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Modulation of Androgen and Progesterone Receptors by Phytochemicals in Breast Cancer Cell Lines

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We have used a tissue culture system based on breast carcinoma cell lines to investigate a large number of naturally occurring compounds and beverages for steroid hormone agonist and antagonist activity. The cell lines used, T-47D and BT-474, produce prostate specific antigen (PSA) upon stimulation with androgens, progestins, glucocorticoids and mineralocorticoids. This biomarker is secreted and can be measured in the tissue culture supernatant with very high sensitivity by an immunofluorometric procedure. Steroid hormone antagonist activity can be assessed with the same system by adding the candidate antagonist first and then stimulating the cells with a known agonist. By using this system we have identified three natural compounds, apigenin, naringenin and syringic acid which exhibited weak progestational activity and eleven other compounds which exhibited weak antiandrogenic/antiprogestational activity. Our study indicates that a significant number of natural compounds have the ability to bind to steroid hormone receptors and act as weak blockers. A fewer number of compounds not only bind to the receptors but they also mediate transcriptional activity, acting as agonists. The agonists and antagonists were active at levels around 10^{-5} M, in accordance with previous reports for other phytochemicals. In comparison to synthetic and natural steroid hormones, the biological activity of these compounds is weaker by a factor of approximately 10^4-fold.

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Recent literature reports indicate that dietary and environmental factors may contribute substantially to cancer incidence. Excellent recent reviews summarize the current knowledge, which was generated by using cell lines, animal models as well as humans (1-5). The role of endogenous and exogenous steroid hormones in the development of breast, prostate and other cancers is well recognized (6-8). More recently, the potential biological impact of environmental and dietary estrogens on human health has generated considerable interest (9-13). These agents include phytoestrogens as well as a variety of synthetic compounds. Many of the known phytoestrogens are either flavonoids, coumestans or resorcylic acid lactones (12,14). Some phytoestrogens appear to be protective against cancer (12,15,16). Anticarcinogenic activity has been noted for a number of other natural products which, in general, have antioxidant activity (17,18). Recently, two reports describe anticarcinogenic and weak estrogenic activities of the phytoalexin resveratrol (19,20). Many other natural compounds were also found to have weak estrogenic activity (21) while others, like quercetin, appear to act as anticarcinogens via different mechanisms one of which is downregulation of mutant oncogenic proteins (22). There is now a large body of evidence indicating that natural compounds can affect signal transduction pathways, cell proliferation rates and cell differentiation and many of these compounds have hormone-like, antioxidant and antimutagenic activity. Some of these compounds may have potential for cancer prevention or even cancer therapy (12,23-25).

We have previously developed a tissue culture system, based on breast carcinoma cell lines which are steroid hormone receptor-positive (26,27). We found that when these cell lines are stimulated by steroid hormones, they can produce prostate-specific antigen (PSA) and secrete it into the culture medium. Since PSA is not produced at detectable amounts in the absence of steroid hor-

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mones, we have proposed this system as a sensitive tool for evaluating the biological activity of candidate androgens, progestins, glucocorticoids and mineralocorticoids. Estrogens do not mediate appreciably PSA production in these cell lines. With a modified procedure, we can utilize the same tissue culture system to assess receptor blocking activity by first exposing the cells to a candidate blocking compound and then inducing them with a known agonist (progestin or androgen). Any blocking activity will manifest as an inhibition of the agonist to upregulate the PSA gene. The system is highly sensitive since no PSA production occurs without steroids and the detection of PSA in the tissue culture supernatant can be accomplished with a highly sensitive and specific immunoassay (28).

In this paper we have assessed the biological activity of various natural products as well as purified compounds using the described tissue culture system. While we have found a number of compounds that can exhibit blocking activity, the only compounds that acted as weak agonists in the system were the 7-hydroxy flavonoids apigenin and naringenin, and the cinnamic acid derivative syringic acid. All three compounds appear to behave as weak progestins.

MATERIALS AND METHODS

Compounds. The compounds and natural products tested are shown in table 1. Steroids used for control purposes were obtained from Sigma Chemical Co., St. Louis, MO. Stock solutions of the purified compounds (10^{-2} or 10^{-3} M) were prepared in absolute ethanol. More dilute solutions were also prepared in the same solvent. Beverages (wine, tea and extracts) were used without further dilution.

Stimulation experiments. The steroid hormone receptor-positive breast carcinoma cell line T-47D was obtained from the American Type Culture Collection (ATCC), Rockville, MD. T-47D cells were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mM), bovine insulin (10 mg/L), fetal bovine serum (10%) and antibiotics (penicillin, streptomycin). The cells were cultured to near confluency in plastic culture flasks and then transferred to phenol red-free media containing 10% charcoal-stripped fetal bovine serum with antibiotics. Phenol red-free media were used as phenol red was previously found to have weak estrogenic activity (29) and charcoal-stripped fetal bovine serum is devoid of steroid hormones.

