α levels of the PSG-1 test group were significantly higher than CTX group. At the same time, the combination of PSG-1 and CTX also can restore the IL-2 and TNF-α level to some degree.

Effects of Combined Therapy on the Level of Lipid Peroxidation. The effects of the amixture of PSG-1 and CTX on the level of lipid peroxidation measured in terms of MDA level in serum of tumor-free or tumor-bearing mice were shown in Table 2. Untreated mice bearing solid tumor (control group) showed a marked elevation in MDA contents in serum, as compared with the normal mice (normal group). When the S-180-bearing mice were administrated with the mixture of PSG-1 and CTX, the MDA

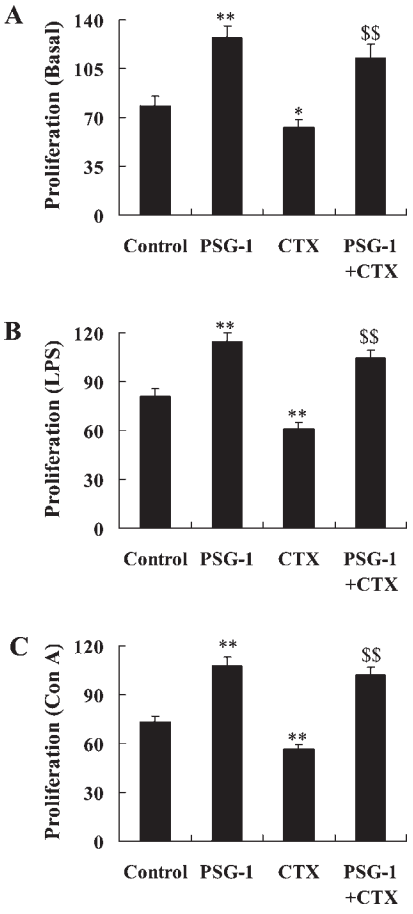

Figure 5. Effects of a combination of PSG-1 with CTX on lymphocytes proliferation in the S-180-bearing mice. (A−C) Basal lymphocyte proliferation and proliferative responses of lymphocytes to both T cell and B cell mitogens (Con A and LPS, respectively) were determined by MTT method. Values are expressed as means ± SDs of eight mice. $^{*}P < 0.05$, and $^{**}P < 0.01$ as compared to control group; $^{\$\$}P < 0.01$ as compared to CTX group.

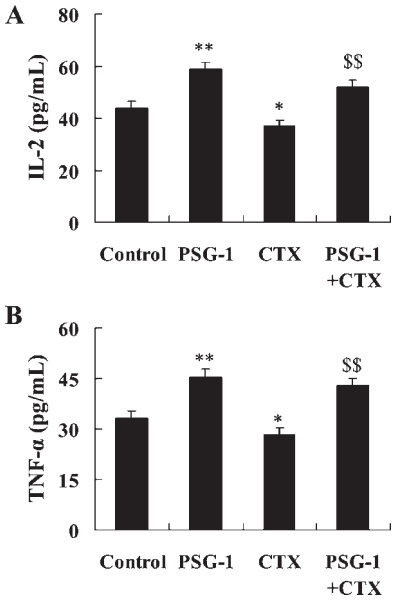

Figure 6. Effect of a combination of PSG-1 with CTX on levels of IL-2 and TNF-α in the serum of S-180-bearing mice. (A−C) Concentrations of TNF-α and IL-2 were determined by ELISA. Values are expressed as means ± SDs of eight mice for each group. $^{*}P < 0.05$, and $^{**}P < 0.01$ as compared to control group; $^{\$\$}P < 0.01$ as compared to CTX group.

contents were decreased more significantly than those of the CTX group and PSG-1 group. These results suggested that PSG-1 could significantly promote CTX-induced reduction of lipid peroxidation in the serum of S-180-bearing mice.

Effects of Combined Therapy on the Activities of Antioxidant Enzymes. We further examined the effect of PSG-1 mixture with CTX on the activity of antioxidant enzymes in normal and S-180-bearing mice. The control group showed a marked decrease in SOD, CAT, and GPx activities in the serum of S-180-bearing mice as compared with the normal group. However, administration of PSG-1 significantly increased the activities of these enzymes in the serum of S-180-bearing mice as compared with the control group. Furthermore, treatment with a mixture of PSG-1 and CTX markedly augmented SOD, CAT, and GPx activities in the serum of S-180-bearing mice as compared with CTX group (Table 2). These data indicated that PSG-1 could advance CTX-induced changes in SOD, CAT, and GPx activities in the serum of S-180-bearing mice.

