CANCER

The body is made up of hundreds of millions of living cells. Normal body cells grow, divide and die in an orderly fashion and during the early years of a person's life, these cells divide faster to allow the person to grow. After the person becomes an adult, most cells divide only to replace worn-out or dying cells or to repair injuries. Cancer begins because of out-of-control growth of abnormal cells in a part of the body leading to many kinds of cancer. Instead of dying, cancer cells continue to grow and form new, abnormal cells. Cancer cells can also invade (grow into) other tissues, something that normal cells cannot do. In a normal cell, when deoxyribonucleic acid (DNA) gets damaged the cell either repairs the damage or the cell dies, however in cancer cells, the damaged DNA is not repaired, but the cell doesn't die like it should. Instead, this cell goes on making undesired new cells, with all having same damaged DNA as the first cell does. Sometimes the cause of the DNA damage is something obvious, like cigarette smoking, tobacco chewing but often no clear cause is found.

In most cases the cancer cells form a tumor. Some cancers, like leukemia, rarely form tumors. Cancer cells sometimes get into the bloodstream or lymph vessels of our body and travel to other parts of the body, where they begin to grow and form new tumors that replace normal tissue. This process is called metastasis. No matter where a cancer may spread, it is always named for the place where it started. For example, breast cancer that has spread to the liver is still called breast cancer, not liver cancer. Likewise, prostate cancer that has spread to the bone is metastatic prostate cancer, not bone cancer. Different types of cancer can behave very differently. For example, lung cancer and breast cancer are very different diseases. They grow at different rates and respond to different treatments. That is why people with cancer need treatment that is aimed at their particular kind of cancer. Not all tumors are cancerous. Tumors that aren't cancerous are called benign. Benign tumors slowly grow in size and can
create pressure on healthy organs and tissues. But they cannot grow into (invade) other tissues and also can’t spread to other parts of the body. These tumors are almost never life threatening.\(^\text{18}\)

The global burden of cancer continues to increase largely because of the aging and growth of the world population alongside an increasing adoption of cancer-causing behaviors, particularly smoking, in economically developing countries. Based on the GLOBOCAN estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world.\(^\text{19}\)

**BREAST CANCER**

Breast cancer is a malignant tumor that starts from cells of the breast. A malignant tumor is a group of cancer cells that may grow into (invade) surrounding tissues or spread (metastasize) to distant areas of the body. The disease occurs almost entirely in women, but men can get it, too.\(^\text{20,21}\)

Estrogens appear to hold the key to the understanding of breast cancer. Before 18\(^{\text{th}}\) century, breast cancer was considered as a systemic disease caused by some problem in body fluids viz. blood and lymph. In 1713, a higher prevalence of breast cancer was observed among nuns in Padua.\(^\text{22}\) Interestingly, 129 years after this observation, Rigoni-Stern found nuns to be at more than three times higher risk of breast cancer compared to other women and linked it with nulliparity.\(^\text{23}\) In 1889, Schinzinger stated that the disease grew more slowly in postmenopausal women and even suggested castration as a mean for hastening the benefit of menopause. Beatson also reported tumour regression in advanced breast cancer patients after surgical castration and for the first time, a systemic treatment became available for patients with breast cancer.\(^\text{24}\)

Breast cancer is a major medical problem with significant public health and social ramifications in females and is the most common cause of cancer in women between the ages of 30-54. It is the second leading cause of cancer deaths in women today (after lung cancer).\(^\text{25}\) A survey of American Cancer
Society has estimated about 207,090 new cases of invasive breast cancer to occur among women. In addition to invasive breast cancer, 54,010 new cases of *in situ* breast cancer are expected to occur and about 39,840 death are expected from breast cancer for the year 2010. If current breast cancer rates remain constant, a woman born today has a one in ten chance of developing breast cancer.\(^{19}\)

The incidence of breast cancer in India is also on the rise and is rapidly becoming the number one cancer in females pushing the cervical cancer to the second spot. It is reported that one in 22 women in India is likely to suffer from breast cancer during her lifetime, while the figure is definitely more in America with one in eight being a victim of this deadly cancer.\(^{26}\)

The death rate for breast cancer has been slowly declining over the past decade and the incidence has remained level since 1988 after increasing steadily for nearly 50 years.\(^{27}\) Twenty five to thirty percentage of women with invasive breast cancer will die of their disease. Mortality rates are highest in the very young (less than age 35) and in the very old (greater than age 75). It appears that the very young have more aggressive disease, and that the very old may not be treated aggressively. Although 60% to 80% of recurrences occur in the first 3 years, the chance of recurrence exists for up to 20 years.\(^{28}\)

**PATHOPHYSIOLOGY**

Breast cancer, like other cancers, occurs because of an interaction between the environment and a defective gene.\(^{29}\) Normal cells divide as many times as needed and stop. They attach to other cells and stay in place in tissues. Cells become cancerous when mutations destroy their ability to stop dividing, to attach to other cells and to stay where they belong. When cells divide, their DNA is normally copied with many mistakes. Error-correcting proteins fix those mistakes. The mutations known to cause breast cancer, such as p53, BRCA1 and BRCA2 occur in the error-correcting mechanisms. These mutations are either inherited or acquired after birth. Presumably, they allow the other mutations, which allow uncontrolled division, lack of attachment and metastasis to distant organs. Normal cells will commit cell suicide (apoptosis) when they are no longer needed. Until then, they are protected from cell
suicide by several protein clusters and pathways. One of the protective pathways is the PI3K/AKT pathway; another is the RAS/MEK/ERK pathway. Sometimes the genes along these protective pathways are mutated in a way that turns them permanently "on", rendering the cell incapable of committing suicide when it is no longer needed. This is one of the steps that causes cancer in combination with other mutations. Normally, the PTEN protein turns off the PI3K/AKT pathway when the cell is ready for cell suicide. In some breast cancers, the gene for the PTEN protein is mutated, so the PI3K/AKT pathway is stuck in the "on" position, and the cancer cell does not commit suicide. Mutations that can lead to breast cancer have been experimentally linked to estrogen exposure. Abnormal growth factor signaling in the interaction between stromal cells and epithelial cells can facilitate malignant cell growth. In breast adipose tissue, over expression of leptin leads to increased cell proliferation and cancer.

RISK FACTORS
Breast cancer incidence is highest in North America and Northern Europe and lowest in Asia and Africa. Environmental and/or lifestyle factors appear to be important determinants of breast cancer risk. Gender is by far the greatest risk factor. Breast cancer occurs 100 times more frequently in women than men. In women, incidence rates of breast cancer rise sharply with age (Table 1) until ages 45 to 50, when the rise becomes less steep. This change in slope probably reflects the impact of hormonal change (menopause) that occurs about this time. By ages 75 to 80, the curve actually flattens and then decreases.

Research has yielded much information about the causes of breast cancer and it is now believed that genetic and/or hormonal factors are the primary risk factors for breast cancer. Breast cancer sensitivity to estrogen has been found to increase with the age of patient, leading to its more common occurrence among postmenopausal women compared to the younger women. Women of advancing age most commonly get breast cancer. Early menarche, late menopause, nulliparity, child birth after the age of thirty, overweight after menopause, alcohol consumption, radiation exposure, all
markers of prolonged ovulatory functions are closely associated with an increased risk of breast carcinoma. Obesity, an indicative of increased plasma concentration of estrogens and application of prolonged hormonal replacement therapy also enhance the risk to breast cancer.\textsuperscript{34} People carrying certain particular set of genes are more susceptible to breast cancer. In particular people with BRCA-1 and BRCA-2 genes have a 30 to 40% increased susceptibility to breast as well as ovarian cancer.\textsuperscript{31}

**Table-1.** Chances of a woman developing breast cancer by age

<table>
<thead>
<tr>
<th>By Age</th>
<th>Normal Risk</th>
<th>Genetic Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>1 in 93 (1%)</td>
<td>42%</td>
</tr>
<tr>
<td>55</td>
<td>1 in 33 (3%)</td>
<td>72%</td>
</tr>
<tr>
<td>65</td>
<td>1 in 17 (6%)</td>
<td>80%</td>
</tr>
<tr>
<td>75</td>
<td>1 in 11 (9%)</td>
<td>84%</td>
</tr>
</tbody>
</table>

Approximately two third of postmenopausal breast cancer patients have hormone-dependent (estrogen-dependent) breast cancer, which requires estrogen for tumor growth. Heightened awareness of breast cancer risk in the past decades has led to an increase in the number of women undergoing mammography for screening, leading to detection of breast cancer in earlier stages and a resultant improvement in survival rate. Still, breast cancer remains the most common cause of death in women.\textsuperscript{35}

**DIAGNOSIS**

Screening of breast cancer is an attempt to find unsuspected cancer. The most common methods of detection are self and clinical breast examination, X-ray, mammography, ultrasound and occasionally Magnetic Resonance Imaging (MRI). Mammography is still the modality of choice for screening of early breast cancer. It is relatively fast, reasonably accurate and widely available. Mammography has been estimated to reduce breast cancer related mortality by 20 to 30%. The American Cancer society recommends that
women 40 years of age or older should have a mammogram every one to two years. Women with one or more first degree relative (mother, sister or daughter) with pre menopausal breast cancer should begin screening at an early age. It is usually suggested to start screening at an age 10 years less than the age the relative was diagnosed with breast cancer.33 Breast cancer is usually diagnosed by the examination of surgically removed breast tissue. However a small number of procedures such as fine needle aspiration cytology can help in making a diagnosis prior to surgery. Imaging tests are sometimes required to detect metastasis (spread) and include chest X-ray, bone scan, CT, MRI and PET scan.36,37

**STAGES**

Stages of breast cancer involve both the size of the tumor and whether or not it has spread to other parts of the body such as the lymph nodes. Staging is very important because it helps healthcare professionals determine the best treatment options to fight against cancer.38

Stages of cancer start at 0 and go up to 4, but they are written in roman numerals (I–IV). The number implies how much the cancer has spread. Generally, a lower number means the cancer has not spread, or has spread less. A higher number means the cancer has spread more.

*Stage 0 (carcinoma in situ)*

The tumor has stayed in the part of the breast where it started. There are 2 types of breast carcinoma in situ: ductal carcinoma in situ (DCIS, cancer in the cells that carry milk) and lobular carcinoma in situ (LCIS, cancer in the cells of the glands that make milk), are different and are treated differently.

*Stage I*

The tumor has spread into nearby parts of the breast, but not outside of the breast. The tumor is 2 centimeters or smaller.

*Stage II*

The tumor is between 2–5 centimeters, or it has spread outside the breast to the lymph nodes under the arm on the same side as the breast cancer.
**Stage III**
The tumor is larger than 5 centimeters, or has spread to areas around the breast, such as the skin of the breast and the collarbone.

**Stage IV**
The tumor has spread beyond the breast and nearby areas, possibly to the base of the neck, lungs, liver, bones or brain.

**TREATMENT STRATEGIES**
The treatment plan differs from patient to patient, from a breast cancer type to another and from a breast cancer stage to another. It depends on the size and location of the tumor in the breast, the results of laboratory tests done on the cancer cells and the stage or extent of the disease. Most breast cancer types in the last stages cannot be cured.

**Local therapy**
Local therapy is used to treat the tumor without affecting the rest of the body. Surgery and radiation therapies are included in this category.

