Anti-Tumor Effects of the Polysaccharide Isolated from *Tarphochlamys Affinis* in H22 Tumor-Bearing Mice

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Key Words
*Tarphochlamys affinis* (Acanthaceae) • Polysaccharide • Anti-tumor • Hepatocarcinoma

Abstract

**Background:** The previous studies have demonstrated that the polysaccharide isolated from *Tarphochlamys affinis* (PTA) exhibits anti-tumor effect on S\textsubscript{180} tumor-bearing mice and protective effects against hepatic injury. **Methods:** In this study, we investigated the anti-tumor activity and possible underlying mechanism of PTA on liver cancer using a murine H22 hepatocarcinoma model. **Results:** PTA was capable of repressing transplanted H22 solid hepatic tumor cell growth in vivo. The relative weight of immune organs (spleen and thymus) and lymphocyte proliferation induced by ConA or LPS were improved after PTA treatment. Furthermore, treatment with PTA promoted immune-stimulating serum cytokine secretion in H22 tumor-bearing mice. Additionally, the percentage of CD4\textsuperscript{+} T lymphocytes, CD8\textsuperscript{+} T lymphocytes and NK cells was increased in tumor-bearing mice following PTA administration. In tumor tissue, PTA significantly up-regulated the expression of Bax and p53 proteins and down-regulated the expression of Bcl-2 protein. In addition, at the therapeutic dose, PTA displayed very few toxic effects to major organs, such as the liver and kidney, in tumor-bearing mice. **Conclusion:** In H22 tumor-bearing mice, PTA exhibited prominent anti-tumor activity in vivo. The possible mechanism of action might be related to enhanced host immune system function and induction of H22 tumor cell apoptosis.

Introduction

Cancer is one of the major public health problem in many parts of the world [1, 2]. Investigations have revealed that hepatocellular carcinoma (HCC) is one of the most frequent deadly malignancies worldwide and accounts for approximately 4.1\% of the annual
increase in malignant tumors [3-6]. At present, the HCC therapeutic schedule is surgery supplemented with chemotherapeutic agents. Although sorafenib has been shown to prolong the almost three-month survival time in advanced HCC patients, the therapeutic success of chemotherapeutic agents for patients with HCC has low sensitivity, severe adverse effects and an unprecedented high cost [7, 8]. Despite endeavors to prevent postoperative metastasis in clinical practice, unfortunately, the high prevalence of metastasis and recurrence account for the poor prognosis in HCC patients, particularly those in advanced stages. There are no feasible approaches for the treatment of HCC; the overall HCC survival rate is still rather dismal, and HCC has become the second leading cause of cancer-related mortality in China [9, 10]. Therefore, there is a critical need to explore and evaluate more effective alternative strategies for the treatment of HCC. The development of potential anti-HCC agents that have a notable therapeutic effect, have no or restricted toxicity to normal cells and that can be used in combination with current existing drugs has become a hot research field for treatment of HCC.

Increasing evidence shows that traditional Chinese medicines (TCM) have multi-level, multi-target and coordinated intervention effects. TCM has also been shown to alleviate the toxic effects of treating several diseases, especially diseases, such as HCC, that cannot be cured with modern synthetic drugs [11-13]. Specifically, natural polysaccharides derived from TCM, such as lentinan, cinobufacini and others, have been used as a source of therapeutic agents for HCC and can be exploited as novel potential anti-HCC agents [14, 15].

Tarphochlamys affinis (Acanthaceae) (TA) has a long history of wide use as a Chinese traditional medicine and has been used to treat tumors, jaundice, acute and chronic hepatitis and other disorders [16]. Our previous studies have shown that more than twenty compounds can be isolated from TA and that polysaccharides have been implicated as the main bioactive ingredient of TA [16]. The polysaccharide of Tarphochlamys affinis (PTA) has been demonstrated to have variety of bioactivities, including antioxidant properties in vitro, protective effects against CCl₄- or ConA-induced hepatic injury and antiviral effects on the hepatitis B virus [16]. Recently, studies have reported that PTA has a significant anti-tumor effect on S₁₈₀ tumor-bearing mice in vivo [16-19]. However, there is no report published on the anti-tumor effect of PTA on HCC. Therefore, to more fully develop the potential use of TA, we focused specifically on elucidating the anti-tumor activity and the possible underlying mechanism of PTA in H22 tumor-bearing mice.