DISCUSSION

Conventional cancer therapies have a limit as single modalities that play an important role in the overall treatment of most solid tumors. Thus, the strategies of cancer treatment using combined therapies are considered more promising for higher efficacy, which could result in better survival. In recent years, more dietary compounds such as polysaccharide have been demonstrated to prevent cancer or/and exert the antitumor activity. Moreover, common cancer therapies combined with these dietary compounds may exert the enhanced antitumor activity.[16] *G. atrum* is a popular functional food that promotes health and has a long history. It has attracted considerable attention because its polysaccharide (PSG-1) has been demonstrated by recent research to possess an antitumor effect.[9] Therefore, the enhancement of PSG-1 on CTX-induced antitumor effect and the molecular mechanisms were investigated in the present study. Our results show that the combination of CTX with PSG-1 enhances the antitumor activity of CTX and produces a dramatic improvement in survival in S-180-bearing mice.

Apoptosis is a very important phenomenon in cytotoxicity induced by anticancer treatment.[17] The present study also indicates that PSG-1 alone or together with CTX inhibits tumor growth in association with apoptosis. Apoptosis is a highly regulated process that occurs under a range of physiological and pathological conditions as part of the cellular mechanism. It is triggered by two major cell death signaling pathways, death receptor-mediated (extrinsic) and mitochondrial (intrinsic) signaling pathways.[11,18] Numerous studies have demonstrated that various stresses, including chemotherapeutic agents, induced cell apoptosis via the mitochondrial-mediated pathway. Mitochondrial changes, including the collapse of the $\Delta\psi$m, result in the release of cytochrome *c* from the mitochondria into the cytosol, which subsequently leads to activation of the "initiator" caspase-9. Activation of caspase-9 results in activation of caspase-3, which cleaves specific substrate to execute apoptosis.[19]

In accordance with these reports, our results have shown that combined therapy rapidly induced apoptosis in the tumor tissue of S-180-bearing mice in association with the loss of $\Delta\psi_m$, the

Table 2. Effects of Combined Therapy on the Level of Lipid Peroxidation and Activities of Antioxidant Enzymes in S 180-Bearing Mice[a]

group	MDA (nmol/mL)	SOD (U/mL)	CAT (U/mL)	GPx (U/mL)
normal	3.55 ± 0.40	108.37 ± 5.8	7.59 ± 0.38	165.17 ± 3.78
control	$7.87 \pm 0.61^{\#\#}$	$72.77 \pm 4.70^{\#\#}$	$4.17 \pm 0.31^{\#\#}$	$125.84 \pm 3.59^{\#\#}$
PSG-1	$4.29 \pm 0.60^{**}$	$100.82 \pm 5.52^{**}$	$7.09 \pm 0.46^{**}$	$142.59 \pm 4.73^{**}$
CTX	5.89 ± 0.58	84.97 ± 5.29	6.40 ± 0.41	139.61 ± 4.31
PSG-1 + CTX	$3.67 \pm 0.49^{\$\$}$	$108.17 \pm 5.67^{\$\$}$	$7.41 \pm 0.47^{\$}$	$155.87 \pm 6.11^{\$\$}$

[a] S180-bearing mice were administered po with the test compounds for 10 days once daily. S180-bearing control group and normal group received the same volume of sodium chloride 0.9% injection. The activities of SOD, CAT and GPx, and MDA contents were determined on day 11. Values are expressed as means ± SDs of eight mice for each group. $^{\#\#}P < 0.01$ as compared to normal group; $^{**}P < 0.01$ as compared to control group; and $^{\$}P < 0.05$, and $^{\$\$}P < 0.01$ as compared to CTX group.

release of cytochrome *c* from mitochondria into the cytosol and the activation of caspase-3 and caspase-9. Together, these observations suggested that PSG-1 enhanced the antitumor effect of CTX via induction of apoptosis mediated by mitochondrial pathways.

Release of cytochrome *c* from mitochondria is a central step in the initiation of mitochondrial apoptotic pathway.[20] Members of the Bcl-2 family control this process. Bax and Bcl-2, the two main members of this family, influence the permeability of the mitochondrial membrane. It has been demonstrated that Bcl-2 prevents the release of cytochrome *c* by stabilizing the membrane permeability. Conversely, the anticytochrome *c* releasing effect of Bcl-2 can be suppressed by activation of the Bax. Generally, the antiapoptotic protein of Bcl-2 is integrated into the mitochondrial membrane, whereas the proapoptotic protein of Bax is localized in the cytosol. Upon apoptotic stimulation, the decrease of mitochondrial Bcl-2 and the translocation of Bax into mitochondria induce the release of cytochrome *c*.[21,22] The results of the present study showed that combined therapy significantly induced the loss of Bcl-2 from the mitoshondria and the translocation of Bax from cytosol to mitochondria, as compared with the use of CTX alone. These interesting findings imply that the enhancement of the triggering of the mitochondrial pathway by CTX may be related to the effects of PSG-1 on the alteration of Bcl-2 family proteins.

