IN VITRO AND *IN VIVO* PRO-APOPTOTIC AND CHEMOSENSITIZING EFFECTS OF ALPHA-TOMATINE ON HUMAN PROSTATE ADENOCARCINOMA

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ABSTRACT

Alpha (α)-tomatine is a major saponin found in tomatoes (*Lycopersicon esculentum*). The present study investigates the molecular mechanisms by which α-tomatine exerts its anti-cancer effect on human prostatic adenocarcinoma cells. Treatment of human androgen-dependent LNCaP and androgen-independent PC-3 prostate cancer cells with α-tomatine resulted in a concentration-dependent inhibition of cell growth with a half-maximal efficient concentration (EC_{50}) value of 2.65 ± 0.1 μM and 1.67 ± 0.3 μM, respectively. PC-3 cells appear to be more sensitive to α-tomatine-induced growth inhibition compared to LNCaP cells. Importantly, α-tomatine treatment is also less cytotoxic to non-tumorigenic human liver WRL-68 and human prostatic epithelial RWPE-1 cells. Due to the higher sensitivity of PC-3 cells to α-tomatine and significant morbidity of metastatic androgen-independent prostate cancer, it is of interest to study in greater detail the mechanisms of action of α-tomatine in PC-3 cells. Results from the present study showed that the inhibitory effect of α-tomatine on PC-3 cell growth was mainly due to the induction of apoptosis via the inhibition of nuclear factor-kappa B (NF-κB) pathway. Alpha-tomatine suppresses both basal constitutive and tumor necrosis factor-alpha (TNF-α)-induced NF-κB activation. The suppression of NF-κB activation by α-tomatine occurs through the inhibition of Akt, leading to the inhibition of IκBα kinase (IKK) activity and subsequently suppression of NF-κB nuclear translocation in PC-3 cells. The inhibition of NF-κB signaling pathway by α-tomatine was accompanied by significant reduction in the expression of NF-κB-dependent anti-apoptotic proteins. The anti-tumor study of α-tomatine against PC-3 cells was extended to subcutaneous xenograft and orthotopic mouse models. Intraperitoneal administration of α-tomatine significantly attenuates the growth of PC-3 cell tumors grown at both sites without significant body weight loss. In agreement to the *in vitro* data, analysis of tumor materials showed an increase in tumor cell apoptosis and a decrease in the basal

nuclear localization of NF-κB p50 and p65. The present study further investigated the efficacy of α-tomatine in combination with low-dose of paclitaxel in PC-3 cells. Treatment with sub-toxic dose of α-tomatine in combination with low-dose paclitaxel resulted in a decrease in cell viability with concomitant increase in apoptosis in PC-3 cells but not in non-tumorigenic human prostatic epithelial RWPE-1 cells. Results from these *in vitro* experiments indicated that the induction of apoptosis by the combined treatment was accompanied by the inhibition of phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pro-survival signaling, which is an upstream mediator of NF-κB and known to confer chemoresistance in prostate cancer. The combined treatment also completely suppressed subcutaneous tumor growth in mouse xenograft without apparent body weight loss. Analysis of tumor materials showed an increase in tumor cell apoptosis with a reduction in the protein expression of activated PI3K/Akt. In summary, results from the present study provide comprehensive evidence that α-tomatine is an effective naturally-derived anti-tumor agent against androgen-independent prostate cancer and when used in combination, it can enhance the efficacy of taxane-based agent. The clinical applications of α-tomatine in prostate cancer treatment should be further explored.

ABSTRAK

Alpha (α)-tomatine merupakan saponin utama yang didapati di dalam buah tomato (*Lycopersicon esculentum*). Kajian ini bertujuan untuk mengkaji potensi terapeutik α-tomatine terhadap sel-sel adenokarsinoma prostat manusia. Rawatan α-tomatine terhadap sel kanser prostat LNCaP yang bergantung kepada androgen dan sel kanser prostat bebas androgen PC-3 telah mengakibatkan perencatan pertumbuhan yang berkadar langsung dengan dos α-tomatine dengan nilai EC_{50} *(half-maximal efficient concentration)* iaitu 2.65 ± 0.1 μM dan 1,67 ± 0.3 μM, masing-masing. Oleh itu, didapati bahawa sel kanser prostat bebas androgen PC-3 adalah lebih sensitif terhadap perencatan pertumbuhan akibat kesan rawatan α-tomatine berbanding dengan sel kanser prostat LNCaP yang bergantung kepada androgen. Lebih menariknya, rawatan α-tomatine tidak memberi kesan negatif terhadap sel hati (WRL-68) dan sel epithelium prostat (RWPE-1) manusia yang normal. Oleh kerana kanser prostat bebas androgen adalah lebih metastatik dan sel PC-3 yang lebih sensitif terhadap rawatan α-tomatine, kajian yang lebih terperinci tentang mekanisme tindakan α-tomatine terhadap sel PC-3 dijalankan dengan selanjutnya. Kajian kami telah membuktikan bahawa α-tomatine merencatkan pertumbuhan sel PC-3 melalui induksi *apoptosis*. Selaras dengan keupayaan α-tomatine yang mendorong sel mati melalui *apoptosis* terhadap sel PC-3, α-tomatine juga boleh mengurangkan pengaktifan laluan nuklear faktor-kappa B (NF-κB) samada secara konstitutif ataupun yang diinduksikan oleh tumor necrosis factor-alpha (TNF-α). Analisis *in vitro* secara terperinci menunjukkan bahawa pengurangan pengaktifan laluan NF-κB oleh α-tomatine adalah melalui perencatan Akt, yang seterusnya membawa kepada perencatan aktiviti kinase IκBα (IKK) dan akhirnya perencatan translokasi nuklear NF-κB di dalam sel PC-3. Keberkesanan α-tomatine di dalam perencatan pengaktifan laluan NF-κB diiringi oleh pengurangan ekspresi protein yang berkaitan dengan proses *anti-apoptosis*. Aktiviti antitumor α-tomatine terhadap sel PC-3 juga diselidik secara *in vivo* dalam model mencit xenograf

subkutan dan orthotopik. Rawatan α-tomatine secara intraperitoneum dapat merencat pertumbuhan tumor subkutan dan orthotopik PC-3 di dalam mencit tanpa menjejaskan berat badan mencit tersebut. Selaras dengan data *in vitro,* analisis tumor yang dirawat dengan α-tomatine juga menunjukkan bahawa terdapat peningkatan di dalam *apoptosis* serta pengurangan translokasi komponen NF-κB p50 dan p65 translokasi nuklear. Seterusnya, kajian terperinci telah dilakukan untuk menyiasat keberkesanan rawatan kombinasi α-tomatine dengan dos rendah paclitaxel di dalam sel PC-3. Rawatan kombinasi α-tomatine dengan dos rendah paclitaxel merencatkan pertumbuhan sel PC-3 tanpa menjejaskan pertumbuhan sel prostat normal RWPE-1. Eksperimen *in vitro* menunjukkan bahawa induksi *apoptosis* oleh kombinasi α-tomatine dengan dos rendah paclitaxel telah diiringi dengan perencatan laluan pro-hidup *phosphatidylinositol-3-kinase* (PI3K)/ *protein kinase B* Akt yang merupakan mediator kepada pengaktifan NF-κB dan terlibat dalam resistan terhadap rawatan kemoterapi untuk kanser prostat. Rawatan kombinasi α-tomatine dengan dos rendah paclitaxel juga merencat pertumbuhan tumor subkutan PC-3 tanpa menjejaskan jumlah berat badan mencit. Analisis daripada sampel tumor PC-3 menunjukkan peningkatan *apoptosis* di dalam sel tumor PC-3 dengan pengurangan ekspresi pengaktifan protein PI3K/Akt. Secara keseluruhannya, kajian kami telah membuktikan bahawa α-tomatine adalah ejen semulajadi yang berkesan untuk merencat pertumbuhan kanser prostat bebas androgen dan juga dapat meningkatkan keberkesanan rawatan paclitaxel apabila digunakan secara kombinasi dengan α-tomatine. Penggunaan α-tomatine di dalam aplikasi klinikal kanser prostat perlu terus diterokai selanjutnya.

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Akt	Protein kinase B
AP-1	Activated protein-1
AR	Androgen receptor
AREs	Androgen response elements
ARGs	Androgen responsive genes
ATCC	American Type Culture Collection
ANOVA	Analysis of variance
Bcl-2	B cell leukaemia-2
Bcl-xL	B cell leukaemia-x long
BPH	Benign prostatic hyperplasia
CI	Combination index
c-IAP1	Cellular inhibitor of apoptosis 1
c-IAP2	Cellular inhibitor of apoptosis 2
DBP	Dibenzo[a,l]pyrene
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMBT1	Deleted in malignant brain tumors 1
DMEM	Dulbecco Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EC_{50}	Half-maximal efficient concentration
ECGC	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal-transition
ERK	Extracellular signal-regulated kinases

FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluoresceinisothiocyanate
FKHR	Forkhead family of transcription factors
FOXM1	Forkhead box protein M1
GSK	Glycogen synthase kinase
GST	Glutathione S-transferase
HCS	High Content Screening
hsp	Heat-shock proteins
IGFR	Insulin-like growth factor receptor
IκB	Inhibitor of kappa B
IKK	IκBα kinase
IPCN	International Prostatitis Collaborative Network
kDa	kilodalton
KGF	Keratinocyte growth factor
LHRH	Luteinizing hormone releasing hormone
LPS	Lipopolysaccharide
MAB	Maximal androgen blockage
MAPK	Mitogen activated protein kinase
MEKK1	MAPK extracellular signaling-regulated kinase kinase-1
MMP	Matrix metalloproteinases
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide
NF-κB	Nuclear factor-kappa B
NIH	National Institute of Health

NSAIDs	Non-steroidal anti-inflammatory drugs
PAP	Prostatic acid phosphatase
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PDGFR	Platelet derived growth factor receptor
PDK	Phosphoinositide-dependent kinase
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PIN	Prostatic intraepithelial neoplasia
PIP2	PtdIns-3,4-P2
PIP3	PtdIns-3,4,5-P3
PKC-α	Protein kinase C-alpha
PSA	Prostatic-specific antigen
PVDF	Polyvinylidene fluoride
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma
REL	Reticuloendotheliosis
RPMI	Roswell Park Memorial Institute
RTCA	Real-time cell analyzer
RTKs	Receptor tyrosine kinases
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TAK1	TGF activated kinase 1
TGF-β	Transforming growth factor-beta

TMB	3,3,5,5 tetramethylbenzidine
TNF-α	Tumor necrosis factor-alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
TSC2	Tuberous sclerosis complex 2
US	United States
XIAP	X-linked inhibitor of apoptosis

LIST OF APPENDICES

INTRODUCTION

The prostate gland is an important organ of the male reproductive system. Its development, growth and differentiation is dependent on androgens. Prostate adenocarcinoma is a common prostate malignancy afflicting men. It is also one of most frequently diagnosed cancer in men worldwide (Jemal *et al.*, 2011). Most patients diagnosed with early stage localized prostate cancer can be cured by prostatectomy and radiation therapy. Nonetheless, a substantial fraction of patients with clinically localized prostate cancer will eventually experience tumor recurrence with metastasis after local therapy with surgery or radiation therapy (Boorjian *et al.*, 2012). Primary tumor that has extended beyond the prostatic capsule following surgery or radiotherapy is generally incurable (Felici *et al.*, 2012; Lassi & Dawson, 2009). Androgen deprivation therapy has been the mainstay of treatment for patients with advanced metastatic prostate cancer (Cannata *et al.*, 2012). Unfortunately, many patients eventually fail this therapy and progress to a stage where the tumor growth becomes unresponsive to hormonal ablation. This stage is termed as androgen-independent or castration-resistant prostate cancer**.** Androgen-independent prostate cancer is an incurable disease with a median overall survival of 16-18 months (Amaral *et al.*, 2012; Harris *et al.*, 2009). It progresses to local invasion of the seminal vesicles, lymph nodes metastasis and eventually develops metastatic bone disease. Progression of prostate cancer to androgen independence is a main barrier of treatment due to the complex molecular mechanisms underlying the evolution of androgen independence. Therefore, there remains an urgent need to find more efficacious treatments for patients with metastatic androgen-independent prostate cancer.

At present, treatment options for metastatic androgen-independent prostate cancer are limited. Systemic chemotherapy with docetaxel in combination with prednisone remains the first-line therapy for patients with symptomatic metastatic

androgen-independent prostate cancer, with response rates of approximately 50% (Dagher *et al.*, 2004). The main obstacles in treating androgen-independent prostate cancer with taxane-based chemotherapy are inherent toxicity associated with their use and short-lived survival benefit of approximately 2 to 3 months largely due to chemoresistance (Dagher *et al.*, 2004). Nonetheless docetaxel-based regimen produces a modest survival benefit in patients, but it is not a curative treatment approach and there is an enlarging subset of patients who exhibit disease progression following docetaxel treatment and require second-line therapy. Cabazitaxel is a novel semisynthetic taxane-based drug that has been approved in combination of prednisone as second-line therapy for patients with docetaxel-refractory disease (de Bono *et al.*, 2010; Pal *et al.*, 2010; Paller & Antonarakis, 2011). Still, the use of cabazitaxel is associated with substantial toxicity, primarily related to myelosuppression (de Bono *et al.*, 2010; Nightingale & Ryu, 2012). This highlights the pressing need to develop novel agents that can provide safer and more efficacious treatment to patients.

The use of phytochemicals in cancer therapy is gaining significant interest owing to their multitarget mechanism of actions and lack of substantial toxicity. A number of preclinical studies have demonstrated the effectiveness of bioactive phytochemicals against human prostate cancer both *in vitro* and *in vivo* with lesser toxicity on normal cells, such as lycopene (Tang *et al.*, 2005), resveratrol (Narayanan *et al.*, 2004; Wang *et al.*, 2008), genistein (Naik *et al.*, 1994; Suzuki *et al.*, 2002) and epigallocatechin gallate (ECGC) (Albrecht *et al.*, 2008; Brusselmans *et al.*, 2003; Lee *et al.*, 2008; Luo *et al.*, 2010). Some of these are being evaluated in clinical trials for prostate cancer treatment (Russo *et al.*, 2010). These phytochemicals function as chemotherapeutic agents by interfering with multiple signaling pathways aberrant in prostate cancer. Moreover, bioactive phytochemicals also synergize with conventional anticancer drug to improve cancer therapeutic efficacy, but reduce the toxic side effects

on normal cells and delay resistance onset. This highlights the promising approach of using phytochemicals for treatment of human prostate cancer.

Alpha (α)-tomatine is the major saponin in tomatoes (*Lycopersicon esculentum*). Previous investigations have reported its cytotoxic effect on different types of human cancer cells (Choi *et al.*, 2012; Friedman *et al.*, 2009; Lee *et al.*, 2004), as well as its anti-metastasis mechanism on lung cancer and breast cancer cells *in vitro* (Shi *et al.*, 2012; Shieh *et al.*, 2011; Shih *et al.*, 2009). However, the therapeutic effect and molecular mechanism of α-tomatine on androgen-independent prostate cancer remain unknown. Issues that require further clarification are whether α-tomatine targets the highly aggressive and invasive phenotype of androgen-independent prostate cancer cells *in vitro* and *in vivo*, and if so what is the molecular target of α-tomatine, and finally does it have chemosensitizing effect on prostate cancer? This study first demonstrates the potent therapeutic effect of α-tomatine as single agent and in combination with paclitaxel against the highly aggressive human androgen-independent prostate cancer PC-3 cells *in vitro* and *in vivo*. This is followed by detailed investigations of the interference by α-tomatine of NF-κB and PI3K/Akt signaling pathways in prostate cancer. It is believed that data from the present study would deliver important insights into therapeutic potential of α-tomatine for the treatment androgen-independent prostate cancer to warrant further clinical investigations.

CHAPTER 1

LITERATURE REVIEW

1.1 The human prostate gland

1.1.1 Overview

The prostate is a large sex gland found only in the male reproductive system. It is approximately the size of a walnut and is located below the bladder and in front of the rectum. The gland surrounds the ejaculatory ducts at the base of urethra. The main function of prostate gland is to secrete seminal fluid that nourishes and protects sperm cells. It is composed of both glandular tissue that produces prostatic secretion and muscle tissue that helps in male ejaculation (Amin *et al.*, 2010).

1.1.2 Macroscopic anatomy of prostate gland

The prostate is made up of anterior, posterior, lateral and median lobes. McNeal (1981) has defined four anatomically and clinically distinct zones within the adult prostatic parenchyma: peripheral, central, transitional and periurethral zones (McNeal, 1981). These zones are distinguished by specific architectural and stromal features, as well as their position relative to the urethra. Both ducts and acini are lined by secretory epithelium in all the zones. The peripheral zone, an outermost part which consists of 70% part of normal prostate gland in an adult and comprises most of the glandular tissues. The central zone accounts for 25% of the normal prostate volume and surrounds the ejaculatory ducts, while the transition zone surrounds the urethra which comprise of 5% of the prostatic glandular tissue and contains the mucosal glands. The minor zone is the periurethral zone, which only consists of mucosal and submucosal glands. McNeal classification of prostate morphology is an important determinant of pathological condition because the prostatic intraepithelial neoplasia (PIN) and prostate adenocarcinoma predominantly arise in the peripheral zone of human prostate gland, whereas the transition zone is the place of origin of benign prostatic hyperplasia (BPH) (McNeal, 1988a, 1992).

1.1.3 Histology of prostate gland

Microscopically, prostate gland consists of two compartments: (1) a surrounding connective tissue, stroma, and (2) epithelial compartment which includes the exocrine glands with their associated ductal structures. Stromal-epithelial interaction via paracrine mechanism is crucial in human normal prostate morphogenesis (Cunha *et al.*, 1987). The stromal layer is composed of extracellular matrix, fibroblasts, lymphocytes, smooth muscle cells and neuromuscular tissues (Coffey, 1992). Stromal cells play a role in regulating the growth and function of epithelial cells by producing growth factor such as keratinocyte growth factor (KGF) (Cunha *et al.*, 2004).

The prostate epithelial cell compartment consists of three cell types: basal cells, luminal cells, and neuroendocrine cells. Androgens including testosterone and dehydroepiandrosterone (DHEA) are essential for proper growth and differentiation of human prostatic epithelium cells during development. Basal cell compartment represent a population of undifferentiated and proliferating cells which forms a continuous layer along basement membrane of each prostatic duct (Bonkhoff & Remberger, 1996; McNeal, 1988b). Basal cells are characterized by their expression of cell surface marker CD44, p53 superfamily member p63, cytokeratins 5 and 14 (Brawer *et al.*, 1985; Liu *et al.*, 1997; Sherwood *et al.*, 1990; Signoretti *et al.*, 2000). It is believed that the presence of pluripotent stem cell population within the basal cell compartment gives rise to terminally differentiated luminal cells and neuroendocrine cells (McNeal, 1988b; Xue *et al.*, 1998). Luminal cell is a fully differentiated secretory cell which represents the predominant cell type within the prostate epithelium (McNeal, 1988b). It forms a layer above basal cells. Secretory luminal cells represent the exocrine compartment of prostate which are responsible to produce prostate-specific secretory proteins including prostatic-specific antigen (PSA) and prostatic acid phosphatase (PAP). Unlike in basal cells, secretory luminal cells express androgen receptor, cell surface marker CD57,

cytokeratins 8 and 18 (Brawer *et al.*, 1985; Lamb *et al.*, 2010; Liu *et al.*, 1997; Sherwood *et al.*, 1990). As these cells express high levels of androgen receptor, therefore they rely on androgens for their survival. Neuroendocrine cells are the androgen-insensitive cells which constitute a relatively minor population within prostatic acini, and scattered throughout the basal layer (McNeal, 1988b). At the molecular level, neuroendocrine cell expresses serotonin, chromogranin A and various peptide hormones with potential growth modulating properties. Immunohistochemical analysis of keratins revealed that prostate cancer is predominantly composed of secretory luminal cells with dispersed neuroendocrine cells. However, several studies have also identified the existence of basal phenotype of androgen-independent intermediate amplifying cell population in androgen-independent prostate cancer cells (DU145 and PC-3) and also within hormone-escaped prostate tumors (van Leenders *et al.*, 2001). It is believed that both basal progenitor and luminal cells can be oncogenically transformed to give rise to prostate tumors (Choi *et al.*, 2012; Taylor *et al.*, 2012).

1.2 Prostate adenocarcinoma

1.2.1 Overview of prostate abnormalities

All men are at risk of prostatic problems, ranging from simple infection to cancer. Every man past of the age of 50 are advised to perform yearly prostate gland examination. The three most common prostatic problems are inflammation, enlargement and cancer.

Prostatitis is an inflammation disease that occurs more often in men younger than 50 years of age, which can be a result of bacterial or nonbacterial infections (Stamey, 1980). In 1998, The International Prostatitis Collaborative Network (IPCN) organized by National Institute of Health (NIH) has documented the classification

system of prostatitis syndromes (Table 1.1). In general, bacterial prostatitis is characterized by positive cultures of urine or prostatic secretions, presence of inflammatory cells in prostatic secretions and symptoms of urinary tract infections. Non-bacterial prostatitis occurs in men with no history of urinary tract infection and negative bacterial cultures of urine and prostatic fluid.

Table 1.1 NIH Prostatitis Classification System (adapted from Krieger *et al.*, 1999)

Category	**Type**
I	Acute bacterial prostatitis
II	Chronic bacterial prostatitis
III IIIA IIIB	Chronic prostatitis/ Chronic pelvic pain syndrome Inflammatory Non-inflammatory
IV	Asymptomatic inflammatory prostatitis

Benign prostatic hyperplasia (BPH) is a non-malignant enlargement of prostate gland which usually occurs in aging males overs 50 years of age. This abnormality is due to hyperplastic changes of the epithelial and stromal cells in the transition zone of prostate gland (Lepor, 2005). It is characterized by nodules of glandular and stromal hyperplasia, as well as diffused non-nodular enlargement (Laczko *et al.*, 2005). Senescence of prostate cells and age-related androgenic change which affect the prostate cell growth are the main factors that contribute to the development of BPH in aging males (Castro *et al.*, 2003; Colombel *et al.*, 1998; Zhang *et al.*, 2006).

Prostate adenocarcinoma is the most common malignancy afflicting men at present. It is a slow growing malignancy that is diagnosed almost exclusively in men over 50 years of age. This malignant tumor arises from glandular epithelium, and hence termed adenocarcinoma (adeno = gland) (Hill & Tannock, 1992; Pierce, 1998). As in

the case of normal prostate development, prostate cancer cells also depend on androgens for growth and survival, and thus androgen deprivation therapy using chemical or surgical castration is the first-line of therapeutic intervention for androgen-dependent tumors. Initial response to hormonal manipulation is favorable with a significant decline in prostate-specific antigen (PSA) levels in most of patients. Unfortunately, remission induced by hormonal treatment is usually short-lived (Singer *et al.*, 2008). Malignancy eventually progresses to metastatic phase and develops resistance to further hormonal manipulation in most patients within 14-30 months after the initiation of therapy (Singer *et al.*, 2008). The progression of prostate tumor to metastasis malignancy is accompanied by elevated serum PSA levels despite castration of the levels of serum testosterone and this is termed metastatic androgen-independent or castration-resistant prostate cancer.

Transition of androgen-dependence to metastatic androgen-independence disease is usually provoked by androgen deprivation therapy, and secondary hormonal manipulation remains as the palliative benefit for patients but this clinical benefit is usually short-lived. In most cases, aggressive malignancy advances to local invasion of the seminal vesicles, lymph nodes metastasis and eventually develops metastatic bone disease which can be deadly. Today, progression of the disease to the metastatic androgen-independent state is the primary reason for prostate cancer-related deaths.

1.2.2 Epidemiology of prostate cancer

According to Global Cancer Statistics, prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in men worldwide in 2008 (Jemal *et al.*, 2011). There is more than 25-fold difference in the worldwide incidence of prostate cancer, with the highest rate recorded primarily in developed countries compared to developing countries (Jemal *et al.*, 2011).

Epidemiology data suggests that increasing age, race and family history of the disease are the only well-established risk factors that contribute to the tumorigenesis of this heterogeneous disease. Approximately 97% of all prostate cancer cases occur in men 50 years of age and older, and 60% of them are 65 years of age and older. The highest prevalence rate in the world is observed in males of African descent in the Caribbean region (Bock *et al.*, 2009; Miller *et al.*, 2003). Asian countries typically have lower prevalence rates compared to Western countries (United States and Europe).

According to National Cancer Registry Report 2007, prostate cancer is the fourth most common cancer in Malaysian males, it accounts for 6.2 % of cancer cases in Malaysian males with the Chinese recording the highest incidence of prostate cancer compared to Malay and Indian (Omar & Tamin, 2011). The incidence of prostate cancer increases after the age of 45 years and 39.1 % of patients were diagnosed with stage IV prostate cancer in 2007 (Omar & Tamin, 2011).

1.2.3 Initiation, promotion and progression of prostate cancer

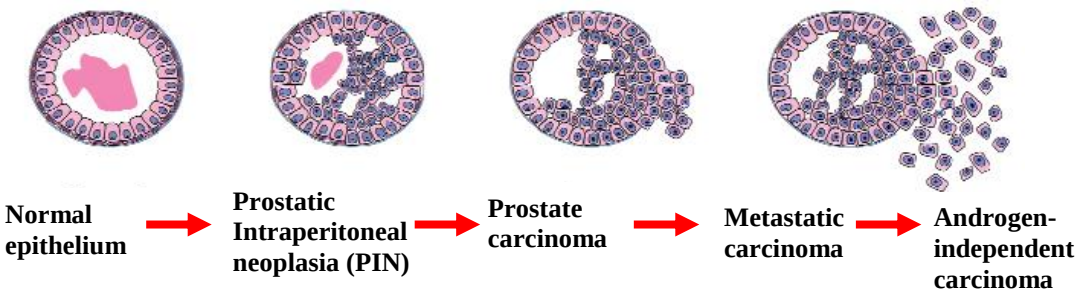


Figure 1.1 Pathway for human prostate carcinogenesis (adapted from Abate-Shen & Shen, 2000).

Prostate adenocarcinoma is a genetically and phenotypically heterogeneous disease. Prostate tumorigenesis is a multistage process involving cellular, biochemical and genetic alterations from an asymptomatic latent carcinoma to clinically metastatic prostatic malignancy. Over 95% of the prostate cancers are adenocarcinomas that arise from the epithelial lining of the prostate gland. Loss of normal glandular structure and destruction of basement membrane resulted from degradation of prostatic architecture occur during prostate tumorigenesis. Extensive studies have identified several important allelic losses of tumor suppressor genes and overexpressed oncoproteins associated with prostate carcinogenesis, and they are further discussed below.

Initiation and development of prostate cancer from a low-grade latent carcinoma to a high-grade metastatic malignancy arises from cellular, biochemical and genetic alterations. PIN is considered as a putative premalignant lesion for clinically significant prostatic carcinoma (Bostwick, 1989; De Marzo *et al.*, 2003; Epstein, 2009). It is composed of dysplastic cells with a luminal secretory cell phenotype, which expresses both PSA and androgen receptor (AR). Histological characteristic of PIN includes the appearance of luminal epithelial hyperplasia, reduction in basal cells, enlargement of nuclei and nucleoli, cytoplasmic hyperchromasia, and nuclear atypia (Bostwick, 1989).

PIN can be categorized into low-grade and high-grade based on the level of cell atypia (Ayala & Ro, 2007). The grade of PIN in prostate biopsy is strongly associated with susceptibility of epithelium cells to neoplastic transformation, and invasive prostate carcinoma (Bostwick & Qian, 2004; McNeal, 1989; McNeal & Bostwick, 1986). High-grade PIN has been increasingly implicated as precursor of early invasive prostate carcinoma (Bostwick, 1995; McNeal & Bostwick, 1986). The continuum from low-grade PIN to high-grade PIN and early invasive prostate carcinoma involves the progression of basal cell layer disruption, loss of secretory differentiation markers, nuclear abnormalities, increased microvessel density, variation in DNA content and allelic loss is implicated in initiation and progression of prostate cancer. Allelic loss of chromosome 8p12-21 (Chang *et al.*, 1994; Macoska *et al.*, 1995; Matsuyama *et al.*, 1994) and its potential candidate gene *NKX3.1*, a tumor suppressor gene is involved in the initiation of prostate carcinoma (Bhatia-Gaur *et al.*, 1999; He *et al.*, 1997). Loss of 8p12-21 has been observed in both PIN lesions and early invasive carcinoma, indicating its role in the initiation stage of prostate carcinogenesis. In addition, nuclear overexpression of MYC oncoproteins due to genomic alteration of chromosome 8q24 region is highly prevalent in both luminal cells of PIN and advanced prostate cancer, suggesting its involvement in the initiation and progression of human prostate cancer (Gurel *et al.*, 2008).

Deletion of a specific region of chromosome 10q and 13q in particular has been identified as one of the mechanisms of prostate cancer progression (Bergerheim *et al.*, 1991; Carter *et al.*, 1990; Cooney *et al.*, 1996; Li *et al.*, 1998; Melamed *et al.*, 1997). Phosphatase and tensin homolog (PTEN) gene maps to 10q23 and MXI1 (encodes a Myc-binding protein) gene maps to 10q24-q25 deletion on chromosome 10q are frequent events in the progression of prostate adenocarcinoma (Bubendorf *et al.*, 1999; Di Cristofano & Pandolfi, 2000; Gray *et al.*, 1995). Loss of heterozygosity at

chromosome 10q is less frequent in PIN lesions, but more frequently found in carcinoma compared to the loss of 8p. Loss of PTEN correlates with high Gleason score and advanced prostate cancer (McMenamin *et al.*, 1999). Therefore it is considered to be a later event in prostate cancer progression (Ittmann, 1996; Trybus *et al.*, 1996). PTEN functions as a negative regulator of Akt, loss of PTEN results in upregulation of Akt pro-survival signaling pathway which confers apoptosis-resistance phenotype to prostate cancer cells (Chen *et al.*, 2006).

Prostate cancer usually progresses to an androgen-independent, highly invasive malignancy with metastatic growth from an androgen-dependent, organ-confined disease. Indeed, analyses of human prostate cancer samples have shown that deletion of retinoblastoma (Rb) tumor suppressor gene that maps to chromosome 13q (Sharma *et al.*, 2010), loss of deleted in malignant brain tumors 1 (DMBT1) tumor suppressor gene (Du *et al.*, 2011), loss of heterozygosity at chromosome 17p deleted a locus for TP53 tumor suppressor gene (Bookstein *et al.*, 1993; Effert *et al.*, 1993), overexpression of forkhead box protein M1 (FOXM1) (Chandran *et al.*, 2007) and B cell leukeimia-2 (Bcl-2) (Fleischmann *et al.*, 2012; McDonnell *et al.*, 1997) are predominately associated with the transition to metastatic androgen-independent stage with poor clinical outcome.

Despite significant allelic losses within several tumor suppressor genes and overexpression of oncoproteins, deregulation of AR signaling and altered apoptotic regulatory genes have been implicated in the mechanism of development of androgen-independent prostate cancer. A majority of patients treated with androgen-ablation therapy ultimately develop androgen-ablation resistance with recurrence of highly aggressive and metastatic androgen-independent prostate cancer. Well-studied molecular processes contributing to aberrant AR signaling and progression of androgen-independent prostate cancer include AR gene amplification, mutations, overexpression

of AR, presence of constitutive AR splice variant, alteration in AR coregulator levels and crosstalk with other growth factor signaling pathways (Hu *et al.*, 2010).

1.2.4 Diagnosis and treatment of prostate cancer

Common diagnostic methods for prostate cancer are digital rectal examination, measurement of serum PSA concentration, transrectal ultrasound and biopsy (Pinthus *et al.*, 2007). PSA is a 34 kDa kallikrein-like serine protease secreted by epithelial cells of the prostate gland (Polascik *et al.*, 1999). PSA is present in small quantities in the serum of men with healthy prostates, but is often elevated in the presence of prostate cancer or other prostatic disorders.

Lack of sufficient sensitivity in detecting early stage of prostate cancer is the main disadvantage of PSA screening. PSA levels can also be increased by various physiological and benign conditions, such as urine retention, PIN, prostatitis, irritation, BPH, and recent ejaculation, giving a false positive result (Herschman *et al.*, 1997; Nadler *et al.*, 1995; Tchetgen & Oesterling, 1997). Hence, it cannot be used to reflect the presence of tumor accurately and has to be used in combination with other diagnostic techniques. Common diagnostic tests used in clinical practice to examine if cancer has spread within the prostate or to other parts of the body include radionuclide bone scan, magnetic resonance imaging, pelvic lymphadenectomy, seminal vesicle biopsy, and computed tomography scan.

Treatment options for prostate cancer are dependent on the stage of prostate cancer. Primary therapies such as watchful waiting, surgery (radical prostatectomy), radiation therapy (external beam radiation and brachytherapy) are used to treat early stage localized prostate cancer (Davidson *et al.*, 1996; Sailer, 2006). However, 30-40% of patients eventually develop recurrent or metastatic disease (Dillioglugil *et al.*, 1997). In 1941, Charles Huggins discovered that deprivation of androgen caused regression of

hormone-responsive metastatic prostate cancer, as prostate cell growth is dependent on androgen hormone (Huggins & Hodges, 1941). Since then, hormone ablation therapies achieved either surgically with bilateral orchiectomy or medically with luteinizing hormone releasing hormone (LHRH) agonists or antagonist, or oral anti-androgens to attain maximal androgen blockage (MAB) have become the frontline treatment for androgen-sensitive metastatic prostate cancer (Loblaw *et al.*, 2007). These hormonal manipulations dramatically suppress gonadal testosterone production, resulting in clinical remissions in the majority of patients (Bracarda *et al.*, 2005). Side effects or complications of these treatments include urinary and erectile difficulties that adversely impact the quality of life.

In androgen-independent metastatic prostate cancer or in the cases where androgen deprivation failed, effective treatment options remain limited. A vaccine-based immune therapy with Sipuleucel-T has been approved by the United States (US) Food and Drug Administration (FDA) for treatment of asymptomatic or minimally symptomatic metastatic androgen-independent prostate cancer in April 2010 (Kantoff *et al.*, 2010; Kawalec *et al.*, 2012). Modest survival advantage with 4.1 month improvement in median overall survival and 22% reduction in risk of death were observed in the phase III clinical trial of sipuleucel-T in patients with asymptomatic or minimally symptomatic metastatic androgen-independent prostate cancer (Kantoff *et al.*, 2010). For patients with symptomatic metastatic androgen-independent prostate cancer which is progressing rapidly, systemic chemotherapy is now considered as the standard of care in these patients. A taxane-based drug, docetaxel in combination with prednisone is the current standard of care in first-line palliative chemotherapy treatment for metastatic hormone-refractory prostate cancer (Petrylak *et al.*, 2004; Tannock *et al.*, 2004). Docetaxel is a semisynthetic derivative of paclitaxel. Both drugs are antimitotic agents, which impair the natural dynamics of microtubules and leading to mitotic block

and apoptosis (Jordan & Wilson, 2004). Chemotherapy with docetaxel regimen improves overall survival and effectively decreases PSA in patients suffering with advanced malignancy compared with mitoxantrone (Petrylak *et al.*, 2004; Tannock *et al.*, 2004). This regimen was approved by the US FDA in 2004. However, inherent toxicity associated with the use of docetaxel and short-lived survival benefit of approximately 2 to 3 months due largely to chemoresistance represent as treatment dilemmas (Chang, 2007; Dagher *et al.*, 2004). Febrile or non-febrile neutropenia, anemia and associated myelotoxicity are the dose-limiting adverse effects of docetaxel regimen that can severely affect the quality of life and consequent survival in elderly patients (Engels & Verweij, 2005). Moreover, there is an ever enlarging subset of patients who exhibit clinical disease progression following taxane-based chemotherapy, and second-line therapy is required to control tumor growth. In 2010, the US FDA has approved cabazitaxel, a second-generation semisynthetic taxane in combination with prednisone for treatment of patients whose disease progresses to standard docetaxel-based therapy (Bilusic & Dahut, 2011). It is the first approved chemotherapeutic drug which has shown an improvement in the overall survival benefit in the post-docetaxel setting (Bilusic & Dahut, 2011). Unfortunately, the use of cabazitaxel regimen in post-docetaxel population is also associated with undesired adverse reactions, including renal failure, neutropenia, leukopenia, anemia, febrile neutropenia, diarrhea, fatigue, and asthenia (Bilusic & Dahut, 2011). Other treatment options for androgen-independent disease include secondary hormonal therapy with abiraterone acetate which has been shown to improve overall survival in patients with progression of metastatic androgen-independent prostate cancer after docetaxel-based treatment (de Bono *et al.*, 2011). Abiraterone acetate, an oral inhibitor of androgen biosynthesis is designed to further inhibit androgen-mediated signaling. It has been approved by US FDA for use in combination with prednisone for treatment in chemo-naive patients and also docetaxel-

refractory patients. However, the survival benefit of this treatment approach for patients is modest and it should be noted that the use of abiraterone acetate can be associated with substantial toxicities (de Bono *et al.*, 2011). Therefore, continued efforts are being focused on development of newer therapeutic agents offering efficacious and safe therapeutic treatment for patients with advanced prostate cancer, either use in frontline chemotherapeutic setting or perhaps in combination with taxane-based regimen.

1.3 Potential therapeutic targets for prostate cancer intervention

1.3.1 Overview

Over the last decade, targeted therapies have emerged as a new and potential strategy for cancer treatment. The hallmarks of cancer are composed of six essential biological capabilities for development of human cancer (Hanahan & Weinberg, 2011). These include sustaining proliferative signaling, resisting cell death, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality and inducing angiogenesis (Hanahan & Weinberg, 2011). Each hallmark is regulated by a number of parallel signaling pathways. Understanding the key signaling pathways implicated in cancer growth and progression is important for the development of mechanism-based targeted therapeutics.

Signaling pathways of great importance are androgen-receptor (AR), nuclear factor-kappa B (NF-κB), and phosphoinositide 3-kinase (PI3K), because deregulation of these signaling pathways frequently occur during prostate cancer progression. Their key roles in the transition of prostate cancer to metastatic androgen-independent state and their therapeutic interventions are further discussed in the following section.

1.3.2 Androgen receptor (AR)

Androgen receptor (AR) is a 110 kDa nuclear receptor, a member of steroid hormone receptor transcription factor family that mediates the actions of androgens (Lindzey *et al.*, 1994). It is composed of three major domains: an N-terminal transcriptional activation domain, a central DNA-binding domain, and a C-terminal steroid-binding domain, localized in the cytoplasm of stromal and secretory epithelial cells (Chatterjee, 2003). AR signaling is important for the development and function of male reproductive organs, including the prostate and epididymis (McPhaul, 2002; Yeh *et al.*, 2002). It is bound to heat-shock proteins (hsp) such as hsp56, hsp70 and hsp90 during inactive state (Yeh *et al.*, 1999). Upon activation by androgens such as testosterone and dihydrotestosterone (DHT), the heat shock proteins dissociate and release AR. AR then translocates into the nucleus, binds to the consensus sequence of androgen response elements (AREs) (Roche *et al.*, 1992), and activates androgen responsive genes (ARGs) involved in diverse biological processes such as proliferation, differentiation, apoptosis, metabolism and secretion (Nelson *et al.*, 2002).

Importantly, AR signaling plays pivotal roles in the prostate carcinogenesis and progression to androgen-independent state, where at this point tumor becomes unresponsive to androgen ablation therapy. Overexpression of AR due to amplification of AR gene is one of the potential mechanisms that has been proposed to explain persistence AR signaling in androgen-independent prostate cancer, as about 30% of androgen-independent prostate cancer have demonstrated an increase in the expression of AR gene (Chen *et al.*, 2004; Koivisto *et al.*, 1997; Linja *et al.*, 2001; Suzuki *et al.*, 2003; Visakorpi *et al.*, 1995). This leads to constitutive activation of the receptor and increases the sensitivity of tumor cells to very low levels of androgens that are produced by the adrenal glands. A recent report also suggests that AR hyperactivation due to Rb depletion significantly contributes to the androgen-independent prostate cancer

transition (Sharma *et al.*, 2010). Mutation in AR gene also allow it to be stimulated by other ligands such as anti-androgens and oestrogens, thereby contributing to androgen-refractory prostate tumor growth (Culig *et al.*, 1993; Gottlieb *et al.*, 2012; Peterziel *et al.*, 1995; Taplin *et al.*, 1995). In addition, several studies have reported that the presence of crosstalk of AR transactivation with other key growth factor signaling events, such as epidermal growth factor receptor (EGFR), mitogen activated protein kinase (MAPK) and mitogen-activated/extracellular signal-regulated kinase kinase 1 (MEKK1), PI3K and NF-κB pathways in the absence of androgen contributes to progression of prostate cancer (Abreu-Martin *et al.*, 1999; Bonaccorsi *et al.*, 2004; Culig, 2004; Culig *et al.*, 2005; Lee *et al.*, 2005; Peterziel *et al.*, 1999). Other documented factors that drive aberrant AR signaling and leads to the progression of androgen-independent prostate cancer are the presence of constitutive AR splice variant (Sun *et al.*, 2010), prostate cancer stem cells (Collins *et al.*, 2005) and the alteration in the expression of coregulators of androgens involved in the regulation of androgen receptor-driven transcription (Comuzzi *et al.*, 2004; Culig *et al.*, 2004).

Current androgen-ablation therapies which suppress AR signaling pathway either by blocking androgen synthesis or blocking androgenic effects have been the cornerstone of treatment for men with metastatic prostate cancer. While initial clinical responses to androgen-ablation therapies are favorable, a vast majority of patients with advanced tumors eventually develop androgen-independent prostate cancer which carries a much poorer prognosis. The resistance to androgen-ablation therapy acquired by tumor cells during androgen deprivation is one of the major challenges in the management of prostate cancer. Several second-line hormone manipulations with agents such as antiandrogens (flutamide, bicalutamide and nilutamide), ketoconazole and abiraterone acetate have been utilized for treatment of metastatic androgen-independent prostate cancer. However, the survival benefit of these treatment approaches for patients

are modest and their responses are usually associated with toxicities. Clearly, discovery of more effective AR inhibitors that will improve clinical outcome for prostate cancer is highly desired.

1.3.3 Nuclear factor-kappa B (NF-κB)

Aberrant regulation of nuclear-factor kappa B (NF-κB) is implicated in the development and perpetuation of a variety of human ailments including autoimmune disorders, cancer, pulmonary, cardiovascular, neurodegenerative, and inflammatory diseases (Ahn *et al.*, 2007; Boissiere *et al.*, 1997; Collister & Albensi, 2005; Sur *et al.*, 2008). Accumulating evidence suggests that the transcription factor NF-κB plays a pivotal role in prostate cancer growth, survival, angiogenesis and metastatic progression (Huang *et al.*, 2001; Surh *et al.*, 2002). NF-κB is an ubiquitous transcription factor that controls the expression of genes involved in diverse biological processes. It regulates the transcriptional activity of over 300 genes involved in growth regulation, immunoregulation, apoptosis, inflammation and carcinogenesis (Sethi & Tergaonkar, 2009). It was discovered as a factor that binds to the promoter of the κ chain of immunoglobulins in B cells (Sen & Baltimore, 1986, 2006). Its activation is stimulated by a divergent of stimuli including pro-inflammatory cytokines, bacterial and viral proteins, carcinogens, tumor promoters, stress, lipopolysaccharide (LPS), chemotherapeutic drugs and ionizing radiation through a wide variety of pathways (Anto *et al.*, 2002; Banerjee *et al.*, 2002; Chen *et al.*, 2002).

In mammalian cells, NF-κB1 (also known as p50 and its precursor is p105), NF-κB2 (also known as p52 and its precursor is p100), Reticuloendotheliosis (REL) A (also known as p65), RELB and c-REL are the five identified NF-κB family members, all of which can form homo- and heterodimeric complexes (Ghosh *et al.*, 1998; Hayden & Ghosh, 2008). Under resting condition, NF-κB dimers are sequestered in cytoplasm and

prevented from DNA binding through the interactions with inhibitor of kappa B (IκB) proteins consisting of IκBα, IκBβ, IκBγ, IκBε and BCL-3 proteins (Gilmore, 2006; Perkins, 2007; Tergaonkar, 2006).

Numerous stimuli activate NF-κB through IκB kinase (IKK)-dependent pathway. The IKK complex consists of 2 catalytic subunits, IKKα and IKKβ, and a non-enzymatic regulatory subunit, IKKγ/ NEMO (Perkins, 2007; Scheidereit, 2006). The signaling of NF-κB is generally mediated through either the canonical (classical) and or non-canonical (alternative) pathway**.** The classical pathway is most widely implicated in human cancer because it is involved in the inhibition of programmed cell death under most conditions (Karin & Delhase, 2000; Karin & Lin, 2002), whereas the non-canonical pathway is crucial for development of secondary lymphoid organs, survival and maturation of premature B cells (Bonizzi & Karin, 2004). In the classical pathway, NF-κB signaling is triggered in response to pro-inflammatory cytokines (e.g.: tumor necrosis factor-α, interleukin-1, pathogen-associated molecular patterns) and microbial or viral infections that activate IKKα /IKKβ heterodimers, leading to phosphorylation of IκB proteins at two crucial serine residues, followed by polyubiquitination by the SCF $^{\beta TrCP}$ (Skp1-Cul1-F box protein) E3 ubiquitin-ligase and degradation of IκB proteins by 26S proteasome (Fuchs *et al.*, 1999; Fuchs *et al.*, 2004; Krappmann & Scheidereit, 2005). This proteolysis allows nuclear translocation of classical NF-κB (p50/RELA), where the free NF-κB dimers function as a transcription factor that induce the expression of proinflammatory cytokines, chemokines, and factors for cell proliferation and survival (Hoffmann & Baltimore, 2006). A heterodimer of RelA and p50 is the most common combination in the canonical NF-κB signaling pathway.

NF-κB can affect all six hallmarks of cancer as described by Hanahan and Weinberg (2011) through the transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis, tumor promotion, inflammation, and suppression

of apoptosis (Basseres & Baldwin, 2006; Burstein & Duckett, 2003; Dutta *et al.*, 2006; Luo *et al.*, 2005). Of great importance, constitutive NF-κB activation has been observed in androgen-independent prostate carcinoma cell lines and the degree of nuclear localization of NF-κB p65 correlated with tumor grade (Fradet *et al.*, 2004; Surh *et al.*, 2002). Activation and localization of NF-κB represent independent risk factors for disease recurrence after radical prostatectomy (Domingo-Domenech *et al.*, 2005; Fradet *et al.*, 2004). Consistently, it has been reported that aberrant IKK activation leads to the constitutive activation of the NF-κB survival pathway in androgen-independent prostate cancer cells (Gasparian *et al.*, 2002). Mutation of inhibitory protein IκBα (Wood *et al.*, 1998), increased level of pro-inflammatory cytokines (O'Connell *et al.*, 1995) and proteosomal activity (Miyamoto *et al.*, 1994) are observed in other types of human cancer with constitutively active NF-κB. In addition, NF-κB appears to mediate transforming growth factor-beta (TGF-β)-induced epithelial-to-mesenchymal-transition (EMT), which is a key process involved in metastatic prostate cancer (Zhang *et al.*, 2009). Hence, it is believed that constitutive NF-κB activation is one of the molecular factors involved in the transition toward metastatic androgen-independent prostate cancer.

Owing to the ability of NF-κB to govern the expression of numerous genes involved in various human physiologies, targeting NF-κB signaling offers an attractive approach for therapeutic development. At present, more than 700 inhibitors of this transcription factor have been described, including natural agents, peptides, synthetic molecules, anti-inflammatory or immunosuppressive agents, viral and microbial proteins (Gupta *et al.*, 2010). NF-κB signaling can be suppressed by targeting various steps of the pathway including upstream signaling of IKKs, IKKs, ubiquitination step, methylation step, nuclear translocation step and DNA binding step. Some molecules

target one specific step leading to NF-κB inactivation, while there are several inhibitors that target multiple steps in the signaling.

To date, NF-κB inhibitors such as are non-steroidal anti-inflammatory drugs (NSAIDs), cyclosporine A and corticosteroids are currently used for treating inflammatory conditions. Unfortunately, these drugs are highly pleiotropic, lack specificity in attenuating NF-κB activity and consequently require high dosage, causing toxicity and adverse effects to patients. A number of clinical trials have been performed with several NF-κB inhibitors for cancer treatment but the most significant clinical data have so far been obtained with bortezomib. Bortezomib is a proteasome inhibitor which was approved by the US FDA in 2003 for second-line therapy of patients with progressive multiple myeloma (Kane *et al.*, 2003). It is a selective inhibitor of the 20S proteasome (Adams *et al.*, 1999), that inhibits the NF-κB signaling by preventing IκBα degradation, and possesses potent anti-tumor activities against various human cancers (Lenz, 2003). The clinical efficacy of bortezomib for treatment of cancer has been thoroughly investigated in Phase I, II and III clinical trials. However, the use of bortezomib is hampered by its severe adverse side effects, unsatisfactory efficacy in treatment of solid tumors and development of drug resistance (Chen *et al.*, 2011). Nevertheless emerging evidences have clearly indicated that NF-κB inhibition is a promising therapeutic strategy for treatment of prostate cancer, and this warrants further efforts to discover highly specific but less toxic NF-κB inhibitors for the treatment of prostate cancer.

1.3.4 Phosphoinositide 3-kinase (PI3K)/Protein kinase B (Akt)

PI3K is a heterodimeric enzyme which plays a central role in several critical cellular processes for cancer survival, growth, metabolism and motility. In mammalian cells, there are multiple isoforms of PI3K and they are subdivided into three classes based on the difference in structure and function. Class I_A PI3Ks is most widely implicated in human cancer (Courtney *et al.*, 2010; Yuan & Cantley, 2008), and is composed of a 110 kDa (p110) catalytic subunit that confers enzyme activity, and a 85 kDa (p85) regulatory subunit (Carpenter *et al.*, 1990). PIK3CA, PIK3CB, and PIK3CD encode p110α, p110β, and p110δ, respectively, which represent the catalytic isoforms (Engelman *et al.*, 2006). The regulatory isoforms, p85α, p85β, and p55δ, are the products of three genes, PIK3R1, PIK3R2, and PIK3R3 (Engelman *et al.*, 2006). The activation of class I_A PI3Ks by growth factor is mediated through receptor tyrosine kinases (RTKs) (Skolnik *et al.*, 1991; Zhao & Vogt, 2008). Examples of RTK include platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), and insulin-like growth factor receptor (IGFR). In response to ligand binding, the p85 regulatory subunit directly binds to phosphotyrosine residues on RTKs, the binding relieves its inhibitory effect on p110 catalytic subunit (Cuevas *et al.*, 2001). Ras oncoprotein (Shaw & Cantley, 2006) and G-protein coupled receptors (Katso *et al.*, 2001) have also been shown to directly activate p110 action. p110 catalytic subunit catalyzes the production of PtdIns-3,4-P2 (PIP2) and PtdIns-3,4,5-P3 (PIP3) that function as second messenger to recruit Akt and phosphoinositide-dependent kinase (PDK) to the plasma membrane (Auger *et al.*, 1989; Cantley & Neel, 1999; Carpenter *et al.*, 1990; Whitman *et al.*, 1988). Akt is phosphorylated and activated in the presence of PDK1 and PDK2. Activated Akt conveys signals through phosphorylating numerous substrates involved in the regulation of cell survival, proliferation and growth (Chan *et*

al., 1999; Duronio *et al.*, 1998). Phosphatase and tensin homolog (PTEN), a tumor suppressor gene terminates PI3K signaling by dephosphorylating PIP3 to PIP2.

Anti-apoptotic mechanism of activated Akt is multifactorial as it directly phosphorylates several substrates involved in cell death. Foremost among these downstream target substrates of Akt are BAD and NF-κB. BAD is one of pro-apoptotic Bcl-2 family members, which promotes cell death by forming a heterodimer with the anti-apoptotic Bcl-2 family member Bcl-xL. Activated Akt inhibits apoptosis cell death by phosphorylating and inactivating BAD, causing its dissociation from the Bcl-xL, and in turn, restores anti-apoptotic function of Bcl-xL (Datta *et al.*, 1997; del Peso *et al.*, 1997; Gross *et al.*, 1999). Similarly, activation of Akt by growth signals can phosphorylate and activate NF-κB signaling through IKK-dependent pathway, which in turn transactivates a number of genes involved in cellular proliferation and apoptosis (Romashkova & Makarov, 1999). Furthermore, it has been shown that PI3K/Akt/NF-κB signaling mediates the process of chemoresistance and radioresistance in various tumor cells, as several genes such as cyclin D1, COX-2, Bcl-2, Bcl-xL, XIAP, and survivin are NF-κB effector genes (Catz & Johnson, 2001; Guttridge *et al.*, 1999; Stehlik *et al.*, 1998; Tamatani *et al.*, 1999; Yamamoto *et al.*, 1995; Zhu *et al.*, 2001). Other Akt target substrates are pro-apoptotic protease caspase-9 and Forkhead family of transcription factors (FKHR), where phosphorylation of these substrates attenuates their pro-apoptotic activities (Brunet *et al.*, 1999; Cardone *et al.*, 1998). Apart from its role in apoptotic machinery, Akt also affects cell growth and proliferation. Akt phosphorylates glycogen synthase kinase-3 beta (GSK-3β) on serine 9, thereby inactivating its kinase activity on cyclin D1 and resulting in cell proliferation (Diehl *et al.*, 1998). Expression of cyclin kinase inhibitors such as p27kip1 and p21Cip/ WAF1 are also negatively regulated by activated Akt (Graff *et al.*, 2000; Li *et al.*, 2002). Mammalian target of rapamycin (mTOR), a serine/threonine kinase which is a downstream effector of Akt is

involved in cell growth (an increase in cell size and mass). Activated Akt promotes the growth function of mTOR by direct phosphorylation inhibiting tuberous sclerosis complex 2 (TSC2), a negative regulator of mTOR (Inoki *et al.*, 2002). Activation of mTORC1 integrates the signals from nutrients and growth factors to initiate cell growth by regulating mRNA translation, ribosome biogenesis, autophagy and metabolism (Kunz *et al.*, 1993; Sarbassov *et al.*, 2005; Wullschleger *et al.*, 2006).

Deregulation of PI3K/Akt pathway is one of the most frequent occurrences in human cancers including prostate cancer (Kreisberg *et al.*, 2004). A study reported that Akt and AR act synergistically to promote the initiation and progression of prostate cancer *in vivo*, and cause the resistance to androgen ablation therapy (Xin *et al.*, 2006). To date, the main documented mechanisms of constitutive PI3K/Akt activation observed in human cancers are genetic mutation and amplification of the specific key components of the signaling and activation by RTKs (Engelman, 2009; Rodon *et al.*, 2013). Mutation in tumor suppressor gene PTEN which functions as a negative regulator of PI3K is commonly observed in prostate cancer samples (Cairns *et al.*, 1997; Komiya *et al.*, 1996; McMenamin *et al.*, 1999; Rodon *et al.*, 2013). A recent study revealed that sustained PI3K activity in PTEN-deficient prostate cancer cells (PC-3 and LNCaP) is mediated through constitutively active form of p110, and is independent of RTK and Ras (Jiang *et al.*, 2010). Studies have shown that prostate epithelium with homozygous deletion of PTEN potentially develop aggressive prostate carcinoma (Trotman *et al.*, 2003; Wang *et al.*, 2003). Indeed, complete loss of PTEN is observed at highest frequencies in androgen-independent prostate cancer accompanied with higher Gleason scores (Bertram *et al.*, 2006; Dreher *et al.*, 2004; Li *et al.*, 1997; McMenamin *et al.*, 1999; Sansal & Sellers, 2004; Schmitz *et al.*, 2007), and is correlated with the incidence of development of lymph node metastases (Schmitz *et al.*, 2007).

To date, a number of potent PI3K pathway inhibitors with improved pharmacological properties have been investigated intensively in both *in vitro* and *in vivo* models of prostate cancer. The most well-studied PI3K pathway inhibitors are LY294002 (Renner *et al.*, 2007), GSK690693 (Rhodes *et al.*, 2008), wortmannin (Powis *et al.*, 1994; Seol *et al.*, 2005), celecoxib (Narayanan *et al.*, 2006; Patel *et al.*, 2005; Zheng *et al.*, 2007), and perifosine (Chee *et al.*, 2007; Posadas *et al.*, 2005). So far only perifosine and celecoxib have been investigated in the clinical settings for treatment of prostate cancer. However, these agents have limited use in clinical practice because they lack specificity in blocking the PI3K/Akt and thus cause devastating side effects to patients. Therefore, there remains a pressing need to discover new, more efficient and less toxic PI3K pathway inhibitor for treatment of prostate cancer.