**Surgery**
Breast surgery is a common part of breast cancer treatment plans. Surgery is performed to remove as much of the breast cancer as possible, and to determine if the cancer has spread to the lymph nodes. Studies have shown that removal of the whole breast (mastectomy) does not prolong life when compared with removal of the cancer lump alone (lumpectomy). As lumpectomy preserves more of the breast, it is associated with a better body image compared with total mastectomy. It is also advisable to remove some lymph glands from the armpit (axillary dissection) if the cancer is invasive. If the lymph glands do not contain cancer, then the outlook for the patient is very good. Removal of the lymph glands increases the chances of arm swelling (lymphedema), arm stiffness and pain. Therefore it is preferable to remove the minimum number of glands and this can be safely achieved through a technique called the sentinel node biopsy.33,39

**Radiation therapy**— Radiotherapy uses high-energy X-rays to destroy cancer cells. It is usually given to the breast area following breast conservation
surgery and sometimes after mastectomy. Radiotherapy is given to prevent a recurrence in the breast area. Radiotherapy is not a systemic treatment and only affects the area that is being treated. It is painless, but there are some side-effects such as skin redness and tiredness, these are usually mild and temporary. Radiotherapy involves sophisticated machinery which directs high energy beams at the cancer and surrounding tissue. Radiotherapy is usually given every day. A course of radiotherapy can last from 3 to 6 weeks. There are two types of radiation therapy.33,40

*External beam radiation* is provided from an external device and focuses on the affected area. The main side effects of this therapy are swelling and heaviness in the breast, sunburn-like skin changes in the treated area, and fatigue.

*Internal radiation device* is placed inside the body into the breast tissue closer to the cancer.

**Systemic therapy**

It includes chemotherapy, hormone therapy and immunotherapy.

**Chemotherapy**

Chemotherapy is the name of a group of anticancer drugs used to kill cancer cells. It is sometimes used as part of the treatment for breast cancer. Some patients require chemotherapy prior to surgery if the tumor is very large. Not all patients will require chemotherapy. This will depend on several factors related to the individual’s breast cancer. Chemotherapy is offered to patients where they are likely to benefit from the treatment. The type of chemotherapy which will be offered is based on the current research, which has shown a benefit for a particular group of patients. There are several different types of chemotherapy drug combinations which are used to treat breast cancer. They are normally given into the vein via a drip. The treatment usually lasts for 4–6 hours and is given in cycles of 2–3 weeks. This is followed by a rest period of 2–3 weeks until the next cycle starts. Chemotherapy is a systemic treatment which affects all the cells in the body. Healthy cells as well as cancer cells are affected and this can cause side-effects. These can include nausea, fatigue
and hair loss. It is important to remember that most side-effects from chemotherapy are temporary as healthy cells recover quickly. Side-effects are managed very well throughout the treatment process to minimize any discomfort. Examples of some of the chemotherapeutic agents are doxorubicin (Adriamycin®), cyclophosphamide (Cytoxan®), methotrexate (Folex®, Mexate®, Amethopterin®) and fluorouracil (5FU).

**Immunotherapy**

Trastuzumab (Herceptin®) is an effective treatment against HER2-positive breast cancer. It is called a monoclonal antibody, which utilizes the natural immune system to kill tumor cells. It binds to the HER2 receptors on the tumor cell surface and stops the receptor signaling of the cell to grow and divide. It is effective in women with metastatic and earlier stages of disease and is given intravenously into the bloodstream once every week and for three weeks. Potential side effects include fever and sweating, running nose, chills, skin flushing, redness, tightness in the chest or difficulty in breathing and discomfort in the throat.

**Endocrine therapy**

Hormonal therapy is a very effective treatment against breast cancer that is hormone-receptor-positive. Hormonal therapy given as a treatment for breast cancer works by blocking the effects of the hormone estrogen, which in some patients can promote the growth of breast cancer. Cancer cells that have estrogen receptors are referred to as hormone receptor positive. If there are no receptors on the cells, this is referred to as hormone receptor negative. Hormonal therapy would not be effective for patients who are hormone receptor negative. The goal of hormonal therapy is to prevent the hormones from being taken up by the cells thereby slowing or stopping the growth of cancer.

Breast cancer endocrine therapies can be classified into three major types of therapeutic interventions: agents that directly target estrogen receptor (ER) through molecules that bind ER and alter ER functions; estrogen deprivation through aromatase inhibition or ovarian ablation or suppression; and sex steroid therapies, including estrogen, progestins and androgens.
Since these approaches have quite different mechanisms of action, they can be used in sequence, recapturing tumor control with each class switch. In addition the side effects of these agents are quite distinct, with very important consequences for women’s health particularly when they are used for long-term adjuvant therapy.44

**Agents that directly target estrogen receptors**

Tamoxifen (1) is a competitive inhibitor of estrogens binding to the estrogen receptors. Tamoxifen and similar agents are not simply anti-estrogens but selective estrogen receptor modulators (SERMs). Other agents in the SERM class include toremifine (2) and raloxifene (3).44,45 Tamoxifen is a non-steroidal triphenylethylene first synthesized in 1966. Tamoxifen was originally developed as an oral contraceptive but in an early clue to its complicated endocrine properties, tamoxifen was found to induce ovulation. Its activity in metastatic breast cancer was first described in the 1970s.46-48 Response rates ranging from 16 to 56%, and a better toxicity profile than alternative therapies such as high dose estrogen therapy or adrenalectomy resulted in rapid adoption of tamoxifen as the treatment of choice for advanced disease.49,50

Prospective randomized clinical trials of tamoxifen as adjuvant therapy also demonstrated considerable efficacy. Five years of adjuvant tamoxifen therapy reduces the risk of death by 26%. These effects are also seen in bone and the lipid metabolism, favorably affecting bone mineral density and blood lipid profile.51,52

Toremifine is the other agent in the SERM class approved for breast cancer treatment. Similar in structure and mechanism of action to tamoxifen but with a chlorine atom at the fourth carbon, toremifine is metabolized by CYP3A and excreted predominantly in the feces with a half-life of 5 days. Toremifine has similar efficacy and side effects to tamoxifen in the treatment of advanced disease but cross-resistance between this agent and tamoxifen has been reported.53,54

Fulvestrant (4) is an ER antagonist with higher affinity for the estrogen receptor than tamoxifen. In contrast to tamoxifen, fulvestrant exhibits no
agonistic properties. It inhibits dimerization of ER and promotes accelerated receptor turnover. This property suppresses receptor protein levels, reduces shuttling of the receptor from the cytoplasm to the nucleus and inhibits estrogen dependent transcription.\textsuperscript{55,56} Unfortunately the compound is insoluble in water which severely limits oral bioavailability and is therefore administered as a 250 mg intramuscular monthly depot injection.\textsuperscript{57}

**Sex steroid therapies**

*High dose estrogen*

The successful treatment of advanced breast cancer with high doses of estrogen has a long history. This option should only be considered in postmenopausal women since high dose estrogens are ineffective before menopause.\textsuperscript{58} In a randomized trial of diethylstilbestrol (DES) and tamoxifen, tamoxifen was initially favored because high dose estrogen was associated with more adverse cardiac events, edema, nausea and vaginal bleeding. However, long-term follow up demonstrated a significant survival advantage for patients who received DES as their initial treatment.\textsuperscript{59} Recent data
suggests that activation of the FAS cell death receptor may be important in the therapeutic response to estrogen. Estrogen deprived breast cancer cells upregulate FAS receptor and subsequent estrogen treatment induces FAS ligand expression, leading to cell death. High doses of estrogen also reduce plasma levels of cell survival enhancing cytokines such as insulin-like growth factor 2 (IGF2) and free IGF1. Most of the data with high dose estrogen is from patients receiving DES as first line therapy for advanced disease, but patients with previous exposure to multiple endocrine therapies (tamoxifen, megestrol acetate and an aromatase inhibitor) may still respond to diethylstilbestrol. In a phase II trial DES conferred 37.5% clinical benefit rate suggesting that high dose estrogen remains a valuable alternative to chemotherapy in selected patients. Side effects include hot flashes, breast tenderness, vaginal discharge, and, more seriously, congestive cardiac failure and venous thrombosis. Estrogens are contraindicated if the patient has a thromboembolic or cardiac disorder.

Progestins

Progestins have activity in advanced breast cancer by unclear mechanisms that may involve aromatase inhibition, increased estrogen turnover, or direct actions on steroid receptors. Available agents for breast cancer include the orally available megestrol acetate (Megace) and medroxyprogesterone which are equally efficacious. However, megestrol has a more adverse side effect profile with weight gain, nausea, fluid retention, vaginal bleeding, thromboembolic events and lower quality of life.

Androgens

Testosterone and its analogues have demonstrated response rates of 20% in metastatic breast cancer. Androgen side effects include edema, hot flashes, and jaundice. They have much reduced efficacy after the use of prior endocrine therapy and are inferior to estrogens and tamoxifen.

Estrogen deprivation therapy

Oophorectomy

The initial endocrine therapy of breast cancer was removal of the ovaries.
This can now also be achieved through pharmacological intervention. In the premenopausal woman with hormone receptor positive breast cancer, one of these forms of ovarian ablation is often recommended as primary treatment to allow the patient to be treated according to postmenopausal guidelines. Randomized trials support the combination of ovarian suppression or ablation and tamoxifen as the first line therapy for hormone receptor positive advanced breast cancer in premenopausal women.\textsuperscript{65,66}

**LHRH analogues**

Goserelin is the only LHRH analogue approved for breast cancer therapy in the United States. It is given as a depot injection and acts on the pituitary to initially stimulate and then suppress FSH and LH secretion to menopausal levels in 3–4 weeks.\textsuperscript{65} Gonadotrophin suppression results in reduced estrogen and progesterone production in the ovary. These agents are 50–100 times more potent than the native peptide.\textsuperscript{66,67}

**Aromatase inhibitors (AIs)**

Aromatase inhibitors are being used as endocrine therapy in postmenopausal patients failing anti-estrogen therapy alone or multiple hormonal therapies. There are two classes of AIs, steroidal and nonsteroidal compounds, which cause potent estrogen suppression. The non-steroidal AIs are mostly azole type compounds such as the clinically used anastrazole and letrozole. Steroidal AIs of contemporary importance include exemestane and formestane.\textsuperscript{68}

**AROMATASE INHIBITORS**

Inhibition of aromatase is the another approach for reducing growth-stimulatory effects of estrogens. Effective aromatase inhibitors have been developed as therapeutic agents for controlling estrogen-dependent breast cancer.\textsuperscript{69} Investigations on the development of aromatase inhibitors began in the 1970s and have expanded greatly in the past three decades. Aromatase inhibitors can reduce estrogen production by more than 90%. Unlike tamoxifen, however, AIs lack estrogen-agonist activity. Tamoxifen inhibits the growth of breast tumors by competitive antagonism of estrogen at its receptor
Recent clinical data has shown that these inhibitors have greater efficacy than tamoxifen in late-stage disease and preliminary data indicate that this efficacy extends to early disease. Aromatase inhibitors therefore almost certainly replace tamoxifen as the hormonal agents of choice for the treatment of breast cancer.71

