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# Flavonoids can block PSA production by breast and prostate cancer cell lines

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**Abstract**

*Background*: Prostatic carcinoma is the most commonly diagnosed cancer and the second leading cause of cancer death of North American men. Combined androgen blockade (CAB) is one treatment option for prostate cancer, using estrogen agonists, luteinizing hormone-releasing hormone (LHRH) agonists and non-steroidal anti-androgens such as nilutamide and cyproterone acetate. Since many of these drugs have serious side effects, many patients are searching for ''natural'' alternatives or complements to traditional therapy. These include phytoestrogens found in soy and other plant foods. Such compounds have only started to be evaluated for potential androgen-blocking activity. Inhibition of production of androgen-regulated proteins, including prostate-specific antigen (PSA), is one indicator of androgen blocking. *Methods*: The ability of 72 flavonoids and related compounds to inhibit PSA production in a breast cancer cell line, BT-474, and a prostate cancer cell line (PC-3), transfected with the human androgen receptor cDNA, PC-3$(AR)_2$ was examined. *Results*: Twenty-two of the 72 flavonoids tested were found to significantly block PSA production by the BT-474 cell line at the highest tested concentration ($10^{-5}$ mol/l), with 17 of these compounds inhibiting production of PSA in the PC-3$(AR)_2$ cells as well. *Conclusions*: That several flavonoids may significantly block production of this androgen-regulated protein. It will be worthwhile to examine these compounds as possible candidates for prostate cancer prevention or management. 

*Keywords:* Prostate cancer; Anti-androgens; Androgen receptor; Flavonoids; Steroid hormone receptors; Prostate-specific antigen

*Abbreviations:* CAB, combined androgen blockade; ER, estrogen receptor; PR, progesterone receptor; AR, androgen receptor; ARE, androgen response element; GTP, green tea polyphenols; DHT, dihydrotestosterone.

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## 1. Introduction

Prostatic carcinoma is the most commonly diagnosed cancer in North American men and the second leading cause of cancer death in this population [1]. The incidence of prostate cancer has risen sharply over the last decade [1–4]. Much of this increase can be attributed to better screening techniques, including serum prostate-specific antigen (PSA) analysis [3,5]. Other potential contributing factors include the aging population, increasing rates of obesity, high consumption of meat and fat, combined with reduced physical activity [6–9].

Since most prostate tumors are hormone-dependent, combined androgen blockade (CAB) is a common strategy for primary or secondary therapy [10–12]. Agents used for treatment include diethylstilbestrol (an estrogen agonist) [13–15], luteinizing hormone-releasing hormone (LHRH) agonists [16–18], and anti-androgens, including flutamide, nilutamide and cyproterone acetate [19–22]. Many of these drugs have side effects including gynecomastia, impotence and hepatic toxicity [23–29]. For these reasons, many patients are searching for ''natural'' alternatives or complements to traditional drugs [30–33].

Over the past few years, the beneficial effects of soy and soy isoflavones in the prevention of breast and prostate cancers, as well as of heart disease, have been investigated [34–40]. A body of epidemiological evidence associates consumption of soy, and vegetables in general, with lower prostate cancer risk in Asian populations versus Western countries [34,36,37]. In vitro studies have demonstrated the partial estrogen/anti-estrogen activities of soy isoflavones and other flavonoids [38,39], as well as their antioxidant effects [40,41]. Cytostasis and induction of apoptosis are two mechanisms by which the monoterpene, perillyl alcohol (found in citrus fruit), and the synthetic flavone flavopiridol may be effective against prostate and other cancer cells [42–46]. These compounds are currently in phase II clinical trials [47,48].

To date, few studies have investigated the ability of plant-derived compounds to block androgenic activities, such as expression of androgen-dependent genes. The soy isoflavone, biochanin A, was demonstrated to decrease testosterone-induced production of PSA in the LNCaP prostate cancer cell line, through upregulation of a catabolic enzyme [49]. Resveratrol, the phytoalexin found in red wine, has been shown to have estrogenic and anti-estrogenic activity [50,51] and to downregulate transcription of both androgen receptor (AR) and PSA genes in this cell line [52]. We therefore undertook this study to determine whether various flavonoids and related compounds could inhibit PSA production, a protein regulated by androgens, through the AR.

