Carbohydrate Polymers 98 (2013) 1466–1475

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

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

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ARTICLE INFO

Article history:
Received 23 April 2013
Received in revised form 18 June 2013
Accepted 9 July 2013
Available online 17 July 2013

Keywords:
Polysaccharide
Punica granatum
Cancer
Dalton's lymphoma ascites
Ehrlich's ascites carcinoma

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

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http://dx.doi.org/10.1016/j.carbpol.2013.07.023

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:

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

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

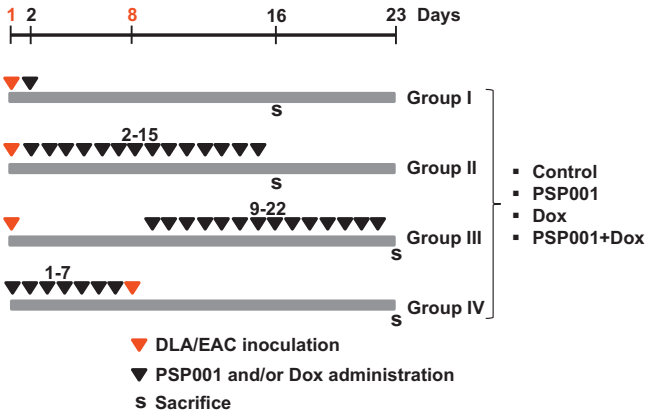

Fig. 1. Schematic for DLA and EAC tumor reduction experiments. Female BALB/c mice were divided into four groups, each group subdivided into 4 treatment options ($n = 6$/treatment group). Tumor cells were inoculated and compounds were administered as shown. Mice were sacrificed on day 16 (groups I–II) or day 23 (groups III–IV) and parameters were assessed.

MTT assays were performed on cancer cell lines with various concentrations of PSP001 ranging from 0.001 μg/ml to 1000 μg/ml over a period of 24–72 h. Doxorubicin was used as the positive control.

2.5. Acridine orange–ethidium bromide staining assay

Acridine orange–ethidium bromide dual staining is the most commonly used method to detect apoptosis and is based on the differential uptake of two fluorescent DNA binding dyes by viable and nonviable cells, respectively (McGahon et al., 1995). The experiment was performed as previously described (Aravind et al., 2012b; Manu et al., 2013). The cells were observed under an inverted fluorescent microscope using a fluorescein isothiocyanate (FITC) filter (Olympus 1X51).

2.6. In vivo toxicity studies

In order to evaluate the toxicity of the compounds on the biological systems, an acute toxicity assay was performed on 5–6 week-old male BALB/c mice as previously described (Aravind et al., 2012a; Manu et al., 2013). PSP001 was dissolved in phosphate buffered saline (PBS) and administered intraperitoneally in increasing doses of 100 mg/kg up to 2000 mg/kg body weight to several groups of experimental animals; one dose was used per group and spaced. Animals were observed continuously for 2 h and then occasionally for an additional 4 h. The mice were housed overnight and mortality, if any, was recorded. Any behavioral changes were tabulated, and the lethal dose causing 50% mortality (LD50) was assessed. All animal studies were performed in accordance with the Institutional Animal Ethical Committee's (IAEC) approval.

2.7. In vivo tumor reduction in murine ascites tumors

Female BALB/c mice were divided into four sets of six animals each ($n = 6$) as shown in Fig. 1. They were maintained in well-ventilated cages with normal mouse food and water provided *ad libitum* in an air conditioned room. DLA or EAC cells were collected from the donor mouse, suspended in sterile isotonic saline, and viable cells were counted (Trypan blue assay) under a light microscope and then adjusted to concentration so that each animal receives 1×10^6 cells/100 μl *i.p.* injection. The mice administered with vehicle (PBS) alone was maintained as the control and experiments were performed as previously described (Aravind et al., 2012a). Doxorubicin (Dox) was used as the positive control for the experiments. The compounds, PSP001 (100 mg/kg), Dox (2.25 mg/kg), and the combination, PSP001 + Dox were administered by *i.p.* injection in treatment groups as summarized in Fig. 1. For groups I, II and III, DLA or EAC cells were inoculated on day 1, while for group IV, cells were inoculated on day 8. The compounds were administered on day 2 (group I), days 2–15 (group II), days 9–22 (group III) and days 1–7 (group IV). Mice were sacrificed on day 16 (groups I and II) and day 23 (groups III and IV). Representative animals from each group were sacrificed by cervical dislocation to determine the tumor volume (Aravind et al., 2012a). Briefly, the mice were euthanized and then the abdomen of each mouse was incised over a clean vessel and the volume of the ascites fluid obtained (V1) was calculated. The peritoneal cavity was washed thoroughly with isotonic saline until the return was clear, and the volume of the added saline (V2) was then noted. The volume of ascites tumor (V3) was calculated using the formula: $V3 = (V1 + V2) - V2$. The mean survival time (MST) and percentage of increase in life span (%ILS) was calculated as previously reported (Atia & Weiss, 1966; Preethi, Kuttan, & Kuttan, 2006). $MST = (A + B)/2$, where A is the day of the first death and B is the day of last death. The percentage of increase in life span was calculated using the following formula: $\%ILS = (T - C)/C \times 100$, where T and C are the MST of treated and control animals, respectively.

