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Aromatase Inhibitors in the Treatment of Breast Cancer

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Abstract

Estradiol, the most potent endogenous estrogen, is biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase. Aromatase is present in breast tissue, and intratumoral aromatase is the source of local estrogen production in breast cancer tissues. Inhibition of aromatase is an important approach for reducing growth stimulatory effects of estrogens in estrogen-dependent breast cancer. Steroidal inhibitors that have been developed to date build upon the basic androstenedione nucleus and incorporate chemical substituents at varying positions on the steroid. Nonsteroidal aromatase inhibitors can be divided into three classes: aminoglutethimide-like molecules, imidazole/triazole derivatives, and flavonoid analogs. Mechanism-based aromatase inhibitors are steroidal inhibitors that mimic the substrate, are converted by the enzyme to a reactive intermediate, and result in the inactivation of aromatase. Both steroidal and nonsteroidal aromatase inhibitors have shown clinical efficacy in the treatment of breast cancer. The potent and selective third-generation aromatase inhibitors, anastrozole, letrozole, and exemestane, were introduced into the market as endocrine therapy in postmenopausal patients failing antiestrogen therapy alone or multiple hormonal therapies. These agents are currently approved as first-line therapy for the treatment of postmenopausal women with metastatic estrogen-dependent breast cancer. Several clinical studies of aromatase inhibitors are currently focusing on the use of these agents in the adjuvant setting for the treatment of early breast cancer. Use of an aromatase inhibitor as initial therapy or after treatment with tamoxifen is now recommended as adjuvant hormonal therapy for a postmenopausal woman with hormone-dependent breast cancer.

Introduction

Estrogens are involved in numerous physiological processes including the development and maintenance of the female sexual organs, the reproductive cycle, reproduction, and various neuroendocrine functions. These hormones also have crucial roles in certain disease states, particularly in mammary and endometrial carcinomas. Cancer is the leading cause of death among women between the ages of 30 and 54, with breast and uterine cancers comprising 28% and 10%, respectively, of all cancers in females per year. An estimated 217,440 new cases of breast cancer will be diagnosed, and 40,580 women in the U.S. will die from breast cancer in 2004 (1). Currently, one out of eight American women will develop breast cancer in her lifetime. Approximately two-thirds of postmenopausal breast cancer patients have hormone-dependent (estrogen-dependent) breast cancer, which contains estrogen receptors and requires estrogen for tumor growth. Estrogens produce normal physiological effects by binding to specific nuclear receptor proteins, estrogen receptor α (ERα) and estrogen receptor β (ERβ) (2). The predominant estrogen receptor in the female reproductive tract and mammary glands is ERα. Following the binding of estrogen to its receptor, the estrogen-receptor complexes form homodimers and interact with sequence specific estrogen response elements (EREs) present in the promoter region of responsive genes in target cell chromatin. Binding of the nuclear steroid-receptor complexes to DNA and interaction with various nuclear transcriptional factors such as steroid receptor co-activator proteins (SRCs) initiate the transcription of the relevant gene to produce messenger ribonucleic acid (mRNA). The elevated mRNA levels result in increased protein synthesis in the endoplasmic reticulum. These proteins include enzymes, receptors, and secreted factors that subsequently result in the steroid hormonal response regulating cell function, growth, differentiation. Estrogens enhance growth and proliferation of certain target

cells, such as breast epithelial cells and estrogen-dependent mammary carcinoma cells, and induce the formation and secretion of various growth factors in established cell lines such as MCF-7, T47D, and ZR-75-1 human mammary carcinoma lines (3).

Aromatase and Estrogen Biosynthesis

A. BIOCHEMISTRY OF AROMATASE

Estradiol is the most potent endogenous estrogen. Estradiol is biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase (4), with the highest levels of enzyme present in the ovaries of premenopausal women, in the placenta of pregnant women, and in the peripheral adipose tissues of postmenopausal women and of men. Aromatase activity has also been demonstrated in breast tissue *in vitro* (5-7). Furthermore, expression of aromatase is highest in or near breast tumor sites (6,8).

The enzyme complex is bound in the endoplasmic reticulum of the cell and is comprised of two major proteins (4,9). One protein is cytochrome $P450_{arom}$, a hemoprotein that converts C_{19} steroids (androgens) into C_{18} steroids (estrogens) containing a phenolic A ring (4,10). The second protein is NADPH-cytochrome P450 reductase, which transfers reducing equivalents to cytochrome $P450_{arom}$. Three moles of NADPH and three moles of oxygen are utilized in the conversion of one mole of substrate into one mole of estrogen product (Figure 1). Aromatization of androstenedione, the preferred substrate, proceeds via three successive oxidation steps, with the first two being hydroxylations of the angular C-19 methyl group. The final oxidation step proceeds with the aromatization of the A ring of the steroid and loss of the C-19 carbon atom as formic acid.

This third and final step in the aromatase reaction oxidatively cleaves the C_{10}-C_{19} bond, although the mechanism of this step remains to be elucidated. A number of mechanisms have been proposed, and one mechanism for the oxidative deformylation step that has received significant favor involves nucleophilic attack of the 19-aldehyde by the reduced ferrous dioxygen, or peroxy, intermediate (Figure 2A). The resulting peroxo hemiacetal is suggested to decay via a process by which the proximal oxygen atom removes the 1β-hydrogen, resulting in aromatization of the steroid A ring and formic acid release (11). Model reaction studies have suggested that formation of the 2,3-enol is a prerequisite for aromatization. A homology model of the aromatase enzyme suggests the presence of an aspartic acid residue near the 2β hydrogen and lysine or histidine residues near the 3-ketone of androstenedione (12). The orientation of these residues is supportive of an enzyme acid-base catalyzed enolization process to selectively remove the 2β hydrogen. Recently, our laboratory has employed computational chemistry approaches to unravel the mysterious third step of aromatase catalysis (13). Recent advances in computational chemistry, including density functional theory (DFT) alone or in combination with molecular mechanical methods, have provided better tools that enable study of the active species in their native protein environment, such as the cytochrome P450 oxidant Compound I (14). A model system of the cytochrome P450 active site and truncated steroid substrates has been studied using DFT calculations and *ab initio* molecular dynamics. Analysis for the reduced ferrous dioxygen (peroxy) intermediate suggests that 1β-hydrogen atom abstraction by the proximal oxygen of the peroxo hemiacetal intermediates encounters a high energetic barrier (> 60 kcal/mol) that is enzymatically inaccessible. Also, the resulting species do not directly fragment to the experimentally observed formic acid and aromatized steroid products. A novel, alternative mechanism was examined, in which the widely accepted cytochrome P450 oxidant

Compound I, the iron oxene catalytic intermediate (Figure 2B), abstracts the 1β-hydrogen atom initiating the aromatization and deformylation cascade. The steroid models which contain the 2,3-enol moiety have a strikingly low barrier for 1β-hydrogen atom abstraction (< 7 kcal/mol) due to the ability of the enolized A-ring to delocalize the impending radical. The transition states containing the 2,3-enol moiety and the 19-gem diol decay directly to the aromatized product, formic acid, and the aqua-bound model cytochrome P450 enzyme. Analysis of the reaction vectors indicate that the second hydrogen transfer occurs with a concerted, non-synchronous mechanism without an energetic barrier. Thus, these calculations support a final catalytic step of aromatase involving the cytochrome P450 oxene intermediate, 1β-hydrogen atom abstraction, and release of formic acid (Figure 2).

B. AROMATASE GENE EXPRESSION

Over the past two decades, knowledge of the biochemistry, molecular biology, and regulation of aromatase has increased greatly. The aromatase gene, designated *CYP19*, encodes the cytochrome $P450_{arom}$, and this gene is located on chromosome 15q21.1. The coding region is approximately 30 kilobases in size, and the regulatory region is ~93 kilobases (9,15). The aromatase gene consists of 10 exons, and its full length cDNA of 3.4 kilobases encodes for a protein of 503 amino acids. The aromatase protein is a glycosylated cytochrome P450 protein with a molecular weight of approximately 58,000 daltons (16). The regulation of aromatase is complex in various tissues, and several tissue-specific promoter regions have been identified upstream from the *CYP19* gene (9,17,18). These tissue-specific promoters include promoter PI.1, PI.3, PI.4, PI.6, PI.7, and PII (Figure 3). Promoter PI.1 is the major promoter used in placental tissues and is the farthest upstream. The PII promoter is utilized in the ovary and in breast cancer

tissues, and it contains a cAMP response element. Promoters PI.3, PI.4, PI.6, and PI.7 are the promoters used in extraglandular sites. Promoter PI.4 is the primary promoter used in normal adipose tissue and is responsive to glucocorticoids and cytokines such as IL-1β, IL-6 and TNFα. Promoter PI.3 is also present in adipose tissues such as normal breast tissue, and PI.3 is elevated in breast cancer tissues.

C. AROMATASE IN BREAST CANCER TISSUES

Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled (6,19,20). Aromatase has been measured in the stromal cell component of normal breast and breast tumors, but the enzyme has also been detected in the breast epithelial cells *in vitro* (5,8,20-22). Furthermore, expression of aromatase is highest in or near breast tumor sites (8,20). The exact cellular location(s) of aromatase must await more rigorous analysis by several labs with a new monoclonal antibody now being developed and evaluated (23).

The increased expression of aromatase cytochrome $P450_{arom}$ observed in breast cancer tissues is associated with a switch in the major promoter region utilized in gene expression, with promoter II being the predominant promoter used in breast cancer tissues (24). As a result of the use of the alternate promoter, the regulation of estrogen biosynthesis switches from one controlled primarily by glucocorticoids and cytokines to a promoter regulated through cAMP-mediated pathways (24). The prostaglandin PGE_2 increases intracellular cAMP levels and stimulates estrogen biosynthesis (24), whereas other autocrine factors such as IL-1β do not appear to act via PGE_2 (25).