## 2. Materials and methods

### 2.1. Materials

The BT-474 human breast cancer cell line was purchased from the American Type Culture Collection (ATCC), Rockville, MD. The levels of ER and PR, as quantified by commercial ELISA assays (Abbott Diagnostics, Abbott Park, Chicago, IL), were 29 and 389 fmol/mg protein, respectively. Although the AR content was not quantified, Northern blot studies indicated that this cell line contains AR [53]. The PC-3 cell line, PC-3$(AR)_2$, transfected with the human full-length AR cDNA, was described elsewhere [54]. AR concentration of this cell line is 491 fmol/mg protein. The parental line has been described as having both glucocorticoid and estrogen receptors, although the latter is controversial [55–57]. All steroids used were from Sigma, St. Louis, MO. Stock, $10^{-2}$ mol/l solutions of steroids were prepared in absolute ethanol. Flavonoids and structurally related compounds were obtained from Indofine Chemical, Summerville, NJ and Sigma. We prepared $10^{-2}$ mol/l stock solutions of these compounds in absolute ethanol. Nilutamide (RU 56187) was a gift from Roussel-UCLAF Romainville, France.

### 2.2. Methods

BT-474 cells were grown to confluency in phenol-free RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 10 mg/ml insulin and 200 mmol/l L-glutamine at 37 °C, 5% $CO_2$. Once confluent, they were subcultured in 24-well microtiter plates using the same media, but with

substitution of charcoal-stripped fetal calf serum for the regular fetal calf serum. PC-3$(AR)_2$ cells were grown under similar conditions, using supplementation of media with 5% fetal calf serum and 100 μg/ml hygromycin-B (Calbiochem-Novabiochem, La Jolla, CA).

BT-474 cells were incubated with a flavonoid or related compound at $10^{-5}$ and $10^{-7}$ mol/l (final concentrations) for 1 h, after which time the cells were stimulated with dihydrotestosterone (DHT) at $10^{-9}$ mol/l. The cells were then incubated for 7 days at the same conditions as above. DHT was also tested alone (no blocker added) to determine maximum production of PSA, and candidate blockers were tested without steroid (DHT), to determine their androgen agonist activity. The blocking activity of nilutamide (an established anti-androgen) used at $10^{-7}$ mol/l served as a positive control for inhibition of DHT-induced PSA production, and ethanol (solvent) was a negative control. After 7 days, the tissue culture supernatants were harvested and analyzed for PSA. Compounds found to have greater than 50% blocking activity of DHT-induced PSA production were then tested for dose–response activity, in the range from $10^{-5}$ to $10^{-8}$ mol/l in both BT-474 and PC-3$(AR)_2$ cell lines, using the same protocol (including that for DHT alone, and nilutamide), outlined above. Estradiol was also tested in both cell lines, at concentrations of $10^{-7}$ to $10^{-9}$ mol/l, to determine whether this steroid had blocking

Table 1
Flavonoids and related compounds that significantly inhibited PSA production in the BT-474 and PC-3$(AR)_2$ cell lines

| Compound[a] | Percentage blocking of PSA production in BT-474[b] | Percentage blocking of PSA production in PC-3$(AR)_2$ |
|---|---|---|
| 6-Bromo-2-naphthol[c] | 100 | 100 |
| 2′,5′-Dimethoxyacetophenone[c] | 100 | 99–100 |
| Flavone | 100 | 80–85 |
| 5-Hydroxyflavone | 100 | 100 |
| 2′-Methoxyflavone | 100 | 60–70 |
| 5-Methoxyflavone | 100 | 100 |
| Harmol | 99–100 | 80–85 |
| 4′-Methoxyflavone | 99–100 | 100 |
| 2′-Hydroxyflavanone | 98–100 | 90 |
| Biochanin A[c] | 98–95 | <50 |
| Mangostine | 81–100 | 100 |
| 7-Hydroxyflavanone[c] | 81–87 | 95 |
| Curcumine | 80–97 | 99–100 |
| Genistein[c] | 76–87 | <50 |
| 4′-Hydroxyflavanone[c] | 74 | 98–100 |
| Resveratrol[c] | 72–99 | 70–75 |
| Harmalol | 67–91 | <50 |
| Naringenin[c] | 65–67 | <50 |
| 6-Hydroxyflavone[c] | 64–92 | <50 |
| Luteolin[c] | 62–81 | 80–85 |
| Chrysin[c] | 57–91 | 55–60 |
| 6-Methoxyflavanone | 55–89 | 90–95 |
| Estradiol[d] | 80–90 | <50 |
| Nilutamide[e] | 97–100 | 80–100 |

