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Inhibition of Aromatase Activity by Flavonoids

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In searching for potent cancer chemopreventive agents from synthetic or natural products, 28 randomly selected flavonoids were screened for inhibitory effects against partially purified aromatase prepared from human placenta. Over 50% of the flavonoids significantly inhibited aromatase activity, with greatest activity being demonstrated with apigenin (IC_{50}: 0.9 μg/mL), chrysin (IC_{50}: 1.1 μg/mL), and hesperetin (IC_{50}: 1.0 μg/mL).

Key words : Aromatase inhibition, Flavonoids, Breast cancer

INTRODUCTION

Approximately one-third of the breast cancers diagnosed in woman are hormone-dependent and regress on blockade of estrogen action or inhibition of estrogen biosynthesis (Santen *et al.*, 1990). Estrogens are biosynthesized from androgens by the microsomal cytochrome P-450 enzyme complex system termed aromatase (Siiteri *et al.*, 1982) (Fig. 1). Recent studies on the fundamental biologic properties of this very complex enzyme system have yielded information concerning genetic control, differential tissue expression, and modulation by a variety of growth factors and cytokines present within the tumor environment (Harvey *et al.*, 1996). For the attempted control of hormone-dependent breast cancer, estrogen receptors can be blocked with antagonists such as tamoxifen, gonadotropin can be inhibited by continuous administration of gonadotrophin-releasing hormone (GnRH) or one of its analogues (Conn and Crowley, 1991), or circulating estrogens can be decreased by inhibition of biosynthesis; the target of such inhibition is the aromatase cytochrome P-450 ($P\text{-}450_{arom}$) enzyme complex (Thompson and Siiteri, 1974a,b). Using monoclonal or polyclonal antibodies against aromatase, Santen *et al.* have demonstrated significant amounts of aromatase in breast tumors and the stroma surrounding breast tumors (Santen *et al.*, 1994). This finding may be of clinical relevance in considering the initiation of breast carcinoma, and in the development of compounds of sufficient potency to effectively lower plasma levels of estrogen by inhibition of synthesis *in situ* (Harvey *et al.*,1996). Since estrogen production is the last step in the biosynthetic sequence of steroid production, selective blockade of aromatase should not interfere with the production of other steroids, such as adrenal corticoids. For these reasons, aromatase is a particularly attractive enzyme target for selective inhibition.

Several classes of aromatase inhibitors such as substrate androstenedione derivatives, nonsteroidal aminoglutethimide and its analogues, several imidazoles, and triazoles have been developed over the past 20 years as potential therapeutic agents (Brodie and Njar, 1996). The first potent and selective aromatase inhibitors identified were androstenedione derivatives that act by competing with the substrate. It is interesting that a number of steroidal

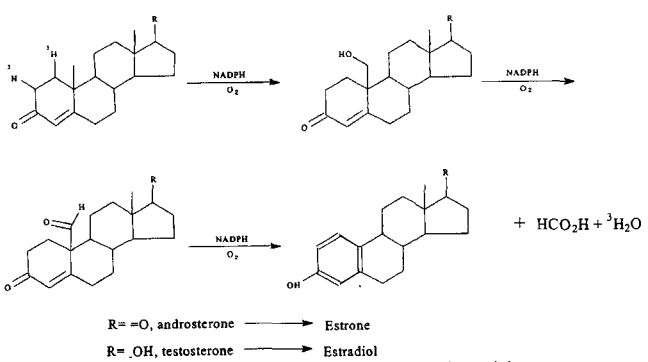

Fig. 1. Biosynthetic pathway of estrogen catalyzed by aromatase

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inhibitors, such as 4-hydroxyandrostenedione, were found to cause inactivation of the enzyme and appear to function as mechanism-based inhibitors. While not intrinsically reactive, compounds of this type are thought to compete with the natural substrate and subsequently interact with the active site of the enzyme, very tightly or irreversibly, thus causing inactivation. Aminogluthetimide was the first aromatase inhibitor widely used for the treatment of metastatic breast cancer. However, this is not an approved indication in the United States, and nonselective effects of aminogluthetimide on other P-450 enzymes lead to significant side effects, including central nervous system toxicities and skin rash and, thus, limited its usefulness. Recently, analogues of antifungal imidazole drugs, such as fadrozole and ORG 33201, were shown to have greater selectivity and potency (Brodie and Njar, 1996).