Local production of PGE_2 via the cyclooxgenase isozymes (constitutive COX-1 isozyme and inducible COX-2 isozyme) can influence estrogen biosynthesis and estrogen-dependent breast cancer. This biochemical mechanism may explain epidemiological observations of the beneficial effects of NSAIDs on breast cancer (26-29). Investigations using human breast cancer patient specimens demonstrated a strong linear correlation between CYP19 expression and the sum of COX-1 and COX-2 expression (30). Gene expression analysis for CYP19, COX-1, and COX-2 were performed in 20 human breast cancer specimens and in 5 normal control breast tissue samples. A positive correlation was observed between CYP19 expression and the greater extent of breast cancer cellularity (Figure 4A), in agreement with literature reports showing that aromatase levels were higher in tumors than in normal tissue. Furthermore, a positive linear correlation was observed between COX-2 expression breast cancer cellularity in each sample. Linear regression analysis using a bivariate model shows a strong linear association between CYP19 expression and the sum of COX-1 and COX-2 expression (Figure 4B). Similar correlations between CYP19 expression and COX-2 expression in breast cancer patient specimens have been confirmed in other laboratories (31). This significant relationship between the aromatase and cyclooxygenase enzyme systems suggests that autocrine and paracrine mechanisms may be involved in hormone-dependent breast cancer development via growth stimulation from local estrogen biosynthesis. In human breast stromal cells, PGE_2 acts via two G-protein coupled receptors, EP_1 and EP_2 receptors, to stimulate aromatase gene expression via protein kinase A and protein kinase C signaling pathways (32). NSAIDs, COX-1 and COX-2 selective inhibitors produce dose-dependent decreases in aromatase activity in breast cancer tissues (Figure 5) (33,34). Real time PCR analysis of aromatase gene expression showed a significant decrease in mRNA levels by these agents, and the effect of COX inhibitors on

aromatase expression occurs through suppression at the tissue specific promoters PI.3, PI.4, and PII. This significant relationship between the aromatase and cyclooxygenase enzyme systems suggests that autocrine and paracrine mechanisms may be involved in hormone-dependent breast cancer development via growth stimulation from local estrogen biosynthesis (Figure 6).

Development of Aromatase Inhibitors

Two primary approaches have been developed to reduce the growth stimulatory effects of estrogens in breast cancer: 1) interfering with the ability of estrogen to bind to its receptor, and 2) decreasing circulating levels of estrogen. Antiestrogens compete for binding to the estrogen receptors and reduce the number of receptors available for binding to endogenous estrogen. This approach has proven effective as an anticancer strategy (35,36) and has led to the development of efficacious antiestrogens, such as the drug tamoxifen, that exhibit minimal toxicity. Inhibition of aromatase is the second approach for reducing growth stimulatory effects of estrogens. Effective aromatase inhibitors have been developed as therapeutic agents for controlling estrogen-dependent breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades. Research summaries of aromatase inhibitors have been presented at international aromatase conferences (37-41) and several reviews have also been published (42-51).

A. STEROIDAL INHIBITORS

1. Competitive enzyme inhibitors. Investigations on the development of aromatase inhibitors began with the synthesis and biochemical evaluation of competitive inhibitors (52-54). Competitive inhibitors are molecules that compete with the substrate androstenedione for

noncovalent binding to the active site of the enzyme to decrease the amount of product formed. The term apparent K_i represents the equilibrium constant for the reversible binding of the enzyme and the inhibitor. The values are utilized in comparisons of inhibitors, and the smaller the K_i value, the better the inhibitor. Steroidal inhibitors that have been developed to date build upon this basic androstenedione nucleus and incorporate chemical substituents at varying positions on the steroid (Figure 7). These inhibitors bind to the aromatase cytochrome P450 enzyme in the same manner as the substrate androstenedione. Initial structure-activity relationships were developed with these early investigations on competitive aromatase inhibitors. In summary, effective inhibition is observed with an androstane steroid molecule possessing an A/B trans ring junction, a ketone functionality at the C-3 position, unsaturation in the steroid nucleus (4-ene, 4,6-diene, or 1,4,6-diene functions), and either a 17-ketone or 17α-hydroxyl moiety.

A limited number of effective inhibitors with substituents on the A ring have been reported. Several steroidal aromatase inhibitors contain modifications at the C-4 position, with 4-hydroxyandrostenedione (4-OHA) being the prototype agent. Initially, 4-OHA was thought to be a competitive inhibitor with an apparent K_i of approximately 170 nM, but the compound was later shown to produce enzyme-mediated inactivation. The spacial requirements of the A-ring for binding of the steroidal inhibitor to aromatase are rather restrictive, permitting only small structural modifications to be made. Incorporation of the polar hydroxyl group at C-4 enhances inhibitory activity. 1-Methylandrosta-1,4-diene-3,17-dione (1-methyl-ADD) is a potent inhibitor of aromatase *in vitro* and *in vivo* (55); on the other hand, bulky substitutents at the 1α-position are poor inhibitors (54).

Extensive structural modifications may be made on the B-ring of the steroid nucleus. Bulky substitutions at the C-7 position of the B-ring have provided several very potent aromatase inhibitors (54). One of the more potent inhibitors, 7α-(4'-amino)phenylthio-4-androstene-3,17-dione (7α-APTA) with an apparent K_i of 18 nM, demonstrated effectiveness in inhibiting aromatase in cell cultures and in treating hormone-dependent rat mammary tumors (56-58). Evaluation of various substituted aromatic analogs of 7α-APTA provided no correlation between the electronic character of the substituents and inhibitory activity. Investigations of various 7-substituted androsta-4,6-diene-3,17-dione derivatives suggest that only those derivatives that can project the 7-aryl substitutent into the 7α-pocket are effective inhibitors (59). Several compounds with substituents at the C-6 position have been described which exhibit irreversible inhibition; these are discussed in a later section. Overall, the most effective B-ring modified aromatase inhibitors are those with 7α-aryl derivatives, with several analogs having 2-10 times greater affinity for the enzyme than the substrate. These results suggest that additional interactions occur between the phenyl ring at the 7α-position and amino acids at or near the enzymatic site of aromatase, resulting in enhanced affinity.

The other position of androstenedione that has received considerable investigation and has resulted in effective inhibitors is the C-19 methyl position, the site of enzymatic oxidation. Competitive inhibitors have been designed to enhance affinity through noncovalent binding of a heteroatom at C-19 with the heme iron of the cytochrome $P450_{arom}$. 19-Substituted aromatase inhibitors include thiiranes and oxiranes (60,61), epoxysteroids (62), and thiol and amino analogs (63,64). The 19R-isomers of the thiiranes and oxiranes were potent inhibitors with an apparent K_i values ranging from 1 to 7 nM, showed affinity 36 to 80-fold greater than the corresponding 19S-isomers, and demonstrated binding to the heme iron in spectroscopic studies. Unique 2,19-

bridged androstenediones have also been reported (65-67). These A-ring bridged steroids consist of both 5-membered and 6-membered ring analogs containing carbon, oxygen, nitrogen, or sulfur atoms. Several of these compounds exhibited apparent K_i values in the low nM range and demonstrated tight-binding competitive inhibition. Thus, effective inhibitors have been prepared with geometrically small functionalities at the C-19 position, suggesting that the enzyme active site can accommodate small changes in structure.

2. Mechanism-based enzyme inhibitors. A mechanism-based inhibitor is an inhibitor that mimics the substrate, is converted by the enzyme to a reactive intermediate and results in the inactivation of the enzyme. The term mechanism-based is used since the inhibitor contains a chemical functionality that is acted upon by the enzyme during the normal catalytic process. A mechanism-based inhibitor produces time-dependent inactivation of the enzyme only in the presence of catalytically active enzyme; omission of a cofactor, such as NADPH, does not produce inactivation. Other terms that are utilized for these inhibitors are enzyme-activated irreversible inhibitors, suicide substrates, and suicide inactivators. Several mechanism-based aromatase inhibitors, structurally related to the natural substrate androstenedione (Figure 8), are initially recognized by the aromatase enzyme as alternate substrates and are then transformed (through an NADPH-dependent mechanism) to reactive intermediates, which bind irreversibly to the enzyme and produce inactivation. Inactivation kinetic values are determined from plots of the half-time of inactivation, i.e., the time required to decrease the enzymatic activity by 50%, versus the reciprocal of the inhibitor concentration. The y-intercept of the resulting line is the half-time of inactivation at infinite inhibitor concentration ($T_{1/2}$) and the rate of inactivation, apparent k_{inact}, is equal to 0.693/ $T_{1/2}$. The most effective mechanism-based inhibitors exhibit short half-times

of inactivation ($T_{1/2}$) and rapid rates of inactivation. Mechanism-based inhibitors have distinct advantages in drug design, since these inhibitors are highly enzyme specific, produce prolonged inhibition, and often exhibit minimal toxicities.

The first compound designed as a mechanism-based inhibitor of aromatase was 10-propargyl-4-estrene-3,17-dione (PED; MDL 18,962); it was synthesized and studied independently by three research groups (68-70). MDL 18,962 has an electron-rich alkynyl function on the C-19 carbon atom, the site of aromatase-mediated oxidation of the substrate, and is an effective inhibitor *in vitro* and *in vivo* (71-74). In biochemical assays, MDL 18,962 exhibited a half-time of inactivation at infinite inhibitor concentration ($T_{1/2}$) of 10.41 minutes and an apparent k_{inact} of 1.11 x 10^{-3} sec^{-1}. Although the identity of the reactive intermediate formed is not known, an oxirene and a Michael acceptor have been suggested.