1.4 Phytochemicals for cancer treatment

1.4.1 Overview

Positive therapeutic outcomes of conventional cancer therapies such as surgery and chemo- and radiotherapy are often limited due to high toxicity and related side-effects, as well as the development of multi-drug resistance. Therefore, there is a pressing need for new therapeutic strategies to ameliorate treatment efficacy but minimize toxicity to normal tissues.

Increasing evidence suggest that phytochemicals with distinct anticancer activity could be considered as potential cancer chemotherapeutic agents (Cragg & Newman, 2009). Phytochemicals are typically secondary metabolites produced by plants for specific functions. They are not essential for normal growth, development and reproduction of normal plant cells, but play a role in protecting the plant against any harm in ecological environment. Phytochemicals are of particular interest for use in preventing cancer prevention or treatment because of their potentially low toxicity

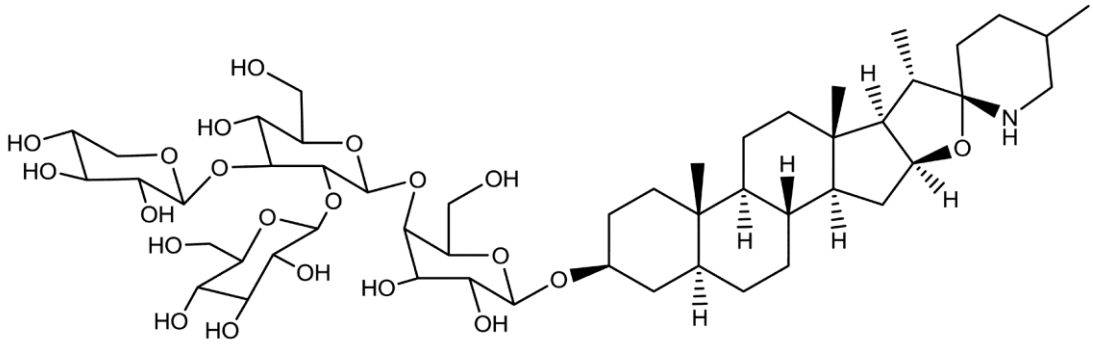
profiles, thus they could be administrated on a long-term basis to either prevent primary tumor formation or tumor growth without adversely affecting the normal tissues (Aggarwal *et al.*, 2007). To date, numerous preclinical studies have identified a number of potential plant-derived agents with lesser toxicity as effective anticancer agents *in vitro* and *in vivo*, such as isothiocyanates, resveratrol, curcumin, sulforaphane, lycopene, genistein and epigallocatechin gallate (ECGC) (Russo *et al.*, 2010). Anti-carcinogenic studies of these plant constituents showed that these phytochemicals either block tumor initiation or suppress tumor growth during the promotion stage of cancer development by modulating signals transduction essential for carcinogenesis. Furthermore, some of these bioactive phytochemicals such as genistein, curcumin, ECGC and lycopene have been shown to improve cancer therapeutic efficacy in association with classical chemotherapeutic drugs and the combined treatment can delay resistance onset (Li *et al.*, 2005; Notarbartolo *et al.*, 2005; Stearns *et al.*, 2010; Stearns & Wang, 2011; Tang *et al.*, 2011).

Indeed, some of these phytochemicals have been evaluated in clinical trials for their efficacy in treating prostate cancer. The clinical data suggest phytochemicals represent good candidates for chemotherapeutic applications. For example, a mixture of green tea catechins was used in a chemoprevention trial on patients with premalignant lesion of prostate cancer (Bettuzzi *et al.*, 2006). The development of prostate cancer from high grade PIN (premalignant lesion of prostate cancer) was 3% in the experimental group and 30% in the placebo group following one year treatments (600 mg/day) (Bettuzzi *et al.*, 2006). A follow-up update to this study reported that the chemopreventive effect of green tea catechins was long-lasting (Brausi *et al.*, 2008). Nonetheless, two clinical studies have reported that green tea catechins have limited benefits in patients with androgen-independent prostate cancer, and the outcomes from these clinical trials do not merit further investigations (Choan *et al.*, 2005; Jatoi *et al.*,

2003). Clinical trials of lycopene conducted on the patients with localized prostate cancer show that lycopene supplementation reduced tumor growth and plasma PSA levels in the intervention group compared with the control group (Kucuk *et al.*, 2002). However, lycopene has also been shown to have unfavorable responses for patients with androgen-independent prostate cancer and it also causes gastrointestinal side effects (Ansari & Gupta, 2004; Jatoi *et al.*, 2007; Schwenke *et al.*, 2009). Other phytochemicals such as genistein (deVere White *et al.*, 2004; deVere White *et al.*, 2010; Kwan *et al.*, 2010; Lazarevic *et al.*, 2011; Pendleton *et al.*, 2008) and pomegranate (Paller *et al.*, 2013; Pantuck *et al.*, 2006) have also been clinically investigated in patients with localized or androgen-dependent prostate cancer, the outcome from these clinical trials suggest their positive effect in reducing serum PSA levels and/ or increase in the PSA doubling time. Despite some successful stories of using phytochemicals as potential treatment for androgen-dependent prostate cancer, there are rooms for the discovery of more bioactive phytochemicals for the development of effective drugs against aggressive form of androgen-independent prostate cancer. The continuing efforts to discover effective phytochemicals will certainly shed light on the improvement of androgen-independent prostate cancer and contribute to impressive therapeutic outcomes.

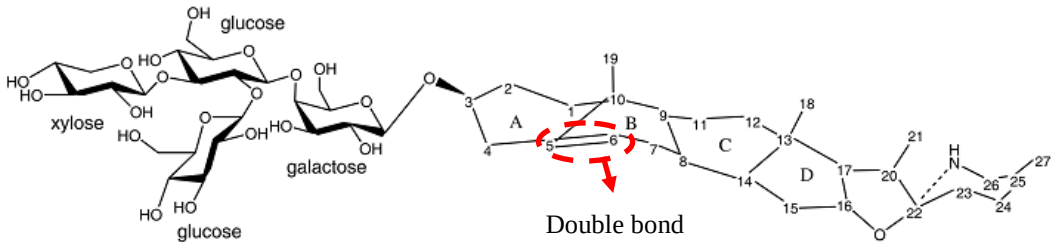
1.4.2 Alpha (α)-tomatine

1.4.2.1 Chemical structure of α-tomatine



Empirical formula: $C_{50}H_{83}NO_{21}$

Figure 1.2 Chemical structure of α-tomatine.



Empirical formula: $C_{50}H_{81}NO_{21}$

Figure 1.3 Chemical structure of dehydrotomatine (adapted from Friedman *et al.*, 2007).

Glycoalkaloid is one of the naturally occurring N-containing secondary plant metabolites in Solanaceous plant species such as egg-plants, potatoes, and tomatoes (*Lycopersicon esculentum*). It is produced by plants in defense against phytopathogens. Fontaine *et al*. (1948) first isolated glycoalkaloids from tomatoes, known as tomatine (Fontaine *et al.*, 1948). Tomatine comprises of ~10:1 mixture of alpha (α)-tomatine and dehydrotomatine, and is present in all parts of the tomato plant (Friedman, 2004; Friedman *et al.*, 1994; Kozukue *et al.*, 2004). Alpha-tomatine consists of an aglycon moiety (tomatidine), and a tetrasaccharide moiety (β-lycotetrose) that contains two molecules of D-glucose and one each of D-galactose and D-xylose (Figure 1.2). The

chemical structure of dehydrotomatine is different from α-tomatine, where dehydrotomatine has a double bond in the steroidal ring B of the aglycon (Figure 1.3). Immature green tomatoes contain up to 500 mg of α-tomatine/ kg of fresh fruit weight. The compound is partly degraded by enzyme synthesized or activated during fruit maturation. As the tomato ripens until at maturity level, there is an approximately 5 mg of α-tomatine/ kg of fresh fruit weight in mature red tomatoes (Friedman & Levin, 1995).

1.4.2.2 Biological activities of α-tomatine

Alpha-tomatine has been shown previously to possess beneficial role in some biological activities including anti-fungal (Ito *et al.*, 2007; Sandrock & Vanetten, 1998), immune-stimulation (Heal & Taylor-Robinson, 2010; Morrow *et al.*, 2004; Yang *et al.*, 2004) and cholesterol lowering activities (Friedman *et al.*, 2000).

Apart from these beneficial biological effects of α-tomatine, previous studies have shown that α-tomatine has *in vitro* cytotoxic effects against the growth of various human cancer cells, including colon cancer HT-29 (Choi *et al.*, 2012; Friedman *et al.*, 2009; Lee *et al.*, 2004), liver cancer HepG2 (Friedman *et al.*, 2009), stomach cancer AGS (Friedman *et al.*, 2009), gastric cancer KATO-III (Choi *et al.*, 2012), leukemia MOLT-4 (Kúdelová *et al.*, 2013), breast cancer MCF-7 (Friedman *et al.*, 2009) and MDA-MB-231 cells (Choi *et al.*, 2012). Based on data derived from *in vitro* cytotoxicity assay, the cytotoxic effect of α-tomatine against the cancer cells was stronger than dehydrotomatine (Friedman *et al.*, 2009) and its hydrolysis products (β_1-tomatine, γ-tomatine, δ-tomatine and tomatidine) (Choi *et al.*, 2010; Lee *et al.*, 2004). *In vivo* study by Friedman *et al.* (2007) showed that α-tomatine has protective effects against dibenzo[a,l]pyrene (DBP)-induced liver and stomach tumors in rainbow trout

without causing significant changes in total weight, liver weight, tissue morphology and mortality (Friedman *et al.*, 2007).

A more recent study reported that exposure of α-tomatine to human chronic myeloid leukemia K562 cell line and acute promyelocytic leukemia HL60 caused growth inhibition and apoptosis (Chao *et al.*, 2012) which is mediated through the release of apoptosis-inducing factor (AIF) from the mitochondria into the nucleus and down-regulated the expression of survivin expression (Chao *et al.*, 2012). The study also showed that α-tomatine significantly inhibited HL60 xenograft tumor growth without significant body weight loss in severe combined immunodeficiency (SCID) mice, accompanied by increased expression of apoptotic marker and decreased expression of survivin (Chao *et al.*, 2012).

In addition, a study has shown that glycoalkaloid α-tomatine inhibits invasion and migration of A549 cells at the sub-toxic concentration by reducing the activities of matrix metalloproteinase (MMP)-2 and MMP-9 (Shih *et al.*, 2009). Further molecular study suggests that anti-metastasis mechanism of α-tomatine on A549 cells is related to its suppression effects on PI3K/Akt, extracellular signal-regulated kinases (ERK) signaling pathways, and the inhibition on binding activities of NF-κB and activated protein-1 (AP-1) transcription factors (Shih *et al.*, 2009). In a similar study, Shieh *et al.* (2011) reported the anti-metastatic activity of α-tomatine on NCI-H460 cells by downregulation of MMP-7 expression. It is proposed that the mechanism of anti-metastasis effect occurs through the inactivation of focal adhesion kinase (FAK)/PI3K/Akt signaling pathway and reducing NF-κB DNA binding activity (Shieh *et al.*, 2011). A more recent study also revealed that α-tomatine suppressed the 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced invasion and migration by blocking the protein kinase C alpha (PKC-α)/ERK signaling followed by NF-κB dependent MMP-2/9 activation on breast cancer MCF-7 cells (Shi *et al.*, 2012). However, the mechanism

of inhibitory effect of α-tomatine on NF-κB DNA binding activity is not reported. These studies proposed that α-tomatine as an anti-metastatic agent against human lung and breast adenocarcinoma.

The present study aimed to investigate the therapeutic efficacy of α-tomatine as single agent and in combination with taxane-based regimen for treatment of androgen-independent prostate cancer. Previous studies have demonstrated the cytotoxic effect of α-tomatine on different types of human cancer cells *in vitro*, as well as its anti-metastatic mechanism on lung and breast cancer cells having wild-type p53 and PTEN tumor suppressor gene. However, the susceptibility of p53/PTEN null androgen-independent prostate adenocarcinoma PC-3 cells to the anti-cancer effect of α-tomatine and the related molecular mechanism remain to be investigated. It is worth noting that different types of human cancer exhibit different histopathologies, genetic and epigenetic alterations, which actually contributes to variable drug response or efficacy. Similarly, there is no one universal chemotherapy drug that is used to treat all types of human cancer at present, and each chemotherapy agent is uniquely effective against specific types of cancer. Therefore, it is essential to further explore the potential of α-tomatine to combat other types of incurable human cancer including androgen-independent prostate cancer.

Androgen-independent prostate cancer PC-3 cells derived from patients with prostate cancer bone metastasis are used as a model test system in the present study because of their highly aggressive proliferative nature. Our preliminary study showed that AR null PC-3 cells were more sensitive to growth inhibition by α-tomatine compared to wild-type p53 and AR expressing androgen-dependent prostate cancer LNCaP cells. PC-3 cell line is used in the present study as it closely mimics the castrate resistant stage of human prostate which is clinically more relevant in discovery of novel therapeutics for androgen-independent prostate cancer. It is expected that findings from

the present study would provide important insights into the therapeutic potential of α-tomatine in androgen-independent prostate cancer that warrants further clinical investigations. More importantly, this will also be the first reported study to investigate the *in vitro* and *in vivo* chemosensitizing anti-cancer efficacy of α-tomatine with taxane-based agent.

1.5 Research objectives

The overall objective of the proposed investigation is to investigate the anti-cancer effect of α-tomatine on the growth of human androgen-independent PC-3 prostate cancer cells. The specific aims of the study are: i) To investigate the *in vitro* anti-cancer effect of α-tomatine on the growth of PC-3 cancer cells, ii) To elucidate the molecular mechanism underlying cytotoxicity of α-tomatine on PC-3 cells, iii) To examine the anti-tumor efficacy of α-tomatine on the subcutaneous and orthotopic tumor growth in immunodeficient mice, and iv) To investigate the *in vitro* and *in vivo* synergistic anti-cancer effect of α-tomatine in combined with paclitaxel against PC-3 cells.

CHAPTER 2

IN VITRO ANTICANCER ACTIVITY OF ALPHA-TOMATINE ON ANDROGEN-INDEPENDENT PROSTATE CANCER PC-3 CELLS

The contents in this chapter have been published in Lee, S. T., Wong, P. F., Cheah, S. C., Mustafa, M. R. (2011). Alpha-tomatine induces apoptosis and inhibits nuclear factor-kappa B activation on human prostatic adenocarcinoma PC-3 Cells. *PLoS ONE*, *6*(4), e18915.

2.1 Abstract

Alpha-tomatine is the major saponin in tomato (*Lycopersicon esculentum*). This study investigates the chemopreventive potential of α-tomatine on androgen-independent human prostatic adenocarcinoma PC-3 cells. Treatment of highly aggressive human prostate cancer PC-3 cells with α-tomatine resulted in a concentration-dependent inhibition of cell growth with a half-maximal efficient concentration (EC_{50}) value of 1.67 ± 0.3 μM. It is also less cytotoxic to normal human liver WRL-68 cells and normal human prostate RWPE-1 cells. Assessment of real-time growth kinetics by cell impedance-based Real-Time Cell Analyzer (RTCA) showed that α-tomatine exhibited its cytotoxic effects against PC-3 cells as early as an hour after treatment. The inhibitory effect of α-tomatine on PC-3 cancer cell growth was mainly due to induction of apoptosis as evidenced by positive Annexin V staining and decreased in mitochondrial membrane potential but increased in nuclear condensation, polarization of F-actin, cell membrane permeability and cytochrome c expressions. Results also showed that α-tomatine induced activation of caspase-3, -8 and -9, suggesting that both intrinsic and extrinsic apoptosis pathways are involved. Furthermore, nuclear factor-kappa B (NF-κB) nuclear translocation was inhibited, which in turn resulted in significant decreases in NF-κB/p50 and NF-κB/p65 in the nuclear fraction of the treated cells compared to the control untreated cells. These results provide further insights into the molecular mechanism of the anti-proliferative actions of a-tomatine. Alpha-tomatine induces apoptosis and inhibits NF-kB activation on prostate cancer cells. These results suggest that α-tomatine may be beneficial for protection against prostate cancer development and progression.

2.2 Introduction

Prostate cancer is one of the leading causes of death in men worldwide with major mortality due to advanced stage of the cancer where androgen-independent cells become unresponsive to hormone ablation therapy (Jemal *et al.*, 2011). There is a pressing need to identify alternative chemopreventive measures as surgery and current chemotherapeutic strategies can be ineffective against the aggressive form of prostate cancer (Neergheen *et al.*, 2010; Russo, 2007; Surh, 2003).

Failure of cells to undergo apoptotic cell death contributes to the development of cancers, hence treatment that induces apoptosis would be a promising anticancer strategy (Hanahan & Weinberg, 2011; Tan *et al.*, 2009). Natural products are important sources of new and less toxic anticancer agents. A variety of dietary agents such as resveratrol, curcumin, sulforaphane, gingerol, indole-3 carbinol, withanolide and green tea catechins can induce apoptosis and are effective against various human cancer cells tested (Khan *et al.*, 2008, 2010; Martin, 2006; Nobili *et al.*, 2009; Tan *et al.*, 2009). These also have an added advantage of lesser toxicity compared to current standard therapeutic drugs (Dorai & Aggarwal, 2004).

The present work investigates the chemopreventive potential of α-tomatine against prostate cancer cells. Alpha-tomatine is the major saponin in tomato (*Lycopersicon esculentum*). It possesses anti-fungal (Ito *et al.*, 2007; Sandrock & Vanetten, 1998), immune-stimulation (Heal & Taylor-Robinson, 2010; Morrow *et al.*, 2004; Yang *et al.*, 2004) and cholesterol lowering activities (Friedman *et al.*, 2000). It is protective against dibenzo[a,l]pyrene (DBP)-induced liver and stomach tumors in rainbow trout (Friedman *et al.*, 2007) and also promotes anti-proliferative effects against human colon HT-29, liver HepG2, breast MCF-7 and stomach AGS cancer cells (Friedman *et al.*, 2009; Lee *et al.*, 2004). Thus far, it is only known that α-tomatine acts on phosphoinositide 3-kinase/ protein kinase B (PI3K/Akt) and extracellular signaling-

regulating kinase (ERK) signaling pathways in lung adenocarcinoma A549 cells (Shih *et al.*, 2009). The present study, hence, seeks to investigate the effect of α-tomatine on the growth of human prostatic adenocarcinoma PC-3 cells and to identify its potential growth inhibitory mechanism. Results from the present study would provide new insights into the beneficial role of α-tomatine for prevention of prostate cancer development and progression.

2.3 Materials and Methods

2.3.1 Phytochemicals, standard drug and reagents

Alpha-tomatine (purity > 97%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Paclitaxel from Ascent Scientific (Weston-SuperMare, UK), a standard drug used in chemotherapy regimen for prostate cancer was used as positive control in this study. Curcumin (Merck, Germany) was used as positive control in NF-κB translocation assay. Dimethyl sulfoxide (DMSO) and tumor necrosis factor-alpha (TNF-α) were purchased from Sigma Aldrich (St. Louis, MO). Both the tested compound and positive controls were prepared in 100% DMSO, stored at -20 ℃, and then diluted as needed in cell culture medium. Penicillin/ streptomycin, Dulbecco Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI 1640), keratinocyte growth medium, fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) were purchased from Invitrogen (Carlsbad, CA).

2.3.2 Cell lines

Prostate cancer PC-3 (CRL-1435), normal human liver WRL-68 (CL-48) and normal human prostate epithelial RWPE-1 (CRL-11609) cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640, DMEM and keratinocyte growth medium, respectively. Both RPMI-1640 and DMEM

were supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at 37 ℃ in a humidified atmosphere of carbon dioxide–air (5:95).

2.3.3 *In vitro* cytotoxicity screening

Briefly, adherent cells were seeded in sterile 96-wells plates at optimum cell density. After 18–24 hours, cells were treated with increasing concentrations (0.16 - 5.0 μM) of α-tomatine for 24 hours. Cells treated with 0.2% DMSO was used as vehicle control. Following treatment, cells were incubated in the dark with 2 mg/ml MTT at 37 ℃ for 2 hours, then the medium was carefully removed and 100 μl of DMSO was added to dissolve the formazan crystals formed. Absorbance was measured at 570nm in a plate reader. Cell viability was calculated using the following formula: (mean absorbance in test wells)/ (mean absorbance in control well) x 100%. Dose-response curves were plotted using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). Half maximal effective concentration (EC_{50}) values were determined using non-linear regression model with sigmoidal dose response (variable slope) following the algorithm of GraphPad Prism 4.

2.3.4 Real time cell proliferation analysis

Real time growth kinetics of PC-3 cells was examined by impedance-based Real-Time Cell Analysis (RTCA) system (Roche Diagnostic, Mannheim, Germany). RTCA utilizes E-plate which contains inter-digitated micro-electrodes on the bottom of the plate that detect local ionic changes as cells proliferate and is measured as electrode impedance. Briefly, 50 μl of medium was added in 16-wells E-plate and background readings were recorded. Cell suspension (50 μl) at cell density of 1.25 x 10^4 cells/well was added to each well of the E-plate. The attachment, spreading and proliferation of

the cells were monitored every 5 minutes intervals. When the cells entered logarithmic growth phase, they were treated with 100 µl of α-tomatine at various concentrations and continuously monitored every 10 minutes for up to 72 hours. Cells treated with 0.2% of DMSO was used as vehicle control and monitored in parallel with the α-tomatine and paclitaxel-treated cells. Cell sensor impedance was expressed as an arbitrary unit called the cell index. The cell index at each time point was defined as $(R_n – R_b) / 15$, where R_n is the cell-electrode impedance of the well and the R_b is the background impedance of the well with the media alone. Growth curves were normalized to the cell index at the last measured time point before compound addition for each well.

2.3.5 Annexin V/propidium iodide (PI) double staining assay

Apoptosis-mediated cell death of tumor cell was examined by a double staining method using FITC-labeled Annexin V/ PI apoptosis detection kit (BD Bioscience, San Jose, CA) according to the manufacturer's instructions. Briefly, control and α-tomatine-treated cells were collected, washed in cold phosphate-buffered saline (PBS) twice, stained with fluoresceinisothiocyanate (FITC)-conjugated annexin V and PI dyes. The externalization of phoshotidylserine and the permeability to PI were evaluated by FACS Calibur flow cytometer (BD Bioscience, San Jose, CA). Data from 10,000 gated events per sample were collected. Cells in early stages of apoptosis were positively stained with annexin V; whereas, cells in late apoptosis were positively stained with both annexin V and PI.

2.3.6 Multiparameter high content screening (HCS) assays

HCS (Cellomics Inc, Pittsburgh, PA, USA) enables concurrent quantitative measurement of multiple independent cellular phenotypes using fluorescent probes. Both Cellomics multiparameter cytotoxicity and apoptosis HitKit (Pittsburgh, PA, USA)

were used to examine cellular changes in the α-tomatine-treated PC-3 cells. Multiparameter cytotoxicity HitKit contains four fluorescent dyes, i.e. blue fluorescent Hoechst 33342, membrane permeability, mitochondrial membrane potential, and cytochrome c dyes. They respectively detect changes in nuclear morphology (nuclear condensation), membrane permeability, mitochondrial membrane potential and expression of cytochrome c. The multiparameter apoptosis kit quantifies three fundamental parameters related to the process of apoptosis, i.e. (i) nuclear condensation, detected by the blue fluorescent nuclear dye, Hoechst 33342; (ii) F-actin content, detected by green fluorescent Alexa Fluor 488 Phalloidin stain; (iii) mitochondrial membrane potential, based on the uptake of MitoTracker Red into mitochondria of cells. Briefly, PC-3 cells were seeded overnight at density of 8000 cells/well into flat-bottomed 96-well plates (Perkin-Elmer Inc., Wellesley, MA, USA). Following treatment with different concentrations of α-tomatine (0.25 to 2.0 μM), fixation and staining for imaging analysis of the PC-3 cells were performed according to the manufacturer's instructions. Cells treated with 0.2% DMSO and 5.0 μM paclitaxel were used as negative and positive controls, respectively. Plates were analyzed using Thermo Scientific ArrayScan VTI HCS Reader (Cellomics Inc, Pittsburgh, PA, USA). This is a computerized automated fluorescence imaging microscope that automatically identifies stained cells and measures the intensity and distribution of fluorescence in individual cells. Images for each fluoroprobe were acquired at different channels using suitable filters with 20x objective at fixed exposure time. The Cell Health Profiling BioApplication software was used for image acquisitions and analysis. For each well, at least 25 fields, corresponding to at least 500 cells were automatically acquired and analyzed. All experiments were performed in triplicate. Cell average intensity (Mean) under the modified object mask within selected range in each channel was used as assay indicator, and reported as average fluorescence intensity.

Cell cycle phase distribution of α-tomatine-treated cells was determined using Cellomics Cell Cycle BioApplication as described previously (Chassoux *et al.*, 1999). It applies similar principles as fluorescence-activated cell sorting (FACS) in assessing cell cycle distribution where the intensity of Hoechst stained nucleus is deemed proportional to the cell's DNA content. Total fluorescence intensity of the dye from the nucleus of each cell typically exhibits a bimodal distribution. The first peak typically contains cells with 2N DNA content (G0/G1phase), the second peak with a double intensity of the first, contains cells with 4N DNA content (G2/M phase). Under normal conditions, there are more cells in the G0/G1 versus the G2/M phase. The region between these two peaks represents cells with DNA content between 2N and 4N (S phase) which are cells in the process of doubling their DNA. Cells found with DNA < 2N distribution are usually apoptotic cells. To identify the range of each of the DNA content categories, the software first identifies 2N (G0/G1 phase) and 4N (G2/M phase) DNA content peaks using vehicle control cells. Then, it automatically classified each cell's nuclear total intensity into one of the categories of DNA content: sub-G0 (DNA<2N), G0/G1 (DNA~2N), S (2N<DNA>4N) and G2/M (DNA~4N) phases in α-tomatine-treated cells. The percentage of each cell cycle phase for vehicle control, paclitaxel-treated cells and α-tomatine-treated cells were then reported.

2.3.7 Caspase activity

The activities of caspase-3, -8 and -9 were measured using the fluorometric assay kit (Calbiochem, USA) following the protocol of the manufacturer. Briefly, cells were treated with α-tomatine (2.0 μM). After treatment, the cells were harvested using trypsinization and cell lysates were prepared as described (Roy *et al.*, 2005). The cell lysates were mixed with reaction buffer and 10 μl of fluorogenic peptide substrate: Ac-DEVD-AMC (caspase-3), Ac-IETD-AMC (caspase-8) and Ac-LEHD-AMC (caspase-

9), and incubated for 2 hours at 37 ℃ in the dark. Inhibitors (caspase-8: z-IETD-FMK; caspase-9: z-LEHD-FMK; caspase-3-like: DEVD-CHO) were added 30 minutes before addition of fluorogenic substrate. Wells containing 50 µl of sample buffer, 50 µl of assay buffer and 10 µl of substrate were used as blank. Purified caspase was used as positive control while untreated cell extract was used as negative control. Fluorescence was then measured at excitation of 390 nm and emission of 500 nm. Fold-increase in the protease activity was determined by comparing the levels of the treated cells with untreated controls.

2.3.8 NF-κB translocation assay

NF-κB translocation in PC-3 cells was examined using NF-κB activation HCS kit which contains Hoechst 33342 and Alexa Fluor 488 conjugated anti-NF-κB dyes. PC-3 cells were seeded into sterile flat-bottomed 96-well plates (Perkin-Elmer Inc., Wellesley, MA, USA) at 8000 cells/well (100µl/well). After 18–24 hours, cells were treated with different concentrations of α-tomatine for 30 minutes, followed by treatment with 10 ng/ml TNF-α for another 30 minutes. Cells pre-treated with 0.2% DMSO and 50 µM curcumin were used as negative and positive inhibitor controls, respectively. Fixation, permeabilization and immunofluorescence staining of cells were performed according to the manufacturer's instructions. For target translocation analysis, cells stained with Hoechst dye were identified as objects in channel 1 and an adjustable mask called Circ was created around every nucleus. In non-activated cells, Alexa Fluor 488-stained NF-κB was detected in channel 2 in the cytoplasm where an annular region called Ring was defined beyond the nuclear region. Images were acquired using suitable filters with 20x objective at fixed exposure times. For each well, at least 500 cells were automatically acquired and analyzed. The output feature MEAN_CircRingAvgIntenDiffCh2 represents the difference between the intensity of

nuclear and cytoplasmic NF-κB associated fluorescence (Nuc-Cyto Diff), reported as translocation parameter, as previously described (Ding *et al.*, 1998).

2.3.9 NF-κB/p50 and NF-κB/p65 transcription factor assay

PC-3 cells at 70-80% confluence were treated with α-tomatine for 30 minutes, followed by treatment with TNF-α (10 ng/ml) for another 30 minutes. The cells were then washed with PBS and the both nuclear and cytoplasmic fractions of the treated cells were extracted using nuclear extraction kit (Cayman Chemical, Ann Arbour, MI). Concentrations of the active forms of NF-κB/p50 and NF-κB/p65 in the both fractions were measured using Cayman NF-κB/p50 and NF-κB/p65 ELISA kits (Ann Arbour, MI) according to the instructions of the manufacturer. The differences in NF-κB/p50 and NF-κB/p65 levels between the nuclear and cytoplasmic fractions were reported.

2.3.10 Statistical analysis

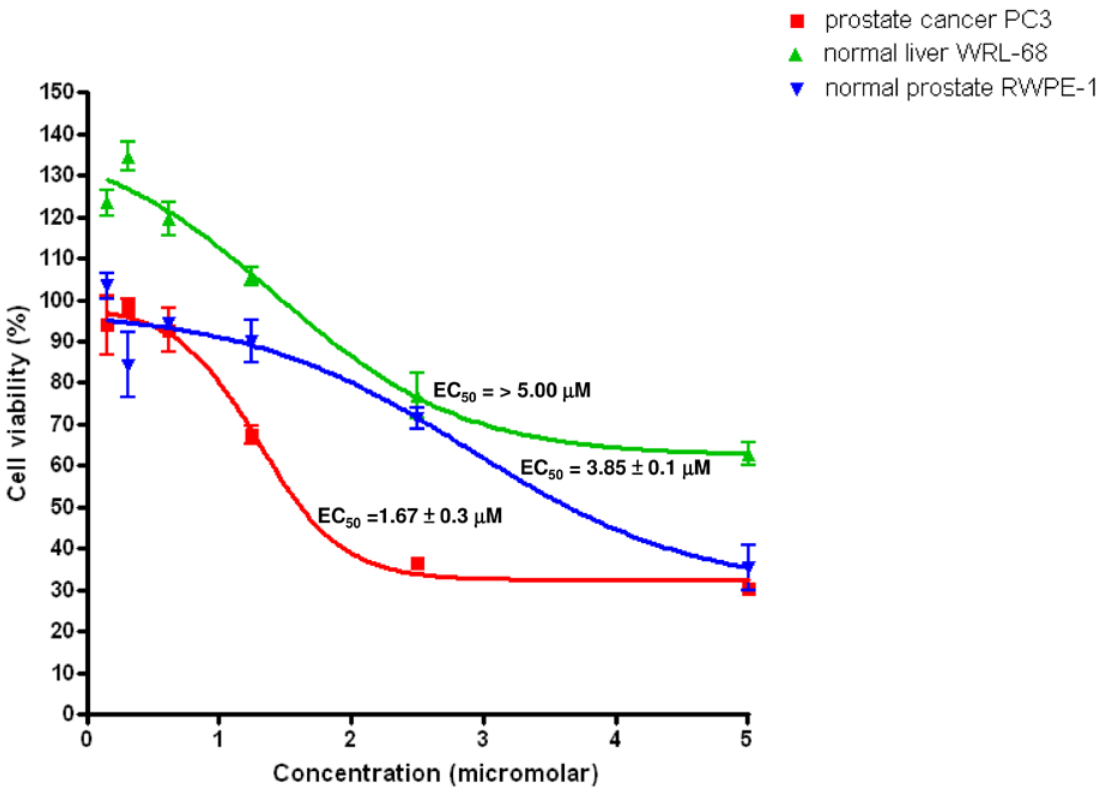
All assays were conducted in at least three separate experiments. Results are expressed as the mean value ± standard error of mean (SEM). Statistical analysis was performed with one-way analysis of variance (ANOVA), with Dunnett's Multiple Comparison Test to identify between-group differences using GraphPad Prism software (version 4.0; GraphPad Software Inc., San Diego, CA). Statistical significance is expressed as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Log EC_{50} calculations were performed using the built-in algorithms from dose-response curves with variable slope.

2.4 Results

2.41 Alpha-tomatine dose-dependently inhibits proliferation of PC-3 cancer cells

To evaluate the effect of α-tomatine on cell viability of PC-3, WRL-68 and RWPE-1 cells, MTT assay was performed. Treatment of α-tomatine to PC-3 cells resulted in a significant dose-dependent (from 0.16 to 5.0 μM) inhibition of cell growth (Figure 2.1). The EC_{50} value at 24 hours post-treatment with α-tomatine for PC-3 cells was estimated at 1.67 ± 0.3 μM. Alpha-tomatine only caused cytotoxicity towards normal prostate RWPE-1 cells at highest concentration (5.0 μM) of α-tomatine, with EC_{50} value of 3.85 ± 0.1 μM (Figure 2.1). Normal human liver WRL-68 cells treated with the highest concentration of α-tomatine tested (5.0 μM) resulted in ~35% of nonviable cells and this suggest that α-tomatine is less cytotoxic against WRL-68 cells when compared to PC3 cells (Figure 2.1). The EC_{50} values of α-tomatine towards WRL-68 cells (>5 μM) and RWPE-1 (3.85 ± 0.1 μM) and were higher than that of PC-3 cells (1.67 ± 0.3 μM), suggesting the selective cytotoxicity of α-tomatine towards prostate cancer PC-3 cells.

Figure 2.1 The effect of α-tomatine on cell viability of PC-3, WRL-68 and RWPE-1 cells. Cells were treated with α-tomatine for 24 hours and cell proliferation was measured using MTT reduction assay. Each value represents the mean ± SEM of three independent experiments, performed in triplicates. EC_{50} values of α-tomatine on PC-3 cancer cell, WRL-68 normal cells and RWPE-1 normal prostate cells at 24 hours treatment were 1.67 ± 0.3 μM, > 5.0 μM and 3.85 ± 0.1 μM, respectively.

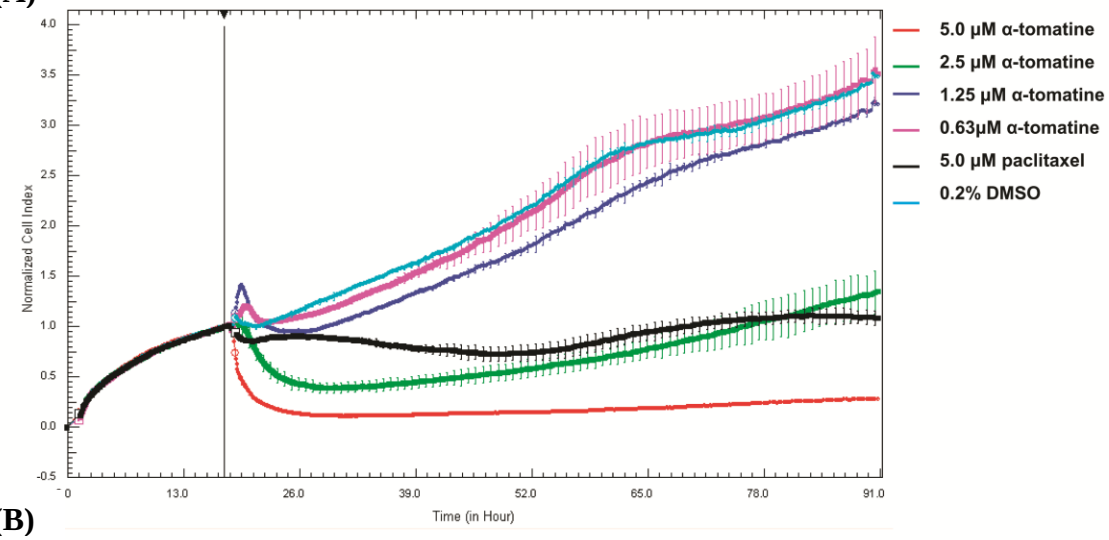

prostate cancer PC3
normal liver WRL-68
normal prostate RWPE-1
Cell viability (%)
Concentration (micromolar)
EC50 = > 5.00 µM
EC50 = 3.85 ± 0.1 µM
EC50 =1.67 ± 0.3 µM

2.42 Real-time growth kinetics analysis of α-tomatine using cell impedance-based analyzer

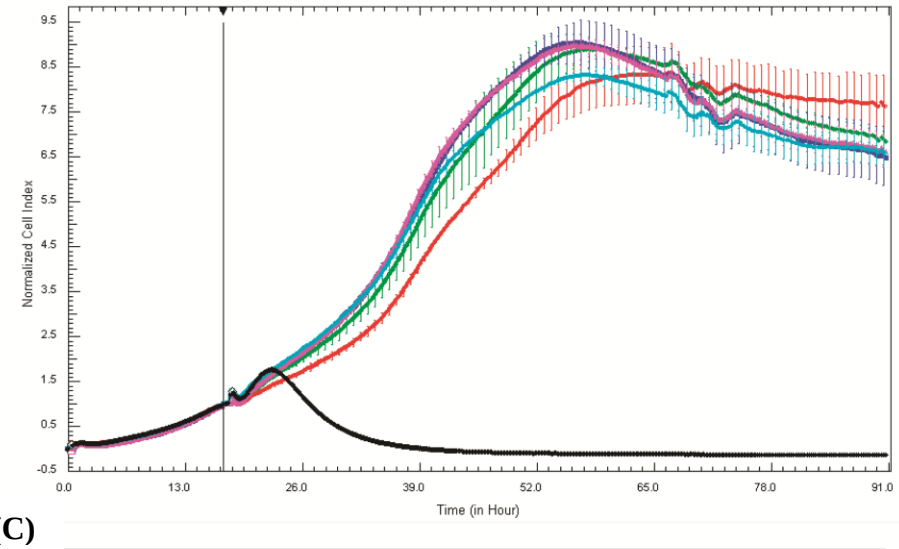
The dynamics of PC-3 cells proliferation on the 16-wells E-plates were monitored at every 5 minutes interval from the time of plating until the cells entered the logarithmic growth phase, following which the cells were treated with different concentrations of α-tomatine. After treatment, cell index values were acquired at every 10 minutes interval for 72 hours. It was observed that a rapid decrease in cell index value occurred as early as an hour after treatment with 5.0 μM and 2.5 μM of α-tomatine on PC-3 cells (Figure 2.2A), suggesting that PC-3 cells were dying from the treatment with these concentrations. PC-3 cells treated at lower concentrations (0.16 - 1.25 μM) of α-tomatine were proliferating in parallel to cells treated with vehicle control as indicated with increase in cell index values. In contrast, WRL-68 cells treatment with α-tomatine showed no reduction in cell index values even at the highest tested concentration of 5.0 μM compared to control levels and it exhibited a continuous rise in cell index values throughout the 72 hours of treatment (Figure 2.2B). This showed that WRL-68 cells growth was not affected by α-tomatine. Treatment with 2.5 μM α-tomatine initially inhibited normal prostate RWPE-1 cell proliferation but eventually recovered to the level of vehicle control cells (Figure 2.2C). Treatment with higher concentration (5.0 μM), however, decreased the cell index values suggesting that α-tomatine inhibited the proliferation of RWPE-1 cells at high concentration. These data suggested that α-tomatine at low concentration (2.5 μM) can inhibit the growth of PC-3 prostate cancer cells but not the normal prostate RWPE-1.

Figure 2.2 Dynamic assessment of cell viability after treatment with α-tomatine. Normalized cell index measured over 72 hours on (A) prostate cancer PC-3, (B) human normal liver WRL-68 and (C) human normal prostate RWPE-1 cells. Briefly, cells at logarithmic growth phase were treated with α-tomatine and cell index values were recorded every 10 minutes intervals using RTCA DP system. Cells treated with 5.0 μM paclitaxel and 0.2% DMSO were used as positive and negative controls, respectively. A change in impedance as the cells spread on the E-plate was displayed as cell index value. Plotted cell index values were normalized to the last time point before addition of treatment.

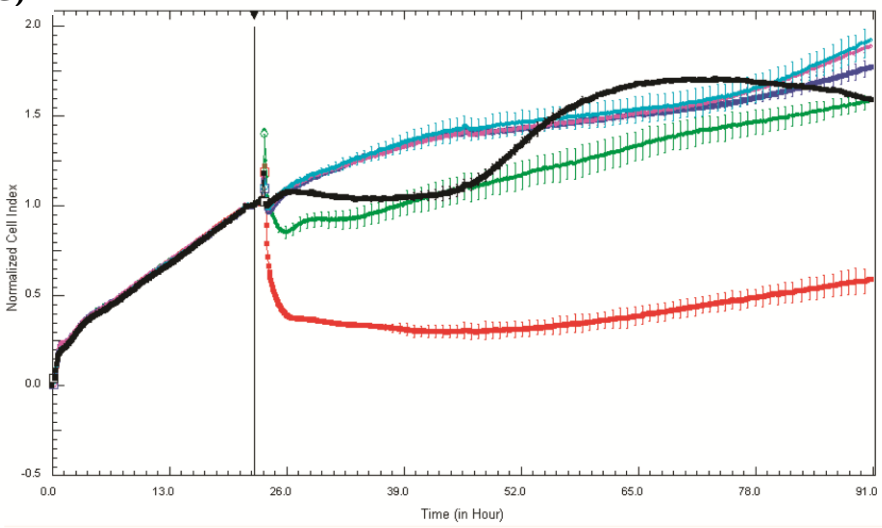
(A)


5.0 μM α-tomatine
2.5 μM α-tomatine
1.25 μM α-tomatine
0.63μM α-tomatine
5.0 μM paclitaxel
0.2% DMSO
Normalized Cell Index
Time (in Hour)

(B)


Normalized Cell Index
Time (in Hour)

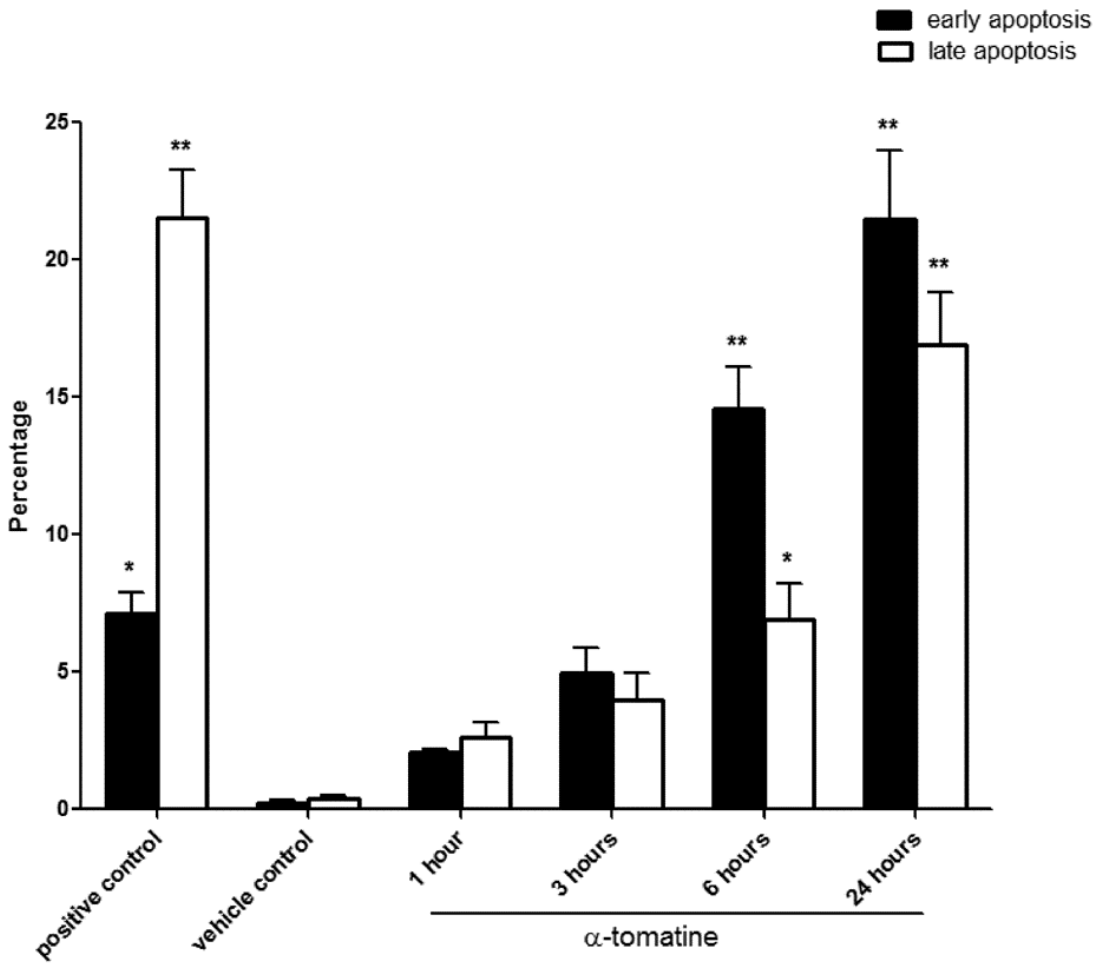
(C)


Normalized Cell Index
Time (in Hour)

2.43 Alpha-tomatine induces apoptosis on PC-3 cancer cells

To determine if α-tomatine induces apoptosis in PC-3 prostate cancer cells, annexin V/propidium iodide (PI) double staining assay and flow cytometry analysis were performed. Treatment with 2.0 μM α-tomatine for 1, 3, 6 and 24 hours resulted in a gradual increase in early apoptotic cells (annexin V positive only) from 2.07 ± 0.12 % to 5.00 ± 0.97 %, 14.57 ± 1.55 %, and 21.50 ± 2.48 %, respectively (Figure 2.3, black bars). The late apoptotic cells (annexin V and PI positive) were also increased significantly, from 2.63 ± 0.56 % to 4.00 ± 0.93 %, 6.93 ± 1.3 %, and 16.9 ± 1.92 % (Figure 2.3, white bars). Positive control cells treated with 5.0 μM paclitaxel for 24 hours also resulted in 7.13 ± 0.75 % of early apoptotic cells (Figure 2.3, black bars) and 21.53 ± 1.74 % of late apoptotic cells (Figure 2.3, white bars) in the culture compared with only 0.23 ± 0.09 % of early apoptotic cells (Figure 2.3, black bars) and 0.37 ± 0.12 % of late apoptotic cells (Figure 2.3, white bars) in the negative control cells treated with 0.2% DMSO. Hence, these results showed that α-tomatine inhibited PC-3 cells growth by inducing apoptosis in these cells.

Figure 2.3 Annexin V/PI double staining assay. Cells were exposed to either 0.2% DMSO (vehicle control), 5.0 μM paclitaxel (positive control), or α-tomatine for the indicated time. The treated cells were stained with annexin V and PI and then subjected to flow cytometric analysis. The percentages of early apoptotic cells (annexin V positive, PI negative) and late apoptotic cells (annexin V and PI positive) observed at different incubation time with α-tomatine are shown. Results are presented as the mean ± SEM of three independent experiments. Statistical significance is expressed as **$p < 0.01$; *$p < 0.05$ versus vehicle control.


early apoptosis
late apoptosis
Percentage
25
20
15
10
5
0
*
**
**
*
**
**
positive control
vehicle control
1 hour
3 hours
6 hours
24 hours
α-tomatine

2.44 Multiparametric HCS assays

To further confirm the induction of apoptosis by α-tomatine, the treated cells were examined for cellular changes associated with apoptosis using HCS analysis. Treatment with 2.0, 1.0 and 0.5 μM α-tomatine resulted in approximately 95%, 83% and 20% cell loss, respectively (Figure 2.4A). In comparison, positive control cells treated with 5.0 μM paclitaxel resulted in only 45% cell loss and none from the vehicle control cells (Figure 2.4A). The α-tomatine treated cells also exhibited a concentration-dependent increase in nuclear chromatin staining with Hoechst 33342 (Figures 2.4B and 2.5C), suggesting an increase in nuclear condensation. Increased in nuclear chromatin staining with Hoechst 33342 was also observed in the paclitaxel positive control cells (Figure 2.4B and 2.5B) whereas the fluorescent intensity was significantly lower and healthy nuclear morphology was observed in the vehicle control cells (Figures 2.4B and 2.5A). Membrane permeability of the treated cells also increased significantly as evidenced by high fluorescent intensity in the cytoplasm of α-tomatine (Figure 2.4C and 2.5C) and paclitaxel- treated cells (Figure 2.4C, and 2.5B) compared to the vehicle control (Figures 2.4C and 2.5A). A reduction in mitochondrial membrane potential is observed in both paclitaxel (Figure 2.4D and 2.5B) and α-tomatine (Figure 2.4D and 2.5C) treated cells compared to intact mitochondrial membrane potential of the vehicle control cells (Figure 2.4D and 2.5A). More specific apoptosis indicators such as release of cytochrome c and F-actin polarization or cleavage were found increased in a concentration-dependent manner in the cytoplasm of PC-3 treated cells, with the highest increase observed at 2.0 μM α-tomatine (Figure 2.4E, 2.4F and 2.5C). These results further confirmed that α-tomatine inhibited PC-3 cell growth by inducing apoptosis.

Figure 2.4 HCS analysis of apoptosis associated cellular morphology on α-tomatine treated PC-3 cells. PC-3 cells were treated with different concentrations of α-tomatine (0.5 - 2.0 μM). Cell morphology changes associated with apoptosis such as (A) percentage of dead cells, (B) alterations in nuclear condensation, (C) membrane permeability, (D) mitochondrial membrane potential, (E) expression of cytochrome c and (F) F-actin contents are reflected by the average fluorescence intensity detected. Cells treated with 0.2% DMSO and 5.0 μM paclitaxel were used as negative and positive controls, respectively. All measured parameters were expressed as average fluorescent intensities and averaged for at least 500 cells per well. Data is representative of three independent experiments. Statistical significance is expressed as $^{**}p < 0.01$; $^{*}p < 0.05$ versus vehicle control.

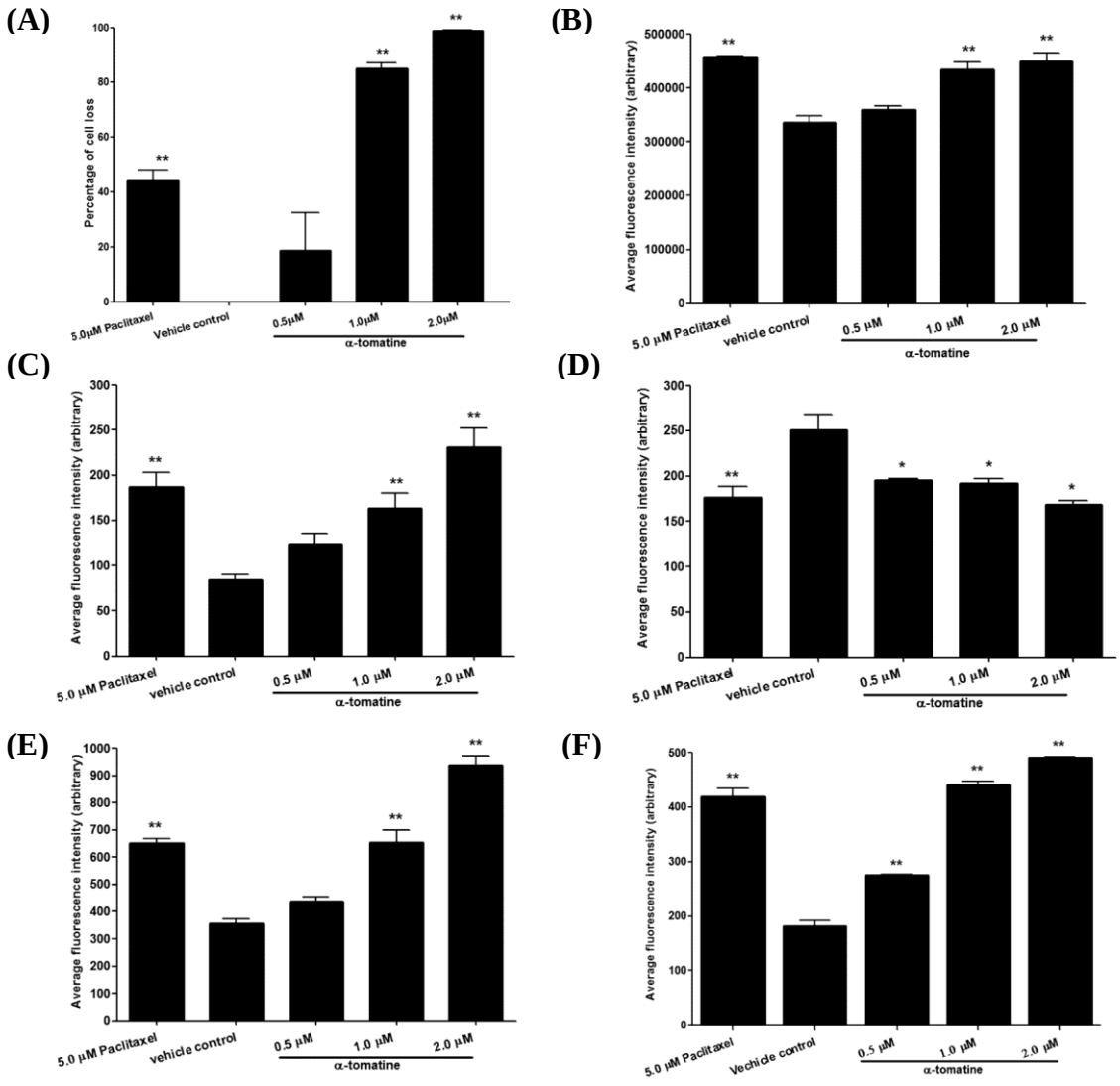
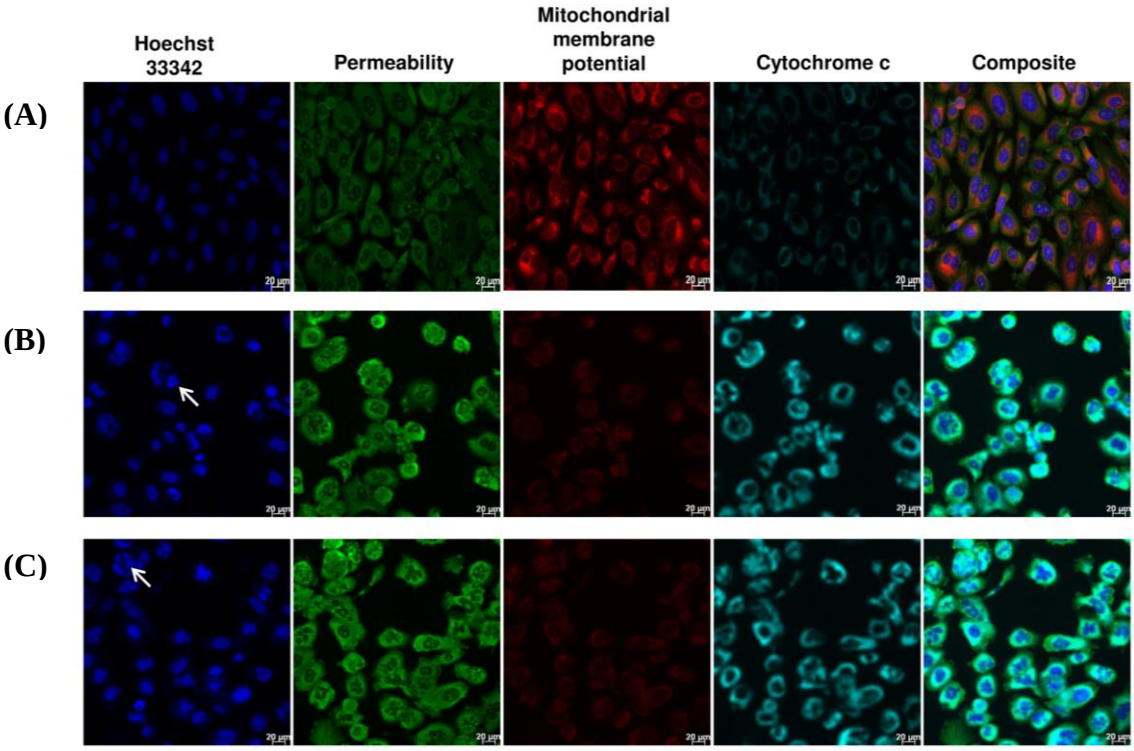

(A)
Percentage of cell loss
5.0μM Paclitaxel
Vehicle control
0.5μM
1.0μM
2.0μM
α-tomatine
(B)
Average fluorescence intensity (arbitrary)
5.0 μM Paclitaxel
vehicle control
0.5 μM
1.0 μM
2.0 μM
α-tomatine
(C)
Average fluorescence intensity (arbitrary)
5.0 μM Paclitaxel
vehicle control
0.5 μM
1.0 μM
2.0 μM
α-tomatine
(D)
Average fluorescence intensity (arbitrary)
5.0 μM Paclitaxel
vehicle control
0.5 μM
1.0 μM
2.0 μM
α-tomatine
(E)
Average fluorescence intensity (arbitrary)
5.0 μM Paclitaxel
vehicle control
0.5 μM
1.0 μM
2.0 μM
α-tomatine
(F)
Average fluorescence intensity (arbitrary)
5.0 μM Paclitaxel
Vechicle control
0.5 μM
1.0 μM
2.0 μM
α-tomatine

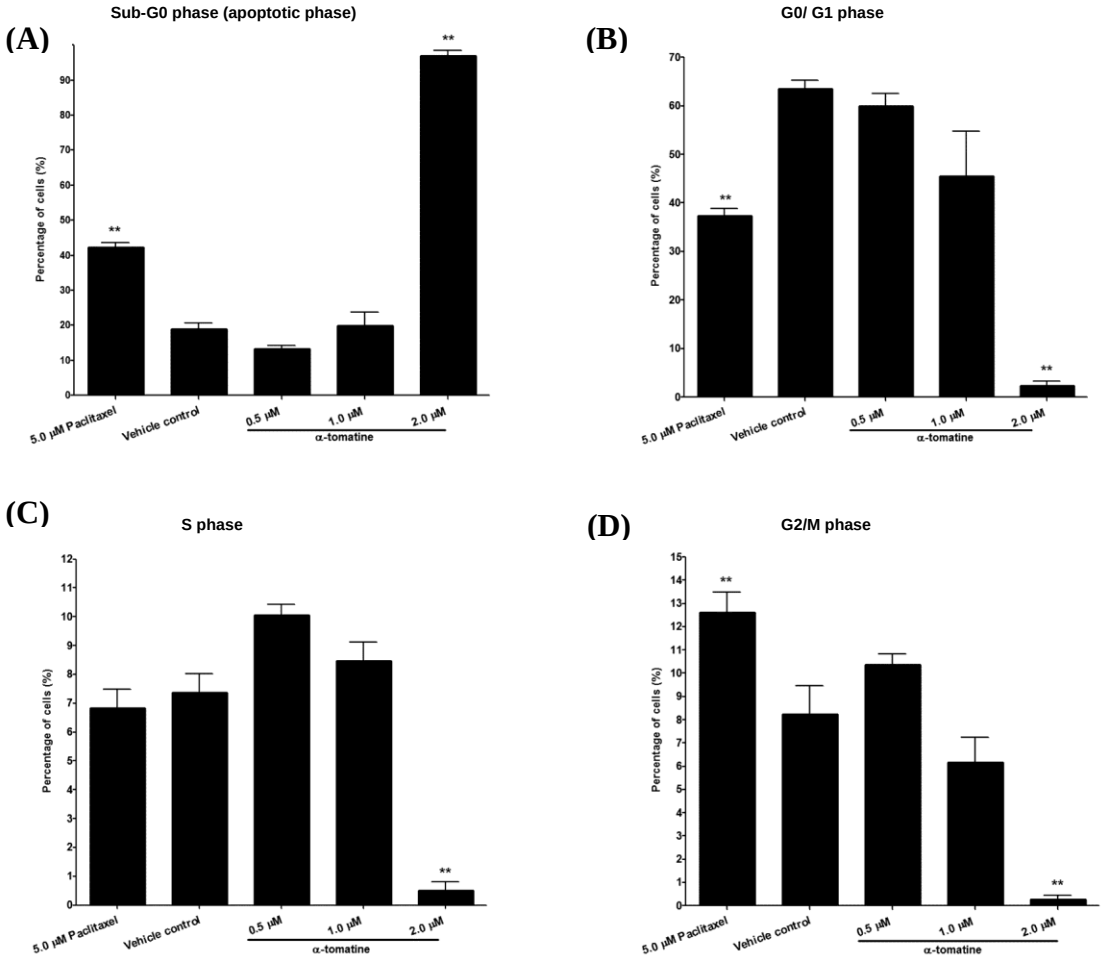
Figure 2.5 Cytotoxic and pro-apoptotic effects of α-tomatine on PC-3 cells. PC3 cells were treated for 24 hours with (A) 0.2% DMSO, (B) 5.0 μM paclitaxel, and (C) 2.0 μM α-tomatine. The treated cells were examined for nuclear condensation using blue Hoechst 33342 stain, changes in membrane permeability using a green fluoroprobe, mitochondrial membrane potential using MitoTracker Red CMXRos dye, and cytochrome c expression using a blue green fluoroprobe. Scale bar indicates 20 μm.


