Aromatase Inhibitors

Structural Features and Biochemical Characterization

YANYAN HONG AND SHIUAN CHEN

Department of Surgical Research, Beckman Research Institute of the City of Hope, Duarte, California 91010, USA

ABSTRACT: Aromatase is the enzyme synthesizing estrogens from androgens. In estrogen-dependent breast tumors, estrogens induce the expression of growth factors responsible for cancer cell proliferation. *In situ* estrogen synthesis by aromatase ¨is thought to play a key role in the promotion of breast cancer growth. Aromatase inhibitors (AIs) provide new approaches for the prevention and treatment of breast cancer by inhibiting estrogen biosynthesis. Through reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical techniques, aromatase has been found to be expressed in many endocrine tissues and tumors originating from these tissues. Unexpectedly, this enzyme is now known to also be expressed in liver, lung, and colon cancers. Such findings suggest a potential role for endocrine manipulation of these types of cancer using AIs. Three Food and Drug Administration (FDA)-approved AIs, anastrozole (Arimidex), letrozole (Femara), and exemestane (Aromasin), effectively challenging tamoxifen, have been used as first-line drugs in the treatment of hormone-dependent breast cancer, and possibly other aromatase-expressing cancers. In addition, natural anti-aromatase chemicals, such as flavones and coumarins, have been identified. Efforts to develop new lines of AIs derived from these phytochemicals have been initiated in several laboratories. Finally, significant progress has been made in the understanding of the structure–function relationship of aromatase. Such information has helped the examination of binding characteristics of AIs, the evaluation of reaction mechanism of aromatase, and the explanation of the molecular basis for a low catalytic activity of the natural variant, M364T.

KEYWORDS: breast cancer; aromatase inhibitors; flavonoid phytoestrogen; mechanism-based inhibitor; structural model

Address for correspondence: Dr. Shiuan Chen, Department of Surgical Research, Beckman Research Institute of the City of Hope, 1500 E. Duarte Rd., Duarte, CA 91010. Voice: 626-359-8111; ext. 63454; fax: 626-301-8972.
e-mail: schen@coh.org

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INTRODUCTION

Aromatase, a cytochrome P450, catalyzes three consecutive hydroxylation reactions converting C19 androgens to aromatic C18 estrogens. Upon receiving electrons from NADPH-cytochrome P450 reductase, aromatase converts androstenedione and testosterone to estrone and estradiol, respectively. The aromatization of androgen is the terminal and rate-limiting step in estrogen synthesis. Pathologically, an abnormal overexpression of aromatase in breast tissue plays an important role in breast cancer development.[1–9] Inhibition of aromatase is a new strategy for reducing growth-stimulatory effects of estrogen in breast cancer by decreasing circulating levels of estrogen. During the last several years, a significant number of articles have been published to demonstrate the presence of aromatase in several endocrine tissues (such as ovary, uterus, prostate, and bone) and cancer associated with these tissues. It is, however, unexpected to find aromatase in cancer not associated with endocrine function, such as that of the liver,[10–13] lung,[14,15] colon,[16,17] and oral keratinocytes.[18] These findings suggest potential endocrine manipulation in the treatment of these types of cancer, and support adjuvant and sequential systemic treatment including AIs. Although AIs are now accepted to be important drugs for hormonal therapy of cancers, their action at the molecular level is not yet described.

FIRST-, SECOND-, AND THIRD-GENERATION INHIBITORS

Effective AIs developed as therapeutic agents are described as first-, second-, and third-generation inhibitors according to the order of their clinical development. The first-generation inhibitor refers to the nonsteroidal inhibitor aminoglutethimide (AG) (FIG. 1), which was the first AI to be studied in patients,[19] but the reports of adrenal insufficiency led to withdrawal from clinical use. AG is less specific and inhibits other CYP450 enzymes involved in cortisol and aldosterone biosynthesis, which results in toxicity. Its efficacy in inhibiting aromatase activity stimulated the development of various new inhibitors during the 1980s and 1990s.

The second-generation inhibitors include the imidazole derivative fadrozole[20] and steroid analogue formestane (4-hydroxyandrostenedione)[21,22] (FIG. 1). Fadrozole is more selective and potent than AG, but it still has inhibitory effects on aldosterone, progesterone, and corticosterone biosynthesis. Formestane was the first selective AI to be used clinically and was effective and well tolerated.[23] However, the fact of its requirement of intramuscular administration limited its clinical use.