A larger number of mechanism-based inhibitors have developed from more detailed biochemical investigations of several inhibitors originally thought to be competitive inhibitors. These inhibitors can be grouped into the general categories of 4-substituted androst-4-ene-3,17-diones, substituted androsta-1,4-diene-3,17-diones, and 6-methylene- or 6-oxo- androst-4-ene-3,17-diones.

4-Hydroxy-4-androstene-3,17-dione (4-hydroxyandrostenedione, 4-OHA, formestane), originally thought to be a competitive inhibitor, produces enzyme-mediated inactivation (75). In enzyme assays, 4-hydroxyandrostenedione exhibited a half-time of inactivation at infinite inhibitor concentration ($T_{1/2}$) of 2.57 minutes and an apparent k_{inact} of 4.50 x 10^{-3} sec^{-1}. *In vivo*, formestane inhibits reproductive processes (76) and causes regression of hormone-dependent mammary rat tumors (77,78). Formestane is effective and well tolerated in the treatment of advanced breast cancer in postmenopausal women (79,80); however, extensive first pass

metabolism of this agent in the liver necessitates intramuscular administration and limits its use (81-83).

Numerous androsta-1,4-diene-3,17-diones have been prepared and have demonstrated mechanism-based or enzyme-mediated inactivation of aromatase *in vitro*. Androsta-1,4-diene-3,17-dione and androsta-1,4,6-triene-3,17-dione were initially thought to be competitive inhibitors, but further examination of the biochemistry of these inhibitors revealed that these compounds demonstrated inactivation of aromatase only in the presence of cofactors (84,85). Introduction of substituents at the 7α-position of both androsta-1,4-diene-3,17-dione and androsta-1,4,6-triene-3,17-dione have resulted in very effective mechanism-based inhibitors of aromatase (57,86-89). 7α-(4'-Amino)phenylthioandrosta-1,4-diene-3,17-dione (7α-APTADD) has high affinity for aromatase, with an apparent Ki of 9.9 nM, and has the most rapid rate of inactivation reported to date with a half-time of inactivation ($T_{1/2}$) of 1.38 minutes and an apparent k_{inact} of 8.40 x 10^{-3} sec^{-1}. More metabolically stable inhibitors were synthesized by replacing the thioether linkage at the 7α-position with a carbon-carbon linkage. The best inactivator of the series was the 7α-phenpropylandrosta-1,4-diene- 3,17-dione, which exhibited a $T_{1/2}$ of 6.08 minutes and an apparent k_{inact} of 1.90 x 10^{-3} sec^{-1}.

An exocyclic double bond at the C-6 carbon atom results in 6-methyleneandrost-1,4-diene-3,17-dione (exemestane), which produces aromatase inactivation *in vitro* and causes regression of hormone-dependent mammary rat tumors (90-92). Exemestane is a potent inhibitor of both human placental aromatase with apparent K_i of 26 nM, a $T_{1/2}$ of 13.9 minutes, and an apparent k_{inact} of 8.30 x 10^{-4} sec^{-1}. Exemestane, when administered subcutaneously or orally, inhibits rat ovarian aromatase (ED_{50} of 1.8 and 3.7 mg/kg, respectively) (90,93).

Despite the large number of effective mechanism-based or enzyme-activated inhibitors that have been prepared, no detailed mechanism(s) of action have been identified. All agents produce time-dependent inactivation of aromatase activity only when incubated with catalytically active enzyme, no inactivation is observed in the absence of cofactors such as NADPH, and co-incubations with the substrate androstenedione decrease the rates of inactivation. Investigations of radiolabeled mechanism-based inhibitors, [^{125}I]-7α-(4'-iodo)phenylthioandrosta-1,4-diene-3,17-dione (7α-IPTADD) and [14C]-MDL 18,962, provided the first evidence of irreversible binding of these inhibitors to the aromatase protein (94). Radiolabeled inhibitors were incubated with purified reconstituted aromatase preparations and NADPH, and the cytochrome $P450_{arom}$ protein was isolated by gel chromatography following the incubation. Subsequent treatments of the cytochrome $P450_{arom}$ protein fractions by precipitation, extensive washings, and SDS polyacrylamide gel electrophoresis demonstrated that the radioactive inhibitors remain bound to the cytochrome $P450_{arom}$ protein. Thus, the mechanism-based inactivation that occurs is due to irreversible, covalent binding of the inhibitors to the enzyme protein. The exact nature of the covalent bound(s), the chemical structures of the bound inhibitors, and the amino acids is yet be elucidated. Additionally, the question of whether the inhibitors are oxidized at the C-19 position in a manner similar to the substrate androstenedione prior to irreversible binding and inactivation remains to be answered.

B. NONSTEROIDAL INHIBITORS

1. First- and second-generation inhibitors. Nonsteroidal aromatase inhibitors possess a heteroatom as a common chemical feature and interfere with steroid hydroxylations by the binding of this heteroatom with the heme iron of the cytochrome P450's (Figure 9). Initial

nonsteroidal inhibitors were less enzyme specific and inhibited, to varying degrees, other cytochrome P450-mediated hydroxylations of steroidogenesis. Aminoglutethimide (AG) was the prototype for nonsteroidal aromatase inhibitors (95). AG was originally an antiepileptic agent which was removed from the market due to serious side effects. AG inhibited cytochrome $P450_{SCC}$ and other enzyme pathways, but was more selective for cytochrome $P450_{arom}$. The racemic mixture (*dl*-AG) inhibits aromatase with an apparent K_i of 700 nM. The *d*-AG stereoisomer is 20 fold more potent than the *l*-AG stereoisomer.

Since aminoglutethimide was the first aromatase inhibitor to be studied in patients, it is referred to as the first-generation aromatase inhibitor. AG has been used in the clinics with some success to treat patients with advanced breast cancer, but it must be administered with corticosteroid due to the inhibitory effects on cortisol and aldosterone biosynthesis (95,96). Although effective at decreasing aromatization, it also inhibited a number of other steroidogenic CYP-450 enzymes, which resulted in significant toxicity. The imidazole compound fadrazole is a potent, competitive inhibitors of aromatase both *in vitro* and *in vivo* (97). Fadrazole, referred to as a second-generation inhibitor, is more selective than AG and its inhibitory activity is 700 times more potent. Clinical studies using fadrazole proved this nonsteroidal inhibitor is effective in the treatment of some postmenopausal women with advanced breast cancer. Both partial and complete responses were observed upon treatment with fadrazole. However, this compound still has some non-selective inhibitory activity with respect to aldosterone, progesterone, and corticosterone biosynthesis. The apparent K_i for the racemate of fadrazole in human placental microsomes is 1.6 nM, and the (-)-S isomer is equipotent to the racemate while the (+)-R isomer has a lower inhibitory activity with an apparent K_i of 39 nM (98).

2. *Third-generation triazole inhibitors.* Several nonsteroidal aromatase inhibitors containing a triazole ring have been successfully developed. Vorozole potently inhibits aromatase in numerous *in vitro* systems, with an apparent K_i of 1.3 nM in human placental microsomes (99). The racemate has been tested thoroughly, but it is known that the (+)-S isomer is 36 times more potent than the (-)-R isomer (100). Regression of tumors in a hormone-dependent rat tumor model has been demonstrated with various doses of vorozole. Another triazole analog is anastrozole, which is an achiral triazole derivative (101). Anastrozole is a potent aromatase inhibitor with an IC_{50} of 15 nM in human placental microsomes. *In vitro*, anastrozole has no effect on numerous other P450 enzymes such as, $P450_{SCC}$, 11β-hydroxylase, 18-hydroxylase, 17α-hydroxylase and lanosterol-14α-demethylase. *In vivo* studies in monkeys showed that anastrozole, at a dose of less than 0.2 mg/kg a day, reduced peripheral aromatase activity by 50-60% (101). The third triazole derivative, letrozole, is a potent inhibitor of aromatase with an IC_{50} of 11.5 nM in human placental microsomes (102). In *in vitro* studies, letrozole has no effect on the biosynthesis of other steroids such as aldosterone, progesterone or corticosterone. *In vivo*, letrozole was determined to be orally active and to cause regression of tumors in the DMBA hormone-dependent rat tumor model, and it demonstrated aromatase inhibition in patients (103). A higher degree of specificity has been reached with the new generation of triazole derivatives (letrozole and anastrozole). These newer agents are 100 to 3,000 times more active than aminoglutethimide and all inhibit whole body aromatization by greater than 96%.

3. *Flavonoid derivatives as inhibitors.* Flavonoids are plant natural products present in many food sources, including fruits, vegetables, legumes, and whole grains. The class of flavonoids encompasses flavones, isoflavones, flavanones, and flavonols, each possessing the

benzopyranone ring system as the common chemical scaffold. Considerable interest in flavonoids in breast cancer has been stimulated by the hypothesis that these natural products, present in soy and in rye flour, are dietary factors that may be responsible for the lower incidence of breast cancer in women from certain regions of the world (104,105). Several flavonoids demonstrate inhibitory activities of the aromatase enzyme, thus lowering estrogen biosynthesis and circulating estrogen levels (Figure 10) (104,106-111). However, these natural products demonstrate numerous biological activities and interact with various enzymes and receptor systems of pharmacological significance, thus limiting their therapeutic usefulness.

Strong evidence for the binding of flavones to the active site of aromatase was obtained by difference spectral absorption studies (106), with 7,8-benzoflavone displacing androstenedione from the aromatase active site and inducing a spectrum consistent with the low-spin state of iron. Reduction of the flavone 4-keto group was detrimental to aromatase inhibition by these compounds (107). Based on data obtained from site directed mutagenesis studies and ligand docking into a homology model of the aromatase protein, a binding orientation was predicted in which the A and C rings of the flavone mimic the C and D rings of the steroid substrate, respectively. The 2-phenyl substituent is oriented in a region similar to that occupied by the A ring of the steroid. This analysis places the flavone 4-keto functionality in the same position as the steroid 19-angular methyl group with respect to the heme iron (110).

