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Arctigenin inhibits prostate tumor cell growth *in vitro* and *in vivo*

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Abstract

The low bioavailability of most phytochemicals limits their translation to humans. We investigated whether arctigenin, a novel anti-inflammatory lignan from the seeds of *Arctium lappa*, has favorable bioavailability/potency against prostate cancer. The anticarcinogenic activity of arctigenin was investigated both *in vitro* using the androgen-sensitive LNCaP and LAPC-4 human prostate cancer cells and pre-malignant WPE1-NA22 cells, and *in vivo* using xenograft mouse models. Arctigenin at lower doses ($< 2\mu M$) significantly inhibited the proliferation of LNCaP and LAPC-4 cells by 30-50% at 48h compared to control, and inhibited WPE1-NA22 cells by 75%, while did not affect normal prostate epithelial cells. Male severe combined immunodeficiency (SCID) mice were implanted subcutaneously with LAPC-4 cells for *in vivo* studies. In one experiment, the intervention started one week after tumor implantation. Mice received arctigenin at 50mg/kg (LD) or 100mg/kg (HD) b.w. daily or vehicle control by oral gavage. After 6 weeks, tumor growth was inhibited by 50% (LD) and 70% (HD) compared to control. A stronger tumor inhibitory effect was observed in a second experiment where arctigenin intervention started two weeks prior to tumor implantation. Arc was detectable in blood and tumors in Arc groups, with a mean value up to 2.0 μM in blood, and 8.3 nmol/g tissue in tumors. Tumor levels of proliferation marker Ki67, total and nuclear androgen receptor, and growth factors including VEGF, EGF, and FGF-β were significantly decreased by Arc, along with an increase in apoptosis marker of Bax/ Bcl-2 ratio. Genes responsive to arctigenin were identified including TIMP3 and ZNF185, and microRNAs including miR-126-5p, and miR-21-5p. This study provides the first *in vivo* evidence of the strong anticancer activity of arctigenin in prostate cancer. The effective dose of arctigenin *in*

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vitro is physiologically achievable *in vivo*, which provides a high promise in its translation to human application.

Keywords

Arctigenin; prostate cancer; SCID mice; chemoprevention; phytochemical

Introduction

Prostate cancer is the most commonly diagnosed male malignancy and the third-leading cause of cancer death among men in the United States [1]. Most prostate tumors take a period of about 20-25 years to develop from a focal lesion to clinically detectable phenotype, and many patients die with prostate cancer without any symptoms [2]. However, there are cases of aggressive prostate cancer that metastasize rapidly particularly to the bones and lymph nodes, often before symptoms are noticed [3]. It remains a challenge to predict the type of cancer and separate aggressive prostate tumors from the localized disease. Patients may be over-treated with conventional methods such as surgery and androgen deprivation therapy, and suffer side effects like incontinence, impotence, and osteoporosis [4]. Since prostate cancer is typically diagnosed at elderly population with a relatively slow rate of growth and progression, it may be an ideal candidate disease for chemoprevention to suppress or reverse tumor progression [5]. Even a slight delay in the disease development may result in substantial reduction in incidence of clinically detectable disease [5].

Natural products from diet and plants have become a major resource for developing chemopreventive agents considering both their efficacy and safety. Evidence from preclinical studies demonstrates the potential of several phytochemicals such as green tea polyphenols in cancer chemoprevention [6]. However, translation of these findings into clinical application is limited, mainly due to the relatively low bioavailability and potency of these natural compounds [6,7]. The effective anti-cancer doses as they demonstrate *in vitro* can barely be achieved in vivo by oral consumption of safe doses [6,8]. Therefore it is in urgent need to identify novel phytochemicals of higher potency for chemoprevention.

Arctigenin (Arc, structure in Fig. 1A) is a novel anti-inflammatory lignan derived mainly from the seeds of *Arctium lappa* [9,10]. In the plant Arc is present mainly as glucoside (arctiin) and Arc is released during the digestive process [11]. Both were detected in rat plasma after oral administration of arctiin and distributed widely in different tissues including small intestine, stomach, lung and kidney [11]. The anti-carcinogenic activity of Arc has been demonstrated *in vitro* and in a few animal studies in several cancers, including pancreatic, breast, and lung cancer, associated with the induction of apoptosis, inhibition of proliferation and modulations of multiple signaling pathways [12-14]. We previously demonstrated *in vitro* in prostate cancer that the combination with Arc at lower doses significantly enhanced the anti-proliferative effect of several other phytochemicals like curcumin, green tea polyphenols, and quercetin [15,16]. The present study provides *in vivo* evidence of the potent anti-tumor strength of Arc in mouse models and provides insight into the underlying

molecular mechanisms. To our knowledge, this is the first report of the *in vivo* efficacy of Arc in prostate cancer.

