INTERRELATIONSHIPS BETWEEN AROMATASE AND CYCLOOXYGENASE-2
AND THEIR ROLE IN THE AUTOCRINE AND PARACRINE
MECHANISMS IN BREAST CANCER

DISSERTATION

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By

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****

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ABSTRACT

Breast cancer is the most common cancer among women, and ranks second among cancer deaths in women. Approximately 60% of all breast cancer patients have hormone-dependent breast cancer, which contains estrogen receptors and requires estrogen for tumor growth. Estradiol is biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase. Previous studies suggest a strong association between aromatase (CYP19) gene expression and the expression of cyclooxygenase (COX) genes. Our hypothesis is that higher levels of COX-2 expression result in higher levels of prostaglandin E2 (PGE₂), which in turn increases CYP19 expression through increases in intracellular cyclic AMP levels and activation of promoter II. This biochemical mechanism may explain the beneficial effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on breast cancer. The effects of NSAIDs (ibuprofen, piroxicam, and indomethacin), a COX-1 selective inhibitor (SC-560), and COX-2 selective inhibitors (celecoxib, niflumic acid, nimesulide, NS-398, and SC-58125) on aromatase activity and expression were studied. To determine if aromatase activity is decreased by COX inhibitors, SK-BR-3 cells were treated for 24 hours with the different concentrations of the inhibitors. The data from these experiments revealed dose-dependent decreases in aromatase activity following treatment with all agents. IC₅₀ values ranged from 1 µM to 977 µM for the inhibitors. To
measure changes in aromatase gene expression, cell cultures were treated with the inhibitors at concentrations near the IC_{50} values. Real time PCR analysis of aromatase gene expression showed a significant decrease in mRNA levels when compared to control (vehicle) for all agents. These results were consistent with enzyme activity data, suggesting that the effect of COX inhibitors on aromatase starts at the transcriptional level. To investigate which specific promoter regions are involved in this molecular mechanism, we performed exon-specific real time PCR. The results from these experiments suggest that exon I.4 and promoter II are involved in this process. Thus, PGE_2 produced via COX may act locally in an autocrine fashion to increase the biosynthesis of estrogen by aromatase in hormone-dependent breast cancer development.

The IC_{50} value for NS-398 was found to be significantly lower when compared to the rest of the COX inhibitors studied. Although the suppression of aromatase activity of NS-398 is thought to involve PGE_2-dependent pathways, it is unclear whether COX-2 inhibition is the only requirement for aromatase suppression. To test this hypothesis we synthesized NS-398 analogs lacking COX-2 activity and their effect was studied on aromatase activity and expression. Our results showed suppression in aromatase activity and decreases in mRNA levels when compared to control similar to those found in NS-398. This separation of activities may provide a molecular basis for the development of NS-398 analogs as selective aromatase modulators.

Peroxisome proliferator-activated receptor (PPAR)-γ is a nuclear receptor that has an essential role in adipogenesis and fat metabolism and is now believed to have a role in tumorigenesis. Studies have shown that while aromatase and COX-2 are induced and PPARγ is inactivated in human breast cancer. To provide information on the
association between aromatase and PPARγ in human breast cancer, this study examines the effect of PPARγ ligands on aromatase and COX-2 activity and expression in normal and breast cancer cells. To examine the effect of PPARγ ligands (ciglitazone, rosiglitazone and troglitazone) in the presence of 9-cis retinoic acid (RA) and PPARγ antagonists (badge and GW9662) on aromatase activity, SK-BR-3 cells were treated for 24 hours with the different compounds. The data from these experiments revealed a decrease in aromatase activity when treating cell cultures with the combination of PPARγ ligands and 9-cis-retinoic acid. Interestingly, PPARγ antagonists also resulted in a decrease in aromatase activity. Real time PCR analysis of aromatase gene expression showed that changes in mRNA expression were associated with comparable changes in aromatase activity data. These results provide information on the association between PPARγ and the aromatase enzyme in breast cancer.
Dedicated to my loving parents, Carmen L. Cruz de Diaz and Santiago Diaz Rosa, for their support, blessings and unconditional love.
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# LIST OF ABBREVIATIONS

4-OHT – 4-hydroxytamoxifen  
7α-APTADD - 7 alpha-(4'-Amino)phenylthio-1,4-androstadiene-3,17-dione  
AG - aminoglutethimide  
ATCC – American Type Culture Collection  
Bt2 cAMP – dibutyryl-cyclic adenosine monophosphate  
BADGE – Biphenol A diglycidyl ether  
cAMP – cyclic adenosine monophosphate  
cDNA – complementary deoxyribonucleic acid  
COX - cyclooxygenase  
DEX – dexamethasone  
DMSO – dimethyl sulfoxide  
dNTP - deoxynucleoside triphosphate  
DNA – deoxyribonucleic acid  
DTT – dithiothreitol  
E2 - estradiol  
ED50 – 50% effective dose  
EGFR – epidermal growth factor receptor  
DBD – DNA binding domain  
DMSO – dimethyl sulfoxide  
DTT – dithiothreitol  
ER – estrogen receptor  
FBS – fetal bovine serum  
Forsk or F – forskolin  
GEN - genistein  
GR – glucocorticoid receptor  
IC50 – 50% inhibitory concentration  
LBD – ligand binding domain  
MAPK – mitogen activated protein kinase  
mRNA – messenger ribonucleic acid  
NADPH - nicotinamide adenine dinucleotide phosphate  
NF-κB – nuclear factor-kappaB  
NSAID – nonsteroidal anti-inflammatory drug  
OSUCCC – The Ohio State University Comprehensive Cancer Center  
PBS – phosphate-buffered saline  
PCR – polymerase chain reaction  
PGE2 – prostaglandin E2
pII – promoter II
PKA – protein kinase A
PKC – protein kinase C
PLC – phospholipase C
PPAR – peroxisome proliferator activated receptor
RA – retinoic acid
RAL - raloxifene
RBA – relative binding affinity
RNA – ribonucleic acid
rRNA – ribosomal RNA
RT-PCR – reverse transcriptase polymerase chain reaction
RXR – retinoic X receptor
SERM – selective estrogen receptor modulator
TAM – tamoxifen
TBP – TATA-box binding protein
TGF – tumor growth factor
TNFα - tumor necrosis factor α
TPA or T - 12-O-tetradecanoylphorbol-13-acetate
CHAPTER 1

THE ROLE OF AROMATASE AND CYCLOOXYGENASES IN THE
INITIATION AND DEVELOPMENT OF BREAST CANCER

1.1. Breast Cancer: Overview and Statistics

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. Adenocarcinoma of the breast is the most common cancer in women in the United States and ranks second only to lung cancer as a cause of cancer-related mortality. A total of approximately 1,372,910 new cancer cases are expected to be diagnosed in the year 2005. An estimated 211,240 new cases of invasive breast cancer, in addition to 58,490 new cases of \textit{in situ} breast cancer are expected to occur among women, and an estimated 40,870 deaths are anticipated from breast cancer in the United States during 2005 (1).

The ductal structure of the breast consists of a continuous layer of epithelial cells that are responsible for milk synthesis and release into the lumen (Figure 1.1). A second layer of myoepithelial cells contacts the basement membrane. These two cell layers, together with the fibroblasts surrounding them, form the basis of the ducts. Epithelial cells seem to be the main site of estradiol action in the breast. Histologically, it is the luminal epithelial cells that are responsible for most breast tumors (2). Although the
pathway to breast cancer development is not clear, some evidence indicates that it might begin with hyperproliferation of the epithelial cells, progressing to ductal carcinoma \textit{in situ}, and ending with invasive breast cancer.

Figure 1.1: Anatomy of the human mammary gland. Each mammary gland contains 15-20 lobes, each lobe containing a series of branched ducts that drain into the nipple. The ducts consist of a layer of epithelial cells, and are surrounded by fibroblasts from the stroma. Adapted from Ali and Coombes (3).

Breast cancer treatment may involve lumpectomy (local removal of the tumor), mastectomy (surgical removal of the breast), removal of the axillary (underarm) lymph nodes, radiation therapy, chemotherapy, and/or hormonal therapy (tamoxifen; aromatase inhibitors).
1.2. Aromatase

1.2.1 Aromatase and Its Gene

Approximately 60% of all breast cancer patients have hormone-dependent breast cancer, which contains estrogen receptors and requires estrogen for tumor growth. Estrogens are biosynthesized from androgens by the aromatase enzyme, the product of the \textit{CYP19} gene and a member of the cytochrome P450 enzyme superfamily (4). The enzyme complex is bound to the endoplasmic reticulum of the cell and is composed of two major proteins (4, 5). One is the hemoprotein cytochrome P450\textsubscript{arom} that converts C\textsubscript{19} steroids (androgens) into C\textsubscript{18} steroids (estrogens) containing a phenolic A ring (4, 6). The second protein is NADPH-cytochrome P450 reductase, which transfers reducing equivalents to cytochrome P450\textsubscript{arom}. Three moles of NADPH and three moles of oxygen are utilized in the conversion of one mole of substrate into one mole of estrogen product. Aromatization of androstenedione, the preferred substrate, proceeds via three successive oxidation steps, with the first two being hydroxylations of the angular C-19 methyl group. The final oxidation step proceeds with the aromatization of the A ring of the steroid and loss of the C-19 carbon atom as formic acid (Figure 1.2).

The aromatase gene, designated \textit{CYP19}, encodes the cytochrome P450\textsubscript{arom}, and this gene is located on chromosome 15q21.2 (4, 7). The aromatase gene consists of 10 exons, and its full length cDNA of 3.4 kilobases encodes for a protein of 503 amino acids. The coding region spans 9 exons and is approximately 30 kilobases in size, and the regulatory region is about 93 kilobases (5, 8). The aromatase protein is glycosylated with a molecular weight of approximately 58,000 daltons (9). The heme-binding region
is located on the last coding exon (X), and the transcription initiation site is located in exon II. Upstream of exon II are located a number of untranslated exons which are spliced into the 5’ ends of transcripts in a tissue-specific fashion.

Figure 1.2: Aromatase enzyme reaction. Aromatase catalyzes a sequence of two carbon hydroxylations followed by an oxidative carbon-carbon bond cleavage. Three molecules of oxygen and three of NADPH are required for this catalytic sequence.
1.2.2. Tissue-specific Gene Expression of Aromatase

Regulation of aromatase expression in human tissues is quite complex, involving alternative promoter sites that provide tissue-specific control (Figure 1.3). The tissue-specific promoters include; PI.1, P2a, PI.4, PI.5, PI.7, PI.f, PI.2, PI.6, PI.3, PI.7, and PII.

![Figure 1.3: Structure of CYP19 gene. CYP19 is located on chromosome 15. The gene spans a total length of approximately 120 kilobases. Its regulatory region contains distinct promoters regulated in a tissue-specific manner, nine of which are shown here. Adapted from Bulun et al (8).](image)

Placental expression of aromatase in the human is driven from a powerful distal placental promoter I.1 upstream of untranslated exon I.1. Two other minor promoters (2a and I.2) for human placenta have also been identified. In ovarian granulosa cells,
aromatase expression is regulated primarily by promoter II under the control of the follicle-stimulating hormone (FSH), whose action is mediated by cAMP (10). Estrogens play an important role in bone mineralization, where aromatase is mainly expressed through promoter I.6. Various aromatase transcripts have been reported in the brain, including the brain specific promoter I.f (11). Promoter I.5 is mainly found in fetal tissues, while promoter I.7 is mainly found in vascular endothelial cells.

Although there are other promoters involved in the aromatase expression in adipose tissue, the main promoter is I.4. Expression of aromatase through promoter I.4 is stimulated by class I cytokines and TNFα, in addition to this, there is an obligatory requirement of glucocorticoids (12). In the normal breast, aromatase is primarily localized in the adipose tissue and as a result it is expressed through promoter I.4. In breast cancer tissues, aromatase expression is driven by promoters I.4, I.3, PII and I.7 (8, 13, 14).

1.2.3. Aromatase in Breast Cancer

The presence of aromatase in the tumor and surrounding areas suggests that locally produced estrogen may play a role in stimulating neoplastic growth and development (15). It is known that the concentration of estradiol, the most potent endogenous estrogen, is higher in the tumor tissue than in the normal areas of the breast (16). In the normal breast, aromatase is localized in the epithelial cells (17, 18) and also in the surrounding stromal cells of the adipose tissue (18, 19). Aromatase transcript expression (20) and activity (16, 21) in the tumor is greater than that in the normal breast tissue. Studies with tissue specimens from human subjects have helped identify the association between aromatase and breast cancer. When evaluating breast tissue samples
using RT-PCR, Brueggemeier et al. found that aromatase is present in tumor tissues (22). Immunohistochemistry studies of human tissues have shown that in breast cancers, aromatase is expressed mainly in epithelial cells as well as surrounding stromal cells (17, 23, 24). Epithelial cells in the region displaying higher aromatase expression are at increased risk of malignant transformation (15), and even higher levels have been observed in breast tissue with evidence of invasion (22).

Stromal cells may be the main site of aromatase in breast cancers (25, 26). The presence of aromatase in stromal cells but not in carcinoma cells in breast tumor tissues indicates that locally synthesized estrogens function in a paracrine fashion (27). This could also suggest that carcinoma cells may secrete some factors which stimulate aromatase expression in the surrounding stromal cells. In breast cancer patients, total aromatase transcript levels seem to be significantly higher in adipose tissue proximal to a tumor in comparison with adipose tissue distal to a tumor (15, 20, 28). This suggests that the presence of a breast tumor enhances the local expression of aromatase in the surrounding breast adipose tissue. Estrogens synthesized by the tumor aromatase can stimulate breast tumor growth in both an autocrine and a paracrine manner. For extensive reviews on this topic refer to the following publications (27, 29, 30).