2.8. In vivo tumor reduction in EAC induced solid-tumors

Viable EAC cells (1×10^6 cells) were injected subcutaneously into the hind limb of mice with a very fine needle (31G). Solid tumor reduction experiments were also performed in mice as described above with slight modifications in the drug and cell injections for groups II and IV. For group II, cells were inoculated on the 1st day and compounds were administered by *i.p.* injection daily for 14 consecutive days starting on the 8th day. Tumor volume was calculated every 3rd day starting on the 8th day up to the 32nd day. For group IV, EAC cells were inoculated on the 8th day after pre-administration of the compounds for 7 consecutive days and then tumor volume was determined every 3 days starting on the 15th day up to 39th day. The radii of the developing tumors were measured using vernier calipers and the tumor volume was calculated using the formula: $V = 4/3\pi {r_1}^2 r_2$, where r_1 and r_2 represent the radii from two different sites (Atia & Weiss, 1966; Preethi et al., 2006; Guruvayoorappan & Kuttan, 2007), respectively, which were compared with the vehicle-treated control.

2.9. Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of three replicates and were analyzed using GraphPad PRISM software version 5.0 (GraphPad Software Inc., San Diego, CA). One-way analysis of variance (ANOVA) was used for the repeated measurements, and the differences were considered to be statistically significant if $P < 0.05$. SPSS 17 software (IBM Inc., NY) was used for Kaplan–Meier survival analysis. The IC50 values were calculated using the Easy Plot software (Spiral Software, MD).

3. Results and discussion

3.1. Galactomannan polysaccharide from PSP001

Isolation and structural characterization of PSP001 was previously reported and was found to be a galactomannan with a molecular weight of 110 kDa and a β-1 → 3 galactopyranose backbone with β-D mannopyranose and α-D mannopyranose side chains (Sreelekha et al., 2008). The polysaccharide was found to be pH neutral, and the total sugar content was 97.5% with 2%

yield. PSP001 successfully isolated, purified, and characterized from pomegranate was found to be water soluble. Water soluble and biodegradable polysaccharides have been shown to have numerous pharmacological applications as thickeners, gelling agents, carriers of hydrophobic drugs (Marathe, Annapure, Singhal, & Kulkarni, 2002), and nanoparticle carriers, as they can prolong residence time (Rubinstein, 2000).

3.2. Structural elucidation and analysis of PSP001

The homogeneity of the purified compound was tested using sodium dodecylsulphate polyacrylamide gel electrophoresis and was verified to be homogeneous. Molecular weights determined by gel filtration chromatography using Sephadex G-200 for PSP001 was 110,000 Da. Complete hydrolysis and thin layer chromatographic analysis and co-chromatography using standard sugars revealed the constituent galactose for PSP001 to be glucose and mannose. Partial hydrolysis, periodate oxidation, methylation, hydrolysis, acetylation and trimethylsilylation were also carried out and analyzed by various spectroscopic methods as mentioned before. Data for characterization and the proposed structure of PSP001 are summarized in Supplementary file.

3.3. In vitro anticancer potential of PSP001

The cytotoxicity of PSP001 was found to be significant ($P < 0.001$) when evaluated on cancer cell lines over a wide range of concentrations (0.001–1000 μg/ml) compared to the standard anticancer agent doxorubicin (Dox) as a positive control. The melanoma cell line A375 and colon cancer cell line HCT116 were growth-arrested after treatment with PSP001, which showed IC50 values of 86.7 ± 1.3 μg/ml and 46.3 ± 1 μg/ml, respectively, after 72 h of incubation. In contrast, Dox induced IC50 values of 154 ± 1.4 μg/ml and 189 ± 1.1 μg/ml after 24 h incubation for the two cell lines, respectively (Fig. 2a and b). The cytotoxic potential of PSP001 increased in a dose-dependent manner up to 200 μg/ml for A375 cells, and then gradually decreased at higher concentrations. For HCT116 cells, a gradual dose-dependent increase in the cytotoxicity was observed irrespective of the incubation time. In the hepatocellular carcinoma cell line HepG2, PSP001 exhibited an optimum cytotoxicity around 200 μg/ml, with an IC50 of 94 ± 1.2 μg/ml at 72 h compared to the Dox IC50 of 174 μg/ml at 24 h (Fig. 2c). PSP001 also exhibited significant cytotoxic potential against the murine cancer cell lines, DLA and EAC, where a dose- and time-dependent increase in cytotoxicity was observed. The IC50 values of PSP001 were 309 ± 1.4 μg/ml (DLA) and 258 ± 1.7 μg/ml (EAC) at 48 h, while for Dox, IC50 values were 374 ± 1.7 μg/ml (DLA) and 375 ± 1.2 μg/ml (EAC) at 24 h (Fig. 2d and e). Taken together, these results indicate that PSP001 has significant therapeutic potential against various types of cancer. Like PSP001 and PST001, various other polysaccharides were previously reported to have both anticancer and immunomodulatory effects (Franz, 1989; Liu et al., 1997; Ooi & Liu, 2000). Modifying or encapsulating these polysaccharides with nanoparticles has been promising in several anticancer studies. Our recent studies demonstrate that the nanoparticle formulation of PST001 exhibited significant anticancer and immunomodulatory effects at lower drug concentrations and shorter incubation periods (Manu et al., 2013).

