

Repression of Cell Proliferation and Androgen Receptor Activity in Prostate Cancer Cells by 2'-Hydroxyflavanone

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Abstract. *Background: Prevention of the development of castration-resistant from hormone-naïve prostate cancer is an important issue in maintaining the quality of life of the patients. We explored the effect of 2'-hydroxyflavanone on proliferation and androgen responsiveness using prostate cancer cell lines. Materials and Methods: To investigate the effect of 2'-hydroxyflavanone on proliferation, prostate cancer cells were treated with 2'-hydroxyflavanone. Androgen-responsiveness in LNCaP cells was confirmed by luciferase assay after transfection of luciferase reporter driven by prostate specific antigen promoter. To detect androgen receptor (AR) expression, reverse transcriptase polymerase chain reaction and western blot analysis were conducted. Results: 2'-Hydroxyflavanone inhibited the proliferation of PC-3 and DU145 cells by induction of apoptosis. 2'-Hydroxyflavanone inhibited the proliferation of LNCaP cells stimulated by androgens and attenuated androgen-responsiveness through down-regulation of AR protein. Conclusion: 2'-Hydroxyflavanone not only inhibited proliferation of prostate cancer cells, but also repressed androgen-responsiveness, suggesting that it might be an useful agent in preventing recurrence of prostate cancer.*

Since the androgen receptor (AR) axis is the main route for development and progression of prostate cancer (PCa), androgen-deprivation therapy (ADT) is conducted as a first-

line hormonal therapy using medical castration, such as luteinizing hormone releasing hormone (LH-RH) agonists, LH-RH antagonist, and antiandrogen for advanced PCa. After an initial response to ADT, however, PCa eventually loses responsiveness to ADT and progresses into what is termed castration-resistant PCa (CRPC).

The AR axis also plays an important part in the process when hormone-sensitive PCa become CRPC. Although serum testosterone decreases to less than 5% before starting ADT, PCa adapts to low serum testosterone level by several mechanisms. One other important factor is the adrenal androgen, dehydroepiandrosterone (DHEA). DHEA is metabolized into testosterone, and then converted to dihydrotestosterone (DHT) by 5 α -reductase in PCa tissue, which then activates the AR. In fact, the concentration of DHT in PCa tissue remains 20 to 40% of pretreatment values (1-3). Interaction of epithelial and stromal cells plays an important role in the production of DHT in PCa tissue. After castration, adrenal androgen DHEA is metabolized into DHT in stromal cells and epithelial cells coordinately (4). Moreover, CYP17A inhibitors, abiraterone acetate and TAK-700, which inhibit conversion from pregnenolone into DHEA, is effective for more than 70% of CRPC after docetaxel-treatment. These results indicate that the AR axis affects the recurrence of PCs even in patients resistant to docetaxel.

Important enzyme in intratumoral androgen synthesis mediating through interaction of epithelial and stromal cells are type 3 and type 5 17 β -hydroxysteroid dehydrogenase (HSD17B3 and HSD17B5) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD). In particular, HSD17B3 catalyzes the formation of testosterone from 4-androstenedione (adione) in the testis and peripheral tissues (5). However, the function of the testes is lost in PCa after ADT, the main androgen synthesis enzyme in CRPC is HSD17B5. It is known that AKR1C3 aldo-keto reductase acts as a HSD17B5 (6, 7).

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AKR1C3 was found to be up-regulated in patients with PCa, especially, in those with metastatic PCa and CRPC (8-11). Moreover, overexpression of AKR1C3 promoted PCa proliferation (12). These findings suggest that hyperactivation of AKR1C3 might affect the recurrence of PCa.

Flavonoids are a large group of polyphenolic compounds present in foods and beverages of plant origin, and are subdivided into six subclasses: flavonols, flavones, flavanones, flavan-3-ols, anthocyanidins, and isoflavones (13). Flavonoids display a broad range of pharmacological activity, such as antioxidative, anti-inflammatory, and antiproliferative activities (13-15). Flavonoids have been shown to inhibit AKR1C3 activity *in vitro* (16, 17). 2'-Hydroxyflavanone (2'HF), which is a flavanones in particular had a strong inhibitory effect on AKR1C3 *in vitro* (16).

In the present study, we investigated the effect of 2'HF on proliferation and androgen responsiveness using PCa cell lines.

Materials and Methods

Cell lines and cell proliferation assay. LNCaP and DU145 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin/streptomycin (P/S; Invitrogen, Carlsbad, CA, USA) and 5% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), respectively. PC-3 cells (ATCC) were cultured in RPMI -1640 supplemented with 1% P/S (Invitrogen) and 5% FBS. Twenty-four hours after plating at a density of 5×10^4 cells onto 12-well plates with DMEM-5% charcoal-stripped fetal calf serum (CCS; Thermo Scientific HyClone, UK), cells were treated with ethanol, adione, testosterone, DHT and/or 2'HF in DMEM-5% CCS and the media were changed every two days. In each experiment, cells were harvested and the numbers of the cells were counted in triplicate using a hemocytometer. The data shown represent the means \pm SD of three replicates.

Reverse transcriptase polymerase chain reaction (RT-PCR) and western blot analysis. For RT-PCR, 24 hours after plating at a density of 1×10^5 cells onto 6-well plates with DMEM-5% CCS, cells were treated with or without Adione, DHT and/or 2'HF for 24 hours and total RNA was extracted. Total RNA extraction from cells and RT-PCR for *AR*, *prostate specific antigen (PSA)*, and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was performed as described previously (2, 4).

For western blot analysis, total protein was extracted from cells as described previously (18). Protein was quantified according to the method of Bradford, and equal amounts of protein were electrophoresed on a 10% or 12.5% Ready Gel J (Bio-Rad, Hercules, CA, USA). Membranes were incubated with mouse monoclonal antibody against AR (NH27) (19) and GAPDH (Novus Biologicals, Littleton, CO, USA). Horseradish peroxidase-conjugated secondary antibody against mouse monoclonal or rabbit monoclonal antibody was used and protein bands were visualized and quantitated with chemiluminescent reagent (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA) and ChemiDoc XRS (Bio-Rad).

