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Lignans and Flavonoids Inhibit Aromatase Enzyme in Human Preadipocytes

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Lignans and flavonoids are naturally-occurring diphenolic compounds found in high concentrations in whole grains, legumes, fruits and vegetables. Seven lignans and six flavonoids were evaluated for their abilities to inhibit aromatase enzyme activity in a human preadipose cell culture system. The lignan, enterolactone (Enl) and its theoretical precursors, 3′-demethoxy-3*O*-demethylmatairesinol (DMDM) and didemethoxymatairesinol (DDMM) decreased aromatase enzyme activity, with K_i values of 14.4, 5.0 and 7.3 μM, respectively. The flavonoids, coumestrol, luteolin and kaempferol also decreased aromatase enzyme activity, with K_i values of 1.3, 4.8 and 27.2 μM, respectively. Aminoglutethimide, a pharmaceutical aromatase inhibitor, showed a K_i value of 0.5 μM. Kinetic studies showed the inhibition by all compounds to be competitive. Smaller decreases in aromatase activity were observed with the lignan, enterodiol (End) and its theoretical precursors, *O*-demethylsecoisolariciresinol (ODSI), demethoxysecoisolariciresinol (DMSI) and didemethylsecoisolariciresinol (DDSI). The flavonoids, *O*-demethylangolensin (*O*-Dma), fisetin and morin showed no inhibitory effects. The inhibition of human preadipocyte aromatase activity by lignans and flavonoids suggests a mechanism by which consumption of lignan- and flavonoid-rich plant foods may contribute to reduction of estrogen-dependent disease, such as breast cancer.

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INTRODUCTION

Lignans and flavonoids are naturally-occurring compounds present in fruits, vegetables, legumes and whole grains. Lignans are diphenolic compounds formed from plant precursors by the actions of intestinal bacteria [1]. The primary mammalian lignans are enterolactone (Enl) and enterodiol (End), formed from plant precursors matairesinol and secoisolariciresinol, respectively. Fiber-rich foods such as whole grains are rich in plant precursors [2–4], such that the urinary excretion of mammalian lignans is proportional to dietary fiber consumption [5, 6]. Epidemiological studies showing low urinary lignan excretion in breast cancer patients [3, 6, 7] suggest that consumption of lignans may exert cancer-preventive effects.

Flavonoids are diphenolic compounds widespread in plant foods. It is estimated that a normal human diet contains an average of 1 g of these compounds per day [8]. Specific flavonoids have been shown to inhibit oxidase enzyme activity [9, 10], suppress oxygen-induced cytotoxicity [11, 12] and suppress viral reverse transcriptase [13]. They also have been shown to possess anti-viral [14–16] and anti-mutagenic activity [17–19] and to inhibit tumor induction by carcinogens and growth promoters.

It has been suggested that lignans and flavonoids may contribute to the prevention of breast cancer in part as a result of their antiestrogenic properties [6]. Specific lignans and flavonoids have been shown to inhibit estrogen synthesis in human placental microsomes [20–22] and preadipocytes [23] and to compete with estradiol for the estrogen receptor and type II binding sites [24–27]. Lignans also have been shown to stimulate sex hormone binding globulin production in the liver [27, 28] and to inhibit *in vivo* estrogen-stimulated RNA synthesis [29], proliferation of ZR-75-1 breast cancer cells [30] and estradiol-induced proliferation of MCF-7 cells [31].

The major source of estrogen in postmenopausal women is aromatization of androstenedione to estrone

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in peripheral tissue such as adipose [32, 33]. Inhibition of aromatase enzyme activity, with subsequent reduction in estrogen synthesis, is thought to contribute to prevention of estrogen-dependent cancers, such as breast cancer, in postmenopausal women. In fact, several pharmaceutical aromatase inhibitors have been used successfully in the treatment of estrogen-dependent carcinoma [34].