Hoechst 33342
Permeability
Mitochondrial membrane potential
Cytochrome c
Composite
(A)
(B)
(C)

2.45 Alpha-tomatine-induced apoptosis in PC-3 cells is not associated with cell cycle arrest

To determine whether induction of apoptosis in PC-3 cells by α-tomatine could be related to the cell cycle arrest, cell cycle distribution of the treated cells was assessed by labelling the cell's DNA with blue fluorescent Hoechst 33342 stain, whose intensity is proportional to the cell's DNA content. The sub-G0 population representing apoptotic cells in the 2.0 μM α-tomatine treated cells was markedly increased (95%) compared to positive control cells treated with paclitaxel (40%, Figure 2.6A). This increase was reflected by significant reduction in cells at G0/G1 (Figure 2.6B), S (Figure 2.6C), and G2/M phases (Figure 2.6D). No significant changes in the percentage of population of cells in other phases were observed in 0.5 and 1.0 μM treated cells, suggesting that induction of apoptosis was not mediated by cell cycle arrest.

Figure 2.6 Cell cycle distribution of α-tomatine-treated PC-3 cells. The percentage of α-tomatine-treated cells at (A) Sub-G0, (B) G0/G1, (C) S, and (D) G_2/M phases of PC-3 cells. Each bar represents the mean ± SEM of data from three independent experiments. Statistical significance is expressed as **p < 0.01 versus vehicle control.

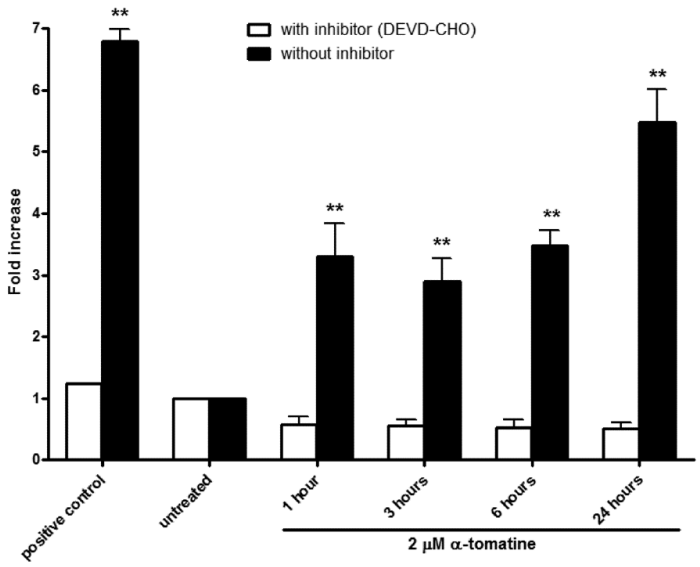

(A)
Sub-G0 phase (apoptotic phase)
Percentage of cells (%)
**
**
5.0 μM Paclitaxel
Vehicle control
0.5 μM
1.0 μM
2.0 μM
α-tomatine
(B)
G0/ G1 phase
Percentage of cells (%)
**
**
5.0 μM Paclitaxel
Vehicle control
0.5 μM
1.0 μM
2.0 μM
α-tomatine
(C)
S phase
Percentage of cells (%)
**
5.0 μM Paclitaxel
Vehicle control
0.5 μM
1.0 μM
2.0 μM
α-tomatine
(D)
G2/M phase
Percentage of cells (%)
**
**
5.0 μM Paclitaxel
Vehicle control
0.5 μM
1.0 μM
2.0 μM
α-tomatine

2.46 Alpha-tomatine induces caspases activation

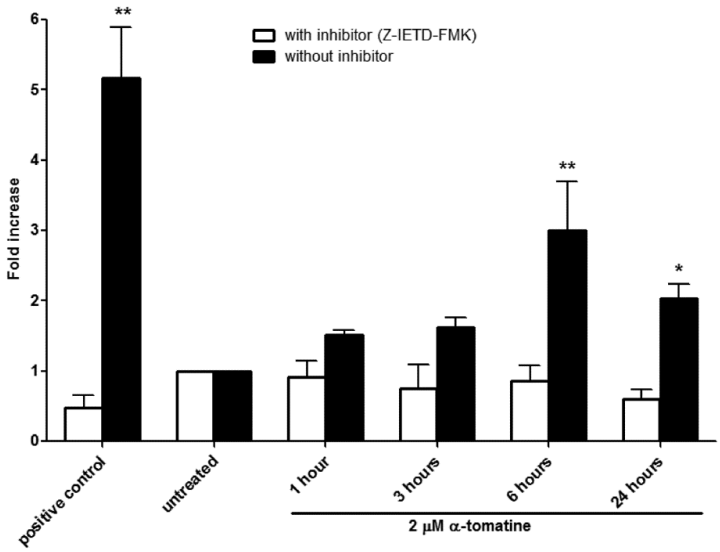
Caspases are present in the proforms (inactive) and become active after site-specific cleavage to participate in the process of apoptosis. To determine whether caspases are involved in apoptosis induction by α-tomatine, the protein levels of active caspases in α-tomatine-treated cells were evaluated. Activation of the executioner procaspase-3 by α-tomatine was found to be time-dependent (Figure 2.7A). Caspase-3 activity was significantly elevated at the first hour of treatment and progressed to a maximal level (6-fold over vesicle control) after 24 hours of incubation (Figure 2.7A). High levels of pro-caspase-8 and pro-caspase 9 were detected as early as 1 hour after the addition of α-tomatine, and reached a maximal level at 6 hours of incubation where the high levels of caspase 8 and 9 persisted over 24 hours of incubation (Figure 2.7B and 2.7C). These findings suggest that α-tomatine activated caspase-3, caspase-8 and caspase-9 as early as 1 hour after treatment. Cell permeable-specific inhibitors of caspase-3-like (DEVD-CHO, Figure 2.7A), caspase-8 (z-IETD-FMK, Figure 2.7B) and caspase-9 (z-LEHD-FMK, Figure 2.7C) were added into α-tomatine treatment to determine whether the activation of caspase-3, -8 and -9 by α-tomatine can be blocked by these inhibitors. In the presence of inhibitors, fold increase of caspase-3 (Figure 2.7A), caspase-8 (Figure 2.7B) and caspase-9 (Figure 2.7C) were reduced. These results further confirmed the activation of caspase-3, -8 and -9 by α-tomatine in PC-3 cells.

Figure 2.7 Effect of α-tomatine on caspases activation. Fold increase of the levels of (A) caspase-3, (B) caspase-8, and (C) caspase-9 in PC-3 cells treated with 2.0 μM α-tomatine, compared to vehicle control. The caspase activities were determined in the presence or absence of specific inhibitors in time course manner. The fluorescence intensity was measured at excitation wavelength of 390nm and emission wavelength of 500nm. The increase of protease activities was determined by comparing the levels in α-tomatine treated PC-3 cells with the vehicle control. Each bar represents the mean ± SEM of data from three independent experiments. Statistical significance is expressed as $^{**}p < 0.01$; $^{*}p < 0.05$ versus vehicle control.

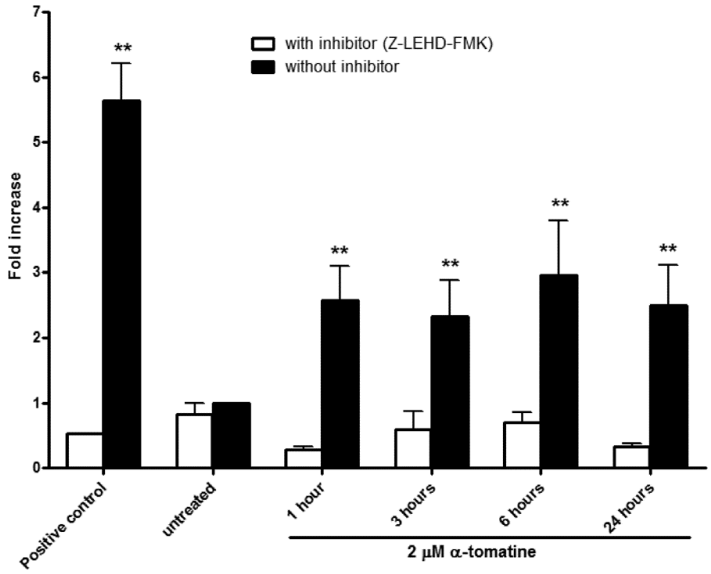
(A)


with inhibitor (DEVD-CHO)
without inhibitor
Fold increase
positive control
untreated
1 hour
3 hours
6 hours
24 hours
2 μM α-tomatine

(B)


with inhibitor (Z-IETD-FMK)
without inhibitor
Fold increase
positive control
untreated
1 hour
3 hours
6 hours
24 hours
2 μM α-tomatine

(C)

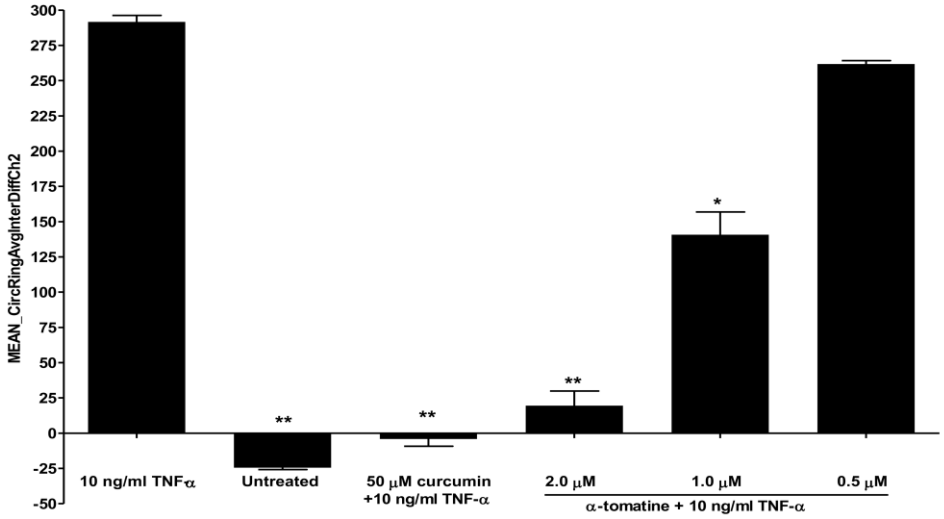

with inhibitor (Z-LEHD-FMK)
without inhibitor
Fold increase
Positive control
untreated
1 hour
3 hours
6 hours
24 hours
2 μM α-tomatine

2.47 Alpha-tomatine inhibits TNF-α-induced NF-κB nuclear translocation

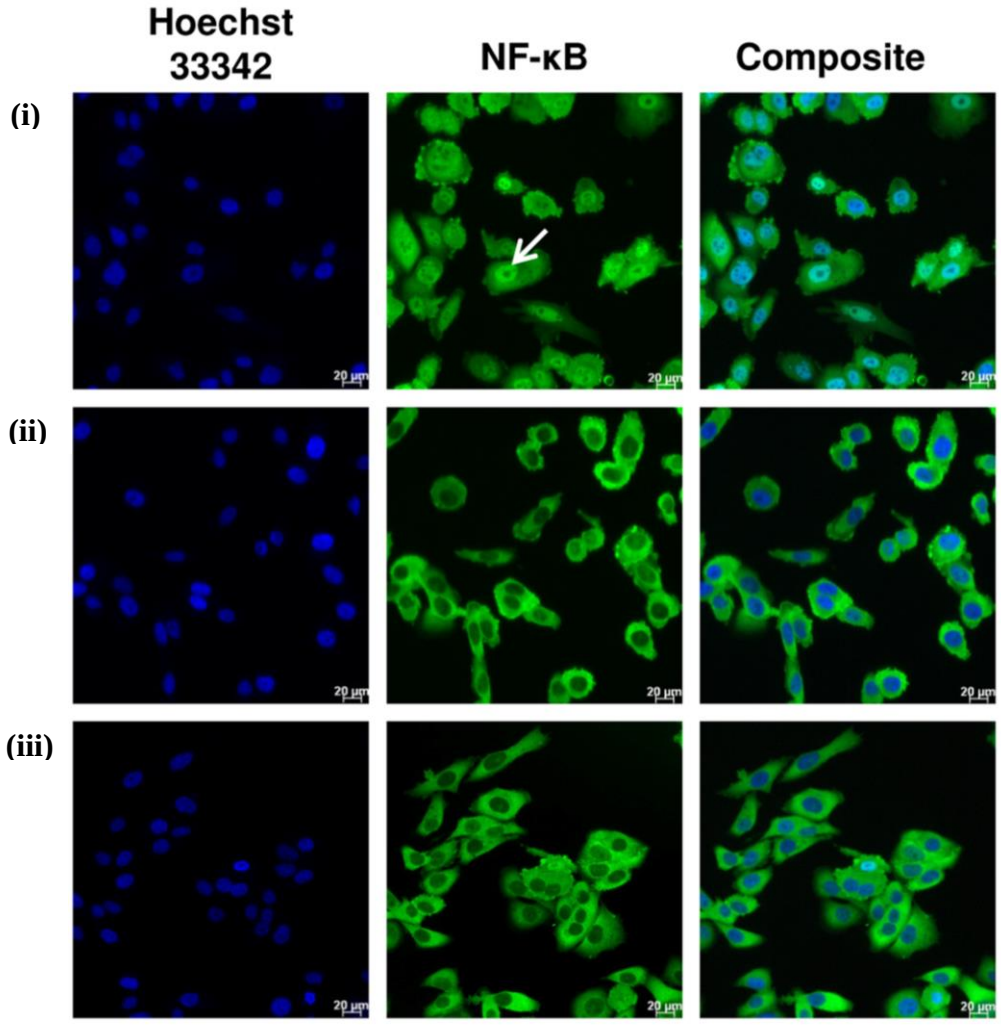
It has been shown that activation of NF-κB blocks apoptosis and promotes cell proliferation (Karin & Greten, 2005). We assessed whether α-tomatine inhibits activation of NF-κB induced by the inflammatory cytokine, TNF-α using Alexa Fluor 488-conjugated anti-NF-κB antibody. In cells treated with medium only (Figure 2.8A and 2.8Bii), high fluorescent intensity of NF-κB was found in cytoplasm but dimly in nuclei, indicating that NF-κB was not activated under resting condition. Following stimulation with TNF-α alone, NF-κB fluorescent intensity significantly increased in nuclei, suggesting that TNF-α stimulation resulted in NF-κB activation and translocation from cytoplasm into nuclei occurred in the stimulated cells (Figure 2.8A and 2.8Bi). However, in PC-3 cells treated with curcumin, a known inhibitor of NF-κB activation, it was observed that significant inhibition of TNF-α-induced NF-κB nuclear translocation as evidenced by low nuclear NF-κB-related fluorescence intensity (Figure 2.8A). Similarly, α-tomatine also inhibited TNF-α-induced NF-κB activation in a dose-dependent manner with strong inhibition observed at 2.0 μM treatment (Figure 2.8A, and 2.8Biii). These results showed that α-tomatine inhibits NF-κB activation.

Figure 2.8 The inhibitory effect of α-tomatine on TNF-α-induced NF-κB nuclear translocation. PC-3 cells were pretreated with α-tomatine for 30 minutes and followed by 10 ng/ml of TNF-α stimulation for 30 minutes. (A) Cells treated with 50 μM curcumin and medium only were used as positive NF-κB inhibitor and negative control, respectively. Cells were fixed, immunostained with anti-NF-κB antibody, and counterstained with Hoechst 33342. NF-κB nuclear translocation index was measured using Thermo Scientific ArrayScan VTI HCS Reader and expressed as the difference between nuclear and cytoplasmic NF-κB related fluorescence intensity. Each bar represents the mean ± SEM of data in triplicate. Statistical significance is expressed as $^{**}p < 0.01$; $^{*}p < 0.05$ versus cells cells treated with TNF-α alone. (B) Images of PC-3 cells treated with 10 ng/mL TNF-α alone (i), medium only (ii), and 2.0 μM of α-tomatine pretreatment before 10 ng/ml TNF-α stimulation (iii). Control and treated PC-3 cells were stained with Hoechst (blue) and the Alexa Fluor 488-conjugated anti-NF-κB antibody (green). Images were acquired for each fluorescence channel, using suitable filters with 20x objective. Scale bar indicates 20 μm.

(A)


MEAN_CircRingAvgInterDiffCh2
300
275
250
225
200
175
150
125
100
75
50
25
0
-25
-50
**
**
**
*
10 ng/ml TNF-α
Untreated
50 μM curcumin
+10 ng/ml TNF-α
2.0 μM
1.0 μM
0.5 μM
α-tomatine + 10 ng/ml TNF-α

(B)

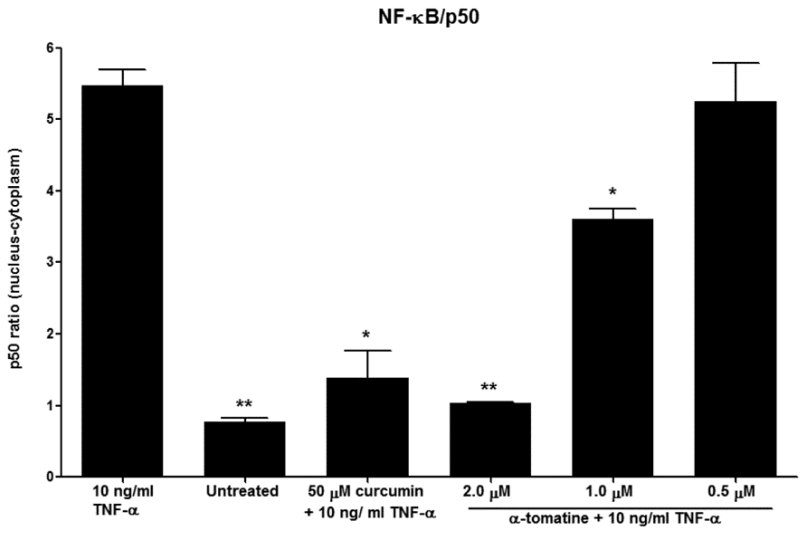

Hoechst 33342
NF-κB
Composite
(i)
(ii)
(iii)
20 μm

2.48 Alpha-tomatine treatment inhibits NF-κB/p50 and NF-κB/p65 nuclear translocation

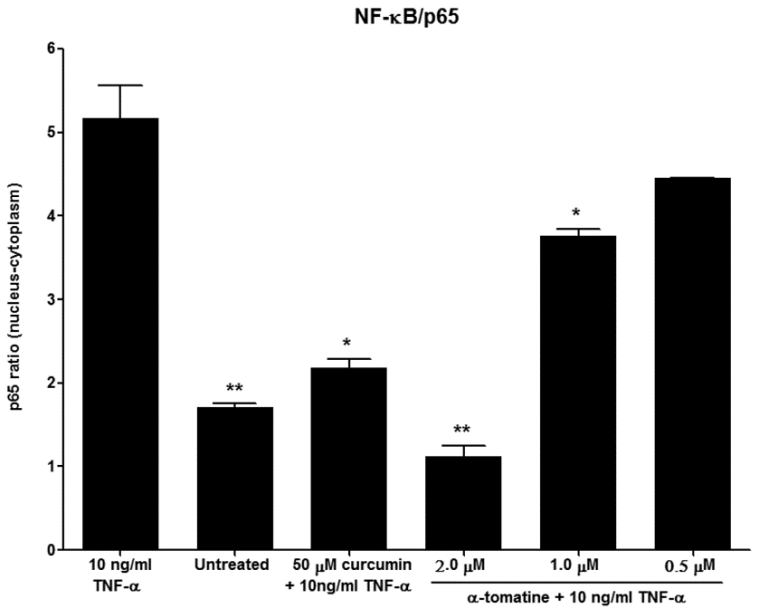
To further confirm TNF-α-induced NF-κB activation was inhibited in α-tomatine treated PC-3 cells through the inhibition of NF-κB nuclear translocation, NF-κB/p50 (Figure 9A) and NF-κB/p65 (Figure 2.9B) in nuclear and cytoplasmic fractions of the treated cells were measured by ELISA. Pretreatment with 2.0 μM of α-tomatine resulted in significant decreased in both NF-κB/p50 (Figure 2.9A) and NF-κB/p65 (Figure 2.9B) level in nuclear fractions, compared to cells treated with TNF-α control (Figure 2.9A and 2.9B). These results showed that α-tomatine inhibits TNF-α-induced NF-κB activation by inhibiting nuclear translocation of NF-κB/p50 and NF-κB/p65 in the treated PC-3 cells.

Figure 2.9 Comparison of NF-κB/p50 and NF-κB/p65 protein levels between nuclear and cytoplasmic fraction. PC-3 cells were treated with 0.5, 1.0, 2.0 μM α-tomatine for 30 minutes, followed by treatment with 10 ng/ml TNF-α for another 30 minutes. Nuclear and cytoplasmic fractions of the PC-3 treated cells were extracted, the concentration of the active form of (A) NF-κB/p50 and (B) NF-κB/p65 in both fractions were measured with ELISA kits. Differences of NF-κB/p50, NF-κB/p65 in the nuclear and cytoplasmic fractions are reported. Each bar represents the mean ± SEM of data in triplicate. Statistical significance is expressed as **$p < 0.01$; *$p < 0.05$ versus cells treated with TNF-α control.

(A)


NF-κB/p50
p50 ratio (nucleus-cytoplasm)
0
1
2
3
4
5
6
**
*
**
*
10 ng/ml TNF-α
Untreated
50 μM curcumin + 10 ng/ ml TNF-α
2.0 μM
1.0 μM
0.5 μM
α-tomatine + 10 ng/ml TNF-α

(B)


NF-κB/p65
p65 ratio (nucleus-cytoplasm)
0
1
2
3
4
5
6
**
*
**
*
10 ng/ml TNF-α
Untreated
50 μM curcumin + 10ng/ml TNF-α
2.0 μM
1.0 μM
0.5 μM
α-tomatine + 10 ng/ml TNF-α

2.5 Discussion

Alpha-tomatine was reported to inhibit growth of human colon HT-29, liver HepG2, breast MCF-7 and stomach AGS cancer cells (Friedman *et al.*, 2009; Lee *et al.*, 2004) but its anti-proliferative mechanisms require further definition. The present study reports that α-tomatine inhibits NF-κB activation and induces apoptosis on androgen-independent human prostatic adenocarcinoma PC-3 cells. The EC_{50} of α-tomatine towards PC-3 cells was estimated at 1.67 ± 0.3 μM and this value is comparable to earlier studies on different cancer cell lines (Friedman *et al.*, 2009; Lee *et al.*, 2004). Dynamic assessment by xCelligence system showed that α-tomatine is a fast-acting compound, inhibiting the cancer cells proliferation as early as an hour after treatment with 2.5 and 5.0 μM α-tomatine and its growth suppressive effect was not fully reversible as the cancer cell proliferation failed to recover throughout 72 hours of treatment. It is also less cytotoxic against human liver normal cells and human prostate normal cells. Although our data showed that α-tomatine inhibited the growth of normal prostate RWPE-1 cells at high concentration (5.0 μM), where utilization of high dose of α-tomatine can be a key consideration for cancer treatment, earlier *in vivo* studies showed no apparent toxic effects in rainbow trout (Friedman *et al.*, 2007) and does not affect body and liver weights of mice (Friedman, 2002; Friedman *et al.*, 1996).

Flow cytometry analysis of annexin V/PI staining showed that α-tomatine conspicuously induced apoptosis. This observation was confirmed by multiparametric cell-based HCS analysis that showed morphological features characteristic of apoptotic cell death such as nuclear condensation, loss of membrane symmetry, polarization of F-actin, release of cytochrome c and reduction in mitochondrial membrane potential. Disruption of the mitochondrial membrane potential is an early event in apoptosis and triggers release of cytochrome c and other apoptogenic molecules from the mitochondria to the cytosol (Ly *et al.*, 2003). These apoptogenic molecules contribute

to the activation of caspases and subsequent cell death. More intensive molecular studies indicate that apoptotic cell death can be triggered either through receptor (extrinsic)-mediated pathway where the ligand-receptor binding activates caspase-8 or the mitochondrial (intrinsic)-mediated pathway where cytochrome c is released from the mitochondrial and activates caspase-9 (Mishra & Kumar, 2005; Schneider & Tschopp, 2000). Both of these mechanisms will eventually merge and lead to the hierarchical activation of the downstream caspase-3, 6 and 7, which are responsible for the characteristic apoptosis-associated morphological changes such as chromatin condensation, membrane blebbing, and loss of overall cell shape (Hengartner, 2000; Stroh & Schulze-Osthoff, 1998). The present study demonstrated that treatment with α-tomatine increased activities of caspase-3 and -9 and caused depolarization of mitochondrial membrane potential and release of cytochrome c. These results suggest that induction of apoptosis is mediated through the intrinsic pathway. Pro-caspase-8 is an intracellular component that directly communicates with the death domain of cell membrane receptors and its activation in the α-tomatine-treated cells suggests that apoptosis is also mediated through the extrinsic pathway which perhaps merged with the intrinsic pathway and activated the executioner caspase 3. Consistent with these data, addition of cell permeable-specific inhibitors of caspase-8 (z-IETD-FMK), caspase-9 (z-LEHD-FMK), or caspase-3-like (DEVD-CHO) enzymes revealed that addition of caspase inhibitors completely decreased α-tomatine-induced activation of caspase-3, -8 and -9, confirmed the role of caspase-3, -8 and -9 in α-tomatine-induced apoptosis. Curcumin is an example of dietary agent that mediates apoptosis via both intrinsic and extrinsic pathways (Karunagaran *et al.*, 2005).

Alpha-tomatine could also significantly alter polymerization of actin filaments in the treated PC-3 cells. This observation is in line with reports that showed polymerization or cleavage of actin cytoskeleton during the early and late phases of

apoptosis (Brown *et al.*, 1997; Gourlay & Ayscough, 2005a, 2005b; Posey & Bierer, 1999). Cisplatin, a well-known anticancer drug is an example of drug that induces F-actin damage prior to changes in nuclear morphology (Kruidering *et al.*, 1998).

While the present study found no evidence of cell cycle arrest by α-tomatine, there are evidence that suggest induction of apoptosis could be mediated through the inhibition of nuclear factor-kappa B (NF-κB) signaling pathway. Constitutive NF-κB activation has been observed in many types of cancer, including androgen-independent prostate cancer (Karin & Greten, 2005). Overexpression of NF-κB/p65 protein was found in the nuclear fraction of prostate cancer clinical specimens (Fradet *et al.*, 2004; Ross *et al.*, 2003), suggesting the pathophysiological role for NF-κB in prostate cancer progression. Constitutive activation of NF-κB in tumor cells could promote cancer cells survival by blocking apoptosis and promote cancer cells growth through angiogenesis, metastasis and invasion (Millera *et al.*, 2010). Accumulating evidence indicate that constitutive activation of NF-κB in tumor cells always contribute to chemoresistance and radioresistance, and represents an independent risk factor for recurrence after radical prostatectomy (Domingo-Domenech *et al.*, 2005; Fradet *et al.*, 2004; Li & Sethi, 2010). Hence, targeting the NF-κB signaling pathway remains an attractive therapeutic option for prostate cancer. Treatment of PC-3 cells with α-tomatine resulted in a strong inhibition of NF-κB activation, which was consistent with a decrease in nuclear levels of NF-κB/p65 and NF-κB/p50. The ability of α-tomatine in inhibiting NF-κB activation by blocking the nuclear translocation of NF-κB/p65 and NF-κB/p50 transcription factors suggests its promising role in prostate cancer prevention. Inhibition of NF-κB activation could indirectly contribute to the pro-apoptotic action of α-tomatine on PC-3 cells as NF-κB controls the transcription of anti-apoptotic and cell proliferation genes, essential for the survival of cancer cells (Sethi & Tergaonkar, 2009).

2.6 Conclusion

In summary, these results show that α-tomatine has good potential as dietary phytochemical with pro-apoptosis activity. Alpha-tomatine can be further explored for its therapeutic potential in the treatment of cancers where constitutive activation of NF-κB and apoptosis resistance remains a major concern in cancer chemotherapy. To establish the relevance of *in vitro* findings, further study is underway to investigate *in vivo* anti-tumor and NF-κB activities of α-tomatine using prostate xenograft cancer model.

CHAPTER 3

ALPHA-TOMATINE ATTENUATION OF *IN VIVO* GROWTH OF SUBCUTANEOUS AND ORTHOTOPIC XENOGRAFT TUMORS OF HUMAN OF PROSTATE CARCINOMA PC-3 CELLS IS ACCOMPANIED BY INACTIVATION OF NUCLEAR FACTOR-KAPPA B SIGNALING

The contents in this chapter have been published in Lee, S. T., Wong, P. F., He, H., Hooper, J. D., Mustafa, M.R. (2013). Alpha-tomatine attenuation of in vivo growth of subcutaneous and orthotopic xenograft tumors of human prostate carcinoma PC-3 cells is accompanied by inactivation of nuclear factor-kappa B signaling. *PLoS ONE, 8*(2), e57708.

3.1 Abstract

Nuclear factor-kappa B (NF-κB) plays a role in prostate cancer and agents that suppress its activation may inhibit development or progression of this malignancy. Alpha (α)-tomatine is the major saponin present in tomato (*Lycopersicon esculentum*) and we have previously reported that it suppresses tumor necrosis factor-alpha (TNF-α)-induced nuclear translocation of NF-κB in androgen-independent prostate cancer PC-3 cells and also potently induces apoptosis of these cells. However, the precise mechanism by which α-tomatine suppresses NF-κB nuclear translocation is yet to be elucidated and the anti-tumor activity of this agent *in vivo* has not been examined. In the present study we show that suppression of NF-κB activation by α-tomatine occurs through inhibition of inhibitor of kappa B alpha (IκBα) kinase (IKK) activity, leading to sequential suppression of IκBα phosphorylation, IκBα degradation, NF-κB/p65 phosphorylation, and NF-κB p50/p65 nuclear translocation. Consistent with its ability to induce apoptosis, α-tomatine reduced TNF-α induced activation of the pro-survival mediator Akt and its inhibition of NF-κB activation was accompanied by significant reduction in the expression of NF-κB-dependent anti-apoptotic (c-IAP1, c-IAP2, Bcl-2, Bcl-xL, XIAP and survivin) proteins. We also evaluated the antitumor activity of α-tomatine against PC-3 cell tumors grown subcutaneously and orthotopically in mice. Our data indicate that intraperitoneal administration of α-tomatine significantly attenuates the growth of PC-3 cell tumors grown at both sites. Analysis of tumor material indicates that the tumor suppressing effects of α-tomatine were accompanied by increased apoptosis and lower proliferation of tumor cells as well as reduced nuclear translocation of the p50 and p65 components of NF-κB. Our study provides first evidence for *in vivo* antitumor efficacy of α-tomatine against the human androgen-independent prostate cancer. The potential usefulness of α-tomatine in prostate cancer prevention and therapy requires further investigation.

3.2 Introduction

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in men worldwide (Jemal *et al.*, 2011). As progression of this malignancy is dependent on the androgen receptor, therapies that target activating ligands (the hormones testosterone and dihydrotestosterone) produce response rates in patients of up to 95% (Bracarda *et al.*, 2005). Unfortunately, nearly all prostate cancer patients develop hormone-refractory prostate cancer (Bracarda *et al.*, 2005). For these patients curative treatments are not available and docetaxel-based chemotherapy provides palliation with response rates of approximately 50% and median survival of 18 to 20 months with survival benefit of about 2 months (Dagher *et al.*, 2004). For patients with hormone-refractory prostate cancer, low toxicity molecular targeting strategies are needed.

Accumulating evidence suggests that the transcription factor nuclear factor-kappa B (NF-κB) plays a pivotal role in prostate cancer growth, survival, angiogenesis and metastatic progression (Huang *et al.*, 2001; Jin *et al.*, 2008; Surh *et al.*, 2002; Sweeney *et al.*, 2004; Yemelyanov *et al.*, 2006). NF-κB consists of a p50/p65 heterodimer that is masked by the inhibitor of NF-κB, inhibitor of kappa B alpha (IκBα) that causes its retention in the cytoplasm under resting condition. Various stimuli, including tumor necrosis-alpha (TNF-α), phorbol ester and lipopolysaccharides (LPS), result in IκBα kinase (IKK) activation, which mediates IκBα phosphorylation at Ser32 and Ser36 followed by its ubiquitination and proteasome-mediated degradation. This releases the NF-κB p50/p65 heterodimer, which then translocates to the nucleus, where it binds to consensus sequence motifs to induce gene transcription. It has been demonstrated that NF-κB is constitutively activated in androgen-insensitive prostate carcinoma cells, and overexpression of NF-κB p65 protein was found in the nuclear fraction of prostate cancer clinical specimens (Fradet *et al.*, 2004; Surh *et al.*, 2002),

suggesting a role for NF-κB in prostate cancer progression. Consistently, it has been reported that aberrant IKK activation leads to the constitutive activation of the NF-κB survival pathway in androgen-independent prostate cancer cells (Gasparian *et al.*, 2002). In addition, activation and localization of NF-κB represent independent risk factors for disease recurrence after radical prostatectomy (Domingo-Domenech *et al.*, 2005; Fradet *et al.*, 2004). Hence, effective inhibition of NF-κB could be a promising strategy for treatment of prostate cancer and prevention of relapse.

Alpha (α)-tomatine is the major saponin in tomato (*Lycopersicon esculentum*). Previous studies have reported its immunopotentiating (Heal & Taylor-Robinson, 2010; Morrow *et al.*, 2004; Yang *et al.*, 2004) and *in vitro* anti-cancer activities (Friedman, 2004; Friedman *et al.*, 2009; Shieh *et al.*, 2011; Shih *et al.*, 2009). It also has protective effects against dibenzo[a,l]pyrene (DBP)-induced liver and stomach tumors in rainbow trout without causing significant changes in total weight, liver weight, tissue morphology and mortality (Friedman *et al.*, 2007). Thus far, the mechanism by which α-tomatine mediates its anti-prostate cancer effect is not well understood. Our previous study reported the pro-apoptotic effect of α-tomatine against androgen-independent human prostatic adenocarcinoma PC-3 cells through the inhibition of TNF-α-induced NF-κB nuclear translocation (Lee *et al.*, 2011). In the present study, the mechanism of the inhibition of α-tomatine on NF-κB signaling pathway is further characterized. For the first time, this study demonstrates the potent anti-tumor activity of α-tomatine against human androgen-independent prostate cancer *in vivo*.

3.3 Materials and Methods

3.3.1 Ethics statement

Experiments with mice were performed in accordance with the protocol approved by the University of Queensland Animal Ethics Committee (AEC Approval Number: MMRI/210/10).

3.3.2 Materials

Alpha-tomatine was purchased from Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO), TNF-α, fetal bovine serum (FBS), 3,3,5,5 tetramethylbenzidine (TMB), Calpain Inhibitor I, ALLN (N-acetyl-leucyl-leucyl-norleucinal) and anti-human beta (β)-actin antibody were purchased from Sigma Aldrich (St. Louis, MO). Penicillin/ streptomycin, Roswell Park Memorial Institute (RPMI-1640) media and 0.4% trypan blue solution were purchased from Invitrogen (Carlsbad, CA). Protein A/G plus agarose beads, Akt inhibitor VIII, and antibodies against p65, p50, IκBα, Akt, IKKα, and IKKβ were obtained from Santa Cruz Biotechnology, CA. The glutathione *S*-transferase-IκBα (GST-IκBα) fusion protein and polyvinylidene fluoride (PVDF) membrane were purchased from Millipore (Bedford, MA). Kinase buffer, antibodies against phospho-specific IκBα (Ser32/36), phospho-specific p65 (Ser536), phospho-specific Akt (Ser473), B cell leukaemia-2 (Bcl-2), B cell leukemia-x long (Bcl-xL), cellular inhibitor of apoptosis 1 (c-IAP1), cellular inhibitor of apoptosis 2 (c-IAP2), survivin, X-linked inhibitor of apoptosis (XIAP), histone H3, cleaved Poly (ADP-ribose) polymerase (PARP) and cleaved caspase-3 antibodies were purchased from Cell Signaling (Beverly, MA). Antibodies against Ki-67 and proliferating cell nuclear antigen (PCNA) were purchased from BD Biosciences (San Diego, CA).

3.3.3 Cell lines

The prostate cancer PC-3 cell line was purchased from the American Type Culture Collection (Manassas, VA). Luciferase-expressing prostate cancer PC-3 cell line was a kind gift of Dr. Patrick Ming Tat Ling, Queensland University of Technology, Australia. Both PC-3 and luciferase-expressing PC-3 cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at 37 ℃ in a 5% CO_2 humidified incubator.

3.3.4 Cell treatment and fractionation

For the *in vitro* assays, PC-3 cells at 70-80% confluence were treated with α-tomatine (2 μM) for 30 minutes, and then exposed to10 ng/ml TNF-α for various time periods. Akt inhibitor VIII (10 μM) which inhibits activation of Akt as evidenced by reduced phosphorylation of this kinase at Thr308 and Ser473 (Barnett *et al.,* 2005) was used as inhibitor control for studying the effect of α-tomatine on Akt activation as described previously (Estrada *et al.*, 2010). Both nuclear and cytoplasmic fractions of treated and vehicle control cells were isolated using a nuclear extraction kit (Cayman Chemical, Ann Arbour, MI) according to the manufacturer's instructions. Briefly, cells were harvested using a cell scrapper then pelleted by centrifugation at 4 ℃ before two washes with ice-cold PBS supplemented with phosphatase inhibitor solution at 4 ℃. Pelleted cells were swollen for 15 minutes in ice-cold hypotonic buffer supplemented with complete protease and phosphatase inhibitors. 10 % Nonidet P-40 assay reagent was then added and cytosolic fractions were collected by brief centrifugation. Pellets were resuspended in ice-cold complete nuclear extraction buffer then vortexed on ice for 30 seconds at highest setting. These cell pelleting and vortexing steps were repeated for a total of 6 cycles. The final pellet was resuspended then centrifuged at 14,000 x g for 10 minutes at 4 ℃. The supernatants containing nuclear fractions were collected.

3.3.5 Cell viability analysis

Cell viability was examined using a trypan blue exclusion assay as described previously (Husain *et al.*, 2011). Briefly, cells in control and treated groups were harvested, stained with 0.4% trypan blue solution and total viable cells were counted using hemacytometer. The proportion of viable cells was calculated by dividing the number of viable test cells by the number of viable control cells at the end of each experimental treatment.

3.3.6 IκBα kinase assay

The effect of α-tomatine on TNF-α-induced IKK activation was analyzed as described previously (Sethi *et al.*, 2007). Briefly, PC-3 cells were preincubated with either 2 μM α-tomatine or 0.1% DMSO (vehicle) for 30 minutes, and then treated with 10 ng/ml TNF-α for the indicated times. IKK complex was immunoprecipitated from whole cell extracts using antibodies against IKKα and IKKβ. Protein A/G plus agarose beads were added and incubated at 4 ℃ for overnight. The beads were washed with lysis buffer and resuspended in a kinase buffer before GST-IκBα as IKK substrate was added. After incubation at 30 ℃ for 30 minutes, the reaction was terminated by addition of Laemmli's loading buffer and heated at 100 ℃ for 5 minutes. Western blot analysis was performed to detect phosphorylated-IκBα (p-IκBα) and to determine the total amounts of IKKα and IKKβ in each sample. To determine whether α-tomatine directly targets IKK, IKKα and IKKβ immunoprecipitated from 10 ng/ml TNF-α and 0.1% DMSO (vehicle) treated cells. *In vitro* kinase assay was performed in the absence or presence of indicated concentrations of α-tomatine at 30 ℃ for 30 minutes.

3.3.7 Subcutaneous and orthotopic implantation of PC-3 cells

Male BALB/c nude mice (6 weeks old) were purchased from the Animal Resources Centre (Canning Vale, Western Australia). For subcutaneous tumor growth study,

luciferase-expressing PC-3 prostate cancer cells (1 x 10^6 in 0.1 ml Dulbecco's PBS) were inoculated subcutaneously into the lower flanks of each mouse. On day 7 after cancer cell inoculation, each mouse had one palpable tumor and were randomly assigned to four groups (n = 8/group). These groups of mice were then given intraperitoneal injections of vehicle solution, 10 mg/kg docetaxel, 5 mg/kg α-tomatine or 10mg/kg α-tomatine thrice a week for an additional 3 weeks. All the mice were monitored weekly for tumor growth and body weight. Tumor dimensions were measured with calipers and volume calculated using a standard formula: (length x width^2) x 0.5 (Plymate *et al.*, 2003). The experiment was terminated 28 days after cancer cell inoculation at which time bioluminescent signals of tumors in live mice were captured on a Xenogen IVIS Spectrum imaging system (Alameda, CA, USA) using Live Imaging Acquisition and Analysis software. Briefly, mice were injected intraperitoneally with luciferin potassium salt, anesthetized and luminescence images acquired. Luminescence signal intensity was quantified as region of interest analysis of total photons per second for each tumor. Tumors were excised, washed with ice-cold phosphate buffered saline (PBS) and stored at -80℃ until examined by Western blot analysis.

Orthotopic growth of PC-3 cells was performed as described previously (Luk *et al.*, 2011). Briefly, under a dissecting microscope (Olympus, Tokyo, Japan), the prostate of 6 weeks old anesthetized SCID mice were exposed through a surgical incision and 2 x 10^5 cells in 10 μl Dulbecco's PBS injected into the dorsal prostate. Organs were then replaced, and the abdomen was closed in two layers with silk sutures. Five days after implantation, mice were randomly assigned to two groups that received vehicle solution or 10 mg/kg α-tomatine intraperitoneally thrice a week for 14 days (n = 6 per group). Body weight was measured weekly. Bioluminescent signal of the PC-3 cell tumor in each mouse was measured at the end of the study using a Xenogen IVIS Spectrum

imaging system as described above. All mice were then sacrificed by cervical dislocation.

3.3.8 Tissue processing and protein extraction

For protein analysis of mouse tumors, tissues were minced, suspended in tissue protein extraction reagent (Thermo-Fisher Scientific, Waltham, MA) supplemented with complete protease and phosphatase inhibitors, and homogenized using a gentleMACS Dissociator (Miltenyi Biotec, Germany). The homogenized tissue was transferred to a pre-chilled microcentrifuge tube, incubated on ice for 30 minutes and then centrifuged at 15,000 x g for 20 minutes at 4 ℃. The supernatant containing total cellular proteins was collected for Western blot analysis. Nuclear proteins were extracted using a nuclear extraction kit according to the manufacturer's instructions. Briefly, hypotonic buffer supplemented with 1 mM DTT and 0.01% Nonidet P-40 per gram of tissues was added to minced tissues, which were then homogenized with a gentleMACS Dissociator followed by incubation on ice for 15 minutes. Cytoplasmic proteins were separated by centrifugation. The pellets were resuspended in ice-cold complete nuclear extraction buffer then vortexed on ice for 30 seconds at highest setting. These cell pelleting and vortexing steps were repeated for a total of 6 cycles. The suspensions were then centrifuged at 14,000 x g for 10 minutes at 4 ℃ and supernatants containing nuclear fractions were collected for Western blot analysis.

3.3.9 Western blot analysis

Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes which were probed with primary antibodies, followed after washes by horseradish peroxidase-conjugated secondary antibodies and visualized colorimetrically after further washes using TMB solution. Protein bands were visualized and quantified using a gel documentation system (BioRad, Richmond, Calif).

3.3.10 Statistical analysis

All assays were performed on at least three separate occasions. Results are expressed as the mean value ± standard error of the mean (SEM). Statistical analysis was performed with one-way analysis of variance, with Dunnett's Multiple Comparison Test to identify between-group differences using GraphPad Prism software (version 5.0; GraphPad Software Inc., San Diego, CA). For *in vivo* tumor growth experiments, statistical significance of differences in tumor volume, body weight and total bioluminescence intensity between control and treatment groups was assessed by two-way ANOVA (GraphPad Software Inc., version 5.0, San Diego, CA), with p values < 0.05 considered significant. Statistical significance is expressed as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

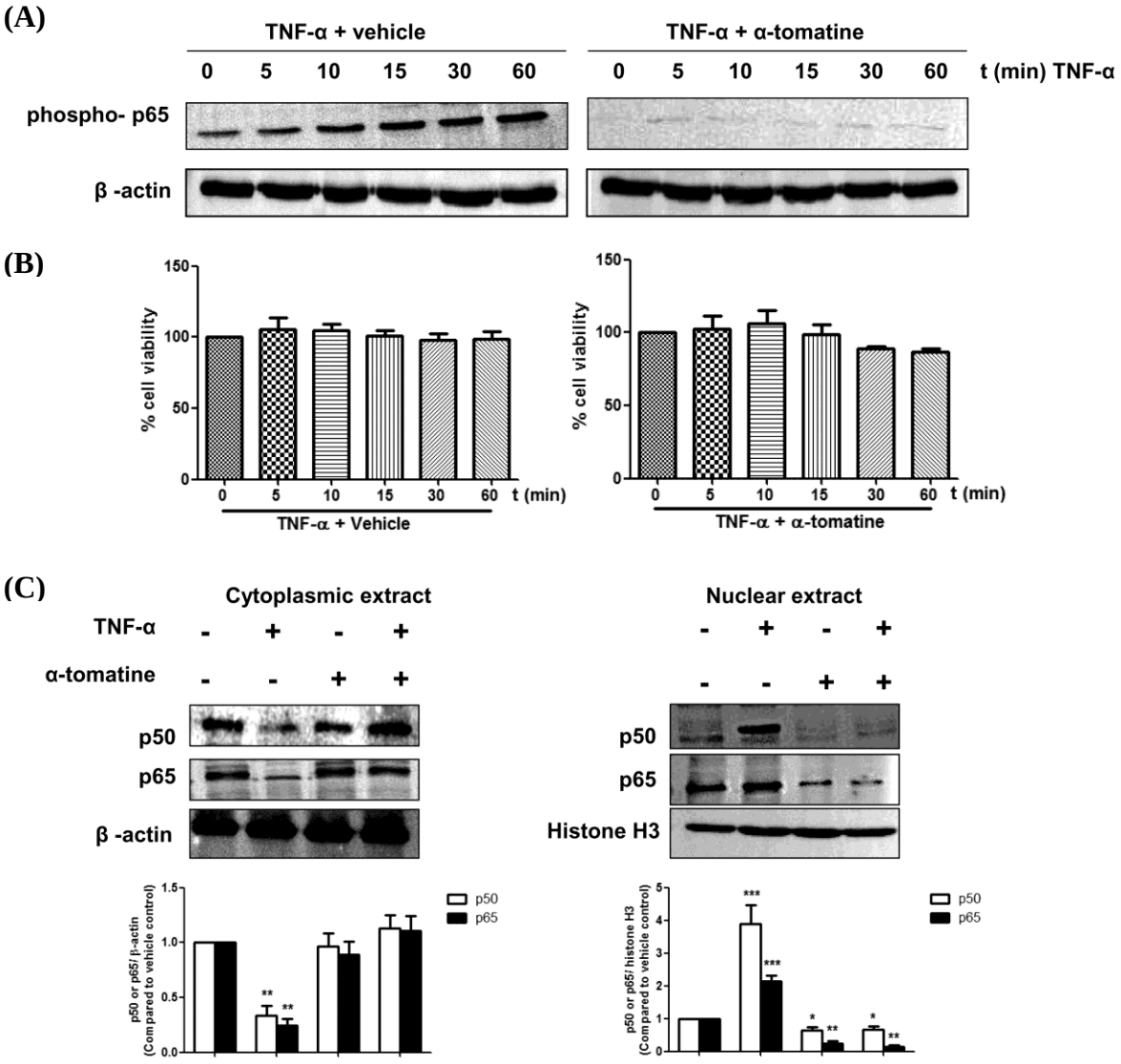
3.4 Results

3.41 Alpha-tomatine inhibits constitutive and TNF-α-induced nuclear translocation of NF-κB p50/p65 and phosphorylation of NF-κB p65

We previously showed that α-tomatine inhibited the growth of androgen-independent human prostatic adenocarcinoma PC-3 *in vitro* with the half maximal effective concentration (EC_{50}) value of 1.67 ± 0.3 μM (Lee *et al.*, 2011). At this chosen dose, α-tomatine was shown to be less cytotoxic to human normal prostate RWPE-1 (EC_{50} = 3.85 ± 0.1 μM) and normal liver WRL-68 cells (EC_{50} > 5 μM). Treatment with 2 μM α-tomatine induces apoptosis and inhibits the TNF-α-induced NF-κB nuclear translocation on PC-3 cells (Lee *et al.*, 2011). In the present study, the mechanism of α-tomatine in inhibition of NF-κB activation was investigated by analyzing its effect on phosphorylation and translocation of NF-κB sub-units in prostate cancer PC-3 cells. PC-3 cell line has been shown to harbor constitutive basal activity of NF-κB due to aberrant IKK activation (Gasparian *et al.*, 2002). As shown in Figure 3.1A, the basal phosphorylation of NF-κB p65 was suppressed by 30 minutes treatment of 2 μM α-tomatine (first lane in right upper panel). The time-dependent phosphorylation of NF-κB p65 induced by TNF-α over a 60 minutes period was also completely suppressed by pretreatment of cells with 2 μM α-tomatine (Figure 3.1A, right upper panel). Examination of treated and control cell populations using a trypan blue exclusion assay indicated that the concentration of α-tomatine and TNF-α used and the time of exposure had minimal effect on cell viability (Figure 3.1B). In addition to the observed loss of p65 phosphorylation, α-tomatine reduced basal nuclear levels of NF-κB p50/p65 subunits (Figure 3.1C, right upper and middle panels, first lane versus third lane) and also suppressed TNF-α induced nuclear translocation of NF-κB p50/p65 subunits (Figure 3.1C, right upper and middle panels, second lane versus forth lane). These findings indicate that α-tomatine impairs the constitutive basal and TNF-α-induced

phosphorylation of NF-κB p65 which in turn prevents the nuclear translocation of NF-κB p50 and p65.

Figure 3.1 Effect of α-tomatine on constitutive and TNF-α-induced phosphorylation of p65 and nuclear translocation of NF-κB p50/p65. (A) PC-3 cells at 70-80% confluency were treated with either 0.1% DMSO (vehicle) or 2 μM α-tomatine in DMSO for 30 minutes, followed by treatment with 10 ng/ml TNF-α for the indicated times. Cytoplasmic extracts were analyzed by Western blot analysis using an antibody against the phosphorylated form of p65. (B) Cell viability was assessed by counting cells that excluded trypan blue using a hemocytometer. (C) Effect of α-tomatine on nuclear and cytoplasmic levels of NF-κB p50 and p65 in human prostate cancer PC-3 cells. Nuclear and cytoplasmic fractions extracted from PC-3 cells treated either 0.1% DMSO or 2 μM α-tomatine in DMSO for 30 minutes, followed by treatment with 10 ng/ml TNF-α for the 30 minutes were analyzed by Western blot analysis with antibodies against NF-κB p50 and p65 proteins. β-actin and histone H3 proteins were loading control for cytoplasmic and nuclear extracts, respectively. Graphical representation of densitometry analysis of NF-κB p50 and p65 Western blot analyses from three independent experiments are shown below each panel. The ratio of the signal intensity of each protein to loading control was normalized to the vehicle control. Statistical significance is expressed as ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ versus vehicle control.


