Coadministrating Luteolin Minimizes the Side Effects of the Aromatase Inhibitor Letrozole

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ABSTRACT

Aromatase inhibitors (AIs) have been used as adjuvant therapeutic agents for breast cancer. Their adverse side effect on blood lipid is well documented. Some natural compounds have been shown to be potential AIs. In the present study, we compared the efficacy of the flavonoid luteolin to the clinically approved AI letrozole (Femara; Novartis Pharmaceuticals, East Hanover, NJ) in a cell and a mouse model. In the in vitro experimental results for aromatase inhibition, the Ki values of luteolin and letrozole were estimated to be 2.44 μM and 0.41 nM, respectively. Both letrozole and luteolin appeared to be competitive inhibitors. Subsequently, an animal model was used for the comparison. Aromatase-expressing MCF-7 cells were transplanted into ovariectomized athymic mice. Luteolin was given by mouth at 5, 20, and 50 mg/kg, whereas letrozole was administered by intravenous injection. Similar to letrozole, luteolin administration reduced plasma estrogen concentrations and suppressed the xenograft proliferation. The regulation of cell cycle and apoptotic proteins—such as a decrease in the expression of Bcl-xL, cyclin-A/D1/E, CDK2/4, and increase in that of Bax—was about the same in both treatments. The most significant disparity was on blood lipids. In contrast to letrozole, luteolin increased fasting plasma high-density lipoprotein concentrations and produced a desirable blood lipid profile. These results suggested that the flavonoid could be a coadjuvant therapeutic agent without impairing the action of AIs.

Introduction

Since estrogen is critical in the initiation and progression of breast cancer, estrogen receptor (ER) antagonism has been a common strategy for treating this disease. Tamoxifen is a widely used ER antagonist in breast cancer therapy. However, it may cause endometrial hyperplasia and elevate the cancer risk at this tissue (Lindahl et al., 2008). Moreover, it also increases the risk of stroke after long-term treatment (Hernandez et al., 2001).

Another type of drug for breast cancer therapy is aromatase or CYP19 inhibitor (AI). Estrogen is converted from cholesterol, and aromatase catalyzes the rate-limiting reaction. AIs, which can be steroidal or nonsteroidal compounds, block the formation of estrogen and reduce the stimulus for growth in ER-positive tumors. Steroidal AIs bind to the active site of aromatase, whereas their nonsteroidal counterparts reversibly interact with the enzyme (Campos, 2004). AIs are superior to tamoxifen in treating advanced breast cancer with fewer side effects (Bonnetere et al., 2001; Cuzick, 2003). Unlike tamoxifen, AIs have no agonistic activity toward the ER in any tissues.

Letrozole (Femara; Novartis Pharmaceuticals, East Hanover, NJ), or 4,4’-((1H-1,2,4-triazol-1-yl)methylene)dibenzoazinitrile, is an approved AI for treating estrogen-sensitive breast cancer after surgery. Because the action of AIs is not tissue-specific, side effects may result from prolonged estrogen deprivation. Increased bone turnover has been observed in women treated with letrozole (McCaig et al., 2010). Postmenopausal women on letrozole therapy could also develop an undesirable blood lipid profile, such as increased levels of total cholesterol, low-density lipoprotein cholesterol, and apolipoprotein B (Elisaf et al., 2001).

Luteolin, or 3’,4’,5’,7’-tetrahydroxylavone, is a common flavonoid which can be isolated from vegetables such as celery, broccoli, carrots, thyme, green peppers, etc. It is one of the most potent aromatase inhibitors in the flavonoid family in vitro (Jeong et al., 1999; van Meeuw et al., 2008). Furthermore, it is capable of containing the transcriptional activity of aromatase.
in MCF-7 cells (Li et al., 2011). In the present study, luteolin’s potency of aromatase inhibition and its side effects were investigated in cell and mouse models. We hypothesized that luteolin could be a therapeutic agent with beneficial effects on the bone and blood lipid.

**Materials and Methods**

**Chemicals.** Luteolin was obtained from Indofine Chemical Co., Inc. (Hillsborough, NJ). Letrozole was purchased from International Laboratory USA (San Bruno, CA). Other chemicals, if not stated, were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell Culture.** The breast cancer cell line MCF-7 was purchased from American Type Culture Collection (Rockville, MD). MCF-7 cells stably transfected with human CYP19 (MCF-7aro) were prepared as previously described (Zhou et al., 1990). The stably transfected MCF-7 cells were maintained in minimum Eagle’s medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen/Life Technologies, Rockville, MD) and the selection antibiotic G418 (Genetin, 500 µg/ml; USB, Cleveland, OH). Cells were incubated at 37°C in 5% carbon dioxide and routinely subcultured when 80% confluency was reached.