**Figure 1. Site of action-aromatase inhibitors and tamoxifen**

AROMATASE

Mammalian cytochromes P450 (CYP) are involved in the biosynthesis of several important hormones, mainly of steroidal nature and in the metabolism of more than 90% of the drugs in current clinical use. In addition, they participate in both toxification and detoxification processes of many xenobiotics. The cytochrome P450 19 (CYP19; EC 1.14.14.1), commonly called aromatase (AR), metabolizes a wide variety of important substrates in many species of bacteria, plants and animals. In the human body, AR catalyzes the conversion of androgens into estrogens, through the aromatization of the A ring of substrates like testosterone and androstenedione.68 In the anti-cancer therapy, AR is an important
pharmacological target because of its critical role in the progression of post-menopausal breast cancer. Actually, the reduction of the levels of circulating estrogens in women with the disease has been demonstrated to be clinically effective.72

**Sources of aromatase**

Aromatase, an enzyme of the cytochrome P-450 superfamily and the product of the CYP19 gene, is highly expressed in the placenta and in the granulosa cells of ovarian follicles, where its expression depends on cyclical gonadotropin stimulation. Aromatase is also present, at lower levels, in several nonglandular tissues, including subcutaneous fat, liver, muscle, brain, normal breast and breast-cancer tissue. Residual estrogen production after menopause is solely from nonglandular sources, in particular from subcutaneous fat. Thus, peripheral aromatase activity and plasma estrogen levels correlate with body-mass index in postmenopausal women.68 At menopause, mean plasma estradiol levels fall from about 110 pg per milliliter (400 pmol per liter) to low but stable levels of about 7 pg per milliliter (25 pmol per liter). In postmenopausal women, however, the concentration of estradiol in breast-carcinoma tissue is approximately 10 times the concentration in plasma, probably in part because of the presence of intratumoral aromatase. Early evidence that intratumoral aromatase activity might help predict the response to aromatase inhibitors remains to be confirmed in large-scale studies.69

**3D-Model of the human aromatase enzyme**

The 3D arrangement of the hAR enzyme is characteristic of this family of CYP enzymes that share the common orthogonal bundle architecture.73 The core of the protein can be approximately located around the heme group, which is comprised between the helices I and L. The I helix, which goes throughout the protein's width, presents a distortion approximately in its center, near the highly conserved T310 residue, which seems to take part in the catalytic mechanism. Seventy percentage of the protein is assembled as α-helices, while 20% of its residues are stabilized as β-sheets. The main difference
between the theoretical model and the template was due to a different length of the F–G loop as shown in figure 2, which is a peculiar feature of the template and within the P450 family seems to be involved in the substrate recognition.\[74,75\]

**Aromatase gene expression**

The aromatase gene, designated CYP19, encodes the cytochrome P450arom, and this gene is located on chromosome 15q21.1. The coding region is approximately 30 kb in size, and the regulatory region is approximately 93 kb. The aromatase gene consists of 10 exons, and encodes for a protein of 503 amino acids. The aromatase protein is a glycosylated cytochrome P450 protein with a molecular mass of approximately 58,000 Da. The regulation of aromatase is complex in various tissues, and several tissue specific promoter regions have been identified upstream from the CYP19 gene.\[^69\] These tissue-specific promoters include promoter Pl.1, Pl.3, Pl.4, Pl.6, Pl.7, and PII as described in figure 3. Promoter Pl.1 is the major promoter used in placental tissues and is the farthest upstream. The PII promoter is
used 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

![Diagram of aromatase gene and promoter region]

**Tissue-specific promoters:**
- **1.1** Placental tissue
- **1.4** Normal adipose tissue
- **1.7** Vascular endothelial tissue
- **1.6** Brain
- **1.3** Adipose tissue; breast cancer
- **II** Ovary; breast cancer

*Figure 3. Aromatase gene and promoter region*

normal adipose tissue and is responsive to glucocorticoids and cytokines—such as IL-1, IL-6, and TNF. Promoter PI.3 is also present in adipose tissues such as normal breast tissue and is elevated in breast cancer tissues.

*Aromatase and estrogen biosynthesis*

Estrogen biosynthesis is catalyzed by a microsomal member of the cytochrome P450 (P450arom), the product of the CYP19 gene. The P450 gene superfamily is a very large one, of which cytochrome P450arom is the sole member of family 19. There is only about 30% homology between P450arom and other cytochrome P450 enzymes. The heme binding region of P450arom has only 17.9 to 23.5% identical amino acids in common with other steroidogenic P450 enzymes. These distinct features impart the selectivity characteristics to the aromatase inhibitors. The aromatase enzyme complex is composed of two polypeptides, aromatase cytochrome P450 and a flavoprotein, NADPH-cytochrome P450 reductase. The enzyme complex is bound in the endoplasmic reticulum of the cell. The androgens
are converted to estrogens by aromatase via three sequential oxidation steps as depicted in figure 4.

Figure 4. Aromatization of androgens to estrogens

The process utilizes three moles each of oxygen and NADPH for the overall conversion.\textsuperscript{81} The first step consists of a typical cytochrome P450 hydroxylation of the angular C-19 methyl group, which stereospecifically forms the 19-hydroxyandrostenedione. The second step is another stereospecific hydroxylation of the C-19 methyl, in which the 19-Pro-R hydrogen is displaced to give a C-19,19-gem diol. The gem diol then can readily dehydrate to produce 19-oxoandrostenedione. The final step consists of another oxidation that results in the elimination of the 1β hydrogen as water and incorporation of the C-19 into formic acid.\textsuperscript{82}

Aromatase in breast cancer tissues

Aromatase is found in the breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled.\textsuperscript{83} 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 \textit{in vitro}.\textsuperscript{84} Furthermore, the expression of aromatase is highest in or near breast cancer sites.\textsuperscript{85}

The increased expression of aromatase observed in breast cancer tissues is associated with a switch in the major promoter region used in gene...
expression, with promoter PII being the predominant promoter used in breast cancer tissues. 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. Prostaglandin E2 (PGE2) increases intracellular cAMP levels and stimulates estrogen biosynthesis.86

Local production of PGE2 via the cyclooxygenase isozymes can influence estrogen biosynthesis and estrogen dependent breast cancer. This biochemical mechanism may explain epidemiological observations of the beneficial effects of nonsteroidal antiinflammatory drugs (NSAIDs) on breast cancer.87,88 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.89 A positive correlation was observed between CYP19 expression and the greater extent of breast cancer cellularity, in agreement with literature reports showing that aromatase levels were higher in tumors than in normal tissue. Similar correlations between CYP19 expression and COX-2 expression in breast cancer patient specimens have been confirmed in other laboratories. This significant relationship between the aromatase and COX enzyme systems suggests that autocrine and paracrine mechanisms may be involved in hormone-dependent breast cancer development. In human breast stromal cells, PGE2 acts via two G protein-coupled receptors, EP1 and EP2 receptors, to stimulate aromatase gene expression via protein kinase A and protein kinase C signaling pathways.90

CLINICAL DEVELOPMENT OF AROMATASE INHIBITORS

The development of aromatase inhibitors and their use in clinical studies was prompted by the recognition that cytochrome P450 inhibitor, aminogluthethimide (5), is effective in advanced breast cancer treatment of postmenopausal women via inhibition of aromatase. Aminogluthethimide was initially developed as an anticonvulsant but was withdrawn from use after the reports of adrenal insufficiency. It was subsequently found to inhibit several cytochrome P450 enzymes involved in adrenal steroidogenesis and was then
redeveloped for use as "medical adrenalectomy" against advanced breast cancer.\textsuperscript{91,92} The significant clinical side effects of aminoglutethimide, its incomplete inhibition of aromatase, poor selectivity and the need for combination with glucocorticoids led to a search of more specific aromatase inhibitors.

**Table-2. Clinical development of various aromatase inhibitors**

<table>
<thead>
<tr>
<th>Generation</th>
<th>Type-I Steroidal aromatase inhibitors (SAI)</th>
<th>Type-II Nonsteroidal aromatase inhibitors (NSAI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>None</td>
<td>Aminoglutethimide</td>
</tr>
<tr>
<td>Second</td>
<td>Formestane</td>
<td>Fadrozole Rogletimide</td>
</tr>
<tr>
<td>Third</td>
<td>Exmestane (Aromasin)</td>
<td>Anastrozole (Armidex) Letrozole (Femara)</td>
</tr>
<tr>
<td>Fourth</td>
<td>None</td>
<td>YM511</td>
</tr>
</tbody>
</table>

The aromatase inhibitors have generally been categorized as first-, second-, and third- generation inhibitors according to the chronological order of their clinical development (Table-2). Aminoglutethimide is recognized as dominant first generation aromatase inhibitor. $\Delta^1$-Testololactone is another first generation aromatase inhibitor.\textsuperscript{93}

The second generation aromatase inhibitors include formestane (6)\textsuperscript{94} and fadrozole (7).\textsuperscript{95} Both have been found to have clinical efficacy but formestane has the disadvantage of requiring intramuscular injection, and
fadrozole causes aldosterone suppression, limiting its use to doses that produce only about 90% inhibition. Some other second-generation aromatase inhibitors have also been investigated clinically but have never been approved for clinical use. In contrast, third generation compounds, the triazoles anastrozole (8) (Arimidex<sup>®</sup>),<sup>96</sup> letrozole (9) (Femara<sup>®</sup>),<sup>97</sup> vorozole (10) and the steroidal agent exemestane (11) (Aromasin<sup>®</sup>)<sup>98</sup> have been found to be highly specific, potent and well tolerated such that they have been usable at dosages that effectively obliterate the activity of aromatase. The orally active exemestane is the main steroidal inhibitor of contemporary importance.

CLASSIFICATION OF AROMATASE INHIBITORS
BASED ON MECHANISM OF INHIBITION
The aromatase inhibitors can be divided into two main groups depending upon their mechanism of inhibition: (a) competitive Inhibitors, and
(b) mechanism based inhibitors.\textsuperscript{99,100} Both these classes contain steroidal and non-steroidal derivatives. Generally, the non-steroidal inhibitors bind to the P450 part of the enzyme and are reversible inhibitors. The steroidal inhibitors bind to the steroid substrate-binding site of the enzyme and are usually quite specific.