In the normal breast, aromatase is mainly localized in the adipose tissue cells which expression is mainly directed by promoter I.4 (31). Examination of adipose tissue from breast cancer patients showed that aromatase expression is mainly directed by promoters I.3 and promoter II (13). These results suggested that there is a switch of promoters directing aromatase expression in normal breast and cancer tissues. This switch in the regulatory mechanism of aromatase expression from normal breast tissue to
cancerous tissue has been extensively investigated (20, 28, 32). While promoter I.4 requires the synergistic actions of glucocorticoids and class I cytokines (33), promoters I.3 and II are both transactivated by protein kinase A adenosine 3’,5’-cyclic monophosphate (cAMP)-dependent signaling pathways (32, 34) (Figure 1.4). Exon I.3 is also regulated by phorbol esters, which activate protein kinase C (28, 35). More recently, the presence of I.7-specific transcripts was demonstrated in cancer samples from patients, whereas breast adipose tissue from cancer-free women did not contain these transcripts (36).
Figure 1.4: Schematic representation of the proposed regulation of aromatase within the normal breast and breast cancer. In the normal breast, aromatase gene expression is stimulated primarily by class I cytokines and TNFα produced locally in the presence of systemic glucocorticoids. In breast cancer, PGE$_2$ produced by epithelial cancer cells and adipose stromal cells is the major factor stimulating aromatase expression.
1.2.4. Aromatase Inhibitors

Estrogens can influence the risk of breast cancer and also the growth of established tumors. Hormone-dependent breast cancer tumors depend of estrogen for growth. Two approaches to treat these cases of breast cancer are either blocking the mechanism of action of estrogens or inhibiting their synthesis. These therapies are particularly helpful in postmenopausal women in whom hormone responsive is common and estrogen synthesis is primarily peripheral (adipose tissue, muscle and breast tissue) rather than in the ovaries. For more than 30 years, the antiestrogen tamoxifen has been the mainstay of hormonal therapy in postmenopausal women with breast cancer. In the late 1970’s, researchers started to investigate the possibility of inhibiting the last step in the biosynthesis of estrogens and focused on aromatase inhibitors. Aromatase inhibitors that have been used clinically can be categorized by generation and by mechanism of action. They are described as first-, second-, and third generation inhibitors according to the order of their clinical development. They can also be classified as type 1 and type 2 according to their mechanism of action. Type 1 inhibitors, also known as steroidal inactivators, bind to the enzyme like the substrate but irreversibly. Type 2 inhibitors, also known as nonsteroidal inhibitors, posses a heteroatom as a common feature that binds reversibly to the heme group of the enzyme (Table 1.1). The aromatase inhibitors have been reviewed in several publications (37-42).
<table>
<thead>
<tr>
<th>Generation</th>
<th>Type 1</th>
<th>Type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Steroidal Inactivator)</td>
<td>(Nonsteroidal Inhibitor)</td>
</tr>
<tr>
<td>First</td>
<td>None</td>
<td>Aminoglutethimide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second</td>
<td>Formestane</td>
<td>Fadrozole</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third</td>
<td>Exemestane</td>
<td>Anastrozole</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Letrozole</td>
</tr>
</tbody>
</table>

Table 1.1: Classification and structures of the main aromatase inhibitors.
Aminoglutethimide, the first aromatase inhibitor, was initially developed as an anticonvulsant agent (43) but was removed from the market due to serious side effects. It was later found to inhibit the aromatase enzyme (44-46), making this compound the first nonsteroidal first-generation aromatase inhibitor. Problems with the selectivity of aminoglutethimide led to the synthesis of the second-generation of aromatase inhibitors. The second-generation of aromatase inhibitors include; formestane, a type-1 steroidal inactivator, and fadrazole, a type-2 nonsteroidal inhibitor. Formestane produces enzyme-mediated inactivation (47), however, extensive first pass metabolism of this agent in the liver necessitates intramuscular administration and this limits its use (48, 49). Fadrazole is a potent competitive inhibitor of aromatase (50) and is more selective than aminoglutethimide (51), however it causes aldosterone suppression.

The third-generation aromatase inhibitors include the type-1 steroidal inactivator exemestane and the type-2 nonsteroidal inhibitors anastrozole and letrozole. Exemestane is a potent inhibitor of human placental aromatase with an apparent $K_i$ of 26 nM (52). When administered orally, exemestane inhibits rat ovarian aromatase with an $ED_{50}$ of 3.7 mg/kg (52, 53). In 1998, a study revealed that exemestane was the first steroidal aromatase inhibitor to inhibit the enzyme almost totally in vivo when administered orally (54). For a more extensive review of exemestane refer to Higa, G.M. (55). Anastrozole is a potent aromatase inhibitor of human placental microsomes with an $IC_{50}$ of 15 nM (56). In animals, it was also found to be selective for the aromatase enzyme with a maximal activity at about 0.1 mg/kg (57). Anastrozole displays no estrogenic, androgenic, progestogenic, glucocorticoid or mineralocorticoid activity in rats making it suitable for the treatment of breast cancer (58). Reviews on anastrozole can be found in Buzdar,
A.U. and Wellington, K. (59, 60). Letrozole is a potent inhibitor of aromatase with an IC\textsubscript{50} of 11.5 nM in human placental microsomes (61). Letrozole suppressed both plasma and urinary levels of estrogen by about 95% and showed no compromise in cortisol and aldosterone output (62). This topic was reviewed by Simpson, D. (63). The third-generation inhibitors possess a specificity that appears to be nearly complete at clinical doses with no effect on aldosterone, progesterone, corticosterone biosynthesis (64). Recent clinical studies have shown that aromatase inhibitors, specially the third-generation, are more effective than tamoxifen in postmenopausal patients with metastatic breast cancer (65-67).

1.3. Cyclooxygenases

1.3.1. Cyclooxygenases: Function and Regulation

Prostaglandin G/H endoperoxide synthase, also known as cyclooxygenase (COX), is a key enzyme which catalyzes the conversion of arachidonic acid to prostaglandins (68). Cyclooxygenase was first purified in 1976 (69, 70) and cloned in 1988 (70). In 1991, several laboratories identified a product from a second gene with COX activity and called it COX-2 (71, 72). Cyclooxygenases are homodimers both functionally and structurally. Each monomer consists of three structural domains: an epidermal growth factor domain, a neighboring membrane binding domain and a large C-terminal globular catalytic domain.

COX-1, the constitutive form, is involved in a range of physiological functions especially in the stomach where it has a cytoprotective role. Inhibition of the gastric constitutively expressed COX-1 results in most of the unwanted side-effects seen in
patients. Cyclooxygenase-2 is inducible, and the inducing stimuli includes pro-inflammatory cytokines and growth factors, implying a role for COX-2 in both inflammation and control of cell growth (73). COX-2 is particularly responsive to growth factors and mediators of inflammation such as interleukin-1 (IL-1), tumor necrosis factor α (TNFα), lipopolysaccharide (LPS), and 12-O-tetradecanoylphorbol 13-acetate (TPA). For all of these factors, there are two shared pathways that are likely to regulate COX-2 transcription; NFκB and mitogen-activated protein kinase (MAPK) signaling pathways.

The two isoforms of COX are almost identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations. The cyclooxygenase active site of PGHS-2 (COX-2) is larger and more accommodating than that of PGHS-1 (COX-1). This size difference has been exploited in developing COX-2-specific inhibitors.

When tissues are exposed to diverse physiologic and pathologic stimuli, polyunsaturated fatty acids such as arachidonic acid are liberated from membrane phospholipids. Arachidonic acid is the major prostanoid precursor. The biosynthesis of prostanoids involves a three-step sequence of stimulus-initiated hydrolysis of arachidonate from glycerophospholipids involving phospholipase A2; oxygenation of arachidonate, yielding prostaglandin endoperoxide H2 (PGH2) by prostaglandin endoperoxide H synthases; and conversion of PGH2 to the most important biologically active end products, PGD2, PGE2, PGF2α, PGI2 (prostacyclin), or TXA2 (thromboxane A2) via specific synthases (68) (Figure 1.5).
Figure 1.5: Biosynthetic pathway for the formation of prostanoids derived from arachidonic acid. Cyclooxygenases catalyze a cyclooxygenase reaction in which arachidonate plus two molecules of O₂ are converted to PGG₂ and a peroxidase reaction in which PGG₂ is reduced to PGH₂ by two electrons. Adapted from Smith et al (68).
Prostaglandins are ubiquitous substances that initiate and modulate cell and tissue responses involved in physiological processes such as platelet aggregation, renin release and inflammation. They display a wide range of other pharmacological activities, including contraction and relaxation of smooth muscle of the uterus, the cardiovascular system, the intestinal tract, and of bronchial tissue, cytoprotective role in the stomach, etc. Their biosynthesis has also been implicated in the pathophysiology of cardiovascular diseases, cancer, Alzheimer’s disease and other inflammatory diseases. Reviewed in (68, 74, 75).

1.3.2. Cyclooxygenases in Breast Cancer

Prostaglandins produced by COX-2, predominantly prostaglandin E2 (PGE2), induce inflammation and are potent mediators of a number of signal transduction pathways that are implicated in cancer development. The involvement of cyclooxygenases, specially COX-2, in breast cancer was first suggested by reports of elevated prostaglandin levels in breast tumors (76). Concentrations of PGE2 in the normal tissue are lower than the levels detected in tumor and metastatic tissues (76, 77).

Molecular evidence using human subjects have shown that COX-2 is present in breast cancer tissue samples but not in the normal breast tissue. Brueggemeier and others found that while COX-2 expression was undetectable in normal breast tissue specimens, breast tissue specimens expressed COX-2 mRNA with a greatest expression in human breast tumors showing signs of metastasis (22). Parret et al. using reverse transcriptase PCR (RT-PCR) found COX-2 expression in 100% of breast cancers studied, with no
detectable expression in normal breast tissue (78). Badawi and Badr found that levels of COX-2 mRNA were higher in tissues obtained from cancer cases compared to controls by RT-PCR (77).

Animal and cell line studies have also contributed to the efforts of associating cyclooxygenases and breast cancer. COX-2 protein overexpression has been mainly detected in the malignant epithelial cells within breast tumors induced in a rat model by various carcinogens (79, 80). In particular, there are significant elevations of COX-2 protein levels in breast tissue with evidence of invasion (22, 78), suggesting that COX-2 is a key player in the process of metastasis. Studies have also shown that the biologically aggressive, invasive MDA-MB-231 cell line, in contrast to MCF-7 cells, possesses a high constitutive level of COX-2 (81).

PGE$_2$ in the presence of epidermal growth factor stimulated the proliferation of mammary epithelial cells (82). Very few other studies have been able to properly examine whether PGE$_2$ could also directly regulate proliferation of mammary epithelial cells. Besides stimulation of cell proliferation, it is also believed that overexpression of COX-2 in the mammary epithelium is sufficient to induce mammary tumorigenesis (83). In an attempt to study the process of how COX-2 transforms the mammary gland into a tumorigenic state, Chang et al. found that the enzymatic activity of COX-2 is critical for the induction of tumorigenic transformation. This process is associated with angiogenic regulatory genes that result in dramatic changes in the mammary gland vasculature (84).

Although COX-1 is present at a constant level in most cells and tissues and is believed to play a housekeeping role, some studies have shown that COX-1 activity and expression is elevated in human breast cancer tumors (78, 85). In fact, transfecting breast
cancer cell lines with cDNA coding for COX-1 resulted in a more invasive behavior of these cells (86). Two reviews provide more information on cyclooxygenases in breast cancer (87, 88).

Epidemiological evidence has also made possible the association of cyclooxygenases and breast cancer. The use of nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 selective inhibitors in breast cancer has been investigated extensively in the past couple of decades. This topic will be addressed later in this chapter.

1.3.3. Cyclooxygenase Inhibitors

In 1971, Vane reported that aspirin and indomethacin inhibit the biosynthesis of prostanoids and shortly after it was established that these inhibitors block the oxygenation of arachidonic acid. COX-1 and 2 are the major targets of NSAIDs including aspirin, ibuprofen, and the new COX-2 inhibitors. Even though cyclooxygenases have both cyclooxygenase and peroxidase activities, NSAIDs only block the cyclooxygenase part. NSAIDs can be grouped into three classes based on their modes of COX inhibition: (a) Class I - simple, competitive, reversible inhibitors that compete with arachidonic acid for binding to the COX active site (e.g. ibuprofen) (89, 90); (b) Class II - competitive time dependent, reversible inhibitors that bind to the COX active site in a first phase, to form reversible enzyme inhibitor complexes, that if retained for a sufficient time, cause a non covalent conformational change in the protein, associated with tighter binding (e.g. indomethacin) (89, 90), or (c) Class III - competitive, time dependent, irreversible inhibitors that form an enzyme inhibitor complex after a covalent conformational change in the protein (e.g. aspirin) (91).
The discovery of COX-2 in 1991, the key enzyme involved in inflammation, set a new goal for researchers. The new goal was to develop COX-2 selective inhibitors to avoid the numerous side effects associated with COX-1 inhibition. The new concept brought another classification for NSAIDs as selective or non-selective depending on their specificity at inhibiting one of the isoforms or both. These efforts have culminated with the introduction of successful drugs like celecoxib as a potent anti-inflammatory agent for the treatment of arthritis (Table 1.2).
<table>
<thead>
<tr>
<th>Class</th>
<th>Non-selective</th>
<th>Selective (COX-2 inhibitors)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I</strong></td>
<td><img src="image" alt="Ibuprofen" /></td>
<td>None</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Piroxicam" /></td>
<td></td>
</tr>
<tr>
<td><strong>II</strong></td>
<td><img src="image" alt="Indomethacin" /></td>
<td><img src="image" alt="NS-398" /></td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Celecoxib" /></td>
<td></td>
</tr>
<tr>
<td><strong>III</strong></td>
<td><img src="image" alt="Aspirin" /></td>
<td>None</td>
</tr>
</tbody>
</table>

Table 1.2: Classification and structures of cyclooxygenase inhibitors.
1.3.4. Association Between Non-steroidal Anti-inflammatory Drugs (NSAIDs) and Breast Cancer

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to decrease inflammation by inhibiting cyclooxygenases. Even though the Nurses Health Study found no difference in aspirin intake among women who developed breast cancer and those who did not (92), a growing body of experimental (79, 93) and epidemiological (93-96) evidence suggests that the use of NSAIDs may decrease the risk of breast cancer. In a case-control study, a 40% reduction in breast cancer incidence was observed in women who reported daily intake of aspirin, ibuprofen, or other NSAIDs for at least 5 years (93). Independent studies found that both indomethacin (97, 98) and ibuprofen (79, 99) reduced the incidence of dimethylbenz[a]anthracene-induced mammary tumors in rats. Celecoxib and ibuprofen produced striking reductions in the incidence of mammary cancer, tumor burden, and tumor volume compared to those seen in the control group in animal models (80).