(A)
TNF-α + vehicle
TNF-α + α-tomatine
0 5 10 15 30 60
0 5 10 15 30 60 t (min) TNF-α
phospho- p65
β -actin
(B)
% cell viability
150
100
50
0
0 5 10 15 30 60 t (min)
TNF-α + Vehicle
TNF-α + α-tomatine
(C)
Cytoplasmic extract
Nuclear extract
TNF-α
α-tomatine
p50
p65
β -actin
Histone H3
p50 or p65/ β-actin (Compared to vehicle control)
p50 or p65/ histone H3 (Compared to vehicle control)
p50
p65

3.42 Alpha-tomatine inhibits constitutive and TNF-α-dependent IκBα phosphorylation and degradation

The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination and proteolytic degradation of IκBα (Zandi *et al.*, 1997). To determine whether the observed inhibition of constitutive and TNF-α-induced NF-κB nuclear translocation caused by α-tomatine was due to inhibition of IκBα degradation, we pretreated cells with α-tomatine and then exposed them to TNF-α stimulation for time periods up to 60 minutes. TNF-α induced IκBα degradation in control cells as early as 5 minutes after stimulation (Figure 3.2A, left upper panel). In contrast, in α-tomatine-pretreated cells, expression of IκBα was sustained under both basal and TNF-α stimulated conditions (Figure 3.2B, right upper panel). The effect of α-tomatine on the constitutive basal and TNF-α-induced phosphorylation of IκBα was examined using an antibody that detects IκBα phosphorylated at Ser32 and Ser36. Calpain inhibitor ALLN was used to prevent degradation of phosphorylated IκBα. As shown in Figure 3.2B, the basal constitutive phosphorylation of IκBα in PC-3 was attenuated by α-tomatine (first lane in right upper panel). Western blot analysis indicated that TNF-α further induced basal phosphorylation of IκBα as early as 5 minutes post-stimulation (Figure 3.2B, left upper panel), but α-tomatine completely suppressed this event (Figure 3.2B, right upper panel). These results indicate that α-tomatine inhibits the basal and the TNF-α-induced phosphorylation of IκBα which in turn prevents the degradation of IκBα as well as subsequent nuclear translocation of NF-κB.

3.43 Alpha-tomatine inhibits the constitutive and TNF-α-induced IKK activation

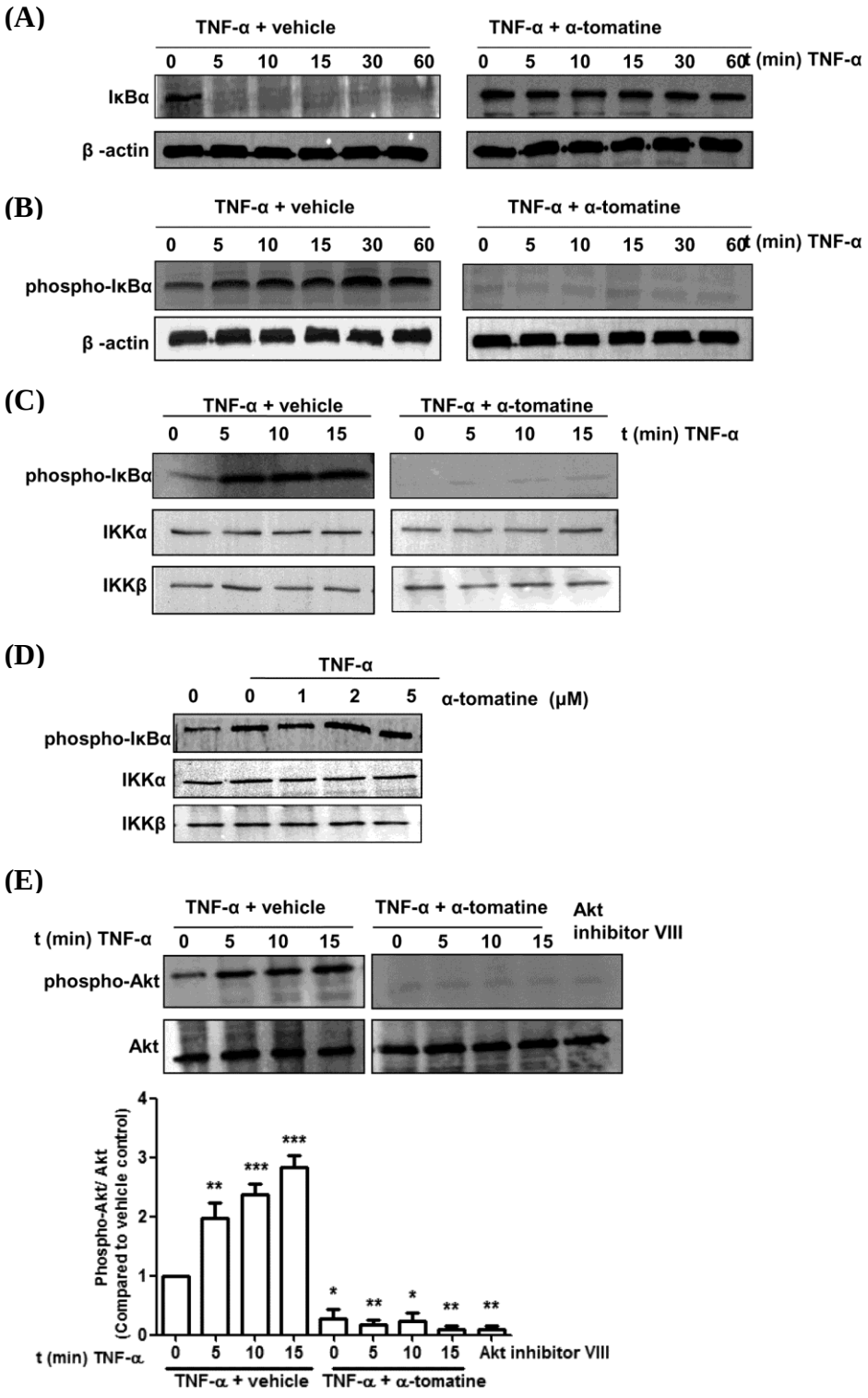
IKK complex is the central regulator of NF-κB activation which phosphorylates and induces degradation of IκBα. TNF-α induces IκBα phosphorylation and degradation via activation of the IKK complex (Zandi *et al.*, 1997). To determine if pretreatment with α-tomatine affects IKK activation, *in vitro* kinase assays were performed using immunoprecipitated components of the IKK complex, and GST-IκBα as the IKK phosphorylation substrate. The phosphorylated substrate was detected by western blotting analysis using a phosphospecific anti-IκBα antibody. As shown in Figure 3.2C, TNF-α treatment further stimulated basal IKK activity as an increase phosphorylated-IκBα was detected as early as 5 minutes after treatment and remained detectable 10 minutes later (Figure 3.2C, left upper panel). In contrast, phosphorylated-IκBα was not detected in cells treated with 2 μM α-tomatine (Figure 3.2C, first lane in right upper panel), and α-tomatine pretreatment also strongly blocked TNF-α-induced IKK activation as no phosphorylated-IκBα was detectable (Figure 3.2C, right upper panel). Western blot analysis demonstrated that TNF-α and α-tomatine had no effect on the expression of the components of the IKK complex, IKKα or IKKβ (Figure 3.2C), indicating that α-tomatine blocked the basal and TNF-α-induced phosphorylation of IκBα by attenuating the action of IKK rather than by causing degradation of this kinase.

In a second set of experiments, we assessed whether α-tomatine suppressed IKK activity by directly binding to IKK by using IKKα and IKKβ immunoprecipitated from cells treated with TNF-α. The kinase reaction mixture was incubated with increasing concentrations of α-tomatine (1, 2 and 5 μM). As shown in Figure 3.2D, whereas TNF-α caused an increase in phosphorylation of IκBα, this was not reduced by inclusion of increasing concentrations of α-tomatine. This suggests that while α-tomatine efficiently inhibits IKK-mediated phosphorylation of IκBα, it did not do this by directly inhibiting IKK.

3.44 Alpha-tomatine inhibits TNF-α-induced Akt activation

It has been reported that the serine-threonine kinase Akt can activate IKK (Ozes *et al.*, 1999). To gain insight into whether this pathway may be relevant in the constitutive and TNF-α–induced activation of NF-κB signaling, we pretreated PC-3 cells with 2 μM α-tomatine for 30 minutes then exposed these cells to TNF-α for time periods up to 15 minutes. In control experiments cells were treated with an Akt inhibitor, Akt inhibitor VIII, at 10 μM for 3 hours as described previously (Estrada *et al.*, 2010). As shown in Figure 3.2E, TNF-α stimulated the basal Akt activation in a time-dependent manner (left upper panel), α-tomatine suppressed both basal and TNF-α-induced Akt activation as effectively as Akt inhibitor VIII with no significant effect on the expression of total Akt protein (right upper and lower panels). This result revealed that α-tomatine impairs the basal and TNF-α-induced IKK activation by suppressing Akt activation.

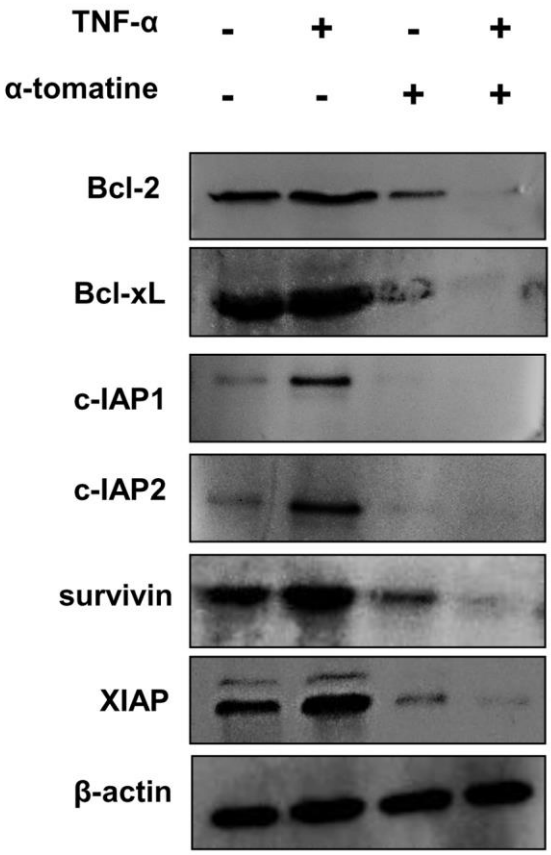
Figure 3.2 Effect of α-tomatine on IκBα Kinase activity. (A) Cells were grown to 70–80 % confluence, treated with either 0.1% DMSO (vehicle) or 2 μM α-tomatine in DMSO for 30 minutes, followed by treatment with 10 ng/ml of TNF-α for the indicated times. The presence of IκBα was detected by Western blot analysis. (B) To determine whether α-tomatine inhibits IκBα degradation by blocking IκBα phosphorylation, cells were treated with either 0.1% DMSO (vehicle) or 2 μM α-tomatine in DMSO for 30 minutes, followed by 50 μg/ml calpain inhibitor ALLN for 30 minutes, and then treated with 10 ng/ml of TNF-α for the times indicated. Anti-phospho-IκBα Western blot analysis was performed on cytoplasmic extracts. β-actin served as a loading control. (C) PC-3 cells were preincubated with either 2 μM α-tomatine or 0.1% DMSO (vehicle) for 30 minutes, and then treated with 10 ng/ml TNF-α for the indicated times. IKKα and IKKβ were immunoprecipitated from lysates from cells and *in vitro* kinase assays were performed using GST-IκBα as substrate. Western blot analysis was performed to detect phosphorylated IκBα. (D) IKK complex immunoprecipitated from vehicle and TNF-α-treated PC-3 cell extracts with an anti-IKKα and IKKβ antibodies was assayed for IKK activity. The kinase reaction mixture was incubated with α-tomatine as indicated. The expressions of phosphorylated IκBα, total IKKα and IKKβ were examined by Western blot analysis. (E) PC-3 cells were pretreated with 2 μM α-tomatine for 30 minutes, and then treated with 10 ng/ml TNF-α for the indicated times. Lysates extracted from cells treated with 10 μM Akt inhibitor VIII for 3 hours serve as inhibition control. The expressions of phosphorylated IκBα, total IKKα and IKKβ were examined by Western blot analysis. Graphical representation of densitometry analysis of phospho-Akt Western blot analysis from three independent experiments is shown below the panel. The ratio of the signal intensity of each protein to loading control was normalized to the vehicle control. Statistical significance is expressed as ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ versus vehicle control.


(A)
TNF-α + vehicle
TNF-α + α-tomatine
0 5 10 15 30 60
0 5 10 15 30 60
t (min) TNF-α
IκBα
β -actin
(B)
TNF-α + vehicle
TNF-α + α-tomatine
0 5 10 15 30 60
0 5 10 15 30 60
t (min) TNF-α
phospho-IκBα
β -actin
(C)
TNF-α + vehicle
TNF-α + α-tomatine
0 5 10 15
0 5 10 15
t (min) TNF-α
phospho-IκBα
IKKα
IKKβ
(D)
TNF-α
0 0 1 2 5
α-tomatine (μM)
phospho-IκBα
IKKα
IKKβ
(E)
TNF-α + vehicle
TNF-α + α-tomatine
Akt inhibitor VIII
t (min) TNF-α
0 5 10 15
0 5 10 15
phospho-Akt
Akt
Phospho-Akt/ Akt (Compared to vehicle control)
0 1 2 3 4
**
***
***
*
**
*
**
**
t (min) TNF-α
0 5 10 15 0 5 10 15
Akt inhibitor VIII
TNF-α + vehicle
TNF-α + α-tomatine

3.45 Alpha-tomatine represses TNF-α-induced NF-κB dependent expression of pro-survival proteins

Several studies have indicated that the transcription factor NF-κB regulates the expression of proteins implicated in facilitating tumor cell survival including Bcl-2, Bcl-xL, c-IAP, survivin and XIAP (Aggarwal, 2004; Plati *et al.*, 2011). Accordingly, we next examined whether α-tomatine inhibition of NF-κB nuclear translocation is accompanied by alterations in the expression of these pro-survival proteins. In these experiments cells were pretreated with α-tomatine (2 μM) for 30 minutes before induction of TNF-α-induced effects for 6 hours. This time period was selected to permit accumulation of levels of pro-survival proteins sufficient for Western blot analysis. As during this time period α-tomatine treatment resulted in varying levels of cell rounding and detachment in treated and control cells, both adherent and non-adherent cells were collected for protein extraction. As shown in Figure 3.3, Western blot analysis revealed that TNF-α induced marked upregulation of c-IAP1 and c-IAP2 while increases in expression of Bcl-xL, survivin and XIAP were also apparent but at lower levels and Bcl-2 expression was marginally increased in response to this cytokine. It was striking that α-tomatine caused sharp down-regulation of each of these mediators of cell survival under basal conditions as well as completely blocking TNF-α-induced upregulation of each protein (Figure 3.3). These data indicate that, consistent with its ability to inhibit TNF-α-induced NF-κB nuclear translocation, α-tomatine blocks the expression of the pro-survival mediators typically upreguated by this cytokine.

Figure 3.3 α-tomatine represses TNF-α-induced NF-κB dependent expression of pro-survival proteins. PC-3 cells grown to 70–80 % confluence were treated with either 0.1% DMSO or 2 μM α-tomatine for 30 minutes, 10 ng/ml TNF-α was then added and the cells were incubated for a further 6 hours. Whole-cell extracts were prepared, and were analyzed by Western blot analysis using antibodies against Bcl-2, Bcl-xL, c-IAP1, c-IAP2, XIAP and survivin. The results shown here are representative of three independent experiments.


TNF-α - + - +
α-tomatine - - + +
Bcl-2
Bcl-xL
c-IAP1
c-IAP2
survivin
XIAP
β-actin

3.46 Alpha-tomatine attenuates growth of PC-3 cell xenograft tumors in mice

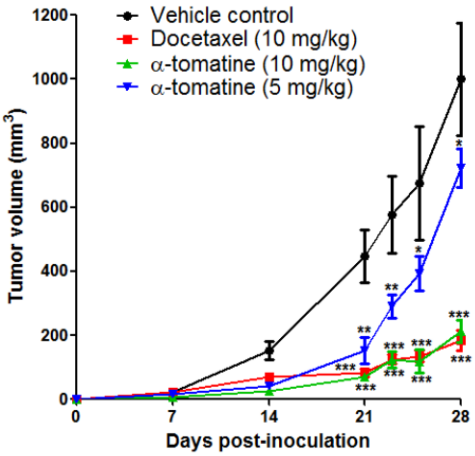
To examine the effect of α-tomatine on tumor growth *in vivo*, we challenged PC-3 cell tumors grown subcutaneously in mice. In these experiments tumors were allowed to establish for 1 week before being challenged 3 times per week for 3 weeks with α-tomatine (5 or 10 mg/kg/mouse) or the prostate cancer therapeutic docetaxel (10 mg/kg/mouse) (Tang *et al.*, 2011) and vehicle solution. Figure 3.4A shows that 10 mg/kg α-tomatine was as efficient as 10 mg/kg docetaxel at retarding growth of PC-3 cell subcutaneous mouse tumors. In addition, there was also a marked decrease in tumor volume in response to lower dose of α-tomatine (5 mg/kg). Specifically, 3 weeks after the commencement of drug challenge the average tumor volume in vehicle control mice (1000 ± 180 mm^3), was approximately 1.4 fold higher as compared to mice treated with 5 mg/kg α-tomatine (720 ± 60mm^3), and approximately 4.7 fold higher compared with mice treated with 10 mg/kg of α-tomatine (210 ± 35 mm^3) or docetaxel (183 ± 32 mm^3) (Figure 3.4A). Of note, α-tomatine treatment at both 5 mg/kg (Figure 3.4B, blue) and 10 mg/kg (Figure 3.4B, green) did not provoke body weight loss in contrast with docetaxel (Figure 3.4B, red) which caused a reduction in body weight of ~10% more than those seen in untreated tumor bearing mice (Figure 3.4B, black). In fact, both control and docetaxel treatment groups showed a trend of decreasing body weight after 14 days of cancer cells inoculation, potentially due to high tumor burden and toxicity of docetaxel, respectively (Figure 3.4B). Consistent with the tumor volumes determined from caliper measurements, the intensity of bioluminescence measured on day 28 was significantly lower in both docetaxel and 10 mg/kg α-tomatine treatment groups (Figure 3.4C and 3.4D). These results suggest that α-tomatine retards the growth of PC-3 cell subcutaneous xenograft tumors at an effective dose of 10 mg/kg.

We also used an orthotopic mouse model to examine the effect of α-tomatine on prostate tumor growth. In this experiment, PC-3 cell tumors were allowed to grow in

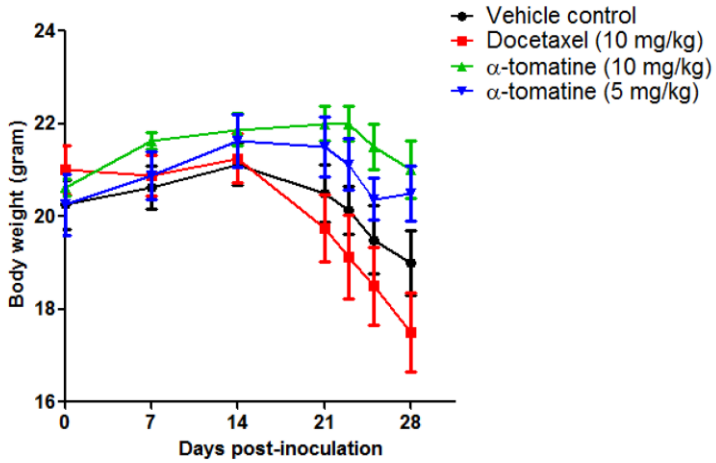
mice for 5 days before thrice weekly treatment for 2 weeks with 10 mg/kg α-tomatine. As shown in Figure 3.5A, and consistent with the data obtained from the xenograft mouse model, α-tomatine significantly suppressed the tumorigenicity of PC-3 cell orthotopic tumors. In addition, the total body weight of mice was not adversely affected by α-tomatine treatment (Figure 3.5B). The images in Figure 3.5C shows strong bioluminescent signal from the prostate of control mice, demonstrating that the tumor cells were successfully implanted. In addition, 4 out of 6 α-tomatine treated mice showed only weak bioluminescent signals and another 2 mice had no signal. These findings demonstrate that α-tomatine has potent anti-tumor effects against mouse xenograft and orthotopic PC-3 cell tumors.

Figure 3.4 Anti-tumor activity of α-tomatine against subcutaneous PC-3 cell tumors. Luciferase expressing PC-3 cell xenograft tumors established in male nude mice (n = 8 per treatment group) for 1 week were treated thrice weekly for 3 weeks with vehicle, docetaxel (10 mg/kg) or α-tomatine (5 or 10 mg/kg). (A) Graph of tumor volume in each treatment group versus the number of days after initial injection of PC-3 cells. (B) Graph of mean body weight for each treatment group versus the number of days after initial injection of PC-3 cells. (C) Bioluminescence intensities emitted from PC-3 cell xenograft tumors at the end of the experiment for each treatment group. (D) Bioluminescence images of PC-3 subcutaneous xenografts. The first row shows the vehicle control group; middle row shows the docetaxel treatment group; bottom row shows the 10 mg/kg α-tomatine treatment group. Each bar or point represents the mean ± SEM of data (n=8). Statistical significance is expressed as ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ versus vehicle control.

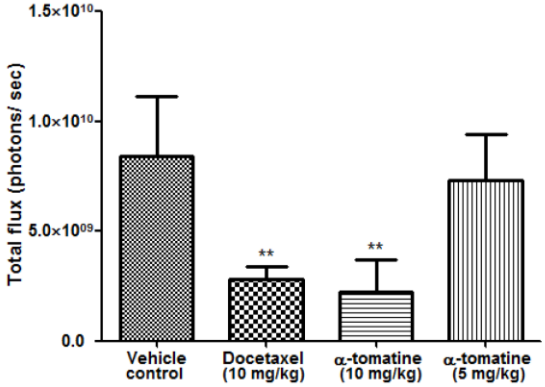
(A)


Vehicle control
Docetaxel (10 mg/kg)
α-tomatine (10 mg/kg)
α-tomatine (5 mg/kg)
Tumor volume (mm3)
Days post-inoculation

(B)


Vehicle control
Docetaxel (10 mg/kg)
α-tomatine (10 mg/kg)
α-tomatine (5 mg/kg)
Body weight (gram)
Days post-inoculation

(C)


Total flux (photons/ sec)
Vehicle control
Docetaxel (10 mg/kg)
α-tomatine (10 mg/kg)
α-tomatine (5 mg/kg)

(D)

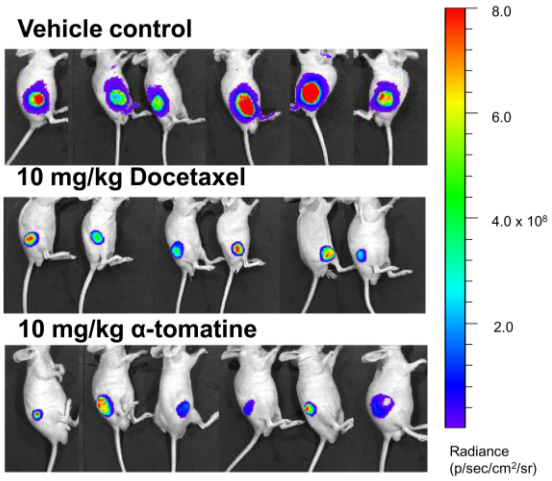
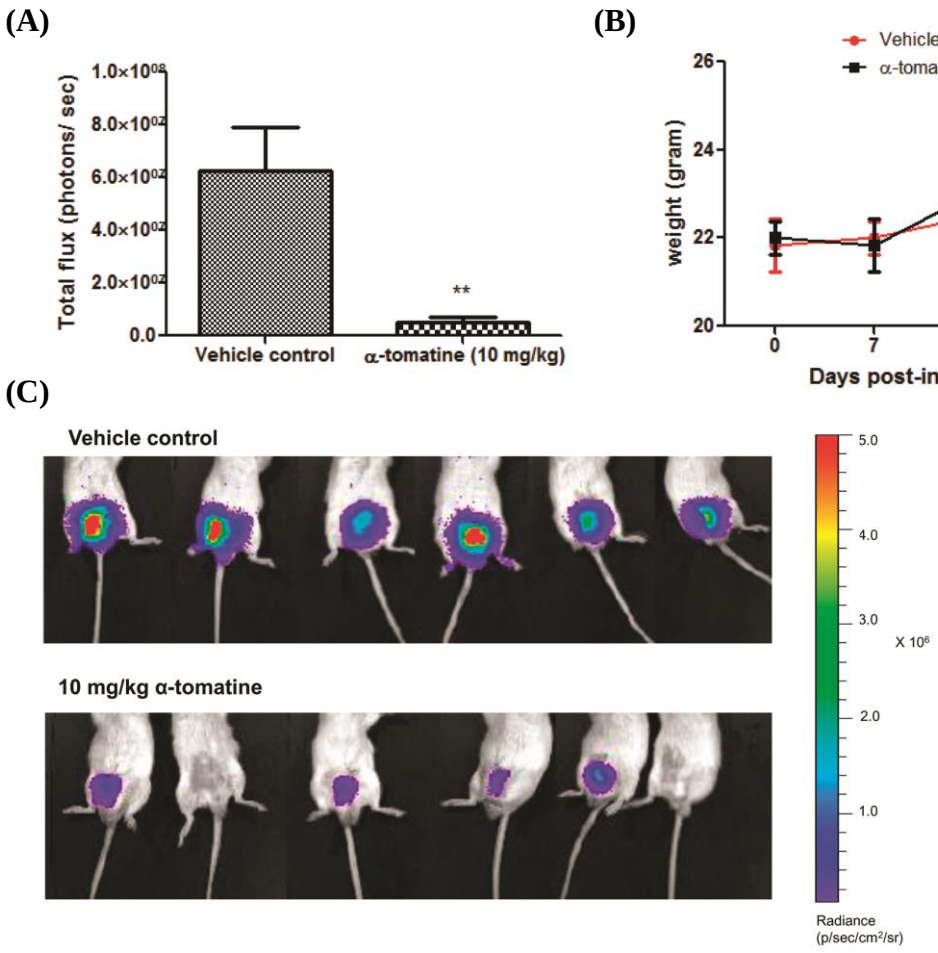

Vehicle control
10 mg/kg Docetaxel
10 mg/kg α-tomatine
Radiance (p/sec/cm²/sr)

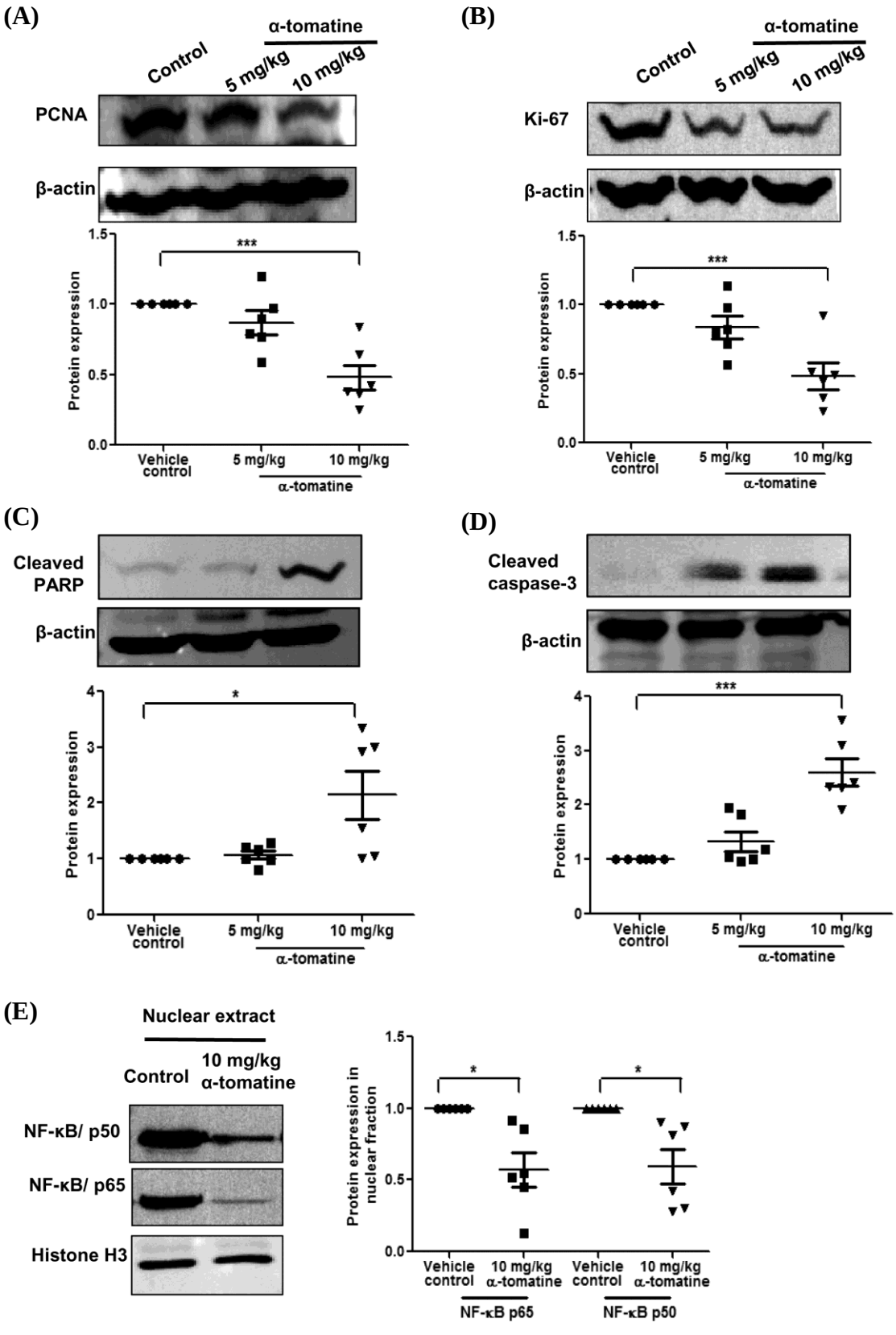
Figure 3.5 Anti-tumor activity of α-tomatine against orthotopic PC-3 cell tumors. Luciferase expressing PC-3 cell orthotopic tumors established in male SCID mice (n = 6 per treatment group) for 5 days were treated thrice weekly for 2 weeks with vehicle or α-tomatine (10 mg/kg). (A) Bioluminescence intensities emitted from PC-3 cell orthotopic tumors for each treatment group after 14 days of treatment. (B) Bioluminescent images at the end of the experiment of SCID mice carrying orthotopic tumors of luciferase expressing PC-3-luc cells. The upper row shows the vehicle control group, whereas the bottom row shows the α-tomatine (10 mg/kg) treatment group. (C) Graph of mean body weight for each treatment group versus the number of days after initial injection of PC-3 cells. Each bar or point represents the mean ± SEM of data (n=6). Statistical significance is expressed as $^{**}p < 0.01$ versus vehicle control.


(A)
Total flux (photons/ sec)
1.0×10^08
8.0×10^07
6.0×10^07
4.0×10^07
2.0×10^07
0.0
**
Vehicle control
α-tomatine (10 mg/kg)
(B)
Vehicle control
α-tomatine (10 mg/kg)
weight (gram)
26
24
22
20
0
7
14
21
Days post-inoculation
(C)
Vehicle control
10 mg/kg α-tomatine
5.0
4.0
3.0
X 10^6
2.0
1.0
Radiance
(p/sec/cm²/sr)

3.47 Alpha-tomatine reduces expression of proliferation markers, increases expression of apoptosis markers and inhibits nuclear translocation of NF- κB in xenograft tumors

To examine the mechanism by which α-tomatine suppressed the growth of PC-3 cell tumors in mice, we next examined tumor tissue recovered from mice carrying subcutaneous tumors for expression of markers of proliferation (PCNA and Ki-67) and apoptosis (cleaved PARP and cleaved caspase-3). This analysis was not possible for orthotopic tumors as insufficient material was available for the α-tomatine treated mice. As shown in Figure 3.6, Western blot analysis of lysates from six independent tumor samples indicated that while both markers of proliferation (PCNA and Ki-67) decreased in response to α-tomatine treatment (Figure 3.6A and 3.6B), levels of both markers of apoptosis (cleaved PARP and caspase-3) increased (Figure 3.6C and 3.6D). These data suggest that both anti-proliferative and pro-apoptotic effects of α-tomatine contribute to the reduced growth of PC-3 cell tumors in mice. As NF-κB translocation to the nucleus is important for promoting increased cell proliferation and survival and we have shown that this translocation event is reduced *in vitro* by α-tomatine, we were interested to examine recovered subcutaneous mouse tumors for localization of NF-κB. As shown in Figure 3.6E, Western blot analysis from six independent tumor samples indicated that there was a distinct decrease in the levels of NF-κB components in the nucleus of tumor cells in response to α-tomatine treatment. These data suggest that the anti-tumor effects of α-tomatine *in vivo* may be due to its ability to block the proliferative and anti-apoptotic effects of NF-κB signaling by reducing its translocation to the nucleus.

Figure 3.6 Western blot analysis of PCNA, Ki-67, cleaved-PARP, cleaved-caspase-3 and NF-κB in PC-3 tumor tissues samples. (A-D) PC-3 tumors excised from mice treated with vehicle, 5 mg/kg or 10 mg/kg of α-tomatine were lysed and examined by Western blot analysis. Representative images are shown. Markers of proliferation (PCNA and Ki-67). Markers of apoptosis (cleaved PARP and cleaved caspase-3). β-actin was used as loading control. (E) Western blot analysis on nuclear extracts probing for NF-κB p50 and p65 levels. Equal loading of protein was confirmed by stripping and reprobing the blots with histone H3 antibody. Graphical representation of densitometry analysis of each Western blot data from six independent tumor samples is shown below each panel. The ratio of the signal intensity of each protein to loading control was normalized to the vehicle control. Statistical significance is expressed as ***$p < 0.001$; *$p < 0.05$ versus vehicle control.


(A)
α-tomatine
Control
5 mg/kg
10 mg/kg
PCNA
β-actin
Protein expression
***
Vehicle control
5 mg/kg
10 mg/kg
α-tomatine
(B)
α-tomatine
Control
5 mg/kg
10 mg/kg
Ki-67
β-actin
Protein expression
***
Vehicle control
5 mg/kg
10 mg/kg
α-tomatine
(C)
Cleaved PARP
β-actin
Protein expression
*
Vehicle control
5 mg/kg
10 mg/kg
α-tomatine
(D)
Cleaved caspase-3
β-actin
Protein expression
***
Vehicle control
5 mg/kg
10 mg/kg
α-tomatine
(E)
Nuclear extract
Control
10 mg/kg α-tomatine
NF-κB/ p50
NF-κB/ p65
Histone H3
Protein expression in nuclear fraction
*
*
Vehicle control
10 mg/kg α-tomatine
Vehicle control
10 mg/kg α-tomatine
NF-κB p65
NF-κB p50

3.5 Discussion

We provide the first evidence that the saponin α-tomatine can efficiently inhibit the growth of prostate cancer tumors *in vivo* without inducing overt toxicity. Our analysis of recovered mouse tumors suggest that mechanistically α-tomatine mediates its anti-prostate cancer effects *in vivo* by blocking, at least in part, the proliferative and anti-apoptotic effects of NF-κB signaling by reducing translocation of this protein complex to the nucleus. Our detailed *in vitro* analyses indicate that α-tomatine suppression of NF-κB activation occurs through indirect inhibition of IKK kinase activity, leading to suppression of IκBα phosphorylation and degradation, NF-κB/p65 phosphorylation and NF-κB p50/p65 nuclear translocation. Consistent with its ability to induce apoptosis, α-tomatine inhibition of NF-κB activation *in vitro* was accompanied by significant reduction in expression of TNF-α induced pro-survival mediators c-IAP, Bcl-2, Bcl-xL, survivin and XIAP.

The NF-κB signaling pathway is an important target for disease treatment because its dysregulation is required for inappropriate inflammatory responses as well as cancer and other ailments (Sethi & Tergaonkar, 2009). In response to most activating stimuli, NF-κB signaling occurs through sequential activation of IKK, phosphorylation of IκBα at serine 32 and 36, leading to its degradation, and the nuclear translocation of NF-κB where it regulates transcription of a range of genes including those that promote cell proliferation and survival (Karin & Lin, 2002). Consistent with our data from α-tomatine, suppression of NF-κB activation has been shown to be a critical mechanism of action of several plant-derived anticancer agents, such as curcumin, lycopene, silibinin, genistein, resveratrol and green tea polyphenols (Gupta *et al.*, 2010). In the present study, we showed that α-tomatine is an indirect inhibitor of IKK. Presumably, α-tomatine inhibits upstream signaling components that lead to activation of the IKK complex, such as Akt serine-threonine kinase, NF-κB inducing kinase, mitogen-

activated/extracellular signal-regulated kinase kinase 1 (MEKK1), MEKK3, TGF activated kinase 1 (TAK1) and glycogen synthase kinase-3 beta (Aggarwal *et al.*, 2004; Hoeflich *et al.*, 2000; Lee *et al.*, 1998; Sakurai *et al.*, 1999; Yang *et al.*, 2001). Indeed, we found that α-tomatine inhibits TNF-α-induced Akt activation, although further experiments are needed to address if this effect is responsible for the changes we observed in phosphorylation and translocation of NF-κB components or the anti-tumor actions of α-tomatine against subcutaneous and orthotopic tumors grown in mice. These results are in agreement with those of previous studies that suggest that α-tomatine suppresses invasion and migration of human lung cancer cells *in vitro* through the inhibition of the Akt (Shieh *et al.*, 2011; Shih *et al.*, 2009). As conventional chemotherapeutic agents, including docetaxel target normal as well as tumor cells and lead to deleterious effects for prostate cancer patients there is a pressing need to identify less toxic agents to control this disease. In recent years, a number of naturally occurring dietary agents of reduced toxicity have been reported to induce apoptosis and inhibit tumor growth, highlighting the promise of using naturally derived agents for chemotherapy and chemoprevention of prostate and other cancers (Cragg *et al.*, 2009; Khan *et al.*, 2010; Nobili *et al.*, 2009).

Furthermore, it is also possible that an inhibitor of NF-κB activation could be an adjuvant for overcoming tumor resistance to radiation and chemotherapies, such as paclitaxel, doxorubicin, 5-fluorouracil, and vinca alkaloids (vinblastine and vincristine), that occur via NF-κB activation (Das & White, 1997; Uetsuka *et al.*, 2003; Wang *et al.*, 2003). It is thought that this induced resistance in a wide variety of tumor cells occurs via induction of NF-κB effector genes, including Bcl-2 (Catz & Johnson, 2001), Bcl-xL (Tamatani *et al.*, 1999), survivin (Zhu *et al.*, 2001), XIAP (Stehlik *et al.*, 1998), c-IAP1 (Gill *et al.*, 2009) and c-IAP2 (Gill *et al.*, 2009) that are known to mediate protective responses to chemotherapeutic agents and radiation. Therefore, targeting NF-κB

through the actions of α-tomatine, which we have shown to block NF-κB activation and transcription of NF-κB effector genes, may result in improvements in treatment of prostate cancer. In support of this, other dietary agents, including resveratrol, curcumin, genistein, (−) epigallocatechin gallate and soya isoflavone that can block various steps leading to NF-κB activation and sensitize tumor cells to the beneficial effects of chemotherapeutic drugs and radiation in treatment of cancer (Akimoto *et al.*, 2001; Fulda & Debatin, 2004; Komatsu *et al.*, 1997; Kunnumakkara *et al.*, 2009; Li *et al.*, 2005; Sandur *et al.*, 2009; Zoberi *et al.*, 2002).

We previously reported that α-tomatine induces caspase-dependent death of PC-3 cells *in vitro* accompanied by increased caspase-3 activity and the release of cytochrome c (Lee *et al.*, 2011). Caspases have been shown to be involved in apoptosis through activation of PARP downstream molecule (Kaufmann *et al.*, 1993). Importantly, here we have demonstrated the pro-apoptotic effect of α-tomatine *in vivo* by showing a significant increase in the cleavage of PARP and caspase-3 in α-tomatine-treated tumors. In addition, our data show that α-tomatine elicits anti-proliferative effects *in vivo* as we observed reduced levels of the markers of proliferation Ki-67 and PCNA. Our data from PC-3 tumors also suggest that these pro-apoptotic and anti-proliferative effects of α-tomatine are mediated, at least in part, by reduced nuclear translocation of NF-κB p50 and p65. Indeed our *in vitro* studies clearly demonstrate that α-tomatine is very effective at blocking activation and translocation of the components of the NF-κB complex providing support for the possibility that this mechanism is also important for its effects *in vivo* against prostate cancer PC-3 cell tumors.

3.6 Conclusion

In summary, we present the first evidence that α-tomatine is an effective anti-tumor compound against prostate cancer xenograft and orthotopic tumors. This agent may prove to be useful in the prevention and treatment of androgen-independent prostate cancer and this warrants further investigation.

CHAPTER 4

ALPHA-TOMATINE SYNERGISES WITH PACLITAXEL TO ENHANCE APOPTOSIS OF ANDROGEN-INDEPENDENT HUMAN PROSTATE CANCER PC-3 CELLS *IN VITRO* AND *IN VIVO*

The contents in this chapter have been published in Lee, S. T., Wong, P. F., Hooper, J. D., Mustafa, M. R. (2013). Alpha-tomatine synergises with paclitaxel to enhance apoptosis of androgen-independent human prostate cancer PC-3 cells *in vitro* and *in vivo*. *Phytomedicine, 20*(14), 1297-1305.

4.1 Abstract

Alpha (α)-tomatine, a major saponin found in tomato has been shown to inhibit the growth of androgen-independent prostate cancer PC-3 cells. The effects of α-tomatine in combination with the chemotherapeutic agent paclitaxel against PC-3 cells were investigated in the present study. Combined treatment with a sub-toxic dose of α-tomatine and paclitaxel significantly decreased cell viability with concomitant increase in the percentage of apoptotic PC-3 cells. The combined treatment, however, had no cytotoxic effect on the non-neoplastic prostate RWPE-1 cells. Apoptosis of PC-3 cells was accompanied by the inhibition of PI3K/Akt pro-survival signaling, an increase in the expression of the pro-apoptotic protein BAD but a decrease in the expressions of anti-apoptotic proteins, Bcl-2 and Bcl-xL. Results from a mouse xenograft model showed the combined treatment completely suppressed subcutaneous tumor growth without significant side effects. Consistent with its *in vitro* anti-cancer effects, tumor materials from mice showed increased apoptosis of tumor cells with reduced protein expression of activated PI3K/Akt. These results suggest that the synergistic anti-cancer effects of paclitaxel and α-tomatine may be beneficial for refractory prostate cancer treatment.

4.2 Introduction

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in men worldwide (Jemal *et al.*, 2011). The majority of patients with advanced prostate cancer respond well to initial treatment with anti-hormonal therapies, but almost all of these patients will eventually develop hormone-refractory prostate cancer and bone metastatic disease (Bracarda *et al.*, 2005). Taxane-based drugs such as paclitaxel and docetaxel are potent chemotherapeutic agents used for the treatment of hormone-refractory prostate cancer (Mancuso *et al.*, 2007). These

drugs are microtubule inhibitors which bind to β-tubulin resulting in microtubule stabilization, disruption of mitosis and ultimately apoptosis (Schiff *et al.*, 1979). The main limitations of taxane-based drugs include inherent toxicity associated with their use and short-lived survival benefit of approximately 2 to 3 months largely due to chemoresistance (Chang, 2007; Dagher *et al.*, 2004). Accordingly, there remains an unmet need for the identification and development of novel agents that can be used to improve the treatment outcomes of chemotherapy.

We are interested in the utility of naturally occurring plant compounds as agents to safely augment the anti-cancer activity of standard chemotherapies. Alpha (α)-tomatine, a major saponin present in tomato has been shown to have potent *in vitro* and *in vivo* anti-tumor activity against human prostate cancer PC-3 cells (Lee *et al.*, 2011; Lee *et al.*, 2013). These reports indicate that α-tomatine-induced apoptosis of PC-3 cells is mediated, at least in part, through the inhibition of nuclear factor-kappa B (NF-κB) nuclear translocation (Lee *et al.*, 2011; Lee *et al.*, 2013). In the present study, we examined the ability of α-tomatine to augment the anti-cancer actions of a taxane-based drug, paclitaxel. Accordingly, we sought to examine the effect of the combined α-tomatine and paclitaxel treatment on constitutively active phosphoinositide 3-kinase (PI3K)/Akt signaling, which is an upstream mediator of NF-κB and confers chemoresistance in advanced prostate cancer (Lee *et al.*, 2004; Madrid *et al.*, 2000).

4.3 Materials and Methods

4.3.1 Materials

Alpha-tomatine was purchased from Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), 3,3,5,5 tetramethylbenzidine (TMB), paclitaxel and anti-human beta (β)-actin antibody were purchased from Sigma-Aldrich (USA). Penicillin/streptomycin, Roswell Park Memorial Institute (RPMI-1640), keratinocyte growth medium, fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) were purchased from Invitrogen (USA). An antibody against Akt was obtained from Santa Cruz Biotechnology, USA. An Akt kinase assay kit, phosphospecific anti-Akt (Ser473), phosphospecific anti-PI3K p85 (Tyr458)/p55 (Tyr199), anti-PI3K**,** anti-Bcl-2, anti-Bcl-xL, anti-BAD, anti-cleaved Poly (ADP-ribose) polymerase (PARP), and anti-cleaved caspase-3 antibodies were purchased from Cell Signaling Technologies (USA). Antibody against proliferating cell nuclear antigen (PCNA) was purchased from BD Biosciences (USA).

4.3.2 Cell lines

Androgen-independent prostate cancer PC-3, androgen-dependent prostate cancer LNCaP and non-neoplastic human prostate epithelial RWPE-1 cell lines were obtained from the American Type Culture Collection. The luciferase-expressing PC-3 cell line was a kind gift from Dr. Patrick Ming Tat Ling, Queensland University of Technology, Australia. PC-3, LNCaP and luciferase-expressing PC-3 cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. RWPE-1 cells were cultured in keratinocyte growth medium. All cells were maintained at 37 ℃ in a 5% CO_2 humidified incubator.

4.3.3 *In vitro* cytotoxicity assay

Briefly, 18–24 hours after seeding, adherent cells in sterile 96-wells plates were treated for 24 hours with α-tomatine and paclitaxel alone or in combination at concentrations shown in the relevant figure legend. Cell viability was determined following 24 hours treatment using the MTT assay as described previously (Lee *et al.*, 2011).

4.3.4 Assessment of the effect of combined drug treatments in PC-3 cells

The effect of combined treatment of α-tomatine and paclitaxel was quantified by determining the combination index (CI) according to the algorithm of Chou and Talalay (Chou, 2010) using CalcuSyn software (Biosoft, Inc., Cambridge, United Kingdom). A CI value of 1 indicates an additive effect whereas < 1 represents synergism and >1 antagonism (Chou, 2010).

4.3.5 Cell cycle analysis

Cell cycle distribution was examined by propidium iodide (PI) staining of nuclei acids using a BD Cycletest Plus DNA Reagent Kit as described previously (Dasmahapatra *et al.*, 2004).

4.3.6 Assessment of apoptosis by annexin V/propidium iodide (PI) double staining assay

Apoptosis-mediated death of tumor cells was examined by a double staining method using a fluoresceinisothiocyanate (FITC)-labeled annexin V/PI apoptosis detection kit (BD Bioscience, San Jose, CA) as described in our previous paper (Lee *et al.*, 2011).

4.3.7 Cell lysis

PC-3 cells at 70-80% confluence were treated with vehicle solution, 1 μM α-tomatine, 5 nM paclitaxel or α-tomatine and paclitaxel in combination. Protein extraction was performed as described in our previous paper (Lee *et al.*, 2013).

4.3.8 Western blot analysis

Separation of proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and densitometric scanning of the membranes were performed as described in our previous paper (Lee *et al.*, 2013). Signal intensity of the respective protein was normalized to loading control and expressed as a percentage of vehicle treated control.

4.3.9 Assessment of Akt kinase activity

Akt kinase activity was quantified using an Akt kinase assay kit (Cell Signaling Technology, Inc.), following the instructions of the manufacturer. Protein lysates from treated PC-3 cells were incubated overnight with an immobilized anti-phospho-Akt monoclonal antibody (bead conjugate). The enzymatic activity of immunoprecipitated phosphorylated Akt was then assessed *in vitro* using the supplied glycogen synthase kinase (GSK)-3α/β fusion protein as substrate, in the presence of ATP. Phosphorylation of GSK-3α/β was detected using an anti-phospho-GSK-3α/β antibody. The signal intensity of phospho-GSK-3α/β was normalized to total GSK-3α/β and presented as a percentage of vehicle treated control.

4.3.10 Growth of subcutaneous PC-3 cell tumors in mice

Experiments with mice were performed in accordance with a protocol approved by the University of Queensland Animal Ethics Committee. Male BALB/c nude mice (6 weeks old) were purchased from the Animal Resources Centre (Canning Vale, Western Australia). Luciferases-expressing PC-3 cells were inoculated subcutaneously into the lower flanks of each mouse as described in our previous paper. On day 7 after cancer cell inoculation, each mouse had one palpable tumor and were randomly assigned to four groups (n = 6/group). These groups of mice were then given intraperitoneal injections of vehicle solution, 5 mg/kg paclitaxel, 5 mg/kg α-tomatine or 5 mg/kg α-tomatine + 5 mg/kg paclitaxel thrice weekly for an additional 3 weeks. The mice were monitored weekly for tumor growth and body weight. Tumor dimensions were measured with calipers and the tumor volumes were calculated using a standard formula: (length x width^2) x 0.5 (Plymate *et al.*, 2003). The experiment was terminated 28 days after cancer cell inoculation, from which the time bioluminescent signals of tumors in live mice were first captured using a Xenogen IVIS Spectrum imaging system (USA) as described in our previous paper (Lee *et al.*, 2013).

4.3.11 Tissue processing and protein extraction

Tissue processing and protein extraction were performed as described in our previous paper (Lee *et al.*, 2013).

4.3.12 Statistical analysis

In vitro assays were performed in at least three separate experiments. Results are expressed as the mean value ± standard error of the mean (SEM). Statistical analysis was performed with one-way analysis of variance (ANOVA), with Dunnett's Multiple Comparison Test to identify between-group differences using GraphPad Prism software

(San Diego, CA). For *in vivo* tumor growth experiments, statistical significance of the differences in tumor volume, body weight and total bioluminescence intensity between control and treatment groups was assessed by two-way ANOVA using GraphPad Prism software (San Diego, CA), with p values < 0.05 considered significant. Statistical significance is expressed as ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$.

4.4 Results

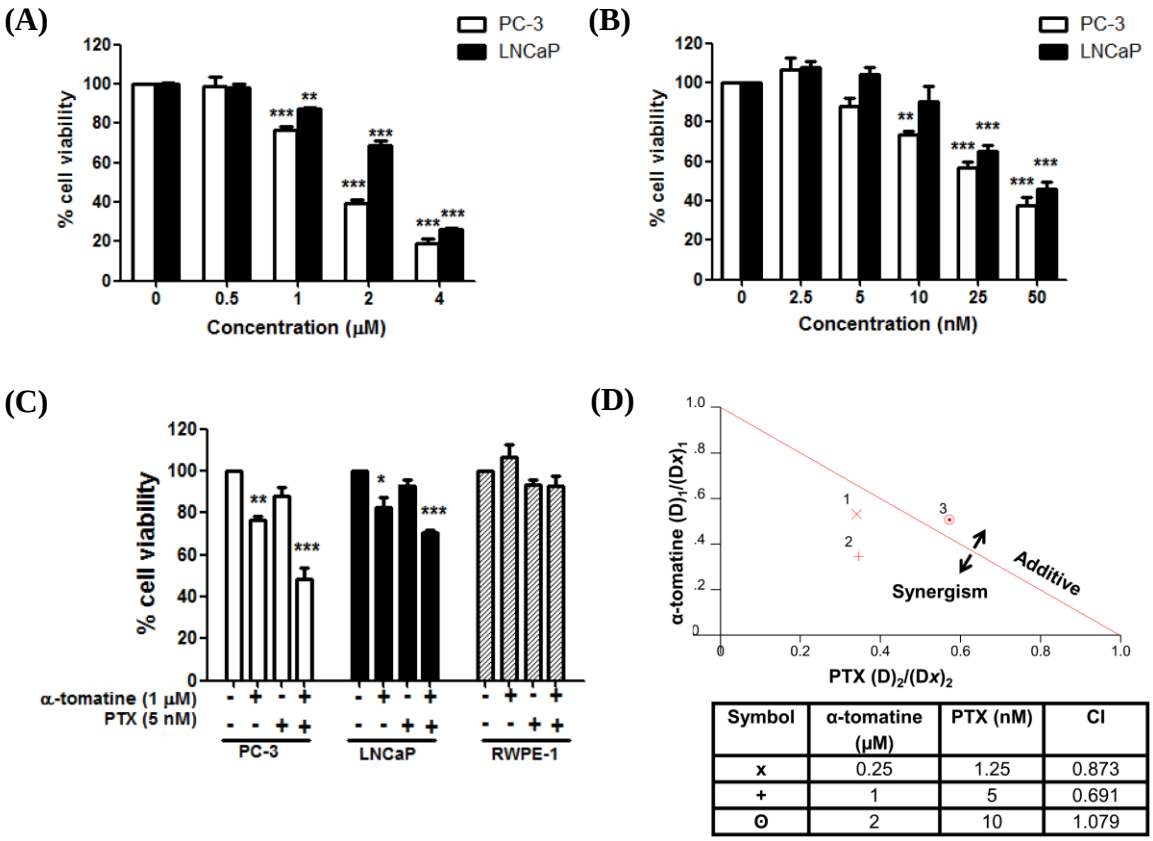
4.4.1 Alpha-tomatine acts synergistically with paclitaxel to inhibit the *in vitro* growth of PC-3 cells

The *in vitro* growth inhibitory effects of α-tomatine and paclitaxel on prostate cancer cells was firstly evaluated as single agent at concentrations of 0.5, 1, 2 and 4 μM for α-tomatine and 2.5, 5, 10, 25 and 50 nM for paclitaxel on PC-3 and LNCaP cancer cells. The α-tomatine concentrations were chosen based on our previous report of half maximal concentration (EC_{50}) in PC-3 cancer cells at 1.67 ± 0.3 μM (Lee *et al.*, 2011). As shown in Figure 4.1A, the growth of PC-3 and LNCaP cells were significantly inhibited by α-tomatine in a dose-dependent manner. Treatment with α-tomatine for 24 hours inhibited the proliferation of LNCaP cells with an EC_{50} value of 2.65 ± 0.1 μM. PC-3 cells, hence, were more sensitive to growth inhibition by α-tomatine compared to LNCaP cells.

Paclitaxel inhibited the proliferation of PC-3 and LNCaP cells in a dose-dependent manner, with an EC_{50} at 32.11 ± 4.39 nM for PC-3 cells and 44.80 ± 2.49 nM for LNCaP cells at 24 hours exposure (Figure 4.1B). We next tested the effect of combined drug treatment at sub-maximal levels of toxicity (1 μM α-tomatine and 5 nM paclitaxel) on PC-3, LNCaP and non-neoplastic prostate RWPE-1 cells. MTT assay revealed that this combination of treatment doses resulted in greater growth inhibition with PC-3 cell viability reduced to ~50% (Figure 4.1C). In contrast, the combined treatment reduced LNCaP cell viability to only ~30% (Figure 4.1C). Viability of non-neoplastic prostate cells was largely unaffected by single agent or combined drug treatments (Figure 4.1C). These results showed that a greater anti-proliferative effect of α-tomatine was observed in paclitaxel-treated PC-3 cells compared to treatments using α-tomatine or paclitaxel alone and in the treated LNCaP cells. Hence, subsequent mechanistic and animal studies were performed only on PC-3 cells.

Chou-Talalay method was used to determine whether the combined drugs action are antagonistic, additive or synergistic (Chou, 2010). CI values obtained using this method were plotted and tabulated in Figure 4.1D. These compounds were found to act synergistically against PC-3 cells at the dose of 1 μM α-tomatine and 5 nM paclitaxel with a CI value of 0.691 (Figure 4.1D).