Materials and methods

Cell line and cell culture

The androgen-sensitive LNCaP and LAPC-4 human prostate cancer cell lines were purchased from ATCC (Chicago, IL, USA). Both cell lines were maintained in RPMI 1640 medium, supplemented with 10% (v:v) of fetal bovine serum (FBS), 100 IU/mL of penicillin and 100 μg/mL of streptomycin at 37 °C in a 5% CO_2 incubator. The pre-malignant WPE1-NA22 cell line (ATCC), which mimics a stage of prostatic intra-epithelial neoplasia (PIN), was cultured in keratinocyte serum free medium supplemented with 0.05 mg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor (Invitrogen, Carlsbad, CA). Normal prostate epithelial PrEC cells (Lonza Walkersville, Inc., Walkersville, MD) were cultured in PrEGM medium (Lonza Walkersville, Inc.).

Cell proliferation assay

Cells were seeded into opaque-wall 96-well plates at a density of 8×10^3 per well. LNCaP, LAPC-4 or WPE1-NA22 cells were treated with Arc (Sigma-Aldrich, St. Louis, MO) at 0.5μM, 1μM, 2μM or vehicle control (DMSO). PrEC cells were treated with Arc at 1μM and 10μM to determine the cytotoxicity in normal cells. Cell proliferation was measured at 48h with adenosine triphosphate (ATP) assay using the CellTiter-Glo Luminescent cell viability assay kit (Promega Corporation, Madison, WI). The experiment was done in quadruplicate and repeated twice.

PCR array analysis of mRNA expression in cultured cells

Total RNA was extracted from LNCaP and LAPC-4 cells using a miRNeasy mini kit (Qiagen, Valencia, CA). A polymerase chain reaction (PCR) array assay was performed to simultaneously measure the mRNA expression of 84 genes involved in prostate cancer development and progression, using a RT[2] Profiler™ PCR Array Human Prostate Cancer Kit (Qiagen) following manufacturer's instruction. Briefly, RNA samples were converted into first-strand cDNA using the RT[2] First Strand Kit (Qiagen). The cDNA was mixed with RT[2] SYBR Green Mastermix for quantitative real-time (qRT) PCR analysis. The experiment was repeated thrice.

Animal study

All procedures carried out in mice were approved by the Institutional Animal Care and Use Committee at Charles R. Drew University of Medicine and Science. Male severe combined immunodeficiency (SCID) mice at age of 5-7 weeks (Charles River Laboratories) were acclimatized on sterilized AIN-93G diet (Dyets Inc., Bethlehem, PA) and water for one week. Mice were inoculated subcutaneously with 5×10^5 LAPC-4 cells. In the first study, the intervention started one week later when tumors reached a volume of around 10mm³. Mice were randomly assigned to: 1) control, receiving vehicle (2% DMSO in corn oil) through oral gavage; 2) low dose (LD) Arc, receiving Arc at 50mg/kg body weight (b.w.); 3) high dose (HD) Arc, receiving Arc at 100mg/kg b.w., with 10 mice per group. Tumor size was

measured twice a week using calipers. Tumor volume was calculated with the formula: length × width × height × 0.5236 [17]. Mouse body weight was measured twice a week. The intervention lasted for 6-weeks.

A second study was performed with an early start of intervention which was started two weeks prior to LAPC-4 cell inoculation. Mice were randomly assigned to control or LD Arc group, receiving vehicle or Arc at 50mg/kg b.w. by oral gavage. The treatment lasted for 8 weeks after tumor inoculation.

Immunohistochemical analysis of Ki67 and androgen receptor

A section of each tumor was fixed in 10% phosphate buffered formalin and paraffin-embedded for tissue microarray and immunohistochemical determination with procedures described before [18]. Briefly, the tissue arrays were assembled with 6 cylindrical cores from each tumor. Slides were incubated with monoclonal anti-human Ki67 or androgen receptor (AR) antibody (DAKO North America Inc., Carpineteria, CA), and counterstained with hematoxylin. Slides were digitized and morphometric analysis performed with Definiens' Tissue Studio (Definiens Inc., Parsippany, NJ) to determine the percentage of Ki67 positive cells, and total (cytoplasmic and nuclear) or nuclear AR positive cells in a non-biased method.

Western blot analysis of apoptosis related proteins in tumor tissues

Total protein was extracted from tumor tissues and the procedure for Western blot analysis was described before [19]. Membranes were incubated with primary antibody for detection of Bax (sc-493) or Bcl-2 (sc-509, Santa Cruz, CA). GAPDH was used as loading control.

Measurement of growth factors in tumor tissues

Tumor levels of 8 growth factors were measured using a Human Growth Factor ELISA Strip II kit (Signosis, inc., Santa Clara, CA) following manufacturer's instruction. Briefly, 10μg of total protein in 100μl volume was loaded on the 8-wells strip coated with different antibodies, followed by a biotin-labeled antibody and then streptavidin-HRP conjugate. After adding a HRP substrate the luminescence intensity was measured which is proportional to the concentrations of growth factors.

Detection of Arc in blood and tumor tissues

Blood and tumor samples from the first intervention study were used for detection of Arc following a pervious procedure [19] with modifications. Briefly, 200 μl of serum was extracted twice with 1mL of ethyl acetate. For tumor Arc analysis, about 150 mg of tumor tissues was homogenized in 200 μl of water containing 2% ascorbic acid, and extracted twice with 1mL of ethyl acetate. The supernatant was pooled, dried in vacuum and reconstituted for high-performance liquid chromatography (HPLC) and CoulArray electrochemical detection (ESA, Chelmsford, MA).

PCR array analysis of microRNA expression in tumor tissues

Total RNA was extracted from tumor tissues using a miRNeasy mini kit (Qiagen), and reversely transcribed to cDNA using miScript II RT kit. A PCR array assay was performed

to simultaneously measure 84 microRNAs (miRNA) involved in prostate cancer development and progression using a miScript miRNA PCR Array Human Prostate Cancer Kit (Qiagen). Each sample was done in duplicate.

Statistical analysis

SPSS software (Version 20, Chicago, IL) was applied for statistical analysis. Mean values and standard deviation (SD) were calculated. Comparison of means was performed by two independent samples t-test or one-way analysis of variance (ANOVA) with Tukey's posttest for paired comparison. Differences were considered significant if $P<0.05$.

Results

Anti-proliferative effect of Arc in vitro

Arc at lower doses (0.5-2 μM) dose-dependently inhibited the proliferation of LNCaP and LAPC-4 cells by 30-50% at 48h (Fig. 1B and C). The pre-malignant WPE1-NA22 cells (Fig. 1D) were more sensitive to Arc treatment compared to LNCaP and LAPC-4 cells, and were inhibited by 70% with 2μM of Arc. However, there was no inhibition observed in normal prostate epithelial PrEC cells with Arc treatment up to 10μM (Fig. 1E).

Modulation of mRNA expression in LNCaP and LAPC-4 cells

PCR array analysis of the expression of 84 prostate cancer related genes demonstrate that the profiles of genes in response to Arc treatment are different between LNCaP and LAPC-4 cells (Table 1). The expression of TIMP3 and ZNF185 was increased in both cell lines. However, a significant downregulation of AR, CAMKK1, IGF1, and LGALS4 and upregulation of DLC1, RASSF1, SOCS3 and TFPI2 were mainly observed in LAPC-4 cells, while upregulation of PPP2R1B and TP53 only observed in LNCaP cells.

Inhibition of xenograft tumor growth in SCID mice

In the first intervention experiment, tumor growth was significantly inhibited after 3 weeks of intervention (Fig. 2A). After 6 weeks of intervention (at week 7), tumor growth was inhibited by 50% and 70%, respectively, by LD and HD Arc (Fig. 2A). A stronger tumor inhibitory effect was observed in the second experiment where Arc treatments were started prior to tumor cell implantation. The tumor growth was inhibited by 60% at week 7 and 70% at week 8 by LD Arc (Fig. 2C). A consistent pattern between tumor volume and tumor weight was observed in both experiments (Fig. 2B and D). There was no significant difference in body weight, food or water consumption between control and Arc groups during the interventions.

Blood and tissue levels of Arc

Arc existed in all of the blood and tumor samples from Arc groups, while not detectable in control group. The blood concentration of Arc was 0.62 ± 0.07 μmol/L in LD Arc group, and 2.0 ± 0.6 μmol/L in HD group (Fig. 2E). Arc was found abundant in the tumor tissues with a concentration of 4.7 ± 3.3 nmol/g tissue in LD group and 8.3 ± 2.9 nmol/g tissue in

HD group (Fig. 2E). The tumor concentrations of Arc were significantly correlated with that in the blood.

Downregulation of growth factors in tumor tissues

Growth factors play important roles in tumor cell proliferation and angiogenesis. The analysis of tumor levels of 8 growth factors showed that the levels of VEGF, EGF, NGF-β, TNF-α, and FGF-β were significantly reduced by Arc treatments compared to control (Fig. 2F). Although not statistically significant, a trend to decrease was observed with PDGF-BB by Arc treatment (Fig. 2F). There was no significant change in SCF or TGF-β level with Arc treatment (data not shown).

Modulation of proteins involved in proliferation and apoptosis

Immunohistochemical analysis of mouse tumor array demonstrated that Arc treatment significantly reduced the nuclear expression of proliferation marker Ki67 compared to control (Fig. 3A and B). In addition, both total and nuclear levels of AR were significantly reduced by Arc treatments in a dose-dependent manner (Fig. 3A and B). A significant increase in apoptosis marker as represented by the ratio of Bax to Bcl-2 protein was observed in tumor tissues by Arc treatments compared to control (Fig. 3C and D).

Modulation of miRNA expression in tumor tissues

MiRNAs are a class of small non-coding RNAs that interact with target mRNAs to negatively regulate gene expression at the post-transcriptional level [20]. The dysregulation of miRNA expression has been widely observed in tumor tissues associated with tumorigenesis and disease progression [20]. Through PCR array profiling of miRNA expression in the mouse tumor tissue, we identified a signature of 2 miRNAs downregulated and 5 miRNAs upregulated by at least 50% in response to Arc treatment (Table 2).

Discussion

The present study demonstrates that Arc at low doses significantly inhibited prostate tumor cell growth both *in vitro* and *in vivo* in xenograft mouse models. The antitumor potency of Arc as demonstrated in this study is about 10-20 fold stronger than that of several commonly studied phytochemicals, such as green tea polyphenols and curcumin, based on early studies from our group and other investigators [18,21,22]. Importantly, our results show that the effective doses of Arc as demonstrated *in vitro* (0.5-2 μmol) were physiologically achievable *in vivo* in mouse blood. In addition, Arc was efficiently absorbed into tissues and the concentrations of Arc in the xenograft tumors were about 50-fold higher than that of green tea polyphenols when orally consumed at the same level of doses based on our previous studies on green tea [6,23]. With these advantages in potency and bioavailability in addition to its safety, Arc is highly promising to succeed in prostate cancer chemoprevention and treatment.

Multiple Arc-responsive signaling molecules were identified in the present study, involved in tumor proliferation, apoptosis, angiogenesis, and invasion, suggesting the potential of Arc to exert a systemic control of the disease. A strong induction of the tumor suppressor TIMP3

and ZNF185 was observed in both LNCaP and LAPC-4 cells. TIMP3 is an inhibitor of matrix metalloproteinases (MMPs), and it also blocks the binding of VEGF to VEGF receptor-2 to inhibit angiogenesis [24]. TIMP3 is found frequently downregulated in cancers, associated with accelerated tumor progression and invasion [25]. The general profiles of Arc-responsive genes differ between AR mutated LNCaP cells and AR wild-type LAPC-4 cells (Table 1). This was also observed in our previous studies [16]. In xenograft tumors, Arc significantly reduced both the total and nuclear AR expression. AR is a key modulator of growth and progression of prostate cancer, which makes it an important target in prostate cancer chemoprevention and treatment [26]. In addition, Arc significantly reduced the expression of multiple growth factors in tumor tissues, which may, at least in part, lead to the downregulation of the proliferative signaling and upregulation of apoptotic signaling in tumors.

The importance of miRNA dysregulation in cancer development and progression has been demonstrated widely [27]. The present study demonstrates that treatment with Arc significantly decreased the expression of pro-angiogenic miR-126-5p and miR-21-5p in tumors, and increased the expression of five putative tumor suppressor miRNAs, including miR-135a-5p [28], miR-205-5p [29], miR-22-3p [30], miR-455-5p [31], and miR-96-5p [32]. The expression of miR-126-5p enhances angiogenesis by repressing the expression of Spred-1, an intracellular inhibitor of angiogenic signaling, leading to enhanced signaling of angiogenic growth factors including VEGF and FGF [33]. MiR-21-5p promote angiogenesis partly through directly targeting TIMPs leading to enhanced VEGF signaling and endothelial cell migration [34]. Together with our observation of reduced expression of VEGF and FGF-β in tumor tissues, it suggests that an anti-angiogenic activity may be involved in the tumor inhibitory effect of Arc.

In summary, Arc at lower doses significantly inhibited prostate tumor cell growth both *in vitro* and *in vivo* in SCID mice in a non-toxic manner. The anti-carcinogenic activity of Arc was associated with the regulation of multiple signals in proliferation, apoptosis, angiogenesis and invasion. This study provides a highly promising agent for prostate cancer chemoprevention as well as treatment at early stage. These results warrant future clinical trials to test Arc in humans.

Acknowledgments

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Abbreviations

|	
AR	androgen receptor
Arc	Arctigenin
EGF	epidermal growth factor
FGF	fibroblast growth factor
HD	high dose
IGF1	insulin like growth factor 1
LD	low dose
NGF	nerve growth factor
PDGF	platelet-derived growth factor
SCF	stem cell factor
TGF	transforming growth factor
TNF	tumor necrosis factor
TIMP3	TIMP metallopeptidase inhibitor 3
VEGF	vascular endothelial growth factor
ZNF185	zinc finger protein 185
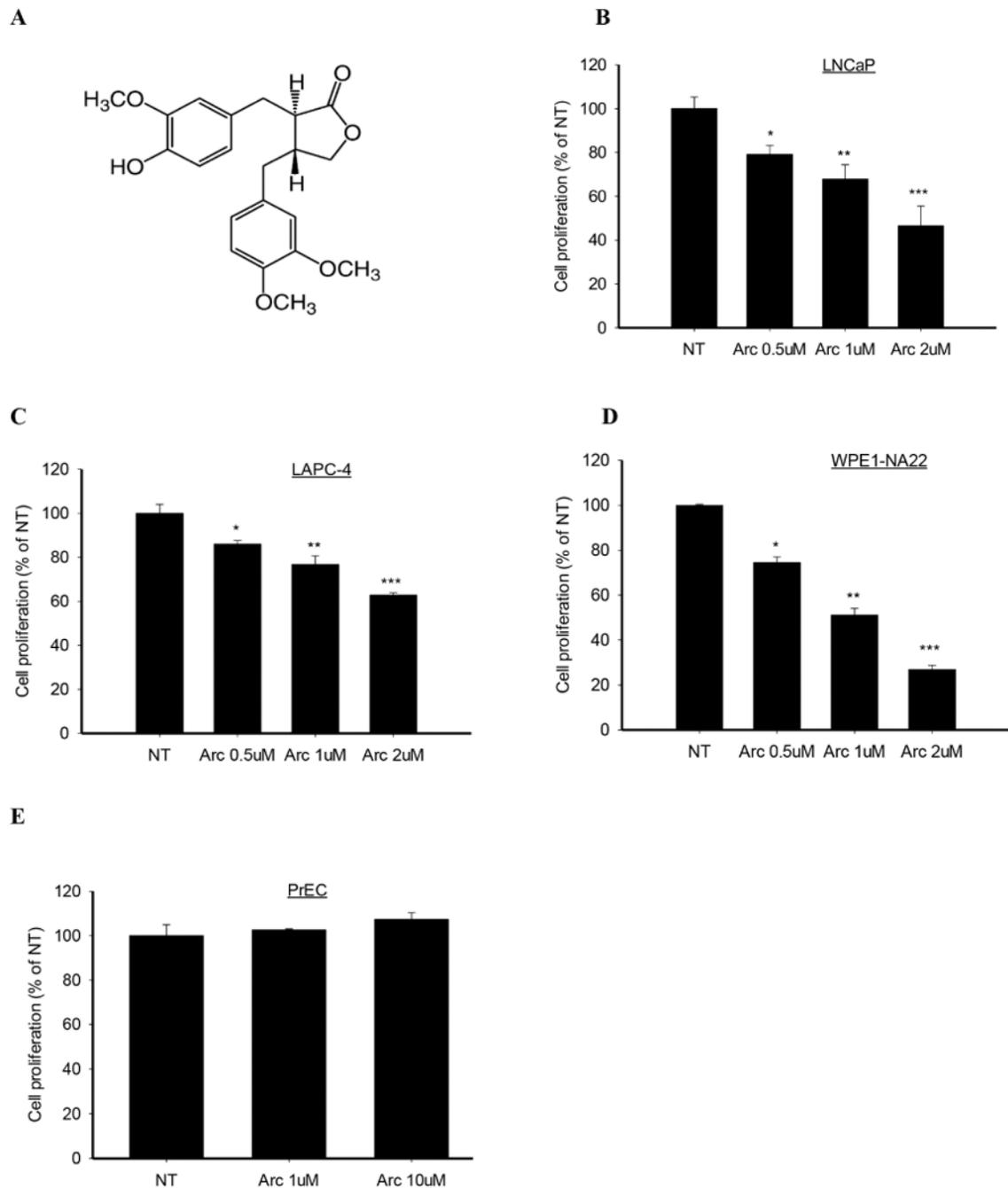
Figure 1.
In vitro anti-proliferative effect of arctigenin. A shows the chemical structure of Arc. Androgen sensitive LNCaP (B) and LAPC-4 (C) prostate cancer cells, prostate pre-malignant WPE1-NA22 cells (D), and normal prostate epithelial PrEC cells (E) were treated with the indicated concentrations of arctigenin for 48h. Cell proliferation was measured by adenosine triphosphate (ATP) assay. Data are presented as mean ± SD. NT: non-treatment, DMSO control; Arc: arctigenin. Compared to NT, *$P<0.05$, **$P<0.01$, ***$P<0.001$.
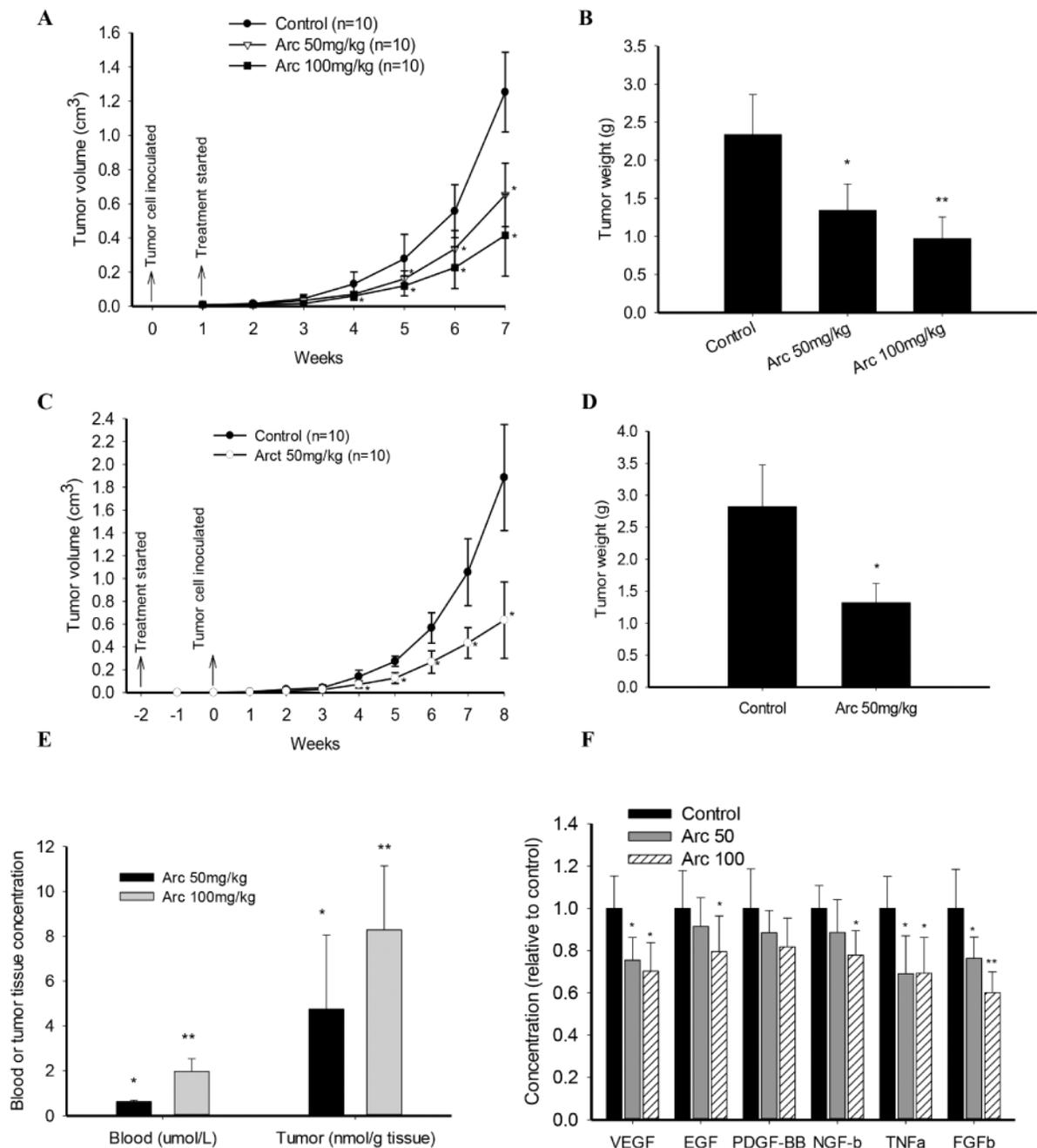
Figure 2.
Inhibition of xenograft tumor growth, blood and tissue levels of arctigenin, and reduced expression of growth factors in SCID mice. Male SCID mice (n=10 per group) were inoculated subcutaneously with 5×10^5 androgen-sensitive LAPC-4 prostate tumor cells. The intervention treatments started either one week after (A, B) or two weeks prior to (C, D) the tumor cell inoculation. Arctigenin was administered daily through oral gavage at dose of 50mg/kg or 100mg/kg bw. Tumor size was measured twice a week using calipers and tumor volume calculated using the formula: length $\times$ width $\times$ height $\times$ 0.5236. Tumor weight was measured at mouse sacrifice. Blood and tumor levels of arctigenin (E) was detected with

HPLC coupled with CoulArray electrochemical detector after extraction with ethyl acetate. The concentrations of 8 growth factors in tumor tissues were simultaneously measured using a Human Growth Factor ELISA Strip II kit (F). Data are presented as mean ± SD. Arc: arctigenin. Compared to control, *$P<0.05$, **$P<0.01$.
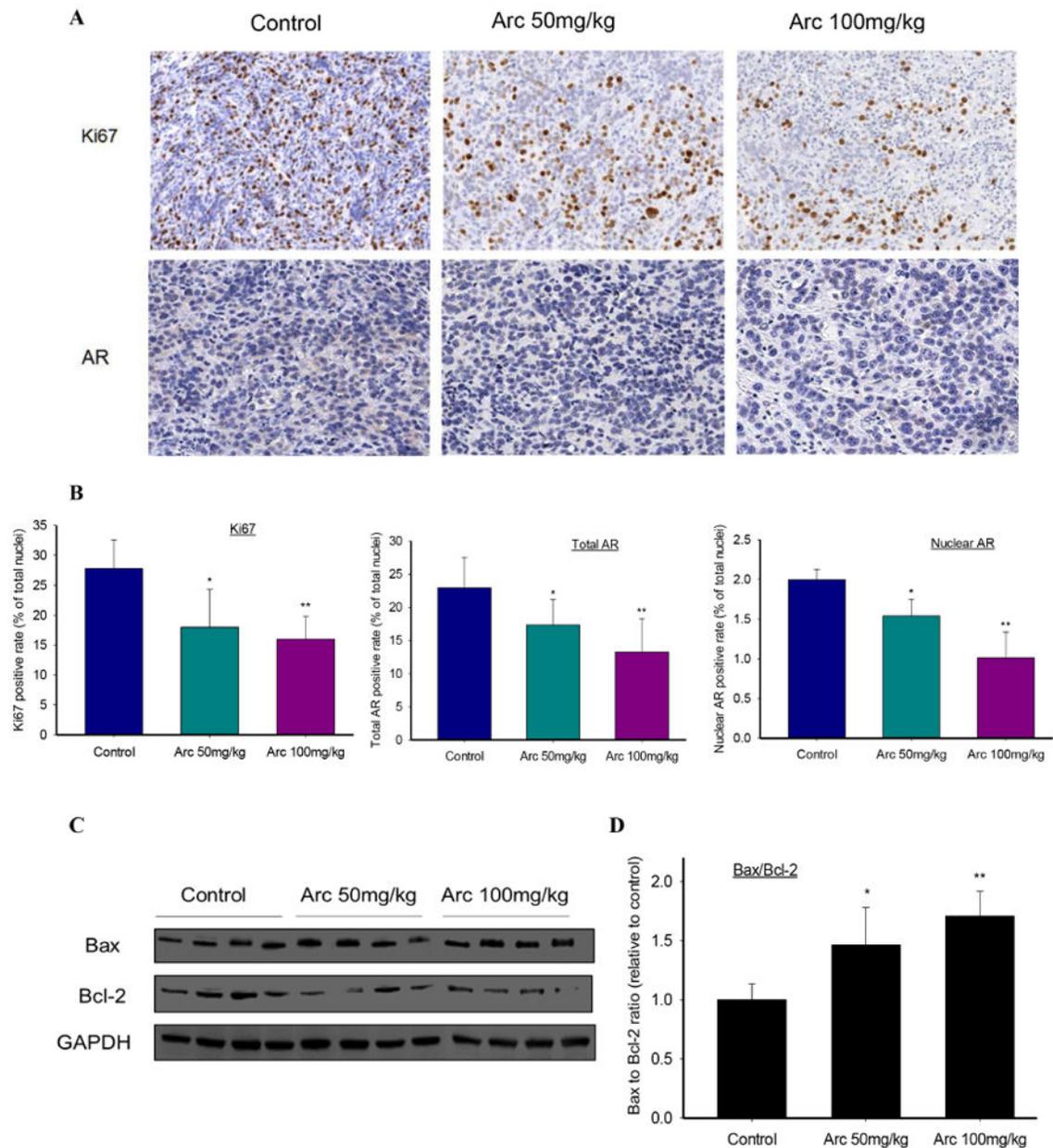
Figure 3.
Decreased Ki67 and AR and increased Bax/Bcl-2 ratio in tumor tissues by arctigenin treatment. A section of tumor tissue was formalin-fixed and paraffin-embedded for tissue microarray and immunohistochemical detection. Slides were cut and incubated with monoclonal antibodies for Ki67 and AR (A). Slides were counterstained with hematoxylin. Nuclei were stained in blue and Ki67 or AR in brown color. The positive rates of Ki67 nuclear staining, total and nuclear AR staining are presented as mean ± SD (B). The protein expression of Bax and Bcl-2 in tumor tissues was detected by Western blot. Four

representative protein bands from each group are demonstrated (C). The ratios of Bax to Bcl-2 are presented as mean ± SD (D). Arc: arctigenin. Compared to control, *P<0.05, **P<0.01.

Table 1

Modulation of mRNA expression in cultured LNCaP and LAPC-4 cells by arctigenin treatment.

Genes	Fold Change (Arc vs. Con)		Gene Function in Cancer	Reference
	LNCaP	LAPC-4		
TIMP3	9.3[*]	3.2[*]	Inhibiting invasion	[25]
ZNF185	2.2[*]	2.1[*]	Inhibiting proliferation	[35]
AR	1.0	0.2[*]	Promoting cell proliferation and survival	[36]
CAMKK1	1.1	0.5[*]	Promoting proliferation and survival through activating Akt pathway	[37]
DLC1	1.4	1.7[*]	Inhibiting invasion through inducing E-cadherin expression	[38]
IGF1	1.4	0.1[*]	Positively associated with prostate cancer risk	[39]
LGALS4	1.0	0.2[*]	Involved in tumor proliferation, migration, angiogenesis and immunity	[40]
PPP2R1B	1.5[*]	0.9	Negative control of cell growth and division	[41]
RASSF1	1.0	2.9[*]	Inducing cell cycle arrest and apoptosis	[42]
S0CS3	0.8	3.0[*]	Inhibiting inflammatory signal transduction	[43]
TFPI2	1.4	5.0[*]	Inhibiting proliferation and invasion and inducing apoptosis	[44]
TP53	2.4[*]	0.8	Inducing cell cycle arrest and apoptosis	[36]

LNCaP and LAPC-4 cells were treated with vehicle control (DMSO) or 1 μM of arctigenin (Arc) for 48h. Total RNA was extracted for quantitative real-time PCR (qRT-PCR) analysis of mRNA expression of 84 genes involved in prostate cancer development and progression using a RT[2] Profiler PCR Array Human Prostate Cancer kit (Qiagen, Valencia, CA). Data are presented as fold change of gene expression by Arc treatment relative to control (Con). TIMP3, TIMP metallopeptidase inhibitor 3; ZNF185, zinc finger protein 185; AR, androgen receptor; DLC1, deleted in liver cancer 1; IGF1, insulin like growth factor 1; LGALS4, lectin, galactoside-binding, soluble; PPP2R1B, protein phosphatase 2 regulatory subunit A, beta; RASSF1, Ras association domain family member 1; SOCS3, suppressor of cytokine signaling 3; TFPI2, tissue factor pathway inhibitor 2; TP53, tumor protein p53.

[*] Compared to control, P<0.05.

Table 2

Modulation of microRNA expression in xenograft tumor tissues by arctigenin treatment in SCID mice.

miRNA	Fold change (Arc vs. Con.)	
miR-126-5p	0.5	399
miR-21-5p	0.5	400
miR-135a-5p	2.0	401
miR-205-5p	1.5	402
miR-22-3p	1.7	403
miR-455-5p	1.6	404
miR-96-5p	1.6	405

Total miRNA was extracted from xenograft tumor tissues from both the control and LD arctigenin (Arc, 50mg/kg bw) groups (n=6 per group), and reversely transcribed to cDNA. The expression of 84 miRNAs involved in prostate cancer development and progression was measured by qRT-PCR using a miScript miRNA PCR Array Human Prostate Cancer kit (Qiagen). Mature miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method in normalization to human RNU6-2 snRNA. Each sample was done in duplicate. Data are presented as fold change of gene expression in Arc group relative to control. Only miRNAs with significant changes in expression (P<0.05 compared to control) were listed.