Biochemical and Biological Characterization of a Novel Anti-aromatase Coumarin Derivative*

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Estrogen stimulates the proliferation of estrogen receptor (ER)-positive breast cancer cells. Aromatase is the enzyme responsible for the conversion of androgens into estrogens, and synthetic aromatase inhibitors such as letrozole, anastrozole, and exemestane have proven to be effective endocrine regimens for ER-positive breast cancer. In a recent study, we have found that 4-benzyl-3-(4′-chlorophenyl)-7-methoxycoumarin is a potent competitive inhibitor of aromatase with respect to the androgen substrate. Its 

Furthermore, (−)-epigallocatechin-3-gallate in green tea has been recently reported to be an inhibitor of 5-cytosine DNA methyltransferase (2). It was predicted that (−)-epigallocatechin-3-gallate could prevent or reverse gene silencing by suppressing DNA methylation. In our laboratory, several flavones have been demonstrated to be effective inhibitors of aromatase (estrogen synthetase) (3) and NADPH:quinone reductase 1 and 2 (4–6). These enzymes play important roles in mammary carcinogenesis. Recently, iso flavones and flavonoids have also been shown to be agonists of estrogen-related receptors (ERRs) (7); therefore, these chemicals can modulate the biological activity of these receptors. Furthermore, we have isolated and identified procyramid B dimers from red wine and grape seeds that act as competitive inhibitors of aromatase, and oral intake of these chemicals suppresses the growth of aromatase-mediated breast tumors in nude mice (8). Coumarins are a major type of phytochemicals, and the therapeutic potential of several coumarins have been discussed (9). Furthermore, several coumarin derivatives have been reported to be steroid sulfatase inhibitors and evaluated for breast cancer therapy (10, 11).

However, the interaction between coumarins and aromatase has not yet been reported.

Aromatase, a cytochrome P450, is the enzyme that synthesizes estrogens by converting C19 androgens (androstenedione and testosterone) to aromatic C18 estrogenic steroids (estrone and 17β-estradiol). This and other laboratories have shown that aromatase is expressed at higher levels in breast cancer cells and/or surrounding adipose stromal cells than in noncancerous breast cells (12–15). Aromatase inhibitors have been found to be valuable in treating these estrogen-dependent and aromatase-mediated diseases including breast cancer (16). The United States Food and Drug Administration approved two new aromatase inhibitors, anastrozole and letrozole, for use as first-line agents against estrogen-responsive cancer in postmenopausal women. In the recent arimidex, tamoxifen, alone, or in combination (ATAC) trial, anastrozole was found to be more effective than tamoxifen in the treatment of ER-positive breast cancer in postmenopausal women, and anastrozole treatment was shown to significantly prevent contralateral cancers (17). In addition, letrozole was found to be very effective in treating Her-2 over-expressing and ER-positive breast cancer (18). Therefore, suppression of in situ estrogen formation in the breast of postmenopausal women by aromatase inhibitors is considered to be a useful way to prevent and treat breast cancer in these women.

In this study, we have examined 21 coumarin derivatives and found 4-benzyl-3-(4′-chlorophenyl)-7-methoxycoumarin to be a potent competitive inhibitor of aromatase with respect to the androgen substrate. Its 

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The abbreviations used are: ER, estrogen receptor; ERR, estrogen-related receptor; AR, androgen receptor.

Phytochemicals are powerful food factors, found in fruits, vegetables, herbs, and other whole foods, that elicit profound effects on our health. The research into how phytochemicals work and the role they play in human bodies is expanding rapidly. A significant number of phytochemicals have been found to interact with enzymes and nuclear receptors in specific manners, leading to the modulation of selective physiological mechanisms. For example, genistein in soybeans is known to be an agonist of estrogen receptor (ER)1 (1), thus, this chemical is thought to compete with estrogen for binding to ER.