Recombinant plasmid constructs. Recombinant plasmid pEGFP-fAR that expresses full-length wild-type AR fused with green fluorescent protein (GFP) was constructed by inserting the full-length *AR* cDNA of pSGAR2, which is driven by SV40 promoter (19) (at -24 to 3110 bp of start codon), into pEGFP-C1 (Invitrogen, CA, USA). The insert configurations of fAR cDNAs were confirmed by sequence analysis.

Luciferase assay. To evaluate *AR* transcriptional activity, 24 hours after plating 5×10^4 cells on 12-well plates in DMEM-5% CCS, LNCaP and PC-3 cells were transfected using Lipofectamine transfection reaction (Invitrogen) using 0.5 μ g of luciferase reporter plasmid, pGL3PSAp-5.8, driven by a 5.8 kb *PSA* promoter (20). Twenty-four hours after transfection, cells were treated by the addition of DHT with and without 2'HF for 24 hours. After treated cells were harvested, cells were lysed in luciferase lysis buffer (Promega, Madison, WI, USA) and the luciferase activity was quantitated by a luminometer. For overexpression of EGFP-fAR in LNCaP cells, 5×10^4 LNCaP cells were co-transfected with 0.1 μ g of pEGFP-fAR and 0.4 μ g of pGL3PSAp-5.8, and then cells were further treated with adione, DHT and/or 2'HF for 24 hours.

Apoptosis assay. To investigate whether 2'HF causes PCa cells to undergo apoptosis, the Annexin-V-FLUOS Staining kit (Roche, Mannheim, Germany) was used according to the manufacture's protocol. In brief, 1×10^5 LNCaP, and DU145 cells, and 5×10^4 PC-3 cells were seeded in 6-well plates with DMEM-5% CCS. They were treated with 10 μ M 2'HF for 72 hours. After removing the media and washing by PBS, cells were incubated with 100 μ l Annexin-V-FLUOS labeling solution added with propidium iodide for 15 min at room temperature. The stained cells were analyzed by fluorescence microscope, FSX100 (Olympus, Tokyo, Japan).

Liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS). After plating 5×10^4 cells on 12-well plates in DMEM-5% CCS, PC-3 cells were treated with 10 nM adione or 10 nM testosterone in the absence and presence of 10 μ M 2'HF. Then the media were collected 24 hours later. The concentration of adione, testosterone, and DHT in media was measured by LC-MS/MS (Division of Pharmacological Research, Aska Pharma Medical Co. Ltd., Kawasaki, Japan).

Visualization of AR localization. Twenty-four hours after transfection of pEGFP-fAR into PC-3 cells, cells were treated with or without 10 μ M 2'HF for 24 hours. Consequently cells were cultured in the absence or presence of 10 nM DHT for 8 hours, and fAR fused to green fluorescent protein (GFP) was visualized by FSX100.

Statistical analysis. Statistical significance was determined by using Prism 6.0 software, the χ^2 test was utilized to assess the significance between different proportions. Analysis of continuous variables between different groups was assessed by one-way analysis of variance followed by Fisher's protected least significant difference test. *, **, and *** in Figure represent significant difference $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Results

Effect of 2'HF on PCa cell proliferation. In order to investigate the effect on PCa cell proliferation, androgen-

independent PC-3 and DU145 cells and androgen-sensitive LNCaP cells were treated with 2'HF. As shown in Figure 1A, the proliferation of PC-3 and DU145 cells was inhibited by 2'HF in a dose-dependent manner. In particular 10 μ M 2'HF inhibited the proliferation of PC-3 and DU145 cells to 22% and 31% that of the controls, respectively. To determine whether inhibition of proliferation of PC-3 and DU145 cells by 2'HF was due to apoptosis, we performed annexin V staining. PC-3 cells treated with 2'HF for 72 hours were well-stained with Annexin-V-FLUOS, indicating apoptosis, whereas PC-3 cells cultured in the absence of 2'HF were not stained by green fluorescence. Similar results were observed in DU145 cells (Figure 1B).

As shown in Figure 1C, although 10 μ M 2'HF also inhibited the proliferation of LNCaP cells in the absence of androgens to 59% that of the control, the inhibition of LNCaP cells was less than that of PC-3 and DU145 cells. In contrast to PC-3 and DU145 cells, the treatment of 10 μ M 2'HF did not influence staining by Annexin-V-FLUOS in LNCaP cells, suggesting that apoptosis was not induced in LNCaP cells by 10 μ M 2'HF (Figure 1D). However, 2'HF did inhibit the stimulation of LNCaP cell proliferation by 10 nM adione in a dose-dependent manner. This stimulation was almost abolished to the basal level by 10 μ M 2'HF. Of interest, the proliferation of LNCaP cells stimulated by 1 nM testosterone and 1 nM DHT was also inhibited by 2'-HF, suggesting that 2'HF inhibited the stimulation of cell proliferation by testosterone and DHT without inhibiting testosterone synthesis in LNCaP cells. We then investigated the effect of 2'HF on the expression of PSA. LNCaP cells were treated with 10 nM adione, or 1 nM DHT in the absence and presence of 2'HF. As shown in Figure 1E, the induction of *PSA* mRNA expression not only by adione but also by DHT was repressed by 2'HF in a dose-dependent manner, although the basal level of *PSA* mRNA was not changed by 2'HF. These results suggest that 2'HF represses androgen-responsiveness in LNCaP cells without affecting androgen synthesis.

