

# A Novel Dietary Flavonoid Fisetin Inhibits Androgen Receptor Signaling and Tumor Growth in Athymic Nude Mice

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## Abstract

**Androgen receptor (AR)-mediated signaling plays an important role in the development and progression of prostate cancer (PCa). Hormonal therapies, mainly with combinations of antiandrogens and androgen deprivation, are the mainstay treatment for advanced disease. However, emergence of androgen resistance largely due to inefficient antihormone action limits their therapeutic usefulness. Here, we report that fisetin, a novel dietary flavonoid, acts as a novel AR ligand by competing with the high-affinity androgen to interact with the ligand binding domain of AR. We show that this physical interaction results in substantial decrease in AR stability and decrease in amino-terminal/carboxyl-terminal (N-C) interaction of AR. This results in blunting of AR-mediated transactivation of target genes including prostate-specific antigen (PSA). In addition, treatment of LNCaP cells with fisetin decreased AR protein levels, in part, by decreasing its promoter activity and by accelerating its degradation. Fisetin also synergized with Casodex in inducing apoptosis in LNCaP cells. Treatment with fisetin in athymic nude mice implanted with AR-positive CWR22Rv1 human PCa cells resulted in inhibition of tumor growth and reduction in serum PSA levels. These data identify fisetin as an inhibitor of AR signaling axis and suggest that it could be a useful chemopreventive and chemotherapeutic agent to delay progression of PCa. [Cancer Res 2008;68(20):8555–63]**

## Introduction

Prostate cancer (PCa) is the most frequently diagnosed non-cutaneous male malignancy and the third leading cause of cancer-related death in men in most western industrialized countries (1). It is estimated that around 660,000 men worldwide will be diagnosed with PCa and 250,000 men will die from it in 2010; thus, it will remain a major health problem in coming years (1). Despite an initial efficacy of androgen deprivation therapy, most patients with PCa progress within 2 years from androgen-dependent status to hormone-refractory PCa, for which there is no curative therapy. Androgen receptor (AR) signaling plays a key role in the development of hormone-refractory PCa. Therefore, finding novel and more effective inhibitors of AR signaling is of paramount interest. AR is a member of the nuclear hormone receptor superfamily and a ligand-activated transcription factor. It contains an amino terminus, a central DNA binding domain, and a carboxyl-terminal ligand binding domain (LBD; refs. 2, 3).

Chemopreventive intervention using naturally occurring dietary substances is an attractive option in PCa because of its incidence, prevalence, and disease-related morbidity and mortality (4, 5). Strategies to reduce the morbidity and mortality of metastatic disease depend on curative treatment of early tumors destined to become life-threatening or their prevention. Very few agents, especially naturally occurring, nontoxic dietary components, that inhibit AR signaling have been reported. Thus, a naturally occurring agent that inhibits AR signaling could be extremely useful for patients whose cancers are diagnosed at an early stage.

Fisetin (3,3',4',7-tetrahydroxyflavone; Fig. 1A) is found in fruits and vegetables, such as apple, strawberry, persimmon, grape, onion, and cucumber (6). It has been reported to inhibit the activities of cyclin-dependent kinases causing G<sub>2</sub>-M phase cell cycle arrest and increase in p21/WAF1 levels in HT-29 human colon cancer cells (7). Fisetin has been shown to exert antiproliferative effects on human PCa (LNCaP and PC3) and breast cancer (MCF-7) cell lines (8). Fisetin showed dose-dependent cytotoxic effects on SK-HEP-1 cells, accompanied by DNA fragmentation, induction of caspase-3/CPP32 activity, and increase of p53 protein (9). We have recently reported that treatment with fisetin caused induction of apoptosis, modulation in the expressions of Bcl2-family proteins, activation of caspases, and cell cycle arrest in human PCa cells (10).

We hypothesized that fisetin may act as an inhibitor of AR signaling and, thus, could serve as a therapeutic agent for the management of human PCa. We report here strong antiandrogen and anti-AR activities of the natural product fisetin in PCa cells. Fisetin inhibited the AR transactivation primarily by decreasing its stability. I.p. administration of fisetin to athymic nude mice implanted with AR-positive CWR22Rv1 cells resulted in significant inhibition of tumor growth and secretion of prostate-specific antigen (PSA). The antiandrogen and, hence, the anti-AR activities of fisetin and consequent inhibition of PCa growth described in the present study could have significant implications for the prevention as well as therapy of PCa.

## Materials and Methods

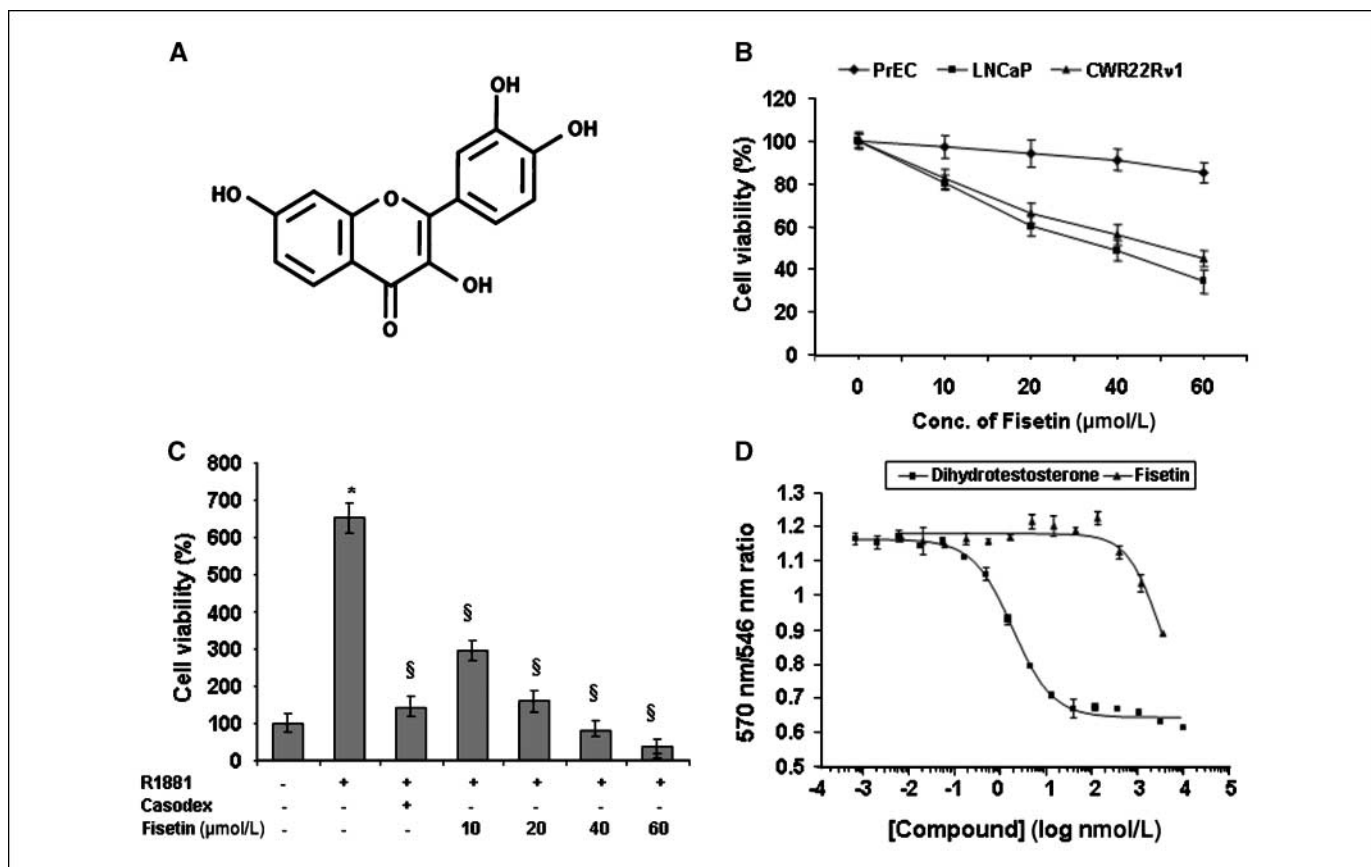
The AR and PSA antibodies were obtained from Santa Cruz Biotechnology, Inc. Fisetin (>99% pure) was purchased from Sigma Chemical Co. Antimouse and antirabbit secondary antibody horseradish peroxidase conjugate was obtained from Amersham Life Science Inc. AR agonist R1881 (methyltrienolone) was from Perkin-Elmer, Inc. Bicinchoninic acid protein assay kit was obtained from Pierce. ELISA kit was purchased from Active Motif. Annexin V-Fluos staining kit was from Roche Diagnostics Corp. Fail-Safe PCR premix kit was purchased from Epicentre Technologies.

