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A galactomannan polysaccharide from *Punica granatum* imparts *in vitro* and *in vivo* anticancer activity

Manu M. Joseph[a], S.R. Aravind[a], Suraj K. George[b], Sheeja Varghese[a], T.T. Sreelekha[a,*]

[a] Division of Cancer Research, Regional Cancer Centre, Thiruvananthapuram 695 011, Kerala, India
[b] Department of Hematopathology, UT MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, United States

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ABSTRACT

Galactomannan polysaccharide (PSP001) was isolated from the fruit rind of *Punica granatum* and was previously reported to have excellent antioxidant and immunomodulatory properties. The cytotoxicity of PSP001 was evaluated in the human cancer cell lines A375, HCT116, and HepG2 as well as the murine cancer cell lines DLA and EAC over a wide range of concentrations. PSP001 exhibited significant cytotoxicity against cancer cells through the induction of apoptosis with no *in vivo* toxicity up to a concentration of 2000 mg/kg body weight when assessed in BALB/c mice. The antitumor efficacy of PSP001 was tested in DLA and EAC murine ascites and EAC solid tumor mouse models. PSP001 alone and in combination with doxorubicin produced a significant reduction in the tumor burden and increased life span in both models compared to the controls. The results suggest that PSP001 has the potential to be developed as an anticancer agent either alone or as an adjuvant to chemotherapy.

1. Introduction

Cancer remains the leading cause of death despite developments in tools for prevention, diagnosis, and treatment. Although chemotherapy appears to be the major treatment modality, it is accompanied with severe side effects, which could lead to opportunistic infections and even death. Evasion of the immune surveillance mechanism of the body by tumor cells through the secretion of immunosuppressive factors that modify the host's immune response is one of the main reasons for the rapid progression of human cancers. One of the main drawbacks of current treatment regimens is the suppression of the immune system (Devasagayam & Sainis, 2002). Immunomodulators are substances that can modify the immune system and many plant products have been shown to have immunomodulatory properties (Guruvayoorappan & Kuttan, 2007). The ability of any compound to selectively inhibit the proliferation of malignant but not normal cells is the hallmark of a promising anticancer therapeutic agent.

Polysaccharides are a class of biological macromolecules that are relatively common in nature with tremendous structural diversity, and thus their biological properties have attracted substantial attention in medicine (Ooi & Liu, 2000; Sinha & Kumaria, 2001). Among the naturally occurring substances, polysaccharides have proven to be useful candidates in the search for effective, non-toxic substances with pharmacological effects and represent a relatively untapped source of new drugs, which may provide exciting new therapeutic opportunities (Beat & Magnani, 2009). Antioxidant, antitumor, immunomodulatory, antimicrobial, antiulcer, and several other pharmacological activities from various polysaccharides have been reported (Franz, 1989; Liu, Ooi, & Chang, 1997). Natural polysaccharides have been used extensively in the design of drug delivery systems due to their excellent biocompatibility, biodegradability, aqueous solubility, and stability (Bhardwaj, Kanwar, Lal, & Gupta, 2000; Liu, Jiao, Wang, Zhou, & Zhang, 2008). Among the biopharmacological properties of polysaccharides, immunomodulatory and antitumor effects are of high priority (Ooi & Liu, 2000), and the majority of these agents act as biological response modifiers, which enhance the immune response (Leung, Liu, Koon, & Fung, 2006). Numerous antitumor polysaccharides have been isolated from plants, mushrooms, yeasts, algae, and lichens, of which the majority have been found to be non-toxic to normal cells and can enhance the immune system of the host (Mahady, 2001). Polysaccharide PST001 was previously isolated from the seed kernel of *Tamarindus indica* by our laboratory and found to possess excellent immunomodulatory properties together with the anticancer properties (Sreelekha, Vijayakumar, Ankathil, Vijayan, & Nair, 1993; Aravind, Manu, Sheeja, Prabha, & Sreelekha, 2012a; Aravind, Manu, Sheeja, Prabha, & Sreelekha, 2012b).

Punica granatum (Pomegranate) belongs to the family of Punicaceae, which grows mainly in the Middle East, India, China, Spain, Israel, and Latin America. Fruits of pomegranate possess a vast

* Corresponding author. Tel.: +91 4712522378; fax: +91 4712447454.
E-mail addresses: manumjoseph2000@yahoo.com (M.M. Joseph), aravind_sr2003@yahoo.co.in (S.R. Aravind), surajrcc@gmail.com (S.K. George), sheejava@gmail.com (S. Varghese), lekhasree64@yahoo.co.in, lekhasree@rcctvm.org (T.T. Sreelekha).

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ethno medical history and represent a phytochemical reservoir of heuristic medicinal value. Pomegranate is a symbol of life, longevity, health, feminity, fecundity, knowledge, morality, immortality, and spirituality (Mahdihassan, 1984). It is an ancient fruit with an illustrious medical history and its peel extracts have been shown to retard proliferation of cells in several different human cancer cell lines (Kawaii & Lansky, 2004). The phytochemistry and pharmacological actions of all *P. granatum* components suggest a wide range of clinical applications for the treatment and prevention of cancer as well as other diseases where inflammation is believed to play an essential etiological role (Ephraim & Robert, 2007).