2'HF represses androgen-induced PSA promoter activity in PCa cells. In order to further investigate the effect of 2'HF on AR activity, we transfected LNCaP cells with a luciferase expression plasmid driven by the *PSA* promoter, pGL3PSAp-5.8, which was induced by androgens, and performed luciferase assay (4). As shown in Figure 2A, 10 μ M 2'HF did not repress the basal level of *PSA* promoter activity. In contrast, 2'HF repressed *PSA* promoter activity induced by 10 nM adione, 1 nM testosterone and by 1 nM DHT in LNCaP cells transfected with pGL3PSAp-5.8 in a dose-dependent manner. Moreover, 10 μ M 2'HF repressed these inductions to the basal level of *PSA* promoter activity. Since the *AR* gene in LNCaP cells is mutated at codon 877, this mutation might affect this repression by 2'HF. To exclude this possibility, we transfected PC-3 cells with a wild-type

AR expression plasmid vector (pSGAR2) and examined the effect of 2'HF (Figure 2B). *PSA* promoter activity induced by 10 nM adione and 1 nM testosterone in the presence of wild-type *AR* was repressed by 10 μ M 2'HF, suggesting that 2'HF might affect *AR* activity directly independently of androgen concentration in the medium.

2'HF does not affect HSD17B and 5 α -reductase activity in PCa cells. Since 2'HF repressed AR activity induced by testosterone and DHT, as well as adione, we determined if 2'HF affected the androgen concentration in medium from cell cultures. We treated PC-3 cells with 10 nM adione or 10 nM testosterone in the absence and presence of 10 μ M 2'HF and measured the concentration of adione, testosterone, and DHT in the medium 24 hours later by Liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS). 2'HF did not change testosterone and DHT concentration after addition of adione, and did not change DHT concentration after the addition of testosterone, suggesting that 2'HF does not affect HSD17B and 5 α -reductase activity *in vitro* (Figure 3).

2'HF inhibited the expression of AR protein but not AR mRNA. To reveal the mechanism of 2'HF repression AR activity, we first investigated the expression level of *AR* mRNA in LNCaP cells. Although 2'HF repressed the expression of *PSA* and other androgen-responsive genes induced by adione, testosterone, and DHT, the expression of *AR* mRNA did not change regardless of the presence or absence of 2'HF (Figure 4A). Next we investigated whether 2'HF affects the expression of AR protein. Western blot analysis revealed 2'HF down-regulated the expression of AR protein in LNCaP cells in a dose-dependent manner (Figure 4B). In addition, the level of exogenous AR in PC-3 cells transfected with pSGAR2 driven by SV40 promoter was also repressed by 2'HF.

Nuclear localization of AR. AR is usually localized in the cytoplasm in the absence of androgen and is translocated into the nucleus in the presence of androgen. We determined whether 2'HF affects AR localization. We transfected PC-3 cells with pEGFP-fAR plasmid that express full-length AR fused to GFP protein and recorded AR localization in the presence and absence of 2'HF. GFP-fAR protein, which was localized in the cytoplasm in the absence of DHT, was translocated into the nucleus in the presence of DHT within 8 hours (Figure 5). This translocation of AR into the nucleus was blocked in the presence of 2'HF and AR was kept staying in the cytoplasm even in the presence of DHT.

Discussion

Once androgen-sensitive PCa becomes CRPC during first-line hormonal therapy, physicians often conduct second-line