Aspirin (100) and flurbiprofen (101) were shown to reduce mammary tumorigenesis. Genetic and pharmacological evidence has shown that specific COX-2 inhibition is more effective than traditional NSAIDs in suppressing polyposis in the mouse (102). SC-560, celecoxib, and indomethacin treatment resulted in statistically significant inhibition of tumor size in comparison with vehicle-treated control animals in a murine model of breast cancer (103). Regular intake of NSAIDs for 2-5 years was associated with a 24% reduction in invasive breast cancer (96). All these findings suggest that cyclooxygenases are involved in the promotion of this type of cancer.
1.4. Relationship Between Cyclooxygenases and Aromatase

PGE₂, the major product of COX-2, binds to cell surface receptors that belong to the family of seven transmembrane G-protein coupled rhodopsin-type receptors, designated as EP₁, EP₂, EP₃, and EP₄. Activation of EP₂ and EP₄ receptors leads to increased intracellular cAMP levels. EP₁ activation results in increased levels of free calcium in the cytosol, while activation of EP₃ receptors results in a decrease in intracellular cAMP. It has been shown that EP₁, EP₂, and EP₄ receptors were elevated, while EP₃ receptor levels were decreased in COX-2 induced mammary tumors (84).

PGE₂ is a powerful stimulator of aromatase activity and expression in human breast adipose stromal cells (104-106). PGE₂ interacts with two receptor subtypes in adipose stromal cells, EP₂ which is coupled to stimulation of cAMP formation and a EP₁ which is coupled to protein kinase C (PKC) activation (104). This suggests that PGE₂ is capable of activating both PKA- and PKC-mediated signaling pathways. PGE₂ is a known stimulant of cAMP in human adipose stromal cells and also breast cancer cells (104). The production of cAMP is higher in high-PGE₂ producing tumors than in low-PGE₂ tumors (107). Dibutyryl-cAMP (Bt₂ cAMP) also stimulates aromatase activity of human adipose stromal cells (104).

The relationship between cyclooxygenases and aromatase was examined in a preliminary study of CYP19 gene expression with COX-1 and COX-2 gene expression in breast cancer patient specimens. This study showed that the levels of CYP19 gene expression remained relatively constant in breast cancer tissue with an increased expression in tissue showing signs of invasion. While COX-1 was present in both normal and cancerous tissue, COX-2 was only present in cancerous tissue with a
significant linear association between tumor cell density and COX-2 gene expression. Regression analysis using a bivariate model showed a strong linear association between the sum of COX-1 and COX-2 expression and \textit{CYP19} expression (22).

Another study using immunohistochemistry staining for aromatase and COX-2 in tumor samples revealed a marked correlation between COX-2 and aromatase expression in tumor samples (24). These data confirm the previous reports showing a positive correlation for \textit{CYP19} and cyclooxygenases.

These observations suggest that both autocrine and paracrine mechanisms may be involved in growth and progression of human breast cancer via stimulation of estrogen production regulated by a high biosynthesis of prostaglandins. Intratumoral aromatase may be important as a source of estrogens for tumor growth and intratumoral COX-2 may be important in the production of prostaglandins (such as PGE$_2$) to direct aromatase gene expression through promoter II (Figure 1.6).
Figure 1.6: Interrelationships between aromatase and cyclooxygenases in breast cancer. PGE$_2$, mainly produced by the epithelial cancer cells, upregulates aromatase expression and activity in the stromal neighboring cells through binding to the EP$_1$ and EP$_2$ receptors. A higher expression of aromatase could result in higher levels of estrogens. The resulting increased estrogen biosynthesis in local sites in turn may result in increased growth and development of the tumor by both paracrine and autocrine actions.
1.5. Estrogen Receptors

1.5.1. Estrogen Receptors and Estrogen Action

Estrogen is primarily synthesized in the ovaries, but ceases at menopause. There is considerable evidence that associates increased breast cancer risk with prolonged exposure to estrogens or estrogenic compounds. The demonstrated role of endogenous sex hormones in supporting the development and growth of breast carcinoma provided a clear and important rationale for the design of therapeutic strategies to interfere with the actions of these hormones at the level of malignant cells. Antiestrogens achieve this interference by competing with estrogens for binding to the steroid receptors.

Estrogen action is mediated by two estrogen receptors, ER$\alpha$ and ER$\beta$. The estrogen receptor (ER) is a member of the nuclear receptor superfamily for steroid and thyroid hormones, vitamin D, retinoids, and prostanoids (108). ERs are located in different tissues in the woman’s body (hypothalamo-pituitary axis, uterus, vagina, liver, bones and breast). ER$\alpha$ was first identified in the 1960’s (109), and almost three decades later ER$\beta$ was discovered (110). The function of ER$\beta$ is still somewhat unclear. The two ERs share a functionally conserved structure consisting of a variable amino terminal region that is involved in transactivation (A/B), a centrally located well conserved DNA binding domain (C), a region involved in dimerization and in binding to heat shock proteins (D), a ligand binding domain (E), which synergizes with the transactivation functions in the A/B region, and a carboxy-terminal region (F) (Figure 1.7). The ER$\alpha$ isoform has two regions called activation functions that contribute to transcriptional activity, AF-1 and AF-2. AF-1 is located in the amino-terminal region within the A/B
region and is associated with basal transcription upon phosphorylation even in the absence of ligand. The AF-2 is located in the carboxy terminus and is ligand-dependent.

**ERα Protein**
595 aa

```
H2N- A/B | C | D | E | F - C OOH
```

Percent Identity 17 97 30 59 18

**ERβ Protein**
485 aa

```
H2N- A/B | C | D | E | F - C OOH
```

Figure 1.7: Structure of estrogen receptors α and β and the percent homology in the different regions. The estrogen receptors are organized into different functional regions (A-F), but the most important are the C region (DNA binding domain) and the E region (ligand binding domain).

The predominant estrogen receptor in the female mammary glands is ERα. ER-positive cells in the normal breast are largely non-dividing. Interestingly, estrogen stimulated proliferation occurs in ER-negative cells that surround the ER-positive luminal epithelial cells. However, proliferation of ER-positive epithelial cells in breast tumors is estrogen regulated in most cases (3). ERα and ERβ differ significantly in their tissue distribution and ligand binding characteristics, therefore affording interesting potential for tissue-selective estrogen action.
Estrogens are carcinogenic in humans but the molecular pathways by which these hormones induce cancer are not well understood (Figure 1.8). One of the mechanisms involves the binding of estrogens to the ER to stimulate cell proliferation and gene expression (111). Another possible mechanism involves the catechol estrogen metabolites, which are less potent estrogens than estradiol but can directly or indirectly damage DNA, proteins, and lipids (112). Catechol estrogens are implicated in tumor initiation via redox cycling mechanisms, inducing different types of DNA damage and reacting directly with DNA bases to form covalent bonds. Efforts to block the effect of estrogens in the process of carcinogenesis present a promising way to treat cancer in women with hormone-dependent breast cancer.
Figure 1.8: Mechanisms in which estrogens are implicated in tumor growth and initiation. Estrogens bind to the estrogen receptor initiating the transcription of genes involved in growth factors production resulting in tumor growth. Estrogens can also be converted into catechol estrogens that can result in DNA damage and initiation of carcinogenesis in the normal cells.

1.5.2. Selective Estrogen Receptor Modulators

Selective estrogen receptor modulators (SERMs) are nonsteroidal compounds that interact with the estrogen receptor and can exert their effects in a tissue specific manner (113). They can show mixed estrogen agonist/antagonist activity. SERMs can demonstrate remarkable differences in efficacy in the various tissues in which estrogens act, functioning as agonists in some tissues but as antagonists in others (114, 115).

Tamoxifen (TAM) (Figure 1.9) is a nonsteroidal drug, the first clinically useful SERM, and is still considered the endocrine therapy of choice for the adjuvant treatment...
of ER-positive breast cancer. In the ER-positive cell line MCF-7, tamoxifen significantly antagonized the E₂ cell growth stimulation. In the ER-negative cell line MDA-MB-231, cell growth was not significantly affected by TAM in the absence or in the presence of estradiol (116). *In vivo* TAM is metabolized to several derivatives, including 4-hydroxytamoxifen (4-OHT) by a cytochrome P450 enzyme (117). 4-OHT has an affinity for the ER equivalent to that of E₂ (118) and is retained in the nucleus via the estrogen receptor. Tamoxifen can also potentially affect cellular functions by binding to calmodulin or inhibiting protein kinase C (119). This led to the study of its ability to inhibit cell growth and to induce apoptosis independently of the presence of estrogen receptors in both ER-positive and ER-negative cell lines. Tamoxifen was found to induce apoptosis on both cell lines, showing that apoptosis can be achieved also independently of the presence of the estrogen receptor (120).
Clinical evidence has shown that a subset of patients fails to respond to TAM, probably due to mixed agonist and antagonist effects displayed by the antiestrogen (121, 122). Moreover, the emergence of drug-resistant clones during TAM treatment represents a further limitation in the treatment of breast cancer patients (123). Long-term adjuvant tamoxifen treatment is associated with a 4-fold increase in the incidence of endometrial carcinoma in postmenopausal women (124, 125). Since breast cancer cells can develop resistance to the antiestrogen TAM or 4-OHT after long-term exposure, and
since about one million patients are taking TAM, the efficacy of pure anti-estrogens is clinically important. These considerations have encouraged the development and testing of new antiestrogens to find drugs with pure antagonist properties.

Raloxifene (Figure 1.9) was developed initially for breast cancer treatment (126), but its use was abandoned in the late 1980’s because clinical trials showed no activity in tamoxifen-resistant patients. The current clinical use of raloxifene is for the treatment and prevention of osteoporosis (127). In 1997, Brzozowski et al. studied the binding of estradiol and raloxifene to the ER (128). They found that the basic chain of raloxifene plays a critical role in tissue selectivity due to a unique conformational change of the ER when compare to estradiol. When raloxifene binds to the ER, its long side chain protrudes from the binding pocket, thereby preventing helix 12 from positioning properly over the ligand binding pocket. This unique conformation may not permit the recruitment of the coactivators that are involved in estrogenic activity in breast tissue.

Steroidal antiestrogens should bind to the estrogen receptor to form an antiestrogen-ER complex that does not promote gene transcription. ICI 182,780, now called fulvestrant (Figure 1.9) was synthesized and shown to display pure antagonist activity in rats and mice (129) and on breast cancer cells (130). In MCF-7 cells, ICI 182,780 in the absence of E2 was able to inhibit cell growth at all tested concentrations. Moreover, the stimulatory effect by E2 was counteracted by the highest concentrations of ICI 182,780. Conversely, ICI 182,780 never inhibited the growth of ER-negative cell lines in the absence of E2 (116). The growth inhibitory action of ICI 182,780 on MCF-7 cells was reversed in a competitive manner by estradiol (131, 132). The maximum growth inhibitory effect of ICI 182,780 exceeded that of 4-OHT (131). The pure
antagonist ICI 182,780 binds to a high-affinity site distinct from the estrogen receptor (133). ICI 182,780 is fully active for the inhibition of cell proliferation and the expression of estrogen-responsive genes in the 4-OHT-resistant MCF-7/LCC2 cell line (134).

1.5.3. Flavonoids

There is a marked difference in the incidence rates of breast cancer in women from western countries compared to those in nonwestern countries. Some investigators have hypothesized that an Asian diet, which is typically high in soy content, may be one factor that explains the lower incidence of breast cancer in those countries compared with other countries on a diet that lacks soy as a common component (135, 136). Soy products are rich in flavonoids.

Flavonoids and isoflavonoids are a diverse group of plant natural products synthesized from phenylpropanoid and acetate-derived precursors. Flavonoids and isoflavonoids exhibit a range of mammalian health-promoting activities that are currently the focus of intense study (137). The isoflavones are strikingly similar in chemical structure to mammalian estrogens. It is no surprise that isoflavonoids exhibit estrogenic and anticancer activities. Experiments demonstrating that phytoestrogens, estrogenic substances produced by plants, could compete with radiolabelled estradiol for binding to the estrogen receptor were important in showing that phytoestrogens and traditional estrogens have a common mechanism of action (138). Flavones were also found to inhibit the aromatization of androstenedione and testosterone to estrogens.
catalyzed by human placental and ovarian microsomes. The kinetic analyses suggest that flavones may inhibit aromatization by competing with androstenedione (and testosterone) for the substrate binding site on the enzyme (139).

The primary isoflavone component of soybeans associated with chemoprevention is genistein. Binding of a selected group of phytoestrogens to ER was found to be of high affinity, and the most potent one was genistein with a dissociation constant \((K_d)\) of 10 nM compared to 0.2 nM for estradiol (140). The same study also showed that ERs complexed to phytoestrogens are processed in the nucleus at about the same rate as the estradiol-bound receptor. The addition of estradiol to breast cancer cells treated with genistein at its IC\(_{50}\) concentration was able to restore cell proliferation to nearly 100% of the control level, as was also observed for tamoxifen (141). This suggests that genistein occupies the estrogen receptor \textit{in vitro}. In a different study, animals receiving the highest concentration of dietary genistein developed the lowest number of mammary tumors per rat, suggesting that dietary genistein reduces susceptibility to mammary cancer in rats (142). In 1997, a study showed that genistein possessed a higher affinity for ERβ than that for ERα (143).

