Flavonoids Inhibit Estrogen Binding to Rat Alpha-Fetoprotein (44238)

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Abstract. **There is considerable interest in the role(s) of plant-derived compounds such as bioflavonoids in regulating steroid hormone action in mammals, and in particular, the possible effects of the bioflavonoids on the growth of steroid-dependent breast and prostate tumors and on possible abnormal development of steroid-sensitive tissues. Studies of the hormone-like actions of bioflavonoids often use fetal or neonatal rats, which contain high levels of serum alpha-fetoprotein (AFP), a protein that binds estradiol with a $K_d \sim 5 \times 10^{-9}$ *M*. Interaction of bioflavonoids with AFP could affect the availability of estrogens to estrogen-responsive cells, as well as the actions of bioflavonoids. These considerations motivated us to study the effect of several flavonoids (quercetin, rutin, naringenin, chrysin, apigenin, kaempferol, myricetin, morin, fisetin) and isoflavonoids (daidzein, genistein) on estrogen binding to rat AFP. We found that naringenin, a flavanone, and quercetin and kaempferol, flavonols, inhibit estrogen binding to AFP with apparent K_ds of about 5×10^{-7} *M*. To our surprise, the two isoflavonoids, daidzein and genistein, have K_ds of about 5×10^{-6} *M* for AFP. This 10-fold difference in affinity for AFP between flavonoids and isoflavonoids suggests that AFP has a specificity for the flavonoid structure. Moreover, the affinities of bioflavonoids for rat AFP are sufficiently high to suggest that flavonoids and isoflavonoids could modulate estradiol and estrone binding to rat AFP *in vivo*, when present at dietary levels. Additionally, the potency of the plant estrogens may be altered by binding to AFP. The flavonoids that we tested have different hydroxyl and glucoside substituents on the A, B, and C rings, which allows us to define some of the spatial requirements for binding to AFP. We find that 5,7-hydroxyl groups in ring A and a 4′-hydroxyl group in ring B are important for binding to AFP. This information, combined with molecular modeling studies, may elucidate the molecular basis for recognition of flavonoids and estrogens by AFP. Also, these findings indicate that the flavonoid levels in the diet need to be considered in studies of the effects of various xenobiotics and endocrine manipulations on experimental animals, particularly during development when serum estrogen binding protein concentrations are often elevated. Finally, bioflavonoids should be useful tools for understanding the variety of estrogen actions initiated by different structural classes of estrogens.**

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The use of plants in medicine goes back at least 5000 years to the dawn of cities in Summaria, China, and India (1–3). Many of the effects of plants resemble those of adrenal and ovarian steroids in humans. This characteristic has stimulated interest in the pharmaceutical community in isolating plant-derived compounds, with the goal of using them for treating diseases, such as cancer, hypertension, diabetes, and arthritis. Concurrently, considerable research is focused on how to add vegetables and fruits that contain medicinally beneficial compounds to human diets for preventing disease. This could serve as an inexpensive means for a widespread improvement in the health of the population. However, additionally, concern has been expressed that some phytoestrogens may disrupt the developing endocrine system similarly to the effects of other estrogens (4).

Considerable effort has been directed toward identifying constituents in diet that either prevent or contribute to disease and to elucidating the role of diet in etiology of

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human disease (4–9). With modern techniques such as high-pressure liquid chromatography (HPLC), gas chromatography, and mass spectrometry, it has been possible to isolate and characterize some biologically active compounds in plants. These analyses have identified as potentially important compounds, the flavonoids and terpenes, which are widely distributed in plants and have been found to have steroid hormone–like activities in humans, mimicking either androgens, estrogens, glucocorticoids, or mineralocorticoids (8–13). The mechanisms of action of these phytohormones are complex because, like steroids, phytohormones may bind to one or more proteins including hormone receptors, hormone-binding proteins in serum, and enzymes that metabolize steroid hormones. The extent of interaction of phytohormones with one or more of these proteins could modulate steroid hormone action to produce profound physiological effects (4–9).