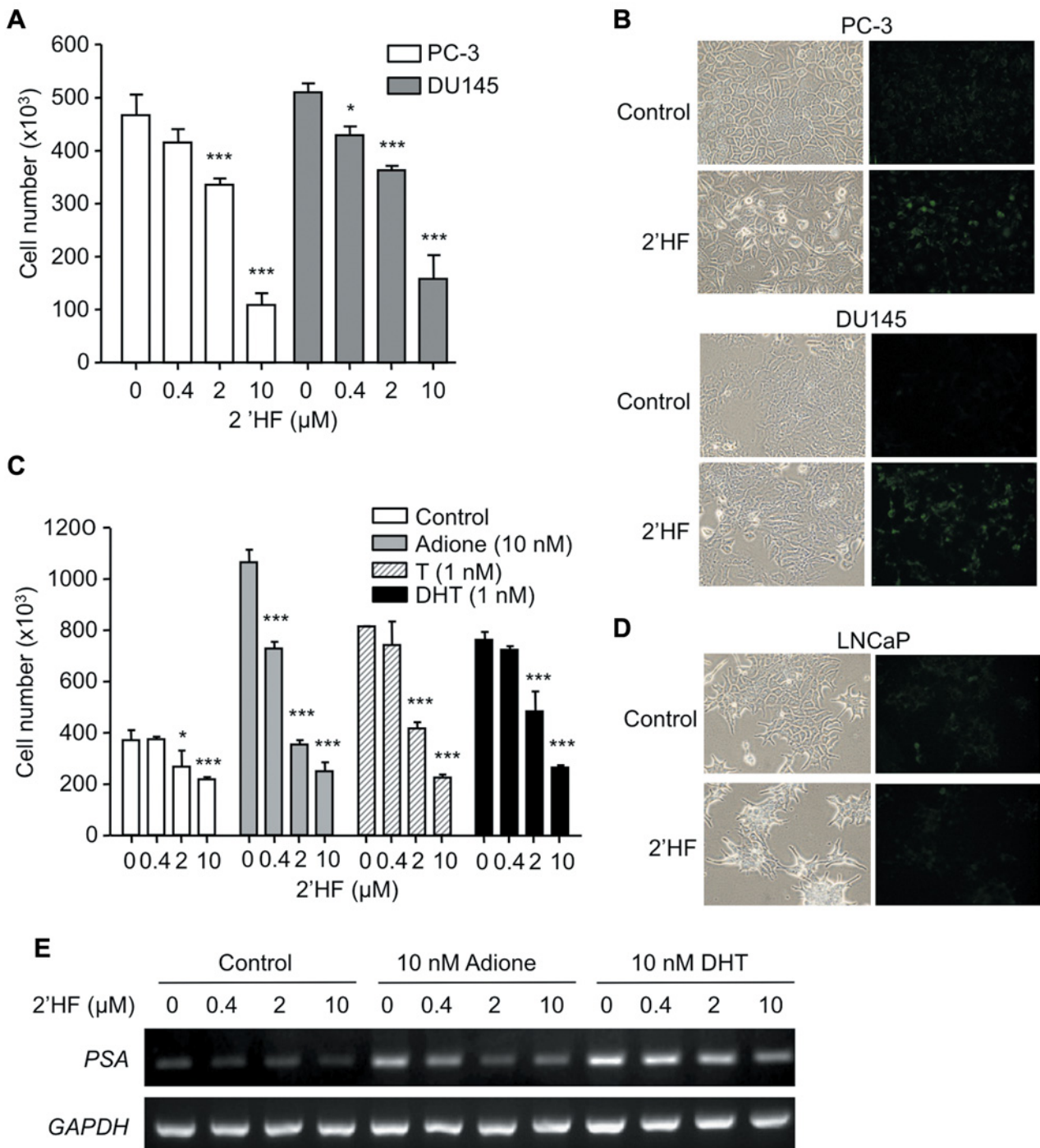


Figure 1. Effect of 2'HF on the proliferation of PC-3, DU145, and LNCaP cells. A: Twenty-four hours after 5×10^4 PC-3 or DU145 cells were plated, cells were treated with the indicated concentrations of 2'HF, then cells were counted. The media were changed every two days. B: After 5×10^4 PC-3 or DU145 cells were plated onto 6-well plates, cells were treated for 72 hours with or without $10 \mu\text{M}$ 2'HF, they were incubated with $100 \mu\text{l}$ Annexin-V-FLUOS labeling solution. They were analysed by phase contrast (left) and fluorescence microscopy (right). C: After LNCaP cells were treated with 0, 10 nM 4-androstenedione (adione), 1 nM testosterone (T), or 1 nM dihydrotestosterone (DHT) in the presence of the indicated concentration of 2'HF for four days, the numbers of LNCaP cells were counted. D: LNCaP cells 1×10^5 were plated onto 6-well plates. Seventy-two hours after treatment with or without $10 \mu\text{M}$ 2'HF, they were incubated with $100 \mu\text{l}$ Annexin-V-FLUOS labeling solution. They were analyzed by phase contrast (left) and fluorescence (right) microscopy. E: Twenty-four hours after seeding of LNCaP cells, cells were treated with 10 nM Adione or 10 nM DHT in the presence of indicated concentrations of 2'HF for 20 hours. The expression level of PSA mRNA was determined by RT-PCR. * $p < 0.05$, *** $p < 0.001$.

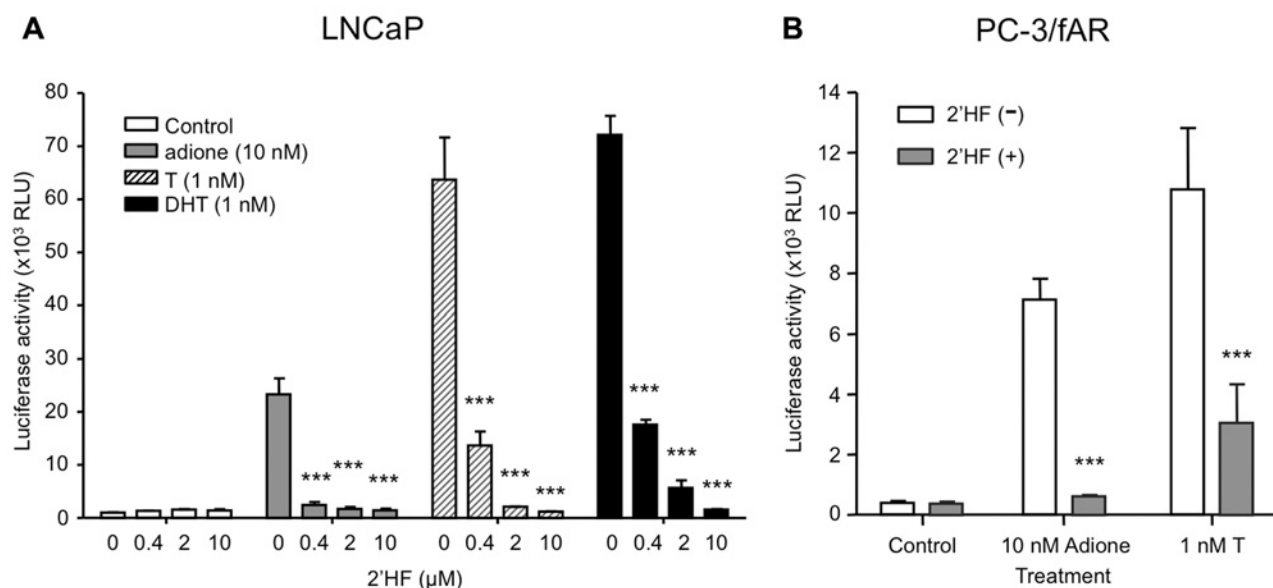


Figure 2. Effect of 2'HF on androgen receptor (AR) activity in prostate cancer cells. A: Twenty-four hours after transfection of LNCaP cells with luciferase reporter plasmid (pGL3PSAp-5.8), cells were treated with 0, 10 nM 4-androstenedione (adione), 1 nM testosterone (T), or 1 nM dihydrotestosterone (DHT) in the presence of the indicated concentrations of 2'HF for 24 hours, luciferase activity was then examined. B: Twenty-four hours after transfection of PC-3 cells with 300 ng pGL3PSAp-5.8 and 200 ng pSGAR2, cells were treated with 0, 10 nM adione, or 1 nM T in the absence or presence of 10 μM 2'HF for 24 hours, then luciferase activity was examined.