Genistein has demonstrated other biological activities not directly associated with the estrogen receptor. Genistein inhibits the growth of MDA-MB-231 breast cancer cells, regulates the expression of apoptosis-related genes, and induces apoptosis through a p53-independent pathway (144). Genistein was shown to be a potent growth inhibitor in both MCF-7 cells and MDA-MB-468 cells, suggesting that isoflavones can act via an ER-independent pathway (145). Genistein has also been shown to inhibit mammalian DNA topoisomerase II in L-1210 cells (146). Tyrosine-specific protein kinase activity is
known to be associated with oncogene products. It is possible that tyrosine phosphorylation plays an important role for cell proliferation and cell transformation. Genistein is a highly specific inhibitor of tyrosine kinases but scarcely inhibits the activity of serine and threonine kinases and other ATP analogue-related enzymes in vitro. Results show that genistein inhibits the tyrosine kinase activity of the EGF receptor in intact A431 cells (147). It is well known that estrogen deficiency induces rapid bone loss during the first decade after menopause. Genistein helps prevent bone loss caused by estrogen deficiency in female mice (148).

1.6. Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) are a member of the orphan nuclear receptor superfamily, which also includes retinoid X receptor (RXR), the thyroid hormone receptor, retinoic acid receptor, liver X receptor, and the vitamin D receptor. There are three subtypes, PPARα, PPARβ/δ and PPARγ. The PPARα is expressed in the liver, heart, muscle, and kidneys where it regulates fatty acid catabolism (149). PPARβ/δ is expressed ubiquitously with a less defined function. PPARγ is highly expressed in adipocytes and macrophages and is involved in adipocyte differentiation, lipid storage, and glucose homeostasis (150). Ligands for PPARγ include the synthetic thiazolidinediones troglitazone and rosiglitazone, as well as 15d-PGJ2 (15-deoxy-Δ(12,14)-Prostaglandin J2), proposed to be a naturally occurring ligand.

PPARs possess the classic domain structure of other nuclear receptors like the estrogen receptor. They are composed of an NH2-terminal region with a ligand-independent transactivation domain (AF-1), a DNA-binding domain (two zinc fingers)
and, at the COOH-terminus, a ligand and dimerization domain and a ligand-dependent activation domain (AF-2) (151). The PPARs bind to the peroxisome proliferator response element on the DNA with the sequence AGGTCANAGGTCA (direct repeat with a single nucleotide spacer) as heterodimers with the 9-cis-retinoic acid receptor (152) (Figure 1.10).

In breast tissue, agonists of PPARγ have been shown to inhibit cell growth (153), inhibit aromatase activity in adipose tissue (154, 155), inhibit estrogen receptor activity (156) and play a role in tumor regression (157).

Figure 1.10: Structure of PPARs and mechanism of activation. The PPARs consist of a ligand-independent domain (AF-1), a DNA binding domain (DBD), a ligand and dimerization binding domain and a ligand-dependent activation domain (AF-2)
1.7. Summary

This introductory chapter discussed three of the most common targets for breast cancer treatment; aromatase, COX-2 and estrogen receptors. While intratumoral aromatase may be an important source of estrogen stimulating tumor proliferation of ER-positive tumors, aromatase may be upregulated in a majority of tumors by factors such as prostaglandin E₂ as indicated by the high correlation between the inducible COX-2 and aromatase in breast tumor specimens. This may provide a growth advantage during initial tumor development by increasing estrogen production and resulting in stimulation of the genes involved in proliferation. Thus, the regulation of both enzymes in breast cancer involves complex paracrine interactions, resulting in significant consequences on the pathogenesis of breast cancer.

Higher levels of COX enzymes expression and increased activity, especially COX-2, could result in higher levels of PGE₂, which in turn could increase aromatase expression through increases in intracellular cAMP levels and activation of promoter II. Breast tumor sites provide a potentially rich source of PGE₂ which can stimulate aromatase expression both in the tumor itself and in the surrounding adipose tissue. A higher expression of aromatase could result in higher levels of estrogens that are available to bind to the ERs. The resulting increased estrogen biosynthesis in local sites in turn may result in increased growth and development of the tumor by both paracrine and autocrine actions.
1.8. References


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CHAPTER 2

STATEMENT OF THE RESEARCH PROBLEM AND SPECIFIC AIMS

2.1. The Research Problem

In the United States, adenocarcinoma of the breast is the most common cancer in women and ranks second as a cause of cancer-related mortality. Approximately 60% of all breast cancer cases are hormone-dependent. The requirement of estrogens for tumor growth makes the estrogen receptors and the aromatase enzyme, the enzyme involved in the last step of the biosynthesis of estrogens from androgens, potential targets for the prevention and treatment of this type of cancer. Additionally, many of these tumors can develop resistance to anti-estrogenic agents like tamoxifen after long-term exposure. This connection has encouraged researchers to develop new anti-estrogens and/or drugs for the treatment of breast cancer.

By comparison, in nonwestern countries – where the diet is very rich in soy – there is a marked difference in the incidence rates of breast cancer as compared to women from western countries. Soy products, themselves, are typically rich in flavonoids which exhibit a range of health-promoting activities. Such activities make this class of compounds the focus of intense study. The striking similarity in structure of
these agents to mammalian estrogens has encouraged scientists to study the possibility of using the isoflavonoid scaffold to develop selective estrogen receptor modulators and aromatase inhibitors.

A growing body of experimental and epidemiological evidence suggests that the use of NSAIDs may decrease the incidence of mammary cancer, tumor burden, and tumor volume. Although this effect has been studied for the past couple of decades, the mechanism by which these benefits occur is unclear.

The regulation of aromatase expression in human tissues is quite complex. Studies have shown that there is a switch in the regulatory mechanism of aromatase expression in breast cancer tissues. In normal breast tissue, aromatase expression is primarily regulated by promoter I.4; but in breast cancerous tissue, aromatase is primarily regulated by promoters I.3 and II. It is also known that promoters I.3 and II are transactivated by cAMP dependent pathways that can be activated by PGE$_2$. There is also evidence that correlates aromatase expression with cyclooxygenase expression in breast cancer tissues. When analyzing this correlation, we question whether the inhibition of cyclooxygenases could explain the chemopreventive properties of NSAIDs.

These observations suggest that both autocrine and paracrine mechanisms may be involved in the growth and progression of human breast cancer through stimulation of estrogen production regulated by a high biosynthesis of prostaglandins. Intratumoral aromatase may be important for tumor growth and intratumoral COX-2 may be important in the production of PGE$_2$ to direct aromatase gene expression through promoter II.
2.2. Specific Aims

1) To provide information on the association between aromatase and COX-2 in human breast cancer development by studying the activity and expression of aromatase *in vitro* after the exposure to cyclooxygenase inhibitors.

2) To investigate whether COX-2 inhibition is the only requirement for aromatase suppression for the selective COX-2 inhibitor NS-398.

3) To study the effect of PPARγ ligands on aromatase activity and expression in breast cancer and adipose stromal cells.

4) To explore the possibility of developing synthetic isoflavonoids as selective estrogen receptor modulators, and study the effect of these agents on cell proliferation in a hormone-dependent cell line.

5) To study the effect of synthetic isoflavonoids on aromatase activity in breast cancer cells.

6) To provide information about which functionalities of the azinomycin molecule are involved in cell cytotoxicity.
CHAPTER 3

THE EFFECTS OF CYCLOOXYGENASE INHIBITORS ON AROMATASE EXPRESSION AND ACTIVITY IN BREAST CANCER CELLS

3.1. Introduction

Every three minutes a woman in the United States is diagnosed with breast cancer. Breast cancer is the second leading cause of death from cancer in American women. Approximately 60% of all breast cancer patients have hormone-dependent breast cancer, which contains estrogen receptors and requires estrogen for tumor growth. Estrogens are biosynthesized from androgens by aromatase, the product of the CYP19 gene and a member of the cytochrome P450 enzyme superfamily (1). Aromatase transcript expression (2) and activity (3, 4) in the tumor is greater than that in the normal breast tissue. Regulation of aromatase expression in human tissues is quite complex, involving alternative promoter sites that provide tissue-specific control. In the normal breast cells, aromatase expression is primarily derived by the tissue-specific promoter I.4 for transcription, whereas expression from breast cancer patients switches from promoter I.4 to promoter I.3 and promoter II (5).

Cyclooxygenase (COX) is a membrane bound enzyme responsible for the oxidation of arachidonic acid into prostaglandins. It is expressed in at least two different
isoforms, a constitutively expressed form, cyclooxygenase-1 (COX-1), and an inducible form, cyclooxygenase-2 (COX-2) (6, 7). Most studies have shown that COX-2 is present in breast cancer tissue samples but not in the normal breast tissue (8-10). Concentrations of PGE$_2$ in the normal tissue are lower than the levels detected in tumor and metastatic tissues (10, 11). Several studies have showed a strong correlation between COX-2 and aromatase expression in tumor samples (8, 12).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to decrease inflammation by inhibiting cyclooxygenases. A growing body of experimental (13, 14) and epidemiological (14-17) evidence suggests that the use of NSAIDs may decrease the risk of breast cancer. Aspirin (18) and flurbiprofen (19) were shown to reduce mammary tumorigenesis. Celecoxib and ibuprofen produced striking reductions in the incidence of mammary cancer, tumor burden, and tumor volume compared to those seen in the control group in animal models (20). Genetic and pharmacological evidence has shown that specific COX-2 inhibition is more effective than traditional NSAIDs in suppressing polyposis in the mouse (21). All these findings indicate that cyclooxygenases are involved in the promotion of this type of cancer. Their role in breast cancer prevention and treatment has yet to be fully characterized.

The present study examines the activity and expression of aromatase in breast cancer cell lines after the exposure to nonselective and isozyme-selective cyclooxygenase inhibitors in order to provide further information on the association between aromatase, COX-2, and human breast cancer development.
3.2. Experimental

3.2.1. Chemicals, Biochemicals and Reagents

Radiolabeled [1β-3H]-androst-4-ene-3,17-dione was obtained from NEN Life Science Products, Boston, MA. The following compounds were purchased from Cayman Chemical, Ann Arbor, MI: niflumic acid, nimesulide, NS-398, SC-560, and SC-58125. The following compounds were purchased from Sigma, St. Louis, MO: ibuprofen, indomethacin, and piroxicam. Celecoxib was a gift from Dr. Ching-Shih Chen, The Ohio State University, College of Pharmacy. Trypsin, TRIzol, and all enzymes were obtained from Invitrogen, Carlsbad, CA. Radioactive samples were counted on a LS6800 liquid scintillation counter, Beckman, Palo Alto, CA. Mixture 3a70B was obtained from Research Prospect International Corp, Mount Prospect, IL.

3.2.2. Cell Culture

JAR, MCF-7, MDA-MB-231, and SK-BR-3 cell lines were obtained from ATCC, Rockville, MD. Cell cultures were maintained in phenol red-free custom media (MEM, Earle’s salts, 1.5x amino acids, 2x non-essential amino acids, L-glutamine, 1.5x vitamins, (Life Technologies, Carlsbad, CA)) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 20 mg/l gentamycin. Fetal calf serum was heat inactivated for 30 min in a 56°C water bath before use. Cell cultures were grown at 37°C, in a humidified atmosphere of 5% CO₂ in a Hereaus CO₂ incubator. For all experiments, cells were plated in either T-25 flasks, 100 mM plates, 96-well plates, or 24-well plates and grown to subconfluency. Before treatment, the media was changed to a defined one containing DMEM/F12 media (Sigma) with 1.0 mg/ml human albumin (OSU Hospital Pharmacy), 5.0 mg/l human transferin and 5.0 mg/l bovine insulin.
3.2.3. Cell Cytotoxicity Assay

Cellular cytotoxicity in the presence and absence of agents was determined using the CellTiter 96® aqueous non-radioactive cell proliferation assay (22). SK-BR-3 cells were plated into 96-well plates (1x10^4 cells/well), and after 24 h the culture medium was removed and cells washed with phosphate-buffered saline (PBS). Culture wells (n=6) were then treated with vehicle or the indicated concentration of agents in defined media at 37°C for 24 h. After incubation, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (Promega, Madison, WI) and phenazine methosulfate (Sigma) were prepared in PBS at a final concentration of 333 µg/ml and 25 μM respectively. These solutions were combined and 20 µl of this mixture were added to each well. After 3 hours of incubation at 37°C, absorbance at 490 nm (reference wavelength is 700 nm) was measured using a SPECTRAmax plate reader.

3.2.4. Tritiated Water-Release Assay

Measurement of aromatase enzyme activity was based on the tritium water release assay (23). Cells in T-25 flasks or 100 mM plates were treated with 0.1% DMSO (control), NSAIDs (ibuprofen, piroxicam, and indomethacin), COX-1 selective inhibitor SC-560, and COX-2 selective inhibitors (SC-58125, NS-398, celecoxib, niflumic acid and nimesulide) at the indicated concentrations. After 24 h, the cells were incubated for 6 hours with fresh media along with 50 nM androstenedione including 2 μCi [1β-3H]-androst-4-ene-3,17-dione. Subsequently, the reaction mixture was removed, and proteins were precipitated using 10% trichloroacetic acid at 42°C for 20 min. After a brief centrifugation, the media was extracted three times with an equal amount of chloroform.
to extract unused substrate and further dextran-treated charcoal. After centrifugation, a 250-µl aliquot containing the product was counted in 5 ml of liquid scintillation mixture. Results were corrected for blanks and for the cell contents of culture flasks, and results were expressed as picomoles of $^3$H$_2$O formed per hour incubation time per million live cells (pmol/h/10$^6$ cells).

### 3.2.5. Diphenylamine DNA Assay

To determine the amount of viable cells in each flask, the cells were trypsinized and analyzed using the diphenylamine DNA assay adapted to a 96-well plate (23, 24). DNA standards (0 – 30 µg) were prepared using double-stranded DNA reconstituted in PBS and added in triplicates directly to the wells. A uniform cell suspension was prepared from the T-25 flasks in 100 µl PBS, and 20 µl of the unknown samples were added in triplicates to separate wells. A solution of 0.16% acetaldehyde in water was prepared and mixed at a 1:5 ratio with perchloric acid (20% vol/vol). This solution (60 µl) was added to each well along with 100 µl of a 4% diphenylamine solution in glacial acetic acid. The plates were incubated at 37°C for 24 hours and the OD$_{595}$ was measured using a microplater reader. The DNA concentration was determined by extrapolation to the standard curve and the amount of cells/flask was calculated using the equation: 1 cell = 7 pg DNA.