**Materials and Methods**

**Materials and chemicals**

Interleukin-2 (IL-2), interleukin-6 (IL-6), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D systems (Minneapolis, MN, USA). DMEM 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). All of the other materials and chemicals were obtained from local commercial sources.

**Preparation of PTA**

Tarphochlamys affinis (Acanthaceae) was purchased from Nanning Qianjinzi Chinese Pharmaceutical Co. Ltd. (Nanning, China). Voucher specimens (LYQ2014011801) were identified by the Guangxi Academy of Chinese Medicine and Pharmaceutical Science. PTA was isolated from TA by the Department of Pharmacology, Guangxi Medical University (Guangxi, China), and was prepared according to the method described in our previous paper with slight modification [16]. Tarphochlamys affinis (1000 g) was dried and powdered, extracted three times with 10000 mL water for 2 h at 95-100°C. The whole extract was filtered, concentrated and centrifuged, and then the supernatant was precipitated fractionally with alcohol. The protein in the crude PTA was removed using the Savag method, and purified using DEAE-52 cellulose ion exchange column chromatography. The content of saccharide in PTA was identified by gas chromatography. The results showed that PTA consists of D-arabinose and D-glucose in molar ratios of 9.75% and 91.06%, with an average molecular weight of 14 241 Da.
Animals and cell lines

Female Kunming mice (20 ± 2 g) were provided by the Experimental Animal Centre at Guangxi Medical University. Animals were maintained at standard specific-pathogen free (SPF) conditions (25 ± 1°C and 55 ± 5% relative humidity) on a 12-h light/12-h dark cycle with standard pellet diet and water supplied ad libitum. Animals were allowed to acclimate in quarantine for a week prior to experimentation. The research was conducted according to protocols approved by the Animal Ethics Committee of Guangxi Medical University.

Murine H22 hepatocarcinoma cells were purchased from Beijing Cowin Biotech Co. Ltd. (Beijing, China) and cultured in DMEM medium supplemented with 10% FBS, 2 mmol/L glutamine, antibiotics (100 mg/L streptomycin and 1×10^5 U/L penicillin) in a humidified atmosphere (5% CO_2, 37°C). The cells were subcultured until reaching logarithmic growth phase and then were maintained by transplanting them into the peritoneal cavities of Kunming mice weekly.

Evaluation of anti-tumor effect in vivo

H22 tumor ascites was taken from mice and mixed with normal saline (NS) to thoroughly dilute the cells into a suspended solution at a concentration of 1 × 10^7 cells/mL. The mice were injected subcutaneously into the right axillary region; to establish a murine solid tumor H22 transplantation model, each mouse received 0.2 mL of the cell suspension (2×10^6 total cells per mouse). Twenty-four hours after inoculation, the H22-bearing mice were randomly divided into five groups (n = 10): the model group, in which mice were given the same volume of physiological saline; the 5-fluorouracil (5-FU) group, in which mice were treated with 5-FU (20 mg/kg) by intraperitoneal injection every other day; and the low-, medium- and high-dosage of PTA-treated groups, in which mice were treated with PTA (70, 140 or 280 mg/kg) by oral administration, respectively. Each mouse was weighed daily. The volume of the solid tumor was measured with a digital caliper every other day and was calculated according to the following equation: tumor volume (mm^3) = [L_a × L_b / 2], where L_a and L_b represent the largest diameter and the smallest diameter, respectively [20, 21].

At the final stage of the experiment, blood samples were collected, and serum was separated for the detection of IL-2, IL-6, IFN-γ, TNF-α and blood physiochemical assays. Each of the mice were then killed, and the segregated tumors, thymuses and spleens of the mice were weighed immediately. The tumor inhibition rate was calculated according to the following equation: inhibition rate = (1−average tumor weight of the treated group/average tumor weight of the model group)×100%. The organ indexes of the thymus and spleen were calculated as follows: organ index = organ weight (mg)/body weight (g) [22].