**Enzyme Kinetic Assay.** To illustrate the aromatase-inhibitory property of luteolin, an enzyme kinetic assay was performed using a previously described method (Wang et al., 2008). MCF-7aro cells were seeded in six-well plates at a density of 5×10^5 per well for 24 hours. The reaction was initiated by replacing serum-free medium containing [1β-3H]4-androstene-3,17-dione and luteolin or letrozole. After incubation for 1/2 hour at 37°C, the medium was removed and mixed with an equal volume of chloroform. The mixture was then centrifuged at 10,000 g for 10 minutes. The aqueous phase was transferred into a tube containing 5% activated charcoal suspension. After 30-minute incubation, the supernatant was aliquoted out for scintillation counting. A bicinchoninic acid kit (Sigma-Aldrich) was used to determine protein content of cells.

**Animal Experimentation.** This mouse model for postmenopausal breast carcinogenesis was adopted from Yue et al. (1994), where the tumor growth was dependent on the aromatase activity. The model was used for testing the effect of luteolin on aromatase, blood lipid profile, and the bone. Six-week-old female athymic mice were acquired from the Animal Facility of Chinese University of Hong Kong. The mice were ovariectomized, and were fed the purified and phytoestrogen-free AIN-93G diet (Reeves et al., 1993). After 2 weeks of recovery, they were transplanted with MCF-7aro cells and randomly assigned into regimens as stated in Table 1. The treatment lasted for 94 days after cell inoculation.

Luteolin was dissolved in ethanol and diluted to 100–200 µl by phosphate-buffered saline (PBS) for gavage, and the final ethanol concentration was 1%. Androstenedione and letrozole were dissolved in 0.1 ml of 0.3% hydroxyl propyl cellulose and given as subcutaneous injections. Control mice received the carrier solvent injection; mice treated with androstenedione (AD), androstenedione plus 2 µg/day letrozole (AD+2 µg LET), and control mice were also given PBS with 1% ethanol by mouth. Before transplantation, MCF-7aro cells were cultured as described earlier. The cells were trypsinized and suspended in Matrigel matrix (10 mg/ml; BD Biosciences, San Jose, CA). Then 0.1 ml of cells (3×10^6 cells/ml) was injected into the two flanks of the animal. Body weight, tumor size, and food intake were monitored weekly throughout the study. Tumor volume was measured by an electronic caliper and estimated according to the following formula: \( V = \frac{1}{6} \times \text{length} \times \text{width} \times \text{height} \), where length, width, and height were the three orthogonal diameters of the tumors. The average volume of two xenografts from the same mice was treated as one data point. At the end of the study, the mice were fasted overnight and euthanized by cervical dislocation. Livers and uteri were excised and weighed. These

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Group</th>
<th>Androstenedione mg/day</th>
<th>Luteolin mg/kg body wt/day</th>
<th>Letrozole µg/day</th>
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<td>1</td>
<td>Control</td>
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<td>0</td>
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</tr>
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<td>5</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>5</td>
<td>AD+LUT 50</td>
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<td>50</td>
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<tr>
<td>6</td>
<td>AD+LUT 20 + 0.04 µg LET</td>
<td>0.1</td>
<td>20</td>
<td>0.04</td>
</tr>
<tr>
<td>7</td>
<td>AD+LUT 20 + 2 µg LET</td>
<td>0.1</td>
<td>20</td>
<td>2</td>
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<tr>
<td>8</td>
<td>AD+2 µg LET</td>
<td>0.1</td>
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![Fig. 1. Enzyme kinetic analysis of luteolin and letrozole on aromatase inhibition.](image-url)
organs were the respective indicators for hepatotoxicity and estrogen status. Tumors and serum were collected and stored at −80°C until assayed. Femurs were removed and soaked in ethanol for scanning. The procedure was approved by the Animal Experimentation Ethics Committee, Chinese University of Hong Kong.

Serum Estradiol Determination. Aromatase is the key enzyme for estrogen synthesis. Serum estrogen levels in the model mice reflected the activity of the enzyme. Serum estradiol concentrations were measured by enzyme-linked immunosorbent assay (Cayman Chemical Company, Ann Arbor, MI). The samples were added into a 96-well plate coated with antibody raised against estradiol. After incubation and development at room temperature, the absorbance at 410 nm was quantified using a microplate reader (FLUOstar; BMG Labtechnologies GmBH, Offenburg, Germany).