**Cell culture and treatment.** The LNCaP and CWR22Rv1 cells were obtained from American Type Culture Collection. The LNCaP cells were cultured in DMEM supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. CWR22Rv1 cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin. Human normal prostate epithelial cells were obtained from Cambrex Bioscience and grown according to the manufacturer's instructions. CV1 cells were grown in DMEM supplemented with 10% FBS and 1%

**Note:** N. Khan and M. Asim contributed equally to this work.

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**Figure 1.** Effect of fisetin on cell viability and its interaction with the LBD of AR. *A*, chemical structure of fisetin. *B*, effect of fisetin on cell growth. As detailed in Materials and Methods, LNCaP, CWR22Rv1, and prostate epithelial cells (*PrEC*) were treated with fisetin for 48 h and the viability of cells was determined by the MTT assay. *Points*, mean percentage of viable cells from three experiments, with each treatment done in multiple wells; *bars*, SE. \*,  $P < 0.001$ , compared with the control (0  $\mu\text{mol/L}$ ) group. *C*, effect of fisetin on R1881-induced cell growth. As detailed in Materials and Methods, LNCaP cells were treated with R1881 (1 nmol/L), Casodex (10–7 mol/L), and the combination of R1881 (1 nmol/L) and fisetin (10–60  $\mu\text{mol/L}$ ) for 48 h, and cell viability was determined by the MTT assay. *Columns*, mean percentage of cell viability from these experiments, with each treatment done in multiple wells; *bars*, SE. \*,  $P < 0.001$ , compared with control (0  $\mu\text{mol/L}$ ); §,  $P < 0.001$ , compared with R1881. *D*, fisetin competes with DHT and physically interacts with the LBD of AR. *Points*, average of two sample wells; *bars*, SE. The curve was fit using a sigmoidal dose-response equation with varying slope using Prism software from GraphPad Software, Inc.

penicillin/streptomycin. All cells were maintained under standard cell culture conditions at 37°C and 5% CO<sub>2</sub> environment. Fisetin dissolved in DMSO was used for the treatment of cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. The cells (60–70% confluent) were treated with fisetin (10–60  $\mu\text{mol/L}$ ) for 48 h in complete growth medium.

**Cell viability assay.** The effect of fisetin on cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) assay. For the second set of experiments, cells were treated with agonistic ligand R1881 (1 nmol/L), Casodex (0.1  $\mu\text{mol/L}$ ), and the combination of R1881 (1 nmol/L) with fisetin (10–60  $\mu\text{mol/L}$ ) for 48 h. After incubation for specified times at 37°C, MTT assay was done as described previously (11).

**Time-resolved fluorescence resonance energy transfer assay.** The Invitrogen method to identify AR ligands was used to test the antiandrogenic potential of Fisetin. The binding assay, which was previously described (12), was followed to determine whether fisetin interacts with the LBD of AR. DHT was included as the control competitor. Briefly, in this assay the ligands for AR are identified by their ability to compete with and displace a strong-affinity AR ligand (called fluormone) from the receptor. A purified, glutathione *S*-transferase (GST)-tagged, AR-LBD (1 nmol/L) is indirectly labeled with a terbium-labeled anti-GST tag antibody (5 nmol/L). Binding of Fluormone AL Red (1  $\mu\text{mol/L}$  solution) to AR is then measured by monitoring fluorescence resonance energy transfer (FRET) from the terbium-labeled anti-GST tag antibody to the red fluorescent ligand, resulting in a high time-resolved FRET ratio (570 nm fluorescent emission/546 nm fluorescent emission). Competitors will displace Fluormone AL Red and disrupt FRET, resulting in a lower time-resolved FRET ratio.

Serial dilutions of DHT and fisetin were done in 100% DMSO at 100× the final screening concentration. Each dilution series was then diluted to 4× the final screening concentration in assay buffer (Invitrogen). To perform the assay, 5  $\mu\text{L}$  of 4× fisetin and DHT were dispensed in the 384-well assay plate. Following this, 5  $\mu\text{L}$  of 4 nmol/L AR-LBD were dispensed in the assay wells following by the addition of 10  $\mu\text{L}$  of 10 nmol/L terbium-anti-GST antibody/20 nmol/L Fluormone AL Red mixture. The plates were incubated for 2 h at room temperature. The plates were read using 546- and 570-nm emission intensities on Tecan Infinite F500 plate reader, following excitation at 340 nm. A 100- $\mu\text{s}$  integration time was used for the time-resolved measurement.

Three-fold recurrently increasing concentrations of reference competitor DHT (starting from 0.00069 to 10,000 nmol/L, 16 different concentrations) and fisetin (starting from 0.00696 to 10,000 nmol/L) were used to test for competition with a constant 10 nmol/L concentration of the labeled competitor Fluormone AL Red, a high-affinity AR ligand. This concentration of tracer corresponds to its  $K_D$  value for binding to our AR-LBD. Three data points (i.e., 100, 33, and 11  $\mu\text{mol/L}$ ) were excluded from data analysis due to compound interference. Compound interference is defined in these cases as >50% quenching of the terbium reference emission intensity measured at 546 nm.

**Western blotting.** Following the treatment of cells as described above, whole cell lysate was prepared and Western blotting was done as previously described (11).

**ELISA.** The human PSA ELISA kit was used for the quantitative determination of PSA levels in culture medium. LNCaP cells were treated

with fisetin (10–60  $\mu\text{mol/L}$ ) for 48 h and assayed by a PSA enzyme immunoassay as per manufacturer's recommendations.

**Apoptosis assessment by Annexin V staining.** The Annexin V-Fluorescein kit was used for the detection of apoptotic cells according to the vendor's protocol. LNCaP cells were treated with fisetin (10 and 20  $\mu\text{mol/L}$ ) and Casodex (0.1  $\mu\text{mol/L}$ ) for 48 h. The fluorescence was measured with a Zeiss 410 confocal microscope.

