

**POLYSACCHAROPEPTIDE ENHANCED THE ANTI-CANCER EFFECT OF GAMMA-TOCOTRIENOL THROUGH ACTIVATION OF AMPK.**

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**Key words:** Polysaccharopeptide; Tocotrienol; AMPK.

**Abbreviations:** Acetyl-CoA carboxylase; AAC, AMP-activated protein kinase; AMPK, Cancer stem cells, CSCs; Gamma-tocotrienols;  $\gamma$ -T3; polysaccharopeptide; PSP.

## **Abstract**

Cancer stem cells (CSCs) are a rare subpopulation of cancer cells which has the ability of self-renewal and repopulating the tumor, and is believed to contribute to maintaining tumor growth and disease metastasis. Unfortunately, conventional therapies against cancer only target the more differentiated tumor cells but spare the CSCs. We recently reported that the two natural compounds PSP (an extract from the mushroom Turkey tail) and Gamma-tocotrienols ( $\gamma$ -T3, one of vitamin E family member) possessed potent anti-cancer activities through targeting of CSCs. In the present study, we showed that PSP significantly enhances the in vitro and in vivo anti-cancer effect of  $\gamma$ -T3. In the presence of PSP,  $\gamma$ -T3 treatment was found to induce a drastic activation of AMP-activated protein kinase (AMPK). This was accompanied with inactivation of acetyl-CoA carboxylase (ACC), as evidenced by the increased phosphorylation levels at Ser 79. In addition, PSP treatment also sensitized cancer cells toward  $\gamma$ -T3-induced cytotoxicity. Furthermore, we demonstrated for the first time that combination of PSP and  $\gamma$ -T3 treatments significantly reduced the growth of prostate tumor in vivo. Our results indicate that PSP and  $\gamma$ -T3 treatments may have synergistic anti-cancer effect in vitro and in vivo, which warrants further investigation as a potential combination therapy for the treatment of cancer.

## **Introduction:**

Cancer remains one of the leading causes of morbidity and mortality worldwide. While most of the cancer can be treated effectively with surgery when the tumor is still localized, the survival rate drops significantly in patients that progressed to metastatic stage. Chemotherapy is usually the last resort for patients with metastatic disease, which is effective in killing actively proliferating cancer cells but often fails to spare normal tissues. Ample evidence supports the idea that cancer originates from a side population of cancer cells known as cancer stem cells (CSCs). CSCs are characterized by their highly invasive characteristics and by their ability to self-renew and differentiate into heterogeneous lineages of cancer cells (1). Similar with normal stem cells, cancer stem cells (CSCs) are capable of entering a quiescent state, thus escape the cytotoxic effect of the conventional chemotherapeutic drugs (2-4) and as a result contributes to treatment failure and disease relapse (5). Therefore, targeting both the bulk tumor cells and the CSCs responsible for tumor regeneration may represent an ideal therapeutic strategy in improving the outcome of cancer patients. We and other have reported that a number of bioactive food compounds have potent anti-CSC effect. For example, we previously demonstrated that gamma-tocotrienol ( $\gamma$ -T3) extracted from palm oil suppresses prostasphere formation and tumor development of prostate cancer cells (6). In addition, a triterpene extracted from fruits was also found to target liver CSCs and sensitize cells to cisplatin treatment (7). More recently, we have shown that polysaccharopeptide (PSP) isolated from Turkey tail (known as *Coriolus versicolor* or Yun-zhi) also inhibit prostate CSC self-renewal in vitro and prevent prostate cancer development in vivo. These findings demonstrated the potential of natural compounds as effective anti-cancer agent against CSCs.

Although natural compounds such as  $\gamma$ -T3 or PSP both demonstrated potent anti-CSC effect, they were found to act through different downstream mechanisms. For example,  $\gamma$ -T3

has been shown to inhibit prostate cancer cell invasion by suppressing the epithelial to mesenchymal transition(8). It was also found to regulate a number of key prosurvival signaling pathways, such as NF-KappaB(9) and PI3K(10), and as a result induce apoptosis of the cancer cells. PSP, on the other hand, was found to inhibit CSC self-renewal without affecting the survival of the cancer cells. In particular, oral intake of PSP was found to have immunomodulatory effect in breast cancer patients, as evidenced by the significant induction of the T-helper lymphocytes and the B-lymphocytes(11). It is thus tempting to speculate that combination of these compounds may achieve a synergistic anti-cancer effect through targeting of multiple signalling pathways.