Immunoblot of Proteins Extracted from Tumor. Western blot analysis was carried out for comparing the cell cycle and death of proteins. The frozen tumors were pulverized in a Dounce homogenizer with added liquid nitrogen. The pulverized samples were then sonicated in lysis buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 40 mg/l phenylmethyl-sulfonyl fluoride, 0.5 mg/l aprotinin, 0.5 mg/l leupeptin, 1.1 mM EDTA, and 0.7 mg/l pepstatin) with a cell disruptor (Branson Ultrasonics Corp., Danbury, CT) on ice for

Fig. 2. Both luteolin and letrozole administration inhibited the growth of MCF-7aro xenograft. Mice were inoculated with MCF-7aro cells at two sites per mouse after ovariectomy, and were treated with letrozole and/or luteolin. (A) Tumor volumes were estimated once a week starting from day 7 after inoculation. (B) The tumor weights were measured on the day of sacrifice. The data were analyzed by one-way ANOVA, followed by Tukey’s multiple comparison test. Values are means ± S.E.M., n = 6–8. Means labeled with different letters are significantly different (order: a > b > c; P < 0.05).

Fig. 3. Reduced plasma estradiol concentration (Conc.) in luteolin-treated mice. Blood was drawn from the animals at euthanasia. Serum estradiol concentration was quantified by enzyme-linked immunosorbent assay. The data were analyzed by one-way ANOVA, followed by Tukey’s multiple comparison test. Values are means ± S.E.M., n = 6–8. Means labeled with different letters are significantly different (order: a > b > c; P < 0.05).

Fig. 4. Luteolin counteracted uterine weight reduction under letrozole treatment. Uteruses of the experimental animals were dissected and weighed at sacrifice. The data were analyzed by one-way ANOVA, followed by Tukey’s multiple comparison test. Values are means ± S.E.M., n = 6–8. Means labeled with different letters are significantly different (order: a > b > c; P < 0.05).
30 seconds for protein extraction. Thirty micrograms of protein extract was separated on 10% SDS-PAGE gel and transferred onto an Immobilon polyvinylidene membrane (Millipore, Bedford, MA). Anti-CDK2 and 4, cyclin A, B1, E, P57, P21 (Santa Cruz Biotechnology, Santa Cruz, CA), anti–cyclin D1 (Abcam, Cambridge, UK), anti–Bcl-xL, Bcl-2, Bax, Bak (Santa Cruz Biotechnology), and anti–β-actin primary (Sigma-Aldrich) and secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) were used for protein detection. The targeted proteins were visualized by autochemiluminochemistry. The optical density readings of the images were analyzed using the computer software ImageJ (NIH, Bethesda, MD), and optical density of the corresponding β-actin was used for normalization.

Bone Microarchitecture Analysis. Bone integrity is a major concern for administering AI, so the status of bone was examined under luteolin and letrozole treatments. High-resolution microcomputed tomography was used for bone microarchitecture evaluation. The femora were placed and affixed in a specimen holder submerged in 70% ethanol. The bone volume fraction, trabecular thickness, and trabecular number were analyzed as previously described (Siu et al., 2004; Shi et al., 2010; Chow et al., 2011).

Analysis of Serum Lipoproteins. Another side effect of AI is in the induction of an undesirable plasma lipid profile. This part of the experiment was to investigate the effect of luteolin on plasma lipids. Plasma total cholesterol (TC) and total triglycerides were measured using the respective commercial kits from Infinity (Waltham, MA) and Stanbio Laboratories (Boerne, TX). For measuring plasma high-density lipoprotein cholesterol (HDL), low-density lipoprotein (LDL) and very-low-density lipoprotein were precipitated by phosphotungstic acid and magnesium chloride (Stanbio Laboratories). HDL in the supernatant was determined using the Infinity kit. The value of LDL cholesterol was estimated by subtracting HDL from TC, i.e., TC – HDL = LDL.

Statistical Analysis. The data were analyzed by the software package GraphPad Prism 5 for Windows, version 5.04 (GraphPad Software, Inc., San Diego, CA). One-way analysis of variance (ANOVA) tests were used for group comparisons; the post-hoc ranking test (Tukey’s multiple comparison test) was performed to differentiate the treatments when P < 0.05. For the tumor volume data, two-way ANOVA was used for analysis. As ANOVA alone would not indicate differences among individual treatments, post-hoc ranking comparison was needed for such differentiation (Figs. 2–7). For instance, means labeled with a were significantly different from those labeled with b but were not different from those labeled with a,b in the present labeling system. The order of magnitude was shown at the end of figure legend in parentheses.
For the enzyme kinetic studies, the data were processed by nonlinear regression and fitted into the built-in mixed model of inhibition in GraphPad Prism 5 with the default setting. No data weight adjustments in the enzyme activity were made. \( K_i \) and \( a \) values were obtained from the program printouts.