Medicinal chemistry approaches to develop synthetic flavonoids, chromone, or xanthone analogs with enhanced aromatase inhibitory activity have identified more selective and/or more potent agents for future development (112-114). Generally, flavones and flavanones have higher aromatase inhibitory activity than isoflavones (Figure 10). The flavone, chrysin, has an IC_{50} value of 0.50 μM; apigenin, flavone, flavanone, and quercetin were less efficacious inhibitors

with IC_{50} values of 1.2, 8, 8, and 12 μM, respectively. Isoflavones are significantly less potent as aromatase inhibitors. The most effective isoflavone inhibitor is biochanin A (BCA) with an IC_{50} value of 113 μM , approximately 20-fold less potent than chrysin in terms of IC_{50} values (110,115). This large difference in potency is the likely reason why there has been little effort to develop aromatase inhibitors on an isoflavone scaffold. On the other hand, we envisioned introduction of the proper functional groups on the isoflavone core could result in the desired aromatase activity. As a proof of principle, a 2-(4-pyridylmethyl)thio functionality was introduced onto the isoflavone nucleus (Figure 10), and this isoflavone modification afforded a 160-fold enhancement in potency compared to the natural product lead, BCA (116). In human placental microsomal assays, the synthetic pyridyl isoflavone analog, 3-phenyl-7-(phenylmethoxy)-2-[(4'-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one, exhibited an IC_{50} value of approximately 210 nM and an apparent K_i value of 220 nM.

Aromatase Inhibitors in Breast Cancer

A. First- and second-generation aromatase inhibitors

1. Aminoglutethimide. Aminoglutethimide was the first of these inhibitors evaluated in clinical studies for treatment of hormone-dependent breast cancer (117). Santen *et al.* extensively evaluated the kinetic and hormonal effects of aminoglutethimide in breast cancer and first proposed aromatase inhibition as the primary mechanism of action of aminoglutethimide therapy (118). Numerous clinical trials performed since the early 1980's have demonstrated the clinical efficacy of combination therapy of aminoglutethimide with corticosteroid replacement (96,119) in treatment of hormone-dependent breast cancer. However, side effects of lethargy,

ataxia, and morbilliform skin rash and the development of more potent aromatase inhibitors resulted in cessation of further development of aminoglutethimide for breast cancer.

2. 4-Hydroxyandrostenedione. The steroidal inhibitor 4-hydroxyandrostenedione, generic name of formestane, was extensively evaluated in clinical trials and was the first aromatase inhibitor approved for general use in Europe. In a series of clinical studies (120), 4-hydroxyandrostenedione treatment in unselected postmenopausal breast cancer patients with either weekly or biweekly intramuscular injections resulted in 26% of patients experiencing complete or partial responses and another 25% exhibiting disease stabilization. Decreased serum estrogen levels have been observed in postmenopausal breast cancer patients. The drug is extremely well tolerated and only a low percentage (13%) of patients experienced pain and/or inflammation at the injection site. Since 4-hydroxyandrostenedione (formestane) was the second aromatase inhibitor to be studied in patients, it is referred to as a second-generation aromatase inhibitor.

B. Third-generation aromatase inhibitors

The third-generation aromatase inhibitors include the nonsteroidal inhibitors anastrozole and letrozole and the steroidal inhibitor exemestane. These third-generation aromatase inhibitors have received extensive clinical evaluations (121-126).

1. Anastrozole. Anastrozole (Arimidex®) is a potent nonsteroidal aromatase inhibitor in patients, decreasing plasma estradiol levels in a dose-dependent manner and producing approximately 97% inhibition of estrogen biosynthesis at the dose of 1 mg/day (127).

Anastrozole was well tolerated in two large, Phase III trials of anastrozole vs. megestrol acetate in patients who progressed on tamoxifen, and the combined analysis demonstrated a clinically significant advantage over megestrol acetate (128-130). Two randomized, double-blind studies demonstrated that anastrozole (1 mg daily) was more effective than tamoxifen (20 mg daily) as first-line therapy in postmenopausal women with advanced breast cancer (131-133). Anastrozole at the recommended therapeutic dose of 1 mg once daily effectively suppressed total-body aromatization, with a mean percentage inhibition of 97.3%, and suppressed plasma estrone and estradiol levels by 81 - 85% in postmenopausal women with metastatic breast cancer (134). In clinical trials with postmenopausal women, no changes were detected in levels of androgens, an increase in the gonadotropins LH and FSH was observed over time, and a decrease in SHBG was also detected (135).

2. Letrozole. Letrozole (Femara®) is a potent nonsteroidal aromatase inhibitor that produces approximately 99% inhibition of estrogen biosynthesis at the dose of 2.5 mg/day in patients (136). Clinical studies, involving postmenopausal women with advanced breast cancer who have had numerous previous endocrine treatments, showed that letrozole produced either a partial response or stabilization of disease in about 40% of the women (137,138). Letrozole is also well-tolerated, causes a marked decrease in serum and urine estrogen levels, and has little effect on other endocrine factors. In clinical trials with postmenopausal women, in plasma levels of androgens were unchanged with letrozole treatment, and increases in LH, FSH and SHBG were detected over time (139). A multicenter, randomized, double-blind study in advanced breast cancer reported letrozole more effective than tamoxifen in response rate, clinical benefit, time to progression, and time to treatment failure (140). Letrozole at the recommended therapeutic dose

of 2.5 mg once daily effectively suppressed total-body aromatization, with a mean percentage inhibition of greater than 99.1%, and suppressed plasma estrone and estradiol levels by 84 - 88% in postmenopausal women with metastatic breast cancer (141).

3. *Exemestane.* Exemestane (Aromasin®) is a potent steroidal inhibitor of human placental aromatase, and a single oral dose of 25 mg exemestane was found to cause a long-lasting reduction in plasma and urinary estrogen levels. Maximal suppression of circulating estrogens occurred two to three days after dosing and persisted for four to five days (142). The lengthy duration of estrogen suppression is thought to be related to the irreversible nature of the drug-enzyme interaction rather than pharmacokinetic properties of the compound. Exemestane is also well tolerated, causes a marked decrease in serum and urine estrogen levels, and has no effect on other endocrine factors (142-146). Increased doses of exemestane can lead to suppression of SHBG (147,148). Exemestane was shown to inhibit peripheral aromatase by 97-98% (144,149).

Table 1 compares the inhibitory activities *in vitro* and *in vivo* of aromatase inhibitors that have been evaluated clinically. As described earlier, anastrozole and letrozole are both nonsteroidal competitive inhibitors of aromatase, while exemestane is a mechanism-based inhibitor. Investigations of these therapeutic agents in cell lines expressing high levels of aromatase, such as JEG-3 and JAr choriocarcinoma cells, have examined the impact of these inhibition characteristics on the residual *in vitro* aromatase activity and protein levels. Several mechanism-based aromatase inhibitors produce prolonged suppression of aromatase catalytic activity for up to 48 hours in cells following short drug exposure (72,94,150). On the other hand, the nonsteroidal inhibitor aminoglutethimide resulted in elevated levels of aromatase enzyme

activity under similar conditions, possibly due to enzyme stabilization (150). In a study comparing letrozole (3 nM), exemestane (20 nM), anastrozole (40 nM), or aminoglutethimide (10 nM), aromatase activity returned to control levels immediately after letrozole exposure and increased by about 60%, 30%, and 20% after 4, 24, and 48 hours, respectively. After preincubation with anastrozole or aminoglutethimide, cellular aromatase also increased. On the other hand, suppression of aromatase activity was maintained for approximately 24 to 48 hours after the removal of exemestane (151). There was no increase in the aromatase protein level in either experiment. In a study with cultured fibroblasts from mammary adipose tissue preincubated with an anti-aromatase agent for 18 hours, removal of aminoglutethimide or anastrozole elicited up to 40% increases in aromatase activity, whereas preincubation with exemestane resulted in a marked suppression (152).

C. CLINICAL STUDIES

The third-generation aromatase inhibitors are approved in the United States for the treatment of postmenopausal women with metastatic estrogen-dependent breast cancer. Both anastrozole and letrozole were more effective than tamoxifen as first-line therapy in postmenopausal women with advanced breast cancer (131-133,153). Exemestane has also shown enhanced efficacy over tamoxifen as first-line therapy in postmenopausal women with advanced breast cancer (154). In these clinical studies, the aromatase inhibitors demonstrated improved clinical efficacy in primary endpoints of objective response rates (complete response, partial response, or disease stabilization), time to progression (TTP), and time to treatment failure (TTF). Patients positive for estrogen receptor (ER+) and/or progesterone receptor (PR+) had better response rates on aromatase inhibitors than the patients treated with tamoxifen.

Overall, the third-generation aromatase inhibitors are well tolerated in these clinical trials of postmenopausal women with hormone-dependent metastatic breast cancer.

Current clinical studies of aromatase inhibitors are focusing on the use of the agents in the adjuvant setting for the treatment of early breast cancer (121,125,155,156). These studies assess the effectiveness of aromatase inhibitors following tamoxifen, of aromatase inhibitors alone, and/or of the combination of aromatase inhibitors and tamoxifen in adjuvant therapy. Table 2 summarizes the key adjuvant trials for the third-generation aromatase inhibitors. Early results from three randomized, adjuvant Phase III clinical trials have recently been published. The largest of these trials, ATAC (Anastrozole, Tamoxifen Alone, or in Combination), recruited 9,366 postmenopausal women with early breast cancer, and the patients were randomized into one of the three treatment arms for 5 years. After a median follow-up of 47 months, the anastrozole arm of the study resulted in statistically significant reduction in breast cancer events and improvement of disease-free survival (157,158). No differences in disease-free survival were observed between the tamoxifen alone arm and the combination arm. The MA-17 trial recruited 5,187 postmenopausal women who had taken tamoxifen for 5 years, and these patients were randomized into two treatment arms of letrozole or placebo for an additional 5 years. After a median follow-up of 2.4 years, the letrozole-treated patients had a significant reduction of breast cancer events (159). At this analysis, the data safety and monitoring committee for the MA-17 study recommended that the trial be halted early and the participants informed of the positive results. The Intergroup Exemestane Study (IES) enrolled 4,742 postmenopausal women who had taken tamoxifen for 2 or 3 years, and these patients were randomized into two treatment arms of tamoxifen for a total of 5 years or exemestane to complete the 5-year hormonal therapy. Exemestane therapy after two to three years of tamoxifen therapy significantly reduced breast

cancer recurrence and contralateral breast cancer as compared with the standard five years of tamoxifen treatment (160). Based on the results of these multiple, large randomized trials, the American Society of Clinical Oncology (ASCO) technology assessment panel (161) recommends that the "optimal adjuvant hormonal therapy for a postmenopausal woman with receptor-positive breast cancer includes an aromatase inhibitor as initial therapy or after treatment with tamoxifen."

These multiple, large randomized trials also enabled a more thorough analysis of the tolerability and adverse events of the aromatase inhibitors. In general, patients on aromatase inhibitors experienced less gynecologic symptoms such as endometrial cancer, vaginal bleeding, and vaginal discharges. Fewer cerebrovascular events and venous thromboembolic events were also observed with patients receiving aromatase inhibitors. No information is yet available on the effects of aromatase inhibitors on serum lipid levels, cardiovascular disease, and coronary heart disease risk. On the other hand, musculoskeletal effects and bone toxicity are associated with aromatase inhibitors. The percentages of musculoskeletal effects, which include increased arthritis, arthralgia, and/or myalgia, were small but showed statistically significant increases with aromatase inhibitors than with tamoxifen. All three aromatase inhibitors were associated with increased fractures when compared to tamoxifen or placebo. The ATAC trial reported a 7.1% fracture incidence in the anastrozole arm vs. tamoxifen at 4.4% (157,158). In the MA-17 study, fractures in the letrozole-treated patients were 3.6% compared to 2.9% in placebo, after 2 or 3 years of prior tamoxifen treatment (159). Exemestane was associated with osteoporosis and/or increased fractures (7.41%) when compared to tamoxifen (5.7%) in the IES trial. Baseline bone mineral density evaluations and potential bisphosphonate therapy are recommended (161).

Other on-going clinical studies are designed to compare the various aromatase inhibitors and/or combination therapies in early stage breast cancer or in the chemoprevention setting. For example, MA-27 is a Phase III adjuvant trial in postmenopausal women with primary breast cancer comparing exemestane with anastrozole, with or without celecoxib, a COX-2 inhibitor. The potential for aromatase inhibitors in the chemoprevention setting in women with increased risk for the development of breast cancer is also being considered. In preclinical models, aromatase inhibitors reduce tumor formation in the carcinogen-induced rat mammary tumor studies (162-165). The International Breast Cancer Intervention Study II (IBIS II) will compare anastrozole vs. placebo in a prevention study, and the accompanying DCIS study will compare tamoxifen vs. anastrozole in women with locally excised ductal carcinoma in situ (DCIS) (166). A Canadian breast cancer prevention study, NCIC MAP3, is a three-arm study of placebo vs. exemestane vs. exemestane and celecoxib(166). Important endpoints in such trials may include not only reduction in tumor incidence but also examine effects of aromatase inhibitors bone mineral density and serum lipid levels.

Conclusions

Aromatase inhibitors, both steroidal and nonsteroidal agents, have been shown to be useful for the treatment of breast cancer. These compounds work by preventing the synthesis of estrogens in the body. Aromatase inhibitors appear to be more effective in postmenopausal women than in premenopausal women due to the fact that the major source of estrogen biosynthesis in postmenopausal women is adipose tissue. Over the years, both steroidal and nonsteroidal inhibitors have developed into very potent compounds that are highly selective for aromatase versus other steroidogenic cytochrome P450 enzymes. The potent, selective and

orally-active third-generation aromatase inhibitors, anastrozole, letrozole, and exemestane, were initially approved for clinical use as endocrine therapy in postmenopausal patients failing antiestrogen therapy alone or multiple hormonal therapies. More recent clinical studies have shown that these aromatase inhibitors are more effective than tamoxifen in postmenopausal patients with metastatic breast cancer, and these agents are the approved therapy for the treatment of postmenopausal women with metastatic estrogen-dependent breast cancer. Furthermore, the third-generation aromatase inhibitors are now being studied in the adjuvant setting either alone or in combination with other agents. Based on the results of these multiple, large randomized trials, the American Society of Clinical Oncology (ASCO) panel recommends that adjuvant hormonal therapy for a postmenopausal woman with receptor-positive breast cancer with an aromatase inhibitor as initial therapy or after treatment with tamoxifen.

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Table 1. Effect of Aromatase Inhibitors on Aromatase Activity of Human Breast Tumors and Human Fibroblasts and Whole Body Aromatization in Postmenopausal Breast Cancer Patients (134,136,141,144-146).

Aromatase Inhibitors	*In Vitro* Inhibition (IC_{50}, nM)		*In Vivo* Inhibition of Whole Body Aromatization	
	Breast tumors	Breast fibroblasts	Oral dose (mg/day)	% Inhibition
First-generation				
Aminoglutethimide	20,000	10,000	1000	90.6
Second-generation				
Fadrozole	-	-	2	82.4
Formestane	30	30	250	84.8
Third-generation				
Letrozole	2	0.8	2.5	98.9
Anastrozole	8	15	1	96.7
Exemestane	15	5	25	97.9

Table 2. Adjuvant Trials of Third-Generation Aromatase Inhibitors (154-160)

Adjuvant Trial	*Organization*	*Design*
ATAC	Cancer Research Campaign Breast Cancer Trials Group	**Anastrozole** alone (1 mg daily, 5 yr) *vs.* Tamoxifen alone (20 mg daily, 5 yr) *vs.* **Anastrozole** (1 mg daily) and Tamoxifen (20 mg daily, 5 yr)
ARNO	German Breast Cancer Group	Tamoxifen (20 mg daily, 2 yr) followed by Tamoxifen (20 mg daily, 3 yr) *vs*. Tamoxifen (20 mg daily, 2 yr) followed by **Anastrozole** (1 mg daily, 3 yr)
FEMTABIG	Femara-Tamoxifen Breast International Group	**Letrozole** alone (2.5 mg daily, 5 yr) *vs*. Tamoxifen alone (20 mg daily, 5 yr) *vs*. **Letrozole** (2.5 mg daily, 2 yr) and Tamoxifen (20 mg daily, 3 yr) *vs.* Tamoxifen (20 mg daily, 2 yr) and **Letrozole** (2.5 mg daily, 3 yr)
MA-17	National Cancer Institute of Canada-Clinical Trials Group	Tamoxifen (5 yr) followed by **Letrozole** (5 yr) *vs.* Tamoxifen (5 yr) followed by placebo (5 yr)
Intergroup Exemestane Study (IES)	International Collaboration Cancer Group	Tamoxifen (20 mg daily, 2-3 yr) followed by **Exemestane** (25 mg daily, 2-3 yr) *vs*. Tamoxifen (20 mg daily, 2-3 yr) followed by Tamoxifen (20 mg daily, 2-3 yr)
NASBP B-33	National Surgical Adjuvant Breast and Bowel Project	Tamoxifen (20 mg daily, 5 yr) followed by **Exemestane** (25 mg daily, 2 yr) *vs*. Tamoxifen (20 mg daily, 2-3 yr) followed by placebo (2 yr)

Figures

Figure 1.

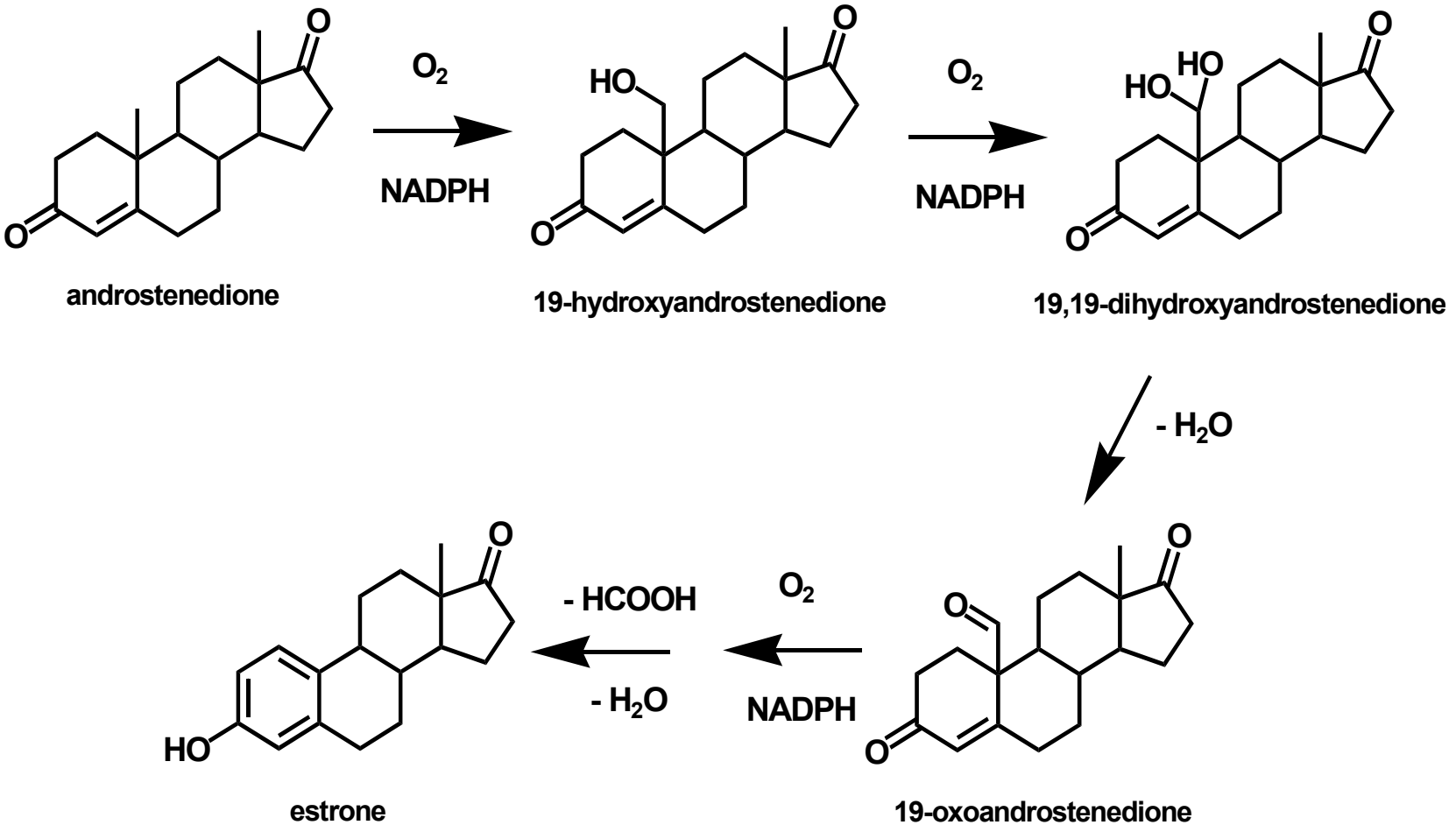
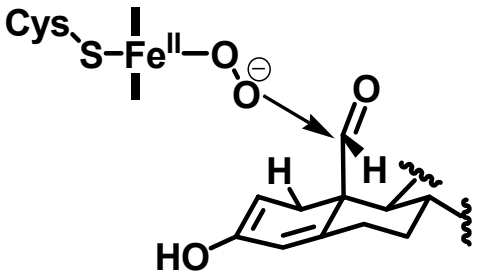

O
O
androstenedione
O2
NADPH
HO
O
O
19-hydroxyandrostenedione
O2
NADPH
HO
HO
O
O
19,19-dihydroxyandrostenedione
- H2O
O
O
O
19-oxoandrostenedione
O2
NADPH
- HCOOH
- H2O
O
HO
estrone

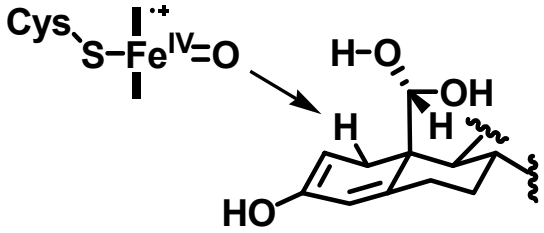
Figure 2.

(A)

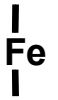


Nucleophilic attack of P450 peroxy intermediate on aldehyde

(B)



P450 Compound I (oxene) intermediate abstracts 1β-hydrogen atom

where  =

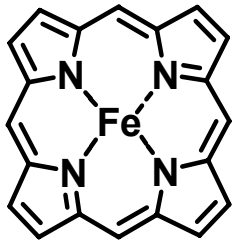
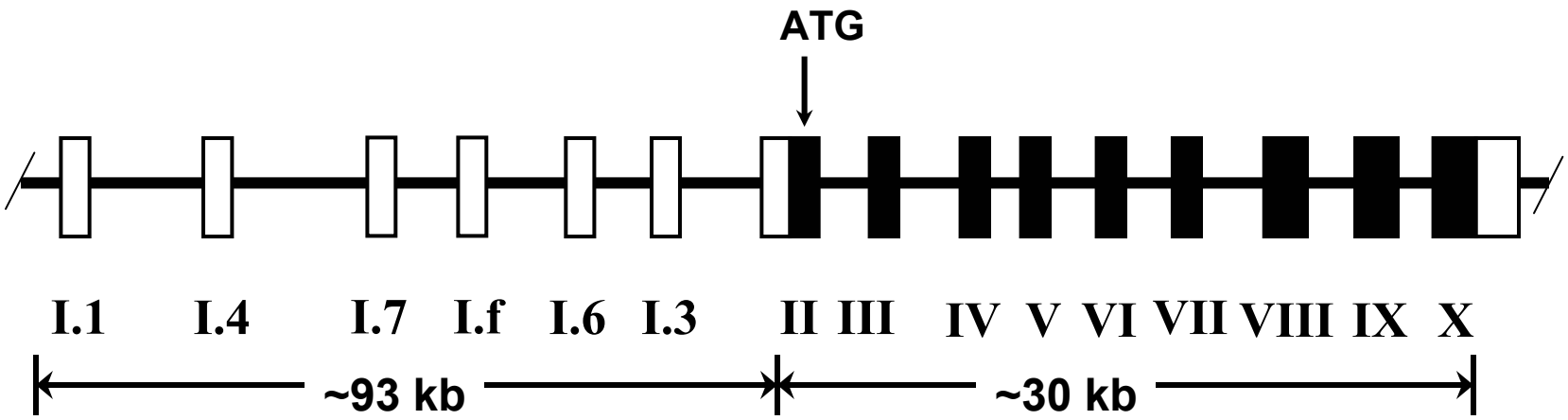


Figure 3.



Tissue-specific promoters:

I.1	placental tissue
I.4	normal adipose tissue
I.7	vascular endothelial tissue
I.f	brain
I.6	bone
I.3	adipose tissue; breast cancer
II	ovary; breast cancer

Figure 4.

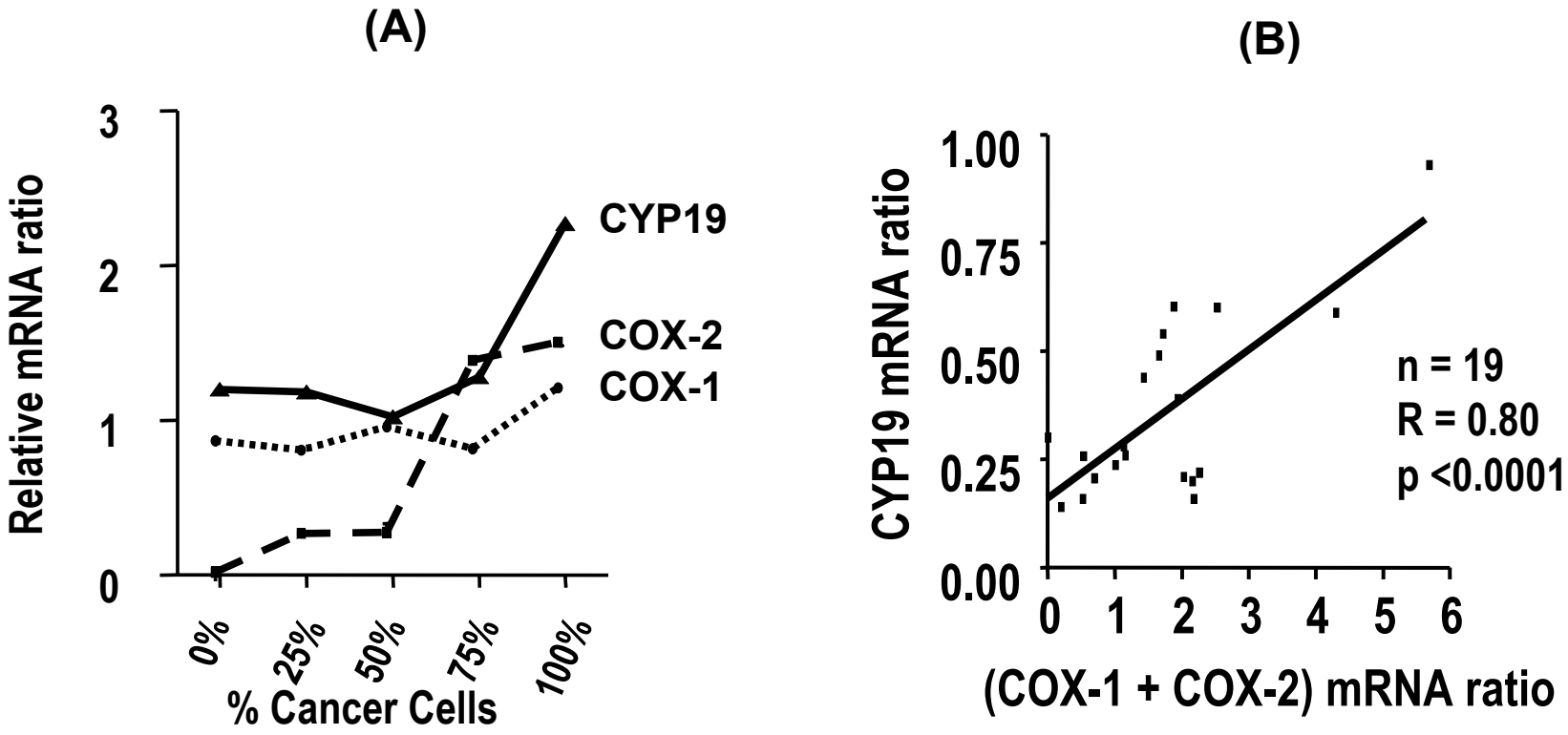
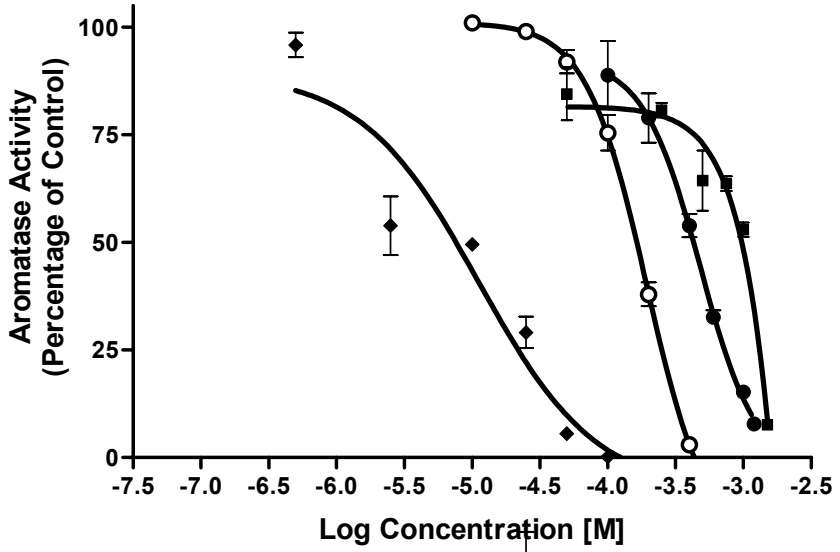

(A)
Relative mRNA ratio
3
2
1
0
CYP19
COX-2
COX-1
0%
25%
50%
75%
100%
% Cancer Cells
(B)
CYP19 mRNA ratio
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
n = 19
R = 0.80
p <0.0001
(COX-1 + COX-2) mRNA ratio

Figure 5.

(A)


Aromatase Activity
(Percentage of Control)
100
75
50
25
0
-7.5
-7.0
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
Log Concentration [M]

(B)

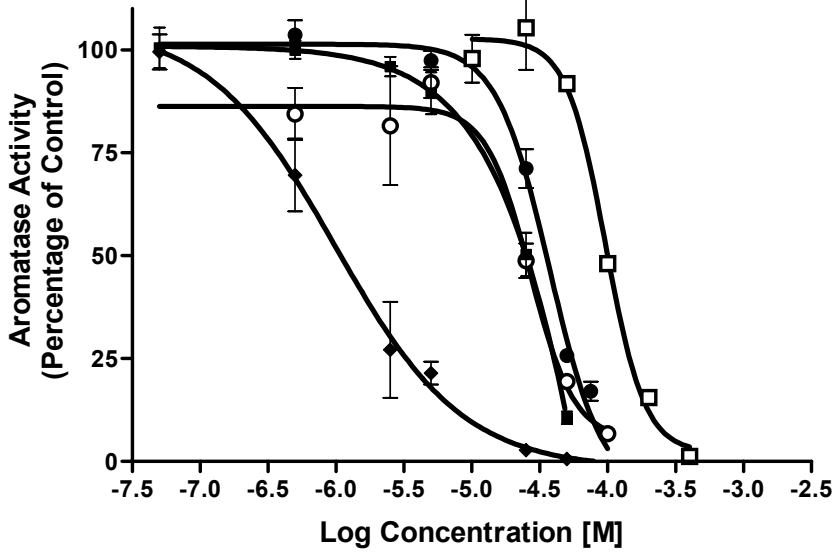

Aromatase Activity
(Percentage of Control)
100
75
50
25
0
-7.5
-7.0
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
Log Concentration [M]

Figure 6.

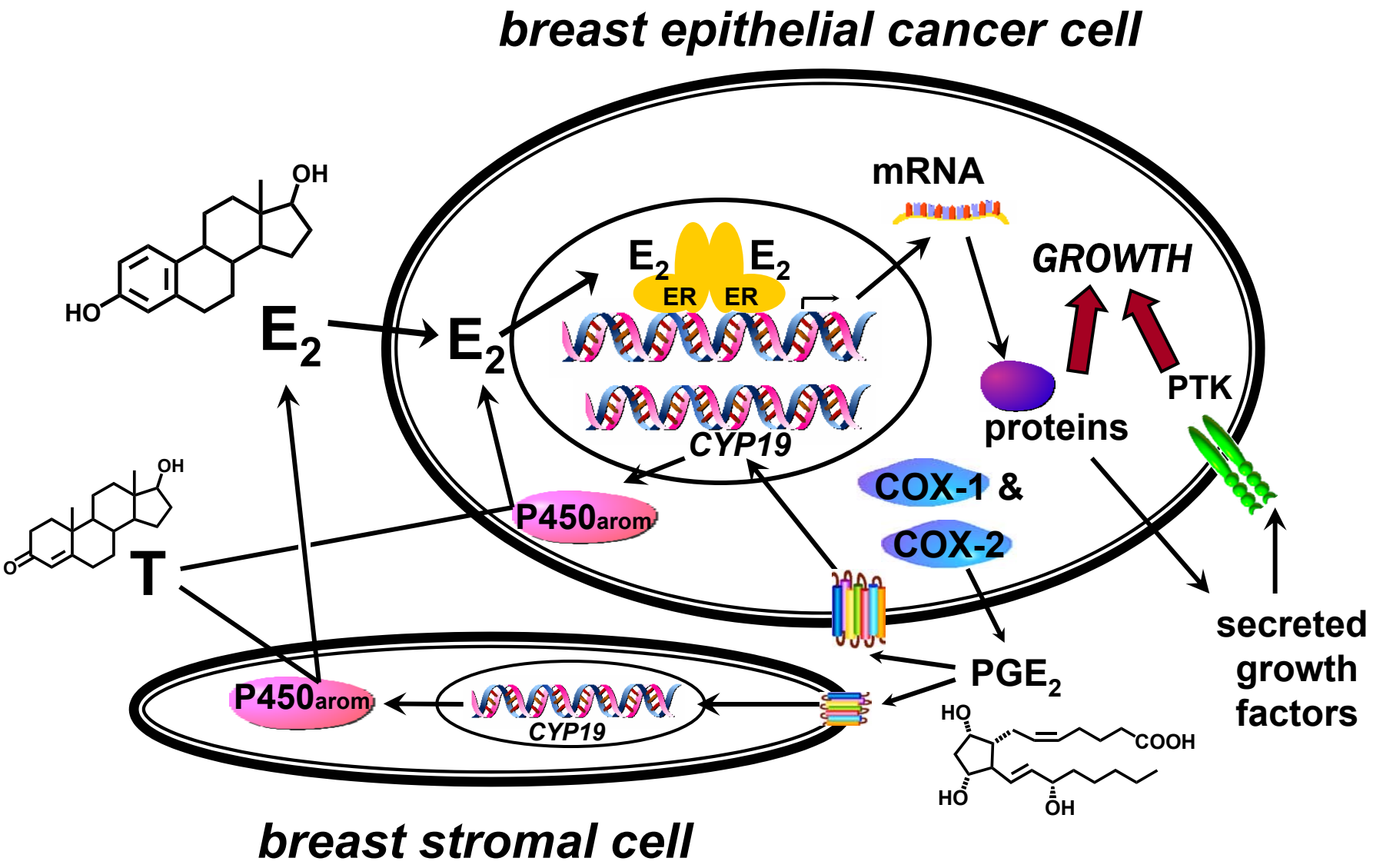
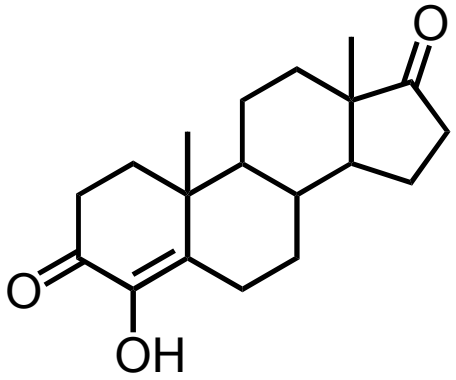
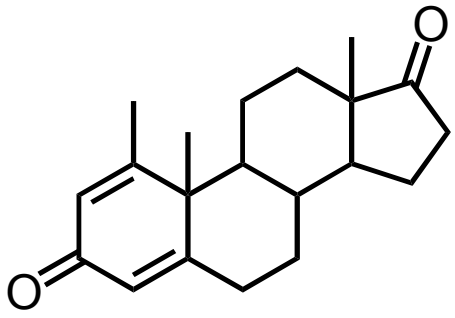

breast epithelial cancer cell
mRNA
GROWTH
PTK
proteins
E2
E2
ER
ER
E2
E2
CYP19
COX-1 &
COX-2
P450arom
T
PGE2
secreted
growth
factors
P450arom
CYP19
HO
COOH
HO
OH
breast stromal cell

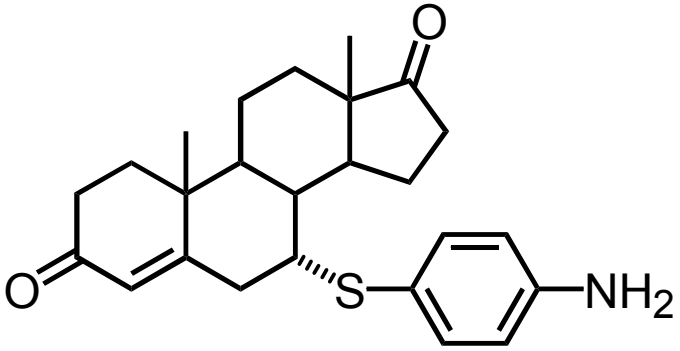
Figure 7.



4-OHA, formestane



1-methyl-ADD



7α-APTA

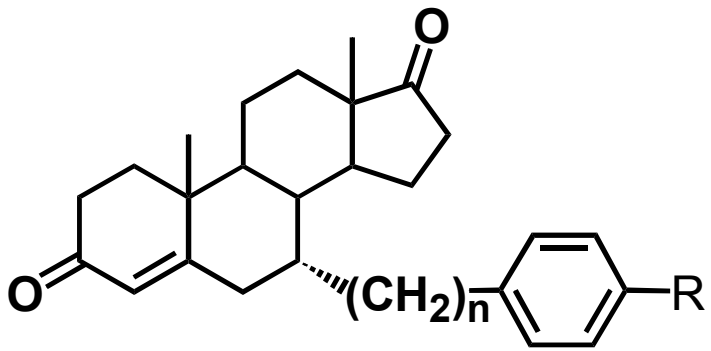
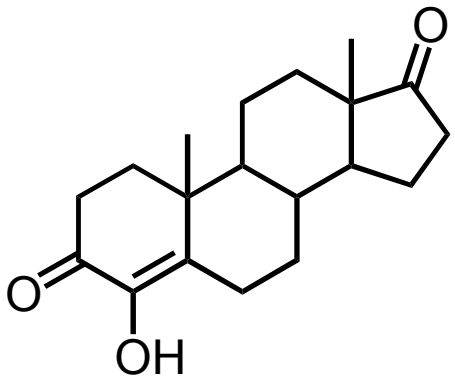
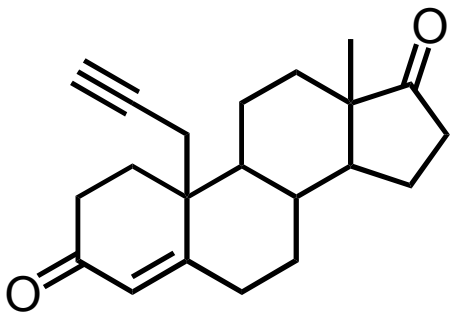


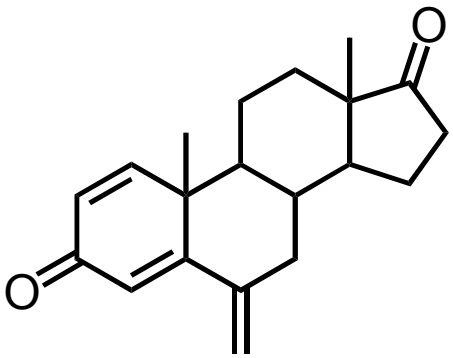
Figure 8.



4-OHA, formestane

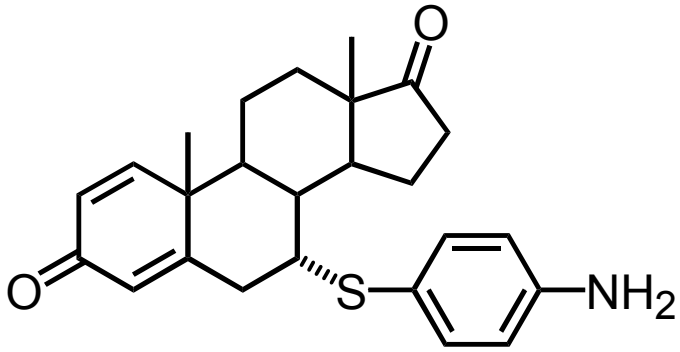


MDL 18,962

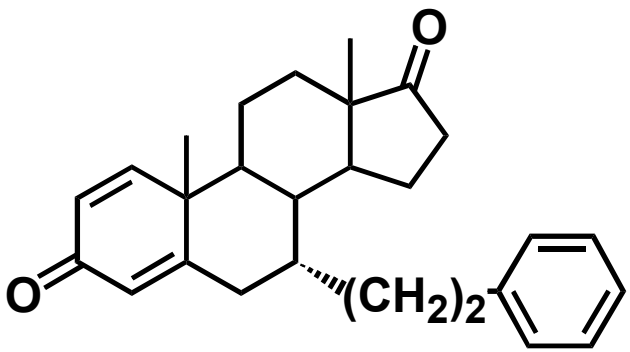


exemestane

Aromasin®



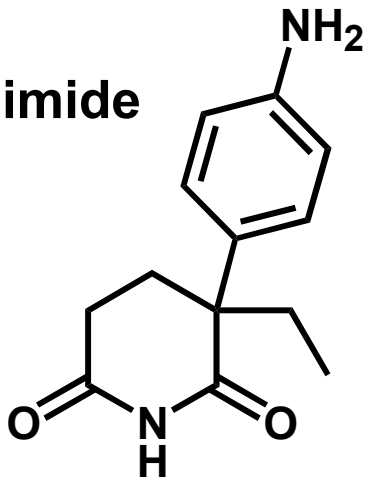
7α-APTADD



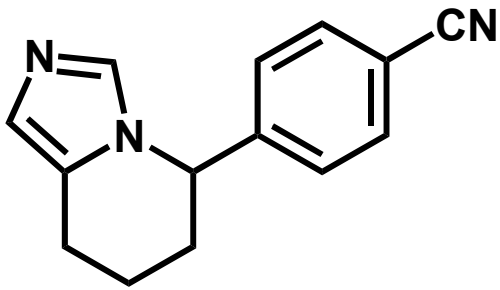
7α-PEADD

Figure 9.

aminoglutethimide

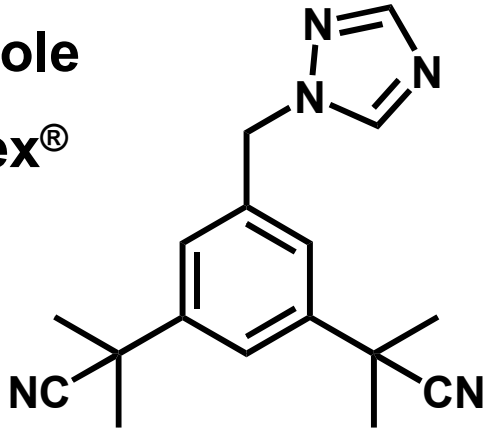


fadrazole



anastrozole

Arimidex®



letrozole

Femara®

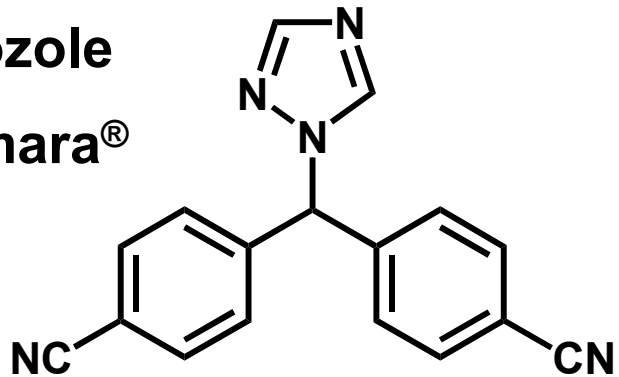
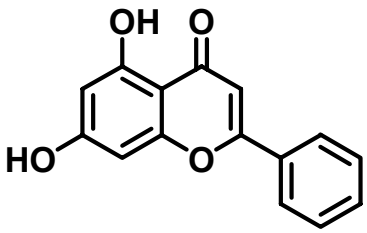
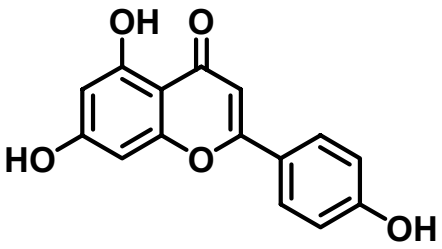


Figure 10.

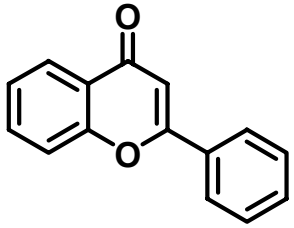
Flavones



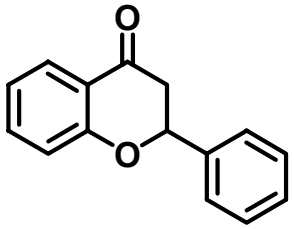
chrysin



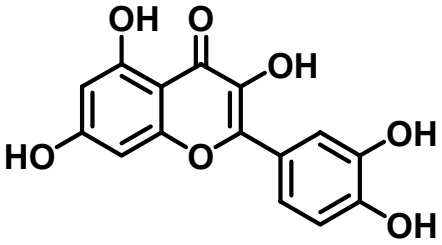
apigenin



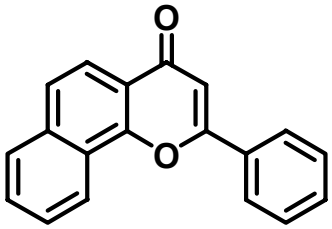
flavone



flavanone

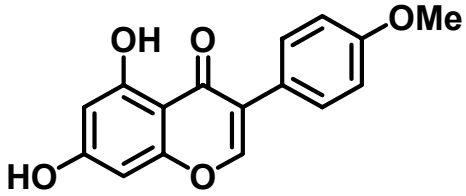


quercetin

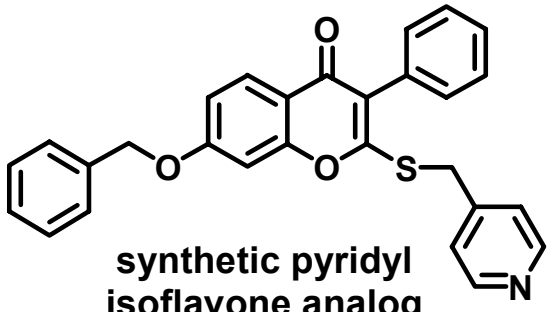


7,8-benzoflavone

Isoflavones



biochanin A



synthetic pyridyl
isoflavone analog