Generation of reactive oxygen species (ROS) combined with potent cytotoxic activity could be exploited for developing novel therapeutic strategies against cancer cells (Pelicano et al., 2003). We previously isolated and characterized the polysaccharide (PSP001) from the fruit rind of *P. granatum*, which is a galactomannan with a molecular weight of 110 kDa and has a β-1 → 3 galactopyranose backbone as well as β-D mannopyranose and α-D mannopyranose side chains (Sreelekha, Vijayan, & Prabha, 2008). Our previous studies have shown that PSP001 exerts *in vitro* cytotoxicity, immunomodulatory, and antioxidant activities capable of free radical scavenging (Manu, Aravind, Sheeja, Mini, & Sreelekha, 2012). However, a thorough understanding of the mode of cytotoxic cell death and the efficacy of antitumor activity *in vivo* has not been investigated. Considering the immense therapeutic value of this polysaccharide, objectives of the current study were to demonstrate the *in vitro* cytotoxic potential of PSP001 in various other cancer cell lines and determine the mechanism of induction of cell death. Additionally, whether PSP001 either alone or in combination with doxorubicin, had the anti-tumor effect *in vivo* were also evaluated. The data suggest the therapeutic efficacy of PSP001 against a wide variety of cancers *via* induction of apoptotic cell death. This drug also decreased tumor burden and increased survival, either alone or in combination with doxorubicin, in mice challenged with ascites and solid tumors.

2. Materials and methods

2.1. Isolation and purification of PSP001 from P. granatum

The fruit rind of *P. granatum* was shade-dried and the powdered material was used for the isolation of polysaccharides with standard protocols previously described (Rao, Ghosh, & Krishna, 1946; Sreelekha et al., 2008). Briefly, 100 g of the powder was extracted initially with 300 ml of petroleum ether (boiling point, 60 °C–80 °C) at room temperature for 72 h, followed by extraction with methanol. These extraction steps were repeated three times until the desired yield was obtained. The extract was filtered, dried and suspended in 1000 ml of cold water with occasional stirring overnight. Thereafter, it was filtered again, concentrated to a reduced volume (~300 ml), under vacuum in a flash evaporator set at 40 °C. Approximately 700 ml of ethanol was added slowly to the previous 300 ml concentrate and stirred for 6 h. This solution was then kept at 4 °C for 24 h, and the precipitate obtained was retained after centrifugation at 20,000 × *g*. This residue was dissolved in a minimum quantity of distilled water (150 ml). The solution was once again precipitated with ethanol (350 ml) and the precipitated polysaccharide was collected by centrifugation at 20,000 × *g*. These steps were repeated three times and finally, the residue obtained was dissolved in distilled water (~50 ml) followed by exhaustive dialysis against several changes of distilled water for 48 h. The contents of the dialysis bags were shaken with chloroform (50 ml) in a separating funnel to remove the denatured protein. This process was repeated until the water–chloroform interphase became clear. Ethanol was then added to the aqueous layer to precipitate the polysaccharide. The yield was further isolated by exhaustive dialysis and freeze drying. Purification was done by gel filtration chromatography using Sephadex G-200 column (Pharmacia Fine Chemicals), Ultrogel AcA-44 chromatography resins (LKB) and 0.001 M phosphate buffered saline (PBS) as the eluent buffer. Briefly, 500 mg of the lyophilized crude sample was suspended in buffer and chromatographed through Sephadex G-200 (3 cm × 75 cm) equilibrated with the buffer. Fractions were monitored at 280 nm, and at 490 nm after mixing with phenol-sulfuric acid reagent. For PSP001, a single peak was obtained for both the columns. The fractions under the peak were pooled, lyophilized and stored at 4 °C for further analysis.

2.2. Chemical characterization of PSP001

To test the homogeneity of the purified polysaccharide, 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Molecular weight of the purified polysaccharide sample was estimated from the gel filtration chromatographic experiments using Sephadex G-200 column (2 cm × 75 cm), which was eluted with 0.001 M PBS at a flow rate of 20 ml per hour. A series of dextrans 10, 20, 40, 70 and T-500 (Pharmacia Fine Chemicals) were used as the reference standards. The molecular weight was estimated from a linear correlation between the logarithm of the molecular weights of the standards and the ratios of their elution volume to the void volume of the column. For identification of the sugar components, complete hydrolysis with 1 N HCl was followed by other techniques such as thin layer chromatography (TLC), partial acid hydrolysis, methylation, reduction, acetylation, proton nuclear magnetic resonance (^{1}H NMR), fast atom bombardment mass spectrometry (FABMS), and gas-chromatography–mass spectrometry (GC–MS) studies. The total carbohydrate content was determined by Dubois's method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using D-glucose as the standard.

2.3. Cell lines

The human cancer cell lines A375 (melanoma), HCT116 (colon cancer), and HepG2 (hepatocellular carcinoma) were kindly provided by Rajiv Gandhi Center for Biotechnology (RGCB), located in Thiruvananthapuram, India. The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2 incubator (Heraeus BB 15). The murine lymphoid cancer cell lines Dalton lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) were procured from Amala Cancer Research Center, Thrissur, India. DLA and EAC were maintained in the peritoneal cavity of mice by intraperitoneal transplantation of 1×10^6 cells/mice.

2.4. In vitro cytotoxicity assay

The growth inhibition capacity of PSP001 was evaluated in cancer cell lines using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay as previously reported (Aravind et al., 2012a,b; Manu, Aravind, Sheeja, Mini, & Sreelekha, 2013). This assay measures cell viability by assessing the cleavage of tetrazolium salt by mitochondrial dehydrogenase. The absorbance was measured at 570 nm using a microplate spectrophotometer (BioTek Power Wave XS). The inhibitory rate on the cells was calculated using the following formulas:

$$\text{Proliferation rate (PR)}\% = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

$$\text{Inhibitory rate (IR)}\% = 100 - \text{PR}$$