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EXPERIMENTAL PROCEDURES

Materials—We have examined the interaction of 21 coumarins with aromatase. The names of these compounds are shown in Table I. These coumarins were purchased from Indofine Chemical Co., Inc. (Somerville, New Jersey). A stable aromatase-expressing the estrogen receptor (i.e., MCF-7aro) has been prepared in our laboratory by aromatase cDNA transfection and G418 (neomycin) selection (19) and was used in this study. Aromatase activity in the MCF-7aro cell line was determined to be 73 ± 6 pmol/mg/h. MCF-7aro cells were cultured in RPMI medium containing 10% fetal bovine serum (Invitrogen), 1× antibiotics, 1× sodium pyruvate, 2 mm l-glutamine, and 15 mm Hepes at 37 °C and 5% CO2. Aromatase wild-type and mutant expression vectors were prepared by Dr. A. S. Novartis, Switzerland, and Dr. Michael Dukes at Zeneca Pharmaceuticals, UK, respectively. The phenol red-free matrigel basement membrane matrix was purchased from Fisher Scientific Co.

Aromatase Assay, Placental Microsomal Assay, and In-cell Assay— Using a human placental microsomal preparation that is enriched with aromatase, aromatase activities (in the presence of various aromatases at five different concentrations) were determined by the tritiated water release method of Thompson and Siiteri (23), with [1H3]-androsten-3,17-dione as the substrate (specific activity 24.1 Ci/mmol) (PerkinElmer Life Sciences). The procedures were described in a published article by Engel et al. (8). The kinetic analyses were performed with the substrate concentrations ranging from 16 to 200 nm. For the inhibition studies, the Ki values of the inhibitors were determined according to a published method (22).

In the “in-cell” aromatase assay, aromatase-expressing cells were plated on 6-well plates in growth medium. When approximately confluent, the cells were washed twice with phosphate-buffered saline and 1 ml of serum-free medium containing inhibitor at various concentrations along with 100 nM [1H3]-androstenedione, as well as 1 µM progesterone, used to suppress the endogenous 5α-reductase that also consumes the androgen substrate. The incubations were carried out for 1 h, and the samples were centrifuged at 1000 × g for 10 min, and the aqueous upper layer from each sample was loaded onto a charcoal–dextran pellet to remove any trace of unreacted substrate. The samples were vortexed and subsequently centrifuged at 15,000 × g for 5 min. Aliquots of the supernatant preparations were counted in a liquid scintillation counter. Aromatase activity was calculated as pmol of tritiated water released/mg of protein/h (pmol/mg/h). The cells in each well were solubilized in 0.5 N sodium hydroxide, and the protein concentrations were quantified by the Bradford method (24).

The tritiated water release assay has been previously validated in our laboratory by the product isoallation assay (25). In addition, the activities of the wild-type aromatase and its mutants have been recently evaluated by the reaction intermediate profile analysis along with the tritiated water release assay (22).

Mammalian Cell Transfection and Luciferase Assays—The expression plasmid for the human ERRa, named pSG5-ERRa, was constructed as described previously (26). The ERRβ and ERRγ expression plasmids, pSG5-ERRβ and pSG5-ERRγ, were generated by Suetsumi et al. (27). Two luciferase reporter plasmids, pGL3(ERE)-luciferase and pGL3(stereiodogenic factor 1 site)-luciferase, was described by Zhou and Chen in a previous publication (28). In addition, the preparation of ERRγ expression plasmid, pSG5-ERRγ, was described by Kinoshita and Chen (29).

HeLa cells were cultured in minimal essential medium Earle’s salts medium and supplemented with a solution of 5% charcoal-dextran-
treated fetal bovine serum for 24 h. Afterward, the cells were transfected with 3 µg of Lipofectin and a total 0.75 µg of plasmid DNA containing various amounts of the test plasmids as indicated in each experiment and appropriate amounts of empty vector, pSG5, to maintain the same overall amount of total DNA in all transfections. After 5 h of incubation, the medium containing Lipofectin and DNA was removed, and the cells were cultured in the 5% charcoal-treated fetal bovine serum containing growth medium with or without ligands. Twenty-four hours after transfection, the cells in each well were lysed in 400 µl of 1× lysis buffer and harvested from the plates by scraping. The luciferase activities in the cell lysate with the same amount of protein were then measured according to the manufacturer’s instruction (Promega). Each experiment was repeated at least three independent times, and each sample was tested in triplicate.