3.4. PSP001 induces anticancer effects through apoptosis

In order to determine the mechanism of cell death induction by PSP001, apoptotic assays were performed. Examination of PSP001-treated cells for apoptosis using acridine orange–ethidium bromide staining showed a change in color from green to yellow/red with associated apoptotic features. Significant changes in fluorescence were observed for A375 cells (Fig. 3a and b), HCT116 cells (Fig. 3c and d), HepG2 cells (Fig. 3e and f), DLA cells (Fig. 3g and h), and EAC cells (Fig. 3i and j) treated with 10 μg/ml PSP001 after 72 h. Morphological phase contrast microscopy evaluation of cells treated with PSP001 (10 μg/ml) for 72 h revealed a decrease in cell number, which was accompanied by salient morphological features of apoptosis, such as distorted shape, membrane blebbing, and the presence of apoptotic bodies compared to the control group (Fig. 3k–t). Apoptosis is the most appreciable mode of cell death, and several polysaccharides have been shown to induce apoptosis in cancer cell lines (Dong, Kwan, Chen, & Yang, 1996). The presence of irregular bulges in the plasma membrane of the cell due to localized decoupling of the cytoskeleton from the plasma membrane is a hall mark of apoptosis. Although the budding phenomenon of apoptotic cells lasts for only a few minutes, the formation of apoptotic bodies remains visible for a few hours (Barres et al., 1992).

3.5. Evaluation of the in vivo toxicity of PSP001

We next assessed the *in vivo* toxicity of PSP001 in 5–6 week-old male BALB/c mice. No behavioral changes or visible toxicity symptoms were observed upon *i.p.* administration of PSP001 up to a concentration of 2000 mg/kg body weight; hence, the LD50 was considered to be more than 2000 mg/kg. Pomegranate fruit rind has been widely used as a traditional medicine for thousands of years, largely without side effects, and thus is considered generally safe. Acute toxicity assays did not show any lethal effect of the compound up to 2000 mg/kg (data not shown), indicating that PSP001 is most likely a non-toxic compound (Fenglin, Zhang, & Zhong, 2011). Most plant-derived polysaccharides are non-toxic in nature and exhibited significant immune stimulatory effects (Guruvayoorappan & Kuttan, 2007). We previously reported that PST001 isolated in our laboratory also showed no toxic effects (Aravind et al., 2012a) and its nanoparticle formulation also lacked toxicity (Manu et al., 2013), as evaluated by both acute and sub-acute toxicity assays.

3.6. Effect of PSP001 on tumor-bearing mice

DLA and EAC ascites tumor-bearing mice were evaluated on the 16th and 23rd day of compound administration for the effects on body weight, tumor volume, tumor cell count, percentage of viable cells, and %ILS. In the DLA mice, there was a significant ($P < 0.001$) reduction in the tumor volume in all groups administered PSP001, with the exception of group I, where the tumor volume reduction was not statistically significant (Fig. 4a and Tables 1 and 2; Supplementary Tables 1 and 2). A similar pattern was observed in EAC tumor-bearing mice with a significant ($P < 0.001$) tumor volume reduction in groups II, III, and IV for all compounds, except for group I, where tumor volume reduction was not significant except for Dox ($P < 0.05$) and PSP001 + Dox ($P < 0.01$) groups (Fig. 5a and Tables 1 and 2; Supplementary Tables 1 and 2). In DLA bearing mice, the best response was observed in group IV, where PSP001 reduced the tumor volume to a level comparable to the Dox administered group. The co-administration of PSP001 and doxorubicin (PSP001 + Dox) produced the best response in all groups compared to either compound administered alone. In the case of EAC mice, PSP001 administration (group IV) demonstrated a greater reduction in tumor volume compared to the Dox and PSP001 + Dox groups. PSP001 gave a better response than Dox in groups II and III and was even better than the combination of drugs (group III). There was a significant ($P < 0.001$) reduction in the viable tumor cell counts for DLA and EAC in all the groups administered compounds (Tables 1 and 2; Supplementary Tables 1 and 2). The %ILS showed a significant ($P < 0.001$) increase in all the groups. For DLA tumor mice, a maximum ILS of $145 \pm 1.9\%$