Figure 4.1 Effect of α-tomatine and paclitaxel on growth of PC-3, LNCaP and RWPE-1 cells *in vitro*. Dose–dependent cytotoxicity of (A) α-tomatine and (B) paclitaxel in PC-3 and LNCaP cancer cells. (C) Cytotoxic effect of combined 1 μM α-tomatine and 5 nM paclitaxel (PTX) treatment in PC-3, LNCaP and RWPE-1 cells. Data are shown as mean ± SEM of three independent experiments. Statistical significance is expressed as ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ versus vehicle control. (D) Top, Isobologram analysis of the cytotoxicity of α-tomatine and paclitaxel (PTX) treatments alone or in combination. Bottom, CI values determined from the formula described in Materials and Methods.



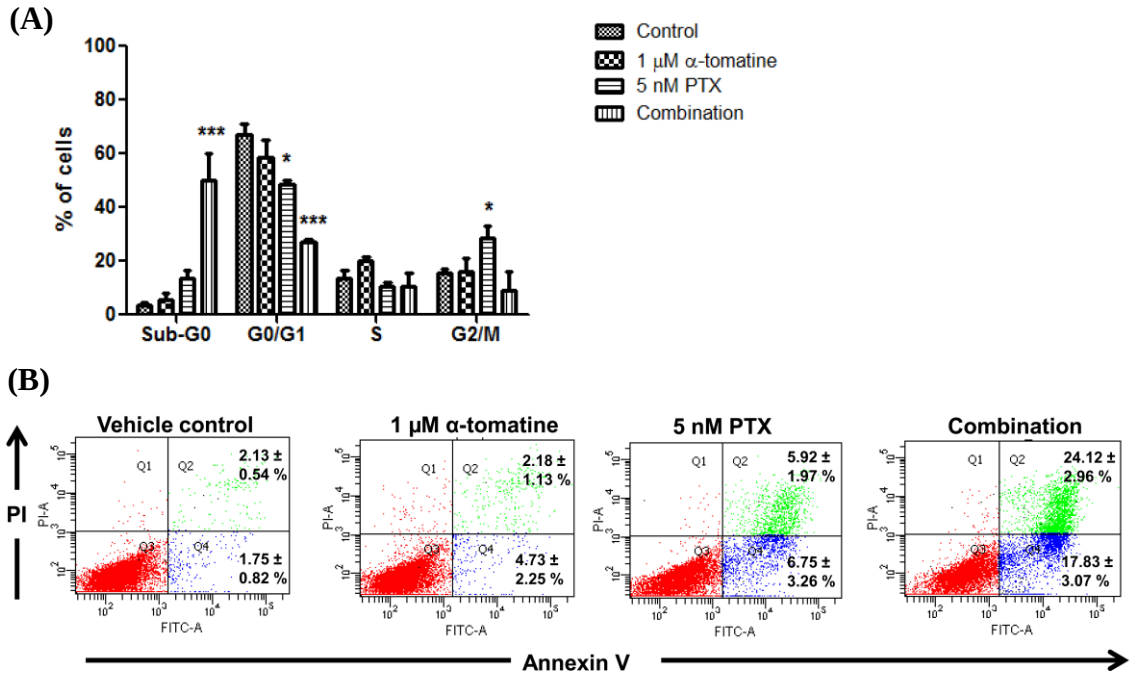
Symbol	α-tomatine (μM)	PTX (nM)	CI
x	0.25	1.25	0.873
+	1	5	0.691
⊙	2	10	1.079

4.4.2 Induction of apoptosis by α-tomatine and paclitaxel in PC-3 cells

Paclitaxel is known to induce G2/M cell cycle arrest (Horwitz, 1992). To examine whether the synergistic effect of paclitaxel and α-tomatine is due to enhancement of paclitaxel-induced G2/M arrest by α-tomatine, we performed flow cytometric analysis of PI-stained PC-3 cells to evaluate the effect of single agent and combination treatments on cell cycle distribution. Combined treatment of α-tomatine and paclitaxel abrogated the G2/M arresting effects of individual paclitaxel treatment. The combined treatment, however, resulted in an increased distribution of cell in sub-G0 phase (50.23 ± 9.86%), compared to 5.39 ± 2.51% and 13.38 ± 3.22% in cells treated with 1 μM α-tomatine and 5 nM paclitaxel alone, respectively (Figure 4.2A), suggesting that there is an increase in apoptotic population.

We next performed annexin V/PI double staining of the treated PC-3 cells to assess the proportion of cells progressing through the phases of apoptotic cell death. In these analyses, cells in the early stages of apoptosis were stained positively with annexin V, whereas cells in late stages of apoptosis were stained positively with both annexin V and PI. As shown in Figure 4.2B, PC-3 cells treated with α-tomatine in combination with paclitaxel showed a marked increase in cells in both the early (17.83 ± 3.07 %) and late (24.12 ± 2.96%) stages of apoptosis compared to cells treated with only α-tomatine (early 4.73 ± 2.25%; late 2.18 ± 1.13%) or paclitaxel (early 6.75 ± 3.26%; late 5.92 ± 1.97%). Taken together, these results showed that combined α-tomatine and paclitaxel treatment induces more cell death than the individual agents.

Figure 4.2 Effect of α-tomatine and paclitaxel (PTX) on cell cycle distribution and apoptosis of PC-3 cells. (A) The percentages of PC-3 cells at sub-G0, G0/G1, S and G2/M phases 24 h after drug treatments. (B) Flow cytometric analysis of apoptotic PC-3 cells using annexin V/PI double staining following 24 hours with the indicated treatments. Data are shown as means ± SEM of three independent experiments. Statistical significance is expressed as ***$p < 0.001$; *$p < 0.05$ versus vehicle control.


(A)
Control
1 μM α-tomatine
5 nM PTX
Combination
% of cells
100
80
60
40
20
0
***
*
***
*
Sub-G0
G0/G1
S
G2/M
(B)
Vehicle control
1 μM α-tomatine
5 nM PTX
Combination
PI
Annexin V
PI-A
FITC-A
Q1
Q2
Q3
Q4
2.13 ± 0.54 %
1.75 ± 0.82 %
2.18 ± 1.13 %
4.73 ± 2.25 %
5.92 ± 1.97 %
6.75 ± 3.26 %
24.12 ± 2.96 %
17.83 ± 3.07 %

4.4.3 Synergism of α-tomatine and paclitaxel growth inhibition is accompanied by the inhibition of PI3K/Akt signaling and altered expression of downstream regulators of apoptosis

We have previously shown that α-tomatine inhibited nuclear translocation of NF-κB in PC-3 cells (Lee *et al.*, 2011). To extend this analysis, we investigated the effect of this combined α-tomatine and paclitaxel treatment on PI3K/Akt, an important determinant of chemoresistance and upstream mediator of NF-κB (Lee *et al.*, 2004; Madrid *et al.*, 2000). The enzymatic activity of Akt was firstly examined by an *in vitro* kinase assay using a GSK-3α/β fusion protein as substrate. PC-3 cells were treated with 1 μM α-tomatine, 5 nM paclitaxel or in combination for 3 hours before the onset of apoptosis. Treated and control cell populations were examined using trypan blue exclusion assay which indicated that this exposure had minimal effect on cell viability (Appendix A). Western blot analysis showed that combined α-tomatine and paclitaxel treatment caused a marked reduction in phospho-GSK-3α/β levels (Figure 4.3A, left) where Akt activity was reduced by ~60% (Figure 4.3A, right). In contrast, single agent treatment with α-tomatine and paclitaxel caused ~28% reduction and ~30% increase in Akt activity, respectively (Figure 4.3A). We also directly assessed the effect of α-tomatine and paclitaxel on Akt activation by detection of phospho-Akt and total Akt on PC-3 cell lysates. As shown in Figure 4.3B, combined α-tomatine and PTX treatment reduced the phosphorylated Akt levels by ~50%, which is close to the 60% reduction in cells treated with PI3K inhibitor wortmannin (1 μM). The effects in this assay of the individual agents on Akt activation (Figure 4.3B), paralleled closely the effects observed from our *in vitro* kinase assay using a GSK-3α/β fusion protein as substrate (Figure 4.3A).

We also examined the direct effect of these agents on PI3K, an upstream regulator of Akt by Western blot analysis. As shown in Figure 4.3C, the effects of single agent treatment with α-tomatine and combined treatment were similar to those observed for

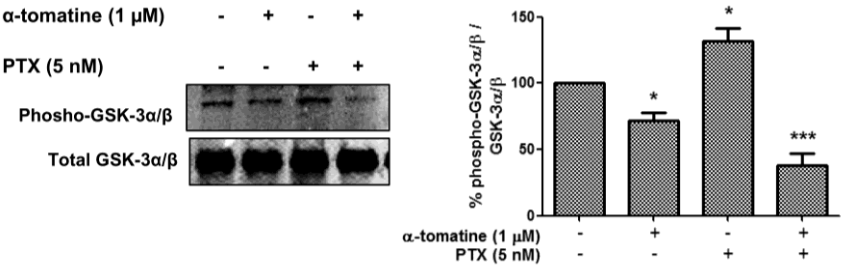
Akt activity (Figure 4.3A, 4.3B), whereas single agent treatment with 5 nM paclitaxel produced no significant change in the protein expression of phospho-PI3K (Figure 4.3C). In addition, neither single agent nor combination treatments had any effect on the levels of total Akt and PI3K (Figure 4.3B, 4.3C).

As we observed that the combined treatment inhibited the constitutive PI3K/Akt activation in PC-3 cells, we were interested in the effect of this combination on downstream regulators of apoptosis including BAD, Bcl-xL and Bcl-2. In cells treated with combined treatment of α-tomatine and paclitaxel for 8 hours, the expressions of anti-apoptosis proteins Bcl-2 and Bcl-xL were significantly downregulated (Figure 4.4A, 4.4Bi, 4.4Bii). However, there was no significant change in the expressions of Bcl-2 and Bcl-xL in response to either single agent treatment (Figure 4.4A, 4.4Bi, 4.4Bii). Conversely, the expression of the pro-apoptotic protein BAD increased in PC-3 cells treated with combined treatment of α-tomatine and paclitaxel (Figure 4.4A, 4.4Biii).

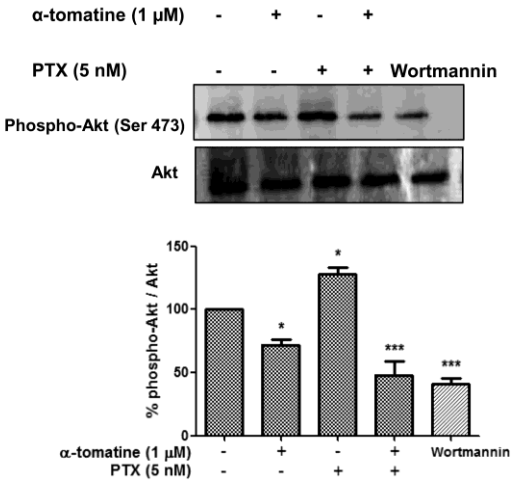
These data suggest that combined treatment with α-tomatine and paclitaxel promotes apoptosis of PC-3 cells by reducing the constitutive PI3K/Akt activity, thereby reducing the expression of Bcl-xL and Bcl-2 pro-survival proteins, and increasing the expression of BAD pro-apoptosis mediator.

Figure 4.3 Inhibitory effect of α-tomatine and paclitaxel (PTX) on PI3K/Akt activity. Protein expressions of (A) phospho-GSK-3α/β (B) phospho-Akt and (C) phospho-PI3K in PC-3 cells treated with the indicated treatments. Bar graphs show densitometry analysis of each Western blot data of three independent experiments. Data are shown as means ± SEM. Statistical significance is expressed as ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ versus vehicle control.

(A)


α-tomatine (1 μM) - + - +
PTX (5 nM) - - + +
Phosho-GSK-3α/β
Total GSK-3α/β
% phospho-GSK-3α/β / GSK-3α/β
150
100
50
0
*
*
***
α-tomatine (1 μM) - + - +
PTX (5 nM) - - + +

(B)


α-tomatine (1 μM) - + - +
PTX (5 nM) - - + + Wortmannin
Phospho-Akt (Ser 473)
Akt
% phospho-Akt / Akt
150
100
50
0
*
*
***
***
α-tomatine (1 μM) - + - + Wortmannin
PTX (5 nM) - - + +

(C)

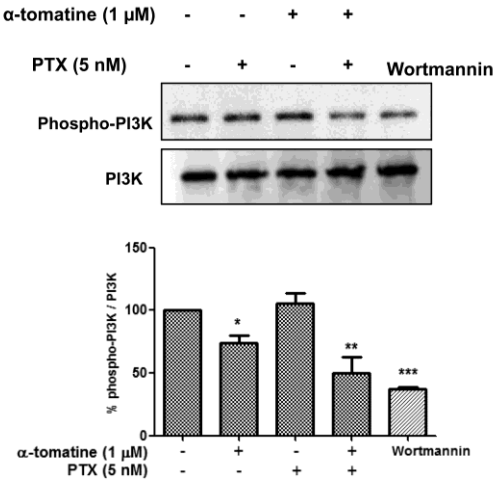
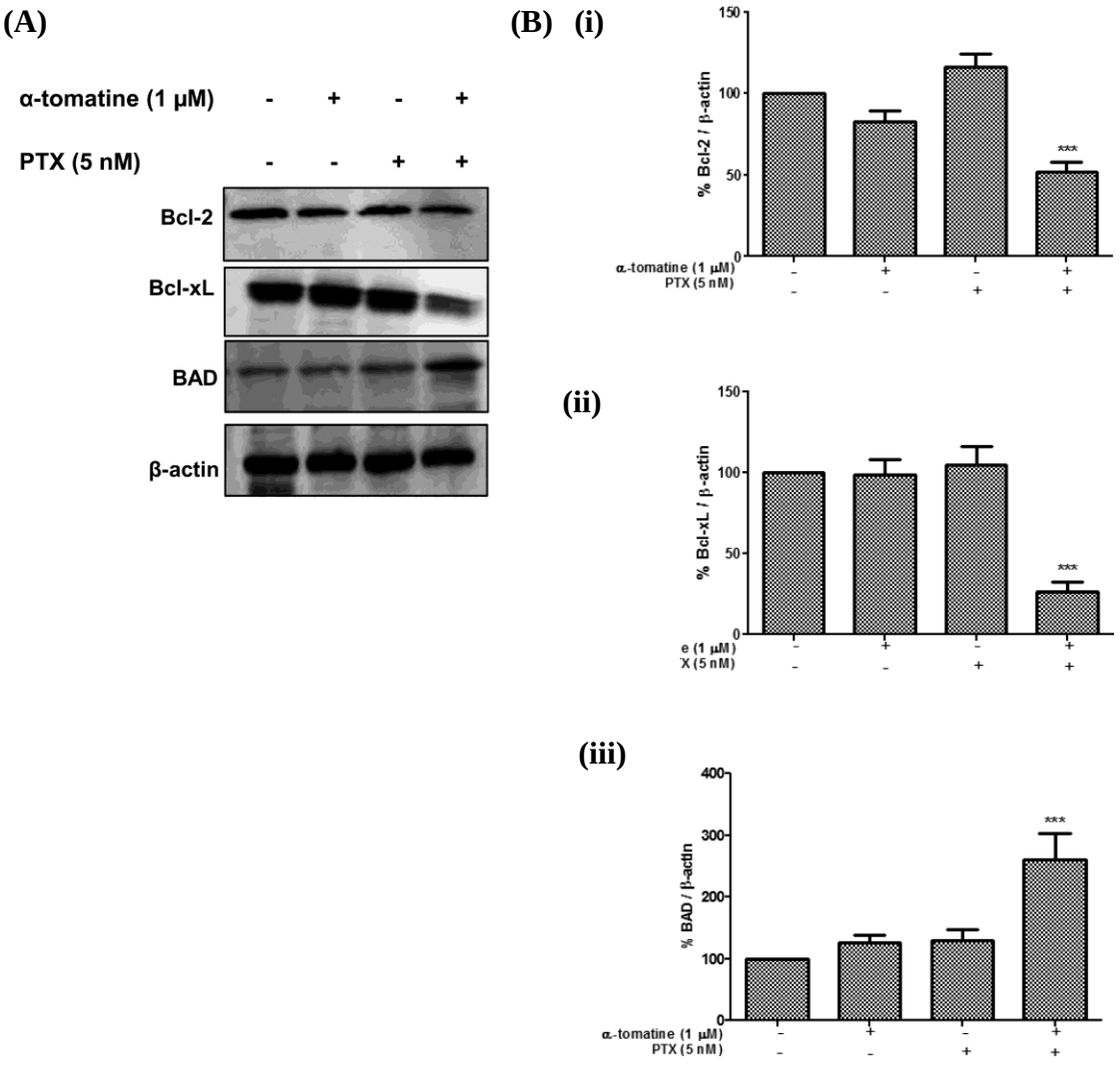

α-tomatine (1 μM) - - + +
PTX (5 nM) - + - + Wortmannin
Phospho-PI3K
PI3K
% phospho-PI3K / PI3K
150
100
50
0
*
**
***
α-tomatine (1 μM) - + - + Wortmannin
PTX (5 nM) - - + +

Figure 4.4 Effect of α-tomatine and paclitaxel (PTX) on the expression of apoptosis mediators in PC-3 cells. (A) Protein expressions of Bcl-2, Bcl-xL and BAD in PC-3 cells treated with the indicated treatments. (B)(i-iii) Densitometry analysis of each Western blot data of three independent experiments. Data are shown as means ± SEM. Statistical significance is expressed as ***p < 0.001 versus vehicle control.

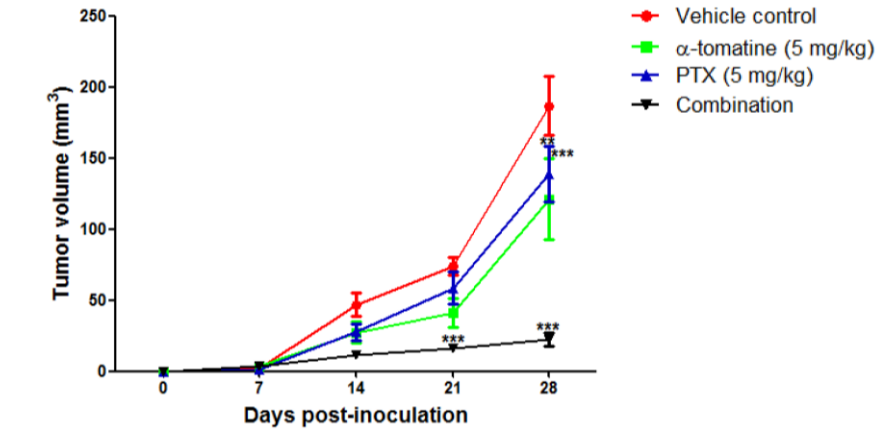

(A)
α-tomatine (1 μM) - + - +
PTX (5 nM) - - + +
Bcl-2
Bcl-xL
BAD
β-actin
(B) (i)
% Bcl-2 / β-actin
150
100
50
0
***
α-tomatine (1 μM)
PTX (5 nM)
(ii)
% Bcl-xL / β-actin
150
100
50
0
***
(iii)
% BAD / β-actin
400
300
200
100
0
***
α-tomatine (1 μM)
PTX (5 nM)

4.4.4 Alpha-tomatine enhances the anti-tumorigenic effects of the paclitaxel against PC-3 tumor xenografts in nude mice

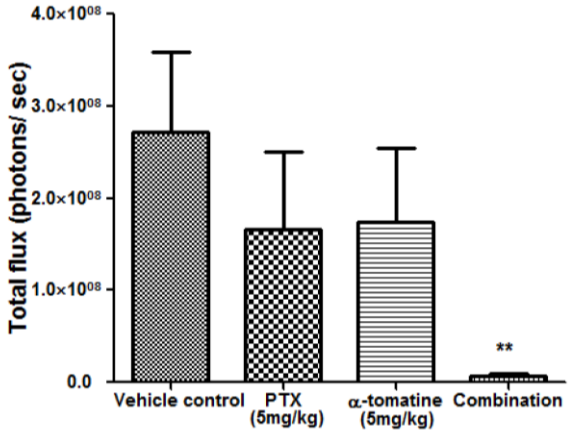
By the end of the experiment, combined treatment of α-tomatine (5 mg/kg) and paclitaxel (5 mg/kg) caused a marked reduction of PC-3 cell tumor volume and bioluminescent signal, whereas individual treatments with α-tomatine and paclitaxel had much less pronounced effects (Figure 4.5A-C). More importantly, none of the treatments caused a reduction in mouse body weight (Figure 4.5D). Taken together, these results suggest that α-tomatine improves the efficacy of paclitaxel in inhibiting PC-3 cell tumor growth *in vivo***.**

Figure 4.5 Alpha-tomatine potentiates paclitaxel (PTX) in inhibiting the growth of subcutaneous PC-3 tumors in mice. (A) Tumor volumes in each treatment group are presented as growth curves. (B) Endpoint bioluminescence intensities of tumors in each treatment group. (C) Representative bioluminescence images of PC-3 subcutaneous xenografts. (D) Graph of mean body weight for each treatment group versus the number of days after initial injection of PC-3 cells. Each point or bar represents the mean ± SEM of data (n = 6 per group). Statistical significance is expressed as ***$p < 0.001$; ** $p < 0.01$ versus vehicle control.

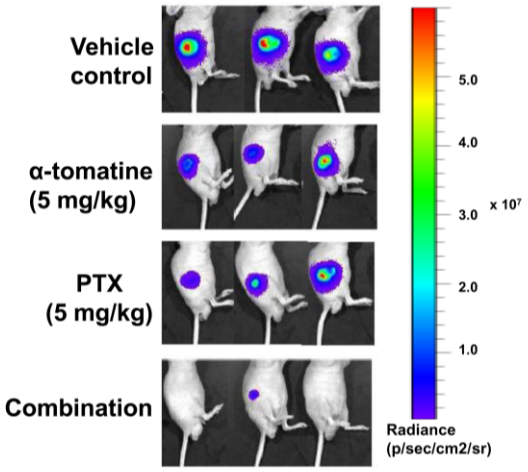
(A)


Vehicle control
α-tomatine (5 mg/kg)
PTX (5 mg/kg)
Combination
Tumor volume (mm3)
250
200
150
100
50
0
0
7
14
21
28
Days post-inoculation
**
***
***
***

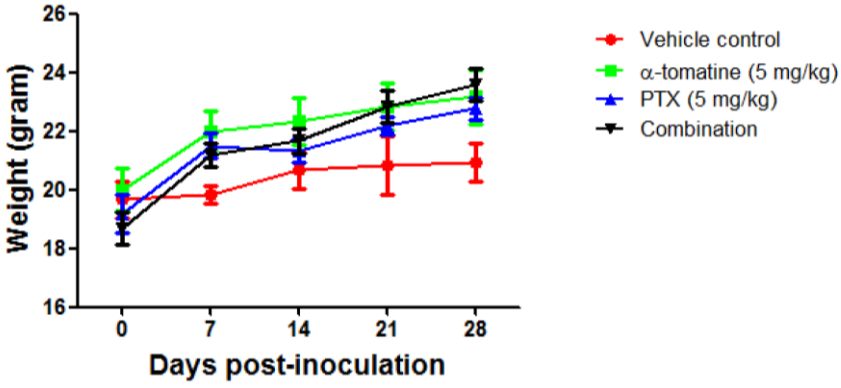
(B)


Total flux (photons/ sec)
4.0×10⁰⁸
3.0×10⁰⁸
2.0×10⁰⁸
1.0×10⁰⁸
0.0
**
Vehicle control
PTX (5mg/kg)
α-tomatine (5mg/kg)
Combination

(C)


Vehicle control
α-tomatine (5 mg/kg)
PTX (5 mg/kg)
Combination
5.0
4.0
3.0
2.0
1.0
x 10⁷
Radiance (p/sec/cm2/sr)

(D)

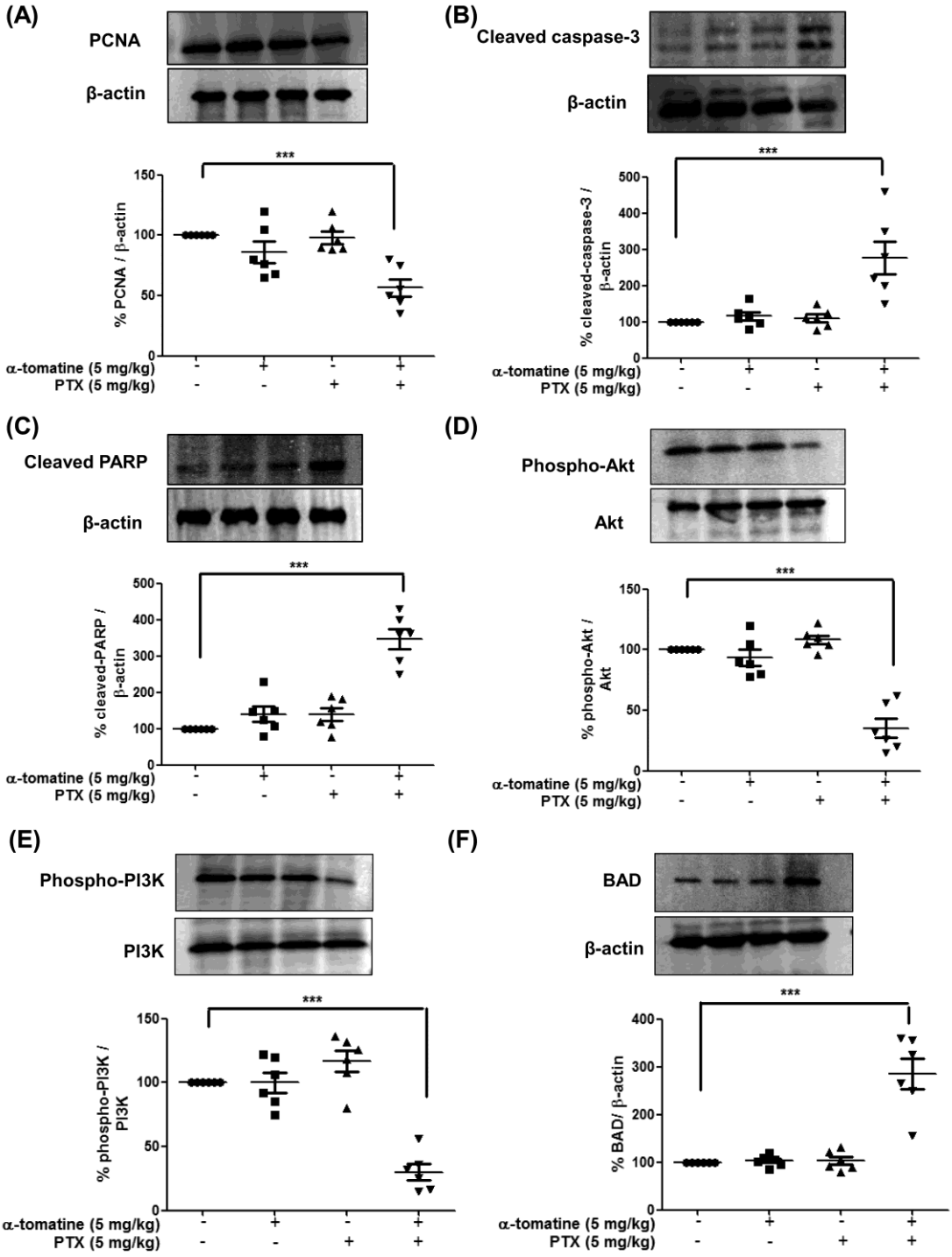

Vehicle control
α-tomatine (5 mg/kg)
PTX (5 mg/kg)
Combination
Weight (gram)
26
24
22
20
18
16
0
7
14
21
28
Days post-inoculation

4.4.5 Combined α-tomatine and paclitaxel treatment inhibits PI3K/Akt signaling and increases apoptosis in PC-3 xenograft tumors

To further assess the effect of the combination treatment on the *in vivo* growth of PC-3 cell mouse xenograft tumors, we examined tumor tissues recovered from mice for the expression of a marker of proliferation (PCNA) and apoptosis (cleaved PARP and cleaved caspase-3). Lysates from six independent tumor samples indicated that while PCNA expression decreased in response to the combination treatment (Figure 4.6A), the levels of cleaved PARP and caspase 3 had increased (Figure 4.6B, 4.6C), compared to those treated with single agents. These data showed that anti-proliferative and pro-apoptotic effects of the combination treatment contribute to the reduced growth of PC-3 cell tumors in mice.

As we have shown that constitutive activation of PI3K/Akt signaling is reduced *in vitro* by the combined treatment, we also examined the tumors recovered from mice for the expression of phospho-PI3K, phospho-Akt and its downstream molecule BAD. As shown in Figure 4.6D and 4.6E, there was a distinct decrease in the levels of phospho-PI3K and phospho-Akt in tumor cells in response to the combined treatment, whereas the expression of BAD increased in response to the combined treatment (Figure 4.6F). These findings support the proposal that the *in vivo* anti-tumor effect of combined α-tomatine and PTX is accompanied with the inhibition of pro-survival PI3K/Akt signaling.

Figure 4.6 Impact of paclitaxel (PTX) and α-tomatine on PI3K/Akt signaling in subcutaneous PC-3 cell tumors. Western blot analysis of (A) PCNA, (B) cleaved caspase-3, (C) cleaved PARP, (D) phospho-Akt, (E) phospho-PI3K and (F) BAD in PC-3 tumor tissues samples. Graphical representation of densitometry analysis of each Western blot data from six independent tumor samples is shown below each panel. Data are shown as means ± SEM. Statistical significance is expressed as ***$p < 0.001$ versus vehicle control.


(A)
PCNA
β-actin
% PCNA / β-actin
***
α-tomatine (5 mg/kg)
PTX (5 mg/kg)
(B)
Cleaved caspase-3
β-actin
% cleaved-caspase-3 / β-actin
***
α-tomatine (5 mg/kg)
PTX (5 mg/kg)
(C)
Cleaved PARP
β-actin
% cleaved-PARP / β-actin
***
α-tomatine (5 mg/kg)
PTX (5 mg/kg)
(D)
Phospho-Akt
Akt
% phospho-Akt / Akt
***
α-tomatine (5 mg/kg)
PTX (5 mg/kg)
(E)
Phospho-PI3K
PI3K
% phospho-PI3K / PI3K
***
α-tomatine (5 mg/kg)
PTX (5 mg/kg)
(F)
BAD
β-actin
% BAD/ β-actin
***
α-tomatine (5 mg/kg)
PTX (5 mg/kg)

4.5 Discussion

Preclinical *in vitro* and *in vivo* studies have indicated that phytochemicals, such as genistein and lycopene can enhance the anti-tumor activity of chemotherapeutic agents (Hwang *et al.*, 2005; Tang *et al.*, 2005). The use of phytochemicals and chemotherapeutic agents in combination has the advantage of low toxicity and potentiation of the actions of toxic chemotherapies thereby providing the opportunity to use chemotherapeutic agents at lower concentrations with fewer unwanted side-effects. We have shown that the tomato saponin, α-tomatine significantly enhanced the *in vitro* and *in vivo* anti-tumor activity of paclitaxel. In particular, α-tomatine acted synergistically *in vitro* with paclitaxel to significantly reduce the viability of PC-3 prostate cancer cells without impacting the viability of non-neoplastic prostate RWPE-1 cells. In addition, α-tomatine potentiated the action of paclitaxel *in vivo* causing regression of PC-3 xenograft tumors in mice without negatively impacting the weight of these animals. These data are suggestive that α-tomatine may be beneficial in clinical settings when combined with conventional chemotherapeutic agents such as paclitaxel.

Drug resistance promoted by activation of pro-survival pathways and inactivation of pro-apoptotic mechanisms is a major problem for treatment of advanced prostate tumors (Seruga *et al.*, 2011). Paradoxically, it is known that several conventional chemotherapeutic drugs, including paclitaxel promotes survival signaling implicated in the development of resistance to chemotherapy (Li & Sethi, 2010). This scenario limits the effectiveness of the chemotherapies and necessitates increases in the dosage or a change in the drug treatment. Accumulating evidence has indicated that activation of the PI3K/Akt pathway in advanced prostate cancer is implicated in the acquisition of resistance to chemotherapeutic drugs (Lee *et al.*, 2004; McCubrey *et al.*, 2006). Previous studies also suggested that activated Akt mediates paclitaxel-induced resistance and thus inhibition of Akt may synergistically increase paclitaxel sensitivity

(Kim *et al.*, 2007; Weng *et al.*, 2009). Consistent with the previous reports, we observed that paclitaxel increased pro-survival Akt activity and promoted the cell survival without affecting the abundant expressions of anti-apoptotic Bcl-2 and Bcl-xL proteins in PC-3 cells. More importantly, the constitutively active PI3K/Akt signaling in PC-3 cells due to the loss of phosphatase and tensin homolog (PTEN), a critical negative regulator of PI3K/Akt (Huang *et al.*, 2001), was most efficiently suppressed by combination treatment with paclitaxel and α-tomatine. Taken together, our data denote that α-tomatine-mediated sensitization of PC-3 cells to paclitaxel involves down-regulation of both constitutive and paclitaxel-induced Akt activity by α-tomatine. Consistent with our findings, α-tomatine has previously been shown to have anti-metastatic effect on lung cancer cells via inactivation of PI3K/Akt signaling (Shieh *et al.*, 2011; Shih *et al.*, 2009). Therefore, therapeutic targeting of PI3K/Akt by α-tomatine presents a promising approach to cancer therapy.

Deregulated anti-apoptotic proteins Bcl-2 and Bcl-xL have been shown to function as oncoproteins in the development of androgen-independent prostate cancer and chemoresistance in prostate cancer (Catz & Johnson, 2003; Lebedeva *et al.*, 2000). Our data indicate that the increased susceptibility of PC-3 cells caused by combined paclitaxel and α-tomatine treatment was accompanied with down-regulation of the Akt-regulated anti-apoptotic proteins Bcl-2 and Bcl-xL and increased expression of the pro-apoptotic protein BAD. These data are consistent with report showing that Akt upregulates expression of Bcl-2 through cAMP-response element-binding protein (Pugazhenthi *et al.*, 2000). Previous study demonstrated that PTEN suppresses the Bcl-2 through inhibiting Akt activity and thereby induces the chemosensitivity (Huang *et al.*, 2001). As constitutively active PI3K/Akt and abundant Bcl-2 protein expression were observed in untreated PTEN null PC-3 cells, reduction of PI3K/Akt activity by α-tomatine may therefore induce chemosensitization of the PC-3 cells to paclitaxel-

induced apoptosis accompanied with downregulation of Bcl-2 protein expression. In addition, activated Akt is known to prevent cell death by phosphorylating several apoptosis regulating proteins including pro-apoptotic Bcl-2 family member BAD, leading to its disassociation from the anti-apoptotic Bcl-2 family member Bcl-xL, and in turn, promotes survival (Datta *et al.*, 1997). Conversely, disruption of this pro-survival pathway will induce the dephosphorylation of BAD leading to the formation of a heterodimer with Bcl-xL and inhibit the anti-apoptosis function of Bcl-xL and thus trigger cell death (Yang *et al.*, 1995). We therefore propose that the enhanced apoptotic effect by the combination of α-tomatine and paclitaxel is attributed to the down-regulation of Akt-regulated pro-survival proteins and upregulation of Akt-regulated pro-apoptotic proteins.

4.6 Conclusion

In conclusion, this study demonstrated that α-tomatine, a phytochemical from tomatoes, improves the ability of paclitaxel to induce *in vitro* and *in vivo* death of androgen-independent prostate cancer PC-3 cells. Our data suggest that this compound has the ability to potentiate sub-lethal doses of paclitaxel to toxic levels, indicating the potential of α-tomatine as an adjunct to taxane-based chemotherapy for the treatment of prostate cancer.

CHAPTER 5

CONCLUSION

Morbidity and mortality from androgen-independent prostate cancer remain a significant health care problem. Patients with androgen-independent prostate cancer have poor prognosis and are more likely to die from complications of metastatic disease. Systemic chemotherapy with docetaxel in combination with prednisone represents the standard first-line treatment. However, chemoresistance, short-lived clinical benefit, and the drug-associated toxicities have limited its clinical application. Discovery of less toxic, more selective and effective agents for combating this malignant form of prostate cancer is highly desirable. Increasing evidence suggests that phytochemicals with distinct anticancer activity are part of cancer therapeutic arsenal, because of their greater efficacy and potentially low toxicity profiles. Alpha-tomatine, a dietary saponin readily available in tomato has gained much research interest. The anti-cancer activities of α-tomatine have been reported against several cancer cells in recent years. As yet, no anticancer activity and chemosensitizing effect of α-tomatine on the androgen-independent prostate cancer has been documented. The present study describes the therapeutic potential of α-tomatine as single agent and in combination with a chemotherapeutic agent for treatment of androgen-independent prostate cancer.

Initial study showed that α-tomatine suppresses the growth of two prostate cancer cell lines but is more potent in suppressing growth of AR-deficient PC-3 cells than the AR-expressing LNCaP cells. The higher sensitivity of α-tomatine in PC-3 compared to LNCaP cells reflects the involvement of AR-independent mechanism in the growth inhibitory effect of α-tomatine on androgen-independent prostate cancer PC-3 cells. Interestingly, this glycoalkaloid is less cytotoxic to both normal prostate epithelial (RWPE-1) and liver (WRL-68) cells, suggesting its specificity for the cancer cells. The cytotoxicity of α-tomatine on the growth of PC-3 cells is attributed to its pro-apoptotic action, as manifested by positive Annexin V staining and decreased in mitochondrial membrane potential but increased in nuclear condensation, polarization

of F-actin, cell membrane permeability, cytochrome c expressions and also caspases activation. Notably, PC-3 cell line is null of p53 tumor suppressor gene, the anti-cancer activity of α-tomatine is independent of p53 status in PC-3 cells, suggesting the involvement of p53-independent apoptosis. Since p53 abnormalities are usually responsible in conferring resistance to cancer chemotherapy and radiotherapy, α-tomatine is believed to exert apoptotic action in androgen-independent prostate cancer cells without conferring resistance to the patients throughout the treatment.

Constitutively active NF-κB inflicted by aberrant IKK activation and upstream signal transduction pathways such as PI3K/Akt is one of hallmarks of androgen-independent prostate cancer that confers pro-survival benefit in cancer cells through transcriptional activation of genes involved cell proliferation and anti-apoptosis. The present study demonstrates that the *in vitro* pro-apoptotic action of α-tomatine in PC-3 cells is mediated, at least in part, by attenuation of basal constitutive and inducible NF-κB activation through indirect inhibition of IKK kinase activity. Indeed, the attenuation of kinase activity of IKK by α-tomatine is attributed to its suppression on Akt activation. Akt is the main downstream target of PI3K and upstream kinase of IKK. Suppression of constitutively active Akt by α-tomatine shuts down the NF-κB pro-survival signal which in turn induces apoptotic machinery in PC-3 cancer cells. Importantly, inhibitory effect of tomatine is not limited to basal constitutive NF-κB activation, but α-tomatine also exerts suppressive effect on TNF-α-induced NF-κB activation. It has been shown that secretion of inflammatory cytokine TNF-α by macrophages and other stromal cells within prostate microenvironment favor tumor growth and metastatic progression through activating NF-κB pro-survival signaling. Elevated serum TNF-α levels in patients with androgen-independent prostate cancers, increased expression of TNF-α and nuclear expression of NF-κB in prostate tumors imply the role of TNF-α-mediated NF-κB signaling in the growth of androgen-independent prostate cancer. Taken together,

these data underscore the fact that α-tomatine overcomes both basal constitutive and TNF-α-induced NF-κB activation through inhibition of Akt, thereby leading to downregulation of NF-κB-regulated anti-apoptotic gene products and consequently apoptosis in PC-3 cells.

Most of the compounds that show prominence in *in vitro* cytotoxicity studies are ineffective when tested *in vivo*. This is because the dosage, absorption, bioavailability, route of administration and metabolism of the drug affect drug effectiveness and potency in human body. Of therapeutic interest, this study also demonstrates the potent anti-tumor activity of α-tomatine against PC-3 tumor in both subcutaneous and orthotopic tumor xenograft mouse models. The noteworthy findings in the present study are that a non-toxic dose of intraperitoneal injection of α-tomatine inhibits the growth of both subcutaneously and orthotopically implanted human prostate adenocarcinoma PC-3 xenograft in immunodeficiency mice. Orthotopic xenograft model which mimics the microenvironment and circulation of primary tumors in humans is another avenue to study the intervention of a potential anticancer agent in the preclinical setting. Suppressive effect of α-tomatine against PC-3 tumors grown subcutaneously and orthotopically *in vivo* therefore provide merit for further clinical investigations. The inhibitory effect of α-tomatine on the *in vivo* growth of PC-3 tumor is also associated with the increased in apoptosis and decreased in NF-κB nuclear translocation. These *in vivo* data strengthen the notion that α-tomatine, by blocking on anti-apoptotic machinery, can be developed as novel treatment for androgen-independent prostate cancer.

Chemoresistance promoted by activation of pro-survival pathways and inactivation of pro-apoptotic mechanisms remains a critical challenge in the management of androgen-independent prostate cancer. The use of phytochemicals and chemotherapeutic agents in combination has the advantage of low toxicity and

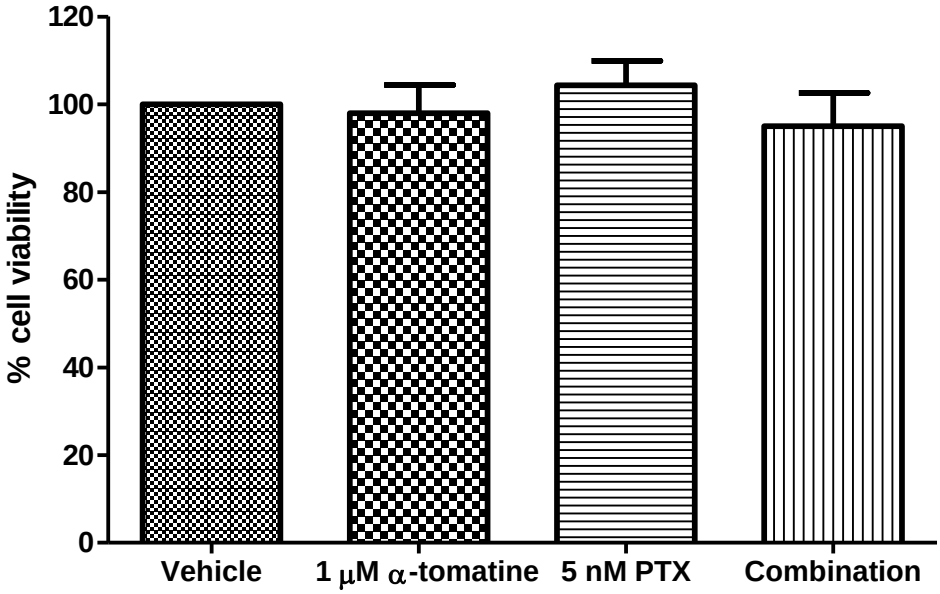
potentiation of the actions of toxic chemotherapies, thereby providing the opportunity to use chemotherapeutic agents at lower concentrations with fewer side-effects. Findings from the present study further revealed the ability of α-tomatine to augment the anti-cancer actions of paclitaxel against PC-3 cells *in vitro* and *in vivo*. *In vitro* study first demonstrated that sub-toxic dose of α-tomatine potentiates the pro-apoptotic action of paclitaxel without increasing cytotoxicity to normal prostate RWPE-1 cells, and isobologram analysis of the enhanced efficacy obtained by combining α-tomatine and paclitaxel indicates synergism. Interestingly, *in vivo* xenograft study further revealed the effectiveness of sub-toxic dose of α-tomatine in augmenting the anti-tumor action of low-dose paclitaxel. The co-administration of α-tomatine and paclitaxel significantly retard the growth of PC-3 xenograft tumors in mice without negatively impacting the weight of these animals. Consistent with our previous observation showing that cytotoxic dose of α-tomatine suppresses NF-κB activation by inhibiting Akt, α-tomatine at sub-toxic dose has synergistic effects with paclitaxel in suppressing upstream mediator of NF-κB, PI3K/Akt. It is believed that α-tomatine at sub-toxic dose partially suppresses the constitutively activated PI3K/Akt in PC-3 cells, thereby increases the susceptibility of PC-3 cells to paclitaxel-induced apoptosis.

Findings from present study are in agreement with the previous reports by Shih *et al.* (2009) and Shieh *et al.* (2011), where sub-toxic dose of α-tomatine suppresses invasion and migration of lung cancer cells having the wild-type PTEN tumor suppressor gene through inactivating PI3K/Akt signaling and reducing NF-κB DNA-binding activity. It is known that PTEN negatively regulates the PI3K/Akt signaling, and deregulated PI3K/Akt signaling through loss of PTEN occurs frequently in androgen-independent prostate cancer. The present study provides further evidence that α-tomatine can also suppress constitutively activated PI3K/Akt signaling in PTEN-deficient PC-3 cells. As emerging evidence have clearly indicated that deregulation of

PI3K/Akt and NF-κB signaling pathways are implicated in androgen-independent prostate cancer and confer chemoresistance, one implication of these observations is that hampering of these oncogenic signalings in PC-3 by α-tomatine represents a promising therapeutic strategy for androgen-independent prostate tumor and improving the efficacy of chemotherapy.

In summary, both *in vitro* and *in vivo* findings of the present study buttress high therapeutic potential of α-tomatine for treatment of androgen-independent prostate cancer. Findings from this preclinical study warrant for further clinical investigations of the efficacy and safety profile of α-tomatine for treatment of androgen-independent prostate cancer. Moreover, combining α-tomatine with paclitaxel may provide clinical benefit for men with androgen-independent prostate cancer. It can be used as adjuvant that potentiates the cytotoxic effects of paclitaxel given at sub-toxic levels as well as to alleviate paclitaxel-associated side effects. Lastly, these preclinical findings also open doors to future pharmacokinetic studies and clinical investigations of α-tomatine for treatment of androgen-independent prostate cancer.

APPENDIX



Appendix A. Cell viability of PC-3 cells in response to 3 hours exposure of the indicated treatments using trypan blue exclusion dye assay. Data are shown as means ± SEM.

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LIST OF SCIENTIFIC PUBLICATIONS

1. Lee, S. T., Wong, P. F., Cheah, S. C., Mustafa, M. R. (2011). Alpha- tomatine induces apoptosis and inhibits nuclear factor-kappa B activation on human prostatic adenocarcinoma PC-3 Cells. *PLoS ONE*, *6*(4), e18915.

2. Lee, S. T., Wong, P. F., He, H., Hooper, J. D., Mustafa, M.R. (2013). Alpha-tomatine attenuation of in vivo growth of subcutaneous and orthotopic xenograft tumors of human prostate carcinoma PC-3 cells is accompanied by inactivation of nuclear factor-kappa B signaling. *PLoS ONE, 8*(2), e57708.

3. Lee, S. T., Wong, P. F., Hooper, J. D., Mustafa, M. R. (2013). Alpha-tomatine synergises with paclitaxel to enhance apoptosis of androgen-independent human prostate cancer PC-3 cells *in vitro* and *in vivo*. *Phytomedicine*. Advance online publication. doi: 10.1016/j.phymed.2013.07.002

4. Cheah, S. C., Appleton, D. R., Lee, S. T., Lam, M. L., Hadi, A. H. A., Mustafa, M. R. (2011). Panduratin A Inhibits the Growth of A549 Cells through Induction of Apoptosis and Inhibition of NF- KappaB Translocation. *Molecules, 16*(3), 2583-2598.

5. Cheah, S. C., Lai, S. L., Lee, S. T., Hadi, A. H. A., Mustafa, M. R. (2013). Panduratin A, a Possible Inhibitor in Metastasize A549 Cells Through Inhibition of NF-Kappa B Translocation and Chemoinvasion. *Molecules, 18*(8), 8764-8778.

LIST OF CONFERENCE PRESENTATIONS

POSTER

1. Involvement of Nuclear Factor-kappa B Signaling in the Induction of Apoptosis and Inhibition of Human Prostate Cancer PC3 Cell Invasion by Alpha-tomatine, Beatson International Cancer Conference, Cancer Research UK, 03 July 2011 to 06 July 2011, International.

2. Evaluation of anti-cancer effect of alpha-tomatine on PC-3 human prostate cancer cells by cell impedance and high content screening assay, 35th Annual Conference of the Malaysian Society for Biochemistry & Molecular Biology, 27 July 2010 to 28 July 2010, National.

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PLoS one

Alpha-Tomatine Induces Apoptosis and Inhibits Nuclear Factor-Kappa B Activation on Human Prostatic Adenocarcinoma PC-3 Cells

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Abstract

Background: Alpha-tomatine (α-tomatine) is the major saponin in tomato (*Lycopersicon esculentum*). This study investigates the chemopreventive potential of α-tomatine on androgen-independent human prostatic adenocarcinoma PC-3 cells.

Methodology/Principal Findings: Treatment of highly aggressive human prostate cancer PC-3 cells with α-tomatine resulted in a concentration-dependent inhibition of cell growth with a half-maximal efficient concentration (EC_{50}) value of 1.67±0.3 μM. It is also less cytotoxic to normal human liver WRL-68 cells and normal human prostate RWPE-1 cells. Assessment of real-time growth kinetics by cell impedance-based Real-Time Cell Analyzer (RTCA) showed that α-tomatine exhibited its cytotoxic effects against PC-3 cells as early as an hour after treatment. The inhibitory effect of α-tomatine on PC-3 cancer cell growth was mainly due to induction of apoptosis as evidenced by positive Annexin V staining and decreased in mitochondrial membrane potential but increased in nuclear condensation, polarization of F-actin, cell membrane permeability and cytochrome c expressions. Results also showed that α-tomatine induced activation of caspase-3, -8 and -9, suggesting that both intrinsic and extrinsic apoptosis pathways are involved. Furthermore, nuclear factor-kappa B (NF-κB) nuclear translocation was inhibited, which in turn resulted in significant decreased in NF-κB/p50 and NF-κB/p65 in the nuclear fraction of the treated cells compared to the control untreated cells. These results provide further insights into the molecular mechanism of the anti-proliferative actions of α-tomatine.

Conclusion/Significance: α-tomatine induces apoptosis and inhibits NF-κB activation on prostate cancer cells. These results suggest that α-tomatine may be beneficial for protection against prostate cancer development and progression.

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Introduction

Prostate cancer is one of the leading causes of deaths in men worldwide with major mortality due to advanced stage of the cancer where androgen-independent cells become unresponsive to hormone ablation therapy [1,2]. There is a pressing need to identify alternative chemopreventive measures as surgery and current chemotherapeutic strategies can be ineffective against the aggressive form of prostate cancer [3–5].

Failure of cells to undergo apoptotic cell death contributes to the development of cancers, hence treatment that induces apoptosis would be a promising anticancer strategy [6,7]. Natural products are important sources of new and less toxic anticancer agents. A variety of dietary agents such as resveratrol, curcumin, sulforaphane, gingerol, indole-3 carbinol, withanolide and green tea catechins can induce apoptosis and are effective against various human cancer cells tested [7–11]. These also have an added advantage of lesser toxicity compared to current standard therapeutic drugs [12].

The present work investigates the chemopreventive potential of alpha-tomatine (α-tomatine) against prostate cancer cells. α-tomatine is the major saponin in tomato (*Lycopersicon esculentum*). It possesses antimicrobial, antifungal, anti-inflammatory and immunopotentiating activities [13–18]. It is protective against dibenzo[a,l]pyrene (DBP)-induced liver and stomach tumors in rainbow trout [19] and also promotes anti-proliferative effects against human colon HT-29, liver HepG2, breast MCF-7 and stomach AGS cancer cells [20,21]. Thus far, it is only known that α-tomatine acts on phosphoinositide 3-kinase and protein kinase B (PI3K/Akt) and extracellular signaling-regulating kinase (ERK) signaling pathways in lung adenocarcinoma A549 cells [22]. The present study, hence, seeks to investigate the effect of α-tomatine on the growth of human prostatic adenocarcinoma PC-3 cells and to identify its potential growth inhibitory mechanism. Results from the present study would provide new insights into the beneficial role of α-tomatine for prevention of prostate cancer development and progression.

Materials and Methods

Phytochemicals, standard drug and reagents

α-tomatine (purity>97%) was purchased from Extrasythese (Genay, France). Paclitaxel from Ascent Scientific (Weston-SuperMare, UK), a standard drug used in chemotherapy regimen

for prostate cancer was used as positive control in this study. Curcumin (Merck, Germany) was used as positive control in NF-κB translocation assay. Dimethyl sulfoxide (DMSO) and tumor necrosis factor-alpha (TNF-α) were purchased from Sigma Aldrich (St. Louis, MO). Both the tested compound and positive controls were prepared in 100% DMSO, stored at −20°C, and then diluted as needed in cell culture medium. Penicillin/streptomycin, Dulbecco Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI 1640), keratinocyte growth medium, fetal bovine serum (FBS) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide) were purchased from Invitrogen (Carlsbad, CA).

Cell lines

Prostate cancer PC-3 (CRL-1435), normal human liver WRL-68 (CL-48) cells, and normal human prostate RWPE-1 (CRL-11609) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640, DMEM and keratinocyte growth medium, respectively. Both RPMI-1640 and DMEM were supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were cultured at 37°C in a humidified atmosphere of carbon dioxide–air (5:95).

In vitro cytotoxicity screening

Briefly, adherent cells were seeded in sterile 96-wells plates at optimum cell density. After 18–24 hours, cells were treated with increasing concentrations (0.16–5.0 µM) of α-tomatine for 24 hours. Cells treated with 0.2% DMSO was used as vehicle control. Following treatment, cells were incubated in the dark with 2 mg/ml MTT at 37°C for 2 hours, then the medium was carefully removed and 100 µl of DMSO was added to dissolve the formazan crystals formed. Absorbance was measured at 570 nm in a plate reader. Cell viability was calculated using the following formula: (mean absorbance in test wells)/(mean absorbance in control well)×100%. Dose-response curves were plotted using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). Half maximal effective concentration (EC_{50}) values were determined using non-linear regression model with sigmoidal dose response (variable slope) following the algorithm of GraphPad Prism 4.

Real time cell proliferation analysis

Real time growth kinetics of PC-3 cells was examined by impedance-based Real-Time Cell Analysis (RTCA) system (Roche Diagnostic, Mannheim, Germany). RTCA utilizes E-plate which contains inter-digitated micro-electrodes on the bottom of the plate that detect local ionic changes as cells proliferate and is measured as electrode impedance. Briefly, 50 µl of medium was added in 16-wells E-plate and background readings were recorded. Cell suspension (50 µl) at cell density of 1.25×10^4 cells/well was added to each well of the E-plate. The attachment, spreading and proliferation of the cells were monitored every 5 minutes intervals. When the cells entered logarithmic growth phase, they were treated with 100 µl of α-tomatine at various concentrations and continuously monitored every 10 minutes for up to 72 hours. Cells treated with 0.2% of DMSO was used as vehicle control and monitored in parallel with the α-tomatine and paclitaxel-treated cells. Cell sensor impedance was expressed as an arbitrary unit called the Cell Index (CI). The CI at each time point was defined as $(R_n-R_b)/15$, where R_n is the cell-electrode impedance of the well and the R_b is the background impedance of the well with the media alone. Growth curves were normalized to the CI at the last measured time point before compound addition for each well.