**AR protein stability assay.** LNCaP cells were treated with 40  $\mu\text{mol/L}$  fisetin and 50  $\mu\text{g/mL}$  cycloheximide for 0, 4, 8, 12, and 24 h, followed by the preparation of whole cell lysates. Cycloheximide was added to the media 30 min before the addition of fisetin. AR protein levels were determined by Western blot analysis with antibody specifically against AR and normalized to  $\beta$ -actin control.

**Reverse-transcription PCR.** Total RNA was isolated from the cells using a commercial RNeasy kit (Qiagen), and RNA concentration was measured spectrophotometrically at 260 nm. Total RNA (1  $\mu\text{g}$ ) was subjected to reverse-transcription PCR (RT-PCR) using Fail-Safe PCR premix kit. For PCR, specific primers for AR, PSA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used: AR (PCR product size, 201 bp), sense 5'-GCGCCAGCAGAAATGATTGCACTA-3' and antisense 5'-ACACTGTCAGCTTCTGGGTTGTCT-3'; PSA (PCR product size, 697 bp), sense 5'-ACACAGGCCAGGTATTTTCAGGTCA-3' and antisense 5'-AAATGCTCCAGAGCCGACTTCCA-3'; GAPDH (PCR product size, 911 bp), sense 5'-AAGTCCGAGTCAACGGATTTGGT-3' and antisense 5'-ACAAAGTGGTCGTTGAGGGCAATG-3'. PCR was done as follows: 4 min denaturation at 94°C, and repeating the cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; the number of cycles was specific for each primer set. PCR products were resolved in a 1.5% agarose gel containing ethidium bromide.

**Transient transfections and reporter assays.** Transient transfection for reporter assays was done using standard calcium phosphate method (13) with minor modifications. LNCaP cells were seeded in six-well dishes in DMEM supplemented with 10% hormone-depleted FBS and were transfected 16 h later with 1  $\mu\text{g}$  of MMTV-Luc, reporter plasmid, and 50 ng of Renilla luciferase plasmid (Promega). After 8 h, cells were given fresh media and treated with R1881 (1 nmol/L) and fisetin (10–60  $\mu\text{mol/L}$ ). Cells were harvested 72 h posttransfection and both luciferase and Renilla luciferase activities were measured using dual luciferase assay reagent kit from Promega.

For PSA-Luc reporter assay, transfection was done with AMAXA electroporation kit (Amaxa Biosystems) with 2  $\mu\text{g}$  of PSA-Luc reporter; after electroporation, cells were treated as above. For AR promoter luciferase assay, a similar procedure as described above was used except that cells were grown in normal 10% FBS-containing media and transfected with 1  $\mu\text{g}$  of pLARS luciferase reporter. For AR amino-terminal/carboxyl-terminal (N-C) interaction assay, CV1 cells were transfected with pSVARN1 (AR-N1) and/or pSVARC (AR-C) plasmid (250 ng each), 1  $\mu\text{g}$  MMTV-Luc, and 100 ng Renilla luciferase plasmid using standard calcium phosphate transfection methods and were treated as described above.

**In vivo tumor xenograft model.** Athymic (*nu/nu*) male nude mice (NxGen Biosciences) were housed under pathogen-free conditions with a 12-h light/12-h dark schedule and fed with an autoclaved diet *ad libitum*. We chose AR-positive CWR22Rv1 cells for determining the *in vivo* effects of fisetin based on the fact that these cells form rapid and reproducible tumors in nude mice and secrete significant amounts of PSA in the bloodstream of the host. CWR22Rv1 tumor xenografts were generated as described previously (14). Twenty-four animals were then randomly divided into two groups, with 8 animals in group 1 and 16 animals in group 2. The first group of animals received i.p. injection of DMSO (30  $\mu\text{L}$ ) and served as control. The animals of groups 2 received i.p. injection of fisetin (1 mg/animal) in 30  $\mu\text{L}$  of DMSO twice weekly. Tumor sizes were measured twice weekly as described previously (14, 15). All animals of group 1 and eight animals from group 2 were sacrificed when tumors reached a volume of 1,200  $\text{mm}^3$  in the control group. The remaining animals in group 2 were sacrificed when tumors reached a volume of 1,200  $\text{mm}^3$ . All procedures conducted were in accordance with the guidelines for the use and care of laboratory animals. Every week, blood samples were collected by the "mandibular bleed." The

sera were separated by centrifuging blood for 5 min at 4°C and then stored at –20°C until assayed for secreted PSA.

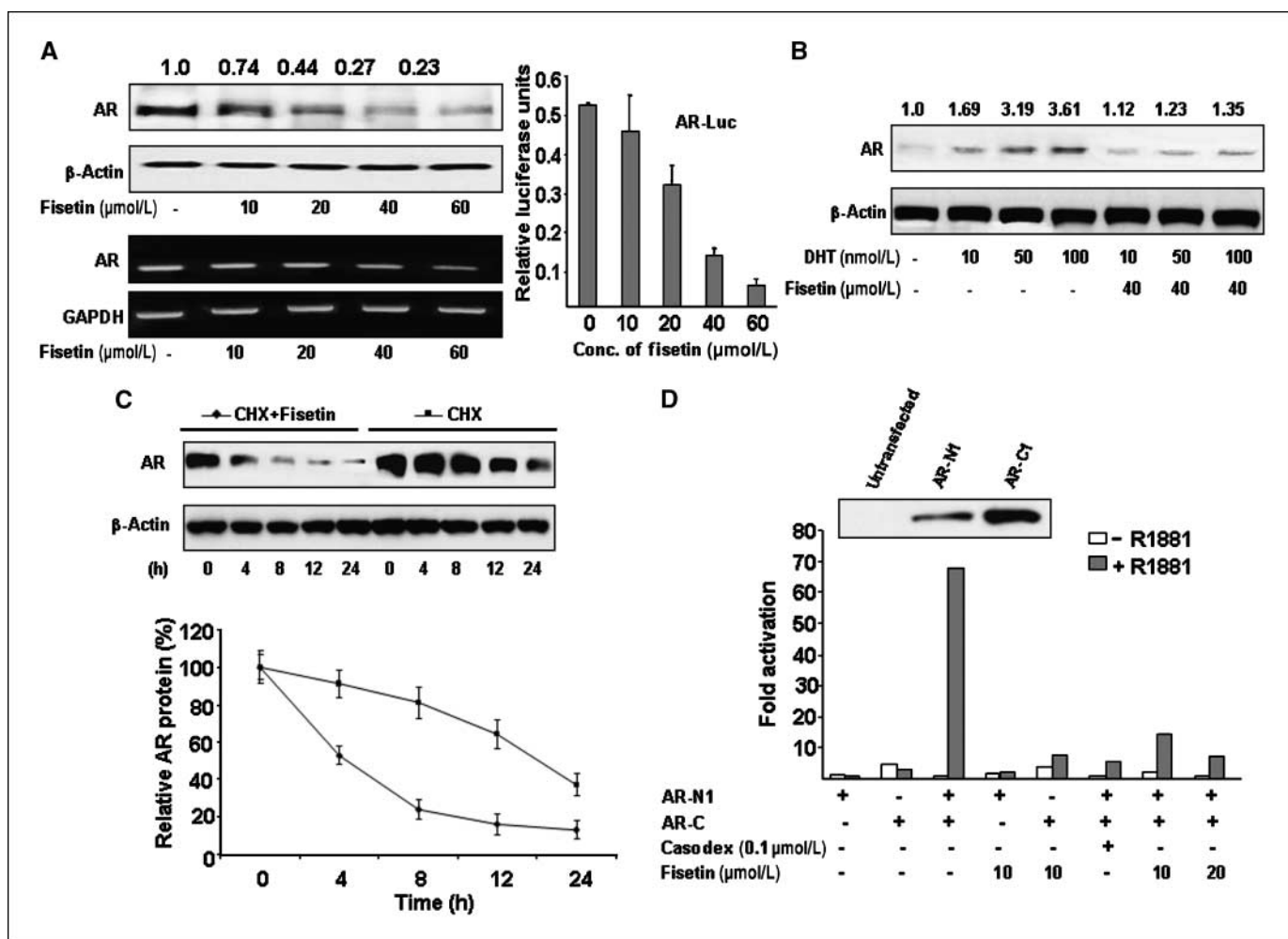
**Statistical analysis.** Results were analyzed using a two-tailed Student's *t* test to assess statistical significance, and  $P < 0.05$  was considered significant.