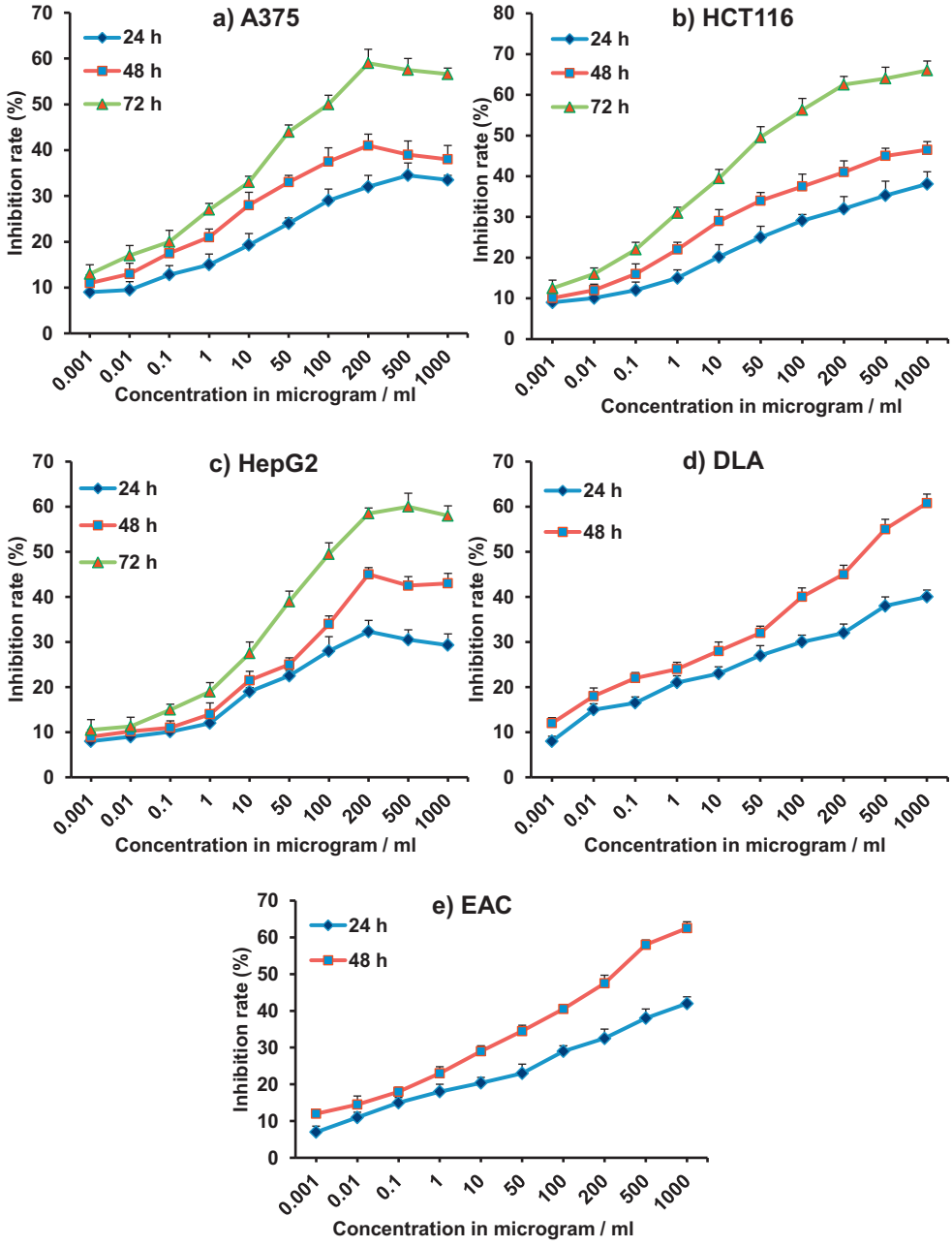

Fig. 2. Cytotoxicity profiling of PSP001 on various cancer cell lines by MTT assay at 24, 48 and 72 h. Inhibition rates are determined for (a) A375, (b) HCT116, (c) HepG2, (d) DLA and (e) EAC cell lines. Results are expressed as the mean ± SD.

Table 1
Evaluation of various parameters in mice injected with DLA/EAC cells on day 1 followed by compound administration on days 2 through 15 (group II). Data were expressed as mean ± SD ($n = 6$).

Parameters	Assessment of antitumor activity in group II							
	DLA				EAC			
	Control	PSP001	Dox	PSP001 + Dox	Control	PSP001	Dox	PSP001 + Dox
Tumor volume (ml)	10 ± 0.5	7 ± 0.8***	3.4 ± 0.3***	3.1 ± 03***	10.5 ± 1	6 ± 0.9***	3.4 ± 0.5***	3.2 ± 0.9***
%TCC (10^6 cells/ml)	166 ± 2.1	90 ± 1.6***	56 ± 1***	48 ± 0.8***	I70 ± 1	77 ± 1.3***	54 ± 0.7***	50 ± 1***
%VTCC	95 ± 1.9	79 ± 1.2***	49 ± 0.6***	50 ± 1***	96 ± 1.2	77 ± 0.9***	45 ± 1.5***	44 ± 1.3***
MST (days)	22 ± 1.1	32 ± 2***	39 ± 1.4***	54 ± 1***	21 ± 1.5	31 ± 1.4***	36 ± 1.2***	44 ± 0.8***
%ILS	0	45 5 ± 1.1***	77 ± 1.2***	145.5 ± 1.9***	0	47.6 ± 1.7***	71 ± 1.4***	109 ± 1.8***

TCC, tumor cell count; VTCC, viable tumor cell count; MST, mean survival time; ILS, increase in life span.
*** Statistically significant differences at $P < 0.001$ compared to the control group.

