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AROMATASE INHIBITION BY FLAVONOIDS

ABDEL-RAHIM IBRAHIM and YUSUF J. ABUL-HAJJ
Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455, U.S.A.

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Summary—Several synthetic flavones were found to inhibit the aromatization of androstenedione to estrone catalyzed by human placental microsomes. Twenty-one compounds were tested and the IC_{50} of the most active were: flavone, 10 μM; 7-hydroxyflavone, 0.5 μM; 7,4′-dihydroxyflavone, 2.0 μM; flavanone, 8.0 μM; and 4′-hydroxyflavanone, 10 μM. Most of the others had IC_{50} values ranging from 80 to >200 μM. These findings show that 4′-hydroxylation results in either no change or very little change in IC_{50} for flavanone, isoflavone and isoflavanone as well as other ring A hydroxylated flavones. Derivatives of flavone with a hydroxyl substituent at position 5, 6 and 7 were also screened. 7-Hydroxyflavone (**11**) was the most effective competitive inhibitor (IC_{50} = 0.5 μM) with an apparent K_i value of 0.25 μM. Compound **11** also induced a change in the absorption spectrum of the aromatase cytochrome *P*-450 which is indicative of substrate displacement. The relative binding affinities of the flavonoid analogs were determined and only ring A and ring B dihydroxylated analogs were found to bind to the estrogen receptor.

INTRODUCTION

Flavonoids are ubiquitous secondary plant metabolites and it is estimated that a normal human diet is likely to contain about 1 g/day of these natural compounds [1, 2]. Fortunately, the vast majority of the flavonoids are nontoxic to humans and animals, and the known pharmacological activities are limited to a few substances of this group. Flavonoids have been shown to possess antiinflammatory, antiallergic, antiviral, antimutagenic, and anticarcinogenic activities [3–5]. Furthermore, some of these compounds were found to have estrogenic or antiestrogenic activities [6, 7] as well as aromatase inhibitory activity [8].

Because flavonoids are consumed in appreciable amount in our diet, knowledge of their pharmacological and physiological properties is of significant importance. Recent studies in our laboratory were carried out on the microbiological transformation of various flavonoids [9–11] and several metabolites were isolated and characterized. Studies by Kellis and Vickery [8] showed that several naturally occurring and synthetic flavones were found to inhibit aromatase. In this paper we report on structure–activity relationships for several flavonoid inhibitors of human placental aromatase.

EXPERIMENTAL

Chemicals

Flavone and flavanone were obtained from Aldrich Chemical Company, Milwaukee, Wis. 5-Hydroxy, 6-hydroxy and 7-hydroxyflavones were purchased from Indofine Chemical Company, Somerville, N.J. Isoflavone was synthesized as described by Varma [12]. All other flavonoids were obtained from previous studies in our laboratory [9–11]. [1β,2β-^{3}H]Androstenedione (sp. act. 43.1 Ci/mmol) was obtained from DuPont, Wilmington, Del. All other chemicals were obtained from Sigma Chemical Co., St Louis, Mo.

Preparation of human placental microsomes

Microsomes were obtained from human placentas after normal full-term delivery and were prepared as described previously [13]. Following isolation of the microsomal pellet (washed twice), they were lyopihilized and stored at −20°C. These preparations can be kept for 6 months without loss of activity.

Aromatase assay

The method of Thompson and Siiteri [14] as modified by Reed and Ohno [15] was used in our studies. This assay quantitates the production of

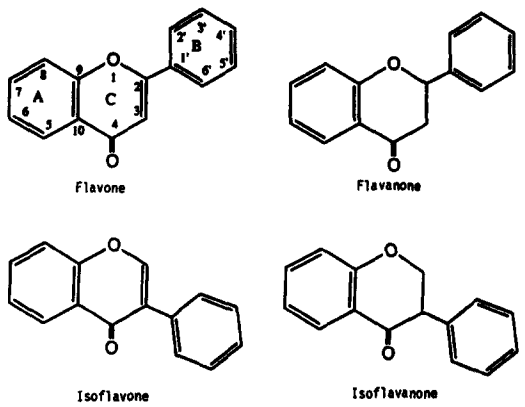

Fig. 1. Structures of flavone, flavanone, isoflavone and isoflavanone.

[^{3}H]H_2O released from [1β,2β-^3H]4-androstenedione after aromatization. All enzymatic studies were performed in 0.1 M phosphate buffer, pH 7.4, at a final incubation volume of 3.0 ml. The incubation mixture contained 2.5 mM glucose-6-phosphate, 0.5 mM NADP, 1 unit glucose-6-phosphate dehydrogenase, 40 nM [1,2-^{3}H]androstenedione, 10 mM EDTA, 10 mM phosphate buffer, 0.05 mg/ml microsomal protein, and, where indicated, inhibitor in 10 μl ethanol. Incubations were carried out for 10 min at 37°C in the air and were terminated by addition of 5 ml of chloroform, followed by vortexing for 40 s. Control samples with no inhibitor present and blank samples containing no microsomes were run simultaneously. After centrifugation at 1500 *g* for 5 min, the aqueous layer was treated with acid-washed charcoal and centrifuged again, and aliquots (0.2 ml) were removed and added to scintillation mixture for determination of ^3H_2O production.