Spleen lymphocyte proliferation assay

Under aseptic conditions, splenocytes were prepared by disrupting half of the spleen with a grinder in phosphate-buffered saline (PBS, pH 7.4) at 1500 rpm for 10 min. Erythrocytes were then lysed using an ammonium chloride reagent. The cells were washed three times with PBS and suspended in cold RPMI-1640 medium with 10% FBS. Lymphocytes were cultured in 96-well plates (1×10^6/well) with concanavalin A (ConA, 2 mg/L) or lipopolysaccharide (LPS, 5 mg/L) and incubated for 48 h at 37°C in a humidified atmosphere with 5% CO_2. After 48 h, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (5 mg/mL) was added to each well (20 μL/well), and the plates were incubated for another 4 h. After precipitated formazan was dissolved in 150 μL DMSO, the absorbance of spleen lymphocytes cells in each well was read on a spectrophotometer at 570 nm using an ELISA reader (Bio-Rad, USA). The stimulation index was calculated as follows: stimulation index (%) = the absorbance value for experimental group/the absorbance value for model group×100%.

Serum cytokine concentration determination

The serum levels of cytokines, including IL-2, IL-6, IFN-γ and TNF-α, were determined by commercial ELISA kits according to the manufacturer’s instructions.

Analysis of liver and kidney function

A series of physiochemical indexes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), uric acid (UA) and creatinine (CRE), were determined using an automatic biochemical analyzer (Sysmex Corporation, Japan) with reagents purchased from Sysmex Corporation.
Analysis of lymphocyte subsets by flow cytometry

Half of the spleen was mashed with a Teflon pestle in RPMI-1640 medium. After filtering the resulting solution and lysing erythrocytes, single-cell suspensions of splenocytes (100 μL, 1×10^6 cells/L) were stained with the following rat anti-mouse monoclonal antibodies: phycoerythrin (PE)-conjugated anti-CD8, fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and PE-conjugated anti-CD49b in the dark for 30 min at 4°C. The percentages of CD4^+ T cells, CD8^+ T cells, and natural killer (NK) cells (indicated by CD49b^+) were determined by flow cytometry (FACS Calibur, Becton Dickinson, USA) and analyzed using CellQuest software.

Western blot analysis for P53, Bax and Bcl-2

Total protein from the tumor tissues was extracted and then quantified using a bicinchoninic acid (BCA) protein concentration assay reagent (Shanghai Haoran Bio Technologies Co., Ltd, China). Sample protein was separated by 15% sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) using a Bio-Rad electrophoresis system (Shanghai Haoran Bio Technologies Co., Ltd, China). Blotted membranes were incubated for 1 h with blocking solution (tris-buffered saline/Tween 20, TBST) containing 5% skim milk (w/v) at room temperature. Subsequently, the membrane was incubated overnight at 4°C with a 1: 500 dilution of the primary P53, Bax and Bcl-2 antibodies and a 1:1000 dilution of β-actin primary antibody. After washing, the membrane was incubated with a horseradish-peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., CA) at a 1:1000 dilution for 1 h at room temperature. The membranes were washed, after which the immunoblot was developed using an ECL chemiluminescent detection kit (Millipore, Bedford, USA) according to the manufacturer’s instructions and analyzed using a GelDoc 2000 system (Bio-Rad Laboratories Inc. USA). To normalize for protein loading, β-actin was used, and the proteins expression levels were expressed as a relative value to that of β-actin.

Statistical analysis

Statistical analysis was performed using SPSS 17 (SPSS, Chicago, IL, USA). The data were expressed as the mean ± S.D. Statistical analysis was performed by using one-way analysis of variances (ANOVA) and student’s t tests. p < 0.05 was considered to be statistically significant.

Results

Effect of PTA on H22 tumor growth

As shown in the curves representing tumor growth volumes (Fig. 1A), from day 2 to day 10, the volume of the tumors in the model group grew rapidly, while that in the PTA-treated groups grew slowly. At the end of the experiment, the average tumor weights in the 5-FU group and PTA-treated groups were significantly decreased compared with that in the model group (P < 0.01) (Fig. 1B). The tumor inhibitory rates for the 5-FU and the PTA-treated groups are shown in Fig. 1C accordingly.

Effect of PTA on the immune organ index

The immune system plays a critical role in anti-tumor defense. Compared to the model group, the indices for the spleen and thymus in the high PTA group were higher (P < 0.05), however, those of the 5-FU group was dramatically decreased (P < 0.01) (Fig. 2).