In order to discover novel cancer chemopreventive agents from natural products, we have evaluated the potential of several classes of compounds to inhibit partially purified aromatase. As a result, several flavonoids were found to demonstrate inhibitory effects. Flavonoids are ubiquitous natural products that mediate a host of activities including anti-inflammatory and anti-oxidant effects. In this paper, the potential importance of flavonoid-mediated inhibition of aromatase activity is presented. These agents should be considered potential candidates as cancer chemopreventive agents, and the relevance of dietary ingestion should be considered.

MATERIALS AND METHODS

Chemicals

Androstenedione, aminoglutethimide and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). [1,2-^{3}H]Androstenedione (42.0 Ci/mmol, 1.0 mCi/ml) was obtained from Dupont/NEN Inc. (Boston, MA). Flavonoids were obtained from Indofine Co. (Somerville, NJ).

Preparation of partially purified human placental microsomes

Freshly delivered human term placenta was washed in cold 0.15 M KCl and the tissue was dissected free of adhering membranes and large blood vessels (Hoffman *et al.*, 1980). The tissue was homogenized with a Polytron homogenizer, using three 20 sec bursts separated by 2 min cooling periods. The homogenized solution was centrifuged at 20,000×g for 30 min to remove mitochondria, nuclei and cell debris. The postmitochondrial supernatant was then subjected to centrifugation at 148,000×g for 45 min to yield a microsomal pellet. The pellet was resuspended in 0.05 M potassium phosphate buffer, pH 7.4, and centrifuged again at 148,000×g for 45 min. This step was repeated twice, and microsomes were finally suspended in a minimal volume of buffer and stored frozen in plastic tubes at -75°C. Protein content was determined using the bichinchonic acid method with bovine serum albumin as a standard protein (Kellis and Vickery, 1987).

Assay for aromatase activity

Reaction mixtures were prepared in glass tubes containing 4 μl of placental microsomes (5 mg/ml), 0.3 μl of [1,2-^{3}H]androstenedione (42.0 Ci/mmol, 1.0 mCi/ml), 5 μl of unlabelled androstenedione (0.875 μM), 5 μl of NADPH (0.48 mM), 10 μl of test sample (dissolved in DMSO), and 0.05 M potassium phosphate buffer, pH 7.4 (500 μl, final volume). After a 4 min incubation at 37°C, the reaction was terminated by adding 3 ml of chloroform. The tubes were centrifuged at 2,000×g for 10 min and then 300 μl of the aqueous phases were transferred to tubes containing 300 μl of charcoal/dextrin solution (5%). Following another 10 min centrifugation at 2,000×g, supernatant fractions (500 μl) were used for determination of radioactivity (Thompson *et al.*, 1974). Inhibition of aromatase activity was calculated using the following equation:

$$\%\ \text{Inhibition} = \left[1 - \frac{\text{Sample (DPM)} - \text{Blank(DPM)}}{\text{DMSO (DPM)} - \text{Blank (DPM)}}\right] \times 100$$

Dose-response curves were prepared and the results were typically expressed as IC_{50} values. Aminoglutethimide was used as a positive control (Rabe *et al.*, 1982).

RESULTS AND DISCUSSION

Prior to searching for inhibitors, preliminary evaluations were carried out to standardize the aromatase assay parameters. This constitutes an important prerequisite for the valid comparison of enzyme inhibitors, since the concentrations of reagents used (especially those of the enzyme and substrate) determine the catalytic efficiency of the enzyme, and ultimately its susceptibility to inhibition by agents with various mechanisms of action.

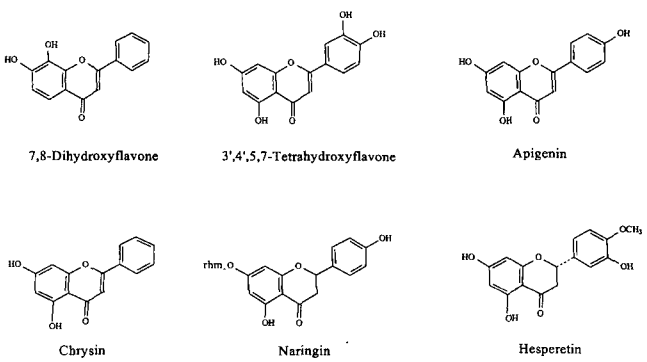

Fig. 2. Structures of major active compounds

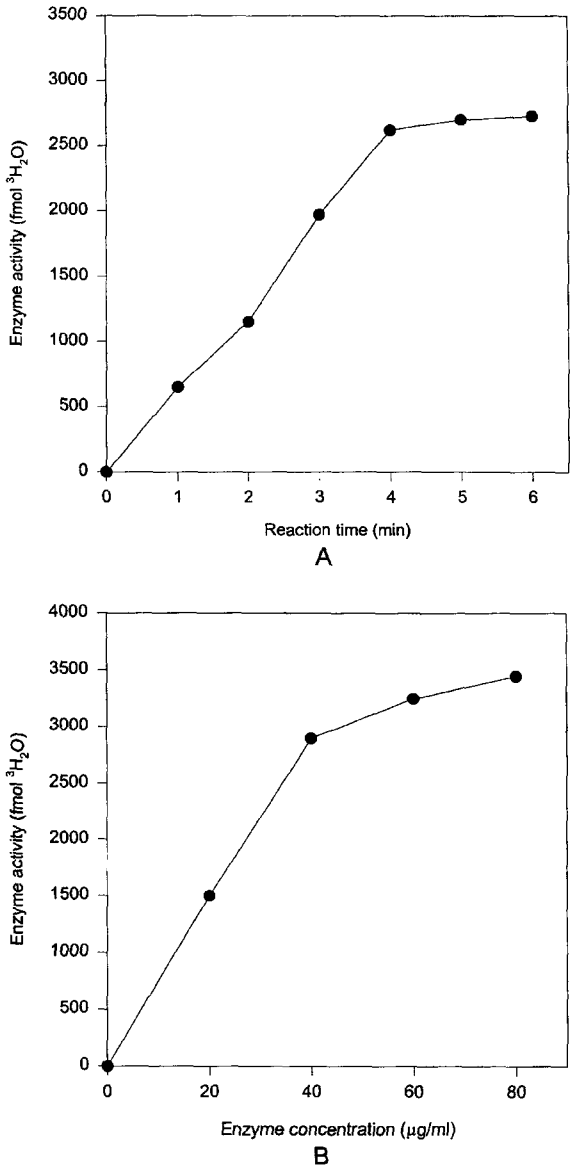

Fig. 3. Dependence of aromatase activity on reaction time and enzyme concentration. Partially purified aromatase (20 μg) was incubated with 14.3 nM [1,2-^{3}H]androstenedione (0.3 μCi) at 37°C for 5 min. Aromatase activity was monitored by measuring formation of ^3H_2O. Reaction times and enzyme concentrations were varied in (A) and (B), respectively.

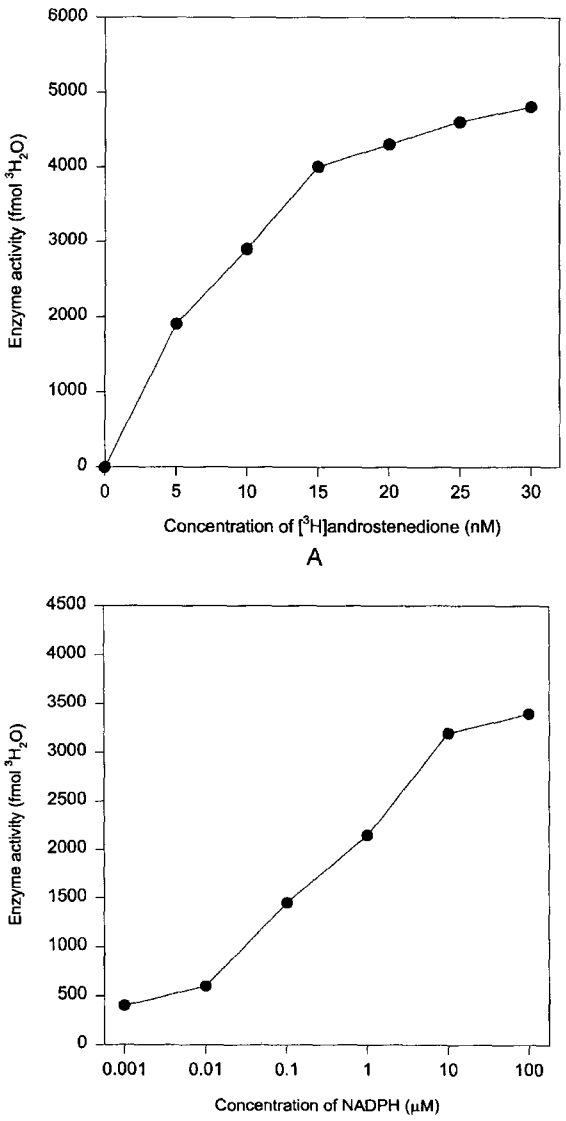

Fig. 4. Dependence of aromatase activity on [1,2-^{3}H]androstenedione and NADPH concentrations. Aromatase activity was monitored by measuring the formation of ^3H_2O. [1,2-^{3}H]Androstenedione and NADPH concentrations were varied in (A) and (B), respectively.

Studies were performed to assess effects of experimental variables such as reaction time, enzyme concentration, substrate and cofactor (NADPH) concentrations, on formation of one of the final products, ^3H_2O (Fig. 3 and 4). The aromatase assay was subsequently established utilizing sub-saturating conditions with respect to all of these parameters. Under these conditions, partially purified aromatase should demonstrate optimum sensitivity to inhibitors acting by diverse mechanisms of action.

Utilizing optimum reaction conditions, 28 flavonoids were evaluated for inhibition of aromatase activity. When tested at a fixed concentration of 80 μg/ml, the following compounds were not active (<50% inhibition): myricitrin, genkwanin, hyperoside, rhamnetine, tangeretin, rutin, formononetin, rhoifolin, luteolin-3',7-diglucose, neohesperidin, flavone, and esculetin. However, the remaining 16 compounds showed significant inhibitory activities (at 80 μg/mL, >50% inhibition) (Table 1). Dose-response activity curves were generated and IC_{50} values were determined (Table 1). Apigenin (IC_{50}: 0.9 μg/mL), chrysin (IC_{50}: 1.1 μg/mL), and hesperetin (IC_{50}: 1.0 μg/mL) showed greatest inhibitory potential.

In conclusion, flavonoid-mediated inhibition of aromatase activity is of potential importance in the field

Table I. Inhibitory effects of flavonoids on aromatase activity[1]

Test Compounds	Inhibition (% at 80 μg/mL)	IC_{50} (μg/mL)
Acacetin	81.2	18.9
Apigenin	70.6	0.9
Biochanin A	86.9	10.2
Chrysin	77.1	1.1
7,8-Dihydroxyflavone	90.3	2.2
Fisetin	90.8	8.5
Flavanone	88.8	8.7
Hesperidine	79.1	40.9
Hesperetin	90.9	1.0
7-Hydroxyflavone	88.0	30.5
Myricetin	69.3	5.6
Naringin	84.6	1.8
Prunetin	85.8	7.8
Robinetine	57.6	45.7
Silymarin	85.4	6.7
3,4,5,7-Tetrahydroxyflavone	84.4	3.3

[1]Aminogluthetimide was used as a positive control: 93.1% inhibition at 80 μg/ml, IC_{50}=0.4 μg/ml.

of cancer chemoprevention. Dietary materials, such as *Citrus* fruits, may be considered relevant, since they are a source of flavonoids including naringin, hesperetin, and hesperidine. Structure-activity relationship (SAR) studies are currently in progress.

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