### 3.2.6. Enzyme Immunoassay of PGE$_2$

To study PGE$_2$ synthesis in cell culture media, experiments were performed in 12-well plates. An aliquot of SK-BR-3 cells (150,000 cells) was added to each well and plates were incubated overnight to allow the cells to adhere to the plates. After this time, cells were serum starved in defined media for 24 h. This step was followed by
replacement of media with fresh media containing either vehicle (DMSO) or the indicated concentration of agents. After 24 h incubation at 37°C the media were collected and the amount of PGE₂ was determined by ELISA (Cayman Chemical) according to the protocol provided by the manufacturer. PGE₂ concentration was normalized to total protein. Total proteins were extracted from adhered cells by 30 min treatment with 0.5 M NaOH at room temperature and shaking. Protein concentrations in these extracts were determined using a protein assay method (Bio-Rad Laboratories, Inc., Hercules, CA).

3.2.7. RNA Extraction

Total RNA was isolated using the TRIzol reagent according to the manufacturer’s protocol. Total RNA pellets were dissolved in DNase, RNase-free water and quantitated using a spectrophotometer. The quality of RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide; the 18S and 28S rRNA bands were visualized under ultraviolet light.

3.2.8. cDNA Synthesis

Isolated total RNA (2 µg) was treated with DNase I, Amplification grade, according to the recommended protocol to eliminate any DNA before reverse transcription. Treated total RNA was denatured at 65°C for 5 min in the presence of 2.5 ng/µl random hexamers and 0.5 mM dNTP mix. The samples were snap-cooled on ice and centrifuged briefly. Complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase according to the recommended protocol. Briefly, the
reactions were conducted in the presence of 1X First-Strand Buffer and 20 mM DTT at 42°C for 50 min and consequently inactivated at 70°C for 15 min. The cDNA generated was used as a template in real-time PCR reactions.

3.2.9. Real-time PCR

Real-time PCR was performed using the Opticon™ 2 system from MJ Research, Waltham, MA. For the COX-2 and CYP19 total gene the PCR reaction mixture consisted of Taqman® Universal PCR Master Mix (Applied Biosystems), 600 nM of each primer (Invitrogen) (Table 3.1), 250 nM Taqman probe, 18S rRNA, Applied Biosystems, Foster City, CA and 2.5 µl of each RT sample in a final volume of 25 µl. The Taqman probe was designed to anneal to a specific sequence of the aromatase gene between the forward and the reverse primers. Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min.

The first step consisted in the optimization of probes and primers for both COX-2 and aromatase to ensure that the concentration was the most appropriate one. The probe concentration was fixed at a concentration of 200 nM and the primer concentration for both sense and antisense primers were tested from 50 – 900 nM. For both genes the combination of 600 nM- 600 nM gave the best repetitive results and the lower Ct value. In the case of the probe concentration optimization process, the concentration of primers was fixed to 600 nM and the concentration of probe varied from 150 – 300 nM. The best results and lower Ct values were obtained with 250 nM concentration of probe.

For the specific exon I promoter regions and TATA-box-binding protein (TBP) the PCR reaction mixture consisted of DyNAmo Hot Start SYBR Green qPCR kit (MJ
Research), 600 nM of each primer (Table 3.1), and 2.5 µl of each RT sample in a final volume of 20 µl. SYBR Green uses a dye that will bind to double stranded DNA, in this methodology the primers are carefully designed to each of the promoter regions of aromatase exon I. Cycling conditions were 95°C for 15 min, followed by 50 cycles at 94°C for 10 s and 60°C for 25 s and 72°C for 30 s.
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<tr>
<th>Gene</th>
<th>Oligonucleotide</th>
<th>Sequences</th>
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<td>CYP19</td>
<td>Primer (S)</td>
<td>5'-TGT CTC TTT GTT CTT CAT GCT ATT TCT C-3'</td>
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<td></td>
<td>Primer (A)</td>
<td>5'-TCA CCA ATA ACA GTC TGG ATT TCC-3'</td>
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<td></td>
<td>Probe</td>
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<td>GGC AAT-3'TAMRA</td>
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<td>Primer (S)</td>
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<td></td>
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<td>Primer (S)</td>
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<td></td>
<td>Primer (A)</td>
<td>5'-CAC ATC ACA GCT CCC CAC CA-3'</td>
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Table 3.1: Oligonucleotide primer and probe sequences for real-time PCR. Total CYP19 and COX-2 gene was analyzed using Taqman methodology and a sequence specific fluorogenic probe. Exon I promoter specific regions and TBP gene were analyzed using SYBR Green methodology. (S) = Sense; (A) = Antisense.

a Reference (25)
b Reference (26)
3.2.10. Statistical Analysis

Statistical and graphical information was determined using GraphPad Prism software (GraphPad Software Incorporated, San Diego, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA). Determination of IC\textsubscript{50} values were performed using nonlinear regression analysis. Statistically significant differences were calculated with the two-tailed unpaired Student’s $t$-test and $P$ values reported at 95% confidence intervals.

3.3. Results

3.3.1 Aromatase Enzymatic Activity and Expression in Human Breast Cancer Cell Lines

Aromatase activity was determined using the “in-cell” tritiated water-release assay and normalized to the number of cells in each flask. Different human breast cancer cell lines (MCF-7, MDA-MD-231, and SK-BR-3) were compared for aromatase activity using the tritiated water release assay (Table 3.2). Cell Line SK-BR-3 showed the highest levels of aromatase activity, followed by MCF-7 and MDA-MB-231. The aromatase activity in the SK-BR-3 cell line is approximately 20 times higher than that in MCF-7 cells and 35 times higher than that in MDA-MB-231 cells. These results agree with previous studies from other researchers (27, 28).

Cell lines JAR, MCF-7, MDA-MB-231, and SK-BR-3 were used to compare $CYP19$ gene expression by real-time PCR (Table 3.2). $CYP19$ expression was normalized relative to 18s rRNA and compared to expression in JAR cells. The choriocarcinoma placental JAR cell line, a cell line that expresses high levels of $CYP19$,
was selected as the calibrator. Results show that basal levels of \textit{CYP19} gene expression in MCF-7 cells are slightly higher than those in SK-BR-3 cells but considerable higher than those in MDA-MB-231 cells. The SK-BR-3 cell line was selected for these studies because this cell line had both a higher aromatase activity and \textit{CYP19} expression than the other breast cancer cell lines.

| Cell Line    | Aromatase Activity (pmol/h/10^6 cells) | \textit{CYP19} Expression Relative to JAR Cells  
\quad \quad \quad \quad \quad (2^{\Delta \Delta Ct})^{c} |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>JAR</td>
<td>6.31 ± 2.17^{d}</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>MCF-7</td>
<td>0.00015 ± 0.00004</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>0.0028 ± 0.0002</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>0.00008 ± 0.00001</td>
<td>0.004 ± 0.001</td>
</tr>
</tbody>
</table>

Table 3.2: Aromatase activity\textsuperscript{a} and expression\textsuperscript{b} in cell lines.
\textsuperscript{a} Values are expressed as pmol/h/10\textsuperscript{6} cells, and reported as mean ± S.D., n = 3.
\textsuperscript{b} \textit{CYP19} expression was normalized relative to 18S rRNA. Values are expressed as \textit{CYP19} expression relative to a calibrator (JAR cells), and reported as mean ± S.D., n = 9.
\textsuperscript{c} 2^{\Delta \Delta Ct} is the amount of the target gene, normalized to an endogenous reference (18S rRNA) and relative to a calibrator (JAR cells)
\textsuperscript{d} (29)
3.3.2. The Effect of Cyclooxygenase Inhibitors on Aromatase Enzymatic Activity in Breast Cancer Cells

In order to optimize our enzyme activity assay methodology, SK-BR-3 cells were treated with increasing concentrations of the irreversible steroidal aromatase inhibitor 7α-(4’-aminophenylthio)-androsta-1,4-diene-3,17-dione (7α-APTADD) (Figure 3.1). Our results, in agreement with previous findings (30), not only demonstrate the inhibitory potency of this compound but also show the utility of this specific cell line to study the effect of agents on aromatase activity inhibition (Figure 3.4).

\[
\text{Figure 3.1: Structure of the irreversible aromatase inhibitor 7α-APTADD.}
\]

The effects of NSAIDs (Figure 3.2), COX-1 and COX-2 selective inhibitors (Figure 3.3) on aromatase activity were determined and the concentration producing a 50% decrease in activity (IC₅₀) for each agent was calculated. All agents decreased aromatase activity under control (vehicle) levels in a dose-dependent manner. NSAIDs (non-selective COX inhibitors) decreased aromatase activity only at high micromolar concentrations (Figure 3.5). Indomethacin was the most potent NSAID in decreasing aromatase activity followed by piroxicam and ibuprofen with IC₅₀ values 157 ± 5.7 µM,
408 ± 23 µM and 809 ± 90 µM, respectively. Cells treated with ibuprofen and piroxicam at concentrations over 750 µM, and indomethacin at concentrations over 200 µM, showed signs of toxicity.

![Chemical structures of ibuprofen, indomethacin, aspirin, and piroxicam](image)

Figure 3.2: Structures of the NSAIDs used in this study.

PGE₂ is a powerful stimulator of aromatase activity and expression in human breast adipose stromal cells (23). To examine the hypothesis that elevated PGE₂ production by COX-2 induces aromatase activity, SK-BR-3 cells were treated for 24 hours with COX-2 selective inhibitors. In fact, all COX-2 selective inhibitors decreased aromatase activity in SK-BR-3 cells in a dose-dependent manner (Fig. 3.6). NS-398 was the most potent agent in decreasing aromatase activity with an IC₅₀ value of 1.0 ± 0.4
Celecoxib and SC-58125, a celecoxib-like agent, showed IC$_{50}$ values of 37 ± 4.5 and 24 ± 1.8 µM, respectively. Nimesulide and niflumic acid also decreased aromatase activity with IC$_{50}$ values of 27 ± 4.7 and 97 ± 8.3 µM, respectively. Aromatase activity was expressed as picomoles of $^3$H$_2$O formed per hour incubation time per million live cells. Some agents (such as ibuprofen, indomethacin and niflumic acid) resulted in cell toxicity at very high concentrations but aromatase activity was normalized by the number of live cells in each assay, to assure that the loss of cells due to any toxicity effects by the agents was taken into account. Treatment of SK-BR-3 cells for 24 hours with SC-560, a COX-1 selective agent, resulted in a decreased of aromatase activity and an IC$_{50}$ value of 5.8 ± 1.2 µM (Figure 3.7).

![Figure 3.3: Structures of the cyclooxygenase selective inhibitors used in this study.](image-url)
Figure 3.4: Suppression of aromatase activity in SK-BR-3 breast cancer cells by 7α-APTADD. SK-BR-3 cells were treated with the indicated concentrations of the agent and aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.003 pmol/h/10$^6$ cells. Each data point represents the mean results of three independent determinations. *, $P < 0.0001$ vs. control by unpaired $t$ test, n = 3.
Figure 3.5: Suppression of aromatase activity in SK-BR-3 breast cancer cells by NSAIDs. SK-BR-3 cells were treated with indomethacin (○), piroxicam (●), or ibuprofen (■), and aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.003 pmol/h/10$^6$ cells. Each data point represents the mean results of three independent determinations.
Figure 3.6: Suppression of aromatase activity in SK-BR-3 breast cancer cells by COX-2 selective inhibitors. SK-BR-3 cells were treated with NS-398 (●), nimesulide (○), SC-58125 (■), celecoxib (●), or niflumic acid (□), and aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle (DMSO). The value of 100% is equal to 0.003 pmol/h/10$^6$ cells. Each data point represents the mean results of three independent determinations.
Figure 3.7: Suppression of aromatase activity in SK-BR-3 breast cancer cells by the COX-1 selective inhibitor SC-560. SK-BR-3 cells were treated at the indicated concentrations and aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle (DMSO). The value of 100% is equal to 0.003 pmol/h/10$^6$ cells. Each data point represents the mean results of three independent determinations.
3.3.3. Levels of PGE$_2$ Production

The production of PGE$_2$ was measured in cells treated with NSAIDs, a COX-1 selective inhibitor, and COX-2 selective inhibitors (Figure 3.8). The levels of cyclooxygenase activity in SK-BR-3 cells are low but detectable, consistent with other studies (31). Even though exogenous arachidonic acid would have increased the production of PGE$_2$, the experimental conditions required the SK-BR-3 cells to utilize the endogenous substrate. This assures that the conditions for the rest of the assays are more consistent and comparable to one another. SK-BR-3 cells were treated for 24 h with the indicated concentration of the agents. All agents resulted in a decrease in PGE$_2$ production in SK-BR-3 cells but only ibuprofen, piroxicam, indomethacin, NS-398, celecoxib, nimesulide, and SC-58125 resulted in significant reductions ($P < 0.05$).
Figure 3.8: Effect of cyclooxygenase inhibitors on prostaglandin E₂ production in SK-BR-3 cells. Cells were treated for 24 hours with the indicated concentrations of agents. Results are expressed as means of the concentration of PGE₂ produced/µg protein ± S.E.M. *, P < 0.05 vs. control by unpaired t test, n = 6.
3.3.4. *CYP19* mRNA Expression by Real-Time PCR

Analysis of total *CYP19* mRNA transcripts was performed using real-time PCR in order to determine whether the decrease in aromatase activity by COX inhibitors in SK-BR-3 cells was due to a down-regulation of aromatase expression at the transcriptional level. SK-BR-3 cells were treated with COX inhibitors for 24 h at concentrations at or near the IC$_{50}$ value for each agent, to assure that the concentration was sufficient to suppress aromatase activity and not result in cell death. In fact, cell cytotoxicity assays showed that the concentrations used for this study were safe and not toxic for the cells (Figure 3.9). Total RNA was extracted at 24 h, and *CYP19* transcript levels were compared to control (vehicle) treatment. NSAIDs and COX-2 selective inhibitors significantly decreased *CYP19* gene expression in SK-BR-3 cells relative to the control (vehicle) treatment (Figure 3.10). The COX-1 selective inhibitor, SC-560, also resulted in a significant decrease in expression suggesting that COX-1 might also be involved in the mechanism. No effect on the expression level of the housekeeping gene 18S rRNA was observed with any of the agents. These results support the aromatase activity results suggesting that COX inhibitors have an effect on aromatase.

