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Effect of flavonoids on androgen and glucocorticoid receptors based on in vitro reporter gene assay

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ABSTRACT

The effect of 32 flavonoids on androgen (AR) and glucocorticoid receptors (GR) was investigated using an MDA-kb2 human breast cancer cell line to predict potential AR and GR activities. Among them, 5-hydroxyflavone (**7**) had the highest AR antagonistic activity with an IC_{50} value of 0.3 μM, whereas 6-methoxyflavone (**11**) had the highest induced luciferase activity with an EC_{150} value of 0.7 μM. Genistein (**2**) and daizein (**1**) showed a sufficient increase of luciferase activities as their concentrations increased with EC_{150} values of 4.4 and 10.1 μM, respectively. These findings provide evidence of a fundamental property of their structure–activity relationship with AR and/or GR.

Phytoestrogens with functional similarity to estrogens have been found to influence a variety of biological processes, including the reduction of cancer risk by modulating estrogen receptors (ER).[1,2] To date, the effect of isoflavones such as genistein and daidzein on the mammalian reproductive system have been well understood; however, few studies of dietary flavonoids derived from plants on their hormonal effects have been reported. Our previous studies revealed that some ER agonists, such as estrone (E1), ethinyl estradiol (EE), estriol, diethylstilbestrol (DES) and alkyl phenols, act as androgen receptor (AR) antagonists.[3] Well-known estrogens agonists, such as isoflavones and related flavonoids, have heightened our interest in the structural requirements necessary to interact with AR and glucocorticoid receptors (GR) using established MDA-kb2 human breast cancer cells with endogenous AR and GR, and stably expressing AR and the GR-responsive luciferase reporter gene, MMTV-neo-luc.[3–6] Thus, this reporter gene assay system elicits an increase in luciferase activity (RLU) in the presence of androgens or glucocorticoids. The assay procedures were described in detail previously.[3–6] Briefly, cells were treated with various concentrations of test chemicals from 10^{-8} to 10^{-4} M to determine the AR or GR agonist and/or antagonist activity in the absence and presence of AR agonist, dihydrotestosterone (DHT) or GR agonist, dexamethasone (Dex) at concentrations of 0.2 and 7 nM, respectively. Data were expressed as induced luciferase activity (%) compared to that of the control, that is, induced luciferase activity (%) = [RLU (test chemical)/RLU (control)] × 100. When a test chemical has no activity, the obtained induced luciferase activity (%) is 100%. When a test chemical has AR and/or GR agonistic activity, its induced luciferase activity (%) increases as its concentration increases in the absence of both DHT and Dex. When a test chemical has AR antagonist activity, its induced luciferase activity (%) decreases as its concentration increases in the presence of 0.2 nM DHT, by which induced luciferase activity was used as a control value. When a test chemical has AR and/or GR agonistic activity, its induced luciferase activity (%) also increases synergistically as its concentration increases in the presence of 0.2 nM DHT or 7 nM Dex. In particular, the value of IC_{50} for AR antagonistic activity is the concentration of the test chemical producing 50% inhibition of 0.2 nM DHT-induced luciferase activity[3] and the value of EC_{150} for AR and/or GR agonistic activity is the concentration of test chemical producing 150% stimulation of luciferase activity in the absence of both 0.2 nM DHT and 7 nM Dex. Since microscope observation of the damage to treated cells was correlated with decreased induced luciferase activity (%) in the absence of both DHT and Dex, induced luciferase activity less than 85% in the absence of both DHT and Dex was assigned for cell toxicity and the corresponding concentrations were not used to calculate IC_{50} in the presence of DHT. The data were analyzed by Student's *t*-distribution with Excel (Microsoft, USA) and *p* values

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less than 0.05 were considered significant. The measured activities represented the means ± SD resulting from at least three separate experiments with quadruplicate wells for each treatment dose. In this assay system, EC_{150} values of DHT and Dex were 0.5 and 194 nM, respectively. Flutamide, which is known as AR antagonist and used to treat prostate cancer, had an IC_{50} value of 0.9 μM.

All four tested isoflavones (daidzein (**1**), genistein (**2**), biocain A (**3**) and puruetun (**4**)) demonstrated a sufficient increase of luciferase activity as their concentrations increased with EC_{150} values of 10.1, 4.4, 4.2 and 3.2 μM, respectively (Table 1), indicating that the hydroxyl group at the 5 position contributed to the increase of their binding affinity to the receptors, while no synergistic effects were observed in the presence of DHT and Dex. The EC_{150} value of genistein was about 10,000-fold less active than that of DHT, but 23-fold less active than that of Dex.

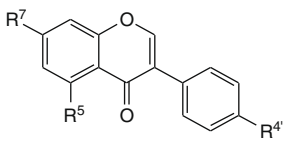
Although no GR antagonistic effects were observed in the presence of Dex, genistein (**2**) and biochanin A (**3**) showed obvious AR antagonistic activities in the presence of DHT, whose calculated values of IC_{50} were 1.3 and 1.6 mM, respectively (Fig. 1a). The IC_{50} value of genistein was 1400-fold less active than that of flutamide. However, this result supports the fact that co-treatment with lycopen, a typical carotenoid and genistein (**2**) induces apoptosis and inhibition of cell growth in androgen-sensitive prostate cancer cell lines (LNCaP), and the possibility of prostate cancer prevention.[7,8]

Based on the X-ray crystal structure of the ligand binding domain of estrogen receptor (ER) beta and genistein, the evidence that the hydroxyl group at the 7 position of genistein (**2**) interacts with His524 and the other hydroxyl group at its 4′ position interacts with Glu353 and Arg394 of ER beta[9] to make hydrogen bond suggests that the hydroxyl groups of 7 and 4′ positions of genistein (**2**) may interact with the corresponding Thr877, Arg752 and Gln711 in the AR ligand binding domain, respectively, by hydrogen bonds. In this study, however, the fact that the methoxy group at the 7 or 4′ position in the isoflavone skeleton did not interfere but increased luciferase activity indicates that the binding form of isoflavones in AR is different from that of ER.

As flavones, 21 compounds were assayed to gain insight into the structure–activity relationship (Table 2). No compounds had the observed GR antagonistic activity in the presence of Dex even at the highest tested concentration. In the presence of DHT, however, flavone (**5**) with no substituents had AR antagonistic activity with an IC_{50} value of 5.9 μM that was only 6.5-fold less active than that of flutamide, while flavone induced luciferase activity in the absence of DHT and even more obviously increased the effect of Dex at the concentration of 1×10^{-4} M (Fig. 1b). In steroid hormone receptors, the steroid hormones are mainly held by the direct hydrogen bonds at the A-ring and D-ring sites. The superposition of AR and GR showed that at A-ring site Arg752 and Gln711 in AR ligand binging domain (LBD) are corresponding to Arg611 and Gln570 in GR-LBD and at D-ring site Asn705 and Thr877 in AR-LBD are corresponding to Asn564 and Cys736 in GR-LBD, respectively.[10] Although the amino acid residues necessary for the hydrogen bond in the ligand binding domains are very similar between AR and GR, the results indicated that flavone (**5**) may interact with AR and GR in a completely different manner from the known steroid hormones, such as DHT and Des. Among the monosubstituted flavones, 6-methoxyflavone (**11**) had the highest luciferase activity with an EC_{150} value of 0.7 μM, while only 3-hydroxyflavone (**6**) lost its luc activity completely. However, 3-hydroxyflavone (**6**) had AR antagonist activity with an IC_{50} value of 23.5 μM, suggesting that 3-hydroxyflavone (**6**) can interact with AR like a pure AR antagonist. The 5-hydroxyflavone (**7**) had the highest AR antagonistic activity with an IC_{50} value of 0.3 μM, which was threefold higher than that of flutamide, while the AR antagonistic activity of 5-methoxyflavone (**8**) was 29-fold lower than that of 5-hydroxyflavone (**7**), although they had the same luciferase activities. This demonstrates that the hydrogen bond at the 5 position plays an important role in the interaction with AR. The monosubstituted flavones at the 6 and 7 positions lost their AR antagonistic activity; however, 6-methoxyflavone (**11**) had the strongest induced luciferase activity among the tested flavones, whose EC_{150} value was 1400-fold less active than that of DHT but it was 3.6-fold less active than that of Dex. The fact indicated that a hydrogen donor is not essential for the interaction with AR and/or GR around the 6-position of flavones. In the presence of 7 nM Dex, 6-hydroxyflavone (**9**) increased its luciferase activity with an EC_{150} value of 4.1 μM (data not shown in Table 2), although its activity was significantly suppressed at a concentration of 3×10^{-5} M in the presence of DHT, indicating its specific interaction with GR (Fig. 1c). In di-substituents, there were no AR and GR antagonists; however, 5,7-dihydroxyflavone (**17**) increased luciferase activity in the presence and absence of DHT, and had little synergetic effect in the presence of Dex compared to the effect of 5-hydroxyflavone (**7**) (data not shown), indicating that the hydroxyl group at the 7 position influenced the AR and GR binding manner. In comparison with 3-hydroxyflavone (**6**), the fact that 3,6-dihydroxyflavone (**15**) increased luc activity in the presence of 7 nM Dex implied that the hydroxyl group at the 6 position is especially important for GR binding. Among compounds with more than tri-substituents (Table 2), 5,6,7-trihydroxyflavone (**19**) decreased its own luciferase activity by 12.6-fold compared with 5,7-dihydroxyflavone (**17**), but increased its activity significantly in the presence of Dex, indicating that the hydroxyl group at the 6 position beneficially worked toward the binding with GR and against that with AR. 4′,5,7-Trihydroxyflavone (**20**) and 3,4′,5,7-tetrahydroxyflavone (**22**) acted as an AR antagonist whose activity was similar to that of flavone (**5**). The introduction of methoxy group at the 4′ position (**21**) lost AR antagonistic activity completely, while its EC_{150} value was about twofold increased compared with that of 4′,5,7-trihydroxyflavone (**20**). 3,3′,4′,7-Tetrahydroxyflavone (**24**) and 3,3′,4′,5,7-pentahydroxyflavone (**25**) did not have any significant activities.

Taken together, the introduction of a hydroxyl group at the 3 position significantly reduced or lost luciferase activities, suggesting that the hydroxyl group at the 3 position interferes with the interaction between AR and the ligand. Meanwhile, the hydroxyl group at the 5 position increased both DHT-induced luciferase activity and AR antagonistic activity, implying that the hydroxyl group at the 5 position positively affects the interaction between AR and the ligand. For AR antagonistic activity, as a methoxy group at the 5 position reduced the activity, this means that the hydrogen

Table 1
Effect of isoflavones on AR and/or GR transcriptional activities



No.	Compound name	R^7	R^5	R^7	EC_{150}[a](μM)	IC_{50}[b] (mM)
1	Daidzein	OH	H	OH	10.1	—[c]
2	Genistein	OH	OH	OH	4.4	1.3
3	Biochanin A	OCH_3	OH	OH	4.2	1.6
4	Prunetin	OH	OH	OCH_3	3.2	—[c]

[a] The EC_{150} value is the concentration of test chemical producing 150% stimulation of induced luciferase activity in comparison with that of vehicle control. The value is the mean of three experiments in quadruplicate.

[b] The IC_{50} value for AR antagonistic activity is the concentration of test chemical producing 50% inhibition of 0.2 nM DHT-induced luciferase activity.[6] The value is the mean of three experiments in quadruplicate.

[c] Not active at the concentration of 1×10^{-4} M.

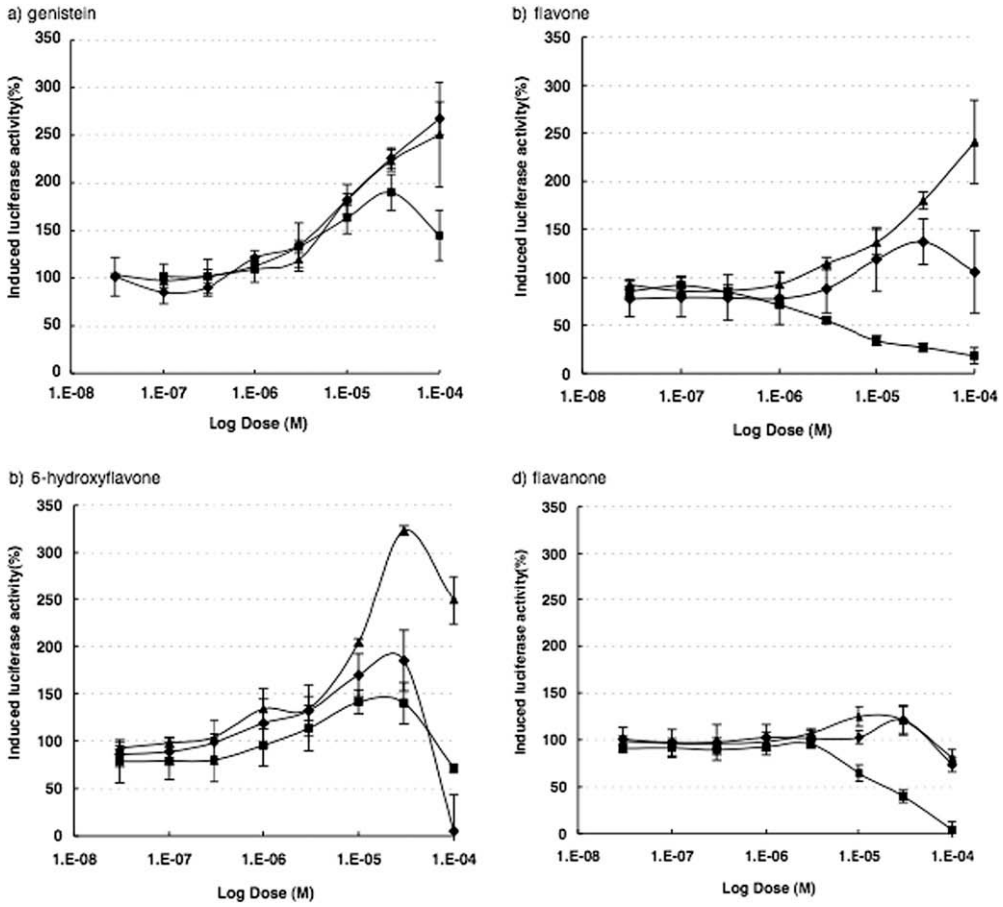


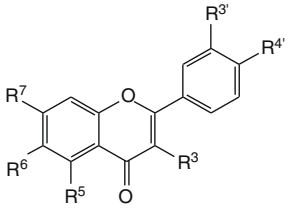
Figure 1. Effects of flavonoids on AR and GR in the absence of both DHT and Dex (diamond), in the presence of 0.2 nM DHT (square) and in the presence of 7 nM Dex (triangle). Data were expressed as the mean of luciferase activity (RLU) of three experiments in quadruplicate, that is, induced luciferase activity (%) = [RLU (test chemical)/RLU (control)] × 100.

bond at this position plays an important role in AR antagonist activity as a hydrogen donor. The introduction of substituents at the 6 and/or 7 positions increased luciferase activities and lost AR antagonistic activities, indicating that flavones with substituents in those positions act as AR and/or GR agonists. As the hydroxyl group at the 4′ position altered luciferase activities and increased AR antagonistic activities, this demonstrated the interaction of the hydroxyl group with AR; however, the introduction of a hydroxyl group at the 3′ position reduced both luciferase activity and AR antagonistic activity, suggesting that this position interferes with the interaction between AR and the ligands.

Flavanones were also assayed and the AR agonistic and antagonistic activities are shown in Table 3. Among flavanones, a flavanone (**26**) with no substituents had obvious AR antagonistic activity with an IC_{50} value of 20 μM in the presence of DHT, even though the steric relationship between A, B and C rings is completely altered by reduction of the 2, 3 double bond compared with the flavone conformation (**5**) (Fig. 1d); however, its remaining activity indicated that there is sufficient space to accept the bulkier flavanone backbone in AR and GR ligand binding regions. As the AR antagonistic activity of 6-hydroxyflavanone (**27**) was increased to sixfold, this means that it interacts with AR directly, while luciferase activity was lost by the introduction of a hydroxyl group into the 6 position of flavanone (**26**); however, introduction of a 6-methoxy group (**28**) decreased the AR antagonistic activity to 12-fold compared with 6-hydroxyflavanone (**27**), suggesting that the hydrogen bond at the 6 position plays a positive role in AR antagonistic activity. Other flavanones (**29**–**32**) lost both AR agonistic and antagonistic activities completely. No compound had GR antagonistic activity in the presence of Dex at the highest concentration.

In summary, we have demonstrated that flavonoids act as novel AR and GR modulators that interact with those receptors in a different manner from known AR and GR agents. Their distance between two functional groups with H-bonding ability, corresponding to 3-carbonyl and 17-OH groups of testosterone and DHT, should be close to 10 Å to maintain favorable H-bond positions, named the near 10 Å polar interaction rule from our previously reported evidence.[3,11] A detailed analysis of the differences between the structures of AR- and/or GR-flavones interaction is of importance to understand how the flavones exert different biological functions through AR and/or GR based on the fundamental function of key residues of the AR- and GR-LBD and their interaction with the ligand. However, it still remains unclear whether the exact structures of AR- and/or GR-flavones is similar to the ER–genistein complex because of lack of X-ray crystal structures of AR- and/or GR-flavones. Nevertheless, in particular, the evidence from this study that flavone without substituents directly interacts with both AR and GR, and that the subtle difference of substituents affects binding activities will lead to the discovery of a new category of AR and GR modulators, although much less is known about their binding mechanism. Furthermore, our findings highlight the careful use of flavonoids as health care supplements in regulating physiological activities of AR and GR in vivo. Therefore, our effort is now focused on new approach to verify their interaction of AR- and/or GR-flavones.

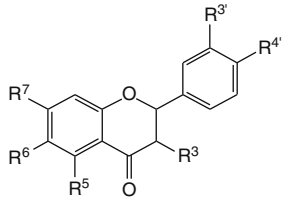
Table 2
Effect of flavones on AR and/or GR transcriptional activities



No.	Compound name	$R^{3'}$	$R^{4'}$	R^3	R^5	R^6	R^7	EC_{150}[a] (μM)	IC_{50}[b] (μM)
5	Flavone	H	H	H	H	H	H	26.1	5.9
6	3-Hydroxyflavone	H	H	OH	H	H	H	–[c]	23.5
7	5-Hydroxyflavone	H	H	H	O H	H	H	6.0	0.3
8	5-Methoxyflavone	H	H	H	O CH_3	H	H	5.5	8.7
9	6-Hydroxyflavone	H	H	H	H	OH	H	7.1	–[c]
10	6-Methylflavone	H	H	H	H	CH_3	H	3.6	–
11	6-Methoxyflavone	H	H	H	H	OCH_3	H	0.7	–
12	6-Chloroflavone	H	H	H	H	Cl	H	12.6	–
13	7-Hydroxyflavone	H	H	H	H	H	OH	3.1	–
14	7-Methoxyflavone	H	H	H	H	H	OCH_3	21.9	–
15	3,6-Dihydroxyflavone	H	H	OH	H	OH	H	1538	–
16	3-Hydroxy-6-methoxyflavone	H	H	OH	H	OCH_3	H	10.3	–
17	5,7-Dihydroxyflavone	H	H	H	OH	H	OH	1.5	*[d]
18	3,5,7-Trihydroxyflavone	H	H	OH	OH	H	OH	633	–
19	5.6,7-Trihydroxyflavone	H	H	H	OH	OH	OH	18.9	–
20	4′,5,7-Trihydroxyflavone	H	OH	H	OH	H	OH	5.2	5.2
21	5,7-Dihydroxy-4′-Methoxyflavone	H	OCH_3	H	OH	H	OH	2.1	–
22	3,4′,5,7-Tetrahydroxyflavone	H	OH	OH	OH	H	OH	55.7	9.7
23	3′,4′,5,7-Tetrahydroxyflavone	OH	OH	H	OH	H	OH	18.0	–
24	3,3′,4′,7-Tetrahydroxyflavone	OH	OH	O H	H	H	OH	–	–
25	3,3′,4′,5,7-Pentahydroxyflavone	OH	OH	O H	OH	H	OH	–	–

[a] The EC_{150} value is the concentration of the test chemical producing 150% stimulation of induced luciferase activity in comparison with that of the vehicle control. The value is the mean of three experiments in quadruplicate.
[b] The IC_{50} value for AR antagonist activity is the concentration of the test chemical producing 50% inhibition of 0.2 nM DHT-induced luciferase activity.[6] The value is the mean of three experiments in quadruplicate.
[c] Not active at the concentration of 1×10^{-4} M.
[d] AR antagonist activity is detected only at the concentration of 1×10^{-4} M.

Table 3
Effect of flavanones on AR and/or GR transcriptional activities



No.	Compound name	$R^{3'}$	$R^{4'}$	R^3	R^5	R^6	R^7	EC_{150}[a] (μM)	IC_{50}[b] (μM)
26	Flavanone	H	H	H	H	H	H	380	20.0
27	6-Hydroxyflavanone	H	H	H	H	OH	H	–[c]	3.3
28	6-Methoxyflavanone	H	H	H	H	OCH_3	H	4077	39.5
29	7-Hydroxyflavone	H	H	H	H	H	OH	–	–
30	7-Methoxyflavanone	H	H	H	H	H	OCH_3	–	–
31	4′,5,7-Trihydroxyflavanone	H	OH	H	OH	H	OH	–	–
32	3,3′,4′,5,7-Tetrahydroxyflavanone	OH	OH	OH	OH	H	OH	–	–

[a] The EC_{150} value is the concentration of the test chemical producing 150% stimulation of induced luciferase activity in comparison with that of the vehicle control. The value is the mean of three experiments in quadruplicate.
[b] The IC_{50} value for antagonist activity is the concentration of the test chemical producing 50% inhibition of 0.2 nM DHT-induced luciferase activity.[6] The value is the mean of three experiments in quadruplicate.
[c] Not active at the concentration of 1×10^{-4} M.

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