Here, we investigated the in vitro and in vivo anti-cancer effect of combining PSP and  $\gamma$ -T3. We found that the PSP significantly enhanced the cytotoxicity effect  $\gamma$ -T3 on cancer cells, which was associated with activation of AMPK. Meanwhile, treatment of the cells with PSP and  $\gamma$ -T3 also lead to inactivation of the acetyl-CoA carboxylase (ACC), a protein known to be upregulated in pluriopotent stem cell and is required for stem cell reprogramming (12). Furthermore, oral supplementation of a combined dosage of PSP and  $\gamma$ -T3 was found to have a more potent anti-tumor effect than either agent alone. Our findings highlight the potential of combining PSP with  $\gamma$ -T3 in the treatment of cancer.

## **Materials and methods:**

### **Polysaccharopeptide (PSP) and gamma-Tocotrienol ( $\gamma$ -T3)**

PSP extracted from Yun-zhi was kindly provided by Wonder Herb Health Products, Ltd. The PSP powder was dissolved in autoclaved Milli Q water at a concentration of 30 mg/ml by mixing in a rotator at room temperature overnight. The PSP solution was stored at -20°C. For cell culture study, PSP stock was sterilized with 0.2  $\mu$ m filtration prior to use. In the animal study, PSP was fed directly to mice.  $\gamma$ -T3 was provided by Davos Life Science Pte. Ltd,

Singapore and was dissolved in absolute ethanol (2.5 µg/ml, 3.5 µg/ml and 5 µg/ml) and stored at -20°C. Using the corresponding T3 isomers as reference standards, the purity of  $\gamma$ -T3 was verified to be  $\geq 97\%$  by high performance liquid chromatography (HPLC).

### **Cell lines and culture conditions**

Prostate cancer cell lines PC3 (ATCC, Rockville, MD) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 1% (w/v) penicillin-streptomycin (Invitrogen, Carlsbad, CA) and 5% fetal bovine serum (Invitrogen, Carlsbad, CA). All cell types were kept at 37°C in a 5% CO<sub>2</sub> environment.

### **Generation of PC-3 cells stably expressing GFP**

A GFP-expressing PC-3 cell line, PC3-GFP, was generated using the Viralpower Lentiviral gene expression system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, supernatant containing the lentivirus was mixed with polybrene (8 µg/ml) and used to infect PC-3 cells. After infection, positive transfectants were selected as a pool by treatment with blasticidine (10 µg/ml) for six days.

### **Western Blotting**

Detailed experimental procedures have been described previously. Briefly, whole cell lysates were prepared by resuspending cell pellets in cell lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml leupeptin). Protein concentration was determined using Pierce BCA Protein Assay Kit (Pierce, Rockford, IL). Protein extract was loaded onto an SDS-polyacrylamide gel, separated by electrophoresis and then transferred to a PVDF membrane (Millipore, Billerica, MA). The membrane was then incubated with

primary antibodies against AMPK $\alpha$ , phospho-AMPK $\alpha$  (Thr172) and phospho-Acetyl-CoA Carboxylase (Ser79) (Cell signaling, Technology Inc, Beverly, MA) and  $\gamma$ -tubulin (Sigma-Aldrich, St Louis, MO) overnight at 4°C. After washing with TBS-T, the membrane was incubated with rabbit IgG secondary antibodies, and the signals were visualized using the ECL western blotting system (Millipore, Billerica, MA).

### **Colony formation assay.**

The colony formation assay was carried out as described previously (13). Briefly, PC3 cells were seeded onto polyHEMA (Sigma-Aldrich, St Louis, MO) coated 12-well plate (100 cells per well). Cells were cultured for 14 days in the presence of  $\gamma$ -T3 at 2.5  $\mu$ g/mL, 3.5  $\mu$ g/mL or 5  $\mu$ g/mL and/or PSP at 30 mg/mL. At the end of the experiment, the number of the colonies formed was counted. Colony forming ability was normalized as a percentage of untreated cells. Each experiment was repeated in triplicate, and each data point represents the mean and standard deviation. Statistical difference was determined by Student *t*-test and was considered as significance if  $p < 0.05$ .

