Published in final edited form as:

Mol Nutr Food Res. 2015 February ; 59(2): 250–261. doi:10.1002/mnfr.201400558.

Arctigenin in combination with quercetin synergistically enhances the anti-proliferative effect in prostate cancer cells

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Abstract

Scope—We investigated whether a combination of two promising chemopreventive agents arctigenin and quercetin increases the anti-carcinogenic potency at lower concentrations than necessary when used individually in prostate cancer.

Methods and results—Androgen-dependent LAPC-4 and LNCaP prostate cancer cells were treated with low doses of arctigenin and quercetin alone or in combination for 48h. The anti-proliferative activity of arctigenin was 10-20 fold stronger than quercetin in both cell lines. Their combination synergistically enhanced the anti-proliferative effect, with a stronger effect in androgen receptor (AR) wild-type LAPC-4 cells than in AR mutated LNCaP cells. Arctigenin demonstrated a strong ability to inhibit AR protein expression in LAPC-4 cells. The combination treatment significantly inhibited both AR and PI3K/Akt pathways compared to control. A protein array analysis revealed that the mixture targets multiple pathways particularly in LAPC-4 cells including Stat3 pathway. The mixture significantly inhibited the expression of several oncogenic microRNAs including miR-21, miR-19b, and miR-148a compared to control. The mixture also enhanced the inhibition of cell migration in both cell lines compared to individual compounds tested.

Conclusion—The combination of arctigenin and quercetin, that target similar pathways, at low physiological doses, provides a novel regimen with enhanced chemoprevention in prostate cancer.

Keywords

Quercetin; arctigenin; prostate cancer; combination; microRNA

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Conflict of Interest Statement: None declared.

1 Introduction

Prostate cancer is the most frequently diagnosed malignancy in men in the United States and the second leading cause of cancer death [1]. Most prostate tumors are slow-growing with a period of about 20-25 years from initiation to the stage when the clinically detectable phenotype can be identified, and many patients die with prostate cancer without any symptoms [2]. However, there are cases of aggressive prostate cancer that metastasize rapidly from the prostate to other parts of the body often before symptoms are noticed [3]. It remains a challenge to identify those aggressive cases from the indolent. Patients may be over-treated with conventional methods such as surgery and androgen deprivation therapy, and suffer side effects from these treatments including incontinence, impotence, and osteoporosis [4, 5]. Prostate cancer is typically diagnosed in a later age and the rate of growth and progression is relatively slow, which makes it an ideal candidate disease for chemoprevention using natural products [6]. Even a slight delay in the disease progression may result in substantial reduction in the incidence of clinically detectable disease [6]. Increasing evidence from preclinical studies is demonstrating a promise of certain bioactive products such as quercetin (Q) in cancer prevention [7, 8]. However translation of these results to clinical studies is limited, mainly due to the low bioavailability of these compounds and their extensive biotransformation *in vivo* into less active metabolites [7, 9]. To overcome these limitations mixtures of bioactive compounds, as traditionally used in Chinese and Indian/Ayurvedic medicine, may be employed.

Q (structure shown in Fig. 1A) is a flavonoid found in a variety of vegetables and fruits particularly in onions, apples, and red wine. Q has been shown to possess antioxidant, anti-inflammatory and anti-proliferative properties [8, 10]. The anti-carcinogenic effect of Q has been demonstrated in several cancers especially in prostate cancer via multiple mechanisms including the inhibition of androgen receptor (AR) and phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathways [8]. AR is a key modulator of growth and progression of prostate cancer through regulating the transcription of target genes that modulate growth and differentiation of prostate epithelial cells [11], thus it is an important target in prostate cancer prevention and treatment [11]. Nevertheless, increasing evidence indicates that PI3K/Akt/mTOR pathway may crosstalk with AR signaling and can directly regulate the expression and activation of AR [11]. Therefore a dual inhibition of AR and PI3K/Akt pathways may significantly enhance the chemopreventive efficacy in prostate cancer. However, limited by its low bioavailability the oral consumption of Q at a safe level may not be able to provide effective concentrations in the body as observed *in vitro* [12]. In addition, Q is extensively methylated, sulfated, or glucuronidated upon uptake [13], which may decrease its bioactivity *in vivo*.

Arctigenin (Arc, structure in Figure 1B) is a novel anti-inflammatory lignan derived mainly from the seeds of the plant *Arctium lappa* [14]. *Arctium lappa* has been widely used in traditional Chinese medicine to treat inflammation related diseases such as cough, cold and swelling of throat [14]. In the plant Arc is present as glucoside (arctiin) and Arc is released during the digestive process [15]. Both have been detected in rat plasma after oral administration of arctiin and distributed widely in different tissues including small intestine, stomach, lung and kidney [15]. The anti-carcinogenic effect of Arc and arctiin has been

demonstrated in several cancers including prostate cancer, associated with the induction of apoptosis, inhibition of proliferation and modulations of multiple signaling pathways particularly the PI3K/Akt pathway [16-19]. Results from our preliminary studies demonstrated that Arc was a strong inhibitor of AR signaling in prostate cancer LAPC-4 cells. Therefore Arc may be an ideal candidate to be combined with Q to enhance the chemopreventive effect through an increased inhibition on both AR and PI3K/Akt pathways at low concentrations of individual compound. The combined effects of Arc and Q on proliferation, apoptosis, and cell migration as well as underlying mechanisms were investigated in the present study using two androgen-dependent prostate cancer cell lines. Results from this study provide a promising novel regimen by combining Arc with Q to enhance chemoprevention in prostate cancer in a non-toxic manner.

2 Materials and Methods

2.1 Cell line and cell culture

The androgen-dependent LNCaP human prostate cancer cell line was purchased from ATCC (Chicago, IL, USA). Androgen-dependent LAPC-4 cell line is a gift from Dr. Charles Sawyers' laboratory previously at UCLA. 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% CO₂ incubator.

2.2 Cell proliferation assay

Both LAPC-4 and LNCaP cells were seeded into opaque-wall 96-well plates at a density of 8×10^3 per well. An inhibition curve was achieved for individual compound including Arc (Sigma-Aldrich, St. Louis, MO) and Q (Sigma-Aldrich) by incubation of both cell lines with multiple doses of Arc or Q for 48h. The doses that led to 10-30% cell growth inhibition by each compound were selected for the combination study. Therefore, cells were treated with Arc at 0.5µM, 1µM, 2µM, Q at 5µM, 10µM, 20µM, or their combinations (total 9 combinations) for 24h and 48h. Cell proliferation was measured with adenosine triphosphate (ATP) assay using the CellTiter-Glo® Luminescent cell viability assay kit (Promega Corporation, Madison, WI). To minimize the effect of hydrogen peroxide (H₂O₂) that may be formed by autoxidation and/or dimerization of phytochemicals in cell culture medium [20], 50 units/mL of catalase was added to the medium prior to Arc and Q in all the experiments in the present study. The experiment was repeated twice with four wells for each treatment in each experiment.

A combination index (CI) was calculated for the combinations of Arc and Q using the CompuSyn software (ComboSyn, Inc., Paramus, NJ), which is based on the widely-accepted Chou-Talalay equation and mass-action law [21]. The value of CI less than 1 indicates a synergistic effect of a combination, equal to 1 additive, and greater than 1 antagonistic [21].

2.3 Intracellular signaling array

A slide-based antibody array was used for simultaneous detection of 18 important and well-characterized signaling molecules when phosphorylated or cleaved using a PathScan Intracellular Signaling Array kit (Cell Signaling Technology, Danvers, MA) following the

manufacturer's instructions. The list of these molecules is shown in Fig. 3C. When 50-60% confluent in T25 Petri dishes, both LAPC-4 and LNCaP cells were treated with vehicle control (DMSO), 1 μ M Arc, 10 μ M Q, or 1 μ M Arc + 10 μ M Q for 48h. Total protein was extracted using RIPA buffer (Santa Cruz Technology, CA), and diluted to 0.5 mg/mL in Array Diluent Buffer provided by the kit. After an overnight incubation of the samples with the array antibodies, a Detection Antibody Cocktail was added to the samples, followed by the addition of HRP-linked Streptavidin and substrate. Protein was visualized using a ChemiDoc XRS chemiluminescence detection and imaging system (Bio-Rad Laboratories, Irvine, CA).

2.4 Western blot analysis of protein markers

When 50-60% confluent in T25 Petri dishes, both LAPC-4 and LNCaP cells were treated with vehicle control (DMSO), 1 μ M Arc, 10 μ M Q, or 1 μ M Arc + 10 μ M Q for 48h. Total protein was extracted using RIPA buffer. The procedure for Western blot analysis was described before [22]. Briefly, 50 μ g of protein was separated on a 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA). Proteins were electrotransferred to nitrocellulose membranes. Membranes were incubated with primary anti-human antibodies for the detection of Bax (sc-493), Bcl-2 (sc-509), AR (sc-7305), PSA (sc-7638), Nkx 3.1 (sc-15022, Santa Cruz Technology), Akt (4685), p-Akt (Ser473, 4058, Cell Signaling Technology). GAPDH or β -actin protein was used as loading control. Protein was visualized and analyzed using the ChemiDoc XRS chemiluminescence detection and imaging system.

2.5 Quantitative real-time PCR analysis of AR downstream gene expression

The mRNA expression of AR regulated genes including the prostate specific antigen (PSA) and Nkx3.1 was analyzed using quantitative real-time PCR (qRT-PCR), to confirm the modulatory effect of the treatments on AR activity. Both LAPC-4 and LNCaP cells were treated with vehicle control (DMSO), 1 μ M Arc, 10 μ M Q, or 1 μ M Arc + 10 μ M Q for 48h. Total RNA was extracted and concentrations measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The PCR procedure was described previously [23]. Briefly, mRNA was transcribed to cDNA by ThermoScriptTM RT-PCR system (Invitrogen). The final volume of the 20 μ L real-time PCR mixture contained 5 μ L of cDNA template, 1 μ L of each primer, 10 μ L of SYBR Green (Qiagen, Valencia, CA), and 3 μ L of nuclease-free water. The primers for PSA included the forward 5'-CAT CAG GAA CAA AAG CGT GA-3' and reverse 5'-AGC TGT GGC TGA CCT GAA AT-3'; for Nkx 3.1 forward 5'-GAA TCC GTA TGC CCC GCT GAA TCT-3' and reverse 5'-ACC CTT GCC AGT GCG TGT GC-3'; and for GAPDH forward 5'-CAT GTT CGT CAT GGG TGT GA-3' and reverse 5'-GGT GCT AAG CAG TTG GTG GT-3'. qRT-PCR was performed on a Bio-Rad iCycler real-time PCR system (Bio-Rad, Hercules, CA). Each sample was measured in triplicate, and non-template negative controls were included in each run. The 2-(-Ct) method was used to normalize the expression of PSA and Nkx 3.1 to GAPDH and to compare the average Ct value.

2.6 qRT-PCR analysis of microRNA expression

Many phytochemicals have been shown to be able to modulate the expression of microRNA (miRNA), a class of small non-coding RNAs that interact with mRNA to regulate the gene

expression post-transcriptionally [24-26]. To investigate whether Arc and Q exert their anti-cancer effects through a modulation of miRNA expression, we selected three candidate miRNAs that have been shown to be involved in prostate cancer, these including miR-21, miR-19b, and miR-148a. LAPC-4 and LNCaP cells were treated with vehicle control (DMSO), 1 μ M Arc, 10 μ M Q, or 1 μ M Arc + 10 μ M Q for 48h. Total miRNA was extracted using a miRNeasy mini kit (Qiagen), and reversely transcribed to cDNA using miScript II RT kit (Qiagen). Specific primers were provided by miScript Primer Assays for miR-21 (MS00009079), miR-19b (MS00031584), and miR-148a (MS00003556). The qRT-PCR was performed using a miScript SYBR Green PCR kit (Qiagen) according to manufacturer's instruction on a Bio-Rad iCycler real-time PCR system. Briefly, the 25 μ L final volume of reaction contained 12.5 μ L of 2 $\times$ QuantiTect SYBR Green PCR Master Mix, 2.5 μ L of 10 $\times$ miScript Universal Primer, 2.5 μ L of 10 $\times$ miScript Primer Assay, 2.5 μ L of template cDNA, and RNase-free water. The reaction mixture was incubated at 95 $^{\circ}$ C for 15 min, followed by 40 cycles of 94 $^{\circ}$ C for 15 s, 55 $^{\circ}$ C for 30 s, and 70 $^{\circ}$ C for 30 s. Mature miRNA expression was calculated using the 2^{-Ct} method in normalization to human RNU6-2 snRNA (MS00033740). Each sample was done in triplicate.

2.7 Wound healing assay

The capacity of these two compounds and their combinations to inhibit cell migration was tested using both wound healing assay and transwell chamber assay. Both LAPC-4 and LNCaP cells were cultured in 24-well plate to 90-100% confluency. Cells were starved in serum-free medium overnight. A line was drawn with a marker pen on the bottom of the plate across the middle of wells. Scratches were made perpendicular to the line using a 200 μ L pipette tip. Three separate wounds were made for each well. The cells were rinsed with PBS and treated with vehicle control (DMSO), 1 μ M Arc, 10 μ M Q, or 1 μ M Arc + 10 μ M Q for 48h. Pictures were taken both above and below each of the intersections using a microscope camera at $\times$ 100 magnification at 0h and 48h (total six pictures for each well at each time point). The gaps were measured and percent wound closure was calculated. The experiment was done in triplicate.

2.8 Transwell chamber assay

The transwell chamber is 24-well plate based with an insert of 8 μ m pore size polyethylene terephthalate membrane (Corning Life Sciences, Tewksbury, MA). LAPC-4 and LNCaP cells were cultured in T25 flasks to 50-60% confluency and treated with vehicle control, 1 μ M Arc, 10 μ M Q, or 1 μ M Arc + 10 μ M Q for 48h. The cells were starved in serum-free medium overnight. After trypsinization 1 $\times$ 10⁵ cells were collected, suspended in 200 μ L serum-free medium and added on the upper well of the chamber. 300 μ L of complete growing medium was added on the bottom. After 20h incubation, cells were fixed with 5% glutaraldehyde and stained with 0.5% toluidine blue as described previously [27]. Cells on the upper membrane were wiped off with a cotton swab. Migrated cells on the lower membrane were counted under a microscope at $\times$ 200 magnification. Three fields for each wells were counted and the experiment was done in triplicate.

2.9 Statistical analysis

SPSS (Version 20.0, Chicago, IL) was used for statistical analyses. Data collected from each experiment was used to calculate the mean values and standard deviations (SD). Comparison of means was performed by one-way analysis of variance (ANOVA) with Tukey's posttest for paired comparison. Differences were considered significant if $P < 0.05$.

3 Results

3.1 Enhanced anti-proliferative effect by combination treatment

Treatment with Arc demonstrated a 10-20 fold stronger potency than Q to inhibit cell proliferation in both LAPC-4 and LNCaP cells (Fig. 2). LAPC-4 cells were more sensitive to Arc treatment than LNCaP cells, and the proliferation of LAPC-4 cells was inhibited by 36% by 1 μ M of Arc at 48h versus 16% in LNCaP cells. The combination of Arc with Q time- and dose-dependently enhanced the anti-proliferative effect in both cell lines. An increased inhibition of cell proliferation by 30% was achieved in both cell lines by a mixture of 1 μ M Arc and 20 μ M Q compared to individual compound (Fig.2). A combination index (CI) was calculated for all the nine combinations (Arc at 0.5 μ M + Q at 5 μ M, 10 μ M or 20 μ M; Arc at 1 μ M + Q at 5 μ M, 10 μ M or 20 μ M; and Arc at 2 μ M + Q at 5 μ M, 10 μ M or 20 μ M) using CompuSyn software. The values of CI were between 0.5-0.7 in LAPC-4 cells, 0.2-0.8 in LNCaP cells.

3.2 Modulations of protein expression and signaling pathways

Results from the intracellular signaling array analysis demonstrated reduced phosphorylation of multiple signaling molecules in LAPC-4 cells, including Akt/mTOR, signal transducer and activator of transcription (Stat)3, AMP-activated protein kinase (AMPK) α , Bad, PRAS40, and glycogen synthase kinase (GSK)-3 β (Fig. 3A). Arc demonstrated a stronger ability to reduce the phosphorylation of these proteins than Q. The inhibitory effect was further enhanced by the combination of Arc with Q. The reduced phosphorylation of Akt and mTOR by Arc + Q treatment was also observed in LNCaP cells. In addition, the mixture decreased the phosphorylation of S6 ribosomal protein kinase in LNCaP cells (Fig. 3B). The phosphorylation of these proteins was inhibited by 50-95% with the combination treatment compared to control in both cell lines. The reduced phosphorylation of Akt was further confirmed using Western blot analysis in both cell lines by the combination treatment compared to Arc or Q alone, along with a decreased expression of Akt protein as observed in LAPC-4 cells (Fig. 4). In addition, a significantly increased ratio of Bax to Bcl-2 protein expression was observed in LAPC-4 cells by the combination treatment compared to Q alone, and a trend to increase in LNCaP cells (Fig. 4).