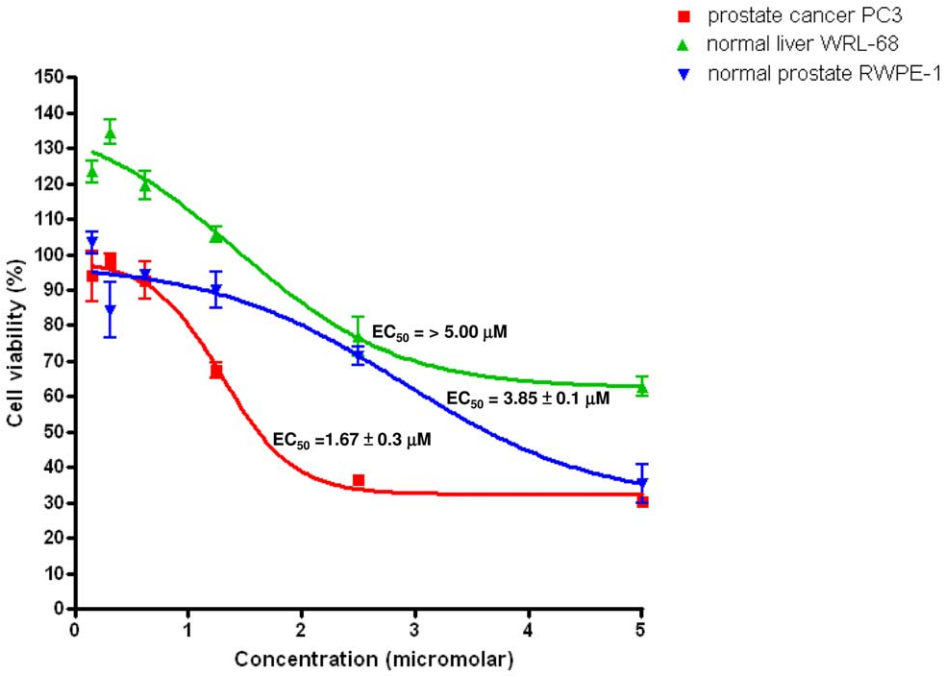

Figure 1. The effect of α-tomatine on cell viability of PC-3, WRL-68 and RWPE-1 cells. Cells were treated with α-tomatine for 24 hours and cell proliferation was measured using MTT reduction assay. Each value represents the mean ± SD of three independent experiments, performed in triplicates. EC_{50} values of α-tomatine on PC-3 cancer cell, WRL-68 normal cells and RWPE-1 normal prostate cells at 24 hours treatment were 1.67±0.3 µM, >5.0 µM and 3.85±0.1 µM, respectively.
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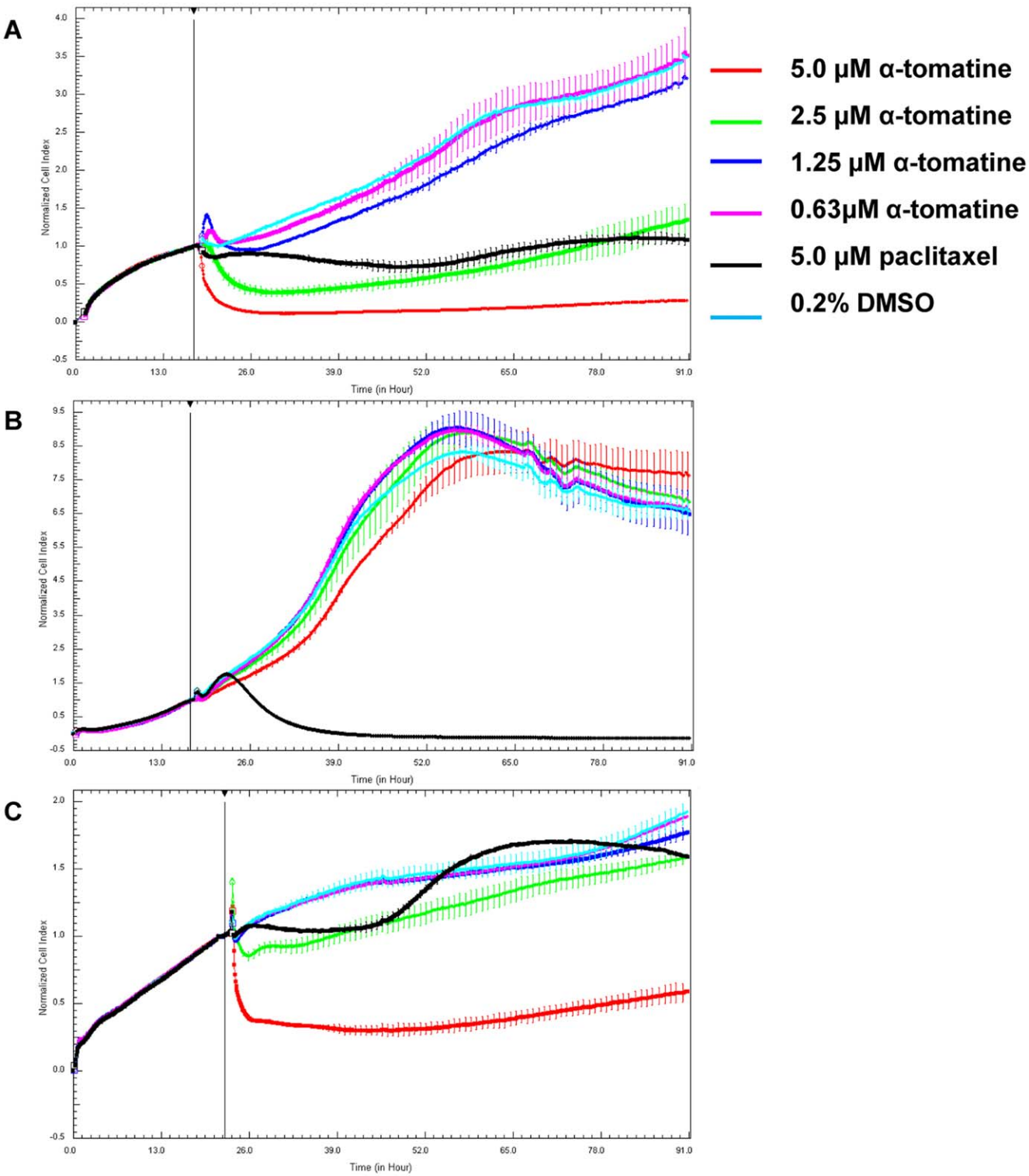


Figure 2. Dynamic assessment of cell viability after treatment with α-tomatine. Normalized Cell Index (CI) measured over 72 hours on A) prostate cancer PC-3, B) human normal liver WRL-68 and C) human normal prostate RWPE-1 cells. Briefly, cells at logarithmic growth phase were treated with α-tomatine and CI values were recorded every 10 minutes intervals using RTCA MP system. Cells treated with 5.0 μM paclitaxel and 0.2% DMSO were used as positive and negative controls, respectively. A change in impedance as the cells spread on the E-plate was displayed as CI value. Plotted CI values were normalized to the last time point before addition of treatment.
doi:10.1371/journal.pone.0018915.g002

Annexin V/propidium iodide (PI) double staining assay

Apoptosis-mediated cell death of tumor cell was examined by a double staining method using FITC-labeled Annexin V/PI apoptosis detection kit (BD Bioscience, San Jose, CA) according to the manufacturer's instructions. Briefly, control and α-tomatine-treated cells were collected, washed in cold phosphate-buffered saline (PBS) twice, stained with fluoresceinisothiocyanate (FITC)-conjugated Annexin V and PI dyes. The externalization of phoshotidylserine and the permeability to PI were evaluated by FACS Calibur flowcytometer (BD Bioscience, San Jose, CA). Data from 10,000 gated events per sample were collected. Cells in early stages of apoptosis were positively stained with Annexin V; whereas, cells in late apoptosis were positively stained with both Annexin V and PI.

Multiparametric High Content Screening (HCS) assays

HCS (Cellomics Inc, Pittsburgh, PA, USA) enables concurrent quantitative measurement of multiple independent cellular phenotypes using fluorescent probes. Both Cellomics multiparametric cytotoxicity and apoptosis HitKit^{TM} (Pittsburgh, PA, USA) were used to examine cellular changes in the α-tomatine-treated PC-3 cells. Multiparametric cytotoxicity HitKit^{TM} contains four fluorescent dyes, i.e. blue fluorescent Hoechst 33342, membrane permeability, mitochondrial membrane potential, and cytochrome c dyes. They respectively detect changes in nuclear morphology (nuclear condensation), membrane permeability, mitochondrial membrane potential and expression of cytochrome c. The multiparametric apoptosis kit quantifies three fundamental parameters related to the process of apoptosis, i.e. (i) nuclear condensation, detected by the blue fluorescent nuclear dye, Hoechst 33342; (ii) F-actin content, detected by green fluorescent Alexa Fluor ® 488Phalloidin stain; (iii) mitochondrial membrane potential, based on the uptake of MitoTracker ® Red into mitochondria of cells. Briefly, PC-3 cells were seeded overnight at density of 8000 cells/well into flat-bottomed 96-well plates (Perkin-Elmer Inc., Wellesley, MA, USA). Following treatment with different concentrations of α-tomatine (0.25 to 2.0 μM), fixation and staining for imaging analysis of the PC-3 cells were performed according to the manufacturer's instructions. Cells treated with 0.2% DMSO and 5.0 μM paclitaxel were used as negative and positive controls, respectively. Plates were analyzed using Thermo Scientific ArrayScan®VTI HCS Reader (Cellomics Inc, Pittsburgh, PA, USA). This is a computerized automated fluorescence imaging microscope that automatically identifies stained cells and measures the intensity and distribution of fluorescence in individual cells. Images for each fluoroprobe were acquired at different channels using suitable filters with 20× objective at fixed exposure time. The Cell Health Profiling BioApplication software

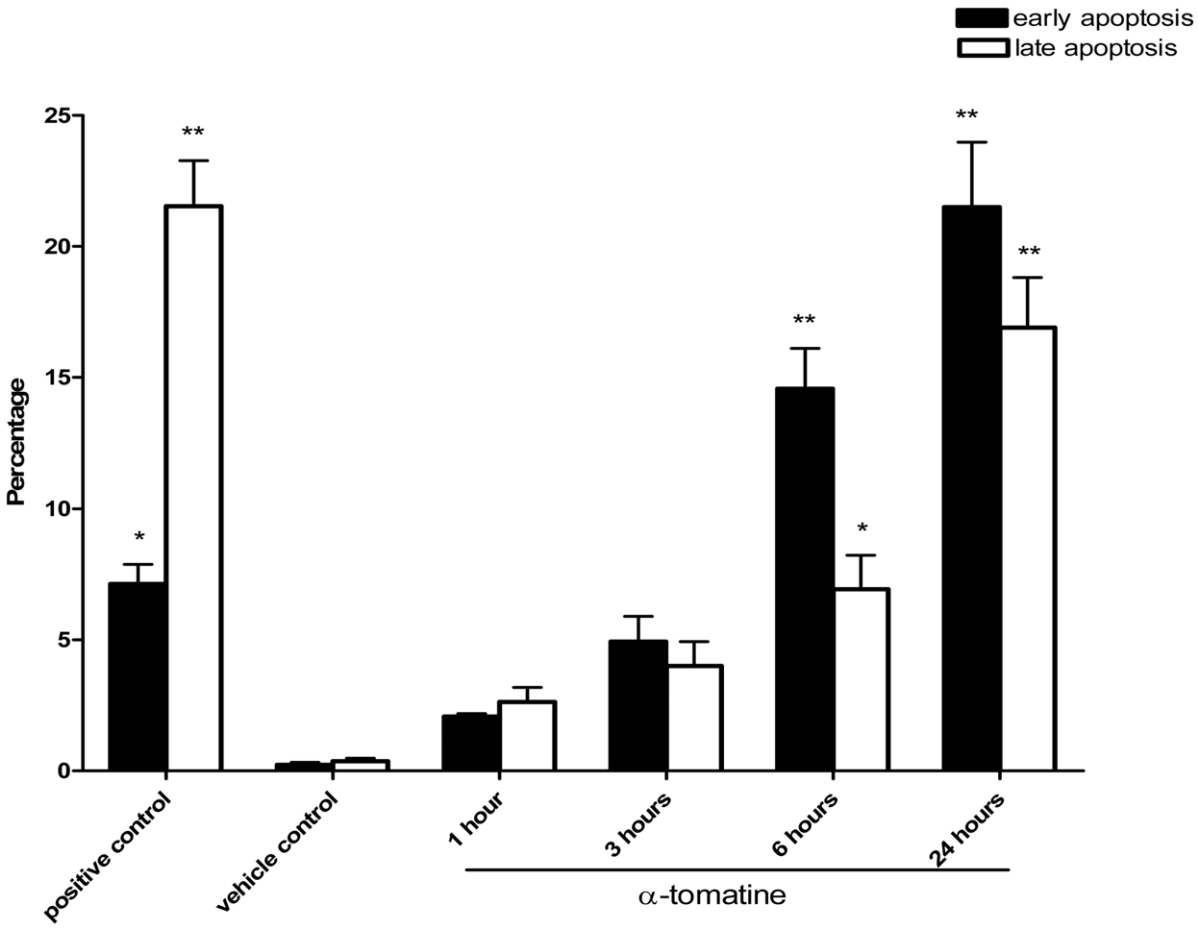


Figure 3. Annexin V/PI double staining assay. Cells were exposed to either 0.2% DMSO (vehicle control), 5.0 μM paclitaxel (positive control), or α-tomatine for the indicated time. The treated cells were stained with Annexin V and PI and then subjected to FACS analysis. The percentages of early apoptotic cells (Annexin V positive, PI negative) and late apoptotic cells (Annexin V and PI positive) observed at different incubation time with α-tomatine are shown. Results are presented as the mean ± SD of three independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* vehicle control.
doi:10.1371/journal.pone.0018915.g003

was used for image acquisitions and analysis. For each well, at least 25 fields, corresponding to at least 500 cells were automatically acquired and analyzed. All experiments were performed in triplicates. Cell average intensity (Mean) under the modified object mask within selected range in each channel was used as assay indicator, and reported as average fluorescence intensity.

Cell cycle phase distribution of α-tomatine-treated cells was determined using Cellomics Cell Cycle BioApplication as

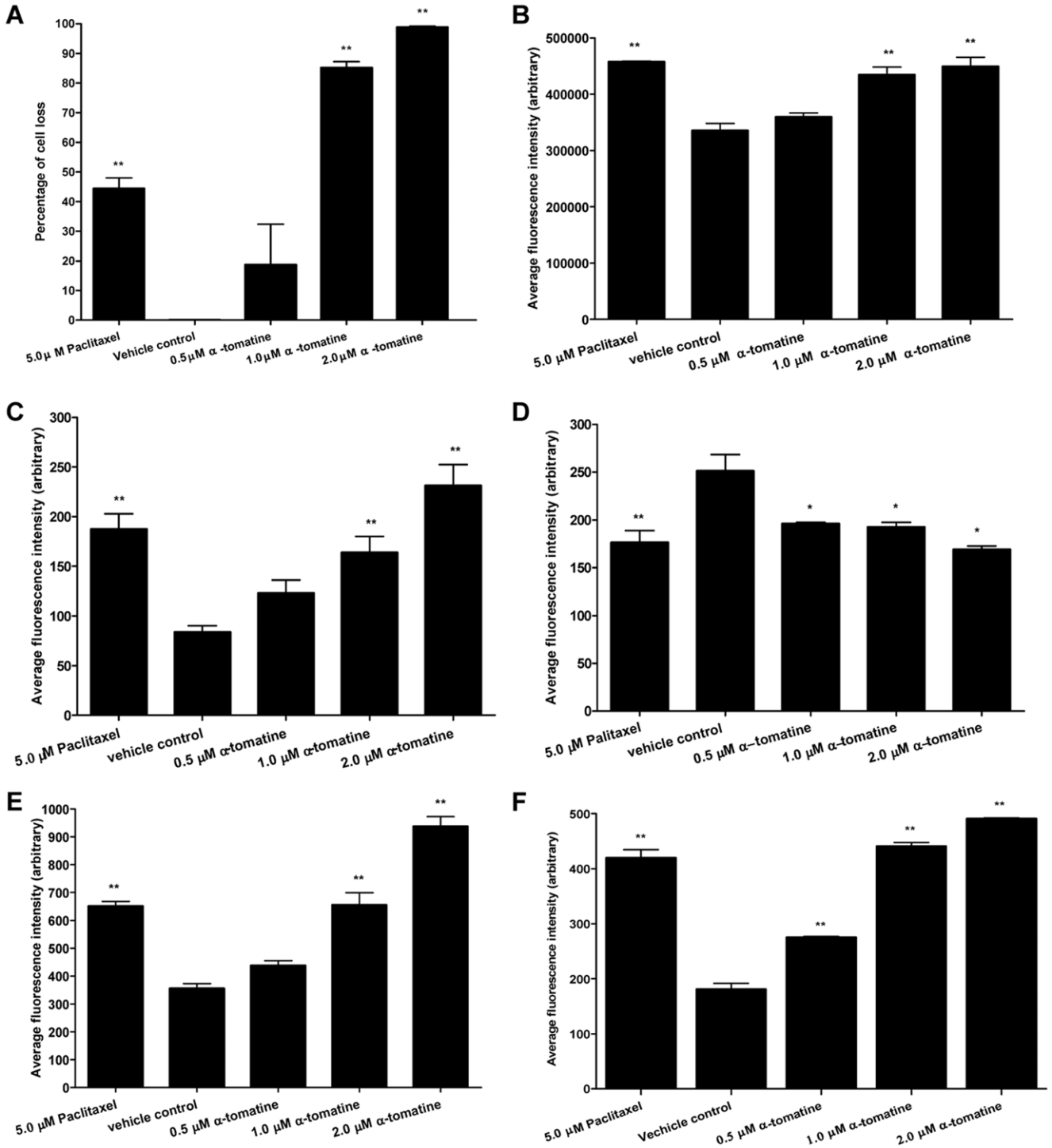


Figure 4. HCS analysis of apoptosis associated cellular morphology on α-tomatine-treated PC-3 cells. PC-3 cells were treated with different concentrations of α-tomatine (0.5–2.0 μM). Cell morphology changes associated with apoptosis such as A) percentage of dead cells, B) alterations in nuclear condensation, C) membrane permeability, D) mitochondrial membrane potential, E) expression of cytochrome c and F) F-actin contents are reflected by the average fluorescence intensity detected. Cells treated with 0.2% DMSO and 5.0 μM paclitaxel were used as negative and positive controls, respectively. All measured parameters were expressed as average fluorescent intensities and averaged for at least 500 cells per well. Data is representative of three independent experiments.* $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* vehicle control.
doi:10.1371/journal.pone.0018915.g004

described previously [23]. It applies similar principles as FACS in assessing cell cycle distribution where the intensity of Hoechst stained nucleus is deemed proportional to the cell's DNA content. Total fluorescence intensity of the dye from the nucleus of each cell typically exhibits a bimodal distribution. The first peak typically contains cells with 2N DNA content (G_0/G_1 phase), the second peak with a double intensity of the first, contains cells with 4N DNA content (G_2/M phase). Under normal conditions, there are more cells in the G_0/G_1 versus the G_2/M phase. The region between these two peaks represents cells with DNA content between 2N and 4N (S phase) which are cells in the process of doubling their DNA. Cells found with DNA<2N distribution are usually apoptotic cells. To identify the range of each of the DNA content categories, the software first identifies 2N (G_0/G_1 phase) and 4N (G_2/M phase) DNA content peaks using vehicle control cells. Then, it automatically classified each cell's nuclear total intensity into one of the categories of DNA content: sub G_1 (DNA<2N), G_0/G_1 (DNA~2N), S (2N<DNA>4N) and G_2/M (DNA~4N) phases in α-tomatine-treated cells. The percentage of each cell cycle phase for vehicle control, paclitaxel-treated cells and α-tomatine-treated cells were then reported.

Caspase activity

The activities of caspase-3, -8 and -9 were measured using the fluorometric assay kit (Calbiochem, USA) following the protocol of the manufacturer. Briefly, cells were treated with α-tomatine (2.0 μM) with or without inhibitors (caspase-8: z-IETD-FMK; caspase-9: z-LEHD-FMK; caspase-3-like: DEVD-CHO). After treatment, the cells were harvested using trypsinization and cell lysates were prepared as described [24]. The cell lysates were mixed with reaction buffer and 10 μl of fluorogenic peptide substrate: Ac-DEVD-AMC (caspase-3), Ac-IETD-AMC (caspase-8) and Ac-LEHD-AMC (caspase-9), and incubated for 2 hours at 37°C in the dark. Inhibitors were added 30 minutes before addition of fluorogenic substrate. Wells containing 50 μl of sample buffer, 50 μl of assay buffer and 10 μl of substrate were used as blank. Purified caspase was used as positive control while untreated cell extract was used as negative control. Fluorescence was then measured at excitation of 390 nm and emission of 500 nm. Fold-increase in the protease activity was determined by comparing the levels of the treated cells with untreated controls.

NF-κB translocation assay

NF-κB translocation in PC-3 cells was examined using NF-κB activation HCS kit which contains Hoechst 33342 and Alexa Fluor 488 conjugated anti-NF-κB dyes. PC-3 cells were seeded into sterile flat-bottomed 96-well plates (Perkin-Elmer Inc., Wellesley, MA, USA) at 8000 cells/well (100 μl/well). After 18–24 hours, cells were treated with different concentrations of α-tomatine for 30 minutes, followed by treatment with 10 ng/ml

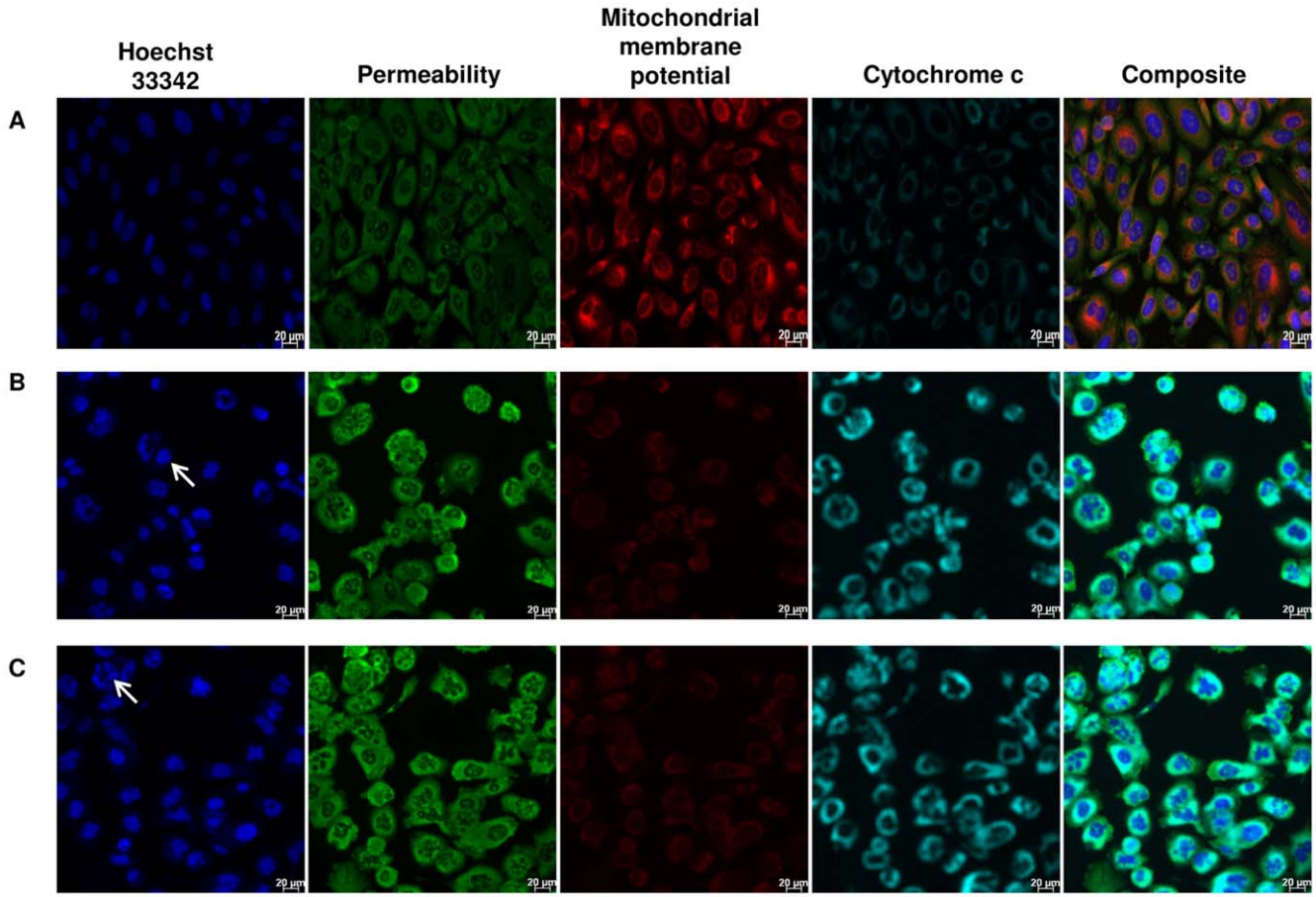


Figure 5. Cytotoxic and proapoptotic effects of α-tomatine on PC-3 cells. PC-3 cells were treated for 24 hours with A) 0.2% DMSO, B) 5.0 μM paclitaxel, and C) 2.0 μM α-tomatine. The treated cells were examined for nuclear condensation using blue Hoechst 33342 stain, changes in membrane permeability using a green fluoroprobe, mitochondrial membrane potential using MitoTracker Red CMXRos dye, and cytochrome c expression using a blue green fluoroprobe. Scale bar indicates 20 μm.
doi:10.1371/journal.pone.0018915.g005

TNF-α for another 30 minutes. Cells pre-treated with 0.2% DMSO and 50 μM curcumin were used as negative and positive inhibitor controls, respectively. Fixation, permeabilization and immunofluorescence staining of cells were performed according to the manufacturer's instructions. For target translocation analysis, cells stained with Hoechst dye were identified as objects in channel 1 and an adjustable mask called Circ was created around every nucleus. In non-activated cells, Alexa Fluor 488-stained NF-κB was detected in channel 2 in the cytoplasm where an annular region called Ring was defined beyond the nuclear region. Images were acquired using suitable filters with 20× objective at fixed exposure times. For each well, at least 500 cells were automatically acquired and analyzed. The output feature MEAN_CircRingAvgIntenDiffCh2 represents the difference between the intensity of nuclear and cytoplasmic NF-κB associated fluorescence (Nuc-Cyto Diff), reported as translocation parameter, as previously described [25].

NF-κB/p50 and NF-κB/p65 transcription factor assay

PC-3 cells at 70–80% confluence were treated with α-tomatine for 30 minutes, followed by treatment with TNF-α (10 ng/ml) for another 30 minutes. The cells were then washed with PBS and the both nuclear and cytoplasmic fractions of the treated cells were extracted using nuclear extraction kit (Cayman Chemical, Ann Arbour, MI). Concentrations of the active forms of NF-κB/p50 and NF-κB/p65 in the both

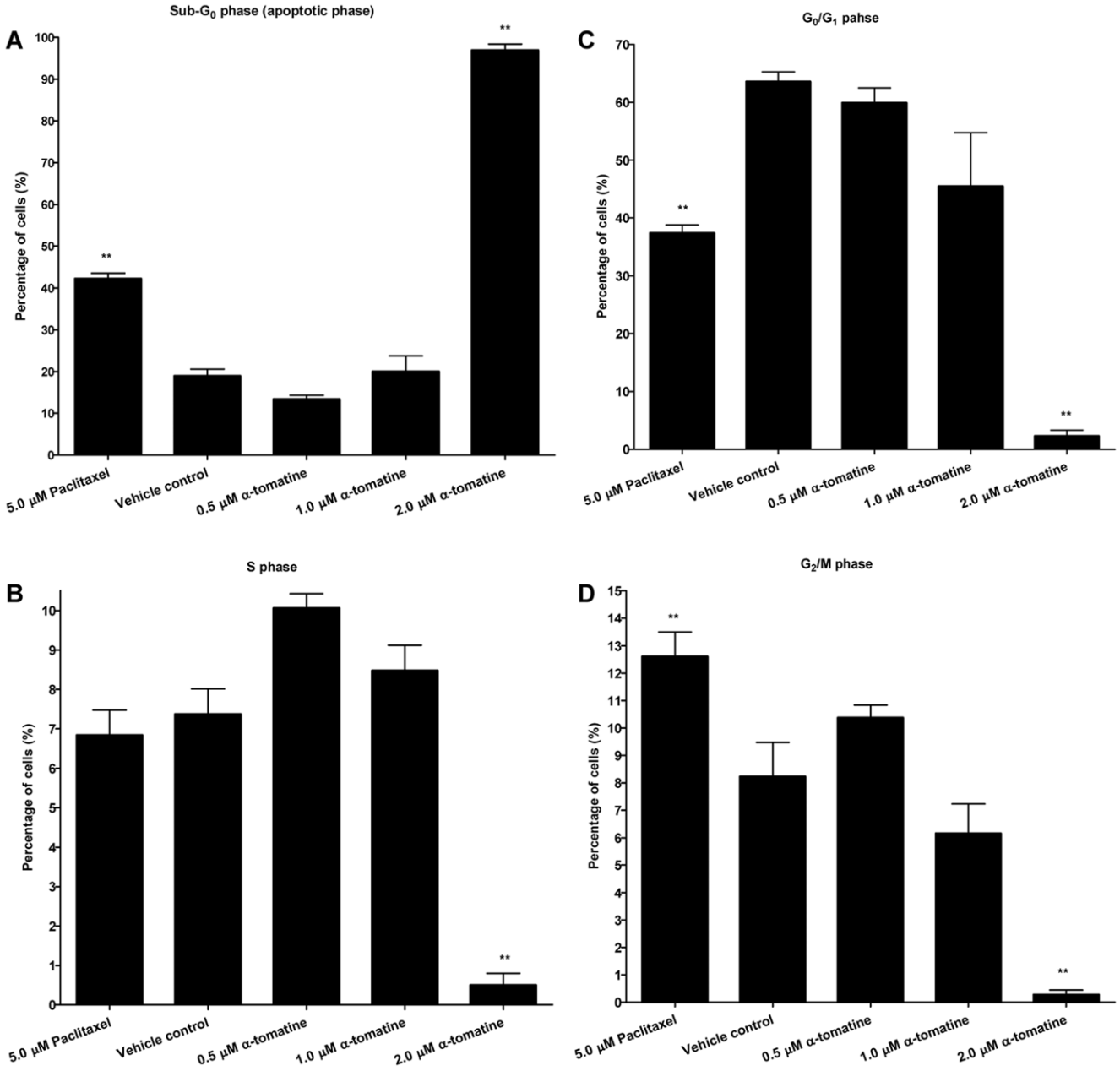
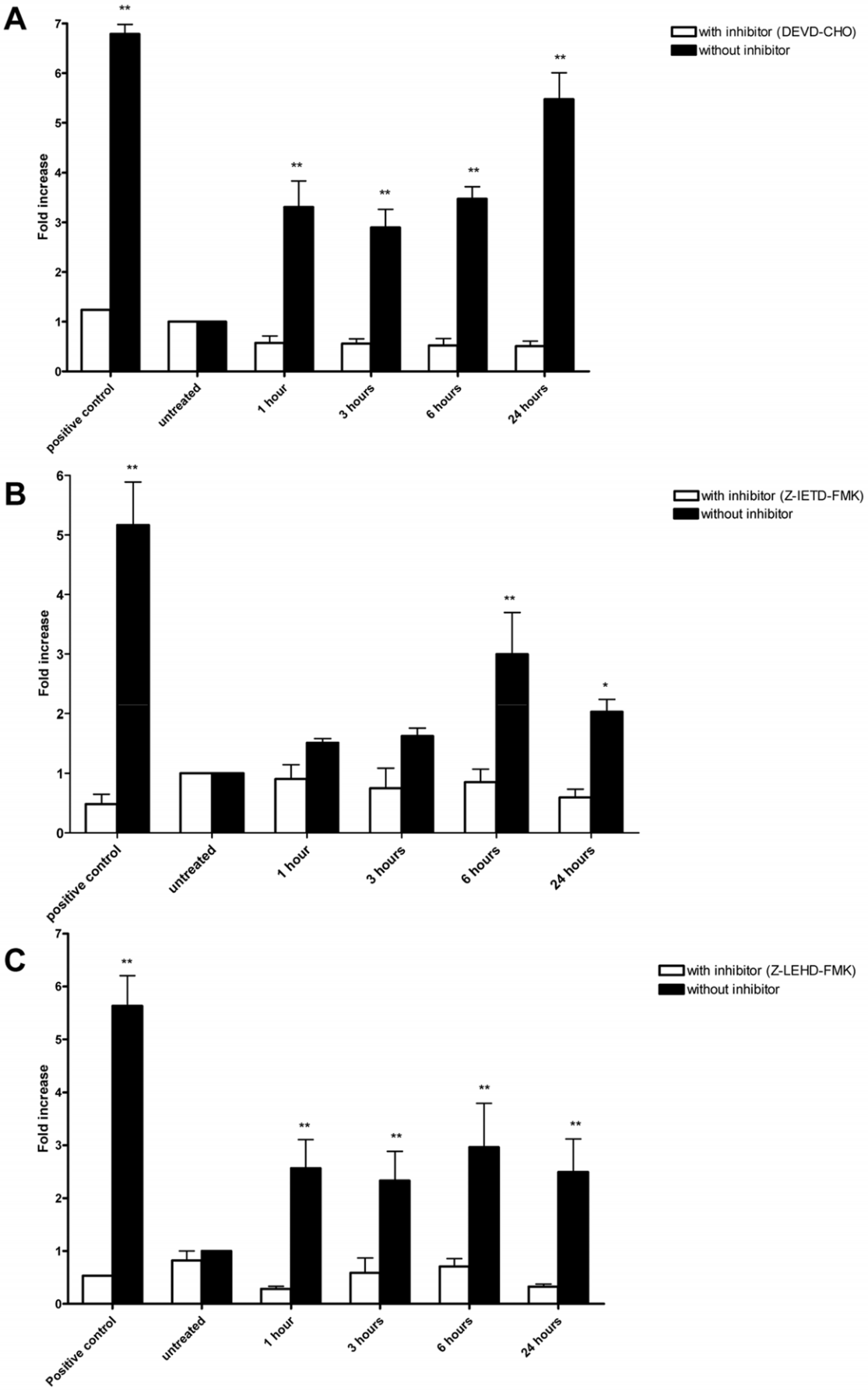


Figure 6. Cell cycle distribution of α-tomatine-treated PC-3 cells. The percentage of α-tomatine-treated cells at A) Sub G_1, B) G_0/G_1, C) S, and D) G_2/M phases of PC-3 cells. Each bar represents the mean ± SD of data from three independent experiments.* $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* vehicle control.
doi:10.1371/journal.pone.0018915.g006


A
with inhibitor (DEVD-CHO)
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1 hour
3 hours
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24 hours
B
with inhibitor (Z-IETD-FMK)
without inhibitor
Fold increase
positive control
untreated
1 hour
3 hours
6 hours
24 hours
C
with inhibitor (Z-LEHD-FMK)
without inhibitor
Fold increase
Positive control
untreated
1 hour
3 hours
6 hours
24 hours

Figure 7. Effect of α-tomatine on caspases activation. Fold increase of the levels of A) caspase-3, B) caspase-8, and C) caspase-9 in PC-3 cells treated with 2.0 μM α-tomatine, compared to vehicle control. The caspase activities were determined in the presence or absence of specific inhibitors in time course manner. The fluorescence intensity was measured at excitation wavelength of 390 nm and emission wavelength of 500 nm. The increase of protease activities was determined by comparing the levels in α-tomatine-treated PC-3 cells with the vehicle control. Each bar represents the mean ± SD of data from three independent experiments.* $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* vehicle control.
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fractions were measured using Cayman NF-κB/p50 and NF-κB/p65 ELISA kits (Ann Arbour, MI) according to the instructions of the manufacturer. The differences in NF-κB/p50 and NF-κB/p65 levels between the nuclear and cytoplasmic fractions were reported.

Statistical analysis

All assays were conducted in at least three separate experiments. Results are expressed as the mean value ± standard deviation (SD). Statistical analysis was performed with one-way analysis of variance (ANOVA), with Dunnett's Multiple Comparison Test to identify between-group differences using GraphPad Prism software (version 4.0; GraphPad Software Inc., San Diego, CA). Statistical significant is expressed as ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$. Log EC_{50} calculations were performed using the built-in algorithms from dose-response curves with variable slope.

Results

α-tomatine dose-dependently inhibited proliferation of PC-3 cancer cells

To evaluate the effect of α-tomatine on cell viability of PC-3, WRL-68 and RWPE-1 cells, MTT assay was performed. Treatment of α-tomatine to PC-3 cells resulted in a significant dose-dependent (from 0.16 to 5.0 μM) inhibition of cell growth (Figure 1). The EC_{50} value at 24 hours post-treatment with α-tomatine for PC-3 cells was estimated at 1.67±0.3 μM. α-tomatine only caused cytotoxicity towards normal prostate RWPE-1 cells at highest concentration (5.0 μM) of α-tomatine, with EC_{50} value of 3.85±0.1 μM. Normal human liver WRL-68 cells treated with the highest concentration of α-tomatine tested (5.0 μM) resulted in ~35% of nonviable cells and this suggest that α-tomatine is less cytotoxic against WRL-68 cells when compared to PC3 cells. The EC_{50} values of α-tomatine towards WRL-68 cells (>5 μM) and RWPE-1 (3.85±0.1 μM) and were higher than that of PC-3 cells (1.67±0.3 μM), suggesting the selective cytotoxicity of α-tomatine towards prostate cancer PC-3 cells.

Real-time growth kinetics analysis of α-tomatine using cell impedance-based analyzer

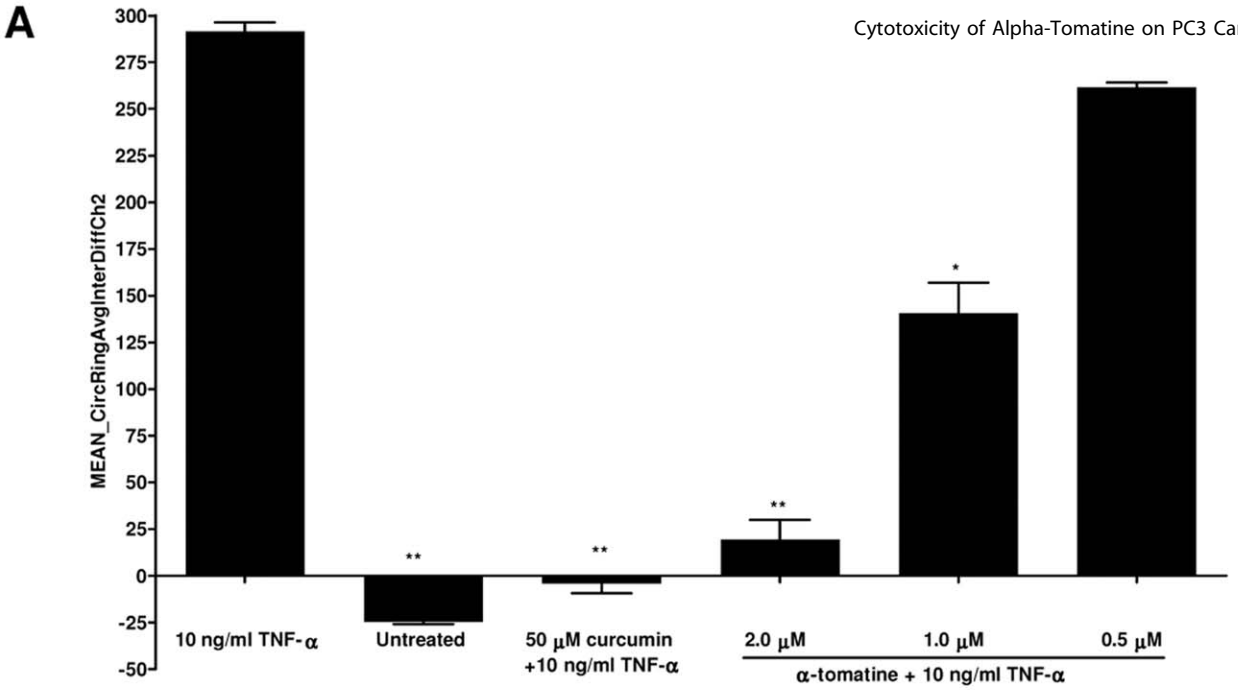
The dynamics of PC-3 cells proliferation on the 16-wells E-plates were monitored at every 5 minutes interval from the time of plating until the cells entered the logarithmic growth phase, following which the cells were treated with different concentrations of α-tomatine. After treatment, CI values were acquired at every 10 minutes interval for 72 hours. It was observed that a rapid decrease in CI value occurred as early as an hour after treatment with 5.0 μM and 2.5 μM of α-tomatine on PC-3 cells (Figure 2A), suggesting that PC-3 cells were dying from the treatment with these concentrations. PC-3 cells treated at lower concentrations (0.16–1.25 μM) of α-tomatine were proliferating in parallel to cells treated with vehicle control as indicated with increase in CI values. In contrast, WRL-68 cells treatment with α-tomatine showed no reduction in CI values even at the highest tested concentration of 5.0 μM compared to control levels and it exhibited a continuous rise in CI values throughout the 72 hours of treatment (Figure 2B). This showed that WRL-68 cells growth was not affected by α-tomatine. Treatment with 2.5 μM α-tomatine initially inhibited normal prostate RWPE-1 cell proliferation but eventually recovered to the level of vehicle control cells (Figure 2C). Treatment with higher concentration (5.0 μM), however, decreased the CI values suggesting that α-tomatine inhibited the proliferation of RWPE-1 cells at high concentration (Figure 2C). These data suggested that α-tomatine at low concentration (2.5 μM) can inhibit the growth of PC-3 prostate cancer cells but not the normal prostate RWPE-1.

α-tomatine induces apoptosis on PC-3 cancer cells

To determine if α-tomatine induces apoptosis in PC-3 prostate cancer cells, Annexin V/propidium iodide (PI) double staining assay and flow cytometry analysis were performed. Treatment with 2.0 μM α-tomatine for 1, 3, 6 and 24 hours resulted in a gradual increase in early apoptotic cells (Annexin V positive only) from 2.07±0.12% to 5.00±0.97%, 14.57±1.55%, and 21.50±2.48%, respectively (Figure 3, black bars). The late apoptotic cells (Annexin V and PI positive) were also increased significantly, from 2.63±0.56% to 4.00±0.93%, 6.93±1.3%, and 16.9±1.92% (Figure 3, white bars). Positive control cells treated with 5.0 μM paclitaxel for 24 hours also resulted in 7.13±0.75% of early apoptotic cells (Figure 3, black bars) and 21.53±1.74% of late apoptotic cells (Figure 3, white bars) in the culture compared with only 0.23±0.09% of early apoptotic cells (Figure 3, black bars) and 0.37±0.12% of late apoptotic cells (Figure 3, white bars) in the negative control cells treated with 0.2% DMSO. Hence, these results showed that α-tomatine inhibited PC-3 cells growth by inducing apoptosis in these cells.

Multiparametric HCS assays

To further confirm the induction of apoptosis by α-tomatine, the treated cells were examined for cellular changes associated with apoptosis using HCS analysis. Treatment with 2.0, 1.0 and 0.5 μM α-tomatine resulted in approximately 95%, 83% and 20% cell loss, respectively (Figure 4A). In comparison, positive control cells treated with 5.0 μM paclitaxel resulted in only 45% cell loss and none from the vehicle control cells (Figure 4A). The α-tomatine treated cells also exhibited a concentration-dependent increase in nuclear chromatin staining with Hoechst 33342 (Figures 4B and 5C), suggesting an increase in nuclear condensation. Increased in nuclear chromatin staining with Hoechst 33342 was also observed in the paclitaxel positive control cells (Figure 4B and 5B) whereas the fluorescent intensity was significantly lower and healthy nuclear morphology was observed in the vehicle control cells (Figures 4B and 5A). Membrane permeability of the treated cells also increased significantly as evidenced by high fluorescent intensity in the cytoplasm of α-tomatine (Figure 4C and 5C) and paclitaxel- treated cells (Figure 4C, and 5B) compared to the vehicle control (Figures 4C and 5A). A reduction in mitochondrial membrane potential is observed in both paclitaxel (Figure 4D and 5B) and α-tomatine (Figure 4D and 5C) treated cells compared to intact mitochondrial membrane potential of the vehicle control cells (Figure 4D and 5A). More specific apoptosis indicators such as release of cytochrome c and F-actin polarization or cleavage were found increased in a concentration-dependent manner in the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**
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*
10 ng/ml TNF-α
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50 μM curcumin
+10 ng/ml TNF-α
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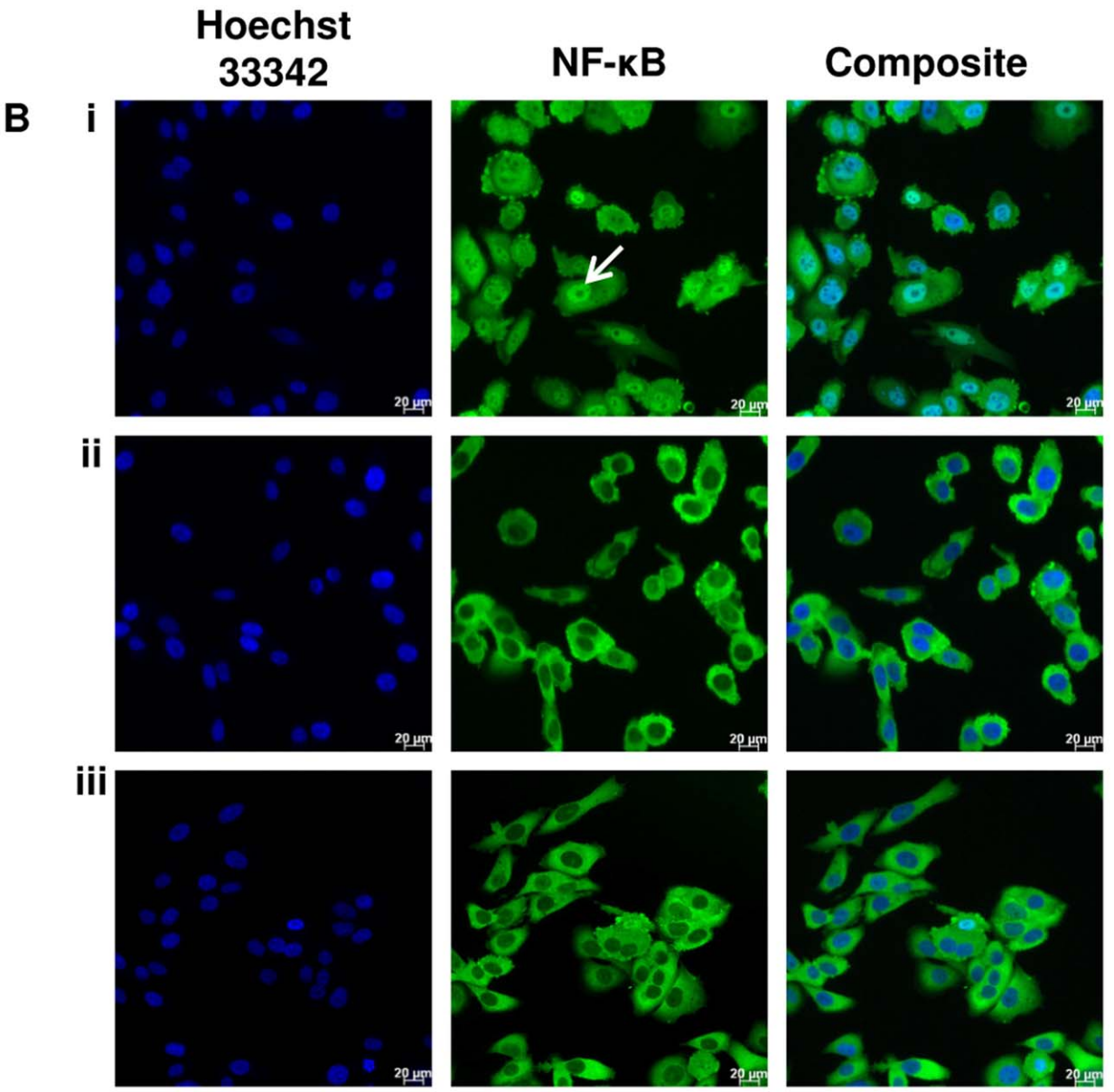
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i
ii
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Figure 8. The inhibitory effect of α-tomatine on TNF-α-induced NF-κB nuclear translocation. PC-3 cells were pretreated with α-tomatine for 30 minutes and followed by 10 ng/ml of TNF-α stimulation for 30 minutes. A) Cells treated with 50 μM curcumin and medium only were used as positive NF-κB inhibitor and negative control, respectively. Cells were fixed, immunostained with anti-NF-κB antibody, and counterstained with Hoechst 33342. NF-κB nuclear translocation index was measured using Thermo Scientific ArrayScan®VTI HCS Reader and expressed as the difference between nuclear and cytoplasmic NF-κB related fluorescence intensity. Each bar represents the mean ± SD of data in triplicate.* $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* cells treated withTNF-α alone. B) Images of PC-3 cells treated with 10 ng/mL TNF-α alone (i), medium only (ii), and 2.0 μM of α-tomatine pretreatment before 10 ng/ml TNF-α stimulation (iii). Control and treated PC-3 cells were stained with Hoechst (blue) and the Alexa Fluor 488-conjugated anti-NF-κB antibody (green). Images were acquired for each fluorescence channel, using suitable filters with 20× objective. Scale bar indicates 20 μm.
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cytoplasm of PC-3 treated cells, with the highest increase observed at 2.0 μM α-tomatine (Figure 4E, 4F and 5C). These results further confirmed that α-tomatine inhibited PC-3 cell growth by inducing apoptosis.

α-tomatine-induced apoptosis in PC-3 cells is not associated with cell cycle arrest

To determine whether induction of apoptosis in PC-3 cells by α-tomatine could be related to the cell cycle arrest, cell cycle distribution of the treated cells was assessed by labelling the cell's DNA with blue fluorescent Hoechst 33342 stain, whose intensity is proportional to the cell's DNA content. The sub G_1 population representing apoptotic cells in the 2.0 μM α-tomatine treated cells was markedly increased (95%) compared to positive control cells treated with paclitaxel (40%, Figure 6A). This increase was reflected by significant reduction in cells at G_0/G_1 (Figure 6B), S (Figure 6C), and G_2/M phases (Figure 6D). No significant changes in the percentage of population of cells in other phases were observed in 0.5 and 1.0 μM treated cells, suggesting that induction of apoptosis was not mediated by cell cycle arrest.

α-tomatine induced caspases activation

Caspases are present in the proforms (inactive) and become active after site-specific cleavage to participate in the process of apoptosis. To determine whether caspases are involved in apoptosis induction by α-tomatine, the protein levels of active caspases in α-tomatine-treated cells were evaluated. Activation of the executioner procaspase-3 by α-tomatine was found to be time-dependent (Figure 7A). Caspase-3 activity was significantly elevated at the first hour of treatment and progressed to a maximal level (6-fold over vesicle control) after 24 hours of incubation (Figure 7A). High levels of pro-caspase-8 and pro-caspase 9 were detected as early as 1 hour after the addition of α-tomatine, and reached a maximal level at 6 hours of incubation where the high levels of caspase 8 and 9 persisted over 24 hours of incubation (Figure 7B and 7C). These findings suggest that α-tomatine activated caspase-3, caspase-8 and caspase-9 as early as 1 hour after treatment. Cell permeable-specific inhibitors of caspase-3-like (DEVD-CHO, Figure 7A), caspase-8 (z-IETD-FMK, Figure 7B) and caspase-9 (z-LEHD-FMK, Figure 7C) were added into α-tomatine treatment to determine whether the activation of caspase-3, -8 and -9 by α-tomatine can be blocked by these inhibitors. In the presence of inhibitors, fold increase of caspase-3 (Figure 7A), caspase-8 (Figure 7B) and caspase-9 (Figure 7C) were reduced. These results further confirmed the activation of caspase-3, -8 and -9 by α-tomatine in PC-3 cells.

α-tomatine inhibits TNF-α-induced NF-κB nuclear translocation

It has been shown that activation of NF-κB blocks apoptosis and promotes cell proliferation [26]. We assessed whether α-tomatine inhibits activation of NF-κB induced by the inflammatory cytokine, TNF-α using Alexa Fluor 488-conjugated anti-NF-κB antibody. In cells treated with medium only (Figure 8A and 8Bii), high fluorescent intensity of NF-κB was found in cytoplasm but dimly in nuclei, indicating that NF-κB was not activated under resting condition. Following stimulation with TNF-α alone, NF-κB fluorescent intensity significantly increased in nuclei, suggesting that TNF-α stimulation resulted in NF-κB activation and translocation from cytoplasm into nuclei occurred in the stimulated cells (Figure 8A and 8Bi). However, in PC-3 cells treated with curcumin, a known inhibitor of NF-κB activation, it was observed that significant inhibition of TNF-α-induced NF-κB nuclear translocation as evidenced by low nuclear NF-κB-related fluorescence intensity (Figure 8A). Similarly, α-tomatine also inhibited TNF-α-induced NF-κB activation in a dose-dependent manner with strong inhibition observed at 2.0 μM treatment (Figure 8A, and 8Biii). These results showed that α-tomatine inhibit NF-κB activation.

α-tomatine treatment inhibited NF-κB/p50 and NF-κB/p65 nuclear translocation

To further confirm TNF-α-induced NF-κB activation was inhibited in α-tomatine treated PC-3 cells through the inhibition of NF-κB nuclear translocation, NF-κB/p50 (Figure 9A) and NF-κB/p65 (Figure 9B) in nuclear and cytoplasmic fractions of the treated cells were measured by ELISA. Pretreatment with 2.0 μM of α-tomatine resulted in significant decreased in both NF-κB/p50 (Figure 9A) and NF-κB/p65 (Figure 9B) level in nuclear fractions, compared to cells treated with TNF-α control (Figure 9A and 9B). These results showed that α-tomatine inhibits TNF-α-induced NF-κB activation by inhibiting nuclear translocation of NF-κB/p50 and NF-κB/p65 in the treated PC-3 cells.

Discussion

α-tomatine was reported to inhibit growth of human colon HT-29, liver HepG2, breast MCF-7 and stomach AGS cancer cells [20,21] but its anti-proliferative mechanisms require further definition. The present study reports that α-tomatine inhibits NF-κB activation and induces apoptosis on androgen-independent human prostatic adenocarcinoma PC-3 cells. The EC_{50} of α-tomatine towards PC-3 cells was estimated at 1.67 ± 0.3 μM and this value is comparable to earlier studies on different cancer cell lines [20,21]. Dynamic assessment by xCelligence system showed that α-tomatine is a fast-acting compound, inhibiting the cancer cells proliferation as early as an hour after treatment with 2.5 and 5.0 μM α-tomatine and its growth suppressive effect was not fully reversible as the cancer cell proliferation failed to recover throughout 72 hours of treatment. It is also less cytotoxic against human liver normal cells and human prostate normal cells. Although our data showed that α-tomatine inhibited the growth of normal prostate RWPE-1 cells at high concentration (5.0 μM), where utilization of high dose of α-tomatine can be a key consideration for cancer treatment, earlier *in vivo* studies showed no apparent toxic effects in rainbow trout [19] and does not affect body and liver weights of mice [14,27].