The T-47D cells were then aliquoted into 24-well tissue culture plates and cultured to confluency with change in media at 3 days. Stimulations were carried out with confluent cells containing 2 mL of phenol red-free media with 10% charcoal-stripped fetal calf serum and antibiotics. Stimulation was initiated by adding 2 μL of each steroid, other compounds or natural products to be tested, either undiluted or diluted in 100% ethanol, and incubating for a certain period of time (usually up to 8 days). Tissue culture supernatants ($\sim$400 μL) were removed for PSA analysis. Appropriate multiple positive controls (with stimulating steroids) and negative controls (only alcohol or nothing added) were included in each experiment. Wells with microbial contamination were excluded from the data analysis.

The BT-474 breast carcinoma cell line, which is also positive for steroid hormone receptors, was used as described above for T-47D cells. Both cell lines have the capability to produce PSA upon stimulation by steroid hormones.

Dose-response experiments. For dose-response experiments, we followed the same protocol as for the general stimulation experiments described above, but the compounds (2 μL per well) were added at various concentrations. The dilution of each stock compound solution in the well was 1000-fold for all experiments. The final concentration of each steroid or compound was used for data interpretation.

Blocking experiments. Blocking experiments were performed for two reasons: (a) To identify if any of the compounds in Table 1 can block the steroid hormone receptors and thus act as antagonists of steroid hormone action; (b) To identify if the compounds that can stimulate PSA production in this culture system are candidate androgens or progestins.

For identifying blocking activity, experiments were performed by parallel examination of the following possibilities: (a) stimulation of the cells with the candidate blocker alone at a final concentration of 10^{-6} M; (b) stimulation with the stimulating steroid alone (dihydrotestosterone, an androgen or norgestimate, a progestin) at a final concentration of 10^{-7} M; (c) adding the candidate blocker to the cells at a final concentration of 10^{-6} M, incubating for 1 h and then adding the stimulating steroid at a concentration of 10^{-7} M. We have also included controls with ethanol only (negative controls). This protocol allows for a direct comparison of the stimulating activity of either the candidate blocker or the stimulating steroid and the effect of the candidate blocker on the ability of the stimulating steroid to induce PSA expression when the blocker is allowed to bind to the receptors at tenfold higher concentration for 1 h before the addition of the stimulating steroid.

For identifying progestational or androgenic activity of the weak agonists apigenin, naringenin and syringic acid, we used the known steroid hormone receptor blockers mifepristone (antiprogestin) and RU 56187 (antiandrogen) at a concentration of 10^{-8} M and the weak agonists at a concentration of 10^{-5} M.

Measurement of PSA. PSA was measured in the tissue culture supernatants with a highly sensitive immunofluorometric procedure described in detail elsewhere (28). This assay can measure PSA at levels of 1 ng/L or higher (up to 10, 000 ng/L) with a precision of $<$10%. All assays were performed in duplicate. Tissue culture supernatants were measured undiluted using 100 μL aliquots per assay.

Calculations. All tissue culture experiments were performed in quadruplicate and the results of PSA analysis are expressed as mean $\pm$ standard error of the mean (SEM). Percent (%) residual activity of the stimulating steroid was calculated by dividing the concentration of PSA produced by the stimulating steroid in the presence of the candidate blocker, and the PSA concentration produced by the stimulating steroid in the absence of the candidate blocker, multiplied by 100. Percent blocking was calculated by subtracting the % residual activity from 100.

RESULTS

We first examined if any of the compounds listed in Table 1 have agonist activity on the steroid hormone receptor system, as revealed by PSA production by the cell lines T-47D and BT-474. Appreciable amounts of PSA were produced by both cell lines upon stimulation by apigenin, naringenin and syringic acid. All other compounds were inactive. The structures of these compounds are shown in Figure 1. The three compounds were active at concentrations around 10^{-5} M; at concentrations $\leq 10^{-6}$ M their agonist activity becomes nondetectable. In comparison to the strong agonists norgestrel (progestin) and dihydrotestosterone (androgen), the three compounds were approximately 10^4-fold less potent since about the same concentration of PSA in

TABLE 1

Compounds and Natural Products Tested in this Study[1]