For the most part, the primary focus has been on understanding phytohormone binding to steroid receptors and determining if the phytohormone acts as either a steroid hormone agonist or antagonist in regulating transcription of steroid-dependent genes. However, as noted above, this is not the only possible mechanism. For example, the triterpenoid glycyrrhizic acid appears to have glucocorticoid and mineralocorticoid activity due to inhibition of 11β-hydroxysteroid dehydrogenase, which converts hydrocortisone to cortisone, an inactive steroid (10–13). This example of an enzyme-mediated activity of a plant-derived compound emphasizes the importance of considering actions of plant-derived compounds with nonreceptor proteins that bind steroids.

Another potentially important class of proteins that could alter hormone-like actions of plant-derived compounds are steroid hormone binding proteins in serum, which include mouse and rat alpha-fetoprotein (AFP), human sex hormone binding globulin (SHBG), albumin, corticosteroid binding globulin, and progesterone binding globulin. Such carrier proteins have at least two roles in hormone action. First, they sequester the hormone, which reduces metabolism by enzymes (14). Second, they modulate the concentration of hormone that is available to the target cell (15, 16). Both activities of carrier proteins would be affected by a bioflavonoid displacing the steroid from its carrier. Of course, binding of bioflavonoids to carrier proteins would also affect the half-life of the bioflavonoid and its accessibility to cells and thus potency. Therefore, the interaction of bioflavonoids with carrier proteins is of importance for both bioflavonoid and steroid hormone action. The widespread use of pregnant rats and their offspring for investigation of estrogenic actions of naturally occurring compounds as well as synthetic compounds for benign and toxic effects requires an understanding of possible compounds that displace estrogen from rat AFP. Moreover, by studying the binding to AFP of compounds with related structures, we could begin to define structure-activity relationships for AFP and accumulate a database of structure-biological activity relationships for improving the evaluation of new compounds regarding their beneficial and toxicological actions in humans as part of a program underway at the National Center for Toxicological Research.

These considerations motivated us to examine the interaction of bioflavonoids with rat AFP, which binds estrogens with high-affinity (17–21) and is present in high concentrations in newborn rats (22) and amniotic fluid.

Methods

[^{3}H]Estrone was purchased from Dupont NEN. Other chemicals were purchased from Sigma Chemical, Co., (St. Louis, MO).

Rat alpha-fetoprotein (AFP) was obtained from amniotic fluid and partially purified by passage through a Cibacron Blue column (Sigma). For most binding assays, AFP was diluted to about 0.9 n*M*, a concentration that is below the K_d of estrone for AFP (19–21). This condition is important for getting accurate inhibition constants from analyses of competition of flavonoids with [^{3}H]estrone for binding to AFP. The AFP was incubated with 3×10^{-9}*M* [^{3}H]estrone (about 50% saturation) in 20 m*M* Hepes pH 7.4 + 50 m*M* NaCl + 20% glycerol (HNG buffer) for 30 min at room temperature and then for 60 min on ice. This time is sufficient for equilibrium binding of estrogens and competing compounds (20, 21). The competing bioflavonoids, (dissolved in ethanol), were added to glass tubes containing 3 n*M* [^{3}H]estrone. 3×10^{-6} *M* estrone was added to a tube with [^{3}H] estrone to determine nonspecific binding, which was about 10%–15% of total binding. Nonspecifically bound counts were subtracted from total counts to yield specifically bound counts. Bound steroid was separated from free using dextran-coated charcoal (20, 21). Measurements were done in duplicate, with variation less than 10%. Each experiment was repeated at least three times.

Scatchard analysis was done as described previously (20, 21, 23). Briefly, rat AFP was incubated with [^{3}H]estrone concentrations of 5×10^{-10} *M* to 1.5×10^{-8} *M*. Separate tubes prepared as above had 3×10^{-6} *M* unlabelled estrone added to determine nonspecific binding. Another set of tubes had AFP, [^{3}H]estrone and 0.75×10^{-6}M quercetin. The specific binding in the tubes with [^{3}H]estrone alone and with quercetin was analyzed by the method of Scatchard. The affinity of quercetin was determined using equations described by Dixon and Webb (24) for analysis of competitive inhibition of enzymes.