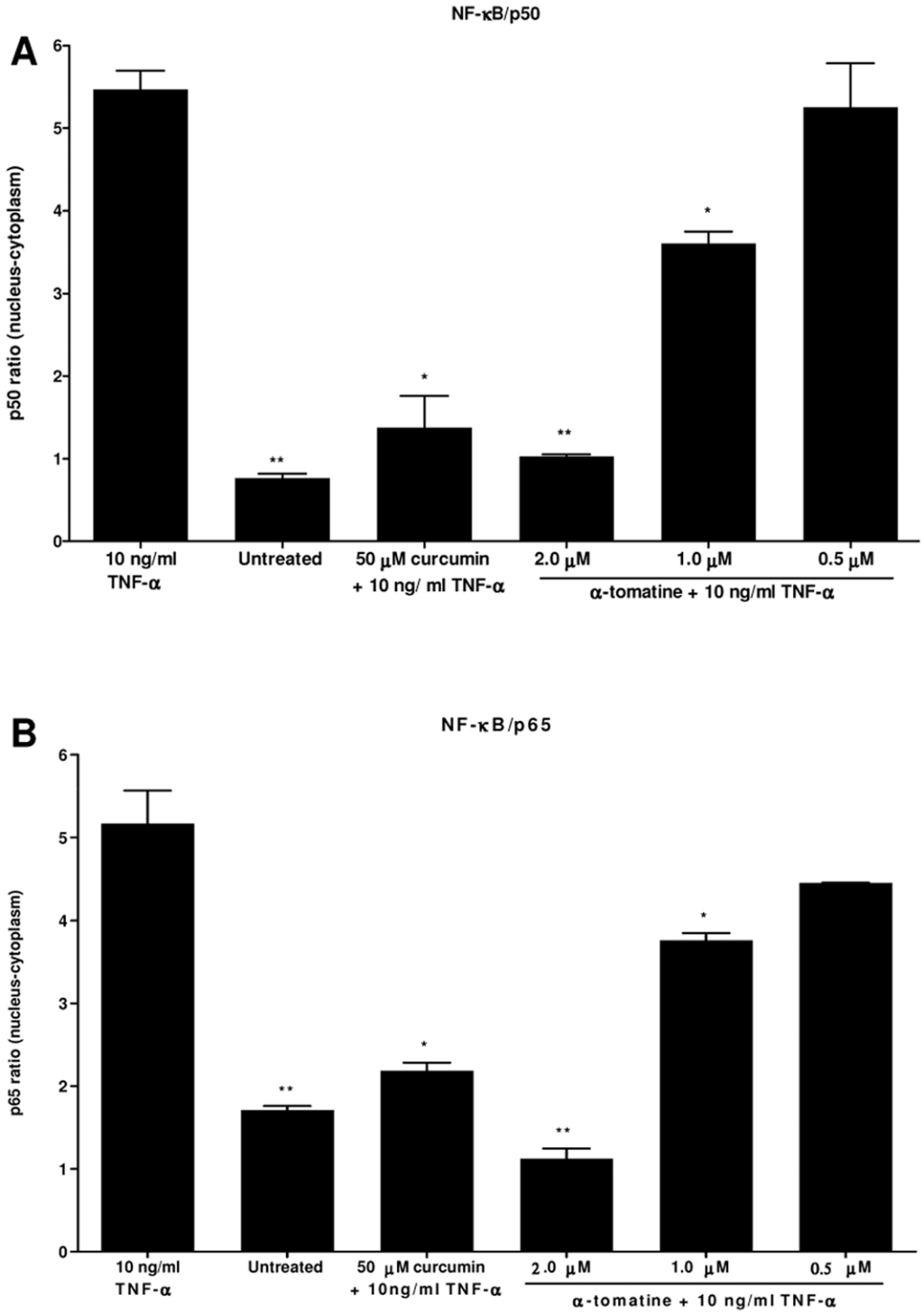

Figure 9. Comparison of NF-κB/p50 and NF-κB/p65 protein levels between nuclear and cytoplasmic fraction. PC-3 cells were treated with 0.5, 1.0, 2.0 μM α-tomatine for 30 minutes, followed by treatment with 10 ng/ml TNF-α for another 30 minutes. Nuclear and cytoplasmic fractions of the PC-3 treated cells were extracted, the concentration of the active form of A) NF-κB/p50 and B) NF-κB/p65 in both fractions were measured with ELISA kits. Differences of NF-κB/p50, NF-κB/p65 in the nuclear and cytoplasmic fractions are reported. Each bar represents the mean ± SD of data in triplicate.* $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* cells treated with TNF-α control.
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Flow cytometry analysis of Annexin V/PI staining showed that α-tomatine conspicuously induced apoptosis. This observation was confirmed by multiparametric cell-based high content screening (HCS) analysis that showed morphological features characteristic of apoptotic cell death such as nuclear condensation, loss of membrane symmetry, polarization of F-actin, release of cytochrome c and reduction in mitochondrial membrane potential. Disruption of the mitochondrial membrane potential is an early event in apoptosis and triggers release of cytochrome c and other apoptogenic molecules from the mitochondria to the cytosol [28].

These apoptogenic molecules contribute to the activation of caspases and subsequent cell death. More intensive molecular studies indicate that apoptotic cell death can be triggered either through receptor (extrinsic)-mediated pathway where the ligand-receptor binding activates caspase-8 or the mitochondrial (intrinsic)-mediated pathway where cytochrome c is released from the mitochondrial and activates caspase-9 [29,30]. Both of these mechanisms will eventually merge and lead to the hierarchical activation of the downstream caspase-3, 6 and 7, which are responsible for the characteristic apoptosis-associated morphological changes such as chromatin condensation, membrane blebbing, and loss of overall cell shape [31,32]. The present study demonstrated that treatment with α-tomatine increased activities of caspase-3 and -9 and caused depolarization of mitochondrial membrane potential and release of cytochrome c. These results suggest that induction of apoptosis is mediated through the intrinsic pathway. Pro-caspase-8 is an intracellular component that directly communicates with the death domain of cell membrane receptors and its activation in the α-tomatine-treated cells suggests that apoptosis is also mediated through the extrinsic pathway which perhaps merged with the intrinsic pathway and activated the executioner caspase 3. Consistent with these data, addition of cell permeable-specific inhibitors of caspase-8 (z-IETD-FMK), caspase-9 (z-LEHD-FMK), or caspase-3-like (DEVD-CHO) enzymes revealed that addition of caspase inhibitors completely decreased α-tomatine-induced activation of caspase-3, -8 and -9, confirmed the role of caspase-3, -8 and -9 in α-tomatine-induced apoptosis. Curcumin is an example of dietary agent that mediates apoptosis via both intrinsic and extrinsic pathways [33].

α-tomatine could also significantly alter polymerization of actin filaments in the treated PC-3 cells. This observation is in line with reports that showed polymerization or cleavage of actin cytoskeleton during the early and late phases of apoptosis [34–37]. Cisplatin, a well-known anticancer drug is an example of drug that induces F-actin damage prior to changes in nuclear morphology [38].

While the present study found no evidence of cell cycle arrest by α-tomatine, there are evidence that suggest induction of apoptosis could be mediated through the inhibition of nuclear factor-kappa B (NF-κB) signaling pathway. Constitutive NF-κB activation has been observed in many types of cancer, including androgen-independent prostate cancer [26]. Overexpression of NF-κB/p65 protein was found in the nuclear fraction of prostate cancer clinical specimens [39,40], suggesting the pathophysiological role for NF-κB in prostate cancer progression. Constitutive activation of NF-κB in tumor cells could promote cancer cells survival by blocking apoptosis and promote cancer cells growth through angiogenesis, metastasis and invasion [41]. Accumulating evidence indicate that constitutive activation of NF-κB in tumor cells always contribute to chemoresistance and radioresistance, and represents an independent risk factor for recurrence after radical prostatectomy [40,42,43]. Hence, targeting the NF-κB signaling pathway remains an attractive therapeutic option for prostate cancer. Treatment of PC-3 cells with α-tomatine resulted in a strong inhibition of NF-κB activation, which was consistent with a decrease in nuclear levels of NF-κB/p65 and NF-κB/p50. The ability of α-tomatine in inhibiting NF-κB activation by blocking the nuclear translocation of NF-κB/p65 and NF-κB/p50 transcription factors suggests its promising role in prostate cancer prevention. Inhibition of NF-κB activation could indirectly contribute to the pro-apoptotic action of α-tomatine on PC-3 cells as NF-κB controls the transcription of anti-apoptotic and cell proliferation genes, essential for the survival of cancer cells [44].

In summary, these results show that α-tomatine has good potentials as dietary phytochemical with pro-apoptosis activity. α-tomatine can be further explored for its therapeutic potential in the treatment of cancers where constitutive activation of NF-κB and apoptosis resistance remain a major concern in cancer chemotherapy. To establish the relevance of *in vitro* findings, further study is underway to investigate *in vivo* anti-tumor and NF-κB activities of α-tomatine using prostate xenograft cancer model.

Author Contributions

Conceived and designed the experiments: STL PFW SCC MRM. Performed the experiments: STL. Analyzed the data: STL PFW SCC MRM. Contributed reagents/materials/analysis tools: MRM. Wrote the paper: STL PFW SCC MRM.

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Alpha-Tomatine Attenuation of *In Vivo* Growth of Subcutaneous and Orthotopic Xenograft Tumors of Human Prostate Carcinoma PC-3 Cells Is Accompanied by Inactivation of Nuclear Factor-Kappa B Signaling

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Abstract

Background: Nuclear factor-kappa B (NF-κB) plays a role in prostate cancer and agents that suppress its activation may inhibit development or progression of this malignancy. Alpha (α)-tomatine is the major saponin present in tomato (*Lycopersicon esculentum*) and we have previously reported that it suppresses tumor necrosis factor-alpha (TNF-α)-induced nuclear translocation of nuclear factor-kappa B (NF-κB) in androgen-independent prostate cancer PC-3 cells and also potently induces apoptosis of these cells. However, the precise mechanism by which α-tomatine suppresses NF-κB nuclear translocation is yet to be elucidated and the anti-tumor activity of this agent *in vivo* has not been examined.

Methodology/ Principal Findings: In the present study we show that suppression of NF-κB activation by α-tomatine occurs through inhibition of I kappa B alpha (IκBα) kinase activity, leading to sequential suppression of IκBα phosphorylation, IκBα degradation, NF-κB/p65 phosphorylation, and NF-κB p50/p65 nuclear translocation. Consistent with its ability to induce apoptosis, α-tomatine reduced TNF-α induced activation of the pro-survival mediator Akt and its inhibition of NF-κB activation was accompanied by significant reduction in the expression of NF-κB-dependent anti-apoptotic (c-IAP1, c-IAP2, Bcl-2, Bcl-xL, XIAP and survivin) proteins. We also evaluated the antitumor activity of α-tomatine against PC-3 cell tumors grown subcutaneously and orthotopically in mice. Our data indicate that intraperitoneal administration of α-tomatine significantly attenuates the growth of PC-3 cell tumors grown at both sites. Analysis of tumor material indicates that the tumor suppressing effects of α-tomatine were accompanied by increased apoptosis and lower proliferation of tumor cells as well as reduced nuclear translocation of the p50 and p65 components of NF-κB.

Conclusion/ Significance: Our study provides first evidence for *in vivo* antitumor efficacy of α-tomatine against the human androgen-independent prostate cancer. The potential usefulness of α-tomatine in prostate cancer prevention and therapy requires further investigation.

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Introduction

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in men worldwide [1]. As progression of this malignancy is dependent on the androgen receptor, therapies that target activating ligands (the hormones testosterone and dihydrotestosterone) produce response rates in patients of up to 95% [2]. Unfortunately, nearly all prostate cancer patients develop hormone-refractory prostate cancer (HRPC) [2]. For these patients curative treatments are not available and docetaxel-based chemotherapy provides palliation with response rates of approximately 50% and median survival of 18 to 20 months with survival benefit of about 2 months [3]. For patients with HRPC, low toxicity molecular targeting strategies are needed.

Accumulating evidence suggests that the transcription factor nuclear factor-kappa B (NF-κB) plays a pivotal role in prostate cancer growth, survival, angiogenesis and metastatic progression [4,5,6,7,8]. NF-κB consists of a p50/p65 heterodimer, that is masked by the inhibitor of NF-κB, I kappa B alpha (IκBα) that causes its retention in the cytoplasm under resting condition. Various stimuli, including tumor necrosis-alpha (TNF-α), phorbol ester and lipopolysaccharides (LPS), result in IκBα kinase activation, which mediates IκBα phosphorylation at Ser32 and Ser36 followed by its ubiquitination and proteasome-mediated degradation. This releases the NF-κB p50/p65 heterodimer,

which then translocates to the nucleus, where it binds to consensus sequence motifs to induce gene transcription. It has been demonstrated that NF-κB is constitutively activated in androgen-insensitive prostate carcinoma cells, and overexpression of NF-κB p65 protein was found in the nuclear fraction of prostate cancer clinical specimens [5,9], suggesting a role for NF-κB in prostate cancer progression. Consistently, it has been report that aberrant IKK activation leads to the constitutive activation of the NF-κB survival pathway in androgen-independent prostate cancer cells [10]. In addition, activation and localization of NF-κB represent independent risk factors for disease recurrence after radical prostatectomy [9,11]. Hence, effective inhibition of NF-κB could be a promising strategy for treatment of prostate cancer and prevention of relapse.

Alpha (α)-tomatine is the major saponin in tomato (*Lycopersicon esculentum*). Previous studies have reported its immunopotentiating [12] and *in vitro* anti-cancer activities [13,14,15,16]. It also has protective effects against dibenzo[a,l]pyrene (DBP)-induced liver and stomach tumors in rainbow trout without causing significant changes in total weight, liver weight, tissue morphology and mortality [17]. Thus far, the mechanism by which α-tomatine mediates its anti-prostate cancer effect is not well understood. Our previous study reported the pro-apoptotic effect of α-tomatine against androgen-independent human prostatic adenocarcinoma PC-3 cells through the inhibition of TNF-α-induced NF-κB nuclear translocation [18]. In the present study, the mechanism of the inhibition of α-tomatine on NF-κB signaling pathway is further characterized. For the first time, this study demonstrates the potent anti-tumor activity of α-tomatine against human androgen-independent prostate cancer *in vivo*.

Materials and Methods

Ethics statement

Experiments with mice were performed in accordance with the protocol approved by the University of Queensland Animal Ethics Committee (AEC Approval Number: MMRI/210/10).

Materials

α-tomatine was purchased from Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO), TNF-α, fetal bovine serum (FBS), 3,3,5,5 tetramethylbenzidine (TMB), Calpain Inhibitor I, ALLN (N-acetyl-leucyl-leucyl-norleucinal) and anti-human beta (β)-actin antibody were purchased from Sigma Aldrich (St. Louis, MO). Penicillin/streptomycin, Roswell Park Memorial Institute (RPMI-1640) media and 0.4% trypan blue solution were purchased from Invitrogen (Carlsbad, CA). Protein A/G plus agarose beads, Akt inhibitor VIII, and antibodies against p65, p50, IκBα, Akt, IKKα, and IKKβ were obtained from Santa Cruz Biotechnology, CA. The glutathione *S*-transferase-IκBα (GST-IκBα) fusion protein and polyvinylidene fluoride (PVDF) membrane were purchased from Millipore (Bedford, MA). Kinase buffer, antibodies against phospho-specific IκBα (Ser32/36), phosphor-specific p65 (Ser536), phosphor-specific Akt (Ser473), B cell leukaemia-2 (Bcl-2), B cell leukaemia-x long (Bcl-xL), cellular inhibitor of apoptosis 1 (c-IAP1), cellular inhibitor of apoptosis 2 (c-IAP2), survivin, X-linked inhibitor of apoptosis (XIAP), histone H3 and cleaved-Poly (ADP-ribose) polymerase (PARP) antibodies were purchased from Cell Signaling (Beverly, MA). Antibodies against Ki-67 and proliferating cell nuclear antigen (PCNA) were purchased from BD Biosciences (San Diego, CA).

Cell line

The prostate cancer PC-3 cell line was purchased from the American Type Culture Collection (Manassas, VA). Luciferase-expressing prostate cancer PC-3 cell line was a kind gift of Dr. Patrick Ming Tat Ling, Queensland University of Technology, Australia [19,20]. Both PC-3 and luciferase-expressing PC-3 cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at 37°C in a 5% CO_2 humidified incubator.

Cell treatment and fractionation

For the *in vitro* assays, PC-3 cells at 70-80% confluency were treated with α-tomatine (2 μM) for 30 minutes, and then exposed to10 ng/ml TNF-α for various time periods. Akt inhibitor VIII (10 μM) which inhibits activation of Akt as evidenced by reduced phosphorylation of this kinase at Thr308 and Ser473 [21] was used as inhibitor control for studying the effect of α-tomatine on Akt activation as described previously [22]. Both nuclear and cytoplasmic fractions of treated and vehicle control cells were isolated using a nuclear extraction kit (Cayman Chemical, Ann Arbour, MI) according to the manufacturer's instructions. Briefly, cells were harvested using a cell scrapper then pelleted by centrifugation at 4°C before two washes with ice-cold PBS supplemented with phosphatase inhibitor solution at 4°C. Pelleted cells were swollen for 15 minutes in ice-cold hypotonic buffer supplemented with complete protease and phosphatase inhibitors. 10 % Nonidet P-40 assay reagent was then added and cytosolic fractions were collected by brief centrifugation. Pellets were resuspended in ice-cold complete nuclear extraction buffer then vortexed on ice for 30 seconds at highest setting. These cell pelleting and vortexing steps were repeated for a total of 6 cycles. The final pellet was resuspended then centrifuged at 14,000 x g for 10 minutes at 4°C. The supernatants containing nuclear fractions were collected.

Cell viability analysis

Cell viability was examined using a trypan blue exclusion assay as described previously [23]. Briefly, cells in control and treated groups were harvested, stained with 0.4% trypan blue solution and total viable cells were counted using hemacytometer. The proportion of viable cells was calculated by dividing the number of viable test cells by the number of viable control cells at the end of each experimental treatment.

IκBα kinase assay

The effect of α-tomatine on TNF-α-induced IKK activation was analyzed as described previously [24]. Briefly, PC-3 cells were preincubated with either 2 μM α-tomatine or 0.1% DMSO (vehicle) for 30 minutes, and then treated with 10 ng/ml TNF-α for the indicated times. IKK complex was immunoprecipitated from whole cell extracts using antibodies against IKKα and IKKβ. Protein A/G plus agarose beads were added and incubated at 4°C for overnight. The beads were washed with lysis buffer and resuspended in a kinase buffer before GST-IκBα as IKK substrate was added. After incubation at 30°C for 30 minutes, the reaction was terminated by addition of Laemmli's loading buffer and heated at 100°C for 5 minutes. Western blot analysis was performed to detect phosphorylated-IκBα (p-IκBα) and to determine the total amounts of IKKα and IKKβ in each sample. To determine whether α-tomatine directly targets IKK, IKKα and IKKβ immunoprecipitated from 10 ng/ml TNF-α and 0.1% DMSO (vehicle) treated cells. *In vitro* kinase assay was performed in

the absence or presence of indicated concentrations of α-tomatine at 30°C for 30 minutes.

Subcutaneous and orthotopic implantation of PC-3 cells

Male BALB/c nude mice (6 weeks old) were purchased from the Animal Resources Centre (Canning Vale, Western Australia). For subcutaneous tumor growth study, luciferase-expressing PC-3 prostate cancer cells (1×10^6 in 0.1 ml Dulbecco's PBS) were inoculated subcutaneously into the lower flanks of each mouse. On day 7 after cancer cell inoculation, each mouse had one palpable tumor and were randomly assigned to four groups (n = 8/group). These groups of mice were then given intraperitoneal injections of vehicle solution, 10 mg/kg docetaxel, 5 mg/kg α-tomatine or 10 mg/kg α-tomatine thrice a week for an additional 3 weeks. All the mice were monitored weekly for tumor growth and body weight. Tumor dimensions were measured with calipers and volume calculated using a standard formula: $(\text{length}\times\text{width}^2)\times0.5$ [25]. The experiment was terminated 28 days after cancer cell inoculation at which time bioluminescent signals of tumors in live mice were captured on a Xenogen IVIS Spectrum imaging system (Alameda, CA, USA) using Live Imaging Acquisition and Analysis software. Briefly, mice were injected intraperitoneally with luciferin potassium salt, anesthetized and luminescence images acquired. Luminescence signal intensity was quantified as region of interest analysis of total photons per second for each tumor. Tumors were excised, washed with ice-cold phosphate buffered saline (PBS) and stored at -80°C until examined by Western blot analysis.

Orthotopic growth of PC-3 cells was performed as described previously [19]. Briefly, under a dissecting microscope (Olympus, Tokyo, Japan), the prostate of 6 weeks old anesthetized SCID mice were exposed through a surgical incision and 2×10^5 cells in 10 µl Dulbecco's PBS injected into the dorsal prostate. Organs were then replaced, and the abdomen was closed in two layers with silk sutures. Five days after implantation, mice were randomly assigned to two groups that received vehicle solution or 10 mg/kg α-tomatine intraperitoneally thrice a week for 14 days (n = 6 per group). Body weight was measured weekly. Bioluminescent signal of the PC-3 cell tumor in each mouse was measured at the end of the study using a Xenogen IVIS Spectrum imaging system as described above. All mice were then sacrificed by cervical dislocation.

Tissue processing and protein extraction

For protein analysis of mouse tumors, tissues were minced, suspended in tissue protein extraction reagent (Thermo-Fisher Scientific, Waltham, MA) supplemented with complete protease and phosphatase inhibitors, and homogenized using a gentleMACS Dissociator (Miltenyi Biotec, Germany). The homogenized tissue was transferred to a pre-chilled microcentrifuge tube, incubated on ice for 30 minutes and then centrifuged at 15,000 x g for 20 minutes at 4°C. The supernatant containing total cellular proteins was collected for Western blot analysis. Nuclear proteins were extracted using a nuclear extraction kit according to the manufacturer's instructions. Briefly, hypotonic buffer supplemented with 1 mM DTT and 0.01% Nonidet P-40 per gram of tissues was added to minced tissues, which were then homogenized with a gentleMACS Dissociator followed by incubation on ice for 15 minutes. Cytoplasmic proteins were separated by centrifugation. The pellets were resuspended in ice-cold complete nuclear extraction buffer then vortexed on ice for 30 seconds at highest setting. These cell pelleting and vortexing steps were repeated for a total of 6 cycles. The suspensions were then centrifuged at 14,000 x g for 10 minutes at 4°C and supernatants containing nuclear fractions were collected for Western blot analysis.

Western blot analysis

Protein samples were separated by SDS-PAGE and then transferred onto PVDF membranes which were probed with primary antibodies, followed after washes by horseradish peroxidase-conjugated secondary antibodies and visualized colorimetrically after further washes using TMB solution. Protein bands were visualized and quantified using a gel documentation system (BioRad, Richmond, Calif).

Statistical analysis

All assays were performed on at least three separate occasions. Results are expressed as the mean value ± standard error of the mean (SEM). Statistical analysis was performed with one-way analysis of variance, with Dunnett's Multiple Comparison Test to identify between-group differences using GraphPad Prism software (version 5.0; GraphPad Software Inc., San Diego, CA). For *in vivo* tumor growth experiments, statistical significance of differences in tumor volume, body weight and total bioluminescence intensity between control and treatment groups was assessed by two-way ANOVA (GraphPad Software Inc., version 5.0, San Diego, CA), with p values < 0.05 considered significant. Statistical significance is expressed as ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$.

Results

α-tomatine inhibits TNF-α-induced nuclear translocation of NF-κB p50 and p65 and phosphorylation of NF-κB p65

We previously showed that α-tomatine inhibited the growth of androgen-independent human prostatic adenocarcinoma PC-3 *in vitro* with the half maximal effective concentration (EC_{50}) value of 1.67 ± 0.3 µM [18]. At this chosen dose, α-tomatine was shown to be less cytotoxic to human normal prostate RWPE-1 (EC_{50} 3.85 ± 0.1 µM) and normal liver WRL-68 cells ($EC_{50} > 5$ µM) [18]. Treatment with 2 µM α-tomatine induces apoptosis and inhibits the TNF-α-induced NF-κB nuclear translocation on PC-3 cells [18]. In the present study, the mechanism of α-tomatine in inhibition of TNF-α-induced NF-κB nuclear translocation was investigated by analyzing its effect on phosphorylation and translocation of NF-κB sub-units in prostate cancer PC-3 cells. As shown in Figure 1A, the time-dependent phosphorylation of NF-κB p65 induced by TNF-α over a 60 minutes period was completely suppressed by pretreatment of cells with 2 µM α-tomatine. Examination of treated and control cell populations using a trypan blue exclusion assay indicated that the concentration of α-tomatine and TNF-α used and the time of exposure had minimal effect on cell viability (Figure 1B). In addition to the observed loss of p65 phosphorylation, α-tomatine prevented TNF-α induced translocation from the cytoplasm to the nucleus of both p50 and p65 NF-κB sub-units (Figure 1C). These findings indicate that α-tomatine inhibits the phosphorylation of NF-κB p65 and prevents the nuclear translocation of NF-κB p50 and p65.

α-tomatine inhibits TNF-α-dependent IκBα phosphorylation and degradation

The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination and proteolytic degradation of IκBα [26]. To determine whether the observed inhibition of TNF-α-induced NF-κB nuclear translocation caused by α-tomatine was due to inhibition of IκBα degradation, we pretreated cells with α-tomatine and then exposed them to TNF-α stimulation for time

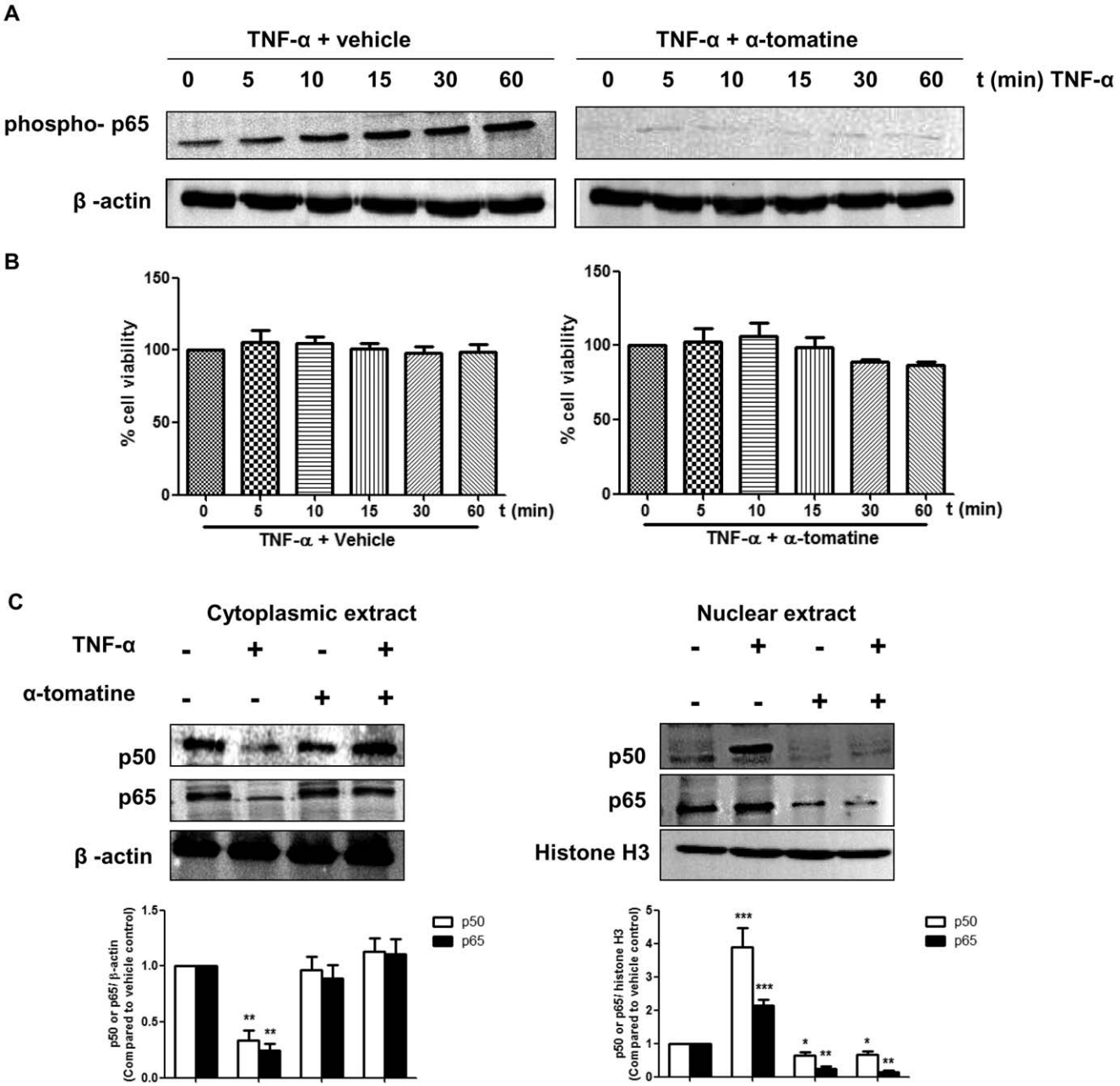

Figure 1. Effect of α-tomatine on TNF-α-induced phosphorylation of p65 and nuclear translocation of NF-κB p50/p65. (A) PC-3 cells at 70–80% confluency were treated with either 0.1% DMSO (vehicle) or 2 μM α-tomatine in DMSO for 30 minutes, followed by treatment with 10 ng/ml TNF-α for the indicated times. Cytoplasmic extracts were analyzed by Western blot analysis using an antibody against the phosphorylated form of p65. (B) Cell viability was assessed by counting cells that excluded trypan blue using a hemocytometer. (C) Effect of α-tomatine on nuclear and cytoplasmic levels of NF-κB p50 and p65 in human prostate cancer PC-3 cells. Nuclear and cytoplasmic fractions extracted from PC-3 cells treated either 0.1% DMSO or 2 μM α-tomatine in DMSO for 30 minutes, followed by treatment with 10 ng/ml TNF-α for the 30 minutes were analyzed by Western blot analysis with antibodies against NF-κB p50 and p65 proteins. β-actin and histone H3 proteins were loading control for cytoplasmic and nuclear extracts, respectively. Graphical representation of densitometry analysis of NF-κB p50 and p65 Western blot analyses from three independent experiments are shown below each panel. The ratio of the signal intensity of each protein to loading control was normalized to the vehicle control. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* vehicle control.
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periods up to 60 minutes. TNF-α induced IκBα degradation in control cells as early as 5 minutes after stimulation (Figure 2A). In contrast, in α-tomatine-pretreated cells, TNF-α stimulation did not result in the degradation of IκBα, instead its expression was sustained up to 60 minutes of treatment (Figure 2B). The effect of α-tomatine on TNF-α-induced IκBα phosphorylation was examined using an antibody that detects IκBα phosphorylated at Ser32 and Ser36. Calpain inhibitor ALLN was used to prevent degradation of phosphorylated IκBα. Western blot analysis indicated that TNF-α induced IκBα phosphorylation as early as 5 minutes post-stimulation, but α-tomatine completely suppressed this event (Figure 2B). These results indicate that α-tomatine

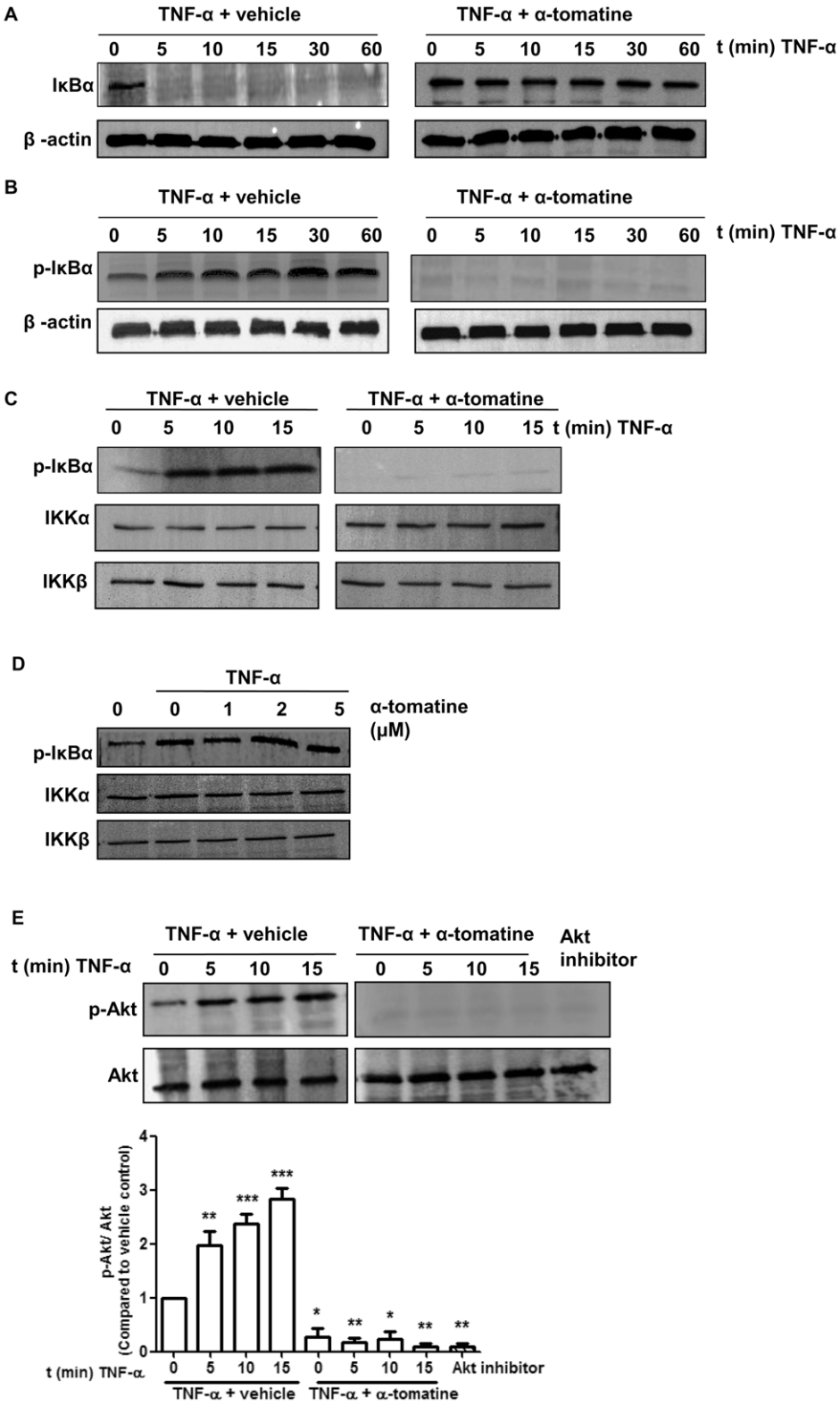

Figure 2. Effect of α-tomatine on IκBα Kinase activity. (A) Cells were grown to 70–80 % confluence, treated with either 0.1% DMSO (vehicle) or 2 μM α-tomatine in DMSO for 30 minutes, followed by treatment with 10 ng/ml of TNF-α for the indicated times. The presence of IκBα was detected by Western blot analysis. (B) To determine whether α-tomatine inhibits IκBα degradation by blocking IκBα phosphorylation, cells were treated with either 0.1% DMSO (vehicle) or 2 μM α-tomatine in DMSO for 30 minutes, followed by 50 μg/ml calpain inhibitor ALLN for 30 minutes, and then treated with 10 ng/ml of TNF-α for the times indicated. Anti-phospho-IκBα Western blot analysis was performed on cytoplasmic extracts. β-actin served as a loading control. (C) PC-3 cells were preincubated with either 2 μM α-tomatine or 0.1% DMSO (vehicle) for 30 minutes, and then treated with 10 ng/ml TNF-α for the indicated times. IKKα and IKKβ were immunoprecipitated from lysates from cells and *in vitro* kinase assays were performed using GST-IκBα as substrate as described in "Materials and Methods". Western blot analysis was performed to detect phosphorylated IκBα.

(D) IKK complex immunoprecipitated from vehicle and TNF-α-treated PC-3 cell extracts with an anti-IKKα and IKKβ antibodies was assayed for IKK activity. The kinase reaction mixture was incubated with α-tomatine as indicated. The expressions of phosphorylated IκBα and IKK were examined by Western blot analysis using anti-phospho-IκBα, anti-IKKα and anti-IKKβ antibodies. To examine the basal level of expression of IKK proteins, whole-cell extracts analyzed by Western blotting using anti-IKKα and anti-IKKβ antibodies. (E) PC-3 cells were pretreated with 2 μM α-tomatine for 30 minutes, and then treated with 10 ng/ml TNF-α for the indicated times. Lysates extracted from cells treated with 10 μM Akt inhibitor VIII for 3 hours serve as inhibition control. Cytoplasmic extracts were used for Western blotting using anti-phosphospecific Akt (Ser473) antibody. The same blot was reprobed with nonphosphorylated Akt antibody. Graphical representation of densitometry analysis of phosphor-Akt Western blot analysis from three independent experiments is shown below the panel. The ratio of the signal intensity of each protein to loading control was normalized to the vehicle control. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* vehicle control.
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inhibited TNF-α-induced phosphorylation and degradation of IκBα and subsequent nuclear translocation of NF-κB.

α-tomatine inhibits TNF-α-induced IKK activation

TNF-α induces IκBα phosphorylation and degradation via activation of the IKK complex [26]. To determine if pretreatment with α-tomatine affects IKK activation, *in vitro* kinase assays were performed using immunoprecipitated components of the IKK complex, and GST-IκBα as the IKK phosphorylation substrate. As shown in Figure 2C, TNF-α treatment stimulated IKK activity as phosphorylated-IκBα was detected as early as 5 minutes after treatment and remained detectable 10 minutes later. In contrast, phosphorylated-IκBα was not detected in cells treated with 2 μM α-tomatine during the 15 minutes period of TNF-α stimulation (Figure 2C). Western blot analysis demonstrated that TNF-α and α-tomatine had no effect on the expression of the components of the IKK complex, IKKα or IKKβ (Figure 2C), indicating that α-tomatine blocked TNF-α-induced phosphorylation of IκBα by attenuating the action of IKK rather than by causing degradation of this kinase. In a second set of experiments, we assessed whether α-tomatine suppressed IKK activity by directly binding to IKK protein by using IKKα and IKKβ immunoprecipitated from cells treated with TNF-α. The kinase reaction mixture was incubated with increasing concentrations of α-tomatine (1, 2 and 5 μM). As shown in Figure 2D, whereas TNF-α caused an increase in phosphorylation of IκBα, this was not reduced by inclusion of increasing concentrations of α-tomatine. This suggests that while α-tomatine efficiently inhibits IKK-mediated phosphorylation of IκBα, it did not do this by directly inhibiting IKK.

α-tomatine inhibits TNF-α-induced Akt activation

It has been reported that the serine-threonine kinase Akt can activate IKK [27]. To gain insight into whether this pathway may be relevant in TNF-α–induced activation of NF-κB signaling, we pretreated PC-3 cells with 2 μM α-tomatine for 30 minutes then exposed these cells to TNF-α for time periods up to 15 minutes. In control experiments cells were treated with an Akt inhibitor, Akt inhibitor VIII, at 10 μM for 3 hours as described previously [22]. As shown in Figure 2E, TNF-α induced Akt activation in a time-dependent manner and α-tomatine suppressed this activation as effectively as Akt inhibitor VIII with no significant effect on the expression of total Akt protein.

α-tomatine represses TNF-α-induced NF-κB dependent expression of pro-survival proteins

Several studies have indicated that the transcription factor NF-κB regulates the expression of proteins implicated in facilitating tumor cell survival including Bcl-2, Bcl-xL, c-IAP, survivin and XIAP [28,29]. Accordingly, we next examined whether α-tomatine inhibition of TNF-α-induced NF-κB nuclear translocation is accompanied by alterations in the expression of these pro-survival proteins. In these experiments cells were pretreated with α-tomatine (2 μM) for 30 minutes before induction of TNF-α-induced effects for 6 hours. This time period was selected to permit accumulation of levels of pro-survival proteins sufficient for Western blot analysis. As during this time period α-tomatine treatment resulted in varying levels of cell rounding and detachment in treated and control cells, both adherent and non-adherent cells were collected for protein extraction. As shown in Figure 3 Western blot analysis revealed that TNF-α induced marked upregulation of c-IAP1 and c-IAP2 while increases in expression of Bcl-xL, survivin and XIAP were also apparent but at lower levels and Bcl-2 expression was marginally increased in response to this cytokine. It was striking that α-tomatine caused sharp down-regulation of each of these mediators of cell survival under basal conditions as well as completely blocking TNF-α-induced upregulation of each protein (Figure 3). These data indicate that, consistent with its ability to inhibit TNF-α-induced NF-κB nuclear translocation, α-tomatine blocks the expression of the pro-survival mediators typically upregulated by this cytokine.

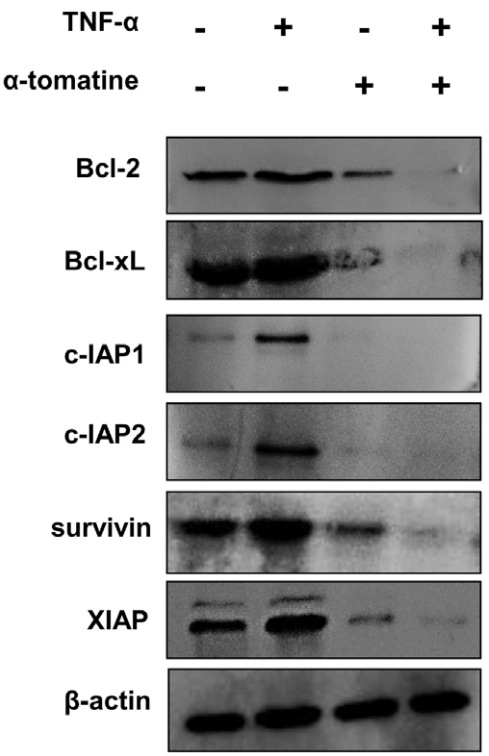

Figure 3. α-tomatine represses TNF-α-induced NF-κB dependent expression of pro-survival proteins. PC-3 cells grown to 70–80 % confluence were treated with either 0.1% DMSO or 2 μM α-tomatine for 30 minutes, 10 ng/ml TNF-α was then added and the cells were incubated for a further 6 hours. Whole-cell extracts were prepared, and were analyzed by Western blot analysis using antibodies against Bcl-2, Bcl-xL, c-IAP1, c-IAP2, XIAP and survivin. The results shown here are representative of three independent experiments.
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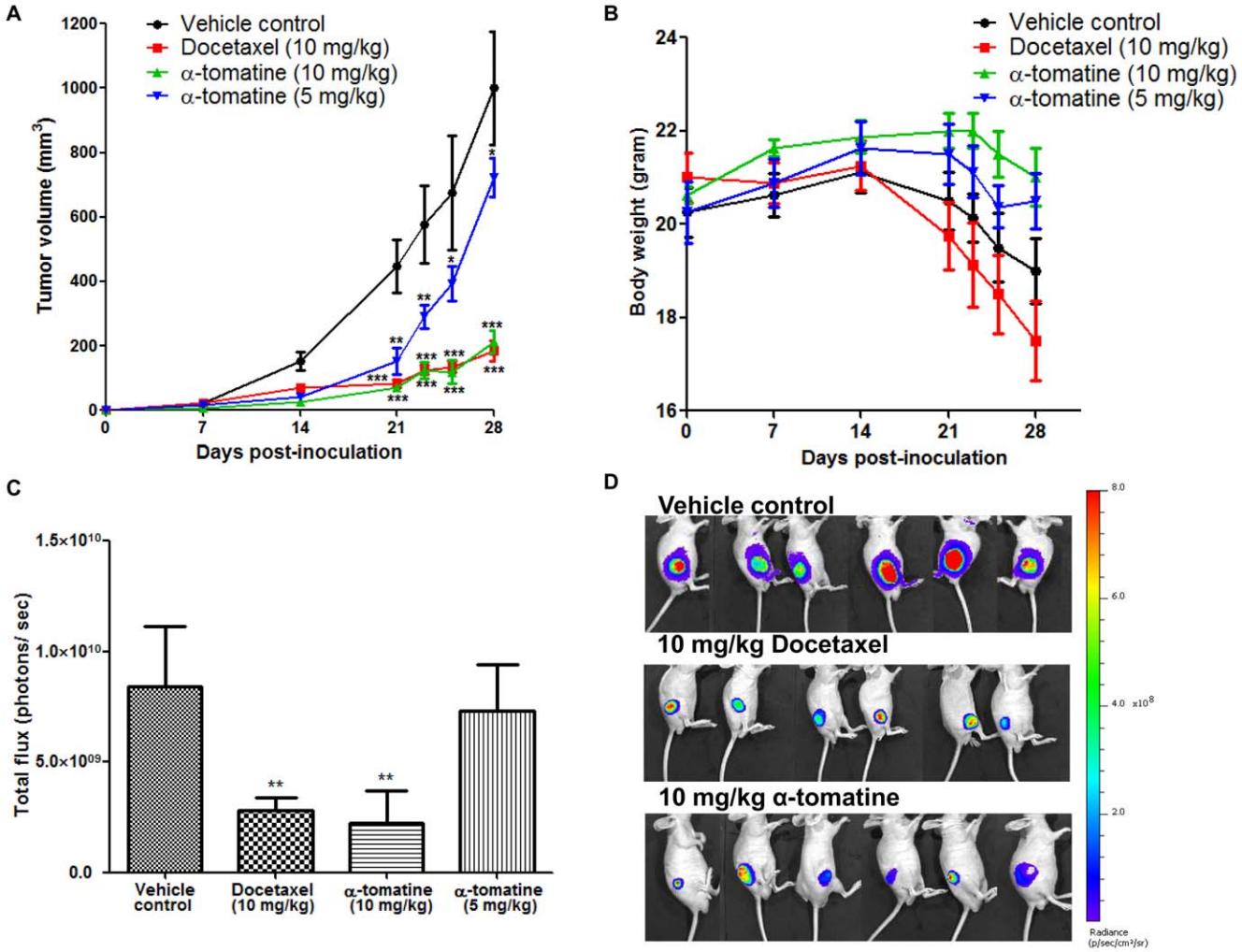

Figure 4. Anti-tumor activity of α-tomatine against subcutaneous PC-3 cell tumors. Luciferase expressing PC-3 cell xenograft tumors established in male nude mice (n = 8 per treatment group) for 1 week were treated thrice weekly for 3 weeks with vehicle, docetaxel (10 mg/kg) or α-tomatine (5 or 10 mg/kg). (A) Graph of tumor volume in each treatment group versus the number of days after initial injection of PC-3 cells. (B) Graph of mean body weight for each treatment group versus the number of days after initial injection of PC-3 cells. (C) Bioluminescence intensities emitted from PC-3 cell xenograft tumors at the end of the experiment for each treatment group. (D) Bioluminescence images of PC-3 subcutaneous xenografts. The first row shows the vehicle control group; middle row shows the docetaxel treatment group; bottom row shows the 10 mg/kg α-tomatine treatment group. Each bar or point represents the mean ± SEM of data (n = 8).* $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* vehicle control.
doi:10.1371/journal.pone.0057708.g004

α-tomatine attenuates growth of PC-3 cell xenograft tumors in mice

To examine the effect of α-tomatine on tumor growth *in vivo*, we challenged PC-3 cell tumors grown subcutaneously in mice. In these experiments tumors were allowed to establish for 1 week before challenge 3 times per week for 3 weeks with α-tomatine (5 or 10 mg/kg/mouse), the prostate cancer therapeutic docetaxel (10 mg/kg/mouse) [30] and vehicle solution. Figure 4A shows that 10 mg/kg α- tomatine was as efficient as 10 mg/kg docetaxel at retarding growth of PC-3 cell subcutaneous mouse tumors. In addition, there was also a marked decrease in tumor volume in response to lower dose of α-tomatine (5 mg/kg). Specifically, 3 weeks after the commencement of drug challenge the average tumor volume in vehicle control mice (1000 ± 180 mm^3), was approximately 1.4 fold higher as compared to mice treated with 5 mg/kg α-tomatine (720 ± 60 mm^3), and approximately 4.7 fold higher compared with mice treated with 10 mg/kg of α-tomatine (210 ± 35 mm^3) or docetaxel (183 ± 32 mm^3) (Figure 4A). Of note, α-tomatine treatment at both 5 mg/kg (Figure 4B, blue) and 10 mg/kg (Figure 4B, green) did not provoke body weight loss in contrast with docetaxel (Figure 4B, red) which caused a reduction in body weight of ~10% more than those seen in untreated tumor bearing mice (Figure 4B, black). In fact, both control and docetaxel treatment groups showed a trend of decreasing body weight after 14 days of cancer cells inoculation, potentially due to high tumor burden and toxicity of docetaxel, respectively (Figure 4B). Consistent with the tumor volumes determined from caliper measurements, the intensity of bioluminescence measured on day 28 was significantly lower in both docetaxel and 10 mg/kg α-tomatine treatment groups (Figure 4C and D). These results suggest that α-tomatine retards the growth of PC-3 cell subcutaneous xenograft tumors at an effective dose of 10 mg/kg.

We also used an orthotopic mouse model to examine the effect of α-tomatine on prostate tumor growth. In this experiment, PC-3 cell tumors were allowed to grow in mice for 5 days before thrice weekly treatment for 2 weeks with 10 mg/kg α-tomatine. As

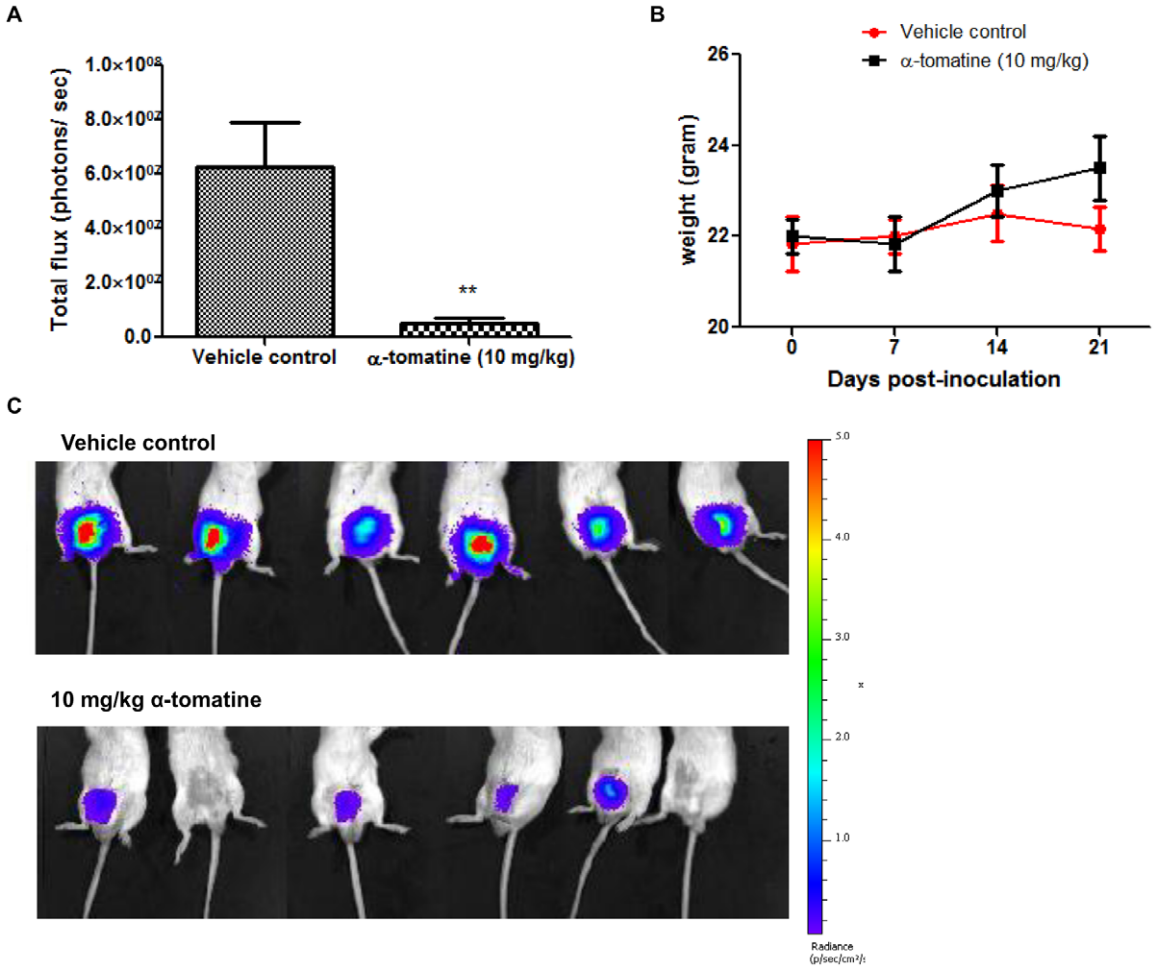

Figure 5. Anti-tumor activity of α-tomatine against orthotopic PC-3 cell tumors. Luciferase expressing PC-3 cell orthotopic tumors established in male SCID mice (n = 6 per treatment group) for 5 days were treated thrice weekly for 2 weeks with vehicle or α-tomatine (10 mg/kg). (A) Bioluminescence intensities emitted from PC-3 cell orthotopic tumors for each treatment group after 14 days of treatment. (B) Bioluminescent images at the end of the experiment of SCID mice carrying orthotopic tumors of luciferase expressing PC-3-luc cells. The upper row shows the vehicle control group, whereas the bottom row shows the α-tomatine (10 mg/kg) treatment group. (C) Graph of mean body weight for each treatment group versus the number of days after initial injection of PC-3 cells. Each bar or point represents the mean ± SEM of data (n = 6).* $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* vehicle control.
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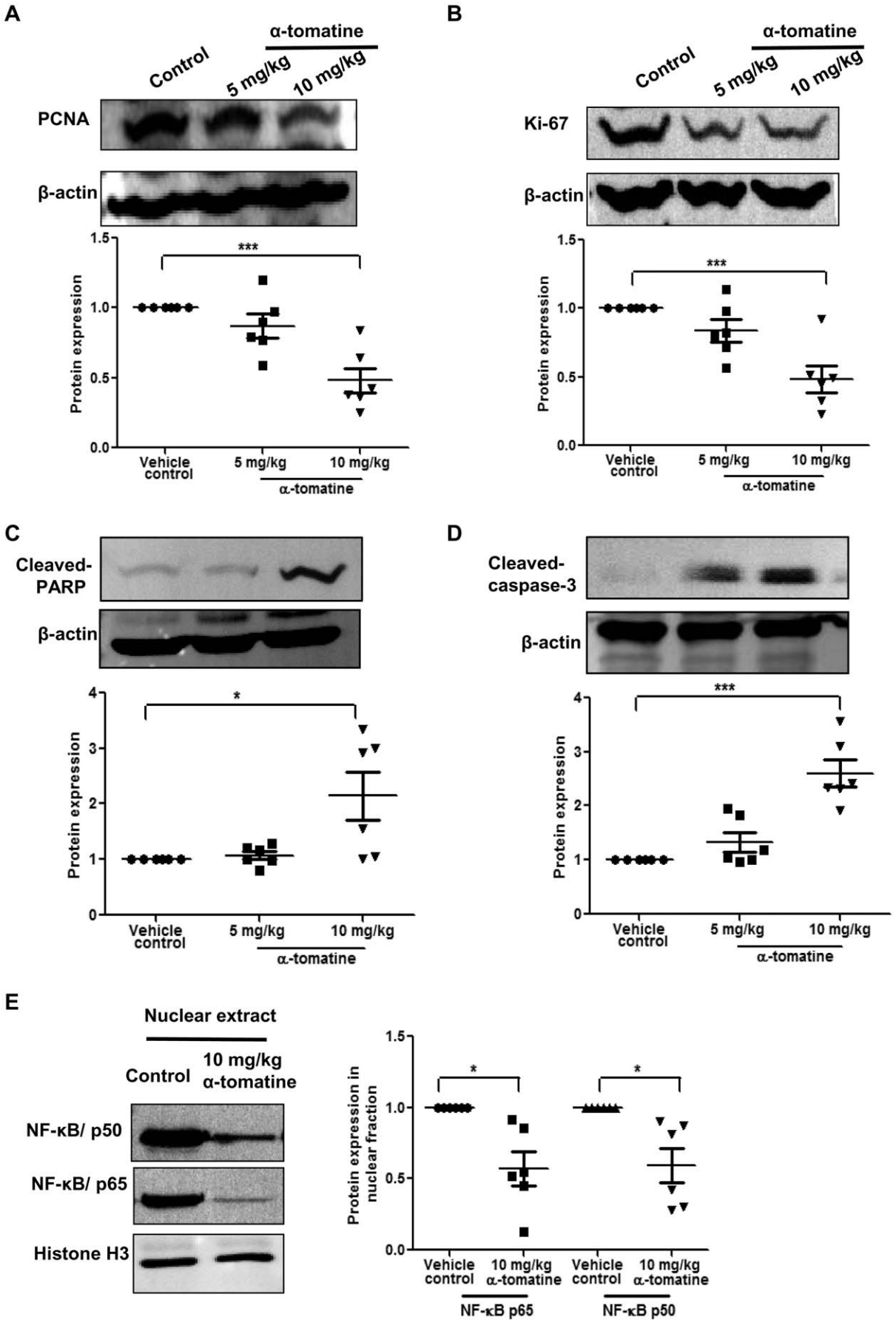
shown in Figure 5A, and consistent with the data obtained from the xenograft mouse model, α-tomatine significantly suppressed the tumorigenicity of PC-3 cell orthotopic tumors. In addition, the total body weight of mice was not adversely affected by α-tomatine treatment (Figure 5B). The images in Figure 5C shows strong bioluminescent signal from the prostate of control mice, demonstrating that the tumor cells were successfully implanted. In addition, 4 out of 6 α-tomatine treated mice showed only weak bioluminescent signals and another 2 mice had no signal. These findings demonstrate that α-tomatine has potent anti-tumor effects against mouse xenograft and orthotopic PC-3 cell tumors.