Effects of PTA on spleen lymphocyte proliferation

Splenic lymphocyte proliferation plays a critical role in the activation of an adaptive immune response. As shown in Fig. 3, compared to the model group, a high and medium dosage of PTA markedly increased the splenic lymphocyte proliferation induced by ConA or LPS in the dose-dependent manner (P<0.05 or P<0.01).

Effects of PTA on serum cytokines levels

As shown in Fig. 4, compared to the model group, the levels of IL-2, IL-6, IFN-γ and TNF-α were higher in H22 tumor-bearing mice after PTA administration in a dose-dependent
manner. This effect is especially pronounced for the high and medium PTA groups (P < 0.05 or P < 0.01), while cytokine levels for the 5-FU group were significantly decreased (P < 0.05 or P < 0.01).

**Effects of PTA on the percentage of splenic CD4⁺, CD8⁺ T lymphocytes, and NK cells**

As shown in Fig. 5, compared to the model group, the proportions of CD4⁺ T lymphocytes, CD8⁺ T lymphocytes and NK cells were increased with PTA administration in a dose-dependent fashion, especially for the high and medium PTA groups (P < 0.05 or P < 0.01). Furthermore, the ratio of CD4⁺ to CD8⁺ T lymphocytes was elevated after treatment with...
PTA. On the contrary, compared to the model group, the 5-FU group displayed much lower percentages of CD4⁺ T lymphocytes, CD8⁺ T lymphocytes and NK cells (P < 0.01).
Effects of PTA on expressions of Bax, P53 and Bcl-2 in the tumor

As shown in Fig. 6, compared to the model group, Bax and p53 proteins were up-regulated dramatically by treatment with 5-FU and PTA (P<0.05 or P<0.01). Bcl-2 expression in the 5-FU and the PTA-treated groups markedly decreased compared to the model group (P<0.05 or P<0.01).

Effects of PTA on hepatic and renal function

To examine for potential hepatic and renal toxicological effects induced by PTA administration in H22 tumor-bearing mice, serum hepatic function markers, including ALT and AST, and serum renal function markers, including BUN, UA and CRE, were determined in the model and treated groups. As shown in Fig. 7, treatment with 5-FU and PTA significantly increased the serum ALT and AST levels compared to the model group (P<0.05 or P<0.01). In contrast, the serum BUN, UA and CRE levels were significantly decreased in the PTA-treated group compared to the model group (P<0.05 or P<0.01).
including BUN, UA and CRE, were evaluated. As shown in Fig. 7, compared to the model group, the levels of serum ALT, AST, BUN, UA and CRE were markedly increased in the 5-FU group \((P<0.05\) or \(P<0.01\)). Contrary to this, there were no significant differences between the PTA groups and the model group, suggesting that PTA exhibits very few toxicological effects in H22 tumor-bearing mice.

**Discussion**

Many studies have documented that polysaccharides are one of the major active ingredients of medicinal plants. Polysaccharides isolated from different Chinese herbs have been reported to possess versatile biological functions, including anti-tumor effects and improved immune function. This has attracted considerable attention in the biomedical field and has become popular today [23-25]. The lentinan, krestin have already been used as immunocceuticals for clinical cancer therapy [26].

In our previous study, PTA was found to have significant anti-tumor effects on S180 tumor-bearing mice; these effects occurred by enhancing immunological function, down-regulation of Bcl-2 oncoprotein expression and up-regulation of Bax oncoprotein expression in vivo [19]. As an extension of our previous investigation, one of our current research priorities is a detailed characterization of the anti-tumor activity and the possible underlying mechanisms of PTA in H22 tumor-bearing mice. Our results show that similar to 5-FU, high, medium and low PTA doses possess a significant inhibitory effect against both the weight and volume of H22 tumors and also similar tumor inhibitory rates. These results demonstrate that PTA possesses notable anti-tumor effects against H22.

Previous studies have reported that the anti-tumor activity of polysaccharides administrated per orally were mediated through immunomodulation effect, such as the astragalus membranaceus polysaccharide [27-29]. And the results of our present study suggested that PTA possesses anti-tumor effects against H22 by similar mechanisms. Spleen lymphocyte proliferation is a crucial event in the activation cascade of both humoral and cellular immune responses [30]. Therefore, we investigated the effect of PTA on splenic lymphocyte proliferation in H22-bearing mice. PTA (140 and 280 mg/kg/day) markedly increased splenic lymphocyte proliferation induced by ConA or LPS in a dose-dependent manner in H22 tumor-bearing mice. This suggests that PTA has a beneficial effect in strengthening immunological responses that repress tumor growth in H22 tumor-bearing mice.