Computer Modeling—To rationalize our experimental results, we superimposed the five coumarins demonstrating the most potent anti-aromatase activity with the natural substrate of aromatase, androstenedione, using the Sybyl FlexS program version 1.10 (30) interfaced with Tripos Sybyl 6.9 (Tripos, Inc., St. Louis, MO) on a Silicon Graphics O2+ work station with the IRIX 6.5 operating system (31). The goal for the alignments is to find three-dimensional structural characteristics that confer biological activity. FlexS makes use of an incremental construction algorithm. The reference molecule is kept rigid, whereas the ligands are suitably imposed as flexible fragments. The first step involves breaking the reference and test ligands down into small rigid fragments, followed by the selection of a core fragment from each ligand. The program aligns these core fragments, and then the remaining fragments are added iteratively. At each step, flexibility is considered by allowing the newly added fragment to adopt a discrete set of conformations. The volume overlap with the reference is checked, and a similarity scoring function is used to score the achieved similarity in a three-dimensional space. The FlexS scoring function is based upon paired intermolecular interactions and overlapping density functions (30). An accurate superposition is a prerequisite for subsequent exploitation of ligand-based computer virtual screening using either three-dimensional QSAR analyses or pharmacophore analyses.

In our case, androstenedione was selected as the reference ligand. Three-dimensional structures of both reference ligand and hope molecule and test compounds (5 coumarins) were generated with the CONCORD program within the Sybyl package (32), and partial atomic charges were calculated within FlexS using the Gasteiger–Huckel algorithm (33). In addition, the superpositioning, all structures were minimized using the Tripos force field with all its parameter settings at their default values. The FlexS alignment for each compound was visually inspected.

Matrigel Thread Three-dimensional Cell Culture—For the preparation of the matrigel thread were similar to those reported by Daly et al. (34). 1.0 × 106 MCF-7aro cells were prepared by a brief trypsinization of cells (at ~80% confluency), which were grown in RPMI medium containing 10% fetal bovine serum, 1× antibiotics (Gemini), 100 units of penicillin (g/ml), 100 µg of streptomycin sulfate/ml, and 0.5 µg of fungizone/ml at 37 °C and 5% CO2. The cell pellet was obtained by centrifugation at 3000 rpm for 10 min was gently mixed with 2 ml of matrigel solution for 10 min on ice so that the agitation would not introduce bubbles. Once the cells were homogenously mixed with the matrigel solution, the mixture was allowed to settle for 30 min on ice for bubble diffusion from the matrigel. Prechilled and sterilized 6-cm × 0.8-mm (inner diameter) Teflon tubes were filled with a cell-matrigel mixture by pulling gently using 35-ml syringe pistons that were attached at the one end of the Teflon tubes. Upon exposing the matrigel-filled Teflon tubes at room temperature for about 2–3 min, the coagulated matrigel threads were extruded out by the pressure created from the syringe in 5 ml of phenol red-free RPMI containing 10% fetal bovine serum (charcoal-coated dextran-treated serum) and delivered into a 6-well plate. After confirmation of homogeneous single cell distribution in the matrigel by microscope, the matrigel threads containing MCF-7aro cells were incubated in 5 ml of phenol red-free RPMI containing 10% fetal bovine serum (charcoal dextran-treated) at 37 °C and 5% CO2 for 24 h. For the positive controls, 10 nM of estradiol or testosterone was added to the culture media. The treated samples were cultured in media containing 100 nM letrozole, anastrozole, ICI 182780, tamoxifen, or coumarins, with or without 10 nM testosterone, with or without 10 nM of androstenedione. The colony formation and cell proliferation of MCF-7aro in the three-dimensional matrigel threads were photographed with 40x magnification using a Polaroid MicroCam (Polaroid Corporation, Cambridge, MA).

To quantify the effects of hormones, aromatase inhibitors, and/or estrogen agonists on the proliferation of MCF-7aro cells in the three-
Therefore, 4-benzyl-3-(4-chlorophenyl)-7-methoxy-3-phenylcoumarin was found to be a very poor inhibitor of aromatase (IC\text{50} value > 50 \mu M). In addition, we found that a compound with a 4-(4-chlorophenyl)-7-methoxy-3-phenylcoumarin was significantly reduced in comparison to that of the wild-type aromatase activity, demonstrating that the mutated amino acid residues are important for the binding of this coumarin (Fig. 3). Glu-302 and Thr-310 of the human aromatase have been suggested to participate in the hydroxylation reactions at the C-19 methyl group of the androgen steroid. Ser-478 and His-480 have been predicted to interact with C-4 and C-7 of the steroid (22). Our results from the aromatase mutant studies support that these coumarin inhibitors are active-site-directed.