### **In vivo treatment of PSP and $\gamma$ -T3**

The xenograft was established in 4- to 6-week-old male athymic nude mice (BALB/*c-nu/nu*) with prostate cancer cell line-labeled PC3 cells (PC3-GFP). Treatment was started once the size of the xenograft reached approximately 4  $\times$  4 mm (length  $\times$  width) in size after subcutaneous injection of  $1 \times 10^6$  of PC3-GFP cells, during which the mice were randomly assigned into four groups and each group consisted of 5 mice :1)  $\gamma$ -T3 (50mg/Kg/day), 2) PSP (300mg/Kg/day), and 3)  $\gamma$ -T3 (50mg/Kg/day) plus PSP (100mg/Kg/day) and 4) DMSO and PBS as a control (group D & E).  $\gamma$ -T3 and PSP were administered to the mice intraperitoneally and orally respectively. After 30 days of various treatments, the effects on

tumor growth were measured and recorded with a Maestro imaging system (CRI Inc. Woburn, MA). Statistical difference was determined by a two-tailed *t*-test and was considered significant if  $p < 0.05$ . All surgical and animal handling procedures were carried out according to the guidelines of the Committee on the Use of Live Animals in Teaching and Research (CULATR), The University of Hong Kong.

### **Immunostaining**

Tissues were formalin fixed and paraffin embedded. Four-micrometer sections were cut, dewaxed in xylene and graded alcohols, hydrated, and washed in PBS. After pretreatment in a microwave oven (12 minutes in sodium citrate buffer [pH 6]), the endogenous peroxidase was inhibited by 0.3% H<sub>2</sub>O<sub>2</sub> for 30 minutes, and the sections were incubated with 10% normal goat serum for 30 minutes. Rabbit polyclonal anti-phospho-AMPK $\alpha$  (Thr172) (1:100) and anti-phospho-acetyl-CoA carboxylase (Ser79) (Cell Signal Technology) was applied overnight in a humidity chamber at 4°C. (A standard avidin-biotin peroxidase technique (Dako) was applied. Briefly, biotinylated goat anti-mouse immunoglobulin and avidin-biotin peroxidase complex were applied for 30 minute each, with 15-minute washes in PBS. The reaction was finally developed with the Dako Liquid DAB+ Substrate Chromogen System (Dako). Slides were imaged on an Aperio Scanscope CS imager, generating 0.43- $\mu$ m/pixel whole slide images.

## **Results:**

### **PSP enhances the cytotoxic effect of $\gamma$ -T3**

To study the synergistic effect between PSP and  $\gamma$ -T3, colony formation assay was performed with prostate cancer cell line PC3 in the presence or absence of PSP and/or  $\gamma$ -T3. As shown in the Figure 1, PSP at 30  $\mu$ g/ml resulted in a decrease of colonies (~50%) when compared to control. Interestingly,  $\gamma$ -T3 at concentrations of 2.5  $\mu$ g/ml and 3.5  $\mu$ g/ml did not have any obvious effect on colony forming ability of the cells. However, when administered simultaneously with PSP, it reduced the number of colonies by approximately 65 % and 85 %, respectively. In addition, 5  $\mu$ g/ml of  $\gamma$ -T3 leads to a 45 % reduction in cell number, which when combined with PSP, almost led to complete inhibition of colony formation. These data suggest that PSP and  $\gamma$ -T3 work synergistically in reducing the viability of the cancer cells.

### **Synergistic effect of PSP and $\gamma$ -T3 on AMPK activation**

To examine the potential mechanism behind the synergistic effect between PSP and  $\gamma$ -T3, we then examine the combine effect of PSP and  $\gamma$ -T3 on a series of cell signalling pathways involved in CSC survival/metabolism. While the effect of  $\gamma$ -T3 on the cell survival pathways such as AKT and JNK was not affected by PSP treatment (data not shown), we found that when the cells were treated with 2.5  $\mu$ g/ml or 5  $\mu$ g/ml of  $\gamma$ -T3 in combination with 30  $\mu$ g/ml of PSP for 24 hr, the phosphorylation levels of AMPK at Thr172 were significantly up-regulated in the PSP and  $\gamma$ -T3 combination group compared with either PSP or  $\gamma$ -T3 (Figure 2). Meanwhile, the total AMPK protein levels were not significantly affected by the treatments, suggesting that the induction of AMPK phosphorylation is not a result of increased AMPK transcriptional level. It is notable that although  $\gamma$ -T3 alone at 5  $\mu$ g/ml also leads to the phosphorylation of AMPK, a more profound effect was observed in the combination therapy group (Figure 2). Strikingly, the combined treatment of PSP and  $\gamma$ -T3



also upregulated the phosphorylation levels of ACC, a direct downstream target of AMPK. Since ACC phosphorylation by AMPK results in inactivation of its activity, our findings suggest that PSP and  $\gamma$ -T3 cooperate to promote AMPK activation, leading to suppression of the ACC function.