## Results

**Fisetin inhibits PCa cell growth and competes with high-affinity androgen to interact with the LBD of AR.** We first determined the effect of treatment with fisetin on the growth of LNCaP, CWR22Rv1, and prostate epithelial cells by MTT assay. Treatment with fisetin (10–60  $\mu\text{mol/L}$ ) for 48 hours decreased cell viability in LNCaP cells (19%, 40%, 49%, and 62%) and CWR22Rv1 cells (18%, 34%, 44%, and 55%) but had minimal effect on prostate epithelial cells at these doses (Fig. 1B). Treatment of LNCaP cells with R1881 led to a 6.5-fold increase in cell growth. Cotreatment of cells with R1881 and either fisetin or Casodex decreased R1881-stimulated growth, suggesting that both Casodex and fisetin might act through a similar pathway (i.e., antagonizing agonist action to decrease cell growth). Interestingly, treatment of LNCaP cells with R1881 (1 nmol/L) and fisetin caused 55%, 76%, 88%, and 94% decrease in cell viability at 10, 20, 40, and 60  $\mu\text{mol/L}$  of fisetin, respectively (Fig. 1C). Because the growth of LNCaP cells depends on functional AR signaling, we next determined if fisetin competes with the AR ligands to inhibit AR function. To test this, fisetin was screened using the AR competitive binding assay in a time-resolved FRET assay. Here, ligands for AR are identified based on their ability to compete and displace a labeled androgen (Fluormone AL Red) from the LBD of AR. Interestingly, fisetin was found to displace Fluormone AL Red from the purified LBD of AR and disrupted FRET, resulting in a lower time-resolved FRET ratio, indicating that it competes with a high-affinity labeled AR ligand (fluormone) and physically interacts with the LBD of AR with an  $\text{IC}_{50}$  of 3.07  $\mu\text{mol/L}$  (Fig. 1D). The natural ligand DHT was used as a reference control.

**Fisetin represses AR expression and inhibits AR interdomain (N-C) interaction.** AR plays a critical role in the development of PCa, and it has been shown that an increased AR protein expression correlates with increase in PCa growth (16, 17). To determine whether fisetin has a negative effect on the expression of AR protein, we conducted Western blot analysis. We found that treatment of LNCaP cells with fisetin for 48 hours resulted in a marked decrease in the protein expression of AR (Fig. 2A, top left). To test whether the observed decrease in AR protein was due to decreased transcription of AR gene, we next evaluated modulation of AR expression by fisetin in LNCaP cells. Although at 10 and 20  $\mu\text{mol/L}$  doses of fisetin, only a marginal decrease in AR mRNA expression was observed, a profound decrease was observed at 40 and 60  $\mu\text{mol/L}$  doses of fisetin (Fig. 2A, bottom left), indicating that the observed decrease in AR protein levels is due, in part, to attenuation of AR gene transcription. Because gene transcription positively correlates with promoter activity, reporter assay using AR gene promoter tagged to luciferase reporter was done. The result indicated that the treatment of LNCaP cells with fisetin strongly decreased AR promoter activity at 40 and 60  $\mu\text{mol/L}$  doses (Fig. 2A, right), in line with mRNA expression data.

Because agonistic ligands such as DHT are known to stabilize AR protein in target cells, we tested whether fisetin, by antagonizing DHT function, could destabilize AR protein in LNCaP cells. LNCaP cells were grown in hormone-free, charcoal-stripped serum-containing media with 10, 50, and 100 nmol/L of DHT. Untreated



**Figure 2.** Effect of fisetin on protein and mRNA expression of AR and its interference with N-C interaction of AR. *A*, effect of fisetin on the protein expression (*top left*) and mRNA expression (*bottom left*) of AR as determined by RT-PCR in LNCaP cells. *Right*, LNCaP cells were transiently transfected with AR promoter reporter (pLARS) along with Renilla luciferase and treated with solvent (DMSO) and fisetin (10–60 μmol/L) for 48 h with or without R1881 (1 nmol/L). *Columns*, average relative luciferase values (normalized to Renilla luciferase) from triplicate experiments; *bars*, SE. *B*, effect of fisetin on DHT-stimulated protein expression of AR in LNCaP cells. The cells were treated with 40 μmol/L fisetin for 48 h and then harvested. Cells were grown in 10% FBS, charcoal-stripped serum with 10, 50, and 100 nmol/L of DHT with or without fisetin (40 μmol/L) for 48 h. The details are described in Materials and Methods. Total cell lysates were prepared and 40 μg of protein were subjected to SDS-PAGE followed by Western blot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for β-actin. The Western blots shown are representative of three independent experiments with similar results. The values above the figures represent relative density of the bands normalized to β-actin. *C*, effect of fisetin on AR protein turnover in LNCaP cells. The cells were treated with 40 μmol/L fisetin and 50 μg/mL cycloheximide for the indicated time periods, followed by the preparation of whole cell lysates. Cycloheximide was added to the media 30 min before the addition of fisetin. AR protein levels were determined by Western blot analysis with antibody specifically against AR and normalized to β-actin control. Western blots were quantified by the relative densities of the bands and the relative percentage of AR protein was plotted against hours after cycloheximide treatment; *bars*, SD. *D*, AR N-C interaction assay was done on CV1 cells as described in Materials and Methods. Eight hours after transfection, cells were washed, given fresh media, and treated with R1881 (1 nmol/L) and/or Casodex (10–7 mol/L) along with 10 and 20 μmol/L of fisetin for 48 h; after which cells were lysed and luciferase activity was measured. The graphs represent the fold hormone induction compared with non-hormone-treated group, which was set as 1.

LNCaP cells exhibited very low AR protein amounts. As expected, cells treated with DHT exhibited enhanced protein expression of AR (Fig. 2B). Cotreatment, however, with DHT and fisetin (40 μmol/L) for 48 hours resulted in decreased AR protein expression, indicating that fisetin functionally antagonizes DHT-mediated AR stabilization. To further test the effect of fisetin binding on AR stability, a pulse chase experiment using a translation inhibitor, cycloheximide, was done. Treatment of LNCaP cells with cycloheximide led to a time-dependent decrease in AR protein expression (Fig. 2C). Interestingly, however, cotreatment of cells with cycloheximide along with fisetin further accelerated the rate of AR decay, indicating that by binding to the LBD, fisetin destabilizes AR protein levels thereby leading to accelerated decrease in its

levels. In cells treated with fisetin + cycloheximide, the half-life of AR protein was reduced from 18 hours to 4 hours and 30 minutes in cycloheximide alone-treated cells, suggesting that AR protein degradation was greatly enhanced in the presence of fisetin.