3.3 Enhanced inhibition of AR signaling

The protein expression of AR was blocked by Arc alone or in combination with Q in LAPC-4 cells (Fig. 4A). A slight inhibition of AR protein expression by 20% was also observed in LNCaP cells by the combination of Arc and Q (Fig. 4B). qRT-PCR analysis of AR regulated genes demonstrated that the mRNA expression of PSA was significantly decreased by the combination treatment compared to both Arc and Q alone in LAPC-4 cells or compared to Arc in LNCaP cells (Fig. 5). In addition, an increased mRNA expression of

Nkx 3.1, an AR-regulated prostate cancer suppressor, was observed in both cell lines by the treatment with Arc and Q alone or in combination compared to control (Fig. 5). The protein expression of PSA and Nkx 3.1 was confirmed by western blot analysis, demonstrating a consistent pattern with their mRNA expression (Fig. 5).

3.4 Modulations of miRNA expression

Arc demonstrated a stronger ability than Q to inhibit the expression of several oncogenic miRNAs including miR21, miR-19b, and miR-148a, in LAPC-4 cells (Fig. 6). The combination of Arc and Q decreased the expression of miR-21 by 40%, and by 70% for both miR-19b and miR-148a in LAPC-4 cells compared to control. There was no change in the expression of these three miRNAs in LNCaP cells with Arc alone. However, the combination of Arc and Q led to an inhibition of all three miRNAs by 20-30% in LNCaP cells compared to control (Fig. 6).

3.5 Enhanced inhibition of cell migration

Both Arc and Q were able to inhibit the wound closure in the two cell lines (Fig. 7A and B). The combination of Arc and Q significantly increased the inhibition compared to Arc or Q alone in LNCaP cells, with a trend to increase the inhibition in LAPC-4 cells. The wound closure was inhibited by 53% in LNCaP cells and 27% in LAPC-4 cells compared to control by the combination treatment (Fig. 7A and B). The inhibitory effect of Arc and Q on cell migration was further confirmed by transwell chamber assay (Fig. 7C and D, Fig. S2). The mixture of Arc and Q significantly enhanced the inhibition in both cell lines compared to Arc or Q alone. The migration of LAPC-4 cells was inhibited by 70% and LNCaP cells by 35% with Arc + Q treatment compared to control (Fig. 7C and D, Fig. S2).

4 Discussion

This study demonstrates that a novel regimen by combining Arc with Q at a low dose synergistically enhanced the anti-proliferative effect in androgen-dependent prostate cancer cells. The low bioavailability of most flavonoids hinders their application in humans, and effective doses as demonstrated *in vitro* can barely be achieved in the human body by oral consumption of safe amount [28]. A characteristic long half-life of Q in plasma, ranging from 11-28h as reported, makes it possible for Q to accumulate in the body with repeated intakes [29]. The consumption of 150mg of Q per day for 2 weeks resulted in 380 nmol/L Q in plasma of healthy humans [30]. The concentration of Q reached 1.5 μ mol/L in plasma of healthy subjects after consumption of 1g of Q per day for 28d [31]. Many bioactive compounds such as Arc and Q share the same molecular anticarcinogenic targets. Therefore, a mixture of these compounds may enhance the effect on their targets at low concentrations of individual compound, thereby overcoming the limitations of low bioavailability in a non-toxic manner. The treatment with Arc and Q alone or in combination did not change the growth of normal prostate epithelial PrEC cells in the present study (Fig. S1). Previously we demonstrated that the combination of green tea polyphenol such as (-)-epigallocatechin gallate (EGCG) with Q significantly enhanced the chemopreventive effect both *in vitro* and in animal models [32, 33]. The present study demonstrates that a 40-fold lower concentration of Arc in combination with Q achieved an anti-proliferative effect comparable

to EGCG combined with Q [32]. This increases the likelihood of success to translate the mixture of Arc and Q to humans.

The combined anti-proliferative activity of Arc and Q was associated with changes in multiple signaling pathways, with a stronger effect in LAPC-4 than in LNCaP cells. Arc treatment significantly inhibited the protein expression of wild-type AR in LAPC-4 cells while mutated AR in LNCaP cells was effected less. The inhibitory effect of Arc and its combination with Q on AR signaling was further confirmed by the changes in mRNA expression of AR regulated genes, represented by a decreased expression of PSA and increased Nkx 3.1. PSA is a commonly used biomarker to indicate prostate tumor growth as well as therapeutic efficacy [34]. Nkx 3.1 is found to be a prostate tumor suppressor, and loss of Nkx 3.1 protein expression is commonly observed in prostate tumors [35]. In addition to AR pathway, a dual inhibition on the PI3K/Akt pathway was observed in both cell lines particularly by the combination treatment. The reduced p-Akt level in LAPC-4 cells may be partly due to an inhibited expression of Akt protein by the combination treatment. The inhibition of PI3K/Akt pathway is important for a sustainable inhibition of AR signaling since PI3K/Akt pathway may be upregulated in response to AR inhibition and in turn activates AR signaling [11, 36]. In addition, the combination of Arc with Q enhanced the ability to modulate several other important signaling pathways in regulating cell proliferation, apoptosis, motility, and survival, particularly in LAPC-4 cells. The reduced phosphorylation of Stat3, AMPK α , Bad, PRAS40, GSK-3 β (Ser 9), and S6-Ribosomal protein kinase has been shown to result in reduced tumor cell growth in response to therapeutic treatment [37-42]. In addition, several of these pathways including Stat3, GSK-3 β and S6-Ribosomal protein kinase may also contribute to the enhanced inhibition of cell migration by the combination treatment, which is important in reducing the risk of tumor invasion and metastasis [37, 38, 42]. The multiple-targeting activity of the mixture of Arc and Q provides a promise for a systemic control of cancer, since a cancer may have hundreds of gene mutations and dysfunctions and many pathways crosstalk with each other in tumor growth [43].

Recent studies have shown that miRNA may be a new and important mechanism and molecular target of phytochemicals in anti-carcinogenesis [44]. Q has demonstrated the ability to regulate multiple mRNAs including the inhibition of miR-19b [44, 45]. Results from the present study demonstrate that the combination of Arc with Q significantly enhanced the inhibition of oncogenic miR21, miR-19b and miR-148a expression compared to Q alone in LAPC-4 cells, and of miR21 and miR-148a in LNCaP cells. The expression of all three miRNAs was reduced by 2-fold with Arc + Q treatment compared to control in LAPC-4 cells. Q alone was unable to inhibit the expression of miR21, but was a stronger inhibitor than Arc to inhibit miR-19b in LNCaP cells. The interactions between Arc and Q may build the basis for the synergy of these two compounds. It has been shown that the expression of both miR-21 and miR-148a is positively regulated by AR [46, 47]. Elevated expression of miR-21 enhanced androgen-dependent tumor growth *in vivo* while inhibition of miR-21 reduced androgen-induced prostate tumor cell proliferation [46]. In addition, Stat3 has been shown to be able to activate miR-21 partly through the inhibition of the phosphatase and tensin homolog (PTEN) [48]. Thus the inhibition of miR-21 by the mixture

of Arc and Q as observed in the present study may be through a dual inhibition of AR and Stat3. PTEN is a tumor suppressor and negative inhibitor of PI3K/Akt/mTOR pathway [36]. The loss of PTEN has been widely found in prostate cancer [36]. In addition to miR-21, the overexpression of both miR-19b and miR-148a has been found to be associated with suppressed PTEN and activated PI3K/Akt signaling [47, 49]. Therefore the reduced activation of PI3K/Akt pathway in the present study may be at least partly attributable to the inhibited expression of these miRNAs.

In summary, a novel combination treatment with Arc and Q synergistically enhanced the anti-proliferative effect in androgen-dependent prostate cancer cells, associated with increased modulations on multiple signaling pathways including a dual inhibition of AR and PI3K/Akt pathways. These results warrant future animal studies and clinical trials and provide a promising non-toxic regimen to enhance chemoprevention in prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (NIH, NCI, NIMHD, NCATS) Grants: U54 CA143931-01, U54MD007598, UL1TR000124 (J.V. Vadgama); and NIH/National Center for Advancing Translational Sciences (NCATS) UCLA CTSI Grant KL2TR000122 (P. Wang).

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Abbreviations

AMPK	AMP-activated protein kinase
AR	androgen receptor
Arc	arctigenin
ATP	adenosine triphosphate
EGCG	(-)-epigallocatechin-3-gallate
GSK	glycogen synthase kinase
miRNA	microRNA
PI3K	phosphatidylinositide 3-kinases
Q	quercetin
Stat	signal transducer and activator of transcription

A

B

Figure 1.
Chemical structures of quercetin and arctigenin. A, quercetin; B, arctigenin.

Figure 2.

Enhanced antiproliferative effect by the combination of arctigenin with quercetin in both LAPC-4 and LNCaP cells. LAPC-4 (A) and LNCaP cells (B) were treated with the indicated concentrations of arctigenin and quercetin alone or in combination for 48h. Cell proliferation was measured by ATP assay. Data are presented as mean $\pm$ SD. NT: non-treatment, DMSO control; Arc: arctigenin. Q: quercetin. a- compared to NT, b- compared to individual compound, $P < 0.05$. CI: combination index, < 1 , synergistic; $= 1$, additive; > 1 , antagonistic effect of the combination.

Figure 3.

Modulations of intracellular signaling pathways in both LAPC-4 and LNCaP cells using antibody array analysis. LAPC-4 (A) and LNCaP cells (B) were treated with vehicle control (DMSO), 1 μ M Arc, 10 μ M Q, or 1 μ M Arc + 10 μ M Q for 48h. Total protein was extracted for the analysis. A slide-based antibody array was used for simultaneous detection of 18 signaling molecules (C) when phosphorylated or cleaved using a PathScan Intracellular Signaling Array kit. Each protein was arranged in duplicate. The names of the proteins with changes in phosphorylation were indicated on the images. NT: non-treatment, DMSO control; Arc: arctigenin. Q: quercetin.

Figure 4.

Enhanced modulation of protein biomarkers related to apoptosis, AR and PI3K/Akt pathways. LAPC-4 (A) and LNCaP cells (B) were treated with vehicle control (DMSO), 1 μ M Arc, 10 μ M Q, or 1 μ M Arc + 10 μ M Q for 48h. The protein biomarkers related to apoptosis (Bax to Bcl-2 ratio), AR and PI3K/Akt pathways were analyzed using Western blot. Data are presented as mean $\pm$ SD. NT: non-treatment, DMSO control; Arc: arctigenin. Q: quercetin. Columns with different letters represent significant difference between treatments ($P < 0.05$).

Figure 6

Figure 5.

Modulations of mRNA expression of AR regulated genes. LAPC-4 (A) and LNCaP cells (B) were treated with vehicle control (DMSO), 1 μ M Arc, 10 μ M Q, or 1 μ M Arc + 10 μ M Q for 48h. Total mRNA was extracted for quantitative real-time PCR analysis of AR down-stream genes PSA and Nkx 3.1. Data are presented as mean $\pm$ SD. NT: non-treatment, DMSO control; Arc: arctigenin. Q: quercetin. Columns with different letters represent significant difference between treatments ($P < 0.05$).

Figure 6.

Enhanced inhibition of oncogenic microRNA expression by combination treatment. LAPC-4 (A) and LNCaP cells (B) were treated with vehicle control (DMSO), 1 μ M Arc, 10 μ M Q, or 1 μ M Arc + 10 μ M Q for 48h. Total miRNA was extracted, reversely transcribed to cDNA and quantified using quantitative real-time PCR. Mature miRNA expression was calculated using the 2^{-Ct} method in normalization to human RNU6-2 snRNA. Each sample was done in triplicate. Data are presented as mean $\pm$ SD. NT: non-treatment, DMSO control; Arc: arctigenin. Q: quercetin. Columns with different letters represent significant difference between treatments ($P < 0.05$).

Figure 7.

Enhanced inhibition of cell migration by combination treatment. For the scratch assay, wounds were made when LAPC-4 (A) and LNCaP cells (B) cells were 90-100% confluent and after an overnight starvation. The cells were treated with vehicle control (DMSO), 1μM Arc, 10μM Q, or 1μM Arc + 10μM Q for 48h. The closure of wounds were imaged and measured at 0h and 48h. For transwell chamber assay, LAPC-4 and LNCaP cells (C) were treated with vehicle control (DMSO), 1μM Arc, 10μM Q, or 1μM Arc + 10μM Q for 48h. The cells were starved in serum-free medium overnight. Then the cells suspended in serum-free medium were seeded on the upper membrane of transwell chamber and incubated for 20h. Complete growth medium was added on the bottom. Cells on the lower membrane of chambers were counted. Data are presented as mean ± SD. NT: non-treatment, DMSO control; Arc: arctigenin. Q: quercetin. *compared to NT, #compared to Arc or Q alone, $P < 0.05$.