\textbf{Competitive inhibitors}

These inhibitors bind reversibly to the active site of the enzyme and prevent product formation as long as the inhibitor occupies the catalytic site of the enzyme. The steroidal inhibitors, although more specific, have the potential to induce unwanted agonist effects especially on estrogen, glucocorticoid, androgen or progesterone receptors. In contrast to this, non-steroidal inhibitors may lack specificity because they have the potential to block several P450 mediated steroid conversions. However, their potential advantage is that they are less likely to exhibit agonist and antagonist properties of steroids and are more likely to be orally absorbed.\textsuperscript{69}

\textit{Type I competitive inhibitors}

Compounds that bind reversibly to the active site of aromatase as steroid substrate analogues, and fail to turn over very rapidly, may be useful inhibitors.\textsuperscript{99} If these compounds bind as normal androgen substrates, they may induce analogous Type 1 changes in the UV-absorption spectrum Soret band of the enzyme bound heme. This phenomenon is a general character for cytochrome P450 enzymes. Type 1 binders induce a shift in the Soret band maximum from about 420 nm to about 390 nm. There are many compounds that have either been demonstrated or are presumed to act as Type 1 inhibitors. Type 1 difference spectra indicate that the steroid inhibits by interacting with the binding site of the enzyme rather than coordinating with heme-iron.\textsuperscript{101}

\textit{Type II competitive inhibitors}

There are some organic compounds containing suitably positioned heteroatoms that are capable of binding to cytochrome P450 enzymes such
that their heteroatom coordinate to the heme iron. This special type of binding is reflected in Soret band changes (usually bathochromic with respect to Type 1 binders). Furthermore, the precise Soret band displacement is often diagnostic of the heteroatom type (N, S, O). The term Type II refers to all the iron-coordinating inhibitors, regardless of the nature of the heteroatoms.69,102

**Suicide inhibitors or mechanism based inhibitors**

They initially compete with the natural substrate (i.e. androstenedione and testosterone) for binding to the active site of the enzyme. The enzyme then, specifically acts on the inhibitor to yield reactive alkylating species, which forms covalent bonds at or near the active site of enzyme and the enzyme is invariably inhibited.99

**BASED ON CHEMICAL STRUCTURE**

**Non-Steroidal aromatase inhibitors**

In the initial attempts to develop aromatase inhibitors for use in the treatment of estrogen dependent breast cancer, steroidal analogues of natural substrates, androstenedione and testosterone were developed, which inhibit the enzyme by binding to the substrate binding site. An alternative approach to the design of aromatase inhibitors was found by the discovery of non-steroidal drugs such as aminoglutethimide (5) and fadrozole (7). These are relatively non specific, competitive and reversible in nature but they provide a basis for the development of safe and effective inhibitors.103 Nonsteroidal aromatase inhibitors (NSAIs) 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 P450s.101

The more recently developed compounds such as anastrozole (8), letrozole (9) and vorozole (10) are more potent with high affinity for the enzyme, in the nano molar range. Overall they are more effective, and have an advantage over standard therapy in terms of toxicity as compared to megestrol acetate or aminoglutethimide. Both anastrozole and letrozole are very potent and selective aromatase inhibitors and are well tolerated by patients.96,97
Aniline derivatives

All the non-steroidal agents are active orally. Until 1992, the only widely available inhibitor was aminoglutethimide. This drug inhibits several cytochrome P450 enzymes, including some involved in steroidogenesis. It inhibited cytochrome P450 csc (CSCC = cholesterol side chain cleavage) and other enzymes but was more selective for cytochrome P450arom. The primary amine on the phenyl ring of aminoglutethimide coordinates with the heme of the active site of aromatase in order to bind to it. It has been used in the clinics with some success to treat patients with advanced breast cancer, but it must be administered with corticosteroids due to the inhibitory effects on cortisol and aldosterone biosynthesis.104

The side effects of aminoglutethimide (5) (mainly skin rashes and neurological symptoms), its lack of specificity (requiring replacement glucocorticoid) and its relatively low potency have been the targets for pharmaceutical improvement and have been well met by the most recent drugs. Attempts have been made to develop analogues with less activity against cholesterol side chain cleavage enzyme. The replacement of aniline ring with a pyridine ring helped to slightly improve selectivity for aromatase but it lowered overall inhibitory activity. Changes were also made in the ethyl side chain, in which it was lengthened or replaced by a cycloalkyl group.105

Relocation of amino group elsewhere in the phenyl ring produces compounds with less inhibitory activity towards aromatase but an enhanced activity towards cholesterol side chain cleavage enzyme.

Replacement of 3-phenyl substituent with a 3-pyridyl group in aminoglutethimide gives compounds, which are stronger bases than aminoglutethimide and so they are expected to be potent aromatase inhibitors. Pyridoglutethimide (12, rogletimide), chemically, 3-ethyl-3-(4-pyridyl) piperidine-2,6-dione is a non steroidal compound resulting from such a modification and has enhanced specificity and lesser side effects than aminoglutethimide.106 Preliminary phase II trials show that pyridoglutethimide is better tolerated than aminoglutethimide.107

As pyridoglutethimide has been found to be a slightly weaker aromatase
inhibitor than aminogluthethimide, attempts were made to produce more potent analogues. Alkyl groups may be substituted on either the piperidine nitrogen atom or the 3-position. These compounds retain the selectivity of rogletimide and show an increased inhibitory activity as the alkyl chain length is increased.

The optimum length of the alkyl substituents has been found to be eight carbon atoms. The n-propyl analogue has the lowest activity against the cholesterol side chain cleavage enzyme and greatest specificity for aromatase. In another series, 3-ethyl group of aminogluthethimide was replaced by other alkyl groups with 2 to 7 carbon atoms with the intention of increasing the inhibition potential.

It is seen from aromatase inhibitory activity of 4-cyclohexylaniline (13) that glutarimide ring is not essential for activity. Further, it has been evidenced that certain side effects of aminogluthethimide, like ataxia, skin rashes, fatigue, hot flashes etc are due to the glutarimide ring of aminogluthethimide. Therefore, some analogues in which the piperidine-2,6-dione ring is replaced by pyrrolidine-2,5-dione ring have been synthesized. Two of these compounds, 3-(4-aminophenyl)-pyrrolidine-2,5-dione (14) and its ethylated analogue have proved to be effective as in vitro inhibitors of aromatase.

Glutarimide ring has also been replaced by substituted or unsubstituted 3-azabicyclo[3.1.0]hexane-2,4-dione rings. Two potent analogues with 3-butyl (15) and 3-pentyl (16) substitution have been synthesized and show a good in vitro inhibitory activity against aromatase and no significant activity against cholesterol side chain cleavage enzyme.
Two novel aromatase inhibitors designated as FCE 24789, \([N\text{-cyclohexyl-2-(4-aminophenyl)propanamide}]\) (17) and FCE-24328, \([\text{cyclohexyl-2-(4-aminophenyl)propionate}]\) (18) have been described.\(^{112}\) These compounds show 10 to 20 folds more potency against aromatase \textit{in vitro} than aminogluthethimide, but have less effect against cholesterol side chain cleavage enzyme.\(^{112}\)

![Chemical structures](image)

Despite these modifications, lack of selectivity remained the major drawback in case of aminogluthethimide and its analogues. Therefore, some newer non steroidal inhibitors were discovered and investigated.

**Indanone and tetralone derivatives**

New attempts were made to obtain the derivatives, which are more selective to P450arom and have greater potency than aminogluthethimide. These include derivatives of tetralone (19), tetralin (20) and indanone (21). 2-(4-Pyridylmethylene) and 2-(4-pyridymethyl)-1-tetralones with methoxy or hydroxy substituents at 5, 6 or 7 positions were synthesized as more selective and potent aromatase inhibitors.\(^{113,114}\)

![Chemical structures](image)

It was found that all the compounds were more potent than aminogluthethimide in exhibiting aromatase inhibitory activity with 5-hydroxy derivative (22) having maximum potency (IC\(50=0.2\) \(\mu\)M). These compounds
were also found to be more selective than aminogluthethimide with no or very less inhibitory activity for cholesterol side chain cleavage enzyme.\textsuperscript{113}

Within the tetralone class derived from (E)-2-(4-pyridylmethylene)-1-tetralone, different modifications were explored, regarding both the heterocycle and the substituents on the aromatic moiety. The aromatase inhibitory potency was increased by replacing the 4-pyridyl with the imidazolyl ring (23, E, IC\textsubscript{50}=0.26 \textmu M). The Z isomer 24 (IC\textsubscript{50}=0.17 \textmu M) of the imidazolyl derivative showed an activity comparable to that of the E isomer.\textsuperscript{115} However, the selectivity made some difference in the biological profiles of the two isomers, as 24 was found to affect other steroidogenic P450 enzymes, like 17α-hydroxylase/C17,20-lyase and steroid 18-hydroxylase. The most potent Al in the tetralone series was the 7-methoxy derivative 25, that showed high aromatase inhibiting potency (IC\textsubscript{50}=0.041 \textmu M) and selectivity.\textsuperscript{116} Indeed, the selectivity issue is extremely important in the Al class, due to the widespread presence in the organism of P450 systems, whose concomitant inhibition could cause serious side effects.

The tetraline series was developed by Hartmann et al. starting from the promising results obtained with 2-(4-pyridyl)methyl derivatives.\textsuperscript{117} The saturated methylene bridge of the latter compounds was constrained by fusing a cyclopropane ring to positions C\textsubscript{1} and C\textsubscript{2} of the tetraline nucleus.\textsuperscript{118} This modification introduced asymmetry in the molecules, and in some cases, the stereoisomers were isolated and tested. Stereospecificity was shown, particularly in the case of the 6-methoxy derivative 26, whose (+)- and (-)-enantiomers had IC\textsubscript{50} value 0.030 \textmu M and 10 \textmu M, respectively.\textsuperscript{118}
The tetralinic skeleton was further varied by changing it into a quinolinic one, in order to pursue the goal of simultaneously blocking both aromatase and another enzyme active in tumor tissues i.e. thromboxane A2 synthase (TxA2). The involvement of TxA2 in the proliferation of tumor cells, as well as the possibility to control the metastasis production by means of TxA2 inhibitors had been earlier demonstrated. The idea was to combine both the aromatase and TxA2 inhibiting abilities in one molecule, to meet the double goal of blocking the breast cancer tissue proliferation and preventing the metastases dissemination. The goal was reached with a number of tetrahydroquinoline derivatives, of which 27 was the most interesting one. This molecule is a potent inhibitor of both aromatase ($IC_{50}=0.50 \, \mu M$) and TxA2 ($IC_{50}=0.29 \, \mu M$), and it is selective with respect to both other steroidogenic P450 enzymes as well as enzymes involved in the arachidonic acid cascade.

In 1998, a French research team published the first article of a series, in which they presented and developed a new class of NSAIs inspired by some indanones previously reported by Hartmann et al. A lead compound was indicated in the 2-pyridylmethylindan-1-one derivative 28 that showed $IC_{50}=3.5 \, \mu M$, a potency comparable with that of the corresponding tetralone and indanone Als. (Z)-tetrahydroindolizinone 29 along with the 3-pyridyl analog was studied in a series of in vitro tests for the inhibition of aromatase both in placental microsomes and in cell culture, and for the cytotoxicity as well. The molecules displayed a submicromolar aromatase inhibiting potency in both tests, and no cytotoxicity. The class was further developed by changing the piperidinone ring into a pyrrolidinone one, and also by introducing different modifications in place of the pyridyl group and on the
phenyl attached in 3-position. Dihydropyrrolizinone derivatives were thus obtained, among which the most potent compound was 30 (IC$_{50}$=0.65 μM).\textsuperscript{125}

An important observation regarding the indolizinone and pyrrolizinone derivatives like 29 and 30 is that they are chiral compounds, but no attempt of resolution of the enantiomers was reported.

A novel dimer of 2-(4-pyridylmethyl)-1-indanone (31) displayed moderate inhibition of human placental aromatase and was found 3 times more potent as compared to aminoglutethimide. It has also been observed that treatment of indanone with various aldehydes having electron-withdrawing substituents at para-position facilitate the formation of dimers in the order pyridine-4-carboxaldehyde > 4-CN > 4-NO$_2$ > 3,4,5-(OCH$_3$)$_3$. The presence of electron-donating groups at the meta-position also facilitate dimer formation as in the case of the 3,4,5-trimethoxy derivative.\textsuperscript{126}

Bansal \textit{et al.} reported the synthesis and aromatase inhibitory activity of a new series of 2-benzylidene indanones.\textsuperscript{127} The imidazolyl-substituted indanones displayed potent aromatase inhibitory activity. The vanilloid-based
derivative 2-[4-(3-imidazol-1-ylpropoxy)-3-methoxybenzyldiene]-indan-1-one (32, IC$_{50}$=0.55 µM) exhibited maximum inhibition of human placental aromatase and was found to be 54 times more potent as compared to aminogluthethimide.