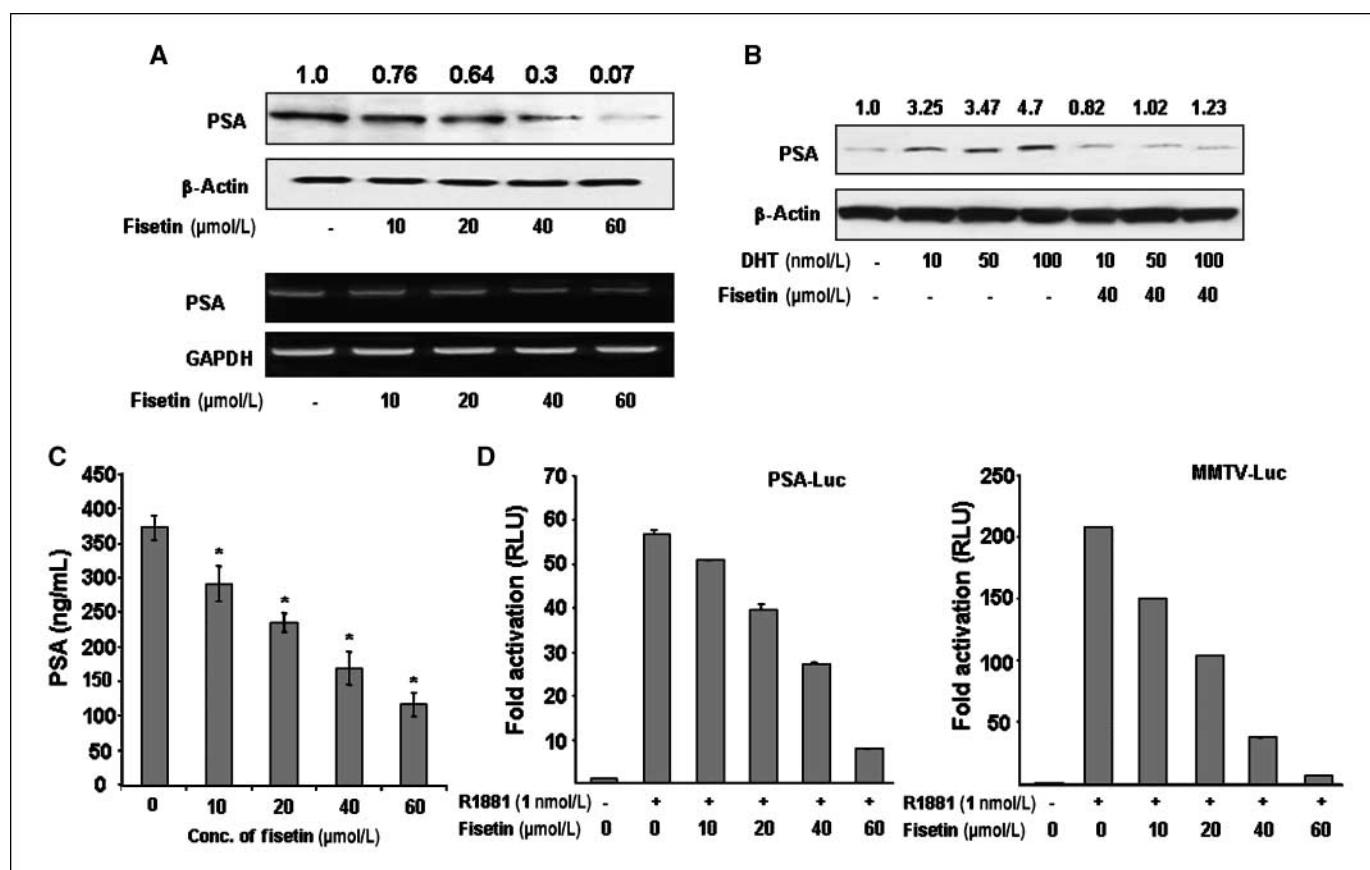
Agonist binding to the LBD of AR leads to N-C interaction between the amino-terminal domain and the carboxyl-terminal LBD of AR (18). This interaction is required not only for optimal or maximal activation of AR but also for AR protein stability. The high-affinity natural ligands such as DHT and the synthetic androgen R1881 at nanomolar concentration induced N-C interaction in mammalian two-hybrid assays, which contributes to stabilization of AR protein (19). AR antagonists inhibit the agonist-induced N-C interaction. To provide a mechanistic insight

into fisetin action, we determined how it modulates AR N-C interaction. We performed transient transfection by transfecting either AR-N1, which codes for the amino terminus of AR, or AR-C, which codes for the DNA binding domain and the carboxyl terminus LBD, or both in combination in functional AR-negative CV1 mammalian cells, as described in Materials and Methods. Both of these constructs were efficiently expressed in CV1 cells (Fig. 2D). Here, the reconstitution of functional receptor strictly depends on the N-C interaction of AR. The results indicate that either the amino-terminal or the carboxyl-terminal constructs alone were incapable of driving the expression of the reporter with or without R1881; cotransfection, however, of AR-N1 and AR-C led to reconstitution of the active receptor, which results in strong transactivation of the reporter in the presence of nanomolar amounts of R1881 (Fig. 2D). Cotreatment of fisetin at both 10 and 20  $\mu\text{mol/L}$  resulted in drastic decrease in this interaction, suggesting that fisetin acts as a potent antagonist by interfering with the N-C interaction. Casodex, which was used as a positive control, also inhibited this interaction. To perform the N-C interaction assay, a nanomolar dose of agonist was used because higher concentration of agonist results in nonspecific inhibition of cell growth and

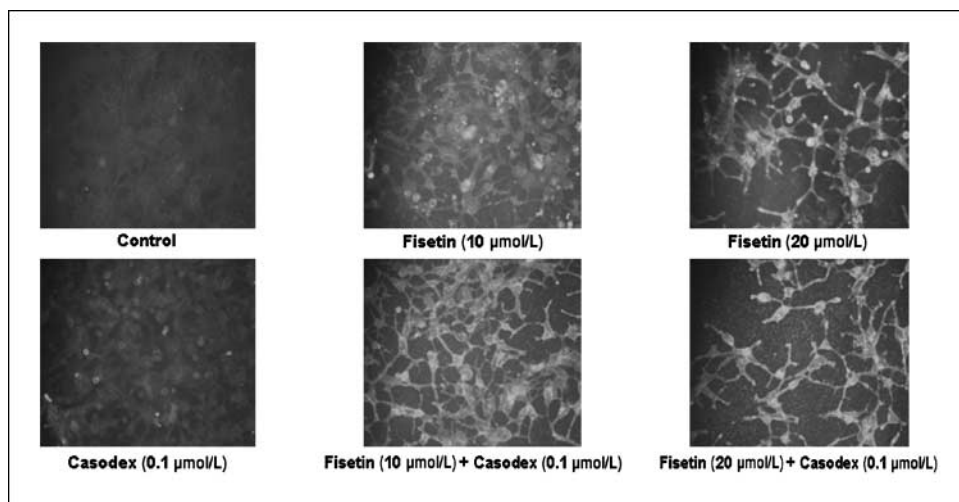
therefore could not serve as a true reflective to evaluate the antagonistic action of fisetin.