To determine if similar results were observed in other cell lines, MCF-7 and MDA-MB-231 cells were treated with selected COX inhibitors. Indomethacin was selected from the group of non-selective inhibitors, NS-398 from the COX-2 selective inhibitors and SC-560 as the COX-1 selective inhibitor. Figure 3.11 shows similar decreases in *CYP19* gene expression in both MCF-7 and MDA-MB-231 cells for all three agents tested.
Figure 3.9: Effect of cyclooxygenase inhibitors and 7-αAPTADD on cell cytotoxicity. SK-BR-3 cells were treated with each of the agents at the indicated concentrations for 24 hours and cell viability was measured as described in the experimental section. The results were normalized against a control treatment with vehicle (DMSO); n = 6.
Figure 3.10: Real-time RT-PCR analysis of CYP19 mRNA expression in SK-BR-3 cells. Cells were treated for 24 hours with the indicated concentrations of agents and total RNA was isolated. Results are expressed as means of CYP19 (normalized to 18S rRNA) ± S.E.M. *, $P < 0.05$ vs. control by unpaired $t$ test, $n = 9$. 
Figure 3.11: Real-time RT-PCR analysis of CYP19 mRNA expression in MCF-7 and MDA-MB-231 cells. Cells were treated for 24 hours with the indicated concentrations of agents and total RNA was isolated. Results are expressed as means of CYP19 (normalized to 18S rRNA) ± S.E.M. *, P < 0.05 vs. control by unpaired t test, n = 9.
3.3.5. \textit{CYP19} Exon-Specific mRNA Expression by Real-Time PCR

PGE₂ is a powerful stimulator of aromatase activity and expression in human breast adipose stromal cells (23, 27, 32). PGE₂ interacts with two receptor subtypes in adipose stromal cells, EP₁ and EP₂. EP₂ is coupled to stimulation of cAMP formation, while EP₁ is coupled to protein kinase C (PKC) activation (32). This suggests that PGE₂ is capable of activating both PKA- and PKC-mediated signaling pathways. Exon-specific real-time PCR was performed to determine if these agents specifically affected expression through promoters I.3 and II, which are the two aromatase promoters directly involved in PKA- and PKC- activation through cAMP.

In a separate experiment, administration of exogenous PGE₂ (1µM) resulted in elevated transcript levels of \textit{CYP19} mRNA through promoter II by approximately 1.5 fold when compared to control in SK-BR-3 cells. In the tritiated water-release assay, treating SK-BR-3 cells with exogenous PGE₂ (1µM) confirmed a 1.5 fold increase in aromatase activity (0.0040 ± 0005 pmol/h/10⁶ cells). The NSAIDs, COX-1 and COX-2 selective inhibitors all demonstrated decreases in aromatase expression specific for promoter II (Figure 3.12). Aromatase expression is also driven through promoter I.3, the other promoter that is directly linked to the \textit{CYP19} switch in aromatase expression in breast tumors. The agents tested (COX-1 and COX-2 selective inhibitors) showed significant decreases in aromatase expression through this promoter (Figure 3.13). To study the possibility of other promoter regions being involved in the proposed mechanism, \textit{CYP19} promoter I.1 and I.4-specific mRNA expression was analyzed. As
expected, these agents had no effect on aromatase expression specific for I.1 (Figure 3.14). On the other hand, these agents showed a decrease in aromatase expression specific for promoter I.4 (Figure 3.15).
Figure 3.12: Real-time RT-PCR analysis of CYP19 promoter II mRNA expression in SK-BR-3 cells. Cells were treated for 24 hours with the indicated concentrations of agents and total RNA was isolated. Results are expressed as means of CYP19 (normalized to TBP) ± S.E.M. *, $P < 0.05$ vs. control by unpaired t test, n = 9.
Figure 3.13: Real-time RT-PCR analysis of CYP19 exon I.3 mRNA expression in SK-BR-3 cells. Cells were treated for 24 hours with the indicated concentrations of agents and total RNA was isolated. Results are expressed as means of CYP19 (normalized to TBP) ± S.E.M. *, $P < 0.05$ vs. control by unpaired $t$ test, $n = 9$. 
Figure 3.14: Real-time RT-PCR analysis of CYP19 exon I.1 mRNA expression in SK-BR-3 cells. Cells were treated for 24 hours with the indicated concentrations of agents and total RNA was isolated. Results are expressed as means of CYP19 (normalized to TBP) ± S.E.M. *, P < 0.05 vs. control by unpaired t test, n = 9.
Figure 3.15: Real-time RT-PCR analysis of CYP19 exon I.4 mRNA expression in SK-BR-3 cells. Cells were treated for 24 hours with the indicated concentrations of agents and total RNA was isolated. Results are expressed as means of CYP19 (normalized to TBP) ± S.E.M. *, $P < 0.05$ vs. control by unpaired t test, n = 9.
3.3.6. COX-2 mRNA Expression by Real-Time PCR

In the process of studying the effect of NSAIDs and COX-selective inhibitors on aromatase activity and expression, the effect of these drugs on COX-2 expression was also assessed. Analysis of COX-2 mRNA transcripts was performed using real-time PCR in SK-BR-3 cells. SK-BR-3 cells were treated with the indicated drugs for 24 h at a concentration of [50 µM]. Total RNA was extracted at 24 h, and COX-2 transcript levels were compared to control (vehicle) treatment. It was found that COX-2 expression was induced by ibuprofen, celecoxib and NS-398 (Figure 3.16). No effect on the expression level of the housekeeping gene 18S rRNA was observed with any of the agents. Similar results have been reported in the literature by other groups (33-35).
Figure 3.16: Real-time RT-PCR analysis of *COX-2* mRNA expression in SK-BR-3 cells. Cells were treated for 24 hours with the indicated concentration of agents and total RNA was isolated. Results are expressed as means of *COX-2* (normalized to 18S rRNA) ± S.E.M. *, *P* < 0.05 vs. control by unpaired *t* test, *n* = 9.
3.4. Discussion

Aromatase is a key enzyme in the synthesis of estrogens (1) and plays an important role in the process of breast carcinogenesis of hormone-dependent breast cancers (3). Cyclooxygenase has also been found to play a key role in this process (9, 36). Furthermore, PGE₂ increases aromatase activity through increases in cAMP (23, 27, 32), and COX enzymes are involved in the synthesis of prostaglandins. To study a possible mechanism of why NSAIDs exert chemopreventive and antitumor properties in human breast cancers, SK-BR-3 cells were treated with these agents and evaluated for aromatase activity and expression. Aromatase activity was decreased in SK-BR-3 cells by NSAIDs and cyclooxygenase selective inhibitors treatment in a dose-dependent manner. COX selective agents are more effective in suppressing aromatase activity, with significant lower IC₅₀ concentrations than those that required for NSAIDs (or non-selective COX inhibitors).

Treatment of SK-BR-3 cells with the various agents at concentrations at or near IC₅₀ values resulted in a decrease production of PGE₂, which supports our hypothesis that PGE₂ is involved in this mechanism. Real-time PCR analysis of aromatase gene expression demonstrated that changes in mRNA expression were consistent with enzyme activity data. The data from these experiments showed a significant decrease in mRNA levels when compare to control (vehicle) for all agents. The COX-1 selective inhibitor SC-560 resulted in a significant decrease in aromatase activity and expression in SK-BR-3 cells, suggesting that COX-1 may also produce prostaglandins that are involved in this mechanism. Although COX-1 has also been implicated in human breast cancers (9, 36), its levels are relatively constant. Aromatase expression in MCF-7 and MDA-MB-
231 cells is low compared to SK-BR-3 cells, treatment of these cells with COX inhibitors also resulted in a decrease in aromatase expression. These results show that COX inhibitors decrease aromatase mRNA expression and that these types of drugs may be potent therapeutic agents in the treatment of breast cancer.

The effect of NSAIDs and COX selective inhibitors on the exon I-specific promoters for aromatase also was investigated using real-time PCR. SK-BR-3 cells contain high abundance of promoter I.1, followed by promoters II and I.4 and relatively low abundance of promoter I.3. When SK-BR-3 cells were treated with NSAIDs, COX-1 and -2 selective inhibitors resulted in decreases in aromatase expression through promoter II. NS-398 showed the most significant effect followed by SC-58125, consistent with the enzyme activity data. Promoter I.3 is the other promoter involved in the promoter switch from normal breast to cancerous cells (5). Decreases in aromatase expression through promoter I.3 were observed with the COX-2 specific inhibitors and the COX-1 specific inhibitor SC-560 as well, even though the levels of promoter I.3 in SK-BR-3 cells are low. As expected, aromatase expression through promoter I.1 was not affected by any of the agents studied.

Promoter I.4 was then studied and once again all agents decreased aromatase expression through this promoter as well. The effect was not as significant as promoter II; nevertheless CYP19 expression through promoter I.4 was affected when treating cells with NSAIDs and COX specific inhibitors. Expression via promoter I.4 requires the action of glucocorticoids and class I cytokines, or TNFα (37). Other studies have shown that PGE2 regulates TNFα at the mRNA and protein level. The pleiotropic effect of prostaglandins in general may be playing a key role in this process. It is possible that the
decrease in prostaglandin production by COX specific inhibitors results in alterations of other biochemical pathways within these cells affecting glucocorticoid and/or class I cytokine action. This would result in a decrease in CYP19 expression through promoter I.4. Further studies are underway to better understand this process.

These results suggest that prostaglandin E\textsubscript{2} produced via cyclooxygenases may act locally in an autocrine fashion to increase the local biosynthesis of estrogen by the aromatase enzyme in hormone-dependent breast cancer development and lead to growth stimulation. Changes in transcript levels were associated with comparable changes in aromatase activity in breast cancer cells, suggesting that the effect of COX inhibitors on aromatase starts at the transcriptional level. In previous preliminary studies conducted in human placental microsomes, NSAIDs and the COX-2 specific inhibitors NS-398 and celecoxib failed to directly inhibit aromatase activity. Only at very high micromolar concentrations these agents showed signs of aromatase activity inhibition. Thus, the effect of NSAIDs and the COX selective inhibitors does not occur through the direct inhibition of the enzyme, but rather as a result of suppressing gene expression.

Based on our results, we propose the following model to explain the interrelationship between aromatase and COX enzymes. Higher levels of COX enzymes expression and increased activity would result in higher levels of PGE\textsubscript{2}. Elevated PGE\textsubscript{2} levels increase intracellular cAMP and result in increased aromatase expression via promoters I.3 and II. Higher levels of aromatase would lead to higher levels of estrogens, resulting in increased growth and development of the tumor by both paracrine and autocrine actions.
Our results show an increase in COX-2 mRNA expression in SK-BR-3 cells treated with NSAIDs and COX-2 selective inhibitors. Further, the different NSAIDs do not enhance COX-2 expression equally. This effect, even though is not well understood, may be a direct effect mediated through peroxisome proliferators-activated receptors (PPARs) (38) or a response to an apoptotic stimulus (39).

Elucidation of the interrelationship between aromatase and cyclooxygenases in breast carcinogenesis could facilitate targeting the enzymes as an effective strategy to prevent and treat breast cancer. We demonstrated that COX inhibitors decrease mRNA expression and aromatase enzyme activity through promoters I.3, I.4 and II. These results support the importance of prostaglandins such as PGE₂. Thus, the breast cancer tissue microenvironment can influence the extent of estrogen biosynthesis and metabolism, resulting in altered levels of hormonally active estrogens and therefore influencing breast tumor development and growth. Furthermore, COX-2 inhibitors suppress CYP19 expression through promoters I.3 and II in a tissue selective manner, without affecting CYP19 expression in other tissues that use different promoters, such as brain and bone. Therapy with currently available aromatase inhibitors and COX-2 inhibitors would affect several of these target pathways. The combination of an aromatase inhibitor with a COX-2 inhibitor may increase efficacy beyond the present treatments for postmenopausal hormone-dependent breast cancer.
3.5. References


CHAPTER 4

THE EFFECTS OF NS-398 ANALOGS ON AROMATASE EXPRESSION AND ACTIVITY IN BREAST CANCER AND ADIPOSE STROMAL CELLS

4.1. Introduction

Approximately 60% of all breast cancer cases are hormone-dependent, in which tumors depend of estrogen for growth. Researchers have found mainly two ways to treat these cases of breast cancer, by either blocking the mechanism of action of estrogens or by inhibiting their synthesis. For more than 30 years, the antiestrogen tamoxifen has been the mainstay of hormonal therapy in postmenopausal women with breast cancer. In the late 1970’s, researchers started to investigate the possibility of inhibiting the last step in the biosynthesis of estrogens and started focusing on aromatase inhibitors.

Even though aromatase inhibitors have been lately recognized as an effective therapy for the treatment of breast cancer patients with hormone-dependent disease, significant clinical side effects have been associated with them. The disadvantage is that these agents inhibit aromatase activity in a global fashion and could adversely impact sites where estrogen is required for normal function. Due to the side effects associated with the use of aromatase inhibitors, other approaches are being investigated to develop agents that can inhibit aromatase expression in a tissue-specific manner.
The concept of selective aromatase modulators (SAMs) is possible since aromatase expression in different tissues is regulated by different tissue-specific promoters and employs different signalling pathways. In breast cancer patients, aromatase expression switches from promoter I.4 to promoter I.3 and II (1, 2). Thus drugs that target promoters I.3 and II-driven expression of aromatase would be more useful as chemotherapeutic agents for the treatment of breast cancer.

The idea that COX-2 inhibition results in the suppression of aromatase in breast cancer is based on the fact that high levels of prostaglandins (primarily PGE₂) are found in or near the breast tumor (3). It has been reported that COX-2 overexpression leads to carcinogenesis and high incidence of metastasis (4, 5).