It has been suggested that naturally-occurring dietary phytoestrogens may contribute to prevention of estrogen-dependent cancers by some of the same mechanisms as pharmaceutical antiestrogens. It is therefore of great interest to identify dietary phytoestrogens, to evaluate their antiestrogenic potencies and to elucidate the mechanisms by which they exert their effects. The purpose of this study was to evaluate the specific effects of lignans and flavonoids on estrogen synthesis in human preadipocytes by measuring aromatase enzyme activity.

Although lignans and flavonoids have been shown to inhibit aromatase enzyme activity in placental microsomes [20–22] and lignans have been shown to inhibit aromatase activity in a human choriocarcinoma cell line [20], little work has been done in whole cells derived from primary cultures of human tissue. We previously reported the flavonoids, α-naphthoflavone (ANF), chrysin, flavone and biochanin A to be inhibitors of aromatase enzyme activity in human preadipocyes [23]. For the present study, we evaluated the effects of six other commonly consumed flavonoids as well as seven lignans, on aromatase enzyme activity in a preadipose cell culture system. The following lignans were studied: Enl and its theoretical precursors, 3′-demethoxy-3*O*-demethylmatairesinol (DMDM) and didemethoxymatairesinol (DDMM); and End and its theoretical precursors, *O*-demethylsecoisolariciresinol (ODSI), demethoxysecoisolariciresinol (DMSI) and didemethylsecoisolariciresinol (DDSI). The flavonoids studied were: the flavonols, morin, fisetin and kaempferol; the flavone, luteolin; and the isoflavones, coumestrol and *O*-demethylangolensin (*O*-Dma). The pharmaceutical aromatase inhibitor, aminoglutethimide (AG), was studied for comparison of inhibitor potency.

EXPERIMENTAL

Chemicals

Aminoglutethimide [3-(4-aminophenyl)-3-ethyl-2,6-piperidinedione], collagenase (clostridiopeptidase A), dexamethasone (9α-fluoro-16α-methylprednisolone), trypan blue stain (0.4%), kaempferol and morin were purchased from Sigma Chemical Company, St Louis, MO, U.S.A. Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Phosphate Buffered Saline (PBS), antibiotic–antimycotic and calf serum were purchased from Gibco BRL, Grand Island, NY. 3 ml disposable extraction columns packed with reversed phase octadecylsilane (C18) bonded to silica gel were purchased from J. T. Baker Inc., Phillipsburg, NY. Tritiated androstenedione ([1β-^{3}H(N)]androst-4-ene-3,17-dione) was purchased from NEN/Du Pont, Wilmington, DE. Fisetin was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. Luteolin was purchased from Carl Roth GmbH and Co., Karlsruhe, Germany and coumestrol was purchased from Eastman Kodak, Rochester, NY. All lignans and *O*-Dma were synthesized in the Department of Chemistry, University of Helsinki, Finland.

Processing of the adipose tissue

The protocol for this experiment was approved by the University of Minnesota Institutional Review Board: Human Subjects Committee. Subcutaneous adipose tissue from abdomen and thigh regions was obtained by liposuction from female patients aged 23–57 years. The procedures for the tissue processing have been described in detail previously [23]. The fresh tissue was digested with collagenase for 30–60 min. After standing for 10 min at 37°C, the aqueous layer was removed and centrifuged. The resulting pellet of vascular/stromal cells was seeded in polystyrene tissue culture flasks containing DMEM and 15% calf serum and grown to confluence in a 5% CO_2 humidified chamber. After the primary culture, cells were passaged approximately once per week.

Aromatase assay

For the aromatase assay, cells were grown to confluence in 25 ml flasks containing DMEM supplemented with 10% calf serum. Prior to the assay, the cells were treated with 100 nM dexamethasone for 18–24 h to induce aromatase activity. At the end of the pre-incubation, the cells were washed twice with PBS and incubated at 37°C for 4 h in the experimental medium, consisting of DMEM containing 1% antibiotic-antimycotic, the desired concentration of ^{3}H-androstenedione and the desired concentration of lignan or flavonoid. In the dose–response experiments, the concentration of ^{3}H-androstenedione was 100 nM and the concentration of lignan or flavonoid ranged from 0 to 100 μM. When inhibition was demonstrated, kinetic studies were carried out to determine the mechanism of inhibition and inhibitor effectiveness. For the kinetic studies, ^{3}H-androstenedione concentrations were 25, 40, 75 and 150 nM and inhibitor concentrations were three levels between 0 and 100 μM.