**Structure-Activity Studies and Computer Modeling Analy-**

To provide some structural explanation of our experimental results, we assessed the shape similarity of the coumarins with four critical mutants, E302D, T310S, S478T, and H480Q, which decrease the aromatase activity significantly, most likely because these point-mutations are located in the active site and involved in the process of aromatization. The activities of these mutants are ~40–50% of that of the wild-type aromatase. Our analysis revealed that the aromatase inhibitory profiles of T310S, S478T, and H480Q by 4-benzyl-3-(4-chlorophenyl)-7-methoxy-3-phenylcoumarin were significantly reduced in comparison to that of the wild-type aromatase activity, demonstrating that the mutated amino acid residues are important for the binding of this coumarin (Fig. 3).

Results of aromatase assay for the 21 coumarin derivatives. —, not active.

| Compound name | IC\text{50} | μM |
|---------------|-------------|----|
| 4-Benzyl-3-(4'-chlorophenyl)-7-methoxy-3-phenylcoumarin | 0.08 | |
| 4-Benzyl-3-(4'-chlorophenyl)-7-hydroxy-3-phenylcoumarin | 0.3 | |
| 4-Benzyl-7-methoxy-3-phenylcoumarin | 1.0 | |
| 3-(4'-Bromophenyl)-7-hydroxy-4-phenylcoumarin | 6.0 | |
| 6-Hydroxy-3-(4'-methoxyphenyl)-4-methylcoumarin | 16.0 | |
| 4-Benzyl-7-hydroxy-3-phenylcoumarin | >50.0 | |
| 3-(2',4'-Dichlorophenyl)-7-hydroxy-4-phenylcoumarin | — | |
| 3-(4',3'-Dinitrophenyl)-7-hydroxy-4-phenylcoumarin | — | |
| 4-(4'-Chlorobenzyl)-7-methoxy-3-phenylcoumarin | — | |
| 4-Benzyl-6-chloro-3-(4'-chlorophenyl)-7-methylcoumarin | — | |
| 4-Benzyl-6-chloro-7-methyl-3-phenylcoumarin | — | |
| 3-(4'-Chlorophenyl)-7-methoxy-4-phenylcoumarin | — | |
| 6-Hydroxy-4-methyl-3-phenylcoumarin | — | |
| 3-(3'-Chlorophenyl)-7-methoxy-4-phenylcoumarin | — | |
| 7-Methoxy-3-(4'-methoxyphenyl)coumarin | — | |
| 7-Hydroxy-3-(4'-methoxyphenyl)coumarin | — | |
| 7-Hydroxy-3-(4'-methoxyphenyl)-4-methylcoumarin | — | |
| 7-Ethoxy-3-(4'-methoxyphenyl)-4-methylcoumarin | — | |
| 3-(2'-Chlorophenyl)-7-methoxy-4-phenylcoumarin | — | |
| 4-Hydroxycoumarin | — | |

**Figure 1. Chemical structures of the anti-aromatase coumarins.**

A three-dimensional matrigril model, the matrigril threads were incubated in the presence of 5 \mu Ci of [\text{methyl-3H}]thymidine for 12 or 30 days at 37 °C in a 5% CO\text{2} environment. Each thread was washed twice with phosphate-buffered saline to reduce the background tritium count caused by nonspecific tritium-labeled thymidine absorption by the matrigril. Each matrigril thread was then placed on a quadruply Kimwip and allowed to completely dry under the hood. The matrigril threads together with Kimwipes were placed in scintillation vials and presoaked in 5 ml of scintillation fluid for 10 min to make sure that they were completely soaked, followed by a [\text{methyl-3H}]thymidine incorporation count. Each treatment was performed in triplicate.

**RESULTS**

**Inhibition of Human Aromatase by Coumarins—** Research from this and other laboratories have identified a number of phytochemicals that can act as inhibitors of aromatase. The anti-aromatase chemicals identified so far are mainly flavones and isoflavones. In search of novel phytochemicals or their derivatives that could selectively inhibit aromatase, we examined 21 coumarins for their anti-aromatase effects. As indicated in Table I, only six compounds were found to be capable of inhibiting aromatase. Their structures are shown in Fig. 1. Three coumarins inhibited aromatase with IC\text{50} values at 1 \mu M or lower and are structurally related (Table I). The three most potent compounds were found to be competitive inhibitors with respect to the androgen substrate. The \text{K}\text{I} values of these compounds were 0.084, 0.23, and 1.1 \mu M, respectively (Fig. 2).

Using the same assay method, these three coumarins were found to be significantly more potent than other reported anti-aromatase chemicals including aminogluthethimide (Table II). Aminogluthethimide was the first aromatase inhibitor approved for use by the Food and Drug Administration for breast cancer treatment. Letrozole, anastrozole, and 4-hydroxyandrostenedione, the new aromatase inhibitors that are approved for breast cancer treatment, are more potent inhibitors than the coumarins. The new aromatase inhibitor development has been based primarily on inhibitor structure-activity relationship studies. Therefore, 4-benzyl-3-(4'-chlorophenyl)-7-methoxy-3-phenylcoumarin could be a lead compound to develop more potent aromatase inhibitors.

The specific interaction of these three compounds with aromatase was further demonstrated by the reduction of their binding by several mutations at the active site region of aromatase. The effects of aromatase inhibition by these compounds were tested with four critical mutants, E302D, T310S, S478T, and H480Q, which decrease the aromatase activity significantly, most likely because these point-mutations are located in the active site and involved in the process of aromatization. The activities of these mutants are ~40–50% of that of the wild-type aromatase. Our analysis revealed that the aromatase inhibitory profiles of T310S, S478T, and H480Q by 4-benzyl-3-(4-chlorophenyl)-7-methoxy-3-phenylcoumarin were significantly reduced in comparison to that of the wild-type aromatase activity, demonstrating that the mutated amino acid residues are important for the binding of this coumarin (Fig. 3). Glu-302 and Thr-310 of the human aromatase have been suggested to participate in the hydroxylation reactions at the C-19 methyl group of the androgen steroid. Ser-478 and His-480 have been predicted to interact with C-4 and C-7 of the steroid (22). Our results from the aromatase mutant studies support that these coumarin inhibitors are active-site-directed.
4-phenyl group, was not able to suppress aromatase. Our results provided strong evidence that these three functional groups (4-benzyl, 3-(4-chlorophenyl), and 7-methoxyl) are critical for the inhibition of aromatase by coumarin derivatives.

We compared the structures of our experimental hits to that of androstenedione using the FlexS program. Fig. 4A shows the alignment of 4-benzyl-3-(4-chlorophenyl)-7-methoxycoumarin (the most potent inhibitor of the compounds we tested) with androstenedione. It is predicted that the coumarin rings mimic the A and B rings, and the 3-(4'-chlorophenyl) group mimics the D ring of the androgen (Fig. 4B). Based on our test set, the substituent groups that appear to be important based on our structure-activity analysis, also align closely with important, known functional groups on the substrate. First, the spatial orientation of the 4-benzyl aligns very closely to the C-19 methyl group of the substrate. The top three inhibitors all have a benzyl group at this position. The importance of this benzyl group became apparent when we found that 3-(4-chlorophenyl)-7-methoxy-4-phenylcoumarin was not able to suppress aromatase activity at all. It is known that the C-19 methyl group of the androgen substrate is pointed toward the heme group of aromatase, and the first and second hydroxylation reactions take place on C-19 (22). It is thought that the space between C-19 and the heme group is not big enough to accommodate a phenyl group. On the other hand, a benzyl group could bend (through the methylene group), and the ring could overlay on top of the 3-(4-chlorophenyl) group. Because a compound with a 4-(4-chlorobenzyl) group instead of a 4-benzyl group (i.e. 4-(4'-chlorobenzyl)-7-methoxy-4-phenylcoumarin) was found not to suppress aromatase, the C-4 of the benzyl group is predicted to situate in a restricted area that prevents the placement of a 4'-chlorobenzyl group. The 7-methoxyl group of the coumarin aligns very closely with the C-3 keto oxygen of the substrate. These groups share the same physicochemical feature of being hydrogen bond donor groups and are superimposed in a similar space. However, it is interesting to find that a 7-hydroxy group instead of 7-methoxy group on the coumarin significantly reduces the inhibitory activity against

| Inhibitor                     | $K_i$ ($\mu M$) |
|-------------------------------|-----------------|
| Letrozole                      | 0.0004          |
| Anastrozole                   | 0.011           |
| 4-Hydroxyandrostenedione      | 0.015           |
| 4-Benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin | 0.084 |
| 4-Benzyl-3-(4'-chlorophenyl)-7-hydroxycoumarin | 0.23 |
| 4-Benzyl-7-methoxy-3-phenylcoumarin | 1.1  |
| Aminogluthethimide            | 2.7             |
| Chrysos                       | 2.6             |
| Naringenin                    | 5.1             |
| 7,8-Dihydroxyflavone          | 10              |
| Biochanin A                   | 12              |

Fig. 2. Competitive inhibition of aromatase. A, 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin. B, 4-benzyl-3-(4'-chlorophenyl)-7-hydroxycoumarin. C, 4-benzyl-7-methoxy-3-phenylcoumarin. The experimental conditions are described under "Experimental Procedures" section.

Fig. 3. Dose-response studies of the inhibition of 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin on the wild-type aromatase and four mutants. The relative aromatase activities (y axis) of the wild-type aromatase and four mutants are shown as total tritiated water release (in cpm/assay).
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A. FlexS alignment of androstenedione with 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin. B. chemical structures of the two compounds.

Although estrogen-related receptors (ERR) indicating that this compound is not an antagonist of ERs. This may explain why 4-benzyl-7-methoxy-3-phenylcoumarin (which is missing an electron-withdrawing group at this position) shows weaker activity. These FlexS alignment results, together with our structure-activity results, strongly suggest that these three functional groups are crucial for the inhibition of aromatase by coumarin derivatives.

Interaction of Coumarins with Other Enzymes and Nuclear Receptors—To assess the potential usage of 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin (the most potent coumarin identified in this study) as an aromatase inhibitor, we need to rule out that this compound is cytotoxic. MCF-7aro cells were cultured for 3 days in the presence of up to 40 \( \mu M \) 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin, letrozole, or anastrozole but in the absence of the androgen substrate. The coumarin did not produce any noticeable cytotoxicity (i.e. suppression of cell proliferation), and this observation was consistent with letrozole and anastrozole, which have been Food and Drug Administration-approved and are currently in use for the treatment of advanced breast cancer. As expected, aromatase inhibitors suppressed breast tumor growth by inhibiting estrogen biosynthesis and should not act as cytotoxic agents that kill cells in a non-selective manner.

Furthermore, the selectivity of this coumarin inhibitor was investigated. We checked the ability of this compound to inhibit other enzymes and hormone receptors, including steroid 5α-reductase (which also utilizes androgen as a substrate), androgen receptor, ERα, ERβ, ERRα, ERRβ, and ERRγ. Both type 1 and 2 steroid 5α-reductase isozymes were inhibited only 11% with 20 \( \mu M \) of 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin.2 Furthermore, through receptor transfection assays, this coumarin was found not to be an agonist of ER and ERR isoforms (Fig. 5). It was also found not to interfere with the interaction of 17β-estradiol (E\( _2 \)) with ERs (data not shown), indicating that this compound is not an antagonist of ERs. Although estrogen-related receptors (ERRα, ERRβ, and ERRγ) share a high amino acid sequence homology with ERs, estrogens are not ligands of ERRs. These receptors have been demonstrated to bind as a monomer, with a high affinity binding site containing the sequence, 5'-TCAAGGTCA-3', that is also recognized by another orphan receptor, steroidogenic factor 1.

2 J. Ye and S. Chen, unpublished results.
ERRs are constitutively active (27). At 20 μM, 4-benzyl-3-(4′-chlorophenyl)-7-methoxycoumarin had minimal effects on the activity of ERRs (Fig. 5). In addition, this coumarin was found not to be a ligand of AR through AR transfection assays. Although we do not know whether this coumarin inhibits any other enzymes or receptor activities, our results provide strong support that this compound inhibits aromatase with a good degree of specificity.

Evaluation of the Anti-aromatase Effects of Coumarins Using Matrigel Thread Cell Proliferation Studies—Cell culture experiments are important for drug evaluation. These experiments allow one to determine whether cells can effectively take up the drugs by measuring the target activity in cultured cells. Of the two types of cell culture, three-dimensional cultures are thought to approximate many conditions of in vivo tumors that are not usually present in monolayer systems in vitro, including three-dimensional intercellular contact, range in pH, oxygen tension, nutrient levels, drug sensitivity, and the ability to be grown in culture for several weeks without trypsinization (35). Matrigel basement membrane matrix is derived from the Englebreth-Holm Swarm tumor that is rich in laminin, collagen IV, heparin sulfate proteoglycans, entactin, nidogen, and growth factors (36, 37). Matrigel has been used as a supporting material for a number of cell culture experiments, such as experiments for studying the effectiveness of the attachment and differentiation of both normal and transformed anchorage tumor cells (38), process of colonic tumorigenesis (39), characterization of metastatic physical properties of cancer cells (40, 41), proliferation of human cancer cells by 31P NMR spectroscopy (34), and the effects of radiation on cancer cells in the matrigel thread in vitro tumor model (42).

To demonstrate further that these anti-aromatase coumarins can suppress the proliferation of aromatase-positive and ER-positive breast cancer cells through the inhibition of estrogen formation, the effects of these compounds were evaluated using a three-dimensional matrigel thread cell culture model.
The individually scattered MCF-7aro cells in the matrigel thread were induced to form large, spherical colonies by either 10 nM estradiol or testosterone (Fig. 6), whereas 100 nM ERα antagonist (ICI 182780) or aromatase inhibitor (letrozole and anastrozole) was able to suppress the testosterone-induced MCF-7aro proliferation in matrigel. 100 nM each of the three coumarins was also found to suppress the proliferation of MCF-7aro cells. Because these coumarins are not ligands of ERs and not cytotoxic at 100 nM, it is thought that the suppression of MCF-7aro proliferation is achieved through the suppression of aromatase or estrogen biosynthesis. These results further indicate that these coumarins can be taken up by the cells in matrigel or in a stromal environment and achieve their anti-aromatase action. Furthermore, the suppression and the hormonal induction of the growth of tumor colonies were clearly demonstrated in [methyl-3H]thymidine incorporation in the presence or absence of 10 nM androgen (Fig. 7) confirming that coumarins indeed suppressed the androgen-dependent MCF-7aro proliferation in matrigel.

**DISCUSSION**

For the first time, a coumarin derivative, i.e. 4-benzyl-3-(4′-chlorophenyl)-7-methoxycoumarin, has been shown to be a potent aromatase inhibitor. Biochemical and biological properties of this coumarin have been evaluated, and it was shown to be a competitive inhibitor with respect to the androgen substrate. The specific interaction with the enzyme was further evaluated through its inability to inhibit aromatase mutants with changes in the active site region. From our structure-activity studies, three functional groups of the coumarin (specifically the 3-(4′-chlorophenyl), 4-benzyl, and 7-methoxyl groups) were shown to be important for its ability to inhibit aromatase. Our computer modeling has revealed that this coumarin aligns well with the androgen substrate, providing evidence for why it is a good inhibitor of aromatase. These computer modeling results serve as a starting point in the process of understanding why these coumarins demonstrate anti-aromatase activity and in general what makes a good aromatase inhibitor. We hope to take what we have learned here, with respect to the structure-activity results, and use this knowledge to predict additional compounds with potential anti-aromatase activity. The FlexX alignment presented here can be used to develop additional ligand-based computer virtual screening strategies, including three-dimensional QSAR analyses and pharmacophore analyses.

In contrast to a strong interaction with aromatase, this coumarin does not have any effect on steroid 5α-reductase, AR, ERα, ERβ, ERα, ERRβ, and ERRγ. These analyses demonstrate that 4-benzyl-3-(4′-chlorophenyl)-7-methoxycoumarin is a potent and selective inhibitor of aromatase. Furthermore, this compound did not demonstrate any cytotoxic effects at concentrations up to 40 μM. Although these findings are promising, we understand the importance of doing more comprehensive dose-dependent studies using additional cells types to verify the lack of cytotoxic effects from this compound. But we have taken the initial first steps to identifying a new aromatase inhibitor with potential clinical efficacy.

Finally, using a three-dimensional matrigel thread cell culture model, we have shown that 4-benzyl-3-(4′-chlorophenyl)-7-methoxycoumarin can suppress aromatase-mediated breast cancer cell proliferation. Our results indicate that this coumarin derivative can be a useful aromatase inhibitor or a lead compound to develop more potent aromatase inhibitors. In addition, our findings indicate that coumarins can be potent inhibitors of aromatase. Because coumarins are a major class of phytochemicals, it is reasonable to predict that some fruits and vegetables may contain potent anti-aromatase coumarins, and consumption of these fruits and vegetables could suppress aromatase *in vivo*.

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