Available online at www.sciencedirect.com
ScienceDirect
Food and Chemical Toxicology 46 (2008) 1825–1833
www.elsevier.com/locate/foodchemtox

Anti-tumor effect of *Coriolus versicolor* methanol extract against mouse B16 melanoma cells: *In vitro* and *in vivo* study

Lj. Harhaji [a], S. Mijatović [a], D. Maksimović-Ivanić [a], I. Stojanović [a], M. Momčilović [a], V. Maksimović [b], S. Tufegdžić [b], Ž. Marjanović [b], M. Mostarica-Stojković [c], Ž. Vučinić [b], S. Stošić-Grujičić [a,*]

[a] *Department of Immunology, Institute for Biological Research "Siniša Stanković", Belgrade University, 11060 Belgrade, Bulevar Despota Stefana 142, Serbia*
[b] *Institute for Multidisciplinary Research, 11030 Belgrade, Serbia*
[c] *Institute of Microbiology and Immunology, School of Medicine, Belgrade University, 11000 Belgrade, Serbia*

Received 27 June 2007; accepted 14 January 2008

Abstract

Numerous studies have shown immunostimulatory and anti-tumor effects of water and standardized aqueous ethanol extracts derived from the medicinal mushroom, *Coriolus versicolor*, but the biological activity of methanol extracts has not been examined so far. In the present study we investigated the anti-tumor effect of *C. versicolor* methanol extract (which contains terpenoids and polyphenols) on B16 mouse melanoma cells both *in vitro* and *in vivo*. *In vitro* treatment of the cells with the methanol extract (25–1600 μg/ml) reduced melanoma cell viability in a dose-dependent manner. Furthermore, in the presence of the methanol extract (200 μg/ml, concentration IC_{50}) the proliferation of B16 cells was arrested in the G_0/G_1 phase of the cell cycle, followed by both apoptotic and secondary necrotic cell death. *In vivo* methanol extract treatment (i.p. 50 mg/kg, for 14 days) inhibited tumor growth in C57BL/6 mice inoculated with syngeneic B16 tumor cells. Moreover, peritoneal macrophages collected 21 days after tumor implantation from methanol extract-treated animals exerted stronger tumoristatic activity *ex vivo* than macrophages from control melanoma-bearing mice. Taken together, our results demonstrate that *C. versicolor* methanol extract exerts pronounced anti-melanoma activity, both directly through antiproliferative and cytotoxic effects on tumor cells and indirectly through promotion of macrophage anti-tumor activity.

Keywords: *Coriolus versicolor*; Methanol extract; B16 melanoma; Tumor apoptosis; Cell cycle; Anti-tumor macrophages

1. Introduction

Malignant melanoma is one of the most aggressive forms of skin cancer with high metastatic potential and extraordinary resistance to cytotoxic agents (Hoang and Eichenfield, 2000). Despite extensive research and partial successes gained by use of platinum analogues, nitrosoureas, taxanes, *Vinca* alkaloids and cytokines (Legha et al., 1998; Sun and Schuchter, 2001), currently there is no effective chemotherapy against invasive melanoma. Therefore, a search for new drugs with greater effectiveness and fewer side effects is necessary.

Anticancer chemotherapeutic agents exert their beneficial effects either directly through induction of tumor cell death or indirectly through stimulation of host immune response. Increasing evidence suggests that certain

Abbreviations: CVme, methanol extract of *Coriolus versicolor*; CV, crystal violet; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TB, trypan-blue.

* Corresponding author. Tel.: +381 112657258; fax: +381 112761433.
E-mail address: duta@EUnet.yu (S. Stošić-Grujičić).