A modification of the tritiated water method of Thompson and Siiteri [35] was used to measure aromatase activity. This modification has been described in detail previously [23]. Following the 4 h incubation with ^{3}H-androstenedione and lignan or flavonoid, the experimental medium was removed and extracted with chloroform. After centrifugation at 1000 *g* for 10 min, the aqueous upper layer was transferred to individual C18 columns for final extraction. Aliquots were

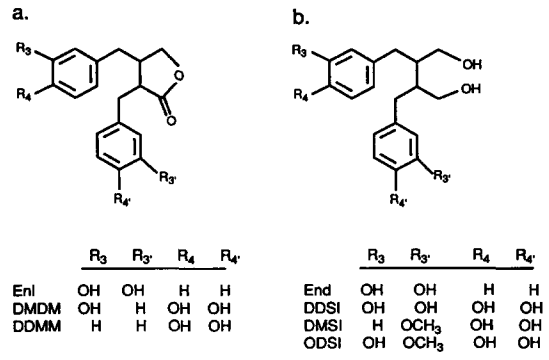


Fig. 1. The structures of Enl (a), End (b) and their theoretical precursors.

Table 1. Kinetic data for aromatase inhibitors

Inhibitor	Percent control V_{max} 1 μM	10 μM	100 μM	I_{50} (μM)	K_i (μM)
Enterolactone	97	89	43	74	14.4
DMDM	104	96	45	84	5.0
DDMM	71	68	44	60	7.3
Enterodiol	100	90	75	>100	—
ODSI	95	96	87	>100	—
DMSI	81	80	77	>100	—
DDSI	100	84	78	>100	—
Coumestrol	119	61	14	17	1.3
Luteolin	90	72	17	25	4.8
Kaempferol	127	107	35	61	27.2
AG	76	39	7	5	0.5

counted in a liquid scintillation counter (Beckman Instruments, Inc. Fullerton, CA). Cell protein was solubilized in sodium hydroxide and quantified by the method of Bradford [36]. Under these conditions, the results fall in the linear range of aromatase enzyme activity for time and cell number.

The effects of the inhibitors on cell viability were evaluated via trypan blue exclusion. Tests were performed after incubation of the cells for 24 hr with DMEM containing 10% calf serum and the highest concentration (100 μM) of inhibitor used in the dose–response experiments.

Data analysis

Double-reciprocal plots were used to characterize the type of inhibition. The K_i values, reflecting inhibitor effectiveness, were determined by graphing the slopes of the double-reciprocal plots vs inhibitor concentration.

RESULTS

In the dose–response studies, a number of lignans and flavonoids were found to be aromatase inhibitors. The lignan, Enl and its theoretical precursors, DMDM and DDMM (Fig. 1), were shown to be moderate aromatase inhibitors (Fig. 2, Table 1), with I_{50} values (inhibitor concentration blocking 50% of V_{max}) of 74, 84 and 60 μM, respectively. The flavonoids, coumestrol, luteolin and kaempferol (Fig. 3) were also found to be aromatase inhibitors (Fig. 2, Table 1), with I_{50} values of 17, 25 and 61 μM, respectively. For comparison, the I_{50} value of AG was 5 μM.