**Fisetin inhibits the protein and mRNA expression of AR target genes.** The expression of PSA is regulated by AR signaling. Studies indicate that the serum PSA level correlates directly with advancing clinical and pathologic disease stage (20). Therefore, PSA has been extensively used as the most reliable biomarker to screen for PCa and is also used as a surrogate marker to monitor response to antiandrogen therapy (21). We found that there was a dose-dependent decrease in the protein expression of PSA in LNCaP cells following treatment with fisetin for 48 hours (Fig. 3A, top). Treatment of LNCaP cells with fisetin also caused dose-dependent decrease in the mRNA levels of PSA as shown by RT-PCR, indicating that the decreased PSA transcription is due to the decrease in AR transactivation function (Fig. 3A, bottom). Treatment of cells with DHT led to an increase in PSA protein expression (Fig. 3B); however, cotreatment of DHT-stimulated cells with fisetin (40  $\mu\text{mol/L}$ ) for 48 hours caused reduction in the increased levels of PSA in LNCaP cells.

We further determined the effect of fisetin on secreted levels of PSA in LNCaP cells. With the use of ELISA, we found that in LNCaP



**Figure 3.** Effects of fisetin on protein and mRNA expression of PSA and AR-regulated reporter genes. *A*, effect of fisetin on the protein expression (top) and mRNA expression (bottom) of PSA as determined by RT-PCR in LNCaP cells. *B*, effect of fisetin on DHT-stimulated protein expression of PSA in LNCaP cells. The cells were treated with 40  $\mu\text{mol/L}$  fisetin for 48 h and then harvested. Cells were grown in 10% FBS, charcoal-stripped serum with 10, 50, and 100 nmol/L of DHT with or without fisetin (40  $\mu\text{mol/L}$ ) for 48 h. The details are described in Materials and Methods. Total cell lysates were prepared and 40  $\mu\text{g}$  protein was subjected to SDS-PAGE followed by Western blot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the Western blots and reprobing them for  $\beta$ -actin. Representative Western blots of three independent experiments with similar results. The values above the figures represent relative density of the bands normalized to  $\beta$ -actin. *C*, effect of fisetin on secreted levels of PSA in LNCaP cells. The cells were treated with fisetin (10–60  $\mu\text{mol/L}$ ) for 48 h and then harvested. The PSA levels were determined by ELISA. The details are described in Materials and Methods; the figures represent the data of three experiments, each conducted in duplicate. \*,  $P < 0.001$ , compared with the control (0  $\mu\text{mol/L}$ ) group. *D*, LNCaP cells transiently transfected with either PSA-Luc (left) or MMTV-Luc (right) reporters were treated 48 h posttransfection with or without 1 nmol/L R1881 and with fisetin (10–60  $\mu\text{mol/L}$ ). The graphs represent the fold hormone induction compared with non-hormone-treated group, which was set as 1.



**Figure 4.** Effect of fisetin on induction of apoptosis. LNCaP cells were treated with fisetin (10 and 20  $\mu\text{mol/L}$ ) and/or Casodex (10–7  $\text{mol/L}$ ) for 48 h. The fluorescence was measured with a Zeiss 410 confocal microscope. The details are described in Materials and Methods. Data are from one representative experiment repeated twice with similar results; magnification,  $\times 400$ .

cells there was dose-dependent decrease in the secreted levels of PSA by 22%, 37%, 58%, and 73% at 10, 20, 40, and 60  $\mu\text{mol/L}$  concentrations of fisetin, respectively (Fig. 3C). Because the growth of LNCaP cells depends on functional AR signaling, the observed inhibitory effects of fisetin on cell growth are largely due to inhibition of AR transactivation and thereby decrease in target gene expression as reflected in decreased PSA expression and secretion.

To further analyze the effect of decreased AR transactivation on target gene expression, transient transfections using two different androgen responsive reporters, PSA-Luc (Fig. 3D, left) and MMTV-Luc (Fig. 3D, right), were carried out. Treatment of LNCaP cells with agonist R1881 led to induction of both the reporters in LNCaP cells, indicating the presence of functionally active endogenous AR. Cotreatment of cells with agonist R1881 and with increasing concentration of fisetin led to a dose-dependent decrease in AR transactivation as reflected in a decrease in the reporter activity. Taken together, these results suggest that fisetin interferes with the hormone-induced transactivation function of AR in LNCaP cells, which leads to a decrease in target gene transcription as reflected in decreases in PSA mRNA and protein levels.

**Fisetin potentiates and synergizes with antiandrogen Casodex to induce apoptosis.** Because Casodex is used for clinical management of PCa, we tested whether fisetin could be used as an adjuvant along with Casodex to improve its therapeutic benefit. Annexin V/propidium iodide staining was done following fisetin treatment on LNCaP cells. When LNCaP cells were stained with Annexin V and examined under a fluorescence microscope, apoptotic cells were found to be increased in fisetin-treated cells. Treatment with specific androgen antagonist Casodex also led to an induction of apoptosis, which was further increased when cells were treated with the combination of Casodex and fisetin (10 and 20  $\mu\text{mol/L}$ ) for 48 hours (Fig. 4), indicating a synergy in their action to promote apoptosis in PCa cells.

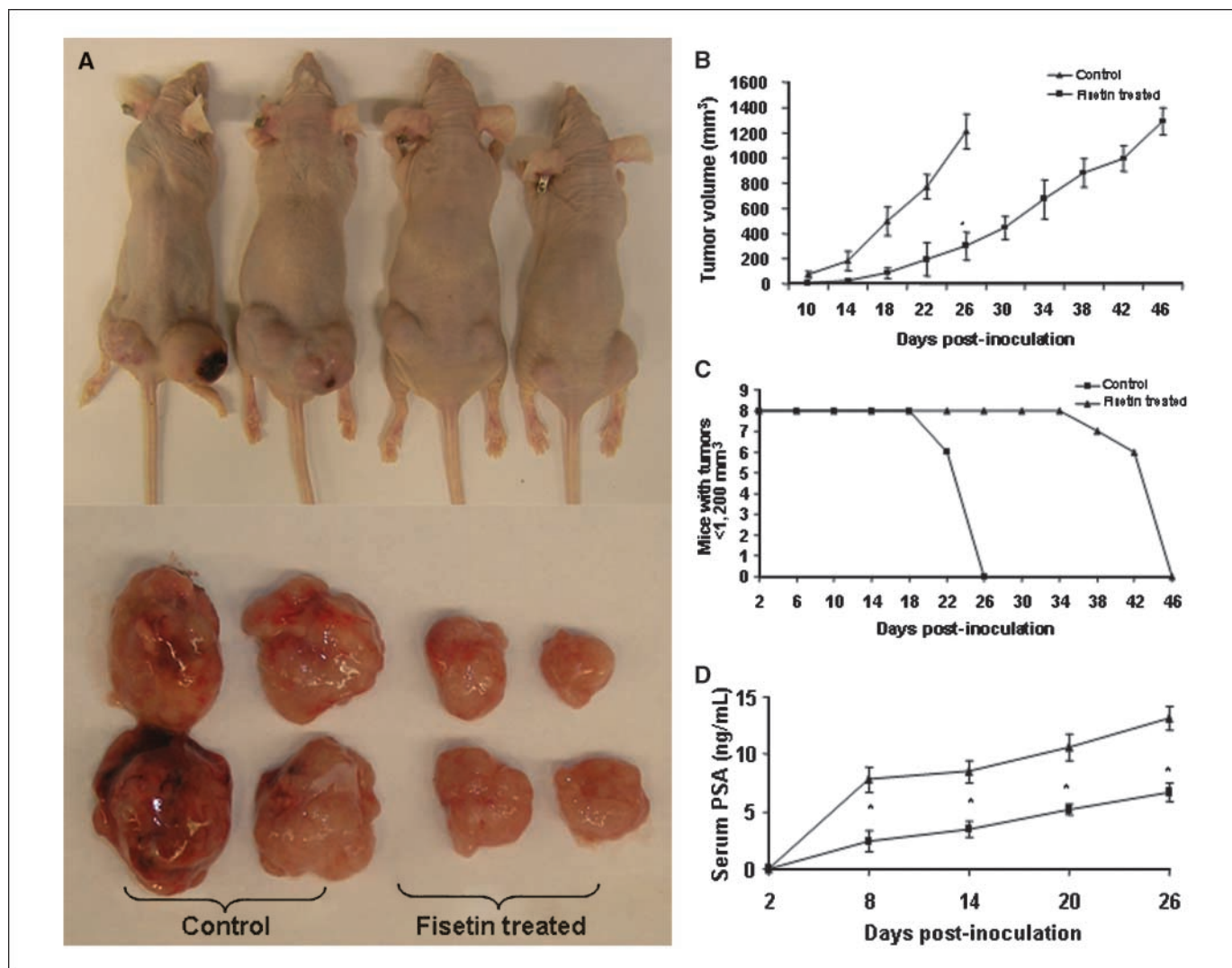
**Fisetin inhibits the growth of human prostate carcinoma CWR22Rv1 cells and PSA secretion in athymic nude mice.** The treatment of athymic nude mice with fisetin resulted in inhibition of AR-positive CWR22Rv1 tumor xenograft growth. The appearance of small solid tumors was observed in animals of the control group receiving DMSO 10 days after cell inoculation. This latency period was prolonged to 18 days in animals receiving fisetin day 1