α-tomatine reduces expression of proliferation markers, increases expression of apoptosis markers and inhibits nuclear translocation of NF- κB in xenograft tumors

To examine the mechanism by which α-tomatine suppressed the growth of PC-3 cell tumors in mice, we next examined tumor tissue recovered from mice carrying subcutaneous tumors for expression of markers of proliferation (PCNA and Ki-67) and apoptosis (cleaved-PARP and cleaved-caspase-3). This analysis was not possible for orthotopic tumors as insufficient material was available for the α-tomatine treated mice. As shown in Figure 6, Western blot analysis of lysates from six independent tumor samples indicated that while both markers of proliferation (PCNA and Ki-67) decreased in response to α-tomatine treatment (Figure 6A and B), levels of both markers of apoptosis (cleaved-PARP and caspase-3) increased (Figure 6C and D). These data suggest that both anti-proliferative and pro-apoptotic effects of α-tomatine contribute to the reduced growth of PC-3 cell tumors in mice. As NF-κB translocation to the nucleus is important for promoting increased cell proliferation and survival and we have shown that this translocation event is reduced *in vitro* by α-tomatine, we were interested to examine recovered subcutaneous mouse tumors for localization of NF-κB. As shown in Figure 6E, Western blot analysis from six independent tumor samples indicated that there was a distinct decrease in the levels of NF-κB components in the nucleus of tumor cells in response to α-tomatine treatment. These data suggest that the anti-tumor effects of α-tomatine *in vivo* may be due to its ability to block the proliferative and anti-apoptotic


A
α-tomatine
Control
5 mg/kg
10 mg/kg
PCNA
β-actin
Protein expression
***
Vehicle control
5 mg/kg
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α-tomatine
B
α-tomatine
Control
5 mg/kg
10 mg/kg
Ki-67
β-actin
Protein expression
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5 mg/kg
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Cleaved-caspase-3
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Control
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Histone H3
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NF-κB p65
NF-κB p50

Figure 6. Western blot analysis of PCNA, Ki-67, cleaved-PARP, cleaved-caspase-3 and NF-κB in PC-3 tumor tissues samples. (A–D) PC-3 tumors excised from mice treated with vehicle, 5 mg/kg or 10 mg/kg of α-tomatine were lysed and examined by Western blot analysis. Representative images are shown. Markers of proliferation (PCNA and Ki-67). Markers of apoptosis (cleaved PARP and cleaved caspase-3). β-actin was used as loading control. (E) Western blot analysis on nuclear extracts probing for NF-κB p50 and p65 levels. Equal loading of protein was confirmed by stripping and reprobing the blots with histone H3 antibody. Graphical representation of densitometry analysis of each Western blot data from six independent tumor samples is shown below each panel. The ratio of the signal intensity of each protein to loading control was normalized to the vehicle control. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs* vehicle control.
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effects of NF-κB signaling by reducing its translocation to the nucleus.

Discussion

We provide the first evidence that the saponin α-tomatine can efficiently inhibit the growth of prostate cancer tumors *in vivo* without inducing overt toxicity. Our analysis of recovered mouse tumors suggest that mechanistically α-tomatine mediates its anti-prostate cancer effects *in vivo* by blocking, at least in part, the proliferative and anti-apoptotic effects of NF-κB signaling by reducing translocation of this protein complex to the nucleus. Our detailed *in vitro* analyses indicate that α-tomatine suppression of NF-κB activation occurs through indirect inhibition of IKK kinase activity, leading to suppression of IκBα phosphorylation and degradation, NF-κB/p65 phosphorylation and NF-κB p50/p65 nuclear translocation. Consistent with its ability to induce apoptosis, α-tomatine inhibition of NF-κB activation *in vitro* was accompanied by significant reduction in expression of TNF-α induced pro-survival mediators c-IAP, Bcl-2, Bcl-xL, survivin and XIAP.

The NF-κB signaling pathway is an important target for disease treatment because its dysregulation is required for inappropriate inflammatory responses as well as cancer and other ailments [31]. In response to most activating stimuli, NF-κB signaling occurs through sequential activation of IKK, phosphorylation of IκBα at serine 32 and 36, leading to its degradation, and the nuclear translocation of NF-κB where it regulates transcription of a range of genes including those that promote cell proliferation and survival [32]. Consistent with our data from α-tomatine, suppression of NF-κB activation has been shown to be a critical mechanism of action of several plant-derived anticancer agents, such as curcumin, lycopene, silibinin, genistein, resveratrol and green tea polyphenols [33]. In the present study, we showed that α-tomatine is an indirect inhibitor of IKK. Presumably, α-tomatine inhibits upstream signaling components that lead to activation of the IKK complex, such as Akt serine-threonine kinase , NF-κB inducing kinase, mitogen-activated protein kinase kinase kinase (MEKK)1, MEKK3, TGF activated kinase 1 (TAK1) and glycogen synthase kinase-3 beta [34,35,36,37,38]. Indeed, we found that α-tomatine inhibits TNF-α-induced Akt activation, although further experiments are needed to address if this effect is responsible for the changes we observed in phosphorylation and translocation of NF-κB components or the anti-tumor actions of α-tomatine against subcutaneous and orthotopic tumors grown in mice. These results are in agreement with those of previous studies that suggest that α-tomatine suppresses invasion and migration of human lung cancer cells *in vitro* through the inhibition of the Akt [15,16]. As conventional chemotherapeutic agents, including docetaxel target normal as well as tumor cells and lead to deleterious effects for prostate cancer patients there is a pressing need to identify less toxic agents to control this disease. In recent years, a number of naturally occurring dietary agents of reduced toxicity have been reported to induce apoptosis and inhibit tumor growth, highlighting the promise of using naturally derived agents for chemotherapy and chemoprevention of prostate and other cancers [39,40,41].

Furthermore, it is also possible that an inhibitor of NF-κB activation could be an adjuvant for overcoming tumor resistance to radiation and chemotherapies, such as paclitaxel, doxorubicin, 5-fluorouracil, and vinca alkaloids (vinblastine and vincristine), that occur via NF-κB activation [42,43,44]. It is thought that this induced resistance in a wide variety of tumor cells occurs via induction of NF-κB effector genes, including Bcl-2 [45], Bcl-xL [46], survivin [47], XIAP [48], c-IAP1 [49] and c-IAP2 [49] that are known to mediate protective responses to chemotherapeutic agents and radiation. Therefore, targeting NF-κB through the actions of α-tomatine, which we have shown to block NF-κB activation and transcription of NF-κB effector genes, may result in improvements in treatment of prostate cancer. In support of this, other dietary agents, including resveratrol, curcumin, genistein, (−) epigallocatechin gallate and soya isoflavone that can block various steps leading to NF-κB activation and sensitize tumor cells to the beneficial effects of chemotherapeutic drugs and radiation in treatment of cancer [50,51,52,53,54,55,56].

We previously reported that α-tomatine induces caspase-dependent death of PC-3 cells *in vitro* accompanied by increased caspase-3 activity and the release of cytochrome c [18]. Caspases have been shown to be involved in apoptosis through activation of PARP downstream molecule [57]. Importantly, here we have demonstrated the pro-apoptotic effect of α-tomatine *in vivo* by showing a significant increase in the cleavage of PARP and caspase-3 in α-tomatine-treated tumors. In addition, our data show that α-tomatine elicits anti-proliferative effects *in vivo* as we observed reduced levels of the markers of proliferation Ki-67 and PCNA. Our data from PC-3 tumors also suggest that these pro-apoptotic and anti-proliferative effects of α-tomatine are mediated, at least in part, by reduced nuclear translocation of NF-κB p50 and p65. Indeed our *in vitro* studies clearly demonstrate that α-tomatine is very effective at blocking activation and translocation of the components of the NF-κB complex providing support for the possibility that this mechanism is also important for its effects *in vivo* against prostate cancer PC-3 cell tumors.

In summary, we present the first evidence that α-tomatine is an effective anti-tumor compound against prostate cancer xenograft and orthotopic tumors. This agent may prove to be useful in the prevention and treatment of androgen-independent prostate cancer and this warrants further investigation.

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Author Contributions

Conceived and designed the experiments: STL PFW JDH HH MRM. Performed the experiments: STL HH. Analyzed the data: STL PFW JDH HH MRM. Contributed reagents/materials/analysis tools: JDH MRM. Wrote the paper: STL PFW JDH.

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Alpha-tomatine synergises with paclitaxel to enhance apoptosis of androgen-independent human prostate cancer PC-3 cells *in vitro* and *in vivo*

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ABSTRACT

Alpha (α)-tomatine, a major saponin found in tomato has been shown to inhibit the growth of androgen-independent prostate cancer PC-3 cells. The effects of α-tomatine in combination with the chemotherapeutic agent paclitaxel against PC-3 cells were investigated in the present study. Combined treatment with a sub-toxic dose of α-tomatine and paclitaxel significantly decreased cell viability with concomitant increase in the percentage of apoptotic PC-3 cells. The combined treatment, however, had no cytotoxic effect on the non-neoplastic prostate RWPE-1 cells. Apoptosis of PC-3 cells was accompanied by the inhibition of PI3K/Akt pro-survival signaling, an increase in the expression of the pro-apoptotic protein BAD but a decrease in the expressions of anti-apoptotic proteins, Bcl-2 and Bcl-xL. Results from a mouse xenograft model showed the combined treatment completely suppressed subcutaneous tumor growth without significant side effects. Consistent with its *in vitro* anti-cancer effects, tumor materials from mice showed increased apoptosis of tumor cells with reduced protein expression of activated PI3K/Akt. These results suggest that the synergistic anti-cancer effects of paclitaxel and α-tomatine may be beneficial for refractory prostate cancer treatment.

Introduction

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in men worldwide (Jemal et al. 2011). The majority of patients with advanced prostate cancer respond well to initial treatment with anti-hormonal therapies, but almost all of these patients will eventually develop hormone-refractory prostate cancer and bone metastatic disease (Bracarda et al. 2005). Taxane-based drugs such as paclitaxel and docetaxel are potent chemotherapeutic agents used for the treatment of hormone-refractory prostate cancer (Mancuso et al. 2007). These drugs are microtubule inhibitors which bind to β-tubulin resulting in microtubule stabilization, disruption of mitosis and ultimately apoptosis (Schiff et al. 1979). The main limitations of taxane-based drugs include inherent toxicity associated with their use and short-lived survival benefit of approximately 2–3 months largely due to chemoresistance (Chang 2007; Dagher et al. 2004). Accordingly, there remains an unmet need for the identification and development of novel agents that can be used to improve the treatment outcomes of chemotherapy.

We are interested in the utility of naturally occurring plant compounds as agents to safely augment the anti-cancer activity of standard chemotherapies. Alpha (α)-tomatine (Fig. 1), a major saponin present in tomato has been shown to have potent *in vitro* and *in vivo* anti-tumor activity against human prostate cancer PC-3 cells (Lee et al. 2011, 2013). These reports indicate that α-tomatine-induced apoptosis of PC-3 cells is mediated, at least in part, through the inhibition of nuclear factor-kappa B (NF-κB) nuclear translocation (Lee et al. 2011, 2013). In the present study, we examined the ability of α-tomatine to augment the anti-cancer actions of a taxane-based drug, paclitaxel (PTX). Accordingly, we sought to examine the effect of the combined α-tomatine and PTX treatment on constitutively active phosphoinositide 3-kinase (PI3K)/Akt signaling, which is an upstream mediator of NF-κB and confers chemoresistance in advanced prostate cancer (Lee et al. 2004; Madrid et al. 2000).

Abbreviations: CI, combination index; EC_{50}, half maximal concentration; FBS, fetal bovine serum; FITC, fluoresceinisothiocyanate; GSK, glycogen synthase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide; NF-κB, nuclear factor-kappa B; PARP, poly (ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; PTX, paclitaxel; PVDF, polyvinylidene fluoride; RIPA, radioimmunoprecipitation assay; TMB, 3,3,5,5 tetramethylbenzidine.

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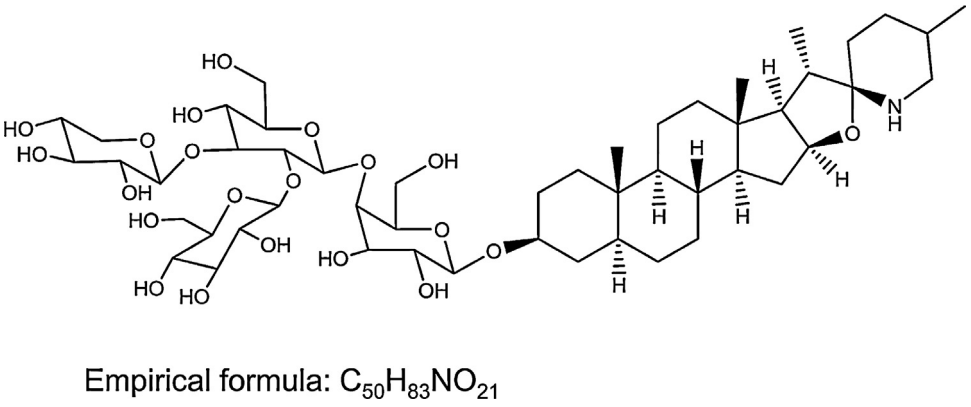

Fig. 1. Chemical structure of α-tomatine.

Materials and methods

Materials

α-Tomatine was purchased from Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), 3,3,5,5 tetramethylbenzidine (TMB), PTX and anti-human beta (β)-actin antibody were purchased from Sigma–Aldrich (USA). Penicillin/streptomycin, Roswell Park Memorial Institute (RPMI-1640), keratinocyte growth medium, fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) were purchased from Invitrogen (USA). An antibody against Akt was obtained from Santa Cruz Biotechnology, USA. An Akt kinase assay kit, phosphospecific anti-Akt (Ser473), phosphospecific anti-PI3K p85 (Tyr458)/p55 (Tyr199), anti-PI3K, anti-Bcl-2, anti-Bcl-xL, anti-BAD, anti-cleaved (ADP-ribose) polymerase (PARP), anti-cleaved caspase-3 antibodies were purchased from Cell Signaling Technologies (USA). Antibody against proliferating cell nuclear antigen (PCNA) were purchased from BD Biosciences (USA).

Cell lines

Androgen-independent prostate cancer PC-3, androgen-dependent prostate cancer LNCaP and non-neoplastic human prostate RWPE-1 cell lines were obtained from the American Type Culture Collection (ATCC) (USA). The luciferase-expressing PC-3 cell line was a kind gift from Dr. Patrick Ming Tat Ling, Queensland University of Technology, Australia. PC-3, LNCaP and luciferase-expressing PC-3 cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. RWPE-1 cells were cultured in keratinocyte growth medium. All cells were maintained at 37 °C in a 5% CO_2 humidified incubator.

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In vitro cytotoxicity assay

Briefly, 18–24 h after seeding, adherent cells in sterile 96-wells plates were treated for 24 h with α-tomatine and PTX alone or in combination at concentrations shown in the relevant figure legend. Cell viability was determined following 24 h treatment using the MTT assay as described previously (Lee et al. 2011).

Assessment of the effect of combined drug treatments in PC-3 cells

The effect of combined treatment of α-tomatine and PTX was quantified by determining the combination index (CI) according to the algorithm of Chou and Talalay (Chou 2010) using CalcuSyn software (Biosoft, Inc., Cambridge, United Kingdom). A CI value of 1 indicates an additive effect whereas <1 represents synergism and >1 antagonism (Chou 2010).

Cell cycle analysis

Cell cycle distribution was examined by propidium iodide (PI) staining of nuclei acids using a BD Cycletest Plus DNA Reagent Kit as described previously (Dasmahapatra et al. 2004).

Assessment of apoptosis by Annexin V/PI double staining assay

Apoptosis-mediated death of tumor cells was examined by a double staining method using a fluoresceinisothiocyanate (FITC)-labeled Annexin V/PI apoptosis detection kit (BD Bioscience, San Jose, CA) as described previously (Lee et al. 2011).

Cell lysis

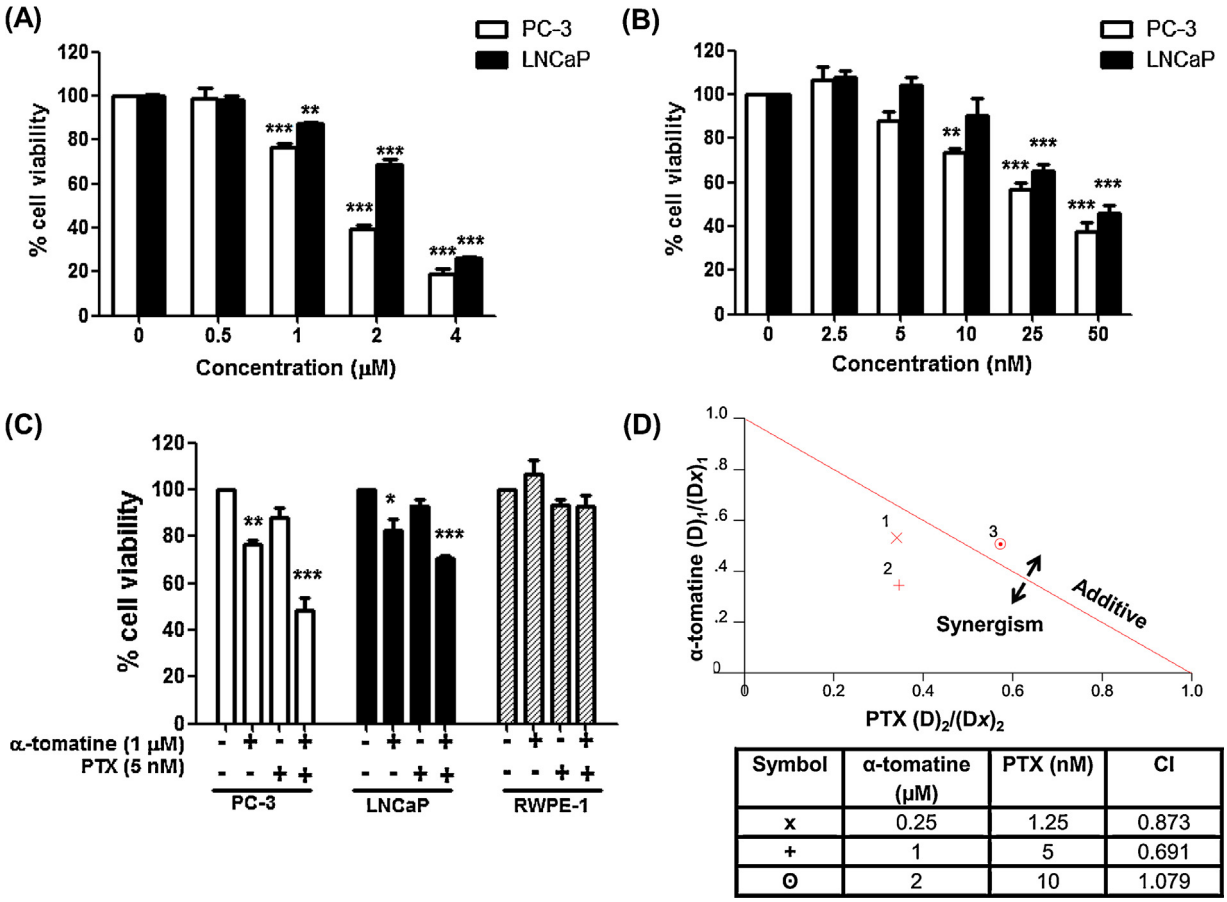
PC-3 cells at 70–80% confluence were treated with vehicle solution, 1 μM α-tomatine, 5 nM PTX or α-tomatine and PTX in combination. Protein extraction was performed as described previously (Lee et al. 2013).

Western blot analysis

Separation of proteins by SDS-PAGE, immunoblotting, and densitometric scanning of the membranes were performed as described previously (Lee et al. 2013). Signal intensity of the respective protein was normalized to loading control and expressed as a percentage of vehicle treated control.

Assessment of Akt kinase activity

Akt kinase activity was quantified using an Akt kinase assay kit (Cell Signaling Technology, Inc.), following the instructions of the manufacturer. Protein lysates from treated PC-3 cells were incubated overnight with an immobilized anti-phospho-Akt monoclonal antibody (bead conjugate). The enzymatic activity of immunoprecipitated phosphorylated Akt was then assessed *in vitro* using the supplied glycogen synthase kinase (GSK)-3α/β fusion protein as substrate, in the presence of ATP. Phosphorylation of GSK-3α/β was detected using an anti-phospho-GSK-3α/β antibody. The signal intensity of phospho-GSK-3α/β was normalized to total GSK and presented as a percentage of vehicle treated control.



Symbol	α-tomatine (μM)	PTX (nM)	CI
x	0.25	1.25	0.873
+	1	5	0.691
⊙	2	10	1.079

Fig. 2. Dose-dependent cytotoxicity of (A) α-tomatine and (B) PTX in PC-3 and LNCaP cancer cells. (C) Cytotoxic effect of combined 1 μM α-tomatine and 5 nM PTX treatment in PC-3, LNCaP and RWPE-1 cells. Data are shown as mean ± SEM of three independent experiments. Statistical significance is expressed as $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ *versus* vehicle control. (D) Top, isobologram analysis of the cytotoxicity of α-tomatine and PTX treatments alone or in combination. Bottom, CI values determined from the formula described in Materials and methods.

Growth of subcutaneous PC-3 cell tumors in mice

Experiments with mice were performed in accordance with a protocol approved by the University of Queensland Animal Ethics Committee. Male BALB/c nude mice (6 weeks old) were purchased from the Animal Resources Centre (Canning Vale, Western Australia). Luciferases-expressing PC-3 cells were inoculated subcutaneously into the lower flanks of each mouse as described previously (Lee et al. 2013). On day 7 after cancer cell inoculation, each mouse had one palpable tumor and were randomly assigned to four groups ($n = 6$/group). These groups of mice were then given intraperitoneal injections of vehicle solution, 5 mg/kg PTX, 5 mg/kg α-tomatine or 5 mg/kg α-tomatine + 5 mg/kg PTX thrice weekly for an additional 3 weeks. The mice were monitored weekly for tumor growth and body weight. Tumor dimensions were measured with calipers and the tumor volumes were calculated using a standard formula: (length × width^2) × 0.5 (Plymate et al. 2003). The experiment was terminated 28 days after cancer cell inoculation, from which the time bioluminescent signals of tumors in live mice were first captured using a Xenogen IVIS Spectrum imaging system (USA) as described previously (Lee et al. 2013).

Tissue processing and protein extraction

Tissue processing and protein extraction were performed as described previously (Lee et al. 2013).

Statistical analysis

In vitro assays were performed in at least three separate experiments. Results are expressed as the mean value ± standard error of the mean (SEM). Statistical analysis was performed with one-way analysis of variance (ANOVA), with Dunnett's Multiple Comparison Test to identify between-group differences using GraphPad Prism software (San Diego, CA). For *in vivo* tumor growth experiments, statistical significance of the differences in tumor volume, body weight and total bioluminescence intensity between control and treatment groups was assessed by two-way ANOVA using GraphPad Prism software (San Diego, CA), with p values <0.05 considered significant. Statistical significance is expressed as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Results

α-Tomatine acts synergistically with paclitaxel to inhibit the in vitro growth of PC-3 cells

The *in vitro* growth inhibitory effects of α-tomatine and PTX on prostate cancer cells was firstly evaluated as single agent at concentrations of 0.5, 1, 2 and 4 μM for α-tomatine and 2.5, 5, 10, 25 and 50 nM for PTX on PC-3 and LNCaP cancer cells. The α-tomatine concentrations were chosen based on our previous report of half maximal concentration (EC_{50}) in PC-3 cancer cells at 1.67 ± 0.3 μM (Lee et al. 2011). As shown in Fig. 2A, the growth of PC-3 and

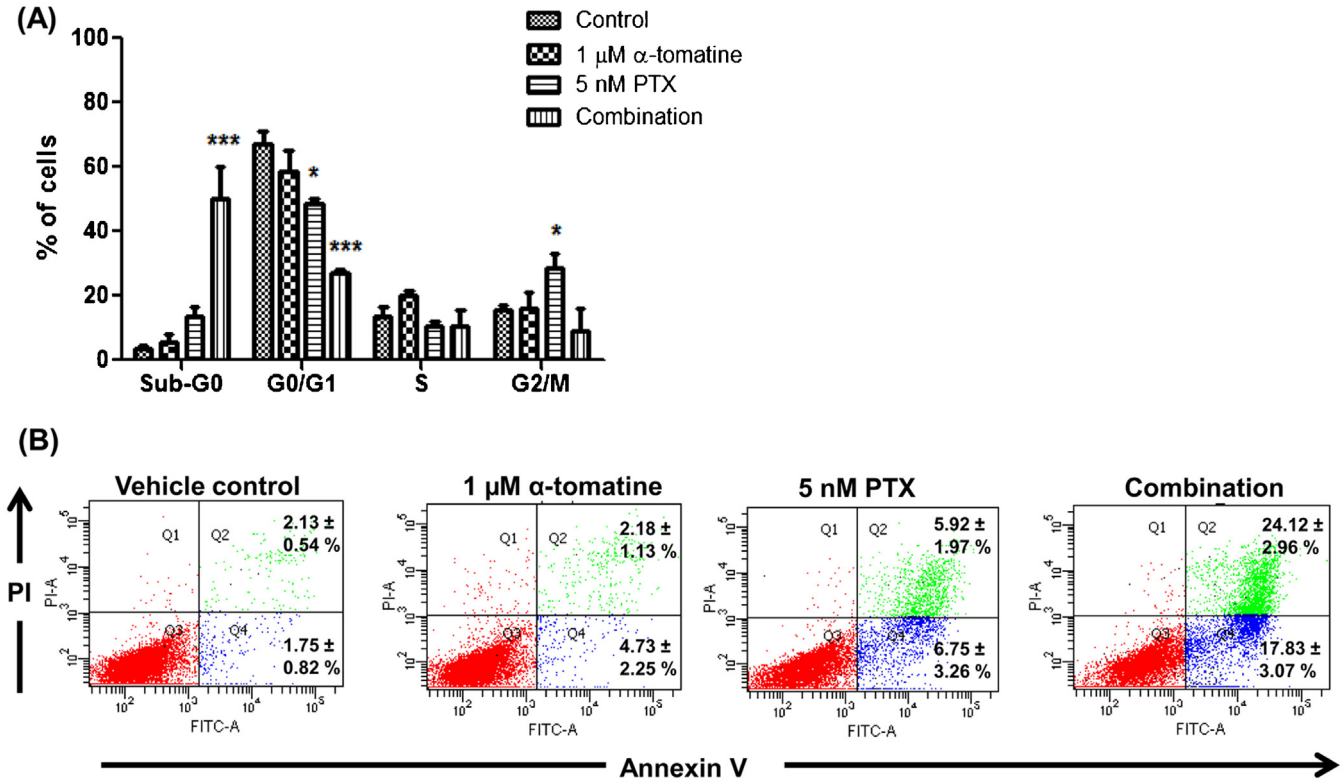


Fig. 3. (A) The percentages of PC-3 cells at sub-G0, G0/G1, S and G2/M phases 24 h after drug treatments. (B) Flow cytometric analysis of apoptotic PC-3 cells using annexin V/PI double staining following 24 h with the indicated treatments. Data are shown as means ± SEM of three independent experiments. Statistical significance is expressed as $^{***}p < 0.001$; $^{*}p < 0.05$ *versus* vehicle control.

LNCaP cells were significantly inhibited by α-tomatine in a dose-dependent manner. Treatment with α-tomatine for 24 h inhibited the proliferation of LNCaP cells with an EC_{50} value of 2.65 ± 0.1 μM. PC-3 cells, hence, were more sensitive to growth inhibition by α-tomatine compared to LNCaP cells.

PTX inhibited the proliferation of PC-3 and LNCaP cells in a dose-dependent manner, with an EC_{50} at 32.11 ± 4.39 nM for PC-3 cells and 44.80 ± 2.49 nM for LNCaP cells at 24 h exposure (Fig. 2B). We next tested the effect of combined drug treatment at sub-maximal levels of toxicity (1 μM α-tomatine and 5 nM PTX) on PC-3, LNCaP and non-neoplastic prostate RWPE-1 cells. MTT assay revealed that this combination of treatment doses resulted in greater growth inhibition with PC-3 cell viability reduced to ~50% (Fig. 2C). In contrast, the combined treatment reduced LNCaP cell viability to only ~30% (Fig. 2C). Viability of non-neoplastic prostate cells was largely unaffected by single agent or combined drug treatments (Fig. 2C). These results showed that a greater anti-proliferative effect of α-tomatine was observed in PTX-treated PC-3 cells compared to treatments using α-tomatine or PTX alone and in the treated LNCaP cells. Hence, subsequent mechanistic and animal studies were performed only on PC-3 cells.

Chou–Talalay method was used to determine whether the combined drug action is antagonistic, additive or synergistic (Chou 2010). CI values obtained using this method were plotted and tabulated in Fig. 2D. These compounds were found to act synergistically against PC-3 cells at the dose of 1 μM α-tomatine and 5 nM PTX with a CI value of 0.691 (Fig. 2D).

Induction of apoptosis by α-tomatine and PTX in PC-3 cells

PTX is known to induce G2/M cell cycle arrest (Horwitz 1992). To examine whether the synergistic effect of PTX and α-tomatine is due to enhancement of PTX-induced G2/M arrest by α-tomatine, we performed flow cytometric analysis of PI-stained PC-3 cells to evaluate the effect of single agent and combination treatments on cell cycle distribution. Combined treatment of α-tomatine and PTX abrogated the G2/M arresting effects of individual PTX treatment. The combined treatment, however, resulted in an increased distribution of cell in sub-G0 phase (50.23 ± 9.86%), compared to 5.39 ± 2.51% and 13.38 ± 3.22% in cells treated with 1 μM α-tomatine and 5 nM PTX alone, respectively (Fig. 3A), suggesting that there is an increase in apoptotic population.

We next performed Annexin V/PI double staining of the treated PC-3 cells to assess the proportion of cells progressing through the phases of apoptotic cell death. In these analyses, cells in the early stages of apoptosis were stained positively with Annexin V, whereas cells in late stages of apoptosis were stained positively with both Annexin V and PI. As shown in Fig. 3B, PC-3 cells treated with α-tomatine in combination with PTX showed a marked increase in cells in both the early (17.83 ± 3.07%) and late (24.12 ± 2.96%) stages of apoptosis compared to cells treated with only α-tomatine (early 4.73 ± 2.25%; late 2.18 ± 1.13%) or PTX (early 6.75 ± 3.26%; late 5.92 ± 1.97%). Taken together, these results showed that combined α-tomatine and PTX treatment induces more cell death than the individual agents.

Synergism of α-tomatine and PTX growth inhibition is accompanied by the inhibition of PI3K/Akt signaling and altered expression of downstream regulators of apoptosis

We have previously shown that α-tomatine inhibited nuclear translocation of NF-κB in PC-3 cells (Lee et al. 2011). To extend this analysis, we investigated the effect of this combined α-tomatine and PTX treatment on PI3K/Akt, an important determinant of

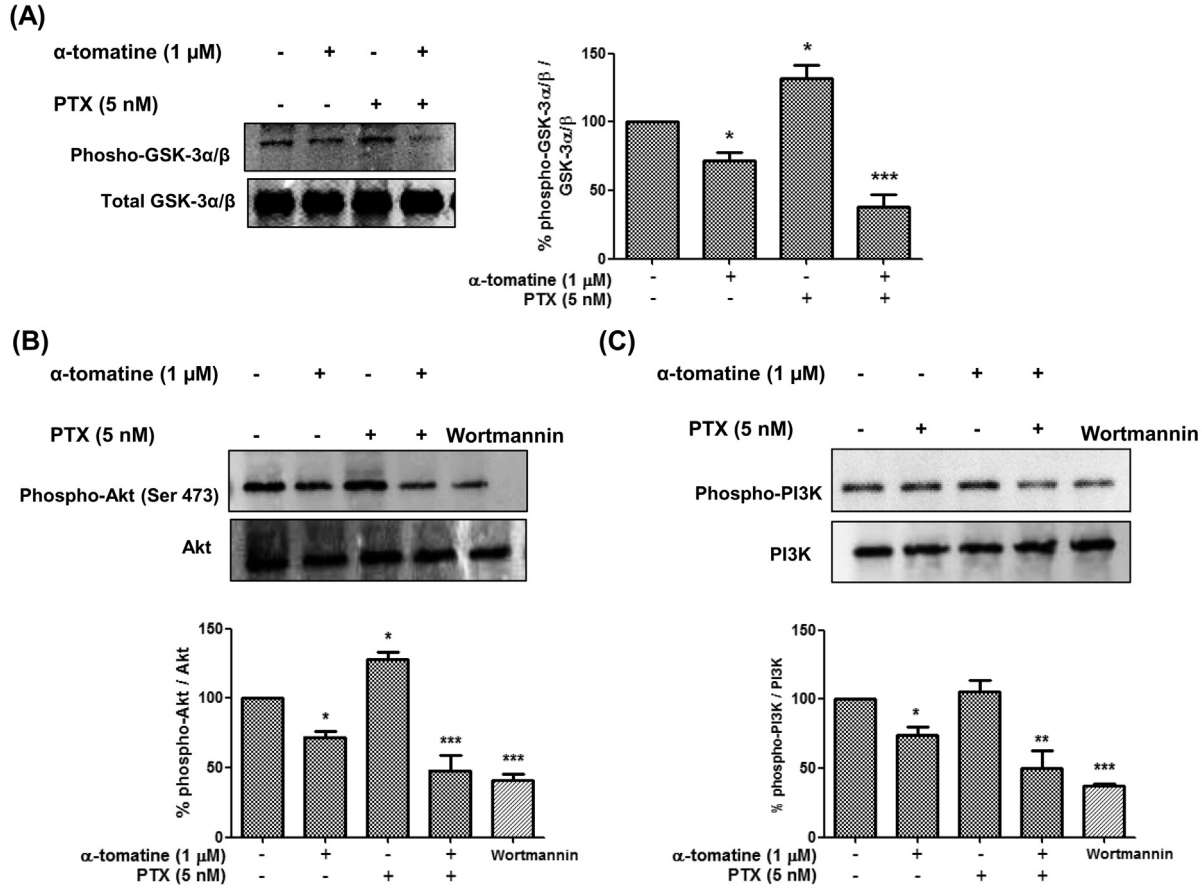


Fig. 4. Protein expressions of (A) phospho-GSK-3α/β (B) phospho-Akt and (C) phospho-PI3K in PC-3 cells treated with the indicated treatments. Bar graphs show densitometry analysis of each Western blot data of three independent experiments. Data are shown as means ± SEM. Statistical significance is expressed as $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ *versus* vehicle control.

chemoresistance and upstream mediator of NF-κB (Lee et al. 2004; Madrid et al. 2000). The enzymatic activity of Akt was firstly examined by an *in vitro* kinase assay using a GSK-3α/β fusion protein as substrate. PC-3 cells were treated with 1 μM α-tomatine, 5 nM PTX or in combination for 3 h before the onset of apoptosis. Treated and control cell populations were examined using trypan blue exclusion assay which indicated that this exposure had minimal effect on cell viability (data not shown). Western blot analysis showed that combined α-tomatine and PTX treatment caused a marked reduction in phospho-GSK-3α/β levels (Fig. 4A, left) where Akt activity was reduced by ~60% (Fig. 4A, right). In contrast, single agent treatment with α-tomatine and PTX caused ~28% reduction and ~30% increase in Akt activity, respectively (Fig. 4A). We also directly assessed the effect of α-tomatine and PTX on Akt activation by detection of phospho-Akt and total Akt on PC-3 cell lysates. As shown in Fig. 4B, combined α-tomatine and PTX treatment reduced the phosphorylated Akt levels by ~50%, which is close to the 60% reduction in cells treated with PI3K inhibitor wortmannin (1 μM). The effects in this assay of the individual agents on Akt activation (Fig. 4B), paralleled closely the effects observed from our *in vitro* kinase assay using a GSK-3α/β fusion protein as substrate (Fig. 4A).

We also examined the direct effect of these agents on PI3K, an upstream regulator of Akt by Western blot analysis. As shown in Fig. 4C, the effects of single agent treatment with α-tomatine and combined treatment were similar to those observed for Akt activity (Fig. 4A and B), whereas single agent treatment with 5 nM PTX produced no significant change in the protein expression of phospho-PI3K (Fig. 4C). In addition, neither single agent nor combination treatments had any effect on the levels of total Akt and PI3K (Fig. 4B and C).

As we observed that the combined treatment inhibited the constitutive PI3K/Akt activation in PC-3 cells, we were interested in the effect of this combination on downstream regulators of apoptosis including BAD, Bcl-xL and Bcl-2. In cells treated with combined treatment of α-tomatine and PTX for 8 h, the expressions of anti-apoptosis proteins Bcl-2 and Bcl-xL were significantly downregulated (Fig. 5A, Bi, and Bii). However, there was no significant change in the expressions of Bcl-2 and Bcl-xL in response to either single agent treatment (Fig. 5A, Bi, and Bii). Conversely, the expression of the pro-apoptotic protein BAD increased in PC-3 cells treated with combined treatment of α-tomatine and PTX (Fig. 5A and Biii).

These data suggest that combined treatment with α-tomatine and PTX promotes apoptosis of PC-3 cells by reducing the constitutive PI3K/Akt activity, thereby reducing the expression of Bcl-xL and Bcl-2 pro-survival proteins, and increasing the expression of BAD pro-apoptosis mediator.

α-Tomatine enhances the anti-tumorigenic effects of the PTX against PC-3 tumor xenografts in nude mice

By the end of the experiment, combined treatment of α-tomatine (5 mg/kg) and PTX (5 mg/kg) caused a marked reduction of PC-3 cell tumor volume and bioluminescent signal, whereas individual treatments with α-tomatine and PTX had much less pronounced effects (Fig. 6A–C). More importantly, none of the treatments caused a reduction in mouse body weight

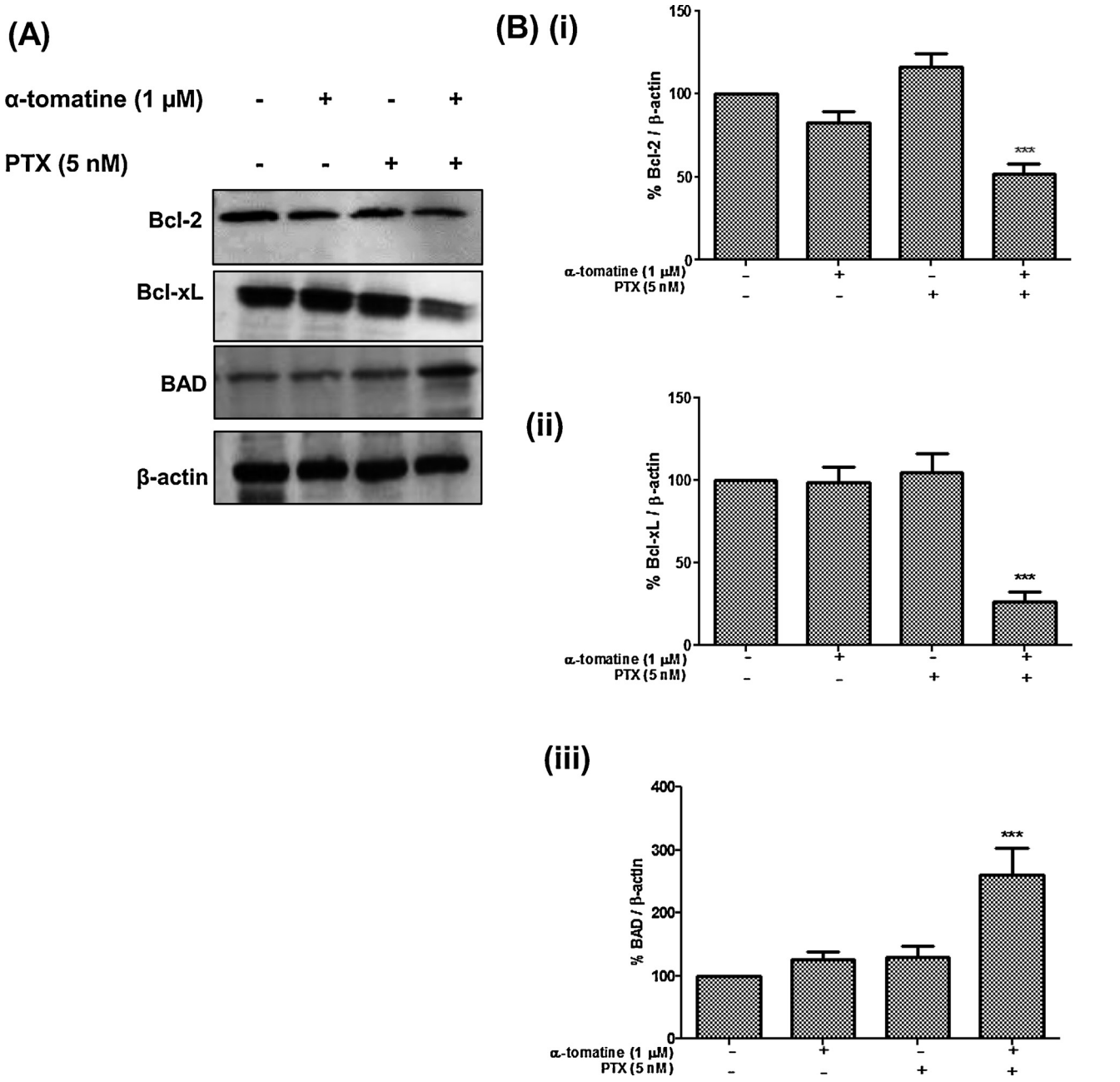


Fig. 5. (A) Protein expressions of Bcl-2, Bcl-xL and BAD in PC-3 cells treated with the indicated treatments. (B) (i–iii) Densitometry analysis of each Western blot data of three independent experiments. Data are shown as means ± SEM. Statistical significance is expressed as $^{***}p < 0.001$ *versus* vehicle control.

(Fig. 6D). Taken together, these results suggest that α-tomatine improves the efficacy of PTX in inhibiting PC-3 cell tumor growth *in vivo*.

Combined α-tomatine and PTX treatment inhibits PI3K/Akt signaling and increases apoptosis in PC-3 xenograft tumors

We examined tumor tissues recovered from mice for the expression of a marker of proliferation (PCNA) and apoptosis (cleaved PARP and cleaved caspase-3). Lysates from six independent tumor samples indicated that while PCNA expression decreased in response to the combination treatment (Fig. 7A), the levels of cleaved PARP and cleaved caspase-3 had increased (Fig. 7B and C), compared to those treated with single agents. These data showed that anti-proliferative and pro-apoptotic effects of the combination treatment contribute to the reduced growth of PC-3 cell tumors in mice.

As we have shown that constitutive activation of PI3K/Akt signaling is reduced *in vitro* by the combined treatment, we also examined the tumors recovered from mice for the expression of phospho-PI3K, phospho-Akt and its downstream molecule BAD. As shown in Fig. 7D and E, there was a distinct decrease in the levels of phospho-PI3K and phospho-Akt in tumor cells in response to the combined treatment, whereas the expression of BAD increased in response to the combined treatment (Fig. 7F). These findings support the proposal that the *in vivo* anti-tumor effect of combined α-tomatine and PTX is accompanied with the inhibition of pro-survival PI3K/Akt signaling.

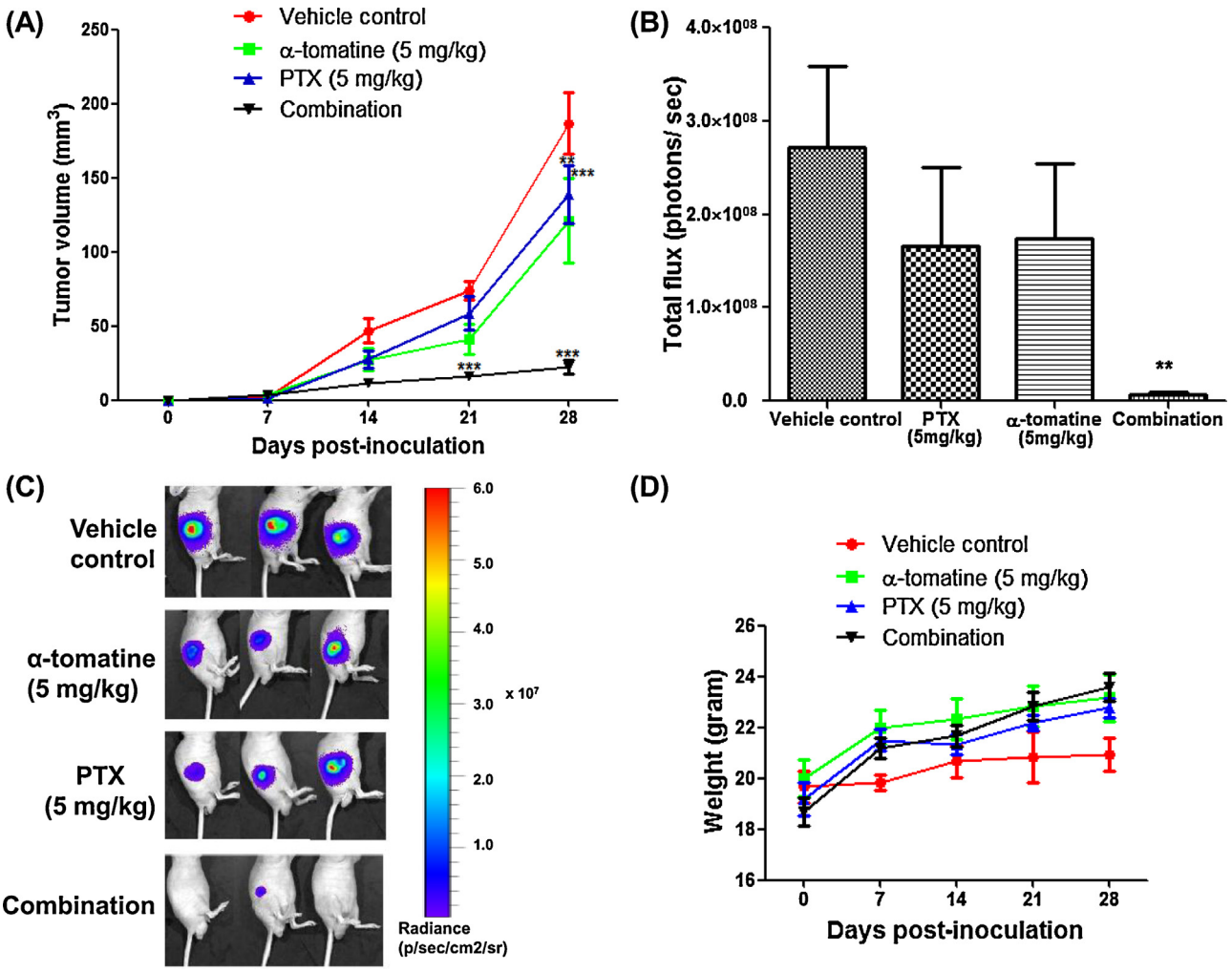


Fig. 6. (A) Tumor volumes in each treatment group are presented as growth curves. (B) Endpoint bioluminescence intensities of tumors in each treatment group. (C) Representative bioluminescence images of PC-3 subcutaneous xenografts. (D) Graph of mean body weight for each treatment group *versus* the number of days after initial injection of PC-3 cells. Each point or bar represents the mean ± SEM of data ($n = 6$ per group). Statistical significance is expressed as $^{***}p < 0.001$; $^{**}p < 0.01$ *versus* vehicle control.

Discussion

Preclinical *in vitro* and *in vivo* studies have indicated that phytochemicals, such as genistein and lycopene can enhance the anti-tumor activity of chemotherapeutic agents (Hwang et al. 2005; Tang et al. 2005). The use of phytochemicals and chemotherapeutic agents in combination has the advantage of low toxicity and potentiation of the actions of toxic chemotherapies thereby providing the opportunity to use chemotherapeutic agents at lower concentrations with fewer unwanted side-effects. We have shown that the tomato saponin, α-tomatine significantly enhanced the *in vitro* and *in vivo* anti-tumor activity of PTX. In particular, α-tomatine acted synergistically *in vitro* with PTX to significantly reduce the viability of PC-3 prostate cancer cells without impacting the viability of non-neoplastic prostate RWPE-1cells. In addition, α-tomatine potentiated the action of PTX *in vivo* causing regression of PC-3 xenograft tumors in mice without negatively impacting the weight of these animals. These data are suggestive that α-tomatine may be beneficial in clinical settings when combined with conventional chemotherapeutic agents such as PTX.

Drug resistance promoted by activation of pro-survival pathways and inactivation of pro-apoptotic mechanisms is a major problem for treatment of advanced prostate tumors (Seruga et al. 2011). Paradoxically, it is known that several conventional chemotherapeutic drugs, including PTX promotes survival signaling implicated in the development of resistance to chemotherapy (Li and Sethi 2010). This scenario limits the effectiveness of the chemotherapies and necessitates increases in the dosage or a change in the drug treatment. Accumulating evidences has indicated that activation of the PI3K/Akt pathway in advanced prostate cancer is implicated in the acquisition of resistance to chemotherapeutic drugs (Lee et al. 2004; McCubrey et al. 2006). Previous studies also suggested that activated Akt mediates PTX-induced resistance and thus inhibition of Akt may synergistically increase PTX sensitivity (Kim et al. 2007; Weng et al. 2009). Consistent with the previous reports, we observed that PTX increased pro-survival Akt activity and promoted the cell survival without affecting the abundant expressions of anti-apoptotic Bcl-2 and Bcl-xL proteins in PC-3 cells. More importantly, the constitutively active PI3K/Akt signaling in PC-3 cells due to the loss of phosphatase and tensin homolog (PTEN), a critical negative regulator of PI3K/Akt (Huang et al. 2001), was most efficiently suppressed by combination treatment with PTX and α-tomatine. Taken together, our data denote that α-tomatine-mediated sensitization of PC-3 cells to PTX involves down-regulation of both constitutive and PTX-induced Akt activity by α-tomatine. Consistent with our findings, α-tomatine has previously been shown to have anti-metastatic effect on lung cancer cells *via* inactivation of PI3K/Akt signaling (Shieh et al. 2011;

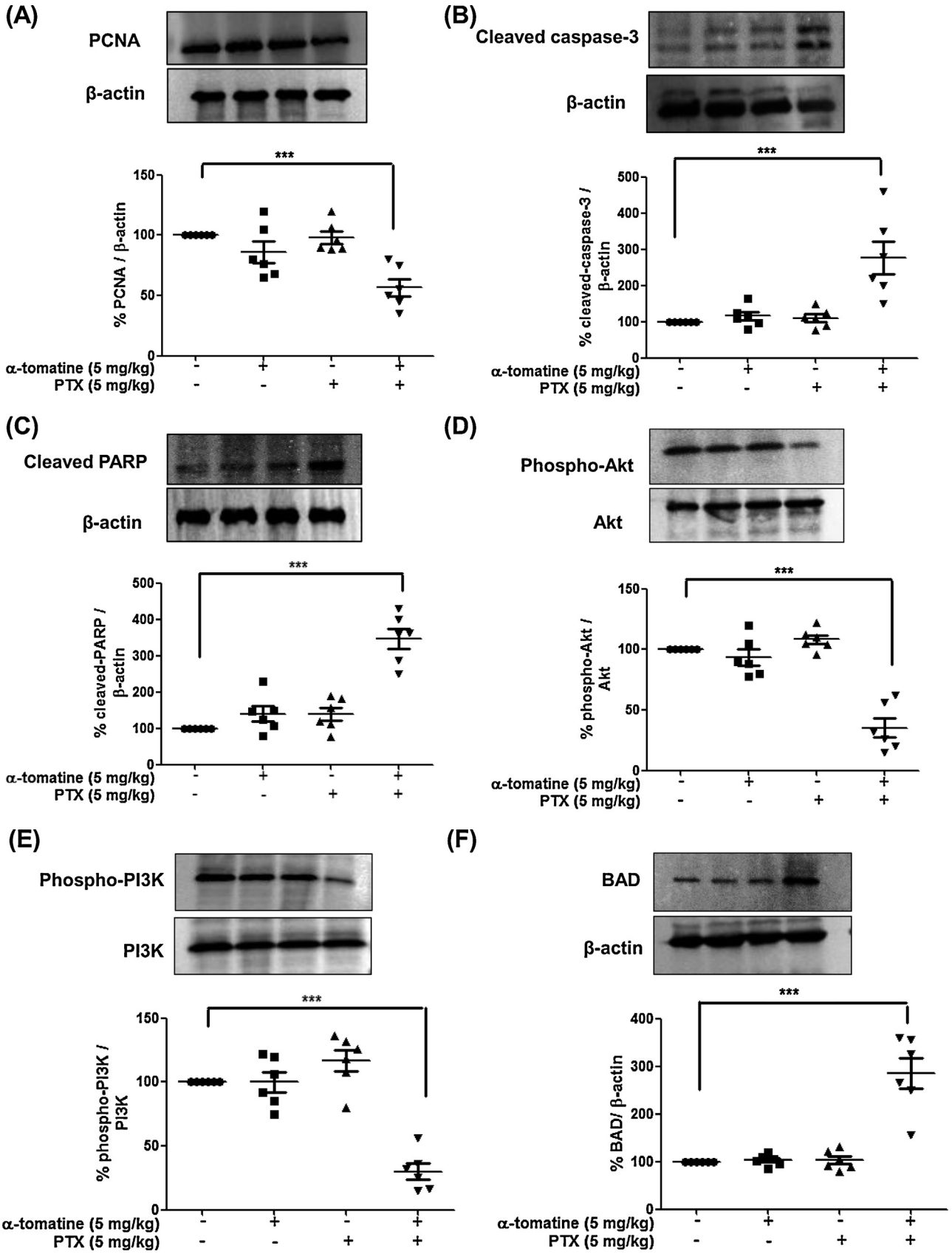


Fig. 7. Western blot analysis of (A) PCNA, (B) cleaved-caspase-3, (C) cleaved-PARP, (D) phospho-Akt, (E) phospho-PI3K and (F) BAD in PC-3 tumor tissues samples. Graphical representation of densitometry analysis of each Western blot data from six independent tumor samples is shown below each panel. Data are shown as means ± SEM. Statistical significance is expressed as $^{***}p < 0.001$ *versus* vehicle control.

Shih et al. 2009). Therefore, therapeutic targeting of PI3K/Akt by α-tomatine presents a promising approach to cancer therapy.

Deregulated anti-apoptotic proteins Bcl-2 and Bcl-xL have been shown to function as oncoproteins in the development of androgen-independent prostate cancer and chemoresistance in prostate cancer (Catz and Johnson 2003; Lebedeva et al. 2000). Our data indicate that the increased susceptibility of PC-3 cells caused by combined PTX and α-tomatine treatment was accompanied with down-regulation of the Akt-regulated anti-apoptotic proteins Bcl-2 and Bcl-xL and increased expression of the pro-apoptotic protein BAD. These data are consistent with report showing that Akt upregulates expression of Bcl-2 through cAMP-response element-binding protein (Pugazhenthi et al. 2000). Previous study demonstrated that PTEN suppresses the Bcl-2 through inhibiting Akt activity and thereby induces the chemosensitivity (Huang et al. 2001). As constitutively active PI3K/Akt and abundant Bcl-2 protein expression were observed in untreated PTEN null PC-3 cells, reduction of PI3K/Akt activity by α-tomatine may therefore induce chemosensitization of the PC-3 cells to PTX-induced apoptosis accompanied with downregulation of Bcl-2 protein expression. In addition, activated Akt is known to prevent cell death by phosphorylating several apoptosis regulating proteins including pro-apoptotic Bcl-2 family member BAD, leading to its disassociation from the anti-apoptotic Bcl-2 family member Bcl-xL, and in turn, promotes survival (Datta et al. 1997). Conversely, disruption of this pro-survival pathway will induce the dephosphorylation of BAD leading to the formation of a heterodimer with Bcl-xL and inhibit the anti-apoptosis function of Bcl-xL and thus trigger cell death (Yang et al. 1995). We therefore propose that the enhanced apoptotic effect by the combination of α-tomatine and PTX is attributed to the down-regulation of Akt-regulated pro-survival proteins and upregulation of Akt-regulated pro-apoptotic proteins.

In conclusion, this study demonstrated that α-tomatine, a phytochemical from tomatoes, improves the ability of PTX to induce *in vitro* and *in vivo* death of androgen-independent prostate cancer PC-3 cells. Our data suggest that this compound has the ability to potentiate sub-lethal doses of PTX to toxic levels, indicating the potential of α-tomatine as an adjunct to taxane-based chemotherapy for the treatment of prostate cancer.

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