NS-398, N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide, is an anti-inflammatory and analgesic agent that inhibits selectively COX-2 without affecting COX-1, resulting in much less gastrointestinal problems. NS-398 is the first documented agent to have selective inhibition for COX-2 (6).

Research in our laboratory demonstrated that the effect of NS-398 on suppression of aromatase activity was significantly stronger than other NSAIDs and COX selective inhibitors (7). This difference suggests other possible mechanisms by which NS-398 may also be acting to suppress aromatase activity and mRNA expression. A small library of NS-398 analogs was synthesized and tested in breast cancer and normal cells to determine whether COX-2 inhibition is the only mechanism by which NS-398 suppress aromatase. The design of these compounds consisted of modifying the scaffold of NS-398 to ensure suppression of aromatase activity and avoid any effects on COX-2 activity. Previous studies have shown that while nimesulide inhibits COX-2 activity in
vitro, N-methylnimesulide fails to do so (8). N-Methylnimesulide was inactive in vitro but resulted in COX-2 inhibition in vivo, suggesting that this compound might be acting as a prodrug of nimesulide. This activity could be explained by metabolism of the product to generate nimesulide by deprotonation of the sulfonamide function. Our drug design rationale was to synthesize analogs of NS-398 and N-methyl NS-398, varying the cyclohexyloxy group present at the 2-position of the methanesulfonamide to test the importance of this bulky substituent on aromatase suppression.

4.2. Experimental

4.2.1. Chemicals, Biochemicals and Reagents

NS-398 analogs were synthesized by graduate student Bin Su. Radiolabeled [1\(\beta\)-\(^3\)H]-androst-4-ene-3,17-dione was obtained from NEN Life Science Products, Boston, MA. NS-398 was purchased from Cayman Chemical, Ann Arbor, MI. Dexamethasone, forskolin and 12-O-tetradecanoylphorbol-1, 3-acetate (TPA) were purchased from Sigma, St. Louis, MO. Trypsin, TRIzol, and all enzymes were obtained from Invitrogen, Carlsbad, CA. Radioactive samples were counted on a LS6800 liquid scintillation counter, Beckman, Palo Alto, CA. Mixture 3a70B was obtained from Research Prospect International Corp, Mount Prospect, IL.

4.2.2. Cell Culture

Adipose stromal cells were isolated from patient breast tissues obtained through the Tissue Procurement Shared Resource Program of the Ohio State University Comprehensive Cancer Center. Samples (~ 1.0 g each) were obtained from cancer-free female patients undergoing reductive mammoplasty. Samples were aseptically minced
and incubated with 1% collagenase for 16 hours at 37°C. The supernatant was recovered and centrifuged at 1500 x g for 5 min. Lipid accumulated at the top was removed and the supernatant containing epithelial cells ad fibroblasts was transferred into a T-75 flask containing DMEM/F12 media supplemented with 10% fetal bovine serum, l-glutamine (5 mM), and gentamicin (0.025%). Under these conditions, any contaminating epithelial component will not plate and will be washed away with regular media changes. Cultures were grown until they reached near confluence and then used in the experiments.

MDA-MB-231 and SK-BR-3 cell lines were obtained from ATCC, Rockville, MD. Cell cultures were maintained in phenol red-free custom media (MEM, Earle’s salts, 1.5x amino acids, 2x non-essential amino acids, l-glutamine, 1.5x vitamins (Life Technologies, Carlsbad, CA)) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine and 20 mg/l gentamycin. Fetal calf serum was heat inactivated for 30 min in a 56°C water bath before use. Cell cultures were grown at 37°C, in a humidified atmosphere of 5% CO2 in a Hereaus CO2 incubator. For all experiments, adipose stromal, MDA-MB-231 and SK-BR-3 cells were plated in either 100 mM plates, 60 mM plates, or 12-well plates and grown to subconfluency. Before treatment, the media was changed to a defined one containing DMEM/F12 media (Sigma) with 1.0 mg/ml human albumin (OSU Hospital Pharmacy), 5.0 mg/l human transferin and 5.0 mg/l bovine insulin.

4.2.3. Tritiated Water-Release Assay in Cells

Measurement of aromatase enzyme activity was based on the tritium water release assay (9). SK-BR-3 cells in 60 mM plates were treated with 0.1% DMSO (control) and the different compounds for 24 h. In the case of adipose stromal cells, cells
were stimulated with 200 nM dexamethasone or 10 nM TPA + 10 µM forskolin for 24 h prior to the addition of the agents. Then, cells were incubated for 6 hours with fresh media along with 50 nM androstenedione including 2 µCi [1β-3H]-androst-4-ene-3,17-dione. Subsequently, the reaction mixture was removed, and proteins were precipitated using 10% trichloroacetic acid at 42°C for 20 min. After a brief centrifugation, the media was extracted three times with an equal amount of chloroform to extract unused substrate and further dextran-treated charcoal. After centrifugation, a 200-µl aliquot containing the product was counted in 5 ml of liquid scintillation mixture. Results were corrected for blanks and for the cell contents of culture flasks, and results were expressed as picomoles of 3H2O formed per hour incubation time per million live cells (pmol/h/10⁶ cells).

4.2.4. Diphenylamine DNA Assay

To determine the amount of viable cells in each flask, the cells were trypsinized and analyzed using the diphenylamine DNA assay adapted to a 96-well plate (9, 10). DNA standards (0 – 30 µg) were prepared using double-stranded DNA reconstituted in PBS and added in triplicates directly to the wells. A uniform cell suspension was prepared from the 60 mM plates in 30 µl PBS, and 10 µl of the unknown samples were added in triplicates to separate wells. A solution of 0.16% acetaldehyde in water was prepared and mixed at a 1:5 ratio with perchloric acid (20% vol/vol). This solution (60 µl) was added to each well along with 100 µl of a 4% diphenylamine solution in glacial acetic acid. The plates were incubated at 37°C for 24 hours and the OD₅₉₅ was measured.
using a microplater reader. The DNA concentration was determined by extrapolation to
the standard curve and the amount of cells/flask was calculated using the equation: 1 cell
= 7 pg DNA.

4.2.5. Triated-Water Release Assay in Human Placental Microsomes

Inhibition of human placental aromatase was determined by monitoring the
amount of $^3$H$_2$O released as the enzyme converts [1$\beta-^3$H] androst-4-ene-3,17-dione to
estrone. Concentrations of NS-398 ranging from 100 µM to 0.78 µM were evaluated.
Aromatase activity assays were carried in 0.1 M potassium phosphate buffer (pH 7.0)
with 5% propylene glycol. All samples contained a NADPH regenerating system
consisting of 2.85 mM glucose-6-phosphate, 1.8 mM NADP$^+$ and 1.5 units of glucose-6-
phosphate dehydrogenase. Samples contained 100 nM androst-4-ene-3,17-dione
(400,000 ~ 450,000 dpm). Reactions were initiated with the addition of 50 µg
microsomal protein. The total incubation volume was 2.0 mL. Incubations were allowed
to proceed for 15 minutes in a shaking water bath at 37°C. Reactions were quenched by
the addition of 2.0 ml of chloroform. Samples were then vortexed and centrifuged for 5
minutes and the aqueous layer was removed. The aqueous layer was subsequently
extracted twice in the same manner with 2.0 ml chloroform. A 0.5 ml aliquot of the final
aqueous layer was combined with 5 ml 3a70B scintillation cocktail and the amount of
radioactivity was determined. Each sample was run in triplicate and background values
were determined with microsomal protein inactivated by boiling.

4.2.6. Enzyme Immunoassay of PGE$_2$

To study PGE$_2$ synthesis in cell culture media, experiments were performed in
12-well plates. An aliquot of MDA-MB-231 cells (150,000 cells) was added to each well
and plates were incubated overnight to allow the cells to adhere to the plates. After this time, cells were serum starved in defined media for 24 h. This step was followed by replacement of media with fresh media containing either vehicle (DMSO) or 25 µM of the indicated agents. After 24 h incubation at 37°C the media were collected and the amount of PGE₂ was determined by ELISA (Cayman Chemical) according to the protocol provided by the manufacturer. PGE₂ concentration was normalized to total protein. Total proteins were extracted from adhered cells by 30 min treatment with 0.5 M NaOH at room temperature and shaking. Protein concentrations in these extracts were determined using a protein assay method (Bio-Rad Laboratories, Inc., Hercules, CA).

### 4.2.7. RNA Extraction

Total RNA was isolated using the TRIzol reagent according to the manufacturer’s protocol. Total RNA pellets were dissolved in DNase, RNase-free water and quantitated using a spectrophotometer. The quality of RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide; the 18S and 28S rRNA bands were visualized under ultraviolet light.

### 4.2.8. cDNA Synthesis

Isolated total RNA (2 µg) was treated with DNase I, Amplification grade, according to the recommended protocol to eliminate any DNA before reverse transcription. Treated total RNA was denatured at 65°C for 5 min in the presence of 2.5 ng/µl random hexamers and 0.5 mM dNTP mix. The samples were snap-cooled on ice and centrifuged briefly. Complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase according to the recommended protocol. Briefly, the
reactions were conducted in the presence of 1X First-Strand Buffer and 20 mM DTT at 42°C for 50 min and consequently inactivated at 70°C for 15 min. The cDNA generated was used as a template in real-time PCR reactions.

4.2.9. Real-time PCR

Real-time PCR was performed using the Opticon™ 2 system from MJ Research, Waltham, MA. For both CYP19 and COX-2 genes the PCR reaction mixture consisted of Taqman® Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 600 nM of each primer (Invitrogen) (Table 4.1), 250 nM Taqman probe, 18S rRNA (Applied Biosystems) and 2.5 µl of each RT sample in a final volume of 25 µl. Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min.
### Table 4.1: Oligonucleotide primer and probe sequences for real-time PCR.

Total *CYP19* and COX-2 gene was analyzed using Taqman methodology and a sequence specific fluorogenic probe. (S) = Sense; (A) = Antisense

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (S)</th>
<th>Primer (A)</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>CYP19</em></td>
<td>5'-TGT CTC TTT GTT CTT CAT GCT ATT TCT C-3'</td>
<td>5'-TCA CCA ATA ACA GTC TGG ATT TCC-3'</td>
<td>6FAM 5'-TGC AAA GCA CCC TAA TGT TGA AGA GGC AAT-3'TAMRA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>5'- GAA TCA TTC ACC AGG CAA ATT G-3'</td>
<td>5'- TCT GTA CTG CGG GTG GAA CA-3'</td>
<td>6FAM 5'-TGG CAG GGT TGC TGG TAG GA -3’TAMRA</td>
</tr>
</tbody>
</table>

4.2.10. Statistical Analysis

Statistical and graphical information was determined using GraphPad Prism software (GraphPad Software Incorporated, San Diego, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA). Determination of IC\textsubscript{50} values were performed using nonlinear regression analysis. Statistically significant differences were calculated with the two-tailed unpaired Student’s $t$-test and $P$ values reported at 99% confidence intervals.
4.3. Results

4.3.1. Effect of NS-398 analogs on aromatase activity in breast cancer cells

A group of NS-398 analogs (Figure 4.1) was synthesized to determine if COX-2 inhibition is the only mechanism by which NS-398 suppresses aromatase in breast cancer cells. Another aim was to study the importance of the cyclohexyloxy group present at the 2-position of NS-398 to inhibit aromatase activity. We performed a 25 µM screening assay in SK-BR-3 breast cancer cells to study the effect of these compounds on aromatase activity (Figure 4.2A). All compounds tested except for compounds SU-3 and SU-5, showed suppression of aromatase activity. A 1 µM screening assay was performed to study the effect of these agents on aromatase activity at a lower concentration (Figure 4.2B). The selective COX-2 inhibitor NS-398 was used as a positive control. Compounds NS-398, SU-9 and SU-20 showed the best results at suppressing aromatase activity showing over 70% reduction when compared to control. Other compounds such as SU-24, SU-12, SU-13, SU-21, SU-17, and SU-11 showed significant suppression of aromatase activity in cells to a lesser extent.

Compounds NS-398 and SU-9 were evaluated for aromatase activity suppression in a dose-dependent manner. The concentrations used for this study ranged from 0.0025 to 50 µM. Aromatase activity was suppressed in a dose-dependent manner by both NS-398 and SU-9 (Figure 4.3). The IC₅₀ values for each compound were calculated using a non-linear regression curve and found to be 0.38 ± 0.20 for NS-398 and 0.44 ± 0.40 for SU-9.
Figure 4.1: Chemical structures of NS-398 and the analogs used in this study.
Figure 4.2: Suppression of aromatase activity in SK-BR-3 breast cancer cells by NS-398 analogs. SK-BR-3 cells were treated with the indicated compounds at (A) 25 µM and (B) 1 µM. Aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.003 pmol/hr/10$^6$ cells. Each data bar represents the mean results of three independent determinations. *, $P < 0.01$ vs. control by unpaired $t$ test, $n = 3$. 
Figure 4.3: IC₅₀ study for the effect of NS-398 and compound SU-9 on aromatase activity in SK-BR-3 cells. SK-BR-3 cells were treated with NS-398 (◊), and SU-9 (■), and aromatase activity was measured as described in the methodology section. Values are expressed as picomoles ³H₂O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.003 pmol/h/10⁶ cells. Each data represents the mean results of three independent determinations.
4.3.2. Effect of NS-398 on aromatase activity in human placental microsomes

To ensure that the aromatase inhibitory effect observed in cells was not due to direct enzyme inhibition, NS-398 was tested in human placental microsomes (Figure 4.4). The IC\textsubscript{50} value for NS-398 was calculated using non-linear regression analysis and found to be 26.5 µM. When comparing this value against the IC\textsubscript{50} value obtained in SK-BR-3 cells (1 µM), our results suggest that the effect on aromatase suppression in cells was not due to a direct inhibition of the aromatase enzyme.