[a] Compounds tested at $10^{-5}$ mol/l final concentration.
[b] Compounds tested at least twice. Blocking expressed as range, where applicable.
[c] Compounds found to be estrogenic in a previous study [64].
[d] Estradiol was tested at $10^{-8}$ mol/l.
[e] Nilutamide was tested at $10^{-8}$ mol/l.

activity. Each experiment was repeated at least twice, and cells were assayed using trypan blue to monitor cell viability.

### 2.3. PSA assay

PSA was quantified using an ELISA-type immunofluorometric procedure described elsewhere [58]. The detection limit of this assay is ~1 ng/l. The total protein concentrations of all cell culture supernatant fluids did not differ significantly (data not shown).

## 3. Results

Our tissue culture testing system is based on the principle that the selected endogenous gene, PSA, is up-regulated by steroid hormones, in this case, DHT. The PSA gene contains multiple androgen response elements (AREs) in its promoter/enhancer region [59]. After binding of the AR to its respective ligand, a conformational change occurs, and the ligand–receptor complex subsequently binds to these AREs of the PSA gene promoter. If other transcription factors are also available, the gene is transcribed to

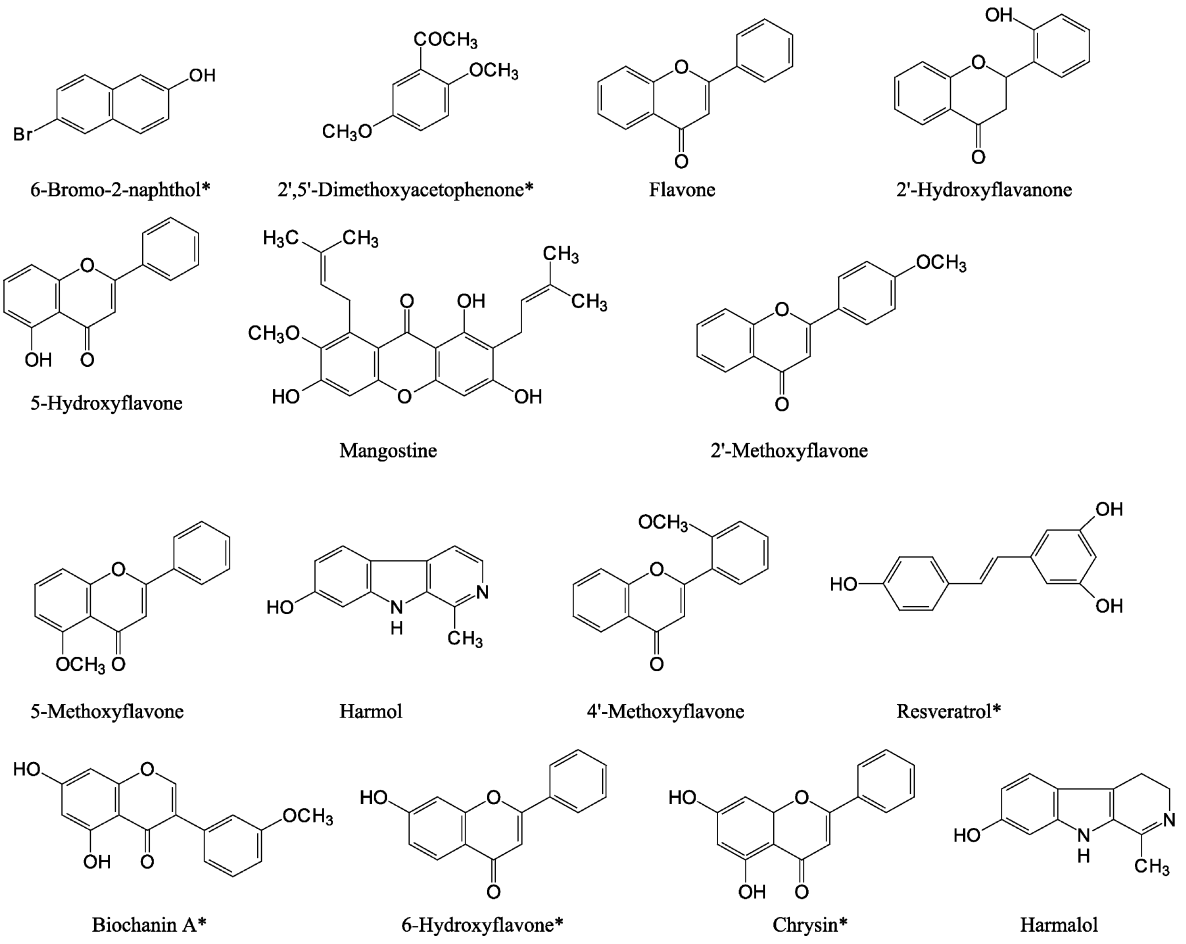


Fig. 1. Structures of flavonoids and related compounds that significantly inhibited PSA production. Significant blocking activity was defined as greater than 50% inhibition of dihydrotestosterone (DHT)-induced prostate-specific antigen (PSA) production. Compounds were tested at $10^{-5}$ mol/l, and DHT was used at $10^{-9}$ mol/l. Asterisks indicate compounds with estrogenic activity as determined in a previous study [64].

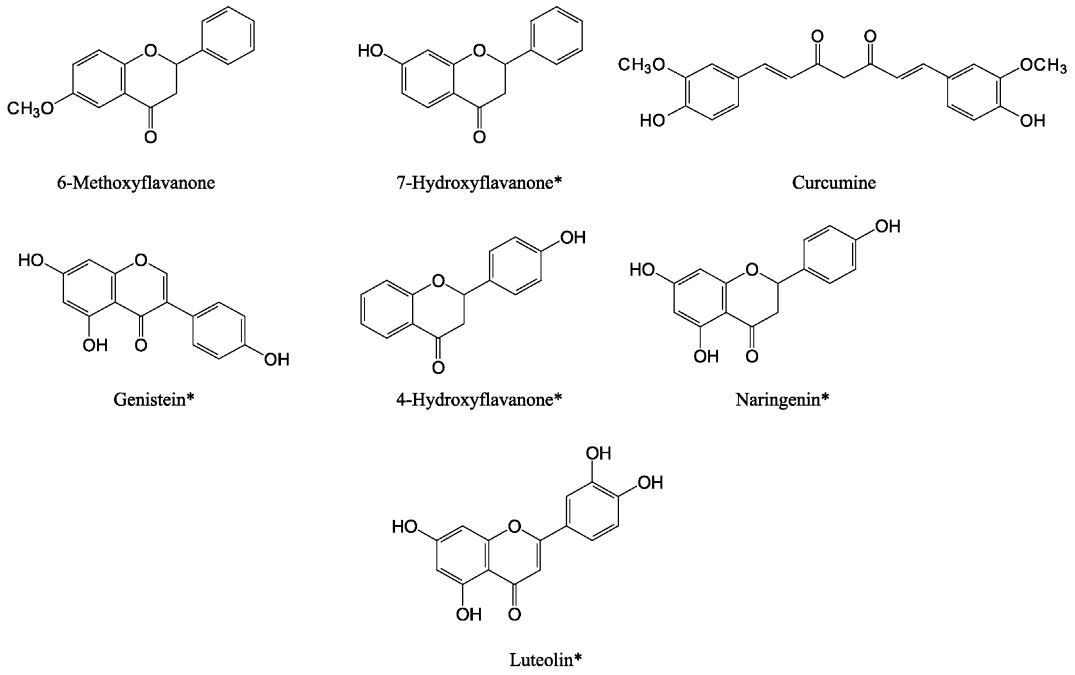


Fig. 1 (*continued*).