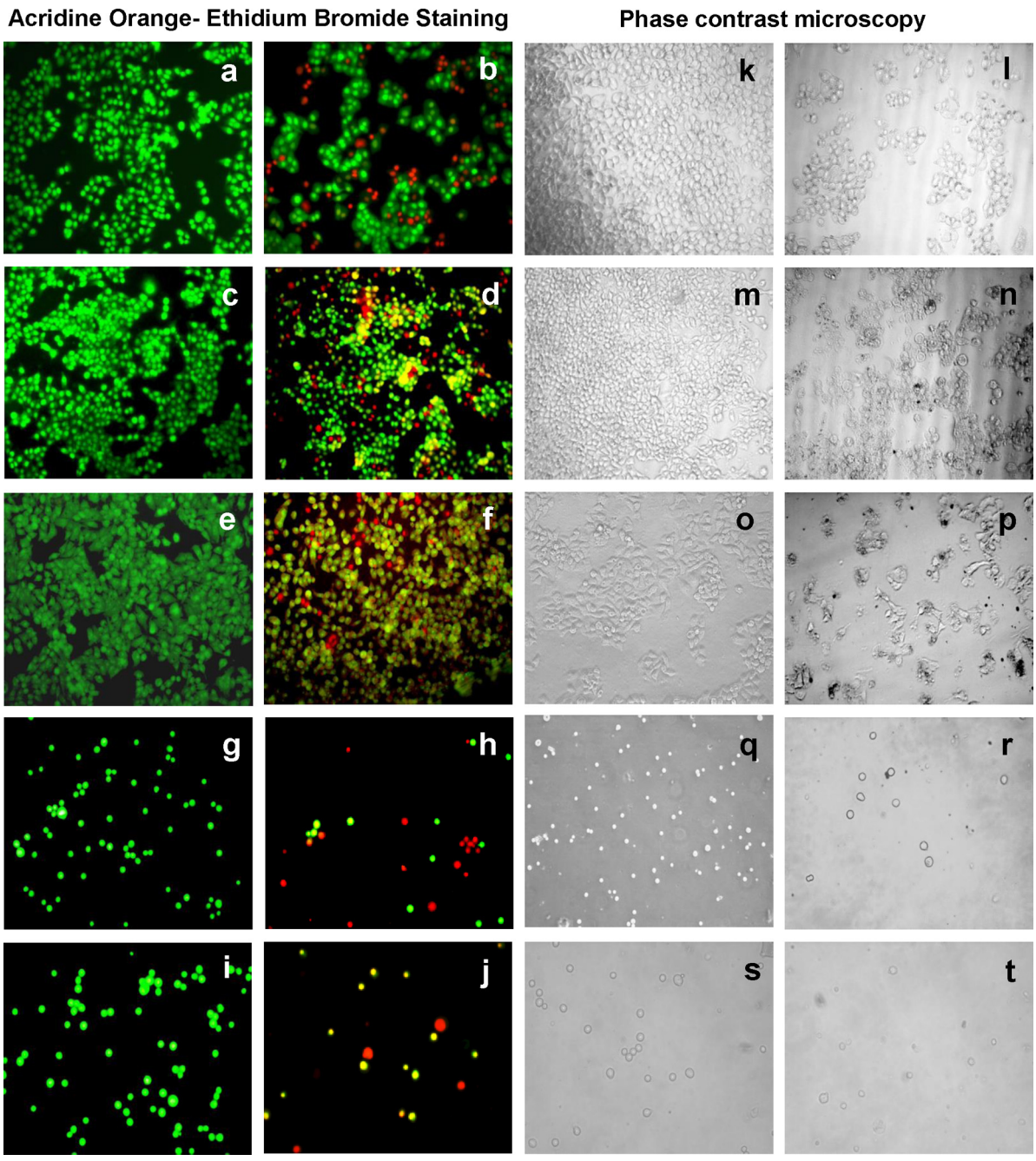

Fig. 3. Representative images of apoptotic evaluation of cell lines treated with 10 μg/ml PSP001 for 72 h by acridine orange–ethidium bromide staining assay (left panel) and phase contrast microscopy (right panel). A375 cells (a and k) control (b and l) PSP001 treated, HCT116 cells (c and m) control (d and n) PSP001 treated, HepG2 cells (e and o) control (f and p) PSP001 treated, DLA cells (g and q) control (h and r) PSP001 treated and EAC cells (i and s) control (j and t) PSP001 treated.

Table 2
Evaluation of various parameters of mice injected with DLA/EAC cells on the 8th day after the administration of compounds for 7 consecutive days and evaluated on the 23rd day (group IV). Data were expressed as mean ± SD ($n = 6$).

Parameters	Assessment of antitumor activity in group II							
	DLA				EAC			
	Control	PSP001	Dox	PSP001 + Dox	Control	PSP001	Dox	PSP001 + Dox
Tumor volume (ml)	11 ± 0.9	4.9 ± 1***	4.8 ± 0.7***	4.1 ± 0.6***	10.5 ± 1	5 ± 1.2***	5 ± 0.5***	4.7 ± 1***
%TCC (10^6 cells/ml)	175 ± 1.8	78 ± 0.8***	75 ± 1.1***	69 ± 0.5***	170 ± 2.1	78 ± 1.4***	80 ± 1***	72 ± 1.9***
%VTCC	95 ± 0.6	79 ± 2.1***	71 ± 0.6***	68 ± 1.5***	94 ± 1.7	79 ± 0.8***	68 ± 1***	70 ± 1.2***
MST (days)	25 ± 1	44 ± 1.4***	36 ± 1.4***	40 ± 2***	24 ± 1.4	44 ± 1.4***	38 ± 1.3***	40 ± 0.8***
%ILS	0	76 ± 1***	44 ± 1.5***	60 ± 1.3***	0	83 ± 0.9***	58 ± 1***	66.6 ± 1.4***

TCC, tumor cell count; VTCC, viable tumor cell count; MST, mean survival time; ILS, increase in life span.
*** Statistically significant differences at $P < 0.001$ compared to the control group.