### **Combination of PSP and $\gamma$ -T3 is a novel strategy against prostate cancer**

We examined the therapeutic role of combined effect of  $\gamma$ -T3 and PSP *in vivo* using PC3-GFP cells. Athymic nude mice were allografted with PC3-GFP cells and were divided into  $\gamma$ -T3 alone, PSP alone, combined  $\gamma$ -T3 and PSP and control (DMSO and PBS). After 30 days of treatment, the tumor was imaged using Maestro imaging system. The representative mice are shown in Figure 3A.  $\gamma$ -T3 reduced the tumor volumes in a manner approximately two folds and the effect is similar to PSP. In addition,  $\gamma$ -T3 combined with PSP had significant inhibitory effects on PC3-GFP growth *in vivo* compared to the DMSO and PBS control group ( $P < 0.01$ ) (B). During the experiment, no signs of toxicity (infection, diarrhea, or loss of body weight) were observed in the animals undergoing treatment with PSP and  $\gamma$ -T3 when compared with DMSO and PBS. To confirm whether the growth suppressive effect  $\gamma$ -T3 in combination with PSP is due to induction of autophagy, we examined and compared the expression of phospho-AMPK $\alpha$  (Thr172) and phospho-acetyl-CoA carboxylase (ser79) in the tumor xenograft tissues of treated and control groups. Consistent to the *in vitro* observation (Figure 2), we found that increased expression in xenograft tumor of both phospho-AMPK $\alpha$  (Thr172) and phospho-acetyl-CoA carboxylase (ser79) in mice treated with  $\gamma$ -T3 and PSP when compared with the control (Figure 4), suggesting that  $\gamma$ -T3 in combination with PSP leads to AMPK activation and subsequent inactivation of acetyl-CoA carboxylase *in vivo*.

## Discussion

PSP and  $\gamma$ -T3 have been studied extensively in separate studies for their potent anti-cancer effects against a wide-range of cancer cells which includes breast (14-16), liver (17), prostate cancer (18) and melanoma. Despite of this, the mechanisms underlying their anti-cancer effects remain poorly understood. Here, we demonstrated for the first time that PSP acts synergistically with  $\gamma$ -T3 to suppress the survival of prostate cancer cells. This combined effect of PSP and  $\gamma$ -T3 was associated with activation of the AMPK signalling pathway, leading to inactivation of the ACC. The synergistic effect of PSP and  $\gamma$ -T3 was further confirmed with a prostate cancer xenograft models.

$\gamma$ -T3 is one of the constituents of Vitamin E which has been shown to possess anti-oxidative, anti-cardiovascular disease, neuroprotective and anticancer effects(19). Recently,  $\gamma$ -T3 was also reported to sensitize cancer cells to chemotherapeutic drugs (8, 20).  $\gamma$ -T3 is known to target a number of key cell signalling pathway frequently activated in cancer cells. Meanwhile,  $\gamma$ -T3 treatment has also been shown to induce autophagy in cancer cells. Although, AMPK signalling cascade plays a key role in the induction of autophagy in normal and cancer cells (21), whether  $\gamma$ -T3 regulates AMPK in cancer cells is currently unclear. The fact that both phosphorylated AMPK and ACC were upregulated by  $\gamma$ -T3 treatment clearly support that  $\gamma$ -T3 activates AMPK and its downstream signalling cascade in cancer cells. Meanwhile, this effect of  $\gamma$ -T3 appears to be dose dependent as a lower dosage of  $\gamma$ -T3 (2.5 $\mu$ g/ml) failed to produce any observable changes on AMPK and ACC phosphorylation.

Unlike  $\gamma$ -T3, PSP has not been shown to regulate either the cell survival signalling pathways or the metabolic pathway like AMPK. As expected, treatment of cancer cells with PSP was unable to induce the phosphorylation of either AMPK or ACC. It is therefore surprising that in the presence of PSP, the effect of  $\gamma$ -T3 was drastically enhanced. Indeed, at a dose of  $\gamma$ -T3 that failed to induce AMPK activation, addition of PSP was found to

significantly upregulate AMPK activation. Since the majority of the research on PSP were restricted on its immunomodulatory effect, it is currently unclear how PSP enhanced the effect of  $\gamma$ -T3. However, considering the important role of AMPK activation in inhibiting stem cell self-renewal (21), it is tempting to speculate that by combining PSP and  $\gamma$ -T3, a more potent anti-CSC effect can be achieved. Indeed, co-treatment of PSP and  $\gamma$ -T3 were found to achieve a significant tumor suppressive effect *in vivo*. Meanwhile, examination of the tumor tissues after the co-treatment confirmed that AMPK and ACC phosphorylation were both upregulated.

In summary, we have demonstrated for the first time that PSP not only enhance the effect of  $\gamma$ -T3 on AMPK activation, but also showed that it effectively inhibits tumor growth *in vivo*. Our results suggest that combination of PSP and  $\gamma$ -T3 may be an effective therapeutic strategy for the treatment of cancers.

A.

### **Figure Legend**

**Figure 1. *Inhibition of colony forming ability by PSP and  $\gamma$ -T3.*** PC-3 cells were allowed to grow under different combinations of PSP and  $\gamma$ -T3. Colonies formed at the end of the experiment were stained and counted. Data represents a mean and SD.

**Figure 2. *Effect of PSP and  $\gamma$ -T3 treatments on AMPK signalling pathway.*** PC-3 were treated with the indicated compounds for 24 hr. Levels of phospho-AMPK $\alpha$  (Thr172), phospho-acetyl-CoA carboxylase (ser79) and tubulin (as loading control) were examined by Western blotting.

**Figure 3. *In vivo treatment of PSP and  $\gamma$ -T3.*** The *in vivo* therapeutic effect of PSP and  $\gamma$ -T3 was examined in a nude mouse model with PC3-GFP cells. Nude mice were randomized into

four groups, each consisting of five animals. Each group was treated daily for 30 days with either  $\gamma$ -T3 (50mg/Kg/day), PSP (300mg/Kg/day),  $\gamma$ -T3 (50mg/Kg/day) plus PSP (300mg/Kg/day and or DMSO and PBS as the control group. **(A)** Tumor volume in each group was evaluated using Maestro imaging system. Representative mice in each group were shown. **(B)**  $\gamma$ -T3 administration reduced the tumor size by ~2 folds as compared with control and its effect was similar to PSP alone. The combination of  $\gamma$ -T3 and PSP exhibited a synergistic effect on tumor suppression ( $P < 0.01$ , *t* test).

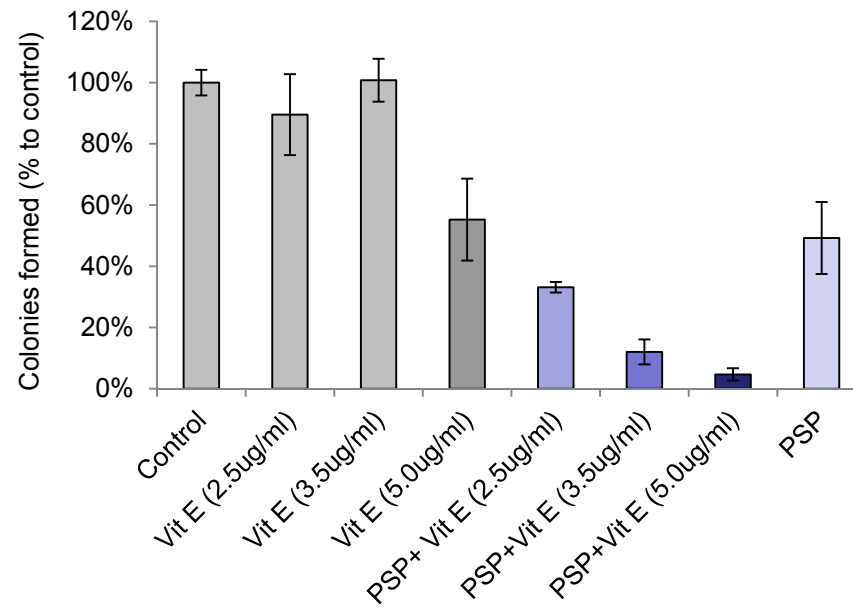
**Figure 4. PSP and  $\gamma$ -T3 activates AMPK signalling pathway *in vivo*.** Immunostaining was performed on xenograft tumor collected from the mice with the indicated treatment. Consecutive sections were stained with antibody against phospho-AMPK $\alpha$  (Thr172) and phospho-acetyl-CoA carboxylase (ser79). Note that an increase in cytoplasmic expression of both proteins was observed in tumors co-treated with  $\gamma$ -T3 and PSP (200 $\times$  magnifications).