Kinetic studies were performed to investigate the mechanism by which the lignans and flavonoids inhibit aromatase enzyme activity. In the absence of inhibitor, the K_m of androstenedione averaged 30 nM. In the presence of 50 and 100 μM Enl, the apparent K_m (K_m^{app}) value increased to 144 and 221 nM, respectively (Fig. 4). In the presence of 50 and 100 μM DMDM, the K_m^{app} increased to 94 and 265 nM, respectively. In

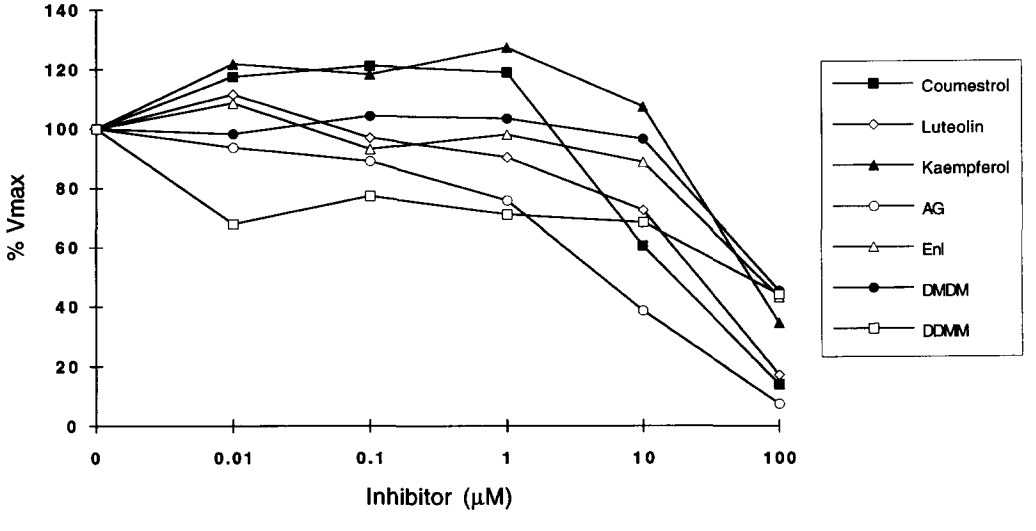
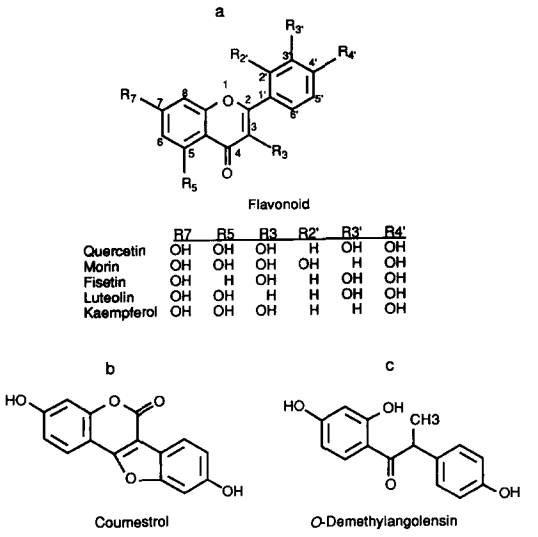


Fig. 2. Dose–response curves showing the effects of inhibitor concentrations on aromatase enzyme activity in human preadipocytes. Aromatase activity was measured after incubation of the cells for 18–24 h with 100 nM dexamethasone. During the 4 h assay, cells were incubated with 100 nM ^{3}H-androstenedione and 0–100 μM inhibitor. Each point represents the mean of three samples.



	R7	R5	R3	R2'	R3'	R4'
Quercetin	OH	OH	OH	H	OH	OH
Morin	OH	OH	OH	OH	H	OH
Fisetin	OH	H	OH	H	OH	OH
Luteolin	OH	OH	H	H	OH	OH
Kaempferol	OH	OH	OH	H	H	OH

Fig. 3. Structures of quercetin and the flavonoids studied. (a) flavonols and luteolin; (b) coumestrol; (c) *O*-Dma.

the presence of 50 and 100 μM DDMM, the K_m^{app} increased to 62 and 94 nM, respectively. In the presence of 15 and 30 μM coumestrol, the apparent K_m (K_m^{app}) increased to 199 and 422 nM, respectively (Fig. 5). In the presence of 25 and 50 μM luteolin, the K_m^{app} increased to 206 and 435 nM, respectively. In the presence of 50 and 100 μM kaempferol, the K_m^{app} of androstenedione increased to 162 and 300 nM, respectively. In the presence of 5 and 10 μM AG, the K_m^{app} increased to 277 and 916 nM, respectively.