**Some more azolyl substituted derivatives**

The research work, which led the Janssen company to the discovery and development of vorozole (10), also produced a number of other potent NSAIs that did not reach the market. One of these compounds was liarozole (33), whose nanomolar level of aromatase inhibitory potency prompted some group to attempt structural modifications aimed at improving its biological profile. In any case, unresolved chiral compounds were obtained.

Le Borgne et al. synthesized a number of liarozole analogs based on an indole nucleus bearing the azolylbenzyl fragment in different positions of the aromatic bicycle. From the several variations introduced (N-substituent, azole ring, phenyl substituents), 34 (IC$_{50}$=0.10 µM)$_{128}$ and 35 (IC$_{50}$=0.04 µM)$_{129}$ are among the most effective compounds. Remarkably, in this series, the imidazole nucleus seems to perform better than the triazole one.

The [(benzofuran-2-yl)phenylmethyl]imidazoles (36) reported by Whomsley et al.$^{130}$ as potent NSAIs were further studied both to assess target
selectivity and enantioselectivity, and to determine the SAR of the class. It was found that removal of the phenyl ring favored the inhibitory potency against P450-17 while its replacement with small alkyl groups, together with the introduction of Br or Cl atoms on the phenyl moiety of the benzofuran ring gave rise to low enantioselectivity. On the other side, replacing the imidazole ring of 36 with a triazole led to a series of compounds, of which 37 was the most potent AI (IC$_{50}$=0.19 μM) even if weaker than the corresponding lead.

A good selectivity was also claimed for 38, although evaluated in in vitro cellular assays by testing the inhibition of the synthesis of other steroids (aldosterone, cortisol, testosterone). An article by Karjalainen et al. reported the results of a program of synthesis and screening of NSAIs carried out at the Orion Pharma. The project started from the observation of the aromatase inhibitory activity of some (α,α-diphenylmethyl)imidazoles resembling letrozole, and led to the development of many diarylalkyl-imidazoles and -triazoles that were tested against aromatase and for the inhibition of both desmolase (P450 11A1) and 7-ethoxycoumarin-O-deethylase (CYP2B1), in order to determine the selectivity. Several structural variations were explored, and the best results in terms of combined potency and selectivity were obtained with the diaryl-alkyl or -alkenylazoles 39 (IC$_{50}$=0.18 μM) and 40 (IC$_{50}$=0.19 μM). The selectivity profile against the other P450 enzymes was favorable, and even better than that of some reference drugs like letrozole and fadrozole. The stereoselectivity issue was partly taken into consideration. With regard to the diarylalkenyl derivatives, E and Z
isomers were identified and separately tested. It was found that the geometric isomerism affects mainly selectivity, not aromatase inhibitory activity.

Two series of para-substituted phenylalkylimidazoles were synthesized and tested for the aromatase inhibition, and all but one displayed greater potency than the aminogluthethimide. Compounds 41 (IC$_{50}$=0.16 $\mu$M) and 42 (IC$_{50}$=0.31 $\mu$M) were the most active ones, showing a substantial equivalence between the 2- or 3-carbon unit spacers. Remarkably, electron-withdrawing para-substituents on the phenyl ring increased the potency with respect to electron-donating ones.$^{135}$

In an article by Cozzi et al., the aromatase inhibiting activity of some 4-phenyl-1,4-dihydropyridine derivatives was reported, and again the molecules were designed with the help of geometrical considerations.$^{136}$ The authors took the distance between the azole lone-pair-carrying nitrogen atom and the cyano group of the most potent azole AIs as the criterion to select molecules able to selectively bind to the enzyme. Remarkably, 43 (IC$_{50}$=3.6 nM) showed quite a high potency without carrying the cyano group. The insertion of CN
functions as in compounds 44 (IC₅₀=1.1 nM) and 45 (IC₅₀=1.8 nM) increased the potency only slightly.

While the clinical efficacy of third-generation Als in the treatment of breast cancer has clearly been demonstrated, there is now abundant and strong evidence to suggest that the deprivation of estrogen levels in patients treated with Als can be increased if steroid sulfatase (STS) is inhibited at the same time. It catalyzes the hydrolysis of steroid sulfates, such as estrone 3-sulfate to estrone, which is the main source of estrogens in tumors.¹³⁷,¹³⁸

Woo et al. reported some compounds with dual aromatase-sulfatase inhibitory activity.¹³⁹ The best dual aromatase-sulfatase inhibitors are 46, (IC₅₀ (aromatase) = 0.82 nM; IC₅₀ (sulfatase) = 39 nM) and 47, (IC₅₀ (aromatase) = 0.77 nM; IC₅₀ (sulfatase) = 590 nM).

In another series of imidazolylmethylbenzophenones, compounds 48 and 49 displayed potent aromatase inhibition.¹⁴⁰

Flavanone derivatives
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. Several flavonoids demonstrate inhibitory activities of the aromatase enzyme, thus lowering estrogen biosynthesis and circulating estrogen levels. 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 (Figure 5) to the active site of aromatase was obtained by difference spectral absorption studies, 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 4-keto group of flavone was detrimental to aromatase inhibition by these compounds.\textsuperscript{151} 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.\textsuperscript{152} 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.\textsuperscript{153-155} Generally, flavones and flavanones have higher aromatase inhibitory activity than isoflavones (Figure 6). The flavone, chrysin, has an IC\textsubscript{50} value of 0.50 \textmuM; apigenin, flavone, flavanone, and quercetin were less efficacious inhibitors. Isoflavones are significantly less potent as aromatase inhibitors. The most effective isoflavone inhibitor is biochanin A with an IC\textsubscript{50} value of 113 \textmuM, approximately 20-fold less potent than chrysin in terms of IC\textsubscript{50} values. This large difference in potency is the likely reason why there has been little effort to develop aromatase inhibitors on an isoflavone scaffold. Introduction of the proper functional groups on the isoflavone core could result in the desired aromatase activity. In human placental microsomal

![Biochanin A and Synthetic pyidyl isoflavone analog](image-url)

*Figure 6. Isoflavones as aromatase inhibitors*

were less efficacious inhibitors. Isoflavones are significantly less potent as aromatase inhibitors. The most effective isoflavone inhibitor is biochanin A with an IC\textsubscript{50} value of 113 \textmuM, approximately 20-fold less potent than chrysin in terms of IC\textsubscript{50} values. This large difference in potency is the likely reason why there has been little effort to develop aromatase inhibitors on an isoflavone scaffold. Introduction of the proper functional groups on the isoflavone core could result in the desired aromatase activity. In human placental microsomal
assays, the synthetic pyridyl isoflavone analogue exhibited an IC\textsubscript{50} value of approximately 210 nM.\textsuperscript{156}

Le Bail \textit{et al.} determined the aromatase inhibitory potency of a number of flavonoids including flavones, flavanones, and chalcones. The latter are precursors of the flavonoids lacking the pyrane ring, and few of them were found to be appreciably potent AIs, in some cases in the micromolar range. The polyhydroxylated derivatives 50 (IC\textsubscript{50}=2.6 \mu M) and 51 (IC\textsubscript{50}=2.8 \mu M) showed the highest potency.\textsuperscript{157}

A series of (di)benzopyranone derivatives, in which the compounds were built by taking the chromone and the xanthone nucleus as molecular skeletons was recently reported.\textsuperscript{158} An azole ring linked to the (di)benzopyranone aromatic moiety by a methylene unit, and an H-bond accepting function located on the aromatic ring at a suitable distance from the azole nitrogen atom carrying the lone pair. The most interesting AIs of the series were 52 (IC\textsubscript{50}=0.043 \mu M) and 53 (IC\textsubscript{50}=0.040 \mu M), which showed good inhibitory potency and selectivity.

Interestingly, by inverting the positions of the azolylmethyl and H-bond accepting groups on the dibenzopyranone nucleus, the selectivity also
inverted, and a promising P450 inhibitor (54, P450 17 IC_{50} = 0.042 \mu M) was discovered.\textsuperscript{158}

**Steroidal aromatase inhibitors**

Initial Structure Activity Relationship (SAR) for steroidal inhibitors was determined by screening numerous available steroids in a human placental microsomal assay for their ability to inhibit aromatase. The SAR for steroidal aromatase inhibitors have become more refined in the past decade, and certain modifications of the steroidal skeleton can be made to keep its affinity for aromatase. Functionalization of different positions of the steroidal nucleus has not only helped to understand the SAR studies but has also given birth to some highly selective and potent aromatase inhibitors. Few of them are presently in the market and others are in various stages of clinical trials for the treatment of breast cancer.\textsuperscript{159}

According to Cole and Robinson\textsuperscript{160} efficient AIs have two domains which play an important role in binding of the steroid substrate with the enzyme, namely, an iron-coordinating domain and a hydrophobic domain. The structural requirements for the latter are considered to be a crucial factor, although chemical details of its specificity are unknown. The introduction into, or removal from, certain positions of the androstenedione (AD) molecule of definite substituents and/or additional double bonds affect the inhibitory activity as indicated by arrows in figure 7.

The steroid molecule must contain at least one keto group, specifically at position 17 to manifest aromatase inhibitory activity. All substituted androstanes sharply decrease their activity as aromatase inhibitor upon reduction of the 17-keto group to the hydroxy group and especially following its esterification.\textsuperscript{161} Evidently, in some cases the carbonyl function at position 3 is not strictly required as a structural element, some 3-deoxy analogues retain high inhibitory activity.\textsuperscript{161,162} Introduction of additional double bonds into the molecule of AD and its substituted analogues enhances their inhibitory activity. It is particularly true for 1,2- and 6,7-double bonds. It is assumed that the presence of a 1,2-double bond in a steroid molecule is responsible for the
irreversible type of inhibition and correspondingly, for the long-lasting effect of aromatase inhibition \textit{in vivo}.\textsuperscript{163}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{The functional groups affecting the inhibitory activity and their position in the androstenedione molecule.}
\end{figure}

Introduction of various substituents into various rings (positions 1, 4, 6, 7, 16, 17 and 19) as well as alkylation or arylation of 4-amino-, -mercapto and hydroxy-androstanes are the most common structural modifications. High affinity for the enzyme has been noted for steroid aromatase inhibitors with small substituents at C\textsubscript{1}, C\textsubscript{4} and C\textsubscript{19} and with bulky substituents at C\textsubscript{7}. Introduction of hydrophobic substituents is a general trend in the design of efficient aromatase inhibitors. It was determined that C\textsubscript{19} steroids with a trans ring juncture between A and B ring was important for good binding to the aromatase enzyme complex.\textsuperscript{164}

Thus, studies on structure activity relationships of steroidal AIs and their interactions with the active site of aromatase provide valuable information for the development of a new drugs with lower side effects for the treatment of breast cancer. On the basis of substitutions in different rings of steroid nucleus, steroidal aromatase inhibitors may be placed in various categories.