![Figure 4.4: IC\textsubscript{50} study for the effect of NS-398 on aromatase activity in human placental microsomes. Microsomes were treated with NS-398 and aromatase activity was measured as described in the methodology section. The results were normalized against a control treatment with vehicle. Each data represents the mean results of three independent determinations.](image)
4.3.3. Effect of NS-398 analogs on COX-2 activity

Nimesulide is a COX-2 selective inhibitor with a similar chemical structure to NS-398. A study showed that adding a methyl group at the methasulfonamide of nimesulide failed to inhibit COX-2 activity \textit{in vitro} (8). We evaluated the effect of a selected group of compounds on COX-2 activity by measuring the production of prostaglandin E$_2$ in MDA-MB-231 cells. MDA-MB-231 cells were chosen for this study due to the high activity and expression of COX-2 when compared to other cell lines (7, 11). Compounds containing a methyl group at the methasulfonamide showed no inhibition of COX-2 activity (SU-9, SU-12, SU-21 and SU-23), whereas those that did not have the methyl group resulted in inhibition (NS-398, SU-17 and SU-20) (Figure 4.5). Compound SU-13 was the exception. It is possible that the extra methyl on SU-13 at the cyclohexyloxy group makes the compound too large to fit properly in the active site of the enzyme resulting in the lost COX-2 inhibitory activity.
Figure 4.5: Effect of NS-398 analogs on prostaglandin E₂ production in MDA-MB-231 cells. Cells were treated for 24 hours with the 25 µM of the indicated agents. Results are expressed as means of the concentration of PGE₂ produced/µg protein ± S.E.M. *, $P < 0.01$ vs. control by unpaired $t$ test, n = 6.
4.3.4. Effect of NS-398 and SU-9 on aromatase activity on SK-BR-3 cells stimulated with TPA and forskolin

SK-BR-3 cells were treated with 1 μM PGE₂, the combination of 10 nM TPA and 25 μM forskolin, and 200 nM dexamethasone to evaluate which pathways are involved in aromatase expression and activity in this cell line (Figure 4.6). Addition of PGE₂ resulted in an increase in aromatase activity by approximately 1.25-fold, whereas addition of TPA and forskolin resulted in a two-fold increase. The synthetic glucocorticoid, on the other hand, failed to increase aromatase activity in this cell line (Figure 4.7).

SK-BR-3 cells were stimulated with a combination of TPA and forskolin for 24 hours. These cells were then treated with NS-398 and compound SU-9 to study their effect on aromatase activity (Figure 4.8). The addition of NS-398 and SU-9 brought the aromatase activity levels of stimulated SK-BR-3 cells back to control levels, suggesting that PKA and PKC pathways may be involved in the mechanism of action for these types of compounds.
Figure 4.6: Chemical structures of the aromatase activity stimulators used in this study.
Figure 4.7: Aromatase activity stimulation of SK-BR-3 cells through PKA and PKC pathways. SK-BR-3 cells were treated with 1 μM PGE$_2$, the combination of 10 nM TPA+25 μM Forskolin, and 200 nM dexamethasone for 24 hours. Aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.003 pmol/hr/10$^6$ cells. Each data bar represents the mean results of three independent determinations. *, $P < 0.01$ vs. control by unpaired $t$ test, $n = 3$. 
Figure 4.8: Effect of NS-398 and SU-9 on aromatase activity through PKA and PKC pathways in breast cancer epithelial cells. SK-BR-3 cells were stimulated with 10 nM TPA + 25 µM forskolin for 24 hours. After the first incubation, cells were treated with TPA and forskolin in addition to 2 µM of NS-398 and SU-9. Aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.003 pmol/hr/10$^6$ cells. Each data bar represents the mean results of three independent determinations. *, $P < 0.01$ vs. control by unpaired $t$ test, n = 3. #, $P < 0.01$ vs. TPA + forskolin treatment by unpaired $t$ test, n = 3.
4.3.5. Effect of NS-398 and SU-9 on aromatase activity on human breast adipose stromal cells stimulated with TPA + forskolin and dexamethasone

Clinical studies have mainly focused on the expression of aromatase in tumor tissues. While most studies have shown that the concentration of estradiol is higher in the tumor tissue than in the normal areas (12), some studies have shown that stromal cells may be the main site of aromatase in breast cancers (13, 14). We believe that the breast microenvironment is such a complex system, that paracrine pathways are as important as autocrine pathways in the process of breast carcinogenesis. A selective aromatase modulator would target not only the breast cancer cells but also the neighboring adipose stromal cells. For this reason, the effect of NS-398 and its analogs were studied in breast adipose stromal cells.

The levels of aromatase activity present in the breast adipose stromal cells were negligible compared to the levels seen in SK-BR-3 cells. However, the addition of TPA and forskolin resulted in an approximately 9-fold increase in aromatase activity (Figure 4.9). These cells were then treated with 2 μM of NS-398 and compound SU-9 to study their effect on aromatase activity. These compounds resulted in a 1.5- and 1.75-fold, respectively, reduction of aromatase activity in adipose stromal cells supporting the involvement of PKA and PKC pathways the mechanism of action of these compounds.

Dexamethasone, at a concentration of 200 nM, resulted in a 55-fold increase in aromatase activity in adipose stromal cells. The addition of 2 μM NS-298 and SU-9 to these cells failed to decrease the levels of aromatase activity suggesting that exon I.4 mediated pathways might not be involved in the mechanism of action of these types of agents (Figure 4.10).
Figure 4.9: Effect of NS-398 and SU-9 on aromatase activity through PKA and PKC pathways in breast adipose stromal cells. Adipose stromal cells were stimulated with 10 nM TPA + 10 µM forskolin for 24 hours. After the first incubation, cells were treated with TPA and forskolin in addition to 2 µM of NS-398 and SU-9. Aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.000125 pmol/hr/10$^6$ cells. Each data bar represents the mean results of three independent determinations. *, $P < 0.01$ vs. control by unpaired $t$ test, n = 3. #, $P < 0.01$ vs. TPA + forskolin treatment by unpaired $t$ test, n = 3.
Figure 4.10: Effect of NS-398 and SU-9 on aromatase activity through exon I.4 mediated pathways in breast adipose stromal cells. Adipose stromal cells were stimulated with 200 nM dexamethasone for 24 hours. After the first incubation, cells were treated with 200 nM dexamethasone in addition to 2 µM of NS-398 and SU-9. Aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.000125 pmol/hr/10$^6$ cells. Each data bar represents the mean results of three independent determinations. *, $P < 0.01$ vs. control by unpaired $t$ test, n = 3.
4.3.6. Effect of NS-398 analogs on CYP19 and COX-2 mRNA expression

Analysis of CYP19 and COX-2 mRNA transcripts was performed using real-time PCR in order to determine whether the decrease in aromatase activity by the NS-398 analogs was due to a down-regulation of aromatase expression at the transcriptional level. It has been shown that treating SK-BR-3 cells with NS-398 significantly decreased CYP19 gene expression relative to control (vehicle) levels (7). A 25 µM screening assay showed that compounds, NS-398, SU-9, SU-21, SU-23 and SU-20, significantly decreased CYP19 gene expression relative to control levels in SK-BR-3 cells after 24 hours (Figure 4.11).

In a similar experiment, the effect of these agents on COX-2 mRNA expression was evaluated. Despite the inhibition of prostaglandin synthesis, the protein and mRNA levels of COX-2 have been shown to increase after the addition of COX-2 inhibitors (15). The treatment of SK-BR-3 cells with NS-398 and SU-9 resulted in an increase COX-2 mRNA expression, whereas treating cells with compounds SU-23 and SU-20 resulted in a decreased expression of COX-2 (Figure 4.12). No effect on the expression level of the housekeeping gene 18S rRNA was observed with any of the compounds tested.
Figure 4.11: Real-time RT-PCR analysis of CYP19 mRNA expression in SK-BR-3 cells. Cells were treated for 24 hours with 25 µM of the indicated agents and total RNA was isolated. Results are expressed as means of CYP19 (normalized to 18S rRNA) ± S.E.M. *, P < 0.05 vs. control by unpaired t test, n = 9.
Figure 4.12: Real-time RT-PCR analysis of COX-2 mRNA expression in SK-BR-3 cells. Cells were treated for 24 hours with 25 µM of the indicated agents and total RNA was isolated. Results are expressed as means of COX-2 (normalized to 18S rRNA) ± S.E.M. *, P < 0.05 vs. control by unpaired t test, n=9.
4.4. Discussion

The need for better treatments for breast cancer has resulted in researchers examining alternative approaches. In an attempt to investigate the interrelationships between cyclooxygenases and aromatase as a possible mechanism by which COX-2 inhibitors exert their beneficial properties in breast cancer, our group studied the effect of these agents on aromatase activity and expression in SK-BR-3 cells. Our results demonstrated that COX inhibitors decrease aromatase mRNA expression and activity, and the selective COX-2 inhibitor NS-398 showed a stronger inhibitory activity than the rest of the agents studied (7).

NS-398 has been shown to induce apoptosis in colon cancer cells by activation of the caspase-9, caspase-3, and poly(ADP-ribose) polymerase components of the cytochrome pathway (16), and also via up-regulation of 15-lipoxygenase-1 in the absence of COX-2 activity (17). NS-398 effectively suppressed NF-κB activity in hepatoma cells (18). Nakatsugi and co-workers demonstrated a clear chemopreventive efficacy of the selective COX-2 inhibitor, nimesulide, against PhIP-induced mammary gland carcinogenesis in rats (19).

A library of NS-398 analogs was synthesized and evaluated for their effect on prostaglandin E$_2$ production, aromatase activity and expression in breast cancer and normal adipose stromal cells. The library was composed of pairs of compounds in which the hydrogen of the methanesulfonamide of one of the compounds was substituted for a methyl group.

All the compounds tested, with the exception of **SU-3** and **SU-5**, suppressed aromatase activity at a concentration of 25 μM in SK-BR-3 cells by almost 70%. In an
effort to discriminate among compounds in this library, a lower concentration screening assay was performed at a 1 µM. Our results suggest that the bulky group on position 2 is important for the suppression of aromatase activity. Compounds containing a methoxy (SU-3 and SU-5), an isopropyl (SU-16 and SU-19), or an extremely long chain (SU-22, SU-25 and SU-26), lost the ability to suppress aromatase activity. The possibility of these agents inhibiting the aromatase enzyme directly is unlikely since NS-398 failed to inhibit aromatase activity in human placental microsomes at the concentrations tested in our experiments.

The best four pairs of compounds – NS-398/SU-9, SU-13/SU12, SU-17/SU-21 and SU-20/SU-23 - were chosen to evaluate their ability to inhibit COX-2 activity in breast cancer cells. The NS-398 analogs containing a methyl group at the methasulfonamide showed no inhibition in the production of prostaglandin E₂ suggesting that COX-2 activity was unaffected. These results proved our hypothesis and our strategy to design this library of compounds.

Figure 4.13 shows the important regulation pathways for aromatase in the normal human breast and in the breast cancer tumor. Aromatase expression can be stimulated by glucocorticoids and class I cytokines through promoter I.4. Class I cytokines activate the JAK1/STAT3 pathway and glucocorticoids bind to the glucocorticoid receptor (GR), which dimerizes and binds to a glucocorticoid response element (GRE) upstream of promoter I.4. The synthetic glucocorticoid dexamethasone (DEX) can also bind to the GR and activate aromatase expression through promoter I.4. Aromatase expression can also be stimulated by prostaglandin E₂ (PGE₂) through promoters I.3 and II. PGE₂ binds to receptor EP₁ resulting in formation of diacylglycerol (DAG) and activation of protein
kinase C (PKC). PGE2 binds to receptor EP2 resulting in formation of cAMP and activation of PKA. Both of these kinases presumably phosphorylate a cAMP-response element binding protein (CREB), resulting in the activation of aromatase expression through promoters I.3 and II. 12-O-Tetradecanoylphorbol-13-acetate (TPA) stimulates phospholipase C (PLC), and forskolin stimulates adenylyl cyclase, resulting in aromatase expression activation.

PGE2 binds to cell surface receptors EP1, EP2, EP3, and EP4. Activation of EP2 and EP4 receptors leads to increased intracellular cAMP levels. However, activation of EP3 receptors leads to a decrease in intracellular cAMP (20). This could explain why PGE2 stimulated aromatase activity to a lesser extent than the phorbol ester and forskolin combination in SK-BR-3 cells. In order to stimulate aromatase through PKA and PKC pathways, the combination of TPA and forskolin was used instead of PGE2 for the subsequent experiments. Our results showed an increase in aromatase activity by 9-fold after the addition of 10 nM TPA and 25 μM forskolin to SK-BR-3 cells.

We observed a slight morphological change under the microscope on adipose stromal cells stimulated with TPA and forskolin when compared to unstimulated cells. The cells became less elongated and more rounded throughout the treatment, but no cells became weak enough to detach from the petri dishes. Other groups have reported the same morphological change for cells stimulated with 10 μM forskolin (21). The combined stimulation of both PKA and PKC pathways with forskolin plus phorbol esters results in maximal expression from promoter II (22, 23). Our results show that the
combination of TPA and forskolin stimulated aromatase activity in stromal cells. These results confirm the importance of promoters I.3 and II in the expression aromatase to maintain the levels of estrogens high in the breast microenvironment.

In the normal breast, aromatase is mainly localized in the adipose tissue cells which expression is mainly directed by promoter I.4 (22). Dexamethasone, a synthetic glucocorticoid, showed a tremendous stimulation of aromatase activity in breast adipose stromal cells, whereas the same treatment failed to stimulate activity on SK-BR-3 cells. This was expected since promoter I.4 is the main promoter driving the expression of aromatase in adipose tissue. These results suggest that a signalling pathway involving cAMP, but not glucocorticoids, regulates the expression of aromatase activity in this cell line.

NS-398 and its analog SU-9 effectively suppressed aromatase activity in both breast cancer and normal adipose stromal cells stimulated with the combination of a phorbol ester (TPA) and forskolin. The same treatment failed to suppress aromatase activity on adipose stromal cells previously stimulated with the synthetic glucocorticoid dexamethasone. Treatment of cells with compounds SU-9, SU-23, SU-20 and NS-398 also resulted in a significant decrease in CYP19 gene expression, suggesting that the effect seen in aromatase activity starts at the transcriptional level.

Our results suggest that these compounds might be acting through PKA and PKC pathways, suggesting that the two promoters involved in their mechanism of action consist of I.3 and II. It is possible that promