## ORIGINAL ARTICLE

## Potential antitumor effects of panaxatriol against DU-15 human prostate cancer cells is mediated via mitochondrial mediated apoptosis, inhibition of cell migration and sub-G1 cell cycle arrest

Rong Yu<sup>1</sup>, Yan Zhang<sup>2</sup>, Ziqiang Xu<sup>2</sup>, Jinjun Wang<sup>2</sup>, Bicheng Chen<sup>3</sup>, Hao Jin<sup>2</sup>

<sup>1</sup>Reproductive Medicine Center, <sup>2</sup>Department of Transplantation, <sup>3</sup>Department of Surgery, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, P.R. China

## Summary

**Purpose:** Prostate cancer is the most frequently diagnosed malignancy in men and the second major reason of cancer death in males. Currently, there are no viable options available for the treatment of advanced-stage prostate cancer. Against this backdrop, the present study aimed to study the anticancer effect of panaxatriol against prostate DU-15 cancer cells.

**Methods:** MTT cell viability assay evaluated the effects of the drug on cell cytotoxicity, while clonogenic assay was used to assess the effects on colony formation in DU-15 cells. Apoptotic effects were evaluated by DAPI staining using fluorescence microscopy. Effects on reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were evaluated by flow cytometry using DCFH-DA and DiOC<sub>6</sub>. Effects on cell cycle were measured by flow cytometry, while cell migration tendency of the cells was evaluated by in vitro wound healing assay.

**Results:** The results indicated that panaxatriol exerts dosedependent cytotoxic effects on prostate DU-15 cancer cells. The  $IC_{50}$  of panaxatriol was 30  $\mu$ M. Panaxatriol was found to exert its anticancer activity through induction of apoptosis. The apoptosis of DU-15 cancer cells was prompted by ROSmediated alterations in mitochondrial membrane potential. Additionally, panaxatriol induced sub-G1 cell cycle arrest and suppressed the DU-15 cell migration ability in a concentration-dependent manner.

**Conclusion:** Taken together, we strongly believe that panaxatriol may prove handy in the treatment and management of prostate cancer and deserves further research.

*Key words:* cell cycle arrest, cell migration, panaxatriol, prostate cancer, ROS

## Introduction

Prostate cancer is the most frequently identified malignancy in men and the second major reason of cancer death in males. Most of the initial tumors are androgen-dependent, thus cutting the supply of tumor of androgens through surgical interventions [1] has proved to be a viable option at initial stages of prostate cancer. However, advanced prostate cancer is difficult to control with such treatments and ultimately relapses with upsetting results [2]. Therefore, there is an

urgent need for development of viable treatment options for prostate cancer and anticancer molecules of plant origin offer many opportunities for the management of this disease. Several plantderived natural products have shown remarkable results in the management of a diversity of cancers. Recently, it has been reported that several triterpenes and their derivatives have exhibited anticancer activities against different cancer cells [3]. Panaxatriol (Figure 1) is one such compound

*Correspondence to:* Hao Jin, MD. Department of Transplantation, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang Province, P. R. China.

Tel/Fax: +86 577 5557 8166, E-mail: HLovewellumays@yahoo.com Received: 19/06/2017; Accepted: 05/07/2017

isolated from *Panax notoginseng*, however it has not be evaluated against prostate cancer [4]. The present study was therefore designed to evaluate the anticancer activity of panaxatriol against a prostate cancer cell line. Moreover, the probable underlying mechanism was assessed with special emphasis on the effect of this natural product on cell migration and cell cycle arrest.



Figure 1. Chemical structure of panaxatriol.

## Methods

#### Chemicals and Reagents

Several chemicals and reagents were used in the present study and included (i) panaxatriol, RNase A, triton X-100 and dimethyl sulfoxide (DMSO) obtained from Sigma-Aldrich Co. (St. Louis, MO, USA); (ii) the fluorescent probes DCFH-DA,  $DiOC_6$ , 4'-6-diamidino-2-phenylindole (DAPI), RPMI-1640 medium, L-glutamine and antibiotics obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

#### Cell line and culture conditions

DU-15 prostate cancer cell line was procured from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and preserved in RPMI-1640 medium tissue culture flasks at 37°C in a humidified atmosphere with 5 %  $CO_2$  and 95 % air.

#### Determination of cytotoxicity

The cytotoxic effect of panaxatriol against prostate cancer cells was determined by MTT assay. DU-15 cells were cultured at the density of  $1 \times 10^6$  cells per well in 96 well plates for 12 hrs and then treated with varying concentrations of panaxatriol (0-120 µM) for 48-72 hrs. MTT solution (20 µl) was added to each well. Afterwards, 500 µl of DMSO was added to solubilize MTT formazan crystals, and ELISA plate reader was used for the determination of optical density. To evaluate the effect of the test compound on the colony formation potential, prostate cancer DU-15 cells were grown to exponential phase and thereafter collected and counted with a hemocytometer. Platting of the cells was done at 200 cells per well and then the cells were incubated for 48 hrs to allow the cells to adhere. This was fol-

lowed by the addition of different doses (0, 15 30 and 60  $\mu$ M) of panaxatriol. After treatment, the cells were again kept for incubation for 6 days, washing was done with phosphate buffered saline (PBS) and methanol was used to fix colonies and then stained with crystal violet for about 30 min before being counted under light microscope.

#### Detection of apoptosis, ROS and MMP

DU-15 cells at a density of  $2 \times 10^5$  cells/well were platted in 6-well plates exposed to 0, 15, 30 and 60 µM panaxatriol for 48 hrs. The cells were then stained with DAPI and each of the cell samples was studied under fluorescence microscopy as described elsewhere [5]. For determination of ROS and MMP, DU-15 cells were platted at a density of  $2 \times 10^5$  cells/well in a 6-well plate, were retained for 24 hrs and treated with 0, 15, 30 and 60 µM panaxatriol for 72 hrs at 37°C in an atmosphere with 5% CO<sub>2</sub> and 95% air. Thereafter cells from all samples were collected, washed twice by PBS and re-suspended in 500 µl of DCFH-DA (10 µM) for ROS estimation and DiOC<sub>6</sub> (1 µmol/l) for MMP at 37°C in the dark for 30 min. The samples were then examined instantly using flow cytometer as described elsewhere [6].

#### *Estimation of cell cycle phase distribution of DU-15 cells*

For estimation of cell cycle phase distribution, the cells were platted in 6-well plates ( $2 \times 10^5$  cells/well) and panaxatriol was added to the cells at 0, 20, 40 and 80 µM doses followed by 24-h incubation. DMSO was used as control. For estimation DNA content, PBS was used to wash the cells and fixed in ethanol at -20°C. This was followed by re-suspension in PBS holding 40 µg/ml propidium iodide (PI) and, RNase A (0.1 mg/ml) and Triton X-100 (0.1%) for 30 min in the dark at 37°C. Afterwards, analysis was carried out by flow cytometry as reported previously [7].

#### Wound healing assay

Prostate cancer DU-15 cells were platted at a density of  $5 \times 10^4$  in 96-well plates and then kept overnight to attach. As they reached confluence, a wound was scratched across each well by wound maker device. Finally, the cells were washed with PBS to remove the detached cells and photographed.

#### Statistics

All experiments were carried out in triplicate and the results were expressed as mean  $\pm$  standard deviation (SD). The results were considered statistically significant at p<0.01, p<0.001 and p<0.001. The statistical analysis was carried out by Graph Pad prism 7.

#### Results

# *Cytotoxicity of panaxatriol on prostate DU-15 cancer cell line*

The results of our study showed that panaxatriol displayed a potent anticancer activity against the prostate cancer DU-15 cells. The cytotoxic activity was found to be concentration-dependent and increased with the increase in the concentration of panaxatriol. The IC<sub>50</sub> of panaxatriol against DU-15 cells was 30  $\mu$ M (Figure 2). Additionally, the cytotoxic potential of panaxatriol was further confirmed by the colony formation assay wherein panaxatriol administration was observed to reduce the number of colonies in a dose-dependent manner (Figure 3).

#### Panaxatriol induced apoptosis in DU-15 cancer cells

Apoptotic cell death induced by panaxatriol was detected by DAPI staining. It was observed that the apoptosis of prostate DU-15 cancer cells increased with panaxatriol treatment. The apoptotic effect was observed to increase in a concentration-dependent manner (Figure 4).



**Figure 2.** Effect of indicated doses of panaxatriol on the viability of DU-15 prostate cancer cells. All experiments were carried out in triplicate and expressed as mean  $\pm$  SD. \*p<0.01, \*\*p<0.001, \*\*\*p<0.0001.



**Figure 3.** Effect of indicated doses of panaxatriol on colony forming potential of DU-15 cells at indicated doses. All experiments were carried out in triplicate. Cancer cells decreased significantly with increasing doses of panaxatriol (crystal violet, original magnification 200x).



**Figure 4.** Induction of apoptosis by panaxatriol at indicated doses as depicted by DAPI staining. All experiments were carried out in triplicate. Apoptosis increased with increasing doses of panaxatriol.



**Figure 5.** Effect of indicated doses of panaxatriol on ROS generation. All experiments were carried out in triplicate and expressed as mean  $\pm$  SD. ROS generation increased significantly with increasing doses of panaxatriol. \*p<0.01, \*\*p<0.001, \*\*\*p<0.0001.

#### Panaxatriol caused ROS production and MMP reduction in DU-15 cancer cells

The apoptosis triggering potential of panaxatriol as evidenced from DAPI staining study indicated that this compound might induce production of intracellular ROS. Therefore, we determined the ROS levels at varying concentrations of panaxatriol for 48 hrs. The results revealed that the intracellular ROS levels of treated cells increased up to 210% at 60  $\mu$ M concentration as compared to untreated cells (Figure 5). Therefore, our results indicated that panaxatriol is a potent molecule for triggering ROS production in DU-15 cells. We also investigated whether panaxatriol induces MMP loss in DU-15 cells exposed at varying concentrations. Panaxatriol-treated DU-15 cells showed a considerable reduction in MMP in a dose-dependent manner (Figure 6).



Figure 6. Effect of indicated doses of panaxatriol on mitochondrial membrane potential (MMP). All experiments were carried out in triplicate and expressed as mean  $\pm$  SD. Increasing doses of panaxatriol led to significant decrease of MMP. \*p<0.01, \*\*p<0.001, \*\*\*p<0.0001.

#### Panaxatriol caused alterations in cell cycle distribution of DU-15 cancer cells

In addition to apoptosis inducing potential of panaxatriol, it was also observed to induce sub-G1 cell cycle phase arrest in DU-15 prostate cancer cells. This effect of panaxatriol was found to be concentration-dependent. The results of the present study showed that panaxatriol administration caused considerable increase of DU-15 prostate cancer cells in sub-G1 phase of cell cycle. Moreover, the highest effect of panaxatriol on cell cycle distribution was observed at the highest dose tested (60  $\mu$ M) (Figure 7).

#### Panaxatriol inhibited cell migration

Panaxatriol was shown to suppress the migration of prostate cancer cells at the  $IC_{50}$  concentration (30 µM). The results of wound healing assays showed that panaxatriol reduced the migratory capability of prostate cancer DU-15 cells (Figure 8), providing strong evidence towards the use of panaxatriol as anti-metastatic agent.

## Discussion

Prostate cancer is the second major causes of death due to cancers in men world over. [1]. Although surgical interventions at initial stage exhibit promising results, there are complete and viable treatment options available for advanced stages [2]. With this background, natural products can offer considerable opportunities for the development of efficient and novel anticancer molecules with low or no associated health risks. In the present study, panaxatriol, a molecule generally isolated from the plants of *Panax notoginseng*, showed potent cytotoxic activity against DU-15 prostate cancer cells as evidenced from the MTT and colony



**Figure 7.** Effect of indicated doses of panaxatriol on cell cycle arrest detected by flow cytometry. All experiments were carried out in triplicate. Increasing doses of panaxatriol led to increased fraction of cells in sub-G1 (cell cycle arrest) causing cell death via cell cycle disruption.



**Figure 8.** Cell migration inhibitory potential of panaxatriol at  $IC_{50}$  concentration (30  $\mu$ M) investigated by wound healing assay. Panaxatriol treatment led to suppression of cancer cell migration after 48 hrs.

formation assays. There is concrete evidence that several anticancer molecules exert their cytotoxic effects through induction of apoptosis, therefore we investigated the apoptosis inducing potential of panaxatriol through DAPI staining. Interestingly, panaxatriol induced apoptosis in DU-15 prostate cancer cells in a dose-dependent manner. Our results are well supported by the existing literature on anticancer drugs [8-13]. For example anticancer cannabinoids have been reported to induce apoptosis in gliomas [8], pheochromocytoma [9], neuroblastoma [10] and hippocampal neurons [11]. Since it is well reported that ROS play an important role in the induction of cell death, we further examined the ROS levels in control and panaxatriol-treated DU-15 prostate cancer cells and we observed that panaxatriol-administered cells showed very high levels of ROS associated with the reduction of MMP. These results are consistent with a study carried out previously [14]. Panaxatriol also caused sub-G1 cell cycle arrest and led to a considerable increase of sub-G1 cells dose-dependently. Cell cycle is considered as an important mechanism for inhibiting cell division. Several natural products have been reported to arrest cell cycle, such as azadirachtin A which has been reported to induce cell cycle arrest in different cell lines [15]. These findings are promising since it is well established that prostate cancer is one of the deadly cancers and panaxatriol could suppress its growth through cell cycle arrest. Additionally, panaxatriol also inhibited the cell migration of DU-15 cells as evidenced from the wound healing assays. Cell migration is the key feature of cancer progression and metastasis [15] and suppression of cell migration may prove crucial in inhibition of metastasis in vivo. This may ensure comparatively longer survival of patients. Therefore, the potential of panaxatriol to suppress

migration of prostate DU-15 cancer cells indicates that this compound may prove as an efficient molecule in inhibiting the metastasis of cancer cells *in vivo* and therefore deserves further investigation.

## Conclusion

We conclude that panaxatriol exerts its anticancer activating through of ROS and MMP mediated apoptosis in prostate DU-15 cancer cells and may prove to be an efficient molecule for the treatment of prostate cancer. However, further studies are required for better evaluation of the molecule.

## Acknowledgements

This research was supported by National Natural Science Foundation of China (Grant No. 81501382) and Zhejiang Provincial Natural Science Foundation of China (Grant No. LQ13H100003 and LQ16H100002).

## **Conflict of interests**

The authors declare no conflict of interests.

## References

- 1. Gnanapragasam VJ, Robinson MC, Marsh C, Robson CN, Hamdy FC, Leung HY. FGF8 Isoform b Expression in Human Prostate Cancer. Br J Cancer 2003;88:1432-8.
- 2. Lara PN Jr, Twardowski P, Quinn DI. Angiogenesis targeted Therapies in Prostate Cancer. Clin Prostate Cancer 2004;3:165-73.
- Bishayee A, Shamina A, Brankov N, Perloff M. Triterpenoids as potential agents for the chemoprevention and therapt of breast cancer. Front Biosci 2011;16:980-86.
- Morikawa T, Kishi A, Pongpiriyadacha Y, Matsuda H, Yoshikawa M. Structures of new friedelane-type triterpenes and eudesmane-type sesquiterpene and aldose reductase inhibitors from Salacia chinensis. J Nat Procl 2003;66:191-6.
- Chiang LC, Ng LT, Lin IC, Kuo PL, Lin CC. Anti-proliferative effect of apigenin and its apoptotic induction in human Hep G2 cells. Cancer Lett 2006;237:207-14.
- Chiang JH, Yang JS, Ma CY et al. Danthron, an Anthraquinone Derivative, Induces DNA Damage and Caspase Cascades-Mediated Apoptosis in SNU-1 Human Gastric Cancer Cells through Mitochondrial Permeability Transition Pores and Bax-Triggered Pathways. Chem Res Toxicol 2011;24:20-9.
- Sun SY, Hail N Jr, Lotan R. Apoptosis as a novel target for cancer chemoprevention. J Natl Cancer Inst 2004;96:662-72.
- Velasco G, Galve-Roperh I, Sanchez C, Blazquez C, Guzman M. Hypothesis: Cannabinoid Therapy for the Treatment of Gliomas. Neuropharmacology 2004;47: 315-23.

- Sarkar KP, Obara S, Nakata M, Kitajima I, Maruyama I. Anandamide Induces Apoptosis of PC-12 Cells: Involvement of Superoxide and Caspase-3. FEBS Lett 2000;472:1039-44.
- Maccarrone M, Lorenzon T, Bari M, Melino G, Finazzi-Agro A. Anandamide Induces Apoptosis in Human Cells via Vanilloid Receptors. Evidence for a Protective Role of Cannabinoid Receptors. J Biol Chem 2000;275:31938-45.
- 11. Chan GC, Hinds TR, Impey S, Storm DR. Hippocampal Neurotoxicity of D9 Tetrahydrocannabinol. J Neurosci 1998;18:5322-32.
- Guzman M, Sanchez C, Galve-Roperh I. Control of the Cell Survival/Death Decision by Cannabinoids. J Molec Med 2001;78:613-25.
- 13. De Petrocellis L, Ligresti A, Schiano Moriello A et al. Non-THC Cannabinoids Inhibit Prostate Carcinoma Growth in Vitro and in Vivo: Pro-Apoptotic Effects and Underlying Mechanisms. Br J Pharmacol 2013;168:79-102.
- Huang JF, Shui KJ, Li HY, Hu MY, Zhong GH. Antiproliferative effect of azadirachtin A on Spodoptera litura Sl-1 cell line through cell cycle arrest and apoptosis induced by up-regulation of p53. Pestic Biochem Phys 2011;99:16-24.
- 15. Lee WJ, Chen WK, Wang CJ, Lin WL, Tseng TH. Apigenin inhibits HGF promoted invasive growth and metastasis involving blocking PI3K/Akt pathway and beta 4 integrin function in MDA-MB-231 breast cancer cells. Toxicol Appl Pharmacol 2008;226:178-91.