\textbf{A-ring derived substrate analogues}

\textit{C\textsubscript{1} and C\textsubscript{2}}-substituted derivatives

Substitution at C\textsubscript{1} position involved both small alkyl and large bulky groups.

\newpage
The bulkier groups such as 1α-substituted thioethers (55)(1α-phenylthio, benzylthio, aminothiophenyl and 4-diethylaminobenzyl derivatives) are poor competitive inhibitors of aromatase\textsuperscript{165} while 1-methylandrosta-1,4-diene-3,17-dione (56) with a smaller alkyl group is the most valuable and perhaps the only potent steroidal aromatase inhibitor of this series. It was synthesized in 1983\textsuperscript{166} and patented under the trade name atamestane in more than a dozen countries. Atamestane is an irreversible inhibitor of estrogen biosynthesis \textit{in vitro} and \textit{in vivo}.\textsuperscript{167} Atamestane has an apparent $K_i$ of 66 nM and has been shown to be an enzyme-activated irreversible inhibitor with a $K_{inact}$ of $1.8 \times 10^{-4}$ sec$^{-1}$.\textsuperscript{168} These observations led to a conclusion that “functionality in this region is expected to interfere with appropriate interaction of the substrate at the active site. Different modes of mechanism have been speculated on the irreversible nature of the 56; first being due to the radical attack of an activated iron-oxygen complex on the C$_1$ and C$_2$-double bond generating a reactive radical species, which in turn covalently binds to the heme, thereby deactivating the catalytic site. This method of deactivation of P450 enzymes has been demonstrated in hepatic microsome preparations and is referred to as prosthetic heme alkylation.\textsuperscript{169} Another possible mechanism could be the two steps catalytic process, i.e. generation of 19-aldehyde intermediate and the third step requires covalent binding to the 19-position followed by the abstraction of a hydrogen atom from the C$_1$-position, which is available in the natural substrate but not in atamestane (56). Demethyl analogue of 56 such as 1,4,6,androstatriene-3,17-dione; ATD; 57 also behaved as irreversible deactivator, $K_{inact} = 1.1 \times 10^{-3}$ sec$^{-1}$ and $t_{1/2}$ of 10.5 min, presumably by the
same mechanism. This way, insertion of double bond between C₁ and C₂ was proved to be important for imparting enzyme-activated irreversible inhibition to a steroid. 1β,2β-Methylene substituted androstenedione, 1-alkyl-androsta-1,4-diene- and 1-alkyl-1,4,6,-triene-3,17-dione derivatives, capable of lowering estradiol levels, have been patented by Schering AG, a research centered German based pharmaceutical company.

The indication of bulk intolerance at these positions was further reflected from the very poor activity of 17β-hydroxy-1α-(trimethylsilyl)- (58) and 17β-hydroxy-2α-[(trimethylsilyl)methyl]androstan-4-en-3-ones (59). However, the lack of activity is also attributed, in part, to the inherent low affinity of the 17β-hydroxy as compared to 17-keto androgens.

2α-Substituted androstenediones were tested for aromatase inhibitory activity in human placental microsomes and also for their ability to serve as a substrate for the enzyme. All of the steroids inhibited the enzyme in a competitive manner with the apparent $K_i$ ranging from 45 to 1150 nM. 2α-Halogeno (60-62) and 2α-alkyl (63 and 64) steroids were powerful to good inhibitors ($K_i = 45–171$ nM) of aromatase. Aromatization of some of the steroids with placental microsomes was analyzed by gas chromatography–mass spectrometry, indicating that the aromatization rate of the 62 was about
two-fold that of the natural substrate androstenedione. Kinetic analysis of the aromatization of androgens revealed that a good substrate was not essentially a good inhibitor for aromatase.\(^\text{175}\)

In another series of 2-substituted steroids, 2-hydroxyandrost-4-ene-3,17-dione and 2-hydroxyandrost-2,4-diene-3,17-dione were found inactive while 2α-mercaptoandrost-4-ene-3,17-dione is a potent suicidal inhibitor of aromatase.\(^\text{176}\)

\textbf{C}_3\text{-Substituted derivatives}

The another alteration at C\(_3\) has been the replacement of the C\(_3\)-keto group with a methylene group (65), resulting in a highly potent competitive inhibitor of aromatase with apparent \(K_i\) value of 4.7 nM.\(^\text{177}\)

\textbf{C}_4\text{-Substituted derivatives}

Of all substituted androstanes, particularly of those substituted at position 4, 4-hydroxyandrost-4-ene-3,17-dione (4-OHA, 6) is the most popular and well-studied. It was first described in 1977 as a competitive AI. Later, it was shown to represent a suicide irreversible AI. 4-OHA is an irreversible inhibitor of aromatase with an apparent \(K_i\) being 50 nM\(^\text{178}\) and also has been shown to rapidly inactivate aromatase in a time dependent manner with \(K_{\text{inact}}\) of 4.5 \(\times\) \(10^{-3}\) sec\(^{-1}\) and \(t_{1/2}\) of 2.1 min.\(^\text{179}\) This compound is known under the trade name 'formestane' and is used for the treatment of hormone-dependent breast cancer in the post-menopausal women.\(^\text{180,181}\)

The mechanism of the inhibitory effect of formestane on aromatase has not been completely elucidated. An inactivation mechanism, which involves the formation of a covalent bond between the enzyme and position 4 of the steroid substrate has been proposed by Covey \textit{et al.}\(^\text{182}\) A great number of
formestane analogues have been synthesised aimed at elucidating the mechanism of its action and obtaining more efficient aromatase inhibitors.

A prominent role among formestane analogues belongs to 4-alkylthio- and 4-arylthio androstenedione. Several compounds of this series were obtained in the 80’s. All of them appeared to be efficient competitive AI with \( K_i \) ranging from 36 to 73 nM. It was found that the chain length at \( C_4 \) of alkylthio analogues of such steroids should not exceed three carbon atoms in order to preserve a sufficiently high inhibitory activity.\(^{183}\)

4-Thio-substituted analogues \(^{66-68}\) were comparable in activity with formestane. The difluoromethyl analogue \(^{68}\) was found to be a reversible AI,

\[
(66) \text{R}=\text{CH}_2\text{F}, (67) \text{R}=\text{CH}_2\text{Cl}, (68) \text{R}=\text{CHF}_2
\]

whereas the fluoromethyl and chloromethyl analogues \(^{66} \text{ and } 67\) are suicide AI with \( K_i = 30 \) nM. \textit{In vivo} activity of compound \(^{66}\) is comparable with that of formestane. It is noteworthy that the presence of an additional 9(11)-double bond in these analogues, like in 19-sulfur-containing steroids, does not influence their affinity and inhibitory activity with respect to aromatase.\(^{184}\)

Replacing the hydroxyl group with an ester as in compound \(^{69}\) did not improve the inhibitory activity of this series of steroids.\(^{185-187}\) Removal of 4-hydroxy group also resulted in dramatic fall in activity. Further, 4-fluoro- \(^{70}\), 19,19-difluoro- \(^{71}\) and 19-nor-4-fluoroandrostenediones \(^{72}\) were prepared to improve the pharmacokinetic profile of 4-OHA \(^{6}\), but these compounds were less potent and found to be androgenic.\(^{188}\) Conjugation of 4-OHA nucleus to the 4,6-diene \(^{73}\), 1,4-diene \(^{74}\) and 1,4,6-triene \(^{75}\) derivatives preserved the activity.\(^{181}\)

Among this class, competitive inhibitors were obtained from the analogues \(^{76} \) and \(^{77}\) of \(^{6}\) without a 10-methyl group. The lack of irreversible
character of these compounds is thought to be due to the need of 10-methyl group to stimulate the substrate analogues for covalent binding to the apoprotein.

\[
\begin{align*}
(69) \quad R &= CH_3, \ X = OCOCH_3 \\
(70) \quad R &= CH_3, \ X = F \\
(71) \quad R &= CHF_2, \ X = OH \\
(72) \quad R &= H, \ X = F
\end{align*}
\]

Of the note, the 4\(\alpha\),5\(\alpha\)- (78) and 4\(\beta\),5\(\beta\)- (79) epoxides were fairly potent despite the disruption of the 3-keto-4-ene conjugation.\(^{161}\)

\[
\begin{align*}
(73) \quad X &= Y = CH_2, \ W = Z = CH, \ R = CH_3, \ R_1 = H \\
(74) \quad X &= Y = CH, \ W = Z = CH_2, \ R = R_1 = H \\
(75) \quad X &= Y = W = Z = CH, \ R = CH_3, \ R_1 = H \\
(76) \quad X &= Y = W = Z = CH_2, \ R = H, \ R_1 = COCH_3 \\
(77) \quad X &= Y = W = Z = CH_2, \ R = H, \ R_1 = H
\end{align*}
\]

Among other 4-substituted androstenedione, special mention should be made of 4-azido-androstenedione and 4-amino-androstenedione prepared from 4-mesyloxy-androstenedione. Highly active 4-amino androstenediones 80 and 81 present special interest. One of them, 4-aminoandrosta-1,4,6-triene-3,17-dione 81, appeared to be an efficient AI and was recommended for treatment of mammary tumours. This compound has passed successfully clinical trials under the trade name of `minametane`.\(^{189,190}\) Studies of SAR revealed the enhanced rate and selectivity of aromatase inhibition upon introduction of additional 1,2- and 6,7-double bonds into the molecule of
4-amino-AD. Steroids 80 and 81 are highly efficient and manifest in vivo antitumour activity both on subcutaneous and oral administration.191

3-Deoxy substrate analogues
Numazawa et al. reported a novel series of 3-deoxy derivatives of androstenedione.192 Androst-4-en-17-one (82), is an excellent competitive inhibitor of aromatase, although this is lacking a carbonyl group at C-3 that is thought to be essential for the proper binding of substrate androstenedione to the active site of the enzyme.192 On the other hand, androst-5-en-17-one (83), an isomer of 3-deoxy steroid 82, is a fair competitive inhibitor. The SAR indicated that a 17-carbonyl function is necessary for an effective binding of 3-deoxy steroids 82 and 83 to the active site and that binding geometries of the two steroids are different in the region of an A-B ring system of the steroid molecule. The binding pocket also tolerates a polar hydroxy group at the 6- or 19-position of 4-ene steroid 82 and at the 4β-position of 5-ene steroid 83. The 4-ene system plays a critical role in the tight binding of a steroid without a carbonyl function at C3 to the active site. In a continuous study of the 3-deoxy steroids as conformational and catalytic probes for the active site of aromatase, 5-ene-4β,19-diol 84 (Ki = 3.4 nM) emerged as a very powerful competitive inhibitor, indicating that a hydrophilic interaction between the
active site and the diol structure plays an important role in the tight binding.\textsuperscript{192}

3-Deoxy steroidal olefin 85 and its epoxide derivative 86 proved to be strong competitive aromatase inhibitors with $K_i = 50$ and 38 nM and $IC_{50} = 225$ and 145 nM, respectively).\textsuperscript{193}

\begin{center}
\includegraphics[width=0.3\textwidth]{85.png}
\includegraphics[width=0.3\textwidth]{86.png}
\end{center}

According to new findings, the C-3 carbonyl group is not essential for anti-aromatase activity, but 5α-stereochemistry and some planarity in the steroidal framework is required.