Results

We investigated the effect on estrogen binding to rat AFP of a variety of bioflavonoids with diverse structures shown in Figure 1 with the goal of elucidating some of their structural requirements for binding to rat AFP. First, we examined the ability of 3×10^{-6}*M* of the various flavonoids to compete with 3×10^{-9} M [^{3}H] estrone for binding to rat AFP. The results are summarized in Table I.

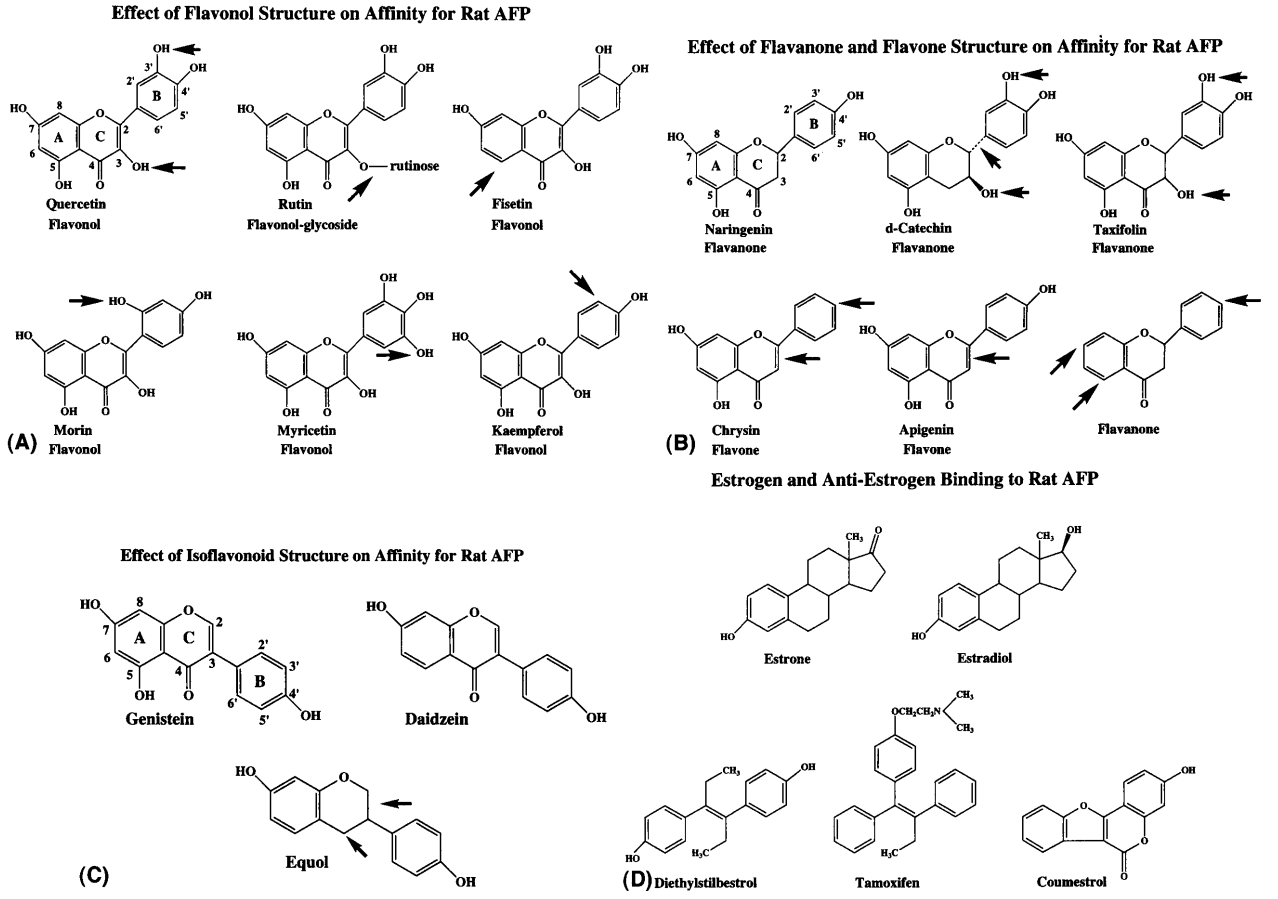

Figure 1. Structures of flavonoids. (A) Flavonols. Compounds are compared to quercetin, which along with kaempferol and naringenin, has the highest affinity for rat AFP among the compounds that we studied. Arrows on the flavonols identify differences with quercetin. Arrows on quercetin compare it to kaempferol and identify the C3-hydroxyl as likely to be important in binding to rat AFP. (B) Flavanone and Flavones. Arrows compare the compounds to naringenin. (C) Isoflavonoids. Arrows show differences in equol that could account for its increased affinity for rat AFP compared to that of daidzein and genistein. (D) Estradiol, synthetic estrogens, and coumestrol. Although diethylstilbestrol has a K_d of less than 1 n*M* for the estrogen receptor, it has a K_d of about 1 μ*M* for rat AFP.

To further quantify the binding affinity of the bioflavonoids, we studied the effect of 0.75 μ*M* quercetin on the binding of [^{3}H]estrone to rat AFP using competitive Scatchard analysis. As shown in Figure 2, in the presence of quercetin the affinity is lowered, and the number of binding sites remains unchanged. The calculation of the K_d of quercetin for AFP from the K_d of estrone alone and in the presence of 0.75 μ*M* quercetin is shown in Table II. The analyses in Figure 2 and Table II indicate that quercetin is a competitive inhibitor with a K_d of about 0.5 μ*M*. This is about 100-fold lower than that of estradiol (17–21) and about the same as that of DES and ethynylestradiol (14).

Discussion

Flavonoid Structure and Binding to Rat AFP. Examination of the structures in Figure 1 and the data in Table I reveals interesting structure-activity relationships among the various bioflavonoids that we tested. The similar affinities for AFP of quercetin, naringenin, and kaempferol, and the lower affinity of taxifolin allow us to understand some of the requirements for binding to rat AFP. It is clear that the unsaturated 2–3 bond in ring C is important if there is a C3-hydroxyl. However, removal of the C3-hydroxyl eliminates the requirement for the 2–3 unsaturated bond in the C ring. The 3′-hydroxyl in the B ring is not essential for binding although movement of the 3′-hydroxyl to the 2′ position in the B ring in morin lowers the affinity for AFP. Similarly, removal of the hydroxyl at position 5 on the A ring, as seen in fisetin, lowers the affinity for AFP. Adding a rutinose substitutent to the hydroxyl at C3 leads to a dramatic reduction in affinity.

Garreau *et al.* (25) found that equol had a K_d of about 6 μ*M* for rat AFP, which in combination with our findings for the other two isoflavonoids, daidzein and genistein, suggests a lower affinity for the isoflavonoid structure compared to the flavonoid structure. It clearly shows that the spatial relationship between the B and C rings is important for flavonoid binding to rat AFP.

Biological Implications. Our finding that some bioflavonoids have affinities of less than 1 μ*M* for rat AFP has important biological implications. This affinity is only 100-fold lower than that of estradiol, which suggests that under certain dietary regimens in which high serum levels are achieved, the concentrations of bioflavonoids could be suf-

Table I. Effect of Bioflavonoids on ^{3}[H] Estrone Binding to Rat AFP

Compound	% Inhibition
A. Flavonols	
Quercetin	72.0
Kaempferol	72.0
Myricetin	42.0
Fisetin	22.5
Rutin	15.0
Morin	14.0
B. Flavanones	
Naringenin	66.5
Taxifolin	23.5
Flavanone	14.5
d-Catechin	9.5
C. Flavone	
Apigenin	43.0
Chrysin	26.0
D. Isoflavonoids	
Equol	22.0
Daidzein	10.5
Genistein	10.0
E. Estrogens	
Diethylstilbestrol	67.0
Coumestrol	35.0
Tamoxifen	23.5

Note. Rat alpha-fetoprotein (AFP) was incubated with 3×10^{-9}M ^{3}[H]estrone in 20 m*M* Hepes pH 7.4 + 50 m*M* NaCl + 20% glcyerol (HNG) for 30 min at room temperature and for 60 min on ice. Competitors were added at 3×10^{-6}*M*. Bound steroid was separated from free using dexteran-coated charcoal. Nonspecific binding, counts in a tube with 3×10^{-6} *M* estrone, was subtracted from total counts to yield specifically bound counts. Measurements were done in duplicate, with variation less than 10%. Each experiment was repeated at least three times.

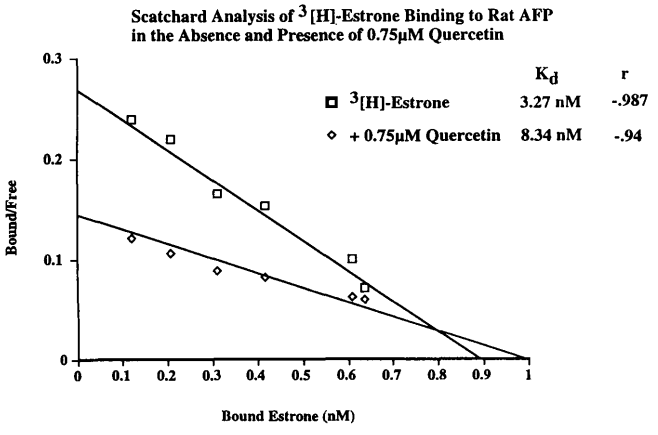

Figure 2. Scatchard analysis of the effect of 0.75 μ*M* quercetin on [^{3}H]estrone binding to rat AFP. AFP was incubated with [^{3}H]estrone (7.5×10^{-10} *M* to 1×10^{-8} *M*) ± unlabelled estrone or 0.75 μ*M* quercetin for 1.5 hr at 0°C. Bound steroid was separated from unbound steroid using dextran-coated charcoal.

ficient to displace estradiol from rat AFP. This could have consequences on estrogen action due to the greater bioavailability of the hormone for both target tissue actions and metabolism (15, 16). Moreover, flavonoid binding to AFP would protect the flavonoid from metabolism which could alter its half-life *in vivo*. However, flavonoid binding to AFP could lower its estrogenic potency compared to chemicals that bind less well. These two competing factors (flavonoid metabolic stability and bioavailability) could modulate flavonoid action in different organs. Additionally, by displacing endogenous estrogens bound to AFP, flavonoids may alter their metabolism and bioavailability.

Table II. Calculation of Quercetin's Affinity for Rat AFP

$$K_d^{Competitor} = K_d C / K_{app} - K_d$$

Where: K_d is the equilibrium dissociation constant of estrone
K_{app} is the apparent dissociation constant in the presence of competitor
C is the concentration of competitor
$K_d^{Competitor}$ is the dissociation constant of the competitor

$$K_d^{Quercetin} = (3.27\ nM)(0.75\ \mu M)/ 8.34\ nM - 3.27\ nM = 0.48\ \mu M$$

As seen in Table I, we find that rutin, the glucoside of quercetin, has low affinity for AFP. However, as with isoflavonoids, bacteria in the gut contain glucuronidases that can metabolize rutin to quercetin.

Garrau *et al.* [25] have also reported that isoflavonoids bind human AFP. However, human AFP binds steroidal estrogens less well than rat AFP. If the bioflavonoids, quercetin, narigenin, and kaemperol, also have μ*M* affinity for human AFP, then bioflavonoid metabolism and bioavailability to the fetus could be modulated by human AFP.

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