after tumor cell implantation. There was significant reduction in growth of prostate tumors in fisetin-treated animals as compared with the control group (Fig. 5A). As depicted in Fig. 5B, tumor growth, as inferred by computed tumor volume, was significantly inhibited in mice receiving fisetin. In the control group, an average tumor volume of 1,200  $\text{mm}^3$  was reached in 26 days after tumor cell inoculation. At this time point, the average tumor volume in the fisetin-treated group was 302  $\text{mm}^3$ . An average tumor volume of 1,200  $\text{mm}^3$  in the fisetin-treated group was achieved in 46 days after tumor cell inoculation (Fig. 5C). Tumor data were analyzed for survival probability by Kaplan-Meier analysis, which indicated that fisetin treatment of prostate tumor-bearing athymic nude mice resulted in increased survival ( $P < 0.0001$ , log-rank test), with a median survival of 46 days (fisetin treated), compared with 26 days in control mice ( $P < 0.0001$ , log-rank test; data not shown).

During the course of tumor growth in animals at days 2, 8, 14, 20, and 26 after inoculation, blood was collected through the mandibular bleed. Quantitative sandwich ELISA was used to determine circulating PSA levels in mouse serum secreted by CWR22Rv1 tumor xenografts. There was significant inhibition of PSA secretion in serum of mice treated with fisetin at all time points examined (Fig. 5D). At 20 days after inoculation, secreted PSA levels were  $10.6 \pm 1.14$   $\text{ng/mL}$  in the control group and  $2.5 \pm 0.58$   $\text{ng/mL}$  in the fisetin-treated group. At 26 days after inoculation, secreted PSA levels were  $13.2 \pm 1.04$  and  $6.7 \pm 0.81$   $\text{ng/mL}$  in the control and fisetin-treated groups, respectively (Fig. 5D). Hence, our results show that treatment of mice with fisetin caused a significant decrease in the serum PSA of animals.

## Discussion

The normal development and maintenance of the prostate is dependent on androgen acting through the AR. The AR remains crucial in the development and functions of the normal prostate and progression of PCa (21–23). AR expression is maintained throughout PCa progression, and the majority of androgen-independent or hormone-refractory PCa express AR. Despite the fact that PCa cells generally become androgen independent, they still depend on functional AR signaling, which is often overexpressed in hormone-refractory PCa, for growth (23), and decreasing AR levels impair their growth (24). This suggests that finding new



**Figure 5.** Effect of fisetin on CWR22Rv1 tumor growth and PSA secretion in athymic nude mice. Twenty-four animals were then randomly divided into 8 animals in group 1 and 16 animals in group 2. Approximately 1 million CWR22Rv1 cells were s.c. injected in each flank of mouse to initiate tumor growth. Twenty-four hours after cell implantation, the eight animals of the first group of animals received i.p. injection of DMSO (30  $\mu$ L) and served as control. The 16 animals of group 2 received i.p. injection of fisetin (1 mg/animal) in 30  $\mu$ L of DMSO twice weekly. Once tumors started to grow, their sizes were measured twice weekly and the tumor volume was calculated. *A*, top, photographs of representative mice with tumors from each group. Bottom, photographs of excised tumors from each group. *B*, average tumor volume of control and fisetin-treated mice plotted over days after tumor cell inoculation. Points, mean of 16 tumors in eight animals; bars, SD. \*,  $P < 0.001$ , versus the control group. *C*, number of mice remaining with tumor volumes of  $\leq 1,200 \text{ mm}^3$  after they received treatment with fisetin for the indicated days. *D*, serum PSA levels were analyzed by ELISA. Points, mean of eight animals; bars, SE. \*,  $P < 0.001$ , versus the control group. Details are described in Materials and Methods.

ways to interfere with the expression and function of AR could dramatically improve the benefits of therapeutic intervention used for the treatment of PCa.

The substantial mortality and morbidity associated with PCa and its treatments have led some medical professionals to shift attention away from diagnosis and treatment and instead focus on prevention. Recently, nuclear factor- $\kappa$ B-regulated cell proliferation was found to be inhibited by fisetin (25). It has also been reported to inhibit epidermal growth factor-induced cell transformation of mouse epidermal JB6 Cl 41 cells (26).

Here for the first time we report that fisetin, a novel dietary flavonoid, inhibits the growth of androgen-dependent PCa cells. More importantly, the advocacy for fisetin to be used for chemoprevention is strengthened from our observation that fisetin only had minimal effect on the growth of prostate epithelial cells (Fig. 1B). Fisetin antagonized androgen action and resulted in

dose-dependent reduction in cell viability. We used androgen agonist R1881 whose nanomolar concentration accelerates the growth of LNCaP cells; combinatorial treatment of LNCaP cells with R1881 and fisetin represses hormone-induced growth of the cells, indicating that fisetin functionally antagonizes androgen function by modulating AR function (Fig. 1C).

Because fisetin decreases the growth of LNCaP cells, which depend on functional AR signaling, we tested and found that fisetin competes with a high-affinity AR ligand and physically interacts with the LBD of AR with an effective  $IC_{50}$  of 3.07  $\mu$ mol/L (Fig. 1D).