Estrogen receptor assay

The method described in our laboratory was used to determine estrogen receptor binding [19].

RESULTS AND DISCUSSION

Twenty synthetic flavonoids (flavones, flavanones, isoflavones and isoflavanones) were tested for aromatase inhibitory activities. Figure 1 shows the hydrocarbon skeletons for the compounds under investigation. Figure 2 shows dose–response curves obtained with the placental system for four flavones and flavanones. Aminoglutethimide is included for comparison purposes. When 40 nM androstenedione was used as the substrate, flavone (**1**) caused 50% inhibition (IC_{50}) at a concentration of 10 μM; flavanone (**13**) at 8.0 μM; 4′-hydroxyflavanone (**14**) at 10 μM; 7-hydroxyflavone (**11**) at 0.5 μM; and 7,4′-dihydroxyflavone (**12**) at 2.0 μM.

Several other flavonoids were also tested as inhibitors of androstenedione aromatization and are shown in Table 1. None of the compounds tested were found to stimulate enzyme activity (concentrations ranged from 0.1 to 50 μM). The inhibitory potency relative to that of flavone is also given in Table 1, with flavone assigned an arbitrary index of 100. The results may be summarized as follows. In general flavones and flavanones have higher aromatase inhibitory activity than isoflavones and isoflavanones. 4′-Hydroxylation in ring B results in a negligible change in inhibitory activity for most compounds studied (e.g. **7** vs **8**, **9** vs **10**, **11** vs **12**, **13** vs **14** and **18** vs **20**) except for flavone in

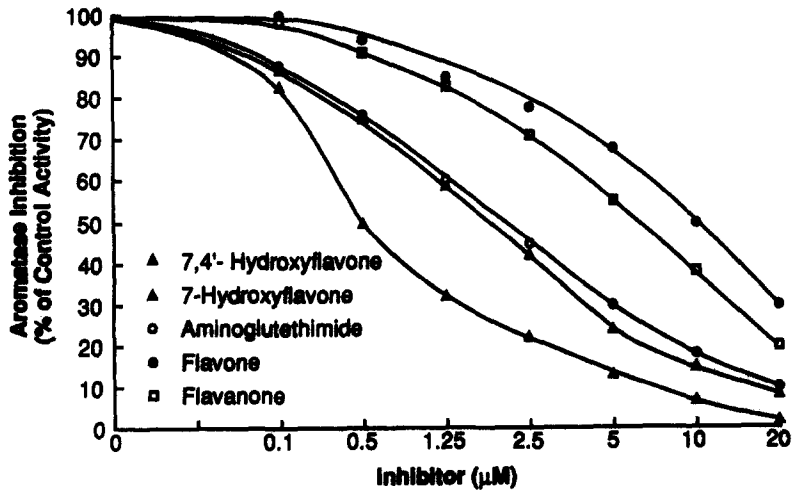

Fig. 2. Dose–response curves for flavonoid inhibition of androstenedione aromatization by human placental microsomes. Standard assay conditions with 40 nM androstenedione were used.

Table 1. Inhibition of aromatase by flavonoids

No.	Compound	IC_{50}[1] (μM)	Relative potency
1	Flavone	10	100
2	3-Hydroxyflavone	140	7
3	4′-Hydroxyflavone	180	6
4	3′,4′-Dihydroxyflavone	90	11
5	Flavan-4-ol	120	8
6	4′-Hydroxyflavan-4-ol	>200	5
7	5-Hydroxyflavone	100	10
8	5,4′-Dihydroxyflavone	120	8
9	6-Hydroxyflavone	80	13
10	6,4′-Dihydroxyflavone	90	11
11	7-Hydroxyflavone	0.50	2000
12	7,4′-Dihydroxyflavone	2.0	500
13	Flavanone	8	125
14	4′-Hydroxyflavanone	10	100
15	3′,4′-Dihydroxyflavanone	160	6
16	Isoflavone	>200	5
17	3′,4′-Dihydroxyflavone	>200	5
18	Isoflavanone	120	8
19	2-Hydroxyisoflavanone	170	6
20	4′-Hydroxyisoflavanone	160	6
21	3′,4′-Dihydroxyisoflavanone	>200	5
22	Aminoglutethimide	2.2	450

[1]Values reported represent the averages of duplicate determinations in two separate experiments.