All inhibitors exerted competitive inhibition, as indicated by the increasing K_m^{app} values and constant V_{max} in the presence of increasing inhibitor concentrations. DMDM was the strongest lignan inhibitor, with a K_i value of 5.0 μM (Table 1). For Enl (Fig. 4, inset) and DDMM, K_i values were 14.4 and 7.3 μM, respectively (Table 1). Coumestrol was found to be the strongest flavonoid inhibitor, with a K_i value of 1.3 μM (Fig. 5, Table 1). Luteolin was next, with a K_i of 4.8 μM, followed by kaempferol, with a K_i of 27.2 μM. Under the same experimental conditions, AG showed a K_i value of 0.5 μM.

The lignan, End and its theoretical precursors, ODSI, DMSI and DDSI (Fig. 1) were weak inhibitors of aromatase enzyme activity. I_{50} values for these compounds were all greater than 100 μM, and K_i values were not determined. The flavonoids, morin, fisetin, quercetin and *O*-Dma showed no inhibitory effects on aromatase enzyme activity.

No obvious morphological changes in lignan- or flavonoid-treated cells were found under microscopic examination. After incubation with 100 μM inhibitor for 24 h, the trypan blue exclusion test showed less than 6% cytotoxicity, with no differences between inhibitor-treated cells and controls. It thus appears that cell viability was not affected by exposure to lignans or flavonoids.

DISCUSSION

We have shown that naturally-occurring lignans and flavonoids are able to pass through the cell membrane