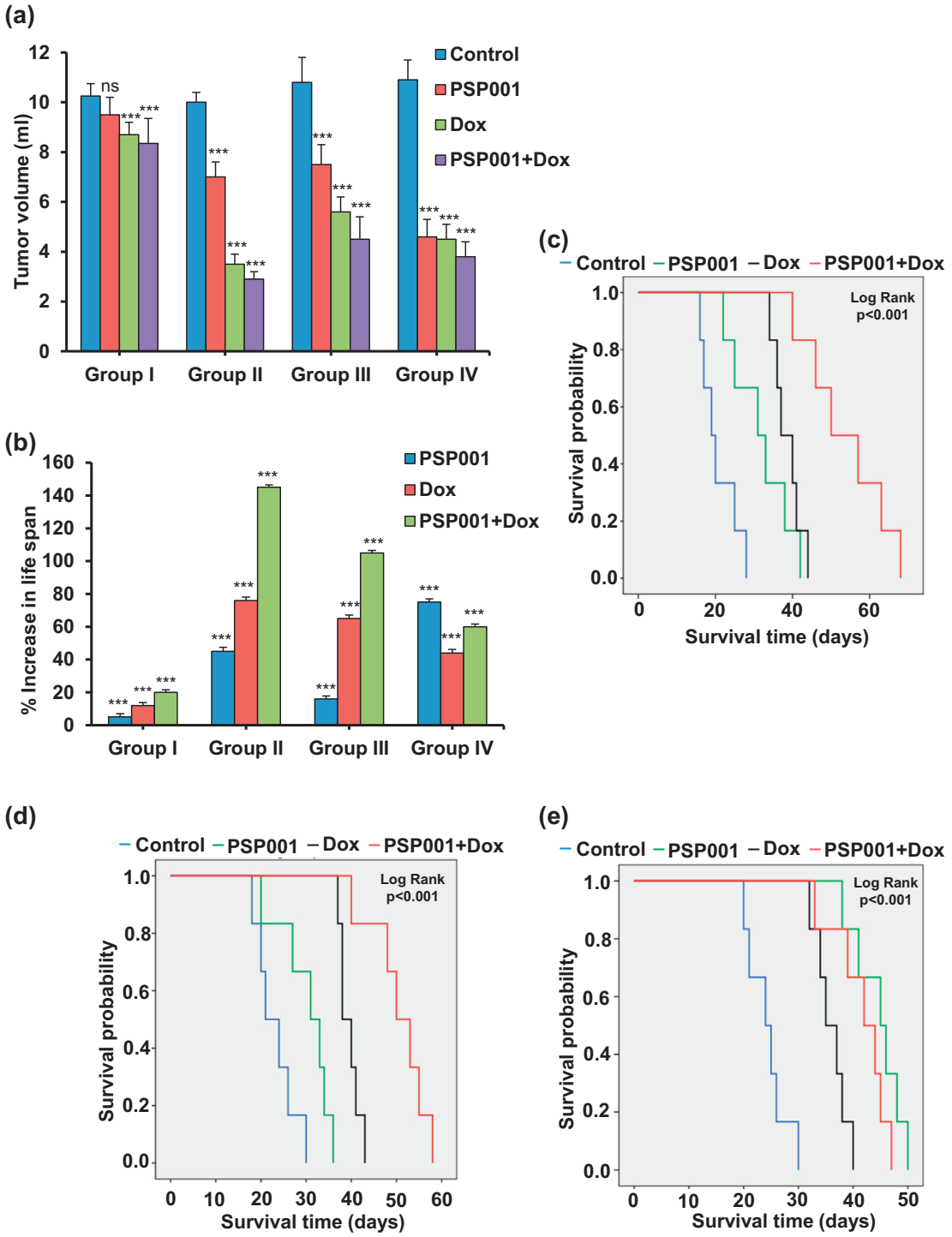

Fig. 4. Antitumor effects of PSP001, Dox and PSP001 + Dox in DLA tumor-bearing mice. (a) Tumor volume measurements between treatment groups (b) Determination of increment in life span. Results are expressed as the mean ± SD. Statistically significant differences at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and ns, non-significant, compared with the control group. Kaplan–Meier survival curve of DLA tumor bearing mice treated with PSP001, Dox, PSP001 + Dox in (c) group II, (d) group III and (e) group IV.

was observed with the PSP001 + Dox administration in group II (Fig. 4b; Tables 1 and 2; Supplementary Tables 1 and 2). PSP001 showed the best response in group IV with a %ILS of 76 ± 1, where 44 ± 1.5 and 60 ± 1.3 were the corresponding values for Dox and the PSP001 + Dox combination, respectively. A similar effect was also observed in EAC tumor mice, with a maximum ILS of 109 ± 1.8% observed for the PSP001 + Dox administration in group II. In group IV, PSP001 showed the best effect with an increase in life span of 83 ± 0.9%, while Dox and PSP001 + Dox showed ILS of 58 ± 1% and 66.6 ± 1.4%, respectively (Fig. 5b and Tables 1 and 2; Supplementary Tables 1 and 2). The Kaplan–Meier survival curves of DLA and EAC groups are shown in Fig. 4c–e and Fig. 5c–e, respectively ($P < 0.001$ *vs.* all groups). We next evaluated the antitumor activity of PSP001 in an EAC-induced solid tumor BALB/c mouse xenograft. There was a significant ($P < 0.001$) reduction in the tumor burden from day 14 in group II by all the compounds, and the PSP001 + Dox combination showed significant responses (Fig. 6a; Supplementary Table 3). In group II, the PSP001 + Dox combination showed a maximum