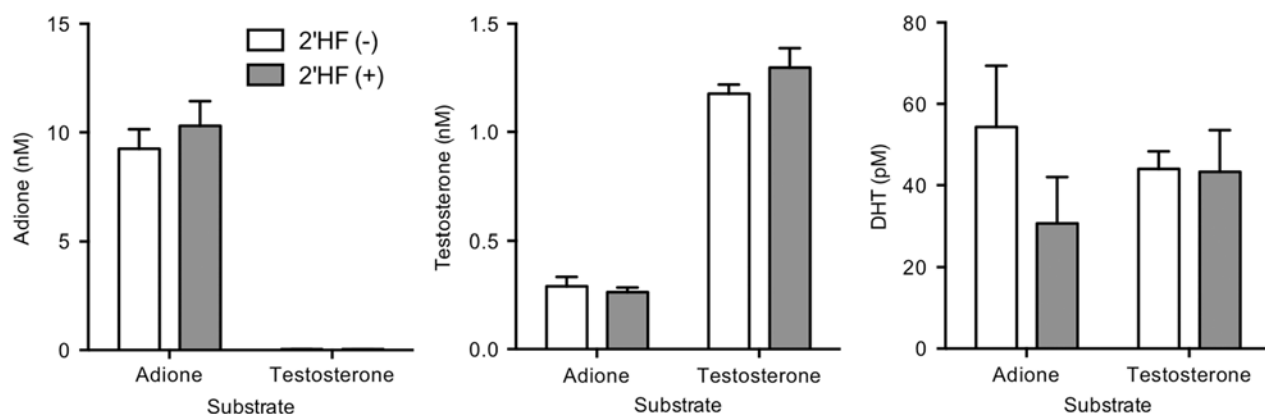


Figure 3. Androgen biosynthesis from adione or testosterone in PC-3 cells. Twenty-four hours after starting culture of 5×10^4 PC-3 cells, 10 nM adione or 10 nM testosterone was added to the medium. Aliquots of medium were collected after 24 hours for measuring the concentration of adione, testosterone and testosterone (T).

hormonal therapy and sequentially conduct chemotherapy using docetaxel. Although docetaxel is effective for 70% of CRPC, CRPC eventually shifts to become docetaxel-resistant. In such a situation, the prognosis of the patients is poor. Therefore, it is extremely important to prevent PCa from recurrence during hormonal therapy, without severe side-effects.

Flavonoids have antitumor activity, inducing apoptosis *via* tumor necrosis factor-related apoptosis-inducing ligand

(TRAIL), nuclear transcription factor-κB (NF-κB) reactive oxygen species, and peroxisome proliferator-activated receptor-gamma (PPARγ)-dependent, and independent pathways (21-24). Recently it was reported that among flavanones, 2'HF showed the most potent tumor inhibitory activity by stimulating caspase-mediated apoptosis of colon cancer cells (25). This apoptosis by 2'HF was associated with up-regulation of nonsteroidal anti-inflammatory drug-activated gene 1 (*NAG-1*) expression through induction of

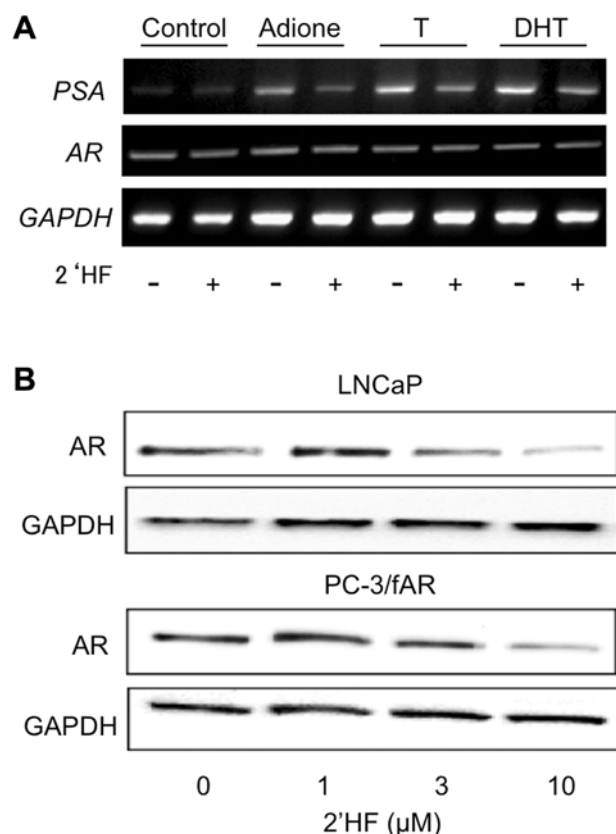


Figure 4. Effect of 2'HF on PSA and AR mRNA and protein expression. A: Twenty-four hours after seeding of LNCaP cells, cells were treated with or without 10 nM adione, 10 nM testosterone (T), or 10 nM dihydrotestosterone (DHT) for 12 hours and harvested to extract total RNA. The expression level of AR, PSA, and GAPDH mRNA was subjected to RT-PCR. B: Twenty-four hours after seeding of LNCaP or PC-3 cells transfected with pSGAR2 vector, cells were treated with indicated concentration of 2'HF for 24 hours and then harvested. The expression level of AR and GAPDH proteins were subjected to western blot analysis.

EGR-1. 2'HF also inhibited cell cycle progression and angiogenesis by reducing vascular endothelial growth factor expression in von Hippel-Lindau (VHL)-mutant renal cell carcinoma (26). In the present study, 2'HF inhibited proliferation of androgen-independent PCa cells *via* apoptosis at least. We are now investigating the mechanism of apoptosis induced by 2'HF in PCa cells.

2'HF was also a candidate for repression of AKR1C3 activity that stimulates biosynthesis of testosterone from adione (16). Contrary to our expectation, however, 2'HF did not have an inhibitory effect on AKR1C3 activity in cultured PCa cells. Instead, 2'HF repressed testosterone and DHT-induced androgen responsiveness. In the present study, 2'HF was shown, to our knowledge for the first time, to down-regulate AR activity. This effect was mediated through down-

regulation of AR protein expression at least. We also previously confirmed whether other candidates of dietary flavonoids AKR1C3 inhibitors could affect AR activity. One such candidate, naringenin, which is a strong AKR1C3 inhibitor, also repressed AR activity in the presence of DHT, as well as adione (data not shown) (16). Naringenin may also repress AR activity similarly to 2'HF without mediating through AKR1C3. Moreover, 2'HF also inhibited AR translocation into the nucleus. However, it is not clear whether this inhibition is a result of inhibition of the nuclear localization signal or of diminished AR expression. We accept that 2'HF may inhibit AKR1C3 activity and inhibit androgen synthesis from adione to testosterone. A higher concentration of 2'HF may inhibit AKR1C3 activity in PCa cells.