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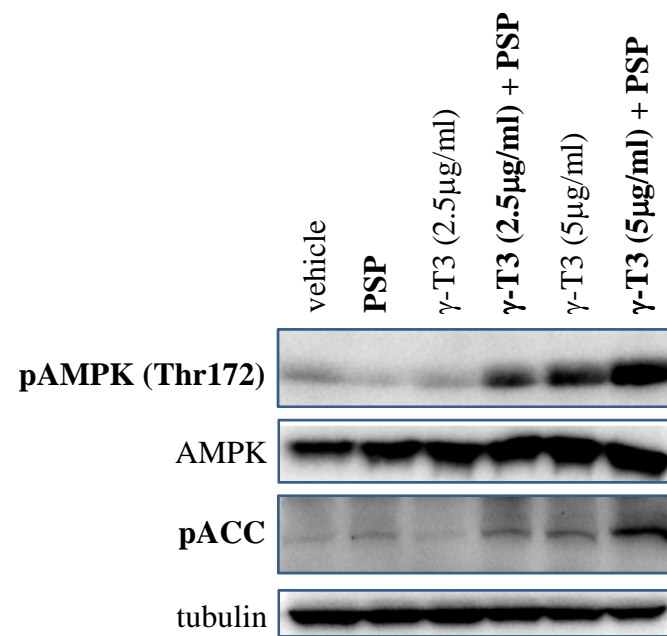
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**Fig. 1**



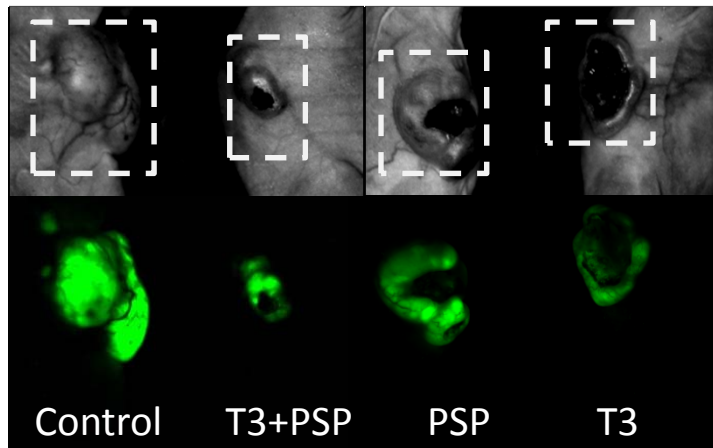
**Fig. 2**



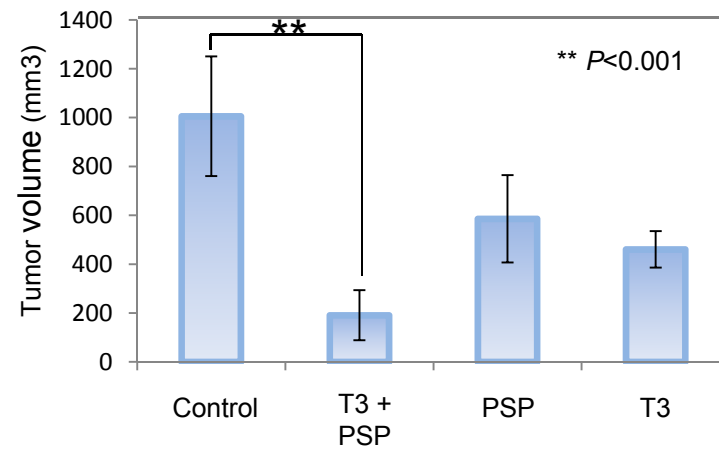


**Fig. 3**

**A.**



**B.**



**Fig. 4**