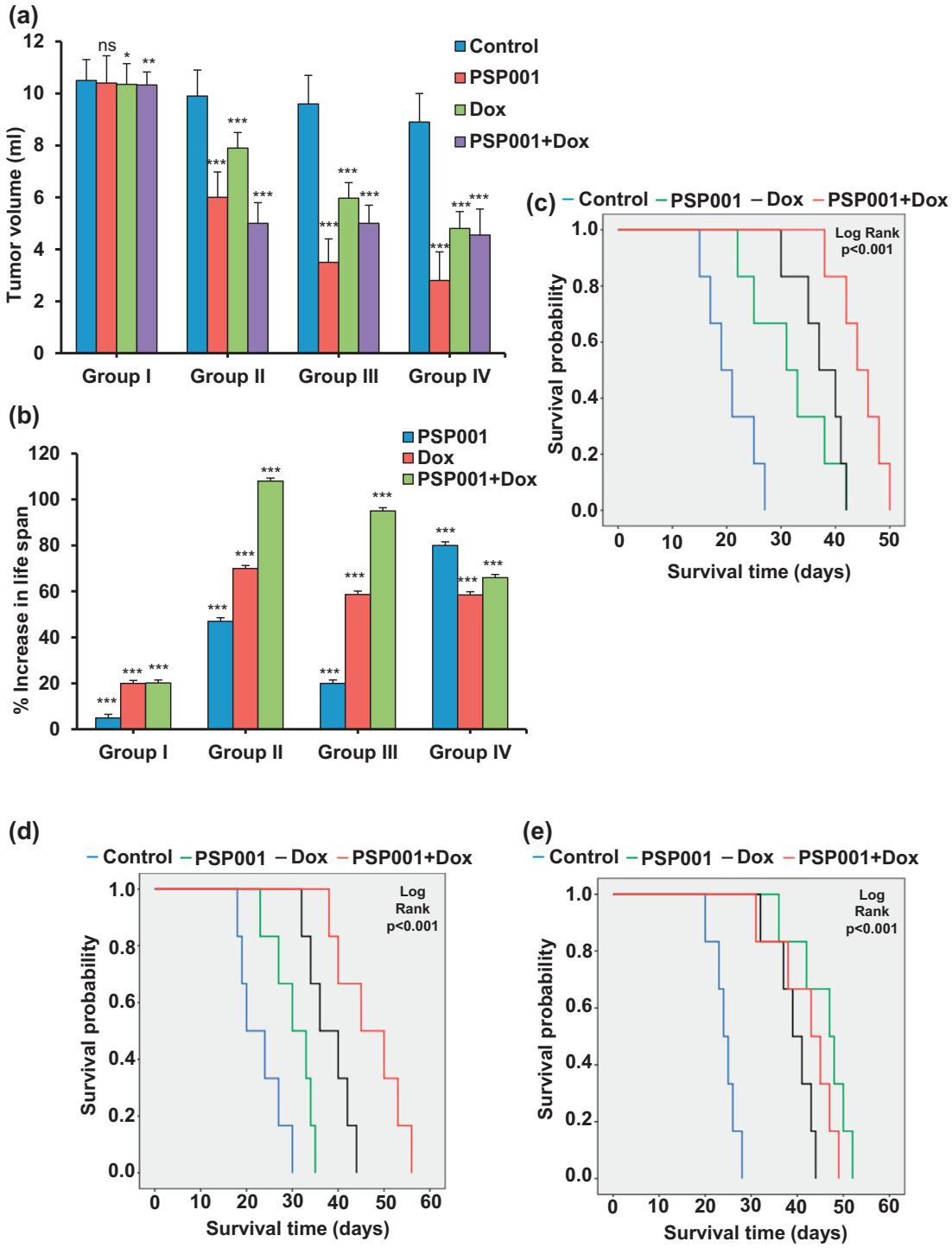

Fig. 5. Antitumor effects of PSP001, Dox and PSP001 + Dox in EAC tumor-bearing mice. (a) Tumor volume measurements between treatment groups (b) Determination of increment in life span. Results are expressed as the mean ± SD. Statistically significant differences at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and ns, non-significant, compared with the control group. Kaplan–Meier survival curve of EAC tumor bearing mice treated with PSP001, Dox, PSP001 + Dox in (c) group II, (d) group III and (e) group IV.

ILS of 83 ± 1.2%, while PSP001 and Dox yielded 54.7 ± 1.5% and 66.6 ± 1.3%, respectively. PSP001 yielded the maximum reduction in tumor volume in group IV, but all combinations showed a significant reduction in tumor volume ($P < 0.001$; Fig. 6b; Supplementary Table 4). PSP001 demonstrated 70 ± 2% increment in lifespan in group IV, whereas Dox and PSP001 + Dox yielded 54 ± 1.3% and 60 ± 0.8%, respectively (Fig. 6c). The Kaplan–Meier survival curves for groups II and IV for EAC solid tumor bearing mice are shown in Fig. 6d and e.

The antitumor effects of PSP001 evaluated in ascites tumor-bearing mice revealed promising results. In the control mice, a rapid increase in ascites fluid was observed. Ascites tumor is the direct nutritional source for tumor cells, and an increased volume indicates the need to meet the nutritional requirements of growing tumor cells (Prasad & Giri, 1994). Treatment with PSP001 significantly reduced the tumor volume, viable tumor cell count, and %ILS of tumor-bearing mice. Although the percentage of viable cells in the ascites fluid was not reduced by PSP001 as much as by Dox