Several mechanisms by which androgen-naïve PCa changes to CRPC have been proposed, such as the existence of hypersensitive AR, promiscuous AR, outlaw AR, bypass AR, and alternative spliced truncated AR (27, 28). Existence of adrenal androgen also affects the mechanisms by which PCa becomes CRPC (29). Moreover, intratumoral androgen synthesis, especially, in PCa-derived stromal cells and bone-derived stromal cells, also play an important role in activation of adrenal androgen (4, 30). Therefore, some strategies to block the androgen AR axis have been considered (31). These include: (i) Inhibition of androgen synthesis enzyme upstream of DHEA synthesis in adrenal gland, such as abiraterone acetate and TAK-700 (32, 33); (ii) use of more potent antiandrogen agents, or inhibition of nuclear translocation of AR, such as enzalutamide (34); (iii) inhibition of androgen synthesis from DHEA in tumor cells or stromal cells, e.g. using 5 α -reductase inhibitors (35). (iv) diminishing the AR level in PCa cells. Clinical evidence that enzalutamide, abiraterone acetate, and TAK-700 are very effective for CRPC even after docetaxel treatment have proven that the androgen AR axis plays an extremely important role in the transformation of PCa to CRPC (29, 36). However, strong inhibitors of androgen synthesis enzymes such as HSD17B (AKR1C3) or HSD3B, in tumor cells have not yet been identified. Moreover, it may be difficult to overcome the progression of CRPC *via* the androgen AR axis completely by inhibition of androgen synthesis. Androgen-independent AR activation is also indicated in the progression of CRPC *via* interleukin-6 (IL-6) activation of signal transducer and activator of transcription 3 (STAT3) (37, 38), and *via* IL-8 signaling tyrosine kinases Src and focal adhesion kinase (FAK) (39, 40). If androgen-independent AR activation by cytokines results in emergence of CRPC, the inhibition of androgen synthesis would not be effective for these patients. In such cases, repression of AR expression by 2'HF might be effective for inhibition of the AR axis.

Recently, ASC-J9 was synthesized, which stimulates AR degradation-repressed androgen responsiveness (41).

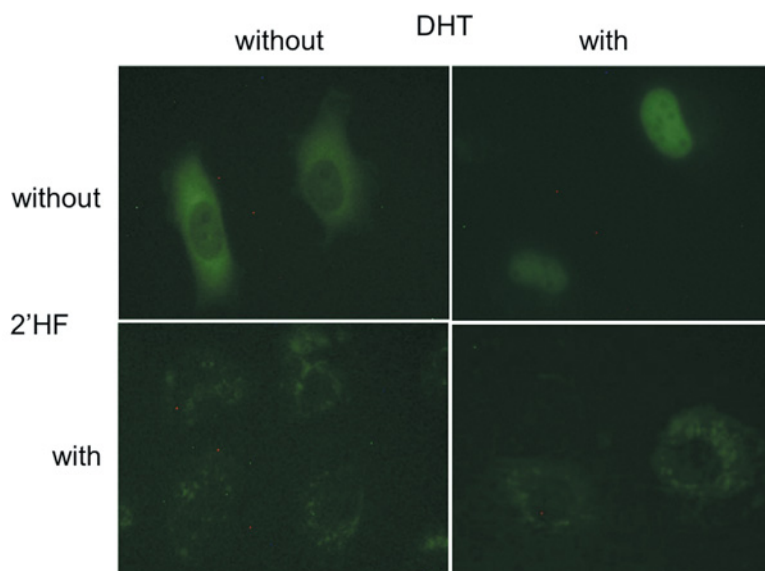


Figure 5. Androgen receptor (AR) localization in PC-3 cells transfected with pEGFP-fAR in the absence and presence of dihydrotestosterone (DHT). After transfection of PC-3 cells with pEGFP-fAR vector, wild-type fAR protein fused to GFP protein were visualized by FSX100 in the absence and presence of DHT with and without 2'HF.

Curcumin which is a major natural yellow pigment in turmeric and is widely used as a spice and coloring agent in several foods such as curry, has also been shown to down-regulate AR protein (42). We revealed that 2'HF similarly represses AR activity by diminishing AR protein in PCa cells. We will investigate whether 2'HF affects AR ubiquitination and degradation of AR or affects AR translation.

When advanced PCa changes to CRPC during hormonal therapy, physicians often conduct docetaxel-based chemotherapy, which can extend the survival period of the patients (43). However, the effective period is limited because of further progression of CRPC. Overcoming docetaxel resistance, therefore, is important to improve the prognosis of patients. One of mechanisms of taxane resistance is up-regulation of P-glycoprotein expression (44). Polymethoxyflavones, which are components of orange juice, inhibit P-glycoprotein-mediated efflux (45). Since flavonoids have a variety of functions for pharmacological activity, 2'HF may also exhibit inhibitory activity on P-glycoprotein and increase the sensitivity to docetaxel. As a result, 2'HF might reduce adverse effects as well as overcoming docetaxel resistance.

In conclusion, we revealed that the natural product, 2'HF, found naturally in fruits and vegetables, can inhibit not only the proliferation of PCa cells but also androgen responsiveness *via* down-regulation of AR protein. If we can prevent progression of PCa during ADT by taking natural products such as 2'HF effectively, patients will be able to

benefit physiologically and economically. Further analyses are needed to reveal the full range of pharmacological activities of 2'HF.