It is well-known that CD4\(^+\) and CD8\(^+\) T lymphocytes are the primary helper and cytotoxic T cells, respectively, which can secrete cytokines and induce the death of tumor cells [31, 32]. Furthermore, NK cells are an important population of lymphocytes and have pivotal roles in both innate and adaptive immunity [33]. Accordingly, CD4\(^+\) T lymphocytes, CD8\(^+\) T lymphocytes and NK cells play pivotal roles in host anti-tumor immune response [34, 35]. Many anti-tumor therapeutic strategies are aimed at stimulating these immune cells to facilitate the destruction of tumor cells and the long-term immunologic memory that is necessary to avoid recurrence of primary tumors or outgrowth of metastases [36]. In this study, PTA (140 and 280 mg/kg/day) enhanced the prevalence of CD4\(^+\) and CD8\(^+\) T lymphocytes and NK cells in the spleen of H22 tumor-bearing mice and elevated the ratio of CD4\(^+\) to CD8\(^+\) T lymphocytes. This is in contrast to the decrease of CD4\(^+\) T lymphocytes, CD8\(^+\) T lymphocytes and NK cells induced by 5-FU. These results suggest that PTA administration could activate these immune cells, allowing them to exert immunomodulatory effects in H22 tumor-bearing mice.

Cytokines including IL-2, IL-6, IFN-\(\gamma\) and TNF-\(\alpha\) have been shown to play a prominent role in the modulation of anti-tumor immune responses that abrogate tumor growth, and have been extensively tested pre-clinically in vitro and in vivo, as well as among patients in the clinic for immunotherapy of malignant diseases [37-40]. The cytokines IL-2 and IL-6 are capable of inducing the proliferation of responsive T-cells. TNF-\(\alpha\) has been
proven to play an important role in anti-tumor activity in vitro and in vivo by inducing apoptotic cell death and tumor necrosis [41]. In our in vivo experiments, H22 tumor-bearing mice that received PTA (140 and 280 mg/kg/day) had significantly increased levels of serum IL-2, IL-6, IFN-γ and TNF-α. This indicated that PTA may indirectly play an anti-tumor role by stimulating the secretion of cytokines in H22 tumor-bearing mice.

To further clarify the mechanisms by which PTA has anti-H22 tumor activity, the expression of proteins Bax, Bcl-2 and p53, which regulate the process of apoptosis in tumor tissues, was explored [42]. p53 is considered to be a pivotal anti-cancer protein and a mainstay of the natural anti-cancer defense; p53 can eliminate and suppress abnormal cell proliferation, thus preventing the development of cancer [43, 44]. p53 can also directly activate pro-apoptotic proteins such as Bax, which can also inactivate anti-apoptotic proteins such as Bcl-2 [45]. Therefore, p53 is a promising target for cancer treatment, and it has been demonstrated that up-regulating the expression of p53 is beneficial for treatment of cancers [46, 47]. Bcl-2 family proteins including Bax, Bcl-2 and others play pivotal roles in intrinsic apoptosis and are recognized as the first regulatory step for inducing mitochondrial apoptosis [48]. In our investigation, PTA significantly up-regulated tumor tissue expression of p53 and Bax proteins but down-regulated Bcl-2 expression. This result revealed that PTA might exert its activity through an apoptosis-associated pathway via the up-regulation of p53 and Bax and down-regulation of Bcl-2.

Perfect anti-HCC chemotherapeutics would include not only the virtue of repressing the growth of malignant cells but also minimum toxicity to normal organs. Blood ALT and AST are considered to be hepatic function parameters, and blood BUN, UA and CRE are considered to be renal function parameters [49]; these parameters were not significant different between the PTA group and the model group in our present study. These results indicate that PTA has no obvious systemic toxicity to major organs in H22 tumor-bearing mice.

In conclusion, our present study demonstrated that PTA had clear anti-tumor effects against H22 in vivo and that the possible mechanism might be related to improving immune functions, up-regulating the expression of p53 and Bax proteins, and down-regulating the expression of Bcl-2 protein. In addition, PTA had no obvious systemic toxicity to major organs in H22 tumor-bearing mice.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

References