In this study, we found dose-dependent decreased expression of AR protein and down-regulation of AR mRNA expression in LNCaP cells on treatment with fisetin (Fig. 2A). AR promoter activity, expressed in luciferase units, was decreased dose-dependently in LNCaP cells on treatment with fisetin, suggesting that fisetin at higher doses indeed modulated the expression of AR gene in

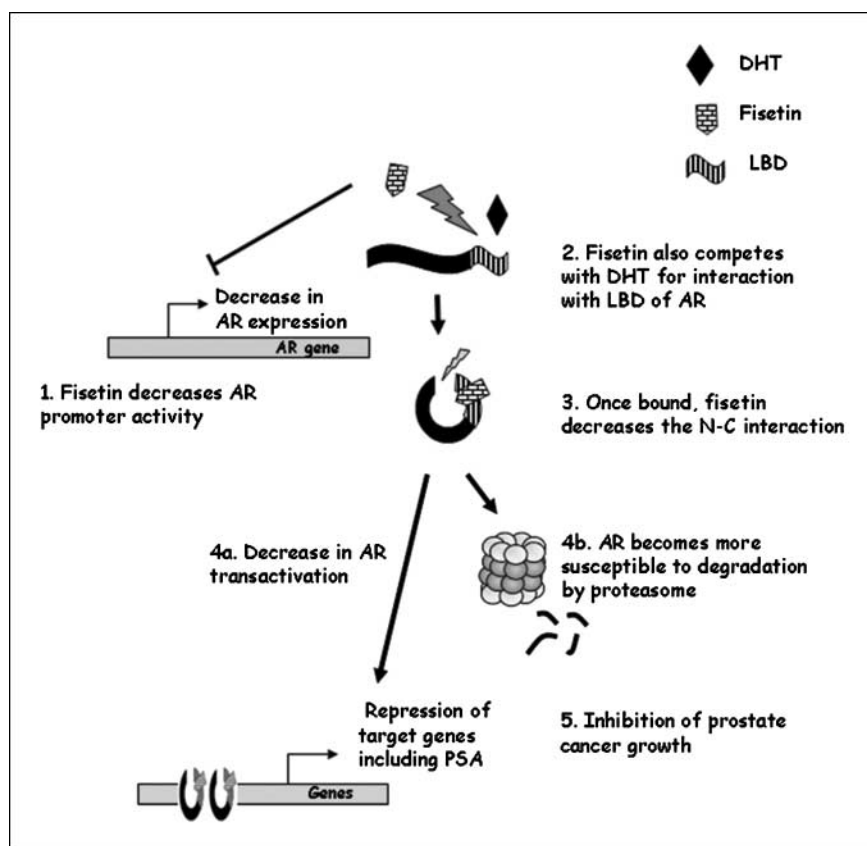
hormone-responsive LNCaP cells presumably by attenuating trans-acting factors required for AR promoter activation (Fig. 2A). In fact, our previous observations suggest that AR gene transcription is not induced by DHT in these cells. In addition, the AR-Luc reporter is not activated by agonist (data not shown), indicating that the observed decrease in AR transcription could result from the decreased access of the trans-acting ancillary factors such as Sp1, and therefore indicates that the antagonistic action of fisetin is not required to repress AR mRNA levels. We also determined the effect of fisetin treatment (40  $\mu\text{mol/L}$ ) on expression of AR protein in DHT-stimulated LNCaP cells. There was increased protein expression of AR with increasing concentration of DHT, which was significantly reduced with 40  $\mu\text{mol/L}$  concentration of fisetin in LNCaP cells (Fig. 2B). Treatment of cells grown in hormone-depleted media with DHT resulted in an increase in AR protein expression. It is known that this increase is not attributed to the increase in AR transcription because in these cells the autoregulation of AR transcription by DHT is not observed; therefore, the observed increase in AR protein expression is due to DHT-mediated stabilization of AR. Further, cotreatment of DHT treated cells with fisetin decreased AR protein expression, which could presumably be, in part, due to a decrease in its stability in addition to a decrease in AR gene transcription by fisetin. Taken together, our data suggest that the effects on cell growth and AR target gene expression are due to the antagonistic action of fisetin (Fig. 2B).

Hence, there exists a possibility that fisetin, by antagonizing AR function through physical interaction with its LBD, could result in the decrease in AR stabilization and hence decrease in its expression. To test the possible mechanism of fisetin-mediated decrease in AR, the pulse chase experiment suggested that fisetin

interaction with AR resulted in its accelerated decay in the absence of protein synthesis (Fig. 2C). Hence, fisetin-mediated decrease in AR protein expression seems to be primarily due to a decrease in AR promoter activity, interference with N-C interaction, and most importantly, its accelerated decay and thereby decrease in trans-activation and protein stability (Fig. 2D) as evident from the decrease in PSA expression and secretion and decrease in tumor growth.

Fisetin caused significant decrease in the protein expression of PSA and down-regulation of PSA mRNA expression in LNCaP cells (Fig. 3A). Treatment with fisetin also caused a decrease in protein expression of PSA in DHT-stimulated cells, reflecting its antagonistic nature (Fig. 3B), and down-regulation of secreted PSA levels in the culture medium (Fig. 3C). Moreover, treatment of LNCaP cells with R1881 caused induction of androgen responsive reporters PSA-Luc (Fig. 3D, left) and MMTV-Luc (Fig. 3D, right), whereas treatment with fisetin led to a dose-dependent decrease in hormone induction. These results indicated that fisetin also decreased the activation of these reporters in a concentration-dependent manner, suggesting the functional attenuation of AR transactivation with decrease in its protein level. In addition, this suggests that these reporters are indeed activated by the hormone R1881, and cotreatment with fisetin dose-dependently represses hormone-induced reporter induction, which further reflects its androgen antagonistic function.

In this study, treatment of LNCaP cells with Casodex caused induction of apoptosis, which was accelerated when cells were treated with the combination of Casodex (0.1  $\mu\text{mol/L}$ ) and 10 and 20  $\mu\text{mol/L}$  of fisetin for 48 hours (Fig. 4). This indicates that fisetin could serve as a more effective antagonist by inhibiting AR



**Figure 6.** Summary of the mechanism of fisetin action in prostate cancer cells. Fisetin decreases AR promoter activity leading to decreased AR expression. In addition, fisetin competes with natural AR agonist DHT to physically interact with the expressed AR protein. Once bound, it decreases the interdomain N-C interaction of AR leading to (a) a decrease in AR stabilization and (b) a decrease in AR transactivation function. This results in decreased expression of AR target genes and, hence, negatively influences the growth of PCa cells *in vitro* and *in vivo*.



signaling at multiple levels, in contrast to Casodex, which serves as an androgen antagonist. However, further studies are required to confirm that fisetin acts as an AR antagonist.

Treatment with fisetin significantly slowed the progression of CWR22Rv1 tumor growth in nude mice (Fig. 5A–C). Importantly, this tumor growth inhibition followed a significant decrease in the serum levels of PSA, a clinical diagnostic serum marker for monitoring the presence and progression of PCa in human patients (Fig. 5D), indicating an AR-specific effect.

The present study is the first report showing the effect of fisetin, a naturally occurring diet-based flavonoid, in inhibiting human prostate carcinoma cell growth in *in vitro* and *in vivo* preclinical models. We have also shown that fisetin effectively inhibits AR transactivation function and inhibits its expression presumably because it interferes with the N-C interdomain interaction of AR

and it competes with the natural ligand DHT to bind to the LBD of AR (Fig. 6). We suggest that fisetin could be developed as a chemotherapeutic agent against PCa.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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