Compound/Product	Present in/Classification
Apigenin	fruits, vegetables and leaves
Ascorbic acid	antioxidant vitamin
Biochanin A	soya
Cabernet franc	red wine
Caffeic acid	coffee
Caffeine	coffee
β-Carotene	antioxidant vitamin
Catechin	tea and red wine
Chlorogenic acid	coffee
Chlorophylline	all green plants and vegetables
m-Coumaric acid	tea and red wine
p-Coumaric acid	tea and red wine
Daidzein	soya
Earl Grey	tea
Ellagic acid	berries
Ferulic acid	tea and red wine
Folic acid	B vitamin
Gallic acid	tea and red wine
Gamay noir	red wine
Genistein	soya
Genistic acid	tea and red wine
Green tea	tea
Hesperetin	citrus fruits
Hesperidin	citrus fruits
Homocysteine	associated with folic acid deficiency
Kaempferol	fruits, vegetables and leaves
Luteolin	fruits, vegetables and leaves
Methylcobalamin	B vitamin
Morin	tea and red wine
Myricetin	fruits, vegetables and leaves
Naringenin	citrus fruits
Naringin	citrus fruits
Oolong tea	tea
Pyridoxine	B vitamin
Quercetin	tea and red wine
Rutin	tea and red wine
Rutin trihydrate	tea and red wine
Salicylic acid	berries
Saw palmetto	fruit extract used in prostate disease
Syringic acid	tea and red wine
Taxifolin	tea and red wine
Theobromine	coffee
Theophylline	coffee
α-Tocopherol	antioxidant vitamin
Vanillic acid	tea and red wine

[1] All compounds were obtained from Sigma Chemical Co., St. Louis, MO, USA except wine, m-coumaric acid, p-coumaric acid, genistic acid, syringic acid, vanillic acid (Liquor Control Board of Ontario), and daidzen, biochanin A and genistein (Indofine Chemical Co., Sommerville, NJ, USA).

the supernatant (~200-500 ng/L at 8 days) could be obtained with norgestrel or dihydrotestosterone concentrations around 10^{-9} M.

In order to examine if the agonist activity was mediated by either the progestin or androgen receptors, we performed blocking experiments with the antiandrogen RU 56,187 and the antiprogestin mifepristone (RU 486). The antiandrogen RU 56,187 was able to block >50% of dihydrotestosterone action (control) but had minimal or no effect on either norgestimate (progestin; also used as a control) or apigenin, naringenin, or syringic acid action. In contrast, mifepristone was able to block significantly the activity of all three natural compounds as well as norgestimate (used as a control). The data of blocking experiments are summarized in Table 2. These data allow us to hypothesize that the three phytochemicals act as weak progestins.

We have further examined if any of the compounds of Table 1 have blocking activity on the steroid hormone receptor system. We have identified 11 plant-derived compounds which exhibited significant antiprogestational and/or antiandrogenic activity, as revealed by their ability to block PSA production by more than 50% under the experimental conditions described. These results are shown in Table 3.

DISCUSSION

There is now considerable interest in naturally occuring or xenobiotic compounds which may exhibit steroid hormone-like activity. Most published work deals with estrogen-like compounds (9-11). This interest stems from the knowledge that such compounds may, on long-term exposure, contribute to the pathogenesis and progression or prevention of hormonally-dependent cancers like breast, prostate and endometrial cancer (30). Natural products that may contain such steroid hormone-like compounds include vegetables, fruits, alcoholic beverages, tea, and wood extracts (31-35).

One of the challenges of such research initiatives is to discover and utilize appropriate techniques for assessing the biological activity of candidate natural and xenobiotic compounds. The demonstration that a compound binds to a steroid hormone receptor is not necessarily related to function since such binding may not lead to any biological effect. Improved systems for assessing biological ac-

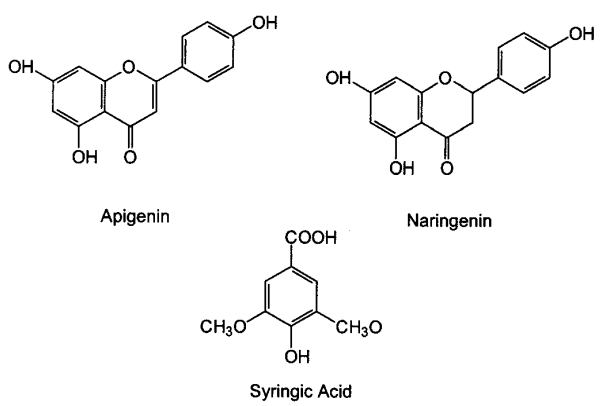

FIG. 1. Structures of Apigenin, Naringenin and Syringic Acid, the only compounds from those of Table 1 which demonstrated weak agonist activity on the steroid hormone receptor system.

TABLE 2

Blocking Experiments to Elucidate the Biological Activity of the Three Phytochemicals in BT-474 Breast Cancer Cells

Stimulating compound[1]	Blocking compound[2]/ % blocking activity	
	RU 56,187	Mifepristone
Dihydrotestosterone (control)	51	18
Norgestimate (control)	0	93
Apigenin	17	95
Naringenin	32	80
Syringic acid	0	94

[1] Added at a concentration of 10^{-9} M for dihydrotestosterone and norgestimate and 10^{-5} M for the three phytochemicals.

[2] Added at concentration of 10^{-8} M one hour prior to the addition of the stimulating compound as described under "Methods". RU 56,187 is an antiandrogen and mifepristone is an antiprogestin.

tivity of compounds include the use of plasmids containing steroid hormone receptor response elements upstream of reporter genes (20,36). These systems may produce artificial results associated with the transfected plasmids and the multiplicity of the response elements that are usually present. A more preferable system includes stimulation of untransfected cells and then monitoring of the endogenous regulation of an indicator gene at either the level of mRNA or protein. Estrogen upregulated genes used for this purpose include the progesterone receptor (20) and pS2 (21).

In our study, we focused on androgen-like and progestin-like agonist and antagonist activity of a relatively large number of compounds (Table 1). Such activity has not been examined in the past as most published studies have focused on estrogenic and antiestrogenic activity. Our experimental system includes two carcinoma cell lines which are steroid hormone receptor-positive. Once stimulated by a progestin or androgen, but not estrogen, these cell lines produce and secrete PSA. The analysis of PSA in the tissue culture supernatant is an indicator of the biological activity of the compounds tested. Once an agonist activity is identified, further experiments with known steroid hormone receptor blockers can reveal if the candidate compound has either progestin-like or androgen-like activity. Similarly, the exposure of the cells to a candidate blocker, followed by stimulation with a known pure agonist (norgestimate, a progestin and dihydrotestosterone, an androgen) can provide information on the blocking activity of these compounds (antiprogestational or antiandrogenic activity).

Among all the compounds tested, only three have shown agonist activity, two of them being flavonoids and the other being a cinnamic acid derivative. Blocking experiments have revealed that these three compounds appear to be weak progestins, acting at concentrations around 10^{-5} - 10^{-6} M . These concentration levels are similar to those found to be necessary for resveratrol action as a weak estrogen (20). The biological activity of the three compounds is about 10^4-fold lower than the activity of synthetic progestins like norgestrel and norgestimate. In addition to this finding, we report considerable blocking activity of eleven phytochemicals on the activity of norgestimate or dihydrotestosterone (Table 3).

It is now realized that hormonally-dependent cancers are not associated with only one class of hormonal stimuli alone but rather, with an integrated environment consisting of various interdependent factors including steroid hormones, growth factors and other mitogens (30). For example, breast and prostate cancers are dependent on the various proportions of steroid hormones with proliferative and differentiating potential i.e. estrogens, progestins and androgens (30). Consequently, the possible diverse activities of natural and xenobiotic compounds should be of interest, and investigations should not be limited to their estrogenic or antiestrogenic activity. In this respect, our study is unique since it has revealed novel biological agonist (progestational) and antagonist (antiprogestational/antiandrogen) activities of a number of natural compounds.

The extrapolation of our findings to human biological effects is difficult to evaluate since the data are derived with a tissue culture system and the details of absorption and pharmacokinetics of the majority of the tested compounds are not known. Similar interpretitive difficulties were discussed by others for different naturally occurring compounds. For example, Jang et al (19) reported anticarcinogenic activities of the trihydroxylstilbene resveratrol, while Gehm et al (20) proposed that this compound may, in fact, promote cancer due to its weak estrogenic activity.

TABLE 3

Blocking of Steroid Hormone Activity by Plant-derived Compounds

Blocking compound	% Blocking ± SEM[1] of	
	Dihydrotestosterone	Norgestimate
β-Carotene	82 ± 3	48 ± 11
Chlorogenic acid	61 ± 2	24 ± 12
Chlorophylline	87 ± 7	94 ± 3
m-Coumaric acid	21 ± 12	53 ± 11
Ferulic acid	44 ± 24	53 ± 15
Folic acid	43 ± 19	52 ± 20
Hesperetin	15 ± 13	79 ± 11
Homocysteine	75 ± 15	67 ± 20
Syringic acid	20 ± 12	72 ± 11
Taxifolin	80 ± 5	58 ± 11
α-Tocopherol	65 ± 10	65 ± 5

[1] SEM, standard error of the mean; four replicates per experiment. The blocking protocol is described under "Methods".

In conclusion, our study contributes data on the possible biological activity of a number of natural compounds and products. It is hoped that when such data become more complete, that these findings may help with a rational approach of cancer chemoprevention, either through better dietary adjustments or supplementation of diet with purified natural, semisynthetic, or synthetic hormone or antioxidant analogues.

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