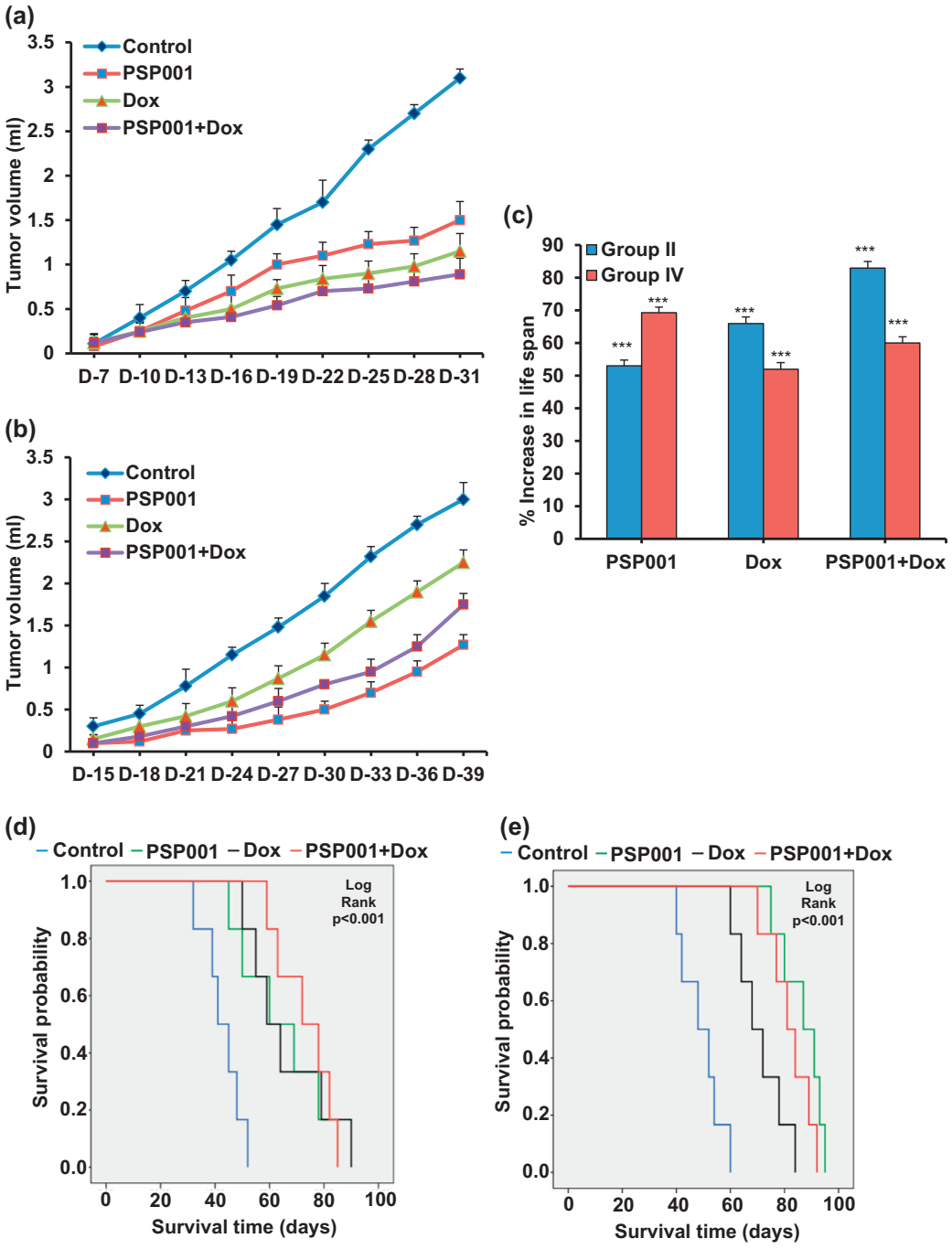

Fig. 6. Antitumor effects of PSP001, Dox and PSP001 + Dox in EAC solid tumor bearing mice. Tumor volume measurements in (a) group II, (b) group IV. Increment in life span in (c) EAC-induced solid tumor bearing mice. Results are expressed as the mean ± SD. Statistically significant differences are at $^{***}P < 0.001$ as compared with the control group. Kaplan–Meier survival curve of EAC-induced solid tumor bearing mice administered with PSP001, Dox and PSP001 + Dox in (d) group II, and (e) group IV.

or the combination treatment, PSP001 significantly reduced the tumor volume and increased the life span as efficiently as the other treatment groups.

A reliable criterion for judging the efficiency of any anticancer drug is the prolongation of lifespan in animals. According to NCI criteria, an ILS exceeding 25% indicated antitumor effectiveness of a drug (Clarkson & Burchenal, 1965), and thus the data in this study indicate that PSP001 may have promising anticancer applications. Although PSP001 did not achieve the desired maximal tumor reduction in certain groups, the %ILS was the highest, which correlates with the immunomodulatory effects of the compound. The better response in group IV for tumor volume reduction and %ILS after PSP001 administration compared to Dox or the combination suggests that PSP001 could also be used as an effective chemopreventive agent. In addition, the antitumor effect of the compounds evaluated in the EAC-induced solid tumor model confirmed that PSP001 is a promising anticancer-immunomodulatory agent that does not exhibit any toxic side effects. The significant reduction in solid tumor size and increase in life span observed with the treatment of PSP001 in group IV once again substantiates its use as an effective natural chemotherapeutic agent. Dox administration produced a significant reduction in tumor

volume in the ascites and solid tumor models in most groups, but the increase in lifespan did not correlate with the reduction in tumor volume observed, which could be due to the severe toxicity associated with doxorubicin (Martin, Konak, & Ulbrich, 2005). The co-administration of PSP001 and Dox in the DLA and EAC ascites tumor model as well as the EAC-induced solid tumor model induced the maximum response in all groups, except for group II, compared to Dox and PSP001 administered alone. It is possible that the combination of PSP001 and Dox may have masked the side effects of Dox, thereby producing the desirable effects observed. Most chemotherapeutic agents have negative side effects that can occur, which limit their widespread clinical application. In addition, macromolecules, such as polysaccharides, possess features that are distinct from low molecular weight drugs mainly with regard to the mechanism of cell entry. When low molecular weight drugs enter the cells by diffusion, macromolecules are internalized by endocytosis and ultimately localize in the lysosomal compartment of the cell (Putnam & Kopecek, 1995). Therefore, the findings of this study indicate that the application of PSP001 either alone or in combination with standard chemotherapeutic drugs, may open up new ways to combat cancer.

4. Conclusion

In conclusion, this study has shown the anticancer potential of a naturally obtained galactomannan polysaccharide from *P. granatum*, PSP001, which has direct cytotoxic effects in several cancer cell lines and ascites tumors. PSP001 also enhanced the survival and tumor reduction in mice bearing transplantable ascites tumors either alone and in combination with doxorubicin. Thus, this study provides evidence of the non-toxic nature of plant-derived compounds, which could be used as an adjuvant or as single agent for the treatment of cancer.

Acknowledgement

We greatly acknowledge the Council of Scientific and Industrial Research (CSIR), Govt. of India, for the research fellowship to the first author (MMJ).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.07.023.

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