mRNA, and then translated to PSA protein, which is secreted. If any of these steps is inhibited, PSA protein production and secretion decrease, and this will be reflected by the PSA concentration in the culture medium. Thus, this tissue culture system is a sensitive indicator of hormone receptor activation and induction of transcription. This system and protocol has been used successfully in gene regulation studies [60–62] and in identifying biological activity of candidate synthetic progestins [63]. The principles of transcriptional activation by steroid hormones have been reviewed by Beato et al. [62]. The BT-474 cell line was used in this system because of its wild-type endogenous AR and PSA gene [53,61].

Inhibition of PSA production was defined as greater than 50% blocking of DHT-induced PSA production. DHT was used in this study in lieu of testosterone because of its high potency and carcinogenicity in prostate cancer. Furthermore, it is not metabolized in this system. Final concentrations used in this study were based on physiological levels of compounds from natural sources (e.g., soy isoflavones) that could be found in the blood when consumed in moderate to high amounts. Twenty-two of the 72 tested flavonoids and structurally related compounds were found to significantly inhibit PSA production in the BT-474 breast cancer cell line (Table 1, Fig. 1). These include the soy isoflavones, biochanin A and genistein, the red wine phytoalexin resveratrol, and naringenin, a flavanone found in citrus fruits. Eight of these compounds showed dose–response blocking of PSA production from $10^{-5}$ down to $10^{-7}$ mol/l (Fig. 2). Eleven of the 22 compounds were previously demonstrated to have estrogen activity, while the other 11 were found to be non-estrogenic (Table 1) [64]. Estradiol also blocked PSA production from $10^{-7}$ down to $10^{-9}$ mol/l in the BT-474 cell line (Fig. 2).

In the PC-3$(AR)_2$ clonal line, several of these compounds showed no significant blocking of PSA production. These included genistein, biochanin A, harmalol, naringenin and 6-hydroxyflavone. Similarly, estradiol did not inhibit PSA production in this

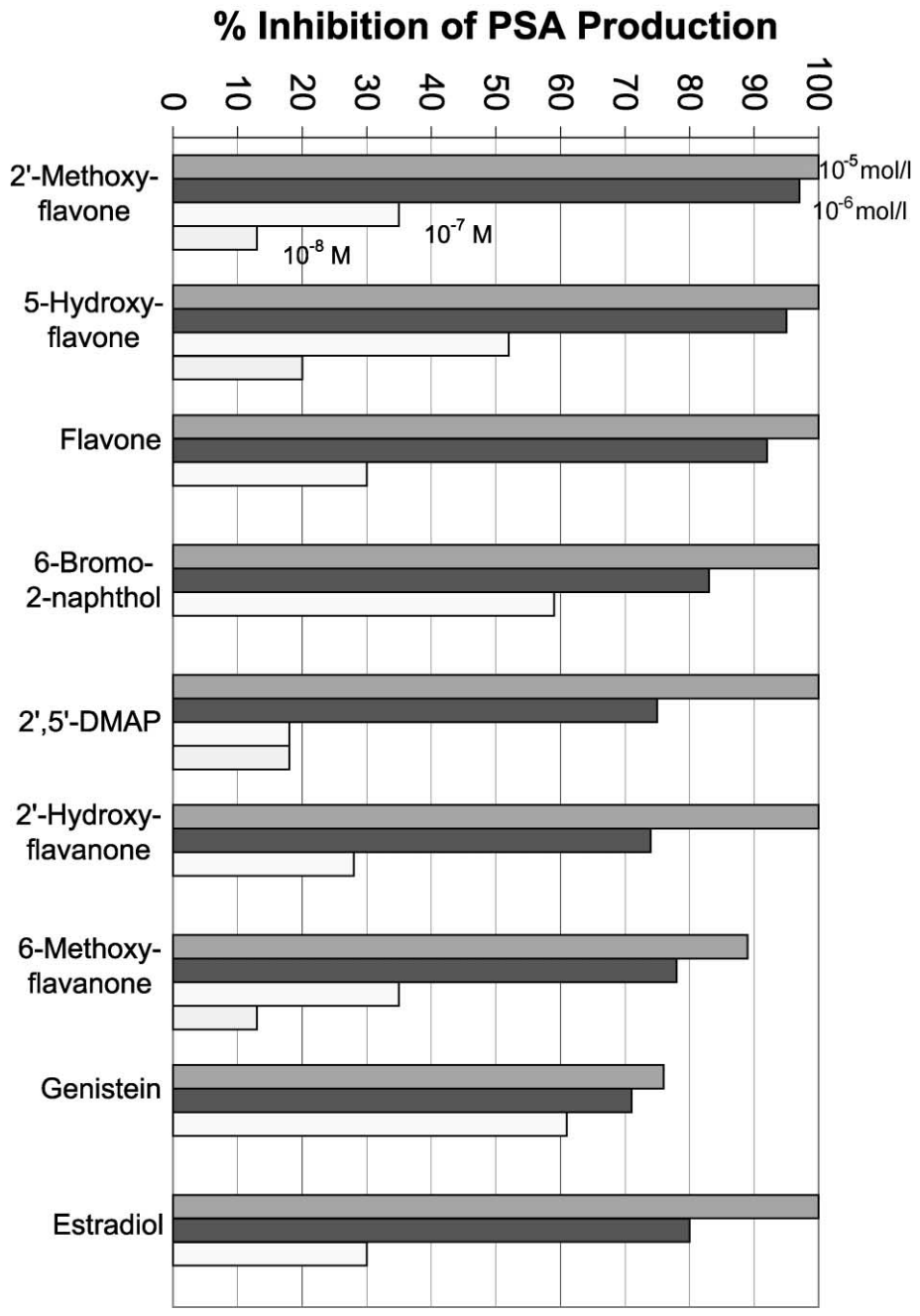


Fig. 2. Dose–response inhibition of PSA production of flavonoids and related compounds in the BT-474 human breast cancer cell line. Significant inhibition is shown as percent blocking of DHT-induced PSA production. Compounds were tested at $10^{-5}$–$10^{-8}$ mol/l, estradiol was tested at $10^{-7}$–$10^{-9}$ mol/l, and DHT was used at $10^{-9}$ mol/l. 2′,5′-DMAP=2′,5′-dimethoxyacetophenone.

Table 2
Flavonoids and related compounds that did not inhibit PSA production

| | | |
|---|---|---|
| Abrine | 7,8-Dihydroxyflavone | 7-Hydroxyflavone |
| Andrographolide | 2′,6′-Dimethoxyacetophenone | Karajin |
| Apigenin | 2,3-Dimethoxybenzaldehyde | 5-Methoxyflavanone |
| Ascorbic acid | 3,4-Dimethoxycinnamic acid | 7-Methoxyflavanone |
| 5,6-Benzoflavone | 7,8-Dimethoxyflavone | 7-Methoxyflavone |
| 7,8-Benzoflavone | Ellagic acid | 6-Methylflavone |
| Bixin | Embelin | L-Mimosine |
| 4′-Bromoacetophenone | Fisetin | Morin |
| Caffeic acid | Flavanone | Naringin |
| Caffeine | Folic acid | Picrotin |
| Chlorogenic acid | Gallic acid | Piperine |
| Conessine | Gardenin | Pongamol |
| Daidzein | Harmaline | Quercetin |
| 2′,4′-Dihydroxyacetophenone | Harmine | Rutin |
| 2′,5′-Dihydroxyacetophenone | 7-Hydroxyflavan | Salicylic acid |
| 2′,6′-Dihydroxyacetophenone | 6-Hydroxyflavanone | Theophylline |
| 2,5-Dihydroxy-1,4-benzoquinone | 3-Hydroxyflavone | |

cell line (Table 1). The compounds listed in Table 2 did not alter PSA production in any of the two cell line systems.