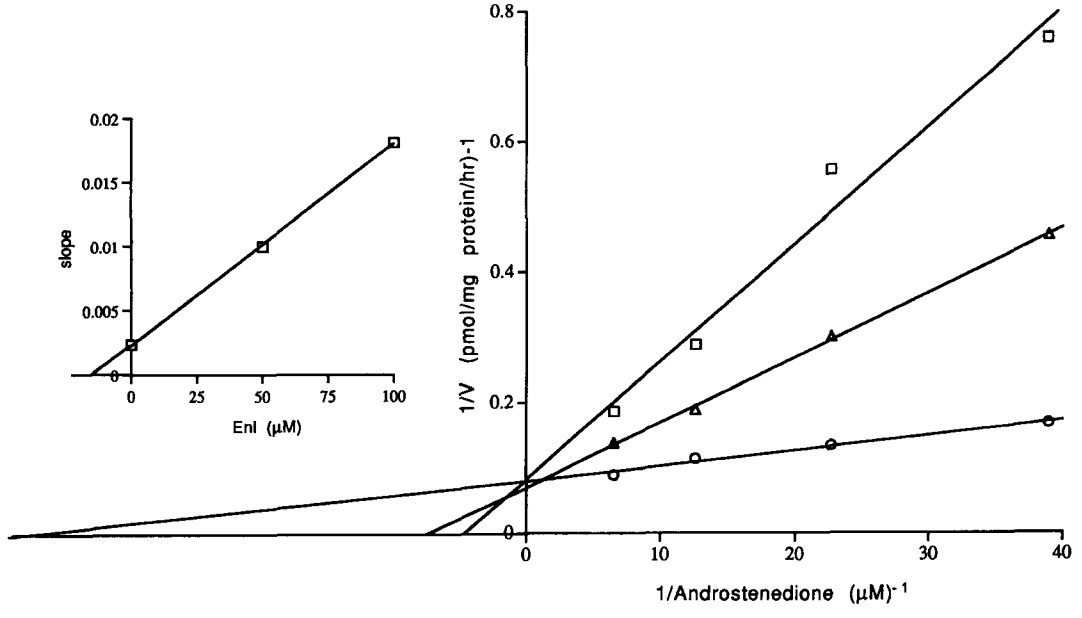


Fig. 4. Double-reciprocal plot showing the effect of substrate (^{3}H-androstenedione) concentration on aromatase activity in the presence of 0 (○), 50 (△) and 100 (□) μM Enl. The K_m is the negative, inverted x intercept and the V_{max} is the inversion of the y intercept. V_{max} values were 13, 14 and 12 pM, and K_m values were 30, 144 and 221 nM, for 0, 50 and 100 μM Enl, respectively. Each point represents the mean of three samples. Inset: Graph showing the effect of Enl concentration on the slope of the double-reciprocal plot lines. The K_i value (negative x intercept), reflecting the inhibitor effectiveness, was 14.4 μM.

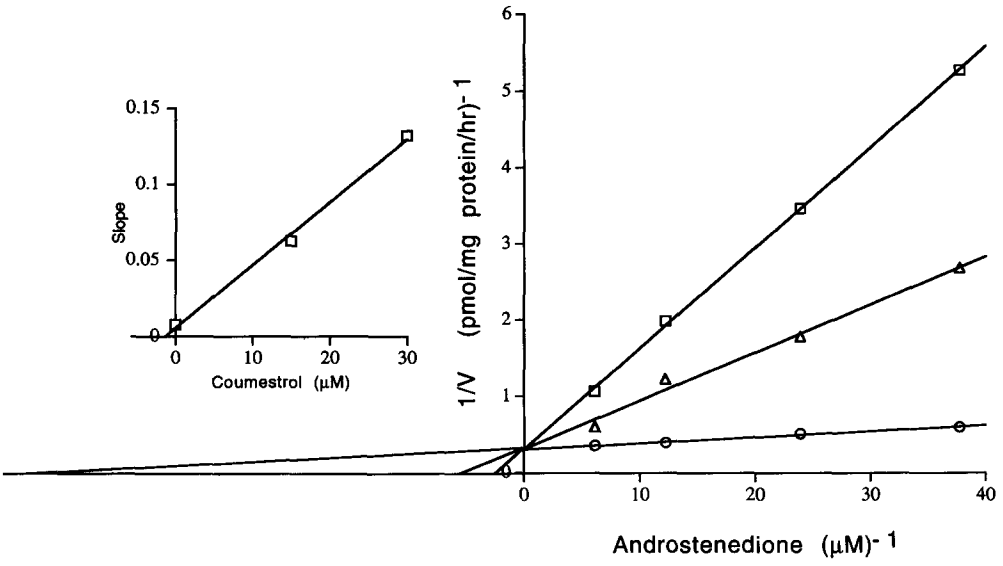


Fig. 5. Double-reciprocal plot showing the effect of substrate (^{3}H-androstenedione) concentration on aromatase activity in the presence of 0 (○), 15 (△) and 30 (□) μM coumestrol. The K_m is the negative, inverted x intercept and the V_{max} is the inversion of the y intercept. The V_{max} was 3.2 pM, and the K_m was 25, 199 and 422 nM, for 0, 15 and 30 μM coumestrol, respectively. Inset. The effect of coumestrol concentration on the slope of the double-reciprocal plot lines. The K_i value (negative x intercept), reflecting inhibitor effectiveness, was 1.3 μM.

to competitively inhibit aromatase enzyme in human predipocytes. Although DMDM and DDMM are theoretical intermediates between matairesinol and Enl and DDSI, DMSI and ODSI are theoretical intermediates between secoisolariciresinol and End, similar intermediates have been identified in primate urine, suggesting that they are true physiological intermediates [37].

Enl and its theoretical precursors were moderate inhibitors, while End and its theoretical precursors were weak inhibitors. The K_i values of the moderate inhibitors were between 10 and 30 times the K_i of AG, suggesting that the affinities of these lignans to aromatase were 10–30-fold weaker than that of AG. At the same time, the K_i values were between 150 and 500 times the K_m of androstenedione, indicating that these compounds bind preadipocyte aromatase less than 1% as tightly as androstenedione binds aromatase.

Using human placental microsomes, Adlercreutz *et al.* [20] first reported that Enl and its theoretical precursors were competitive inhibitors of aromatase. The I_{50} values of Enl and DDMM (4,4′-dihydroxyenterolactone) were 14 and 6 μM, respectively, 5–10 times lower than our results. The K_i value of Enl in placental microsomes was 6 μM, less than half of our finding of 14.4 μM in preadipocytes. The affinity of Enl for aromatase in their study was 1.3% of that of androstenedione, 3–10-fold greater than in our study. Thus, the inhibitor affinity and potency we observed in preadipocytes was 2–10-fold lower than that observed by Adlercreutz *et al.* in placental microsomes. These differences between whole cell and placental microsome systems are similar to those we reported previously in studies of flavonoid aromatase inhibitors [23]. As we suggested earlier, the lower potency of lignans in preadipocytes is possibly due to the incomplete passage of lignans through the cell membrane barrier in whole cells. The intracellular concentration of inhibitor would likely be lower than that in the culture medium. The preadipocyte microsomes would therefore be exposed to lower concentrations of lignan than the placental microsomes, leading to lower inhibitory affinity and efficiency.

The I_{50} value of DDMM (6 μM) was lower than that of Enl (14 μM) in the experiments of Adlercreutz *et al.*, suggesting greater potency with DDMM. Our studies also showed this relationship in preadipocytes, with I_{50} values of 60 and 74 μM, and K_i values of 7.3 and 14.4 μM for DDMM and Enl, respectively.

Adlercreutz *et al.* also tested the effect of Enl on aromatase activity in the human choriocarcinoma cell line JEG-3 [20]. After 1–2 h incubation with Enl, estrone production was 73–78, 61–63 and 24–27% of the control level when Enl concentrations were 1, 10 and 100 μM, respectively. The inhibition in JEG cells was greater than in preadipocytes (aromatase activities of 97%, 89% and 37% of control level at 1, 10 and 100 (μM Enl), most likely reflecting differences in cell type.

Our study confirmed the observations of Adlercreutz *et al.* that End is a somewhat weaker aromatase inhibitor than Enl in placental microsomes [20]. Structural differences may contribute to this observed difference in inhibitor potency between Enl and End. While both are diphenolic compounds, Enl and its theoretical precursors contain a keto-tetrahydrofuran ring, and

End and its theoretical precursors are without such a ring. The presence of this ring structure may either directly increase the affinity of Enl to aromatase enzyme, or it may increase lipid solubility, allowing Enl and its theoretical precursors to enter the cell more easily and gain access to the binding site of aromatase. An effect of the ring on lipid solubility may also explain our finding that the difference in potency between End and Enl is greater in whole cells than in microsomes, with End being a far weaker inhibitor in whole cells.

Our results with flavonoids are the first to demonstrate that the isoflavone, coumestrol, the flavone, luteolin, and the flavonol, kaempferol, are competitive inhibitors of aromatase enzyme activity. K_i values for the flavonoids were 2.6, 9.6 and 54 times the K_i of AG, for coumestrol, luteolin and kaempferol, respectively, reflecting inhibitor potencies that were about 40%, 10% and 2% of the potency of AG. Their affinities to aromatase enzyme were less than 3% that of androstenedione.

Coumestrol is a phytoestrogen known to bind the estrogen receptor [38] and exert estrogenic effects. These include induction of vaginal opening, cornification and cycling; stimulations of uterine metaplasia and promotion of hemorrhagic follicles [39, 40]; stimulation of cytosolic progestin receptors [40]; and proliferation of MCF-7 [41] and other mammary tumor cells [38]. Our findings suggest that the estrogenic effects of coumestrol may be opposed in some cases by its inhibition of estrogen synthesis.