0278-6915/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2008.01.027

mushrooms contain different classes of biologically active compounds with strong immunomodulating and anticancer properties (Moradali et al., 2007). One of the medicinal mushrooms extensively used in both traditional herbalism and modern clinical practice is *Coriolus versicolor* (*C. versicolor*), also known as Yun-Zhi. The chemical composition of the mushroom is very complex. Among various bioactive components derived from hot water and standardized ethanol–water extracts of *C. versicolor*, polysaccharopeptides (PSP) and protein-bound polysaccharides (PSK, also known as Krestin) were found to have the strongest biological activity. Numerous *in vitro* studies have reported that PSP and PSK can inhibit the proliferation of leukemia, lymphoma, hepatoma, breast, lung and prostate tumor cell lines (Dong et al., 1996, 1997; Kidd, 2000; Hsieh and Wu, 2001; Hsieh et al., 2002; Chow et al., 2003). Apart from a direct anti-tumor effect, PSP contribute to tumor eradication by stimulating both humoral and cell-mediated immune responses. They can increase the synthesis of interferon (IFN)-γ and interleukin (IL)-2, enhance T-cell proliferation, stimulate macrophage-derived nitric oxide production and counteract the immunosuppression induced by cytotoxic drugs (Wang et al., 1996; Ooi and Liu, 2000; Qian et al., 1997). Antimetastatic activity of PSK and PSP has also been demonstrated and attributed to their potential for inhibiting metalloproteinases and growth factors involved in the process of metastasis (Ho et al., 2004b; Kobayashi et al., 1995). *In vivo* administration of polysaccharopeptides derived from a water extract to nude mice strongly suppressed the growth of inoculated human hepatoma, lung and prostate adenocarcinoma and extended the survival time (Mickey et al., 1989; Dong et al., 1996; Ng, 1998; Kidd, 2000). In clinical trials, PSP and PSK administration to patients with different forms of cancer, who were receiving radio- or chemotherapy, provided additional benefit by enhancing the ability of the immune system to kill tumor cells (Ng, 1998; Kidd, 2000; Zjawiony, 2004). Moreover, several studies investigated the biological activity of whole standardized ethanol–water extract, which contains triterpenoid compounds as well as polysaccharides. Ethanol–water extracts possess both immunostimulatory capacity (Ho et al., 2004a) and the ability to induce apoptosis in different tumor cells (Ho et al., 2005, 2006). On the other hand, the biological effects of methanol extracts of *C. versicolor* have not been investigated so far, except for antioxidative activity, which was primarily ascribed to the mushroom's phenols (Mau et al., 2002). However, considering the strong anti-tumor effects of methanol extracts derived from other medicinal mushrooms (Ajith and Janardhanan, 2003; Chang et al., 2004; Tomasi et al., 2004), it is reasonable to suppose that methanol extracts of *C. versicolor* also possess an anti-tumor potential. The aim of this study was to investigate the anti-tumor effect of a terpenoid- and phenolic-containing methanol extract of *C. versicolor* (CVme) on B16 mouse melanoma cells both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals

Fetal calf serum (FCS), RPMI 1640, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue (TB), lactic acid, phenazine methosulfate and *p*-iodonitrotetrazolium were obtained from Sigma (St. Louis, MO), propidium iodide (PI) and Annexin V-FITC from BD Pharmingen (San Diego, CA) and (^{3}H)-thymidine and β-nicotinamide adenine dinucleotide (NAD) from ICN (Costa Mesa, CA).

2.2. Preparation and characterization of methanol extracts from C. versicolor

The cultivated fruiting bodies of *C. versicolor* were provided by Agarica (Bela Crkva, Serbia). All solvents used for extraction were pro analysis and were purchased from Merck (Darmstadt, Germany). Fungal sporocarps (50 g) were cut into small pieces and extracted with 100% methanol at 50 °C in an apparatus fitted with a reflux condenser to prevent solvent loss during extraction. The solutions were passed through Macherney-Nagel MN751 filter paper. After removal of methanol under reduced pressure, the product was partitioned between a mixture of organic solvents (chloroform/methanol = 2:1) and water (added up to 30% of the organic solvent volume) (Folch et al., 1957) and concentrated using vacuum evaporation. After purification from residual solvents with anhydrous ethanol, the lyophilized fraction yielded CVme. The lyophilized powder was dissolved in DMSO and analyzed by HPLC. Alternatively, the stock solution was diluted in PBS or culture medium immediately before use, for *in vivo* and *in vitro* treatment, respectively, and the final DMSO concentration never exceeded 0.2%.

The extracts were analyzed on a Hewlett Packard 1100 HPLC (Palo Alto, CA, USA) with a photodiode array detector adjusted at 252 nm for determination of terpenoids. Reversed phase separations were made on a Waters (Milford, MA, USA) Xterra column (250 × 4 mm) with 5 μm particle size and a corresponding precolumn. The mobile phases were 0.3% phosphoric acid (mobile phase A) and acetonitrile (mobile phase B) with the following gradient profile: in the first 30 min from 30% to 40% B, followed by a linear rise to 60% of B in the next 30 min. Acetonitrile (J. T. Baker, USA), p.a. grade phosphoric acid and 18 MΩ deionised water (Millipore, Bedford, MA, USA) were used.

2.3. Mice and tumor cells

C57BL/6 mice were originally purchased from the Charles River Laboratories (France) and then bred in our own colony under conventional conditions. All experiments were performed with groups of 8–10 mice, matched by age (8- to 10-week-old) and weight (20–25 g). The conditions for housing and experiments were as recommended by the Institutional Animal Care and Use Committee at the Institute for Biological Research "Sinisa Stankovic".

The transplantable B16 murine melanoma cell line of C57BL/6 origin was a kind gift from Dr Siniša Radulović (Institute for Oncology and Radiology, Belgrade, Serbia). The cells were maintained by twice-a-week passages in HEPES-buffered RPMI 1640 medium supplemented with 5% FCS, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, antibiotics and 0.01% sodium pyruvate (culture medium) at 37 °C in a humidified atmosphere with 5% CO_2.

2.4. Determination of cell viability by crystal violet, MTT, trypan blue and lactate dehydrogenase release assay

For *in vitro* experiments, B16 cells were cultured in flat-bottomed 96-well plates (10^4 cells/well) overnight, and then treated with various concentrations of CVme for another 24, 48 or 72 h. Afterwards, crystal violet (CV), tetrazolium (MTT), trypan-blue (TB) and lactate dehydrogenase (LDH) release assays were performed.