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References

- 1 Labrie F, Dupont A and Belanger A: Complete androgen blockade for the treatment of prostate cancer. *Important Adv Oncol* pp. 193-217, 1985.
- 2 Mizokami A, Koh E, Fujita H, Maeda Y, Egawa M, Koshida K, Honma S, Keller ET and Namiki M: The adrenal androgen androstenediol is present in prostate cancer tissue after androgen deprivation therapy and activates mutated androgen receptor. *Cancer Res* 64: 765-771, 2004.
- 3 Nishiyama T, Hashimoto Y and Takahashi K: The influence of androgen deprivation therapy on dihydrotestosterone levels in the prostatic tissue of patients with prostate cancer. *Clin Cancer Res* 10: 7121-7126, 2004.
- 4 Mizokami A, Koh E, Izumi K, Narimoto K, Takeda M, Honma S, Dai J, Keller E and Namiki M: Prostate cancer stromal cells and LNCaP cells coordinately activate the androgen receptor through synthesis of testosterone and dihydrotestosterone from dehydroepiandrosterone. *Endocr Relat Cancer* 16: 1139-1155, 2009.

- 5 Labrie F, Luu-The V, Lin SX, Labrie C, Simard J, Breton R and Belanger A: The key role of 17 β -hydroxysteroid dehydrogenases in sex steroid biology. *Steroids* 62: 148-158, 1997.
- 6 Penning TM, Burczynski ME, Jez JM, Hung CF, Lin HK, Ma H, Moore M, Palackal N and Ratnam K: Human 3 α -hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: Functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. *Biochem J* 351: 67-77, 2000.
- 7 Byrns MC, Mindnich R, Duan L and Penning TM: Overexpression of aldo-keto reductase 1C3 (AKR1C3) in LNCaP cells diverts androgen metabolism towards testosterone resulting in resistance to the 5 α -reductase inhibitor finasteride. *J Steroid Biochem Mol Biol* 130: 7-15, 2012.
- 8 Fung KM, Samara EN, Wong C, Metwalli A, Krlin R, Bane B, Liu CZ, Yang JT, Pitha JV, Culkin DJ, Kropp BP, Penning TM and Lin HK: Increased expression of type 2 3 α -hydroxysteroid dehydrogenase/type 5 17 β -hydroxysteroid dehydrogenase (AKR1C3) and its relationship with androgen receptor in prostate carcinoma. *Endocr Relat Cancer* 13: 169-180, 2006.
- 9 Stanbrough M, Bubley GJ, Ross K, Golub TR, Rubin MA, Penning TM, Febbo PG and Balk SP: Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. *Cancer Res* 66: 2815-2825, 2006.
- 10 Mitsiades N, Sung CC, Schultz N, Danila DC, He B, Eedunuri VK, Fleisher M, Sander C, Sawyers CL and Scher HI: Distinct patterns of dysregulated expression of enzymes involved in androgen synthesis and metabolism in metastatic prostate cancer tumors. *Cancer Res* 72: 6142-6152, 2012.
- 11 Hamid AR, Pfeiffer MJ, Verhaegh GW, Schaafsma E, Brandt A, Sweep FC, Sedelaar JP, and Schalken JA: Aldo-keto reductase family 1 member C3 (AKR1C3) Is a biomarker and therapeutic target for castration-resistant prostate cancer. *Mol Med* 18: 1449-1455, 2013.
- 12 Dozmorov MG, Azzarello JT, Wren JD, Fung KM, Yang Q, Davis JS, Hurst RE, Culkin DJ, Penning TM and Lin HK: Elevated AKR1C3 expression promotes prostate cancer cell survival and prostate cell-mediated endothelial cell tube formation: Implications for prostate cancer progression. *BMC Cancer* 10: 672, 2010.
- 13 Romagnolo DF and Selmin OI: Flavonoids and cancer prevention: A review of the evidence. *J Nutr Gerontol Geriatr* 31: 206-238, 2012.
- 14 Pietta PG: Flavonoids as antioxidants. *J Nat Prod* 63: 1035-1042, 2000.
- 15 Coelho RC, Hermsdorff HH and Bressan J: Anti-inflammatory properties of orange juice: Possible favorable molecular and metabolic effects. *Plant Foods Hum Nutr* 68: 1-10, 2013.
- 16 Skarydova L, Zivna L, Xiong G, Maser E and Wsol V: AKR1C3 as a potential target for the inhibitory effect of dietary flavonoids. *Chem Biol Interact* 178: 138-144, 2009.
- 17 Byrns MC, Jin Y and Penning TM: Inhibitors of type 5 17 β -hydroxysteroid dehydrogenase (AKR1C3): overview and structural insights. *J Steroid Biochem Mol Biol* 125: 95-104, 2011.
- 18 Izumi K, Mizokami A, Li YQ, Narimoto K, Sugimoto K, Kadono Y, Kitagawa Y, Konaka H, Koh E, Keller ET and Namiki M: Tranilast inhibits hormone-refractory prostate cancer cell proliferation and suppresses transforming growth factor β 1-associated osteoblastic changes. *Prostate* 69: 1222-1234, 2009.
- 19 Mizokami A, Saiga H, Matsui T, Mita T and Sugita A: Regulation of androgen receptor by androgen and epidermal growth factor in a human prostatic cancer cell line, LNCaP. *Endocrinol Jpn* 39: 235-243, 1992.
- 20 Mizokami A, Gotoh A, Yamada H, Keller ET and Matsumoto T: Tumor necrosis factor- α represses androgen sensitivity in the LNCaP prostate cancer cell line. *J Urol* 164: 800-805, 2000.
- 21 Jin CY, Park C, Hwang HJ, Kim GY, Choi BT, Kim WJ and Choi YH: Naringenin up-regulates the expression of death receptor 5 and enhances TRAIL-induced apoptosis in human lung cancer A549 cells. *Mol Nutr Food Res* 55: 300-309, 2011.
- 22 Kanno S, Tomizawa A, Ohtake T, Koiwai K, Ujibe M and Ishikawa M: Naringenin-induced apoptosis via activation of NF- κ B and necrosis involving the loss of ATP in human promyeloleukemia HL-60 cells. *Toxicology Lett* 166: 131-139, 2006.
- 23 Ko CH, Shen SC and Chen YC: Hydroxylation at C4' or C6 is essential for apoptosis-inducing activity of flavanone through activation of the caspase-3 cascade and production of reactive oxygen species. *Free Radic Biol Med* 36: 897-910, 2004.
- 24 Ghorbani A, Nazari M, Jeddi-Tehrani M and Zand H: The citrus flavonoid hesperidin induces p53 and inhibits NF- κ B activation in order to trigger apoptosis in NALM-6 cells: Involvement of PPAR γ -dependent mechanism. *Eur J Nutr* 51: 39-46, 2012.
- 25 Shin SY, Kim JH, Lee JH, Lim Y and Lee YH: 2'-Hydroxyflavanone induces apoptosis through EGR-1 involving expression of BAX, p21, and NAG-1 in colon cancer cells. *Mol Nutr Food Res* 56: 761-774, 2012.
- 26 Nagaprasanthan LD, Vatsyayan R, Singhal J, Lelsani P, Prokai L, Awasthi S and Singhal SS: 2'-Hydroxyflavanone inhibits proliferation, tumor vascularization and promotes normal differentiation in VHL-mutant renal cell carcinoma. *Carcinogenesis* 32: 568-575, 2011.
- 27 Feldman BJ and Feldman D: The development of androgen-independent prostate cancer. *Nat Rev Cancer* 1: 34-45, 2001.
- 28 Dutt SS and Gao AC: Molecular mechanisms of castration-resistant prostate cancer progression. *Future Oncol* 5: 1403-1413, 2009.
- 29 Ryan CJ and Tindall DJ: Androgen receptor rediscovered: The new biology and targeting the androgen receptor therapeutically. *J Clin Oncol* 29: 3651-3658, 2011.
- 30 Montgomery RB, Mostaghel EA, Vessella R, Hess DL, Kalhorn TF, Higano CS, True LD and Nelson PS: Maintenance of intratumoral androgens in metastatic prostate cancer: A mechanism for castration-resistant tumor growth. *Cancer Res* 68: 4447-4454, 2008.
- 31 Molina A and Belldegrun A: Novel therapeutic strategies for castration-resistant prostate cancer: Inhibition of persistent androgen production and androgen receptor mediated signaling. *J Urol* 185: 787-794, 2011.
- 32 de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones RJ, Goodman OB, Jr., Saad F, Staffurth JN, Mainwaring P, Harland S, Flaig TW, Hutson TE, Cheng T, Patterson H, Hainsworth JD, Ryan CJ, Sternberg CN, Ellard SL, Flechon A, Saleh M, Scholz M, Efsthathiou E, Zivi A, Bianchini D, Loriot Y, Chieffo N, Kheoh T, Haqq CM, and Scher HI: Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 364: 1995-2005, 2011.
- 33 Agarwal N, Sonpavde G and Sternberg CN: Novel molecular targets for the therapy of castration-resistant prostate cancer. *Eur Urol* 61: 950-960, 2012.

- 34 Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, Wasielewska T, Welsbie D, Chen CD, Higano CS, Beer TM, Hung DT, Scher HI, Jung ME and Sawyers CL: Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* 324: 787-790, 2009.
- 35 Shah SK, Trump DL, Sartor O, Tan W, Wilding GE and Mohler JL: Phase II study of Dutasteride for recurrent prostate cancer during androgen deprivation therapy. *J Urol* 181: 621-626, 2009.
- 36 Tsao CK, Galsky MD, Small AC, Yee T and Oh WK: Targeting the androgen receptor signalling axis in castration-resistant prostate cancer (CRPC). *BJU Int* 110: 1580-1588, 2012.
- 37 George DJ, Halabi S, Shepard TF, Sanford B, Vogelzang NJ, Small EJ and Kantoff PW: The prognostic significance of plasma interleukin-6 levels in patients with metastatic hormone-refractory prostate cancer: Results from cancer and leukemia group B 9480. *Clin Cancer Res* 11: 1815-1820, 2005.
- 38 DeMiguel F, Lee SO, Lou W, Xiao X, Pflug BR, Nelson JB and Gao AC: STAT3 enhances the growth of LNCaP human prostate cancer cells in intact and castrated male nude mice. *Prostate* 52: 123-129, 2002.
- 39 Seaton A, Scullin P, Maxwell PJ, Wilson C, Pettigrew J, Gallagher R, O'Sullivan JM, Johnston PG and Waugh DJ: Interleukin-8 signaling promotes androgen-independent proliferation of prostate cancer cells *via* induction of androgen receptor expression and activation. *Carcinogenesis* 29: 1148-1156, 2008.
- 40 Lee LF, Louie MC, Desai SJ, Yang J, Chen HW, Evans CP and Kung HJ: Interleukin-8 confers androgen-independent growth and migration of LNCaP: Differential effects of tyrosine kinases SRC and FAK. *Oncogene* 23: 2197-2205, 2004.
- 41 Yamashita S, Lai KP, Chuang KL, Xu D, Miyamoto H, Tochigi T, Pang ST, Li L, Arai Y, Kung HJ, Yeh S and Chang C: ASC-J9 suppresses castration-resistant prostate cancer growth through degradation of full-length and splice variant androgen receptors. *Neoplasia* 14: 74-83, 2012.
- 42 Ide H, Tokiwa S, Sakamaki K, Nishio K, Isotani S, Muto S, Hama T, Masuda H and Horie S: Combined inhibitory effects of soy isoflavones and curcumin on the production of prostate-specific antigen. *Prostate* 70: 1127-1133, 2010.
- 43 Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Theodore C, James ND, Turesson I, Rosenthal MA and Eisenberger MA: Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 351: 1502-1512, 2004.
- 44 Takeda M, Mizokami A, Mamiya K, Li YQ, Zhang J, Keller ET and Namiki M: The establishment of two paclitaxel-resistant prostate cancer cell lines and the mechanisms of paclitaxel resistance with two cell lines. *Prostate* 67: 955-967, 2007.
- 45 Ikegawa T, Ushigome F, Koyabu N, Morimoto S, Shoyama Y, Naito M, Tsuruo T, Ohtani H and Sawada Y: Inhibition of P-glycoprotein by orange juice components, polymethoxyflavones in adriamycin-resistant human myelogenous leukemia (K562/ADM) cells. *Cancer Lett* 160: 21-28, 2000.

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