The isoflavone, *O*-Dma, did not inhibit aromatase enzyme in preadipocytes. This was not surprising, as the structure of *O*-Dma is similar to that of other isoflavones, such as daidzein and genistein, that we previously reported to be noninhibitors [23]. On the other hand, Adlercreutz *et al.* [20] found *O*-Dma to be a weak aromatase inhibitor in human placental microsomes, with an I_{50} of 160 μM. Under our conditions in whole cells, we have generally found I_{50} and K_i values to be 10-fold greater than those reported in placental microsomes. With an I_{50} of 160 μM in placental microsomes [20], we would be unable to detect inhibition by *O*-Dma in our system.

The flavonols, morin and fisetin, were found to be noninhibitors of aromatase enzyme in our system. Our results with morin differ from those of Moochhala *et al.*, who found morin to bind aromatase enzyme in placental microsomes [42]. These investigators found morin to bind aromatase to a similar degree as does quercetin, a flavonol that we previously reported to be a noninhibitor in preadipocytes [23]. Although morin binds aromatase enzyme weakly in placental microsomes, as do quercetin and *O*-Dma, limited passage through the cell membrane causes these weak inhibitors to fall below the limits of sensitivity of our whole cell system.

Several reports have explored the relationship between flavonoid structure and ability to inhibit aromatase enzyme activity. Ibrahim and Abul-Hajj suggested the importance of C-7 hydroxylation for binding to aromatase [21], while Moochhala *et al.* suggested the importance of 5,7-dihydroxylation [42]. In our study, all of the compounds have a C-7 hydroxyl, while two of the three inhibitors and one of the three noninhibitors have a C-5 hydroxyl. Quercetin, a flavonol previously reported to be a non-inhibitor [23], also has a C-5 hydroxyl. Thus, for this group of compounds, hydroxylation at the 5 or 7 position does not appear to be a major determinant of inhibitory effect. Our findings do, however, support previous reports of decreased inhibition by C-3 hydroxylated flavonoids [21, 23, 42]. In our study, all of the noninhibitor flavonols, as well as the weak inhibitor, kaempferol, have a C-3 hydroxyl, while the stronger inhibitor, luteolin, does not. The increased inhibitory capacity of luteolin, relative to quercetin, strongly supports this hypothesis. In addition, the increased inhibitory capacity of kaempferol relative to quercetin suggests that the presence of a C-3′ hydroxyl reduces inhibitory capacity and the increased inhibitory capacity of kaempferol relative to morin suggests that the presence of a C-2′ hydroxyl reduces inhibitory capacity.

There are no reports of the effects of lignans or flavonoids on aromatase enzyme activity in living organisms. Considering the much lower affinities of these compounds to aromatase than that of the substrate, it is reasonable to assume that very high levels of inhibitor would be required to successfully compete with androstenedione and testosterone. Few data have been reported, however, on the normal circulating levels of these compounds in humans. Adlercreutz *et al.* reported mean plasma levels of End and Enl of 2.5 and 33 nM, respectively, in omnivorous women and 17 and 253 nM in vegetarian women [43]. At 253 nM, the circulating level of Enl in vegetarians is about 50-fold lower than the K_i of Enl, suggesting that the physiological significance of inhibition by Enl alone is unlikely.

Although the concentration of any one compound may be low relative to the individual K_i value, the total effect of many aromatase inhibitors circulating *in vivo* over a lifetime, may be of physiological significance. Additional dietary aromatase inhibitors have been reported, including other lignans [20] and flavonoids such as chrysin and biochanin A [23], hydroxyflavones [21] and apigenin and flavanone [22]. The estrogen-reducing effects of these compounds may be enhanced by their other reported antiestrogenic properties, such as suppression of estrogen action and bioavailability. These effects would be more important in vegetarians, as a result of their higher consumption of plant foods containing lignans and phytoestrogens.

These results provide further evidence that naturally-occurring dietary compounds inhibit aromatase enzyme activity in intact preadipose cells. Our findings suggest that lignans and flavonoids may contribute to

the cancer-preventive properties of plant-based diets by inhibiting estrogen synthesis in adipose tissue. Although it is unlikely that any one compound alone inhibits aromatase inhibitor significantly *in vivo*, the physiological significance of long-term consumption of many such compounds has yet to be explored.

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