The third-generation inhibitors, developed in the early 1990s, including two triazole derivatives anastrozole (Arimidex)[24] and letrozole (Femara)[25] and one steroid analogue exemestane (Aromasin)[26] (FIG. 1), are widely used as the first-line drugs in the endocrine treatment of hormone-dependent breast cancer

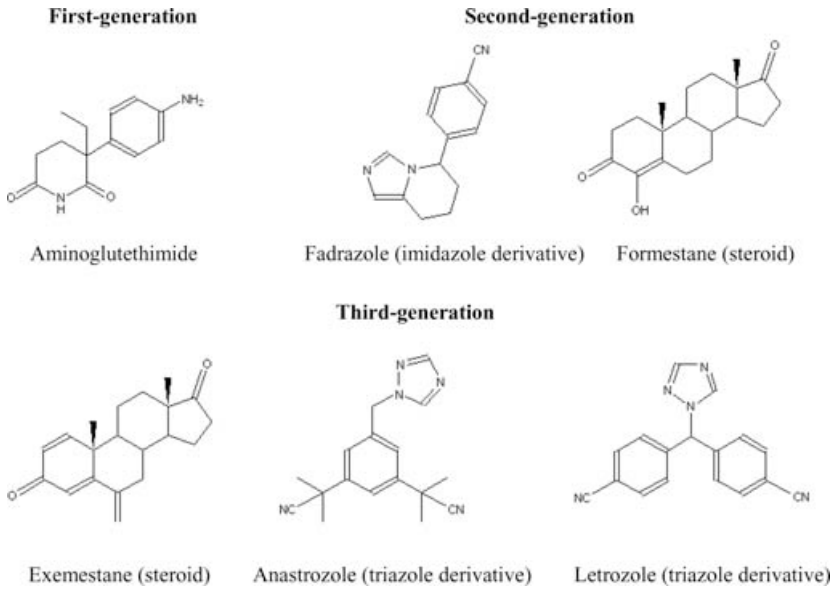

FIGURE 1. First-, second-, and third-generation aromatase inhibitors.

in postmenopausal patients. Anastrozole, letrozole, and exemestane are administered orally with 1 mg, 2.5 mg, and 25 mg once daily, respectively. Compared to the first- and second-generation inhibitors, the third-generation inhibitors produce greater clinical benefit with near-complete specificity at clinical use. These drugs were also found to be better tolerated than tamoxifen and were associated with lower incidences of endometrial cancer, vaginal bleeding and discharge, cerebrovascular events, venous thromboembolic events, and hot flashes.[27,28] In addition, the incidence of contralateral breast cancer occurrence was found to be significantly lower in the AI group than the tamoxifen group.[27,29,30] However, the long-term effects of these drugs on skeletal problems, cardiovascular disease, and Alzheimer's disease need to be carefully followed up.

Anastrozole and letrozole are nonsteroidal derivatives that have the triazole functional which interacts with the heme prosthetic group of aromatase, and they act as competitive inhibitors with respect to the androgen substrates. Exemestane is a steroidal and mechanism-based inhibitor that is catalytically converted into a chemically reactive species, leading to irreversible inactivation of aromatase.

MECHANISM-BASED INHIBITORS

A mechanism-based inhibitor is a steroidal inhibitor that is recognized by the enzyme as a pseudo-substrate, and is then converted to reactive intermediates,

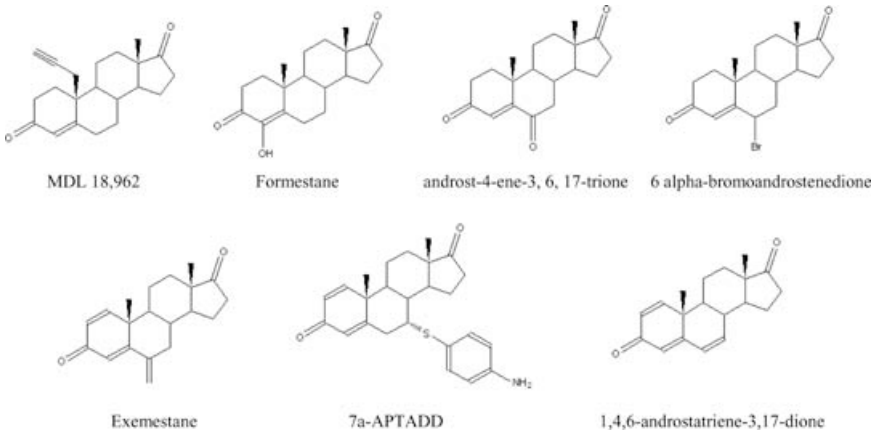

FIGURE 2. Mechanism-based inhibitors.

which irreversibly bind to the enzyme to produce suicide inhibition. Mechanism-based inhibitors cause time-dependent inhibition of aromatase only in the presence of its redox partner NADPH-P450 reductase and co-factor NADPH. These inhibitors are generally classified into several groups (FIG. 2): C-10 substituted androstendione (i.e., MDL 18,962,); 4-substituted androstenedione (i.e., formestane); 6-substituted androstenedione (i.e., androst-4-ene-3, 6, 17-trione, 6 alpha-bromoandrostenedione); substituted androsta-1, 4-diene-3, 17-diones (i.e., 7α-APTADD, exemestane); substituted androsta-1, 4, 6-diene-3, 17-diones (i.e., 1,4,6-androstatriene-3,17-dione). These inhibitors have recently been discussed in a review by Brueggemeier *et al*.[31] Several evidences have supported the fact that the reactive intermediates result from enzymic oxidation at the C-19 group of mechanism-based inhibitors: compounds lacking a C-19 methyl group did not cause a time-dependent inhibition;[32] tritium release from [19-^{3}H]-19,19-difluoroandrost-4-ene-3,17-dione during inactivation of aromatase[33]; and incubation of androst-4-ene-3,6,17-trione (AT) with human placental microsome yielded the 19-hydroxy-AT and 19-oxo-AT.[34] Moreover, experimental results from radioactive inhibitor probe studies demonstrate that a mechanism-based inhibitor can be covalently bound to aromatase.[35] Mechanism-based inhibitors provide promising perspectives for drug design because these steroid analogue inhibitors are highly selective and less toxic.

Among these mechanism-based inhibitors, as discussed above, exemestane has been approved by the FDA for the treatment of hormone-dependent breast cancer. It causes a time-dependent inactivation of human placental aromatase with a $t1/2$ of 13.9 min and K_i of 26 nM.[36] Our laboratory has recently found that exemestane (as well as formestane) can further induce a degradation of aromatase by proteasome, which occurs after the irreversible inactivation

step,[37] indicating that these mechanism-based inhibitors not only can inactivate aromatase, but also can eliminate the enzyme protein. The exact nature of the interaction of these mechanism-based inhibitors with aromatase protein and amino acids involved has yet to be elucidated. The ability of exemestane and formestane to induce enzyme degradation could explain why it has been difficult to identify the amino acids/peptides that participated in the mechanism-based inhibition of aromatase by these inhibitors. However, recent advances in structure–function studies of aromatase have generated valuable structure information for the evaluation of the interaction of steroid ligands (including the substrate and inhibitors such as exemestane) with the aromatase enzyme (discussed in the next section).

BINDING NATURE OF THE ANDROGEN SUBSTRATE/STEROIDAL INHIBITOR

Extensive computer modeling, site-directed mutagenesis, and proteomic analysis from this and other laboratories have helped to define the active site region of aromatase and to evaluate the reaction mechanism of this enzyme.[38–51] On the basis of the crystal structure of human CYP2C9, and taking our laboratory findings into consideration, Favia *et al*.[52] recently generated a three-dimensional model of aromatase. After careful evaluation, we feel that the model by Favia *et al*.[52] is more reliable than those previously generated in this and other laboratories because it can adequately explain most of our experimental data. By carefully examining this new model and the results from our recent structure–function studies of aromatase, a new clamping mechanism of substrate/steroidal inhibitor, binding to the active site, has been proposed.[53] The heme iron is ligated by a conserved cysteine (C437) and the propionates of the heme interact with the side chains of R115, W141, R145, R375, and R435. The steroid substrate/inhibitor sits above the heme, with its C19 methyl group pointing to the heme iron, and is positioned next to the I helix (FIG. 3). Our site-directed mutagenesis data allow us to identify three additional important regions in the active site of aromatase. Together with D309, S478, and H480 in the β-4 sheet at the carboxy-terminus are thought to participate in a charge relay system that lead to the aromatization of the A ring of the substrate.[53] I133 and F134 in the B′-C loop are hypothesized to interact with the D ring of the substrate/inhibitor through van der Waals forces.[53] The 3′-flanking loop (P368-M374) of the K helix is thought to participate in forming the hydrophobic ligand-binding pocket and hence possibly residue V373 interacts specifically with the B ring of the substrate/inhibitor. This loop (P368-M374), together with the B′-C loop and β4-including loop, holds the steroid substrate/inhibitor at the correct orientation. Exemestane, the FDA-approved mechanism-based AI, is hypothesized to be converted to reactive intermediates by heme through the hydroxylation of the C-19 group, helped by D309. Finally, the intermediates,

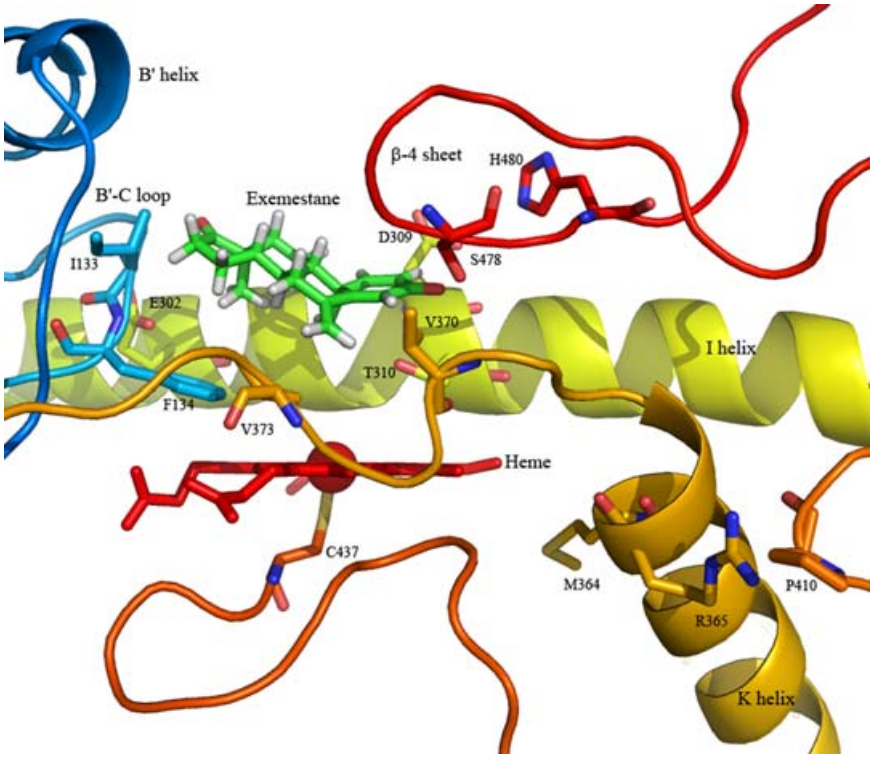

FIGURE 3. Clamping mechanism of exemestane binding provided by the heme, I helix, B′-C loop, β-4 sheet, and the 3′-flanking loop of the K helix.

irreversibly bound to the enzyme, cause suicide inhibition in which D309 may be involved.

In a recent study, Ma *et al*. identified and characterized genetic polymorphisms in the human aromatase gene.[54] There are four coding single nucleotide polymorphisms (cSNPs) in the coding region. These cSNPs alter the following amino acids: W39R, T201M, R264C, and M364T. Interestingly, the M364T variant was found to be less stable and to have significantly lower affinities for the androgen substrate and for the inhibitor exemestane. Our laboratory previously generated two mutants, R365A and R365K.[41] These mutants were not active. The immunoprecipitation analysis revealed that these mutants were expressed, but at levels lower than that of the wild-type enzyme. These results indicate that R365 plays a very critical role during the enzyme catalysis because it cannot be replaced with a lysine residue. Computer modeling analysis has revealed that M364 and R365 are situated in the K helix (FIG. 3). It is thought that the side chain of R365 forms a hydrogen bond with the backbone carbonyl oxygen of P410, which is located in the loop between the β-1/β-2

two sheets (R375-I395) and L helix (G439-R456). C437, the heme-binding cysteine residue, is located at the end of this loop. Possibly, R365 stabilizes this loop structure. M364 faces toward the inside of the active site, although it is not close to the heme and steroidal ligand. It is likely that M364 helps to form the hydrophobic pocket together with the loop P368-M374.

The availability of a reliable three-dimensional model of aromatase has helped us better understand the molecular features of the active site and catalytic mechanism of the enzyme. The model will enable the application of the structure-based design (SBD) of the next generation of selective and potent AIs.

PHYTOCHEMICAL INHIBITORS

Studies have suggested that diet sources, including cruciferous vegetables, soy, rye flour, grapes, and mushrooms, are associated with a decreased risk of breast cancer.[55–59] Bioactive food components present in these diet sources demonstrate various biological activities and are being investigated for the prevention of both ER+ and ER– breast tumors. Some phytochemicals, isolated from the plant kingdom, such as flavonoids and lignans, are known to be competitive inhibitors of aromatase, resulting in a decrease in the level of estrogen. For example, isolicoflavonol/prenylated flavonoid (FIG. 4), isolated from

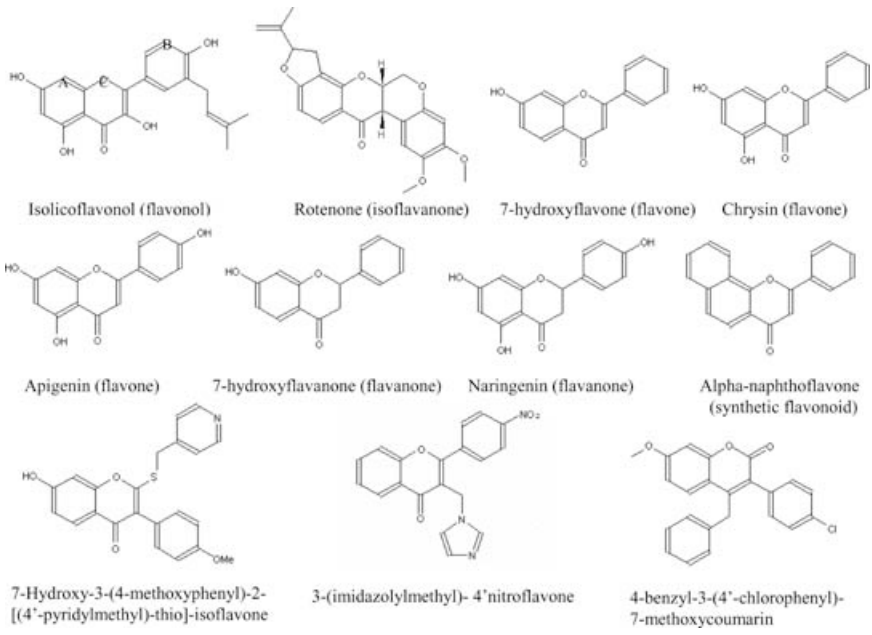

FIGURE 4. Flavonoid aromatase inhibitors.

the paper mulberry, was found to suppress aromatase with IC_{50} values near 0.1 μM[60]; nectandrin-B/lignan, with anti-aromatase activity, was isolated from *Myristicaceae*;[61] Procyanidin B dimers, isolated from red wine and grape seeds, were shown to be aromatase inhibitors; and the *in vivo* studies in an aromatase-transfected MCF-7 breast cancer xenograft model also demonstrated that these chemicals reduced androgen-dependent tumor growth.[58] These studies indicate that procyanidin B dimers could be used as chemopreventive agents against breast cancer.

The most comprehensive studies of phytochemical aromatase inhibitors are focused on flavonoid phytoestrogens. Phytoestrogens are plant-derived nonsteroidal compounds that possess estrogen-like biological activity, and may function as antiestrogens or weak estrogens by competing with estrogens for binding to ER. Flavonoids include flavanones/isoflavanones, flavones/isoflavones, and flavonols/isoflavonols, characterized as containing the benzopyranone ring system. Some flavanoids are capable of inhibiting aromatase (FIG. 4). For example, IC_{50} values for the inhibition of aromatase by the isoflavanone derivative rotenone (from *Derris*) was 0.3 μM; the flavones 7-hydroxyflavone, chrysin (from *Passiflora coerulea*), and apigenin (from *Matricaria chamomilla*) were 4, 7, 20 μM; and the flavanones 7-hydroxyflavanone and naringenin (from *Petunia*) were 65 and 85 μM, respectively.[62] Some flavanoids are poorer inhibitors or possess no effect on aromatase activity. Genistein (FIG. 5), an isoflavone phytoestrogen abundant in soy products with numerous biochemical activities, elevates the conversion of the most physiologically active form of estrogen (estradiol) to an estrogenically weaker metabolite (estrone) by increasing 17-betahydroxysteroid dehydrogenase activity,[63] but slightly inhibits aromatase activity.[63–65] Flavonoids exerting no effect on aromatase activity are baicalein, 6-hydroxylavone, daidzein, quercetin, catechin, and equol (FIG. 5).[64,66] Generally, natural flavones are more potent than isoflavones in inhibiting aromatase. The binding characteristics by flavone and isoflavone have been studied using computer modeling and site-directed mutagenesis. It was found that these compounds bind to the active site of aromatase in an orientation in which their ring–A and –C mimic ring–D and –C of the steroidal substrate, respectively.[64,67] These studies also provide a molecular basis describing why isoflavones are significantly poorer inhibitors of aromatase than flavones.

Although flavonoid natural products have weak aromatase inhibitory activity, the benzopyranone-ring system of flavonoid provides a molecular scaffold for designing potential aromatase inhibitors for future drug development. The synthetic flavonoid, alpha-naphthoflavone (FIG. 4), is a potent aromatase inhibitor with an IC_{50} value of 0.5 μM.[66] Brueggemeier *et al.*[63] reported that several compounds in the initial benzopyranone libraries, which were constructed by diversifying the benzopyranone scaffold and utilizing combinatorial chemistry approaches, inhibit aromatase activity in screening assays. Kim *et al.*[68] reported the synthesis of several pyridine-containing isoflavones.

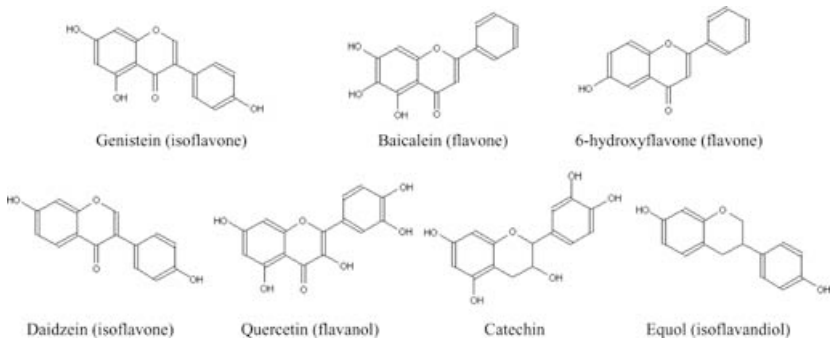

FIGURE 5. Flavonoid noninhibitors of aromatase.

The best inhibitors in this series, such as 7-hydroxy-3-(4-methoxyphenyl)-2-[(4′-pyridylmethyl)-thio]-isoflavone (FIG. 4), inhibited the human placental aromatase with K_i values around 0.3 μM. Gobbi *et al.*[69] also reported the synthesis of a series of flavone derivatives as AIs, with the removal of the 7-methoxy group leading to compounds showing inhibitory activity in the nanomolar range. Among them, 3-(imidazolylmethyl)- 4′nitroflavone (FIG. 4) had an IC_{50} value of 45 nM when the assay was performed with the substrate (androstenedione) concentration of 500 nM. Recanatini *et al.*[70] have synthesized chromone and xanthone derivatives. Several xanthone derivatives were found to inhibit aromatase with IC_{50} values around 50 nM in the presence of 2.5 μM of the androgen substrate. Interestingly, our laboratory has found a series of coumarins that can act as competitive inhibitors of aromatase with respect to the androgen substrate.[71] The best inhibitor in this series, 4-benzyl-3-(4′-chlorophenyl)-7-methoxycoumarin (FIG. 4), inhibits aromatase with a K_i value of 84 nM. These results suggest that it is possible to generate new potent AIs that are derived from natural anti-aromatase chemicals.

There are two considerations for the development of a new generation of AIs from natural anti-aromatase chemicals. The specificity of chemicals has to be carefully examined. Many phytochemicals have been shown to have more than one activity in the body. In addition, it is important to confirm the results generated from the assay using placental microsomes (a noncellular assay) with the in-cell assay. The in-cell assay is performed using cells that express aromatase and will determine whether the inhibitor can enter the cells. Some AIs, identified by noncellular assays, have been found to be inactive when tested with in-cell assay.[72] It is also important to demonstrate that potential AIs can actually suppress androgen (converted to estrogen by aromatase)-mediated cell proliferations. 4-Benzyl-3-(4′-chlorophenyl)-7-methoxycoumarin, an anti-aromatase chemical identified in our laboratory, was found not only to be active by the in-cell assay, but also to suppress the proliferation of aromatase and estrogen receptor–positive MCF-7aro breast cancer cells through a Matrigel thread three-dimensional cell culture.[71]

CHEMOPREVENTION STUDIES USING GRAPE SEED EXTRACT AND MUSHROOMS

Our laboratory has found that grapes, mushrooms, and red wine contain chemicals that can suppress aromatase activity.[57–59,73,74] Therefore, a diet that includes grapes, mushrooms, and red wine would be considered preventative against breast cancer. We are purifying and characterizing these natural anti-aromatase chemicals and evaluating their *in vivo* effects using animal experiments. The active chemicals in grapes and red wine have been found to be procyanidin dimers that are present at high concentrations in grape seeds.[58] Recently, grape seed extract (GSE) was found to inhibit aromatase activity in a dose-dependent manner and reduce androgen-dependent tumor growth in an aromatase-transfected MCF-7 (MCF-7aro) breast cancer xenograft model,[59] agreeing with our previous findings. More interestingly, through the suppression of the expression of CREB-1 and GR, GSE has been found to decrease the expression of aromatase in breast cancer tissue by reducing the activity of promoters I.3, II, and I.4.[59] Therefore, GSE can suppress estrogen production in breast cancer through at least two mechanisms: inhibition of the expression of aromatase and functioning as an AI. On the basis of results of preclinical studies from this and other laboratories, a phase I chemoprevention clinical trial involving GSE in postmenopausal women has been initiated at our institution, the City of Hope (<http://clinicaltrials.coh.org/study_display.aspx?pid=3713861>). GSE is a common dietary supplement that is widely used. Additional experiments to determine the mechanisms of inhibition of aromatase activity and downregulation of its expression through breast cancer–specific promoters by GSE would help in designing prevention strategies that selectively suppress its expression and activity in breast tumor tissue while also maintaining estrogen levels in normal tissues.

PERSPECTIVES

The potent and highly selective third-generation AIs are the approved therapeutic agents for the treatment of estrogen-dependent breast cancer in postmenopausal women. Furthermore, a combination of aromatase inhibitors with signal transduction inhibitors (i.e., HER1/2 kinase inhibitors and COX2 inhibitors) is being developed in an attempt to increase the efficacy of AIs. This type of therapy will also be useful for other aromatase-expressing cancers.

The design and synthesis of derivatives using natural bioactive chemicals as scaffolds with aromatase inhibitory activity would provide a new series of potent and selective agents for cancer prevention and treatment. The availability of the active-site structural information has assisted the examination of binding characteristics of AIs and the evaluation of the reaction mechanism of

aromatase. Such information will also be critical for the design of new selective and potent AIs. At the present time, the weak inhibitory effects on aromatase of natural products may require a high level of exposure to get a significant impact in cancer patients, and the diverse biological activities with various enzymes and receptor systems of these phytochemicals limit their therapeutic use. However, a diet containing bioactive chemicals that decrease aromatase activity and expression offers an intriguing prevention strategy to reduce the incidence of breast cancer and other hormone-dependent cancers. Therefore, identification of diet sources that contain anti-aromatase chemicals is very interesting and important. Furthermore, it is critical to translate the findings from the laboratory research into clinical use. As the first step, our institution has initiated a clinical trial to examine whether GSE intake will result in the reduction of circulating estrogen levels in postmenopausal women, leading to a decrease of the incidence of breast cancer.

ACKNOWLEDGMENTS

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