Chemotherapy-induced immunosuppression leads to significant morbidity and mortality, a major limiting factor in clinical chemotherapy without efficacious remedies. The antitumor effect of CTX is in proportion to the dose of CTX administered, often resulting in immunosuppressive effects.[7,23] *G. atrum* has attracted considerable attention because its polysaccharides have been demonstrated to possess diverse and potentially significant pharmacological activities, especially immunomodulatory activity.[9]

Lymphocyte proliferation ratio is an important immune factor that can represent the immunomodulation ability of the body. The proliferation of T and B lymphocytes is known as a response to the stimulation induced by antigen or mitogens.[24,25] In this work, T and B cell proliferation activities of the CTX group were significantly lower than that in the control group, and this also indicated the side effect of CTX. T and B cell proliferation activities of PSG-1 group were higher than those in the control group. When transplanted S-180 tumor mice ingested the mixture of PSG-1 and CTX, their T and B cell proliferation activities were increased more significantly than those of the CTX group. Thymus and spleen are the major immune organs.[25−27] The mixture of PSG-1 and CTX also can restore thymus and spleen indexes decreased by CTX to some degree. Cytokines, soluble glycoproteins, exert an effect on hematopoiesis, immune process, including acute phase reaction and antitumor defense. IL-2 and TNF-α, two important cytokines, exhibit various bioactivities and are involved in immunomodulation.[28] In the present study, CTX significantly decreased IL-2 and TNF-α levels in the serum. However, the mixture of PSG-1 and CTX can restore IL-2 and TNF-α levels reduced by CTX to some degree. In summary, our results revealed that administration of PSG-1 together with CTX could alleviate CTX-induced the immune dysfunction by enhancing the host immune response. Immunosuppression is thought to be one of the mechanisms by which immunogenic tumors are enabled to escape from host immune surveillance, grow progressively, and metastasize. Tumors can evade destruction by inhibiting immune responses directly. Therefore, enhancement of the host immune responses in tumor bearing host could restore the dynamic balance between the tumor cells and the immune response and thus exert the potential antitumor effects. Thus, our results indicated that enhanced antitumor effects of chemotherapy (CTX) by PSG-1 might be achieved by improving host immune response.

Growing evidence suggests oxidative stress as a mechanism contributing to carcinogenesis. Antioxidants play an important role in scavenging the free radicals, thus providing protection to humans against infection and degenerative diseases. The relationship between the antioxidant systems and the growth of malignant cells is a commonly observed feature.[29−31] A number of studies have indicated that tumor growth can cause antioxidant disturbance and acceleration in lipid peroxidation in vital organs of the tumor host. Consistent with these findings,[32,33] our data showed an increase of lipid peroxidation and a marked decrease of several endogeneous antioxidant enzymes such as SOD, CAT, and GPx in the control group, further suggested that there is a direct correlation between changes in the growth of tumor and changes in the antioxidant activities. Therefore, some anticancer agents could act as antioxidants.

Previous studies have demonstrated that PSG-1 had potent antioxidant activities both in vitro and in vivo.[12,34] These suggest that the PSG-1 could potentially be used as natural antioxidants. We thus chose the antioxidant activities for studying the antitumor mechanisms of combined therapy in S-180-bearing mice. In the present study, we also observed that treatment with PSG-1 significantly improved the activities of antioxidant enzymes (SOD, CAT, and GPx) and decreased the content of MDA in the serum of S-180-bearing mice. Moreover, when PSG-1 and CTX were mixed, the activities of these enzymes in the serum were significantly higher than those in the CTX group. These findings suggested that antioxidant effects of

PSG-1 might be implicated in enhancement of the antitumor activities of CTX.

In conclusion, PSG-1 could produce a dramatic enhancement in the antitumor activity of CTX through mitochondrial apoptotic pathways in S-180-bearing mice. The immune benefits and antioxidant activities of PSG-1 are attributed to its enhancement effects. Therefore, our results could support new evidence for future cancer clinical trials by using CTX combined with PSG-1. However, further investigations should be performed to fully support our strategy for the treatment of patients with tumors.

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ACKNOWLEDGMENT

The financial support for the present study by the National Natural Science Foundation of China (31071532) and National H3igh Technology Research and Development Program of China (863 Program) (2008AA10Z325), Objective-Oriented Project of State Key Laboratory of Food Science and Technology (SKLF-MB-200806), and Key Science and Technique Project of Department of Science, Jiangxi Province, is gratefully acknowledged.

ABBREVIATIONS USED

CTX, cyclophosphamide; $\Delta\psi_m$, mitochondrial membrane potential; ELISA, enzyme-linked immunosorbent assay; ip, intraperitoneally; IL, interleukin; ρNA, ρ-nitroanilide; PI, propidium iodide; PSG-1, *Ganoderma atrum* polysaccharide; Rho, rhodamine; S-180, sarcoma 180; TIR, inhibition rate; TNF, tumor necrosis factor.

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NOTE ADDED AFTER ASAP PUBLICATION

Figures 1 through 4 were incorrect in the version of this paper published March 22, 2011. The correct version published March 31, 2011.