which a drastic decrease in its inhibitory potency was observed (**1** vs **3**). Furthermore, hydroxylation at the 3 position in ring C also results in a significant decrease in enzyme inhibitory activity. Reduction of the carbonyl function in flavone to a 4-hydroxyl group (compound **5**) caused almost complete loss of inhibitory activity. This suggests that the keto group in flavone plays an important role in its interaction with the enzyme. Similar results were obtained by Kellis *et al.* [16] who showed that elimination of the carbonyl function in 7,8-benzoflavone decreased its inhibitory activity. Furthermore, hydroxylation of **5** to the 4′-hydroxy analog (compound **6**) results in even further decrease in inhibitory activity. With respect to hydroxylations in ring A it seems that maximal binding is essential with a 7-hydroxy group in flavone as shown for compound **11**. Indeed, 7-hydroxyflavone (**11**) is 20 times more active than flavone (**1**). However, changing the site of hydroxylation to either position 5 or 6 drastically reduced inhibitory potency. It is interesting to note that the farther the hydroxyl group is from the carbonyl function the higher the inhibitory activity of the compound. Earlier studies [8] showed that chrysin (5,7-dihydroxyflavone) is a potent aromatase inhibitor. Our results showed that 5-hydroxyflavone is inactive as an aromatase inhibitor. Thus, it seems likely that the hydroxy group at position 7 is the substituent essential for enhanced aromatase inhibitory activity in flavone type compounds.

Since many flavonoids are known to have estrogenic or antiestrogenic activity [6, 7], the

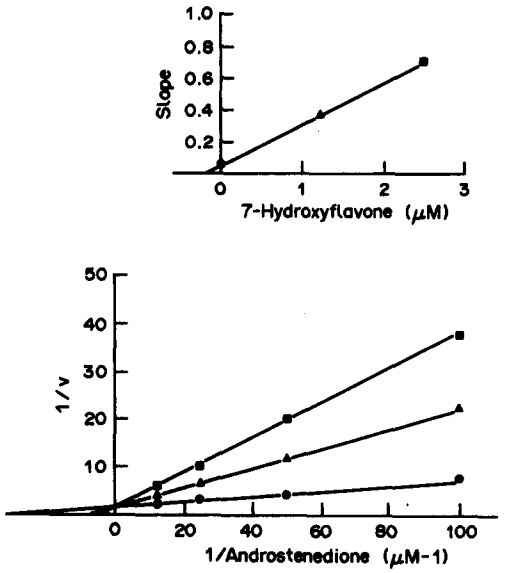

Fig. 3. Kinetic analysis of the mechanism of androstenedione aromatization in human placental microsomes by 7-hydroxyflavone. Standard assay conditions were used. Key: (●) no inhibitor; (▲) 1.25 μM 7-hydroxyflavone; (■) 2.5 μM 7-hydroxyflavone. Inset: slope of lines in double reciprocal plot vs inhibitor concentration.

compounds shown in Table 1 were tested for their estrogen receptor binding affinity. Only three compounds which possessed a hydroxy group in ring A and another hydroxy group at the 4′-position in ring B were found to compete with estradiol for receptor binding. A comparison of the relative binding affinities required to achieve 50% inhibition of the binding of [^{3}H]estradiol to estrogen receptor is shown in Table 2. The remaining compounds that did not have this hydroxylation pattern were virtually inactive.

To investigate the mechanism of aromatase inhibition by the flavonoid analogs, we performed kinetic analyses of their inhibition. Figure 3 shows a Lineweaver–Burk plot of the inhibition of androstenedione aromatization by 7-hydroxyflavone. It can be seen that the binding of 7-hydroxyflavone was competitive with respect to the substrate, androstenedione. The K_i value observed for 7-hydroxyflavone was 0.25 μM. In these studies, the apparent K_m for androstenedione was found to be 58 nM. Also

Table 2. Relative binding affinities (RBA) of flavonoids to estrogen receptor-immature rat uterus

Compound	RBA
Estradiol	100
6,4′-Dihydroxyflavone	1.2
7,4′-Dihydroxyflavone	0.04
6,4′-Dihydroxyisoflavanone	0.13

compounds **1**, **12**, **13** and **14** (data not shown) demonstrated competitive inhibition, as determined from Lineweaver–Burk plots and V_{max} intercepts.

The kinetic analyses suggest that flavonoids may inhibit aromatization by competing with androstenedione for the substrate binding site on the enzyme. To further investigate the mechanism of aromatase inhibition, the interaction between 7-hydroxyflavone and human placental microsomes was studied spectroscopically and the results (data not shown) were essentially identical to those obtained previously by several investigators [8, 16, 17]. The different absorption spectrum induced by 7-hydroxyflavone in the presence of androstenedione reflects substrate displacement and conversion of the high-spin aromatase cytochrome *P*-450 to a low-spin complex [18], that is, displacement of androstenedione. Thus, these results along with those reported previously [8, 16, 17] clearly indicate that flavonoid type compounds appear to act as Type II binders in which the heteroatom coordinates to the heme iron as a sixth ligand. Whether this heteroatom is an oxygen molecule from the flavonoid, or comes from water or an amino acid residue is not fully understood. Further studies on the structural elucidation of the aromatase active site may be helpful in delineating the mechanism of action of the flavonoid aromatase inhibitors.

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