Two series of 3-deoxy androgens, androst-5-en-17-ones with a non-polar alkoxy, alkyl or phenylalkyl group at C-4β and 4-acyloxyandrost-4-en-17-ones were synthesized and evaluated by Nagaoka et al.\textsuperscript{194} 4-Benzoyloxy (87) and 4-acetoxy (88) steroids are among the powerful inhibitors of aromatase (\(K_i = 70\) and 60 nM, respectively). Elongation of an acetoxy group in a series of 4-acyloxy steroids or a methyl group in a series of 4β-alkyl steroids decreased affinity for aromatase principally in relation to carbon number of the acyl or alkyl function.\textsuperscript{194}

**B-ring derived substrate analogues**

*C₆*-substituted derivatives

6-Methyleneandrosta-1,4-diene-3,17-dione (11) synthesized in the late 80's as
an anticancer drug known under the trade name 'exemestane' is the most well known steroidal aromatase inhibitor. Exemestane is shown to be 2.5 times more active than formestane. Exemestane is an orally active and a highly selective irreversible AI. The irreversibility of its inhibitory action is assigned to the presence of a 1,2-double bond in the molecule; the corresponding 1,2-hydrogenated analogues represent reversible AI.

None of the synthetic exemestane analogues displays \textit{in vitro} activity which would be equal or greater than that of exemestane itself. Thus the 17β-hydroxy derivative is active \textit{in vitro}, but its activity is 2.6 fold less than exemestane.

According to their inhibitory activity, exemestane analogues modified at C₆ and having a 17-keto group can be arranged in the order 6-methylene (11) > 6β-hydroxymethyl (90) > 6-hydroxy-6-hydroxymethyl (89) > 6β-carboxy derivatives (91).

17β-Hydroxy analogues 92 and 93 manifest 3-8-fold lower activity than the corresponding 17-oxo compounds.

Isomerisation or reduction of the double bond leading to compounds 94
and 95 decreases their inhibitory activity threefold in comparison with exemestane itself.\textsuperscript{200}

A number of 6-oxygenated derivatives such as 6-oxo (96), \(\beta\)-hydroxy (97), \(\beta\)-hydroperoxy (98) and \(\alpha\)-hydroperoxy (99) have shown a decreasing order of activity against aromatase enzyme.\textsuperscript{201} Further investigation on 6-hydroperoxy derivatives 98 and 99, reported that the time dependent-inactivation of these compounds did not require the presence of NADPH. Therefore, the irreversibility is ascribed to the inherent reactivity of the substrate analogues and did not depend upon enzyme activity. The probable explanation is that the hydroperoxy groups might be reacting with neighboring cysteine residues in the aromatase active site.\textsuperscript{202}

Numazawa \textit{et al.} have combined 6-keto functionality with substitution at either C\textsubscript{2} or C\textsubscript{7} positions. 7-Acetoxyandrostan-4-ene-3,6,17-trione exhibited poor activity while the 2,2-dimethylandrostan-4-ene-3,6,17-trione was a good competitive aromatase inhibitor.\textsuperscript{202}

Various 6-halogenated substrate analogues have also been prepared and screened for aromatase inhibitory activity. Of the interest, 6\(\alpha\)- and 6\(\beta\)-
bromoandrostenedione have exhibited different behavior of inactivation, 6α-isomer being the competitive inhibitor with respect to androstenedione and 6β-isomer as an enzyme-activated irreversible inhibitor.\textsuperscript{203,204} Other 6-fluorinated derivatives, such as 6β-fluoro- and 2β,6β-difluoroandrost-4-ene-3,17-dione, were also found active against aromatase.\textsuperscript{205} Other active substitutions include 6-azido, thiocyanato, and propargyloxy functionalities.

A new series of 6α- and 6β-alkyl-(C\textsubscript{1} to C\textsubscript{6}, isopropyl, vinyl, ethynyl) and aryl-(phenyl and benzyl)-androstenediones (100) was also synthesized by Numazawa et al.\textsuperscript{206} to act as probes of the aromatase active site. The effects of length and configuration of 6-alkyl group has been extensively explored. The SAR between various substituents at C\textsubscript{6}, their inhibitory experiments and conformational analyses have revealed new information regarding the accessible volume of the binding pocket of aromatase. All of the inhibitors synthesized inhibited human placental aromatase in a competitive manner, with apparent \( K_i \) ranging from 4.7 to 54 nM. The inhibitory activities of all the 6-\( n \)-alkylated steroids as well as the 6β-vinyl and 6α-benzyl compounds were very powerful whereas those of the 6-isopropyl, 6-phenyl, 6β-benzyl, and 6β-ethynyl steroids, having a bulky or polar substituents, were relatively weak. The 6β-alkyl steroids essentially had higher affinity for the enzyme than the corresponding 6α-isomers. Same workers have further synthesized C\textsubscript{6} ether and ester substituted androstenedione analogues as an expansion to their studies.\textsuperscript{207}

Another novel modification involves the synthesis of androgens with either a 6α,7α- or 6β,7β-aziridino group (101). This modification proved to cause a large decrease in the ability of the steroid to bind or to inhibit aromatase.\textsuperscript{208}
6-Hydroxyimino-androstenedione 102 and its 17β-hydroxy analogue 103 show a high affinity for human placental aromatase and act as competitive inhibitors of this enzyme.\textsuperscript{209}

\begin{center}
\begin{tikzpicture}
\node (a) at (0,0) {\includegraphics[width=0.4\textwidth]{102}};
\node (b) at (0.5,0) {\includegraphics[width=0.4\textwidth]{103}};
\end{tikzpicture}
\end{center}

\textit{C}_7\text{-substituted derivatives}

Extensive structural modifications at C\textsubscript{7} position, especially with bulkier groups, have afforded numerous potent aromatase inhibitors. Remarkable SAR has been developed by the eminent work of Brueggemeier and coworkers\textsuperscript{210-215} at C\textsubscript{7} position of androgens. A series of C\textsubscript{7}-thioalkyl, -thioaralkyl and -thioaryl androstenediones proved to be a successful probe of the active site and resulted in the synthesis of one of the most potent competitive inhibitors of aromatase. 7α-(4-Aminophenylthio)androst-4-ene-3,17-dione (7α-APTA; 104) has an apparent $K_i$ of 18 nM.\textsuperscript{210} The synthesis of an unsaturated derivative of 104 resulted in conversion of a reversible competitive inhibitor into an enzyme-activated irreversible inhibitor. 7α-(4'-Aminophenyl)thio-1,4-androstadiene-3,17-dione (7α-APTADD; 106) has an apparent $K_i$ value of 10 nM and $K_{\text{inact}}$ of $8.4 \times 10^{-3}$ sec$^{-1}$.\textsuperscript{211-213} The replacement of amino group of 104 with a bromoacetamido group (105), showed an apparent $K_i$ of 93 nM, and the time dependent inactivation did

\begin{center}
\begin{tikzpicture}
\node (a) at (0,0) {\includegraphics[width=0.4\textwidth]{104}};
\node (b) at (0.5,0) {\includegraphics[width=0.4\textwidth]{105}};
\end{tikzpicture}
\end{center}
not require the co-factors. Any other change in the electrons around the aryl ring of 7-APTA found to have no significant effect on the activity profile.

Investigations have also been focused on the synthesis of $7\alpha$-alkyltestosterone derivatives, which were prepared by the conjugate addition of the appropriate Grignard or lithium dialkyl copper reagent to $17\alpha$-hydroxyandrost-4,6-diene-3-one propionate followed by the hydrolysis of the ester. The enzyme was found to tolerate at least the bulk of a hydroxypropyl group at the $7\alpha$-position. It was found that the affinity for the enzyme decreased in the order $7\alpha$-ethyl, -propyl and -butyl substituents. Again, these workers found that the 17-keto compounds were more potent than the corresponding alcohols as aromatase inhibitors.

Efforts were also made to prepare another series of 7-substituted analogues with additional double bond between $C_6-C_7$ positions. 7-Benzylandrosta-4,6-diene-3,17-dione (107) was the best inhibitor of the series with an apparent $K_i$ of 61 nM. The importance of configuration of $C_7$-substituent and the contribution of thioether linkage to impart potency to aromatase inhibitors has been worked up to a great extent by O’Reilly et al. Of the series of $7\alpha$- and $7\beta$-arylaliphatic substituted androstenediones, $7\alpha$-phenethylandrostan-4-ene-3,17-dione (108) was the most potent inhibitor with an apparent $K_i$ of 13 nM, while its $7\beta$-isomer had an apparent $K_i$ of 41 nM. These observations concluded that $7\alpha$-configuration results in maximal binding to the active site and the thioether linkage is also not important for activity since compound 108 is equipotent to $7\alpha$-APTA.
Numazawa et al reported a series of 6α,7α-cyclopropa [6,7]-androst-4-en-17-ones. These compounds proved to be efficient competitive Al. cyclo-propane derivative 109 ($K_i = 5 \text{ nM}$) being the most active one. However, introduction of a 6α,7α-difluorocyclopropane group into the androstenedione molecule as in compound 110 did not affect considerably its affinity for the enzyme.$^{217}$

**D-ring derived substrate analogues**

*C$_{16}$ and C$_{17}$-substituted derivatives*

Very few 16-substituted steroids are reported in the literature as steroidal aromatase inhibitors. 16α-Hydroxytestosterone (111) not only inhibits the aromatization of androstenedione but has also found to act as a substrate for aromatase to be metabolized to estriol. It was observed that at very high concentrations of 111 and a subsaturating concentration of androstenedione, the compound 111 appeared to compete non-competitively with androstenedione the enzyme. The presence of two aromatase P450 enzymes in human placenta was speculated for such behavior. Another rationale may be that compound 111 occupies a secondary binding site at the active site as well as being capable of inhabiting the substrate binding site at the active site.$^{218}$ Further, studies have proved that the 19-hydroxy and 19-oxo
derivatives of 111 are involved in the biosynthetic pathway and that the conversion involves the same enzyme and enzymatic process.\textsuperscript{219} 16\(\alpha\)-Bromoandrostenedione (112) and 16-bromo-6-keto-androstenedione were tested by Bellino et al.\textsuperscript{220} for their ability to inactivate microsomal aromatase from term human placenta. Numazawa et al.\textsuperscript{221} have also reported 3-deoxy-16(\(\alpha/\beta\))-bromoandrostand-4-ene-17-one compounds as competitive inhibitors.

Early work on the inhibition of aromatase and its affinity labeling led to the synthesis of 17\(\beta\)-bromoacetylamino-4-androsten-3-one (113). This derivative along with two other potential affinity labels, the 16\(\alpha\)-bromoacetoxy-4-androst-3,17-dione and 16\(\alpha\)-bromoacetoxy-4-androsten-3,16,17-trione were reported to act as competitive inhibitors although the activity was weaker than the third compound, 17\(\beta\)-bromoacetoxy-4-androsten-3-one (114).\textsuperscript{221}

\begin{align*}
(113) & \quad R=\text{NHCOCH}_2\text{Br}, \quad R_1=\text{H} \\
(114) & \quad R=\text{COCH}_2\text{Br}, \quad R_1=\text{H} \\
(115) & \quad R=\text{OOH} \\
(116) & \quad R=\text{H}
\end{align*}

The inhibition of human placental aromatase has also been studied with in a series of 17-ethynyl-substituted 10-hydroperoxy and related 19-nor steroids 115 and 116.\textsuperscript{222,223}

\textit{D-ring modified steroids}

The potential for the D-modified steroids to act as probes of the aromatase active site is yet to be fully exploited because this category has received hardly any attention. Ring-D lactones related to testosterone proved to be effective aromatase inhibitors\textsuperscript{224} and testolactone (117) was one of the first steroid used in the clinical treatment of breast cancer, although it has been withdrawn before its activity as an aromatase inhibitor had been established. It has been described as a competitive inhibitor of
It is a suicide inhibitor, which inactivates aromatase in a NADPH-dependent process. These findings are again in agreement with the suggestion that double bond between C1 and C2 endeavor to impart the irreversible inhibitory activity to the steroid nucleus either due to the activation of Δ^1-double bond or because of lack of an extractable 1β-hydrogen.

Another interesting compound 118 has been synthesized by combining the important structural features of formestane (6) and testolactone to act as potent aromatase inhibitor. In another approach, B-ring functionalized 2-oxa steroids were modified at D-ring to afford potential aromatase inhibitors,

![Chemical Structures](image)

...potential aromatase inhibitors,

![Chemical Structures](image)

Recently, a new series of D-ring modified androgens has been reported as aromatase inhibitors, in which several 17α-substituted-17β-hydroxy-16-oximino derivatives of 5-androstene and the corresponding D-seco derivatives were explored. The 4-ene-3-keto, 1,4-diene and 1,4,6-triene analogs in D-seco series were also prepared to further investigate the effect of high degree of unsaturation in the A/B ring. Compound 120 expressed the highest inhibition in the denucleated rat ovarian fraction in comparison to other androstene derivatives (IC_{50} = 0.42 μM). The inhibition was competitive with K_i value of 55.
0.27 µM. Surprisingly, introduction of additional units of unsaturation in D-seco derivatives did not increase the anti-aromatase activity.229

**Miscellaneous**

*C_{10} and C_{19}*-substituted derivatives

19-Substituted androstanes are the numerous and diverse aromatase inhibitors.230,231 It is quite natural because many investigators consider the angular 19-methyl group to be the site of the primary enzyme attack leading to aromatisation. Formally, the functionalisation of the 19-methyl group of the steroid molecule is a very difficult task. This class of steroids are synthesised mostly from compounds having a 5(10) double bond or from 19-hydroxy(oxo)androstanes using diverse methods of synthetic organic chemistry for their further transformations.

10β-Propynyl-steroids (19-ethynylandrostanes) synthesised in the 80’s simultaneously and independently in several laboratories,230,231 pertain to irreversible suicide inhibitors.

The steroid 121 and its derivatives have been extensively assayed as AI. It was found that 19-ethynyl-AD 121 and its 19-hydroxy-(122,123) and 19-oxo-

derivatives (124) are irreversible suicide inhibitors of aromatase and manifest high specificity.230
The steroid 121 possesses high activity on oral administration with respect to the inhibition of ovarian aromatase. The allenic derivative of androstenedione 125 also pertains to irreversible Al.\textsuperscript{232}

19-substituted AD 126-128 competitively inhibits aromatase. Of special interest is the 19-methylthio derivative 128, which is the first highly efficient Al ($K_i = 1$ nM); its inhibitory effect is determined not only by its interaction with the steroid binding site of aromatase but also by coordination of the steroid sulfur atom with the heme iron of cytochrome P-450.\textsuperscript{233}

\[ \text{H}_2\text{C}≡\text{C} \quad \text{O} \quad \text{O} \]

(125)

\[ \text{R} \]

(126) $R=\text{CN}$, (127) $R=\text{N}_3$, (128) $R=\text{SMe}$

The attention of several research groups was focussed on 10$\beta$- and 19-sulfur-containing AD derivatives as aromatase inhibitors. Compounds with the sulfur atom linked directly with C$_{10}$ or separated from the C$_{10}$ atom by one or several methylene groups have been synthesised and assayed. Thus the introduction of the SH group at position 10 of androst-4-ene-3,17-dione resulted in an efficient suicide Al 129. In vitro studies of the biological activity of the thiol 129 revealed that the free thiol group is required for the binding to

\[ \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \]

(129)

\[ \text{SH} \]

(130)

the enzyme, because the corresponding acetyltio derivative was inactive.\textsuperscript{230} Steroid 130 represents an irreversible Al. The inhibition constant is 34 nM, its activity is higher than formestane both in vitro and in vivo. However, the thiol 130 has a limited use because of its insufficient pharmacological stability due
to the presence of a free thiol group. To overcome this limitation, its ethylthio derivative, 19-ethyl-thio-androstenedione, has been synthesized.\textsuperscript{234}

The results of \textit{in vitro} and \textit{in vivo} studies of activities of the majority of compounds synthesised by Lesuisse \textit{et al.}\textsuperscript{235} suggest that the steroids 131-133 with one- or two-carbon chains having a terminal thioether function at position 10 are the most efficient AI. The effect of the substituent at the sulfur atom in various

\begin{align*}
(131) \quad R=\text{Me} \\
(132) \quad R=\text{Et} \\
(133)
\end{align*}

androstenedione analogues on the inhibition of aromatase has also been studied. Sulfur-containing steroids with methyl, vinyl or ethynyl substituents at the sulfur atom possessed the highest affinity for the enzyme. Substitution of the ethyl group (132) for the methyl group (131) decreases the activity of the steroid substrate 72-fold. Compounds with hydroxyethyl and cyclopropyl groups are completely devoid of activity, while compounds with allyl, phenyl and methylthiomethyl groups possess certain activity. Compound 131, which is the most active in this series, decreases the estradiol level and inhibits the growth of endocrine tumours \textit{in vivo} when used at a dose of 0.4 mg / kg.\textsuperscript{235,236}

The presence or the absence of the 9(11)-double bond does not practically affect their inhibitory activity. Compound 131 was the most reactive among other compounds assayed and was considered \textsuperscript{237} to be one of the best AI. On the contrary, the steroids with the sulfur atom attached directly to the steroid skeleton (134) or separated from it by three carbon atoms as in compound 135 have practically no affinity for the enzyme. The key property of the efficient AI of this series is the ability of the sulfur atom to bind with the heme iron of the porphyrin moiety of the enzyme (compounds 128, 131 and 133), whereas compound 134 reacts only with the lipophilic steroid-binding centre of the enzyme. This behaviour is consistent with the hypothesis that the incorporation of one methylene group between the sulfur atom and position 10 of the steroid skeleton alters the character
of the interaction of the steroid substrate with the enzyme. The fact is that Al with a two-carbon chain at $C_{10}$ react most efficiently with the heme Fe(III). The 19-hydroxy- and 19-oxo-androgens are considered to be intermediates in the estrogen biosynthesis. Various modifications at $C_{10}$ and $C_{19}$ were designed to explore the different aspects of mechanistic pathway of enzyme inhibition, such as regioselectivity of aromatase, steric constraints and substrate conformation, and to be acquaintance about the active sites of the enzyme. These efforts led to the synthesis of a variety of compounds and many more derivatives. The appropriate homologues extended by a methylene group have been synthesized and evaluated both as possible substrates and inhibitors of aromatase. It was found that steroidal derivative 136 acted as a competitive inhibitor ($K_i = 81 \text{ nM}$) with affinity comparable with that of androstenedione.

Further, heterocyclic rings were experimented at $C_{10}$ with a vision of being potential site for irreversible binding to the enzyme. 10-Oxiranyl- (137) and 10-thiiranyl- (138) estr-4-ene-3,17-diones were shown to be competitive, reversible inhibitors with stereoselectivity. The 19R-isomers were more potent, the oxiranyl species was 36-times more potent than its enantiomer and the thiranyl was 80-fold. The ultraviolet spectral shifts induced in the
aromatase enzyme preparation by the 19R-isomers of both systems suggest that they both interact not only with the substrate binding site but also with the heme-iron complex. The interaction with the heme-iron complex was considered to be via direct heteroatom lone pair-iron bonding. The (19R)-10β-aziridinylestr-4-ene-3,17-dione (139) was also potent aromatase inhibitor with apparent \( K_i \) of 3.4 nM. Increasing the distance of heterocyclic rings and the steroid, by inserting methyl group in between these two groups, resulted in decrease in activity.

A-ring bridged steroids constitute another interesting series of this class, which was developed with an ethano bridge between the axial 19 and 2-positions. These 2,19-bridged steroids, incorporated with a pyran (140) and thiopyran (141) ring, afforded competitive inhibitors while the compound with piperidine ring (142) appears to be non-competitive with the natural substrate. The X-ray analysis reveals that bridge alters considerably the conformation of the steroid A-ring and the 19-carbon leaned towards the centre of the A-ring as compared to the androstenedione. The most potent of this series is with the entire carbon six-membered ring analog 143, which appears to be a tight binding inhibitor with an apparent \( K_i \) of 2.2 nM.

Azolyl substituted derivatives

Nitrogen containing steroids represent an important class of steroids, including natural products, semi-synthetic compounds and synthetic compounds, all of which have been studied intensively. Most modifications are aimed at the A- and D-rings by incorporation of amino groups to the...
Steroids backbone. Nitrogen containing steroids have the ability to regulate a variety of biological processes and thus are potential drug candidates for the treatment of breast cancer.\textsuperscript{243}

![Chemical Structures](144.png) ![Chemical Structures](145.png)

Steroidal epoxy and/or \textit{N}-oxy-17-picolyl and 17-picolinylidene-androst-5-ene derivatives have been prepared and tested on activity against the enzyme aromatase by Djurendic \textit{et al.}\textsuperscript{244} 17-Picolyl derivatives\textsuperscript{144} and\textsuperscript{145} at 50 \(\mu\)M exhibited inhibitory effects 51.5\% and 67.9\%, respectively, against control. Recently, Androst-3,17-dione-4-eno[4,5,6,b,c]pyrole (146) and androst-3,17-dione-4 eno[6,5,4-c,c] isoxazole (147) were found it to be effective inhibitors against aromatase.\textsuperscript{245,246}

![Chemical Structures](146.png) ![Chemical Structures](147.png)

A new series of novel pyrazole and isoxazole derivatives has been synthesized as potent aromatase inhibitors by Yadav \textit{et al.} Compound 148 (IC\textsubscript{50}=0.5 \(\mu\)M) having pyrazole ring at 2,3 position displayed highest aromatase inhibitory activity.\textsuperscript{247}
Pyridine- and other heterocyclic ring-containing derivatives of 2- and 4 amino-estrone, estrone, and estradiol have been recently reported by Numazava et al. Compounds 149 and 150 were fairly potent competitive inhibitors of aromatase with $K_i = 2.1\pm0.14$ and $1.53\pm0.08 \ \mu M$, respectively. However the other compounds did not show, to a significant extent, the aromatase inhibitory activity.  

Literature survey indicates that structural modifications in A, B and D ring of steroid nucleus brings out noticeable changes in aromatase inhibitory potential of steroidal derivatives and provides potent, easily obtainable and structurally simple aromatase inhibitors. In view of this, it was of substantial interest for the present investigator to further structurally modify the steroidal skeleton to synthesize newer potent aromatase inhibitors. The succeeding RESUMÉ AND DISCUSSION section summarizes the synthetic work and pharmacological studies that have been accomplished, followed by the EXPERIMENTAL WORK details.