## 4. Discussion

To date, many anti-hormonal agents are being used to treat prostate cancer, including the anti-androgens nilutamide and cyproterone acetate [19–22]. As these drugs have various side effects [23–29], many patients are searching for ''natural'' alternatives or complements to these therapies. Plant-derived products and compounds, such as flavonoids, have recently grabbed much attention. Of particular attention are the soy isoflavones, such as genistein and daidzein [65]. Flavonoids are now also being evaluated in terms of prostate cancer prevention [66]. The full impact of these compounds, found in fruits, vegetables, tea and others on prostate disease development and treatment has yet to be determined.

The purpose of this study was to evaluate inhibition of androgen-regulated gene expression by flavonoids and structurally related compounds, measured as inhibition of DHT-induced PSA production in two cell line-based systems. Several studies have investigated estrogen activity of similar compounds and natural products containing these polyphenols [64,67,68]. However, very few studies have examined androgenic or anti-androgenic activities [49–52,69].

Our study has demonstrated that several flavonoids can inhibit PSA production, including isoflavones (genistein, biochanin A), flavones (luteolin, chrysin), and flavanones (naringenin), which were hydroxylated (5-hydroxyflavone, 2′-hydroxyflavanone), methylated (6-methoxyflavanone, 5′-methoxyflavone) or neither (flavone). Other compounds that showed significant PSA-blocking include nitrogen-containing (6-bromo-2-naphthol, harmalol, harmol), and carboxyl-containing (curcumine, 2′ 5′-dimethoxyacetophenone) phenols. Several of these compounds were shown to have estrogenic activity in a previous study [64]. However, unlike estrogenic activity, which was determined to follow structure–function relationships, inhibition of PSA production was not observed to possess such relationships.

The ability of flavonoids and other polyphenols in regulating androgenic effects is not well studied. Those that have been conducted have shown several mechanisms by which these compounds inhibited PSA and other androgen-regulated proteins. Three papers have used the human prostate cancer cell line LNCaP, derived from the lymph node metastasis of a prostate cancer patient. The study by Mitchell et al. [52] demonstrated dramatic decreases in androgen-induced PSA and human glandular kallikrein (hK2) production in the presence of resveratrol (50–150

μmol/l). Our data supports this finding, but we used much lower resveratrol concentrations (10 μmol/l), which may be more physiologically relevant. Using a PSA-promoter fragment in front of a luciferase reporter gene, they showed that this phytoalexin abolished androgenic induction of the PSA promoter, resulting in significant decreases in transcription of both AR and androgen-regulated genes.

Similar results were seen with green tea polyphenols (GTP) [69]. It was found that at 20–60 μg/ml of GTP incubated with LNCaP cells 1 h prior to treatment with testosterone significantly reduce the production of ornithine decarboxylase, another androgen-regulated protein. Northern blot analysis revealed that this almost complete inhibition occurred at the transcriptional level. Moreover, in vivo, when GTP was added to drinking water, at 0.2% w/v, a 40% inhibition of ornithine decarboxylase activity was found in castrated and sham-operated rats, compared to controls. These two studies indicate that anti-androgen activity of these compounds must occur through inhibition of transcription of AR and/or androgen-controlled genes, blocking of the receptor, or a combination of these events. These are similar to the potential mechanisms by which the compounds tested in the current study may act to inhibit DHT-induced PSA production.

A study conducted with flavonoids, also in LNCaP cells, found that biochanin A significantly induced activity of UDP-glucuronyl transferase, an enzyme responsible for metabolizing testosterone to inactive products. Through this mechanism, inhibition of PSA production was also found [49]. In this study, a slight increase in binding sites of the AR and AR protein was noted, therefore, inhibition of AR transcription was not a plausible mechanism for the action of these flavonoids. DHT, a non-metabolized androgen in our system, was used, so this mechanism is not relevant to the present study.

In conclusion, we found that several flavonoids and related compounds can significantly inhibit PSA production, an androgen-regulated protein. This inhibition may be reflective of a decrease in androgenic activity of DHT, a carcinogenic androgen to the prostate. Therefore, these plant-derived compounds may thus play a role in the prevention and/or management of prostate cancer. However, more research is needed to determine the mechanisms by which flavonoids inhibit production of this and other androgen-regulated genes, and how these compounds, and foods containing them, may be used in men.

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