### Results

#### Cell Study

**Enzyme Kinetic Results.** Five concentrations, i.e., 0, 1, 2.5, 5, and 10 \( \mu \)M, were selected for kinetic analysis for luteolin, whereas 0, 0.0025, 0.005, and 0.25 \( \mu \)M were used for letrozole. Aromatase enzyme activities were plotted against substrate concentrations for luteolin and letrozole in Fig. 1, A and B, respectively. Mixed-model analysis for luteolin indicated that a competitive type of inhibition on aromatase was apparent with a calculated \( K_i \) value of \( 2.44 \) \( \mu \)M (S.E. = 1.29 \( \mu \)M) and \( a \) value of 26.65 (S.E. = 34.05). Letrozole was also shown to be a competitive inhibitor with an estimated \( K_i \) value of 0.41 nM (S.E. = 0.22 nM) and \( a \) value of 26.21 (S.E. = 17.52) in this system. This result indicated that luteolin was a weak AI compared with letrozole.

#### Animal Study

**Treatments of Luteolin and Letrozole Did Not Affect Body or Liver Weight.** Mouse body weights were monitored weekly. There were no significant differences in body weights or liver weights (data not shown). Treatments of luteolin, letrozole, and the combination at the given concentrations appeared to be tolerable to the mice.

**Luteolin or Letrozole Deterred Xenograft Proliferation.** The tumor size of AD was significantly \((P < 0.05)\) larger than that of control mice starting from day 14 to the end of the experiment. Starting from day 35, tumor volumes in mice receiving luteolin or letrozole were smaller than that in AD mice (Fig. 2A). Among the luteolin treatment groups, a dose-response relationship was observed. The tumor weights measured at sacrifice were consistent with the tumor volume results (Fig. 2B). High doses of luteolin administration could achieve a tumor-suppressing effect comparable to that of letrozole.

**Luteolin Reduced Plasma Estradiol Concentration.** Increased serum estradiol concentration was seen in AD mice as compared with that in control mice. The concentration was suppressed by luteolin, letrozole, or coadministration of the two (Fig. 3). The estradiol concentration in the sham-operated group was the highest among all groups, but was not different \((P > 0.05)\) from the AD group (data not shown). The aromatase-expressing xenografts synthesized a significant amount of estradiol from the substrate, and both letrozole and luteolin could prevent the production of estradiol.

**Luteolin and Letrozole Counteracted Androgen-Induced Uterine Growth.** The uterine wet weight of androstenedione-injected mice displayed a 2-fold increase as compared with that of control mice. Treatment with 2 \( \mu \)g of letrozole or 50 mg/kg luteolin prevented the androstenedione-induced uterine growth (Fig. 4). No significant differences were seen with the other treatments. The uterotrophic effect of luteolin appeared to be coming from aromatase inhibition.

**Cell Cycle and Apoptotic Protein Expression in Tumors.** Luteolin at 50 mg/kg per day and letrozole at 2 \( \mu \)g/day altered the...
proteins of the cell cycle (Fig. 5) and apoptosis (Fig. 6) in a comparative pattern. Both drugs increased Bax and prevented the androstenedione-induced cyclin A and E, Cdk2 and 4, and Bcl-xL expression in the tumor samples. However, the suppression of letrozole on Cdk4 and Bcl-xL was significantly stronger than that curbed by luteolin. In addition, reduced expression of cyclin D1 was only observed in letrozole-treated samples. Although the xenograft growth was suppressed by both letrozole and luteolin, the mechanisms could be different.

**Luteolin Did Not Change the Bone Status.** Compared with the ovariectomized control mice (control), AD mice exhibited a 10% increase in trabecular number (ThN). The estrogen status was consistent with the ThN results. Letrozole reduced ThN, and luteolin administered at a high dosage apparently induced a similar reduction, although the decrease was not significant (data not shown). The result of bone volume fraction (data not shown) showed a similar pattern as that of ThN. The bone status would not benefit from luteolin administration.

**Luteolin Increased HDL Levels in Mice.** Luteolin did not change plasma TC or total triglycerides (data not shown), but increased HDL levels in a dose-dependent manner (Fig. 7A). The levels in mice treated with 50 mg/kg luteolin (AD + LUT 50) were higher than those treated with androstenedione or AD + 2 μg LET (Fig. 6A). When expressed as the ratio of HDL to TC, the values of groups of AD + LUT 20 and AD + LUT 50 were significantly higher than that of AD + 2 μg LET (Fig. 7B). On the other hand, the ratio of LDL/HDL in AD + LUT 20 and AD + LUT 50 was significantly lower than that in AD + 2 μg LET (Fig. 7C). No matter whether administered alone or together with letrozole, luteolin could favorably change the plasma lipid profile.

**Discussion**

AI is an adjuvant therapeutic drug for estrogen-responsive breast cancer. However, it may induce adverse effects on blood lipids (Confavreux et al., 2007; Rabaglio et al., 2009). Some
flavonoids are able to improve plasma lipid profiles. However, the isoflavone genistein may also compromise the action of letrozole (Ju et al., 2008). By comparing the estimated $K_i$ values in the present study, the aromatase-inhibitory effect of luteolin was about 5900-fold weaker than that of letrozole. Unlike the antagonistic effect seen in the aforementioned study of genistein, luteolin would not weaken the activity of AI.

Estrogen is known to influence the blood lipid profile, such as increasing plasma HDL and decreasing LDL (Espeland et al., 1998; Nasr and Breckwoldt, 1998). Increases in total serum cholesterol, LDL, and serum lipid have been observed in breast cancer patients receiving AI (Nicolaides et al., 2000; Elisasf et al., 2001). Another study has found that exemestane and letrozole may differentially affect HDL and LDL; nevertheless, the ultimate LDL/HDL ratios are significantly increased in patients treated with the two AIs (Bell et al., 2012).

The effect of luteolin on lipid metabolism has been reported previously. Luteolin or its conjugate reduced plasma LDL and TC in diabetic rats (Wang et al., 2012) and normal adult rats (Azevedo et al., 2010), and elevated HDL levels in the study by Wang et al. (2012). The flavone also prevents lipid accumulation in cultured hepatic cells (Liu et al., 2011). These properties may reduce the risk of cardiovascular disease. In the present study, luteolin administration could increase HDL levels and suppress the ratio of LDL/HDL in the blood. Moreover, the coadministration of luteolin and letrozole reduced the LDL/HDL ratio without elevating serum estradiol and the xenograft growth rate. This suggested that luteolin could be a cotherapeutic agent to AIs.

Bone homeostasis is regulated by the activities of osteoclasts and osteoblasts. Estrogen can directly or indirectly prevent bone resorption by suppressing the formation and survival of osteoclasts. AI therapy would induce bone loss by depriving the hormone (Pfeilschifter and Diel, 2000). Luteolin has previously been shown to induce G1 arrest (Husdal et al., 2006), and transition from G1 into the S phase is regulated by the activities of cyclin-associated kinases (Morgan, 2000). Cyclins in the G1 phase of the cell cycle are overexpressed in breast cancer patients, with cyclin A and cyclin E overexpressing in breast cancer patient (Keyomarsi et al., 2002; Lim et al., 2007). In the present study, increased expression levels of cyclins A, D1, and E, and the related CDR2 and 4, were observed in androstenedione-treated mice. Treatment of luteolin or letrozole could reverse the expression levels of these genes.

These results also show that these agents may influence apoptosis. Estrogen may alter Bcl-2 family gene expression in favor of cell survival (Leung and Wang, 1999). In the current study, androstenedione induced Bcl-xL and Bcl-2 and suppressed Bax in the tumor samples. Letrozole or luteolin reversed the expression of Bcl-xL and Bax with no effect on Bcl-2.

Although luteolin is widely distributed in plant foods, the dosages used in the current study were above the normal levels that humans consume. Nevertheless, this study could provide the scientific basis for nutraceutical or pharmacological development of this flavone.

In conclusion, our results demonstrated that luteolin suppressed MCF-7arxo xenograft growth while raising HDL. As a high LDL/HDL ratio is usually associated with long-term use of AI, the administration of luteolin can be a potential compensation mechanism without compromising aromatase inhibition.

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Authorship Contributions

Participated in research design: Leung, Li, Wong, Lin.

Conducted experiments: Li, Wong, Lin, Chow.

Contributed new reagents or analytic tools: Leung, Chan, Chen, Cheung.

Performed data analysis: Leung, Li, Lin, Chow, Wong.

Wrote or contributed to the writing of the manuscript: Leung, Li, Wong.

References


Luteolin Inhibits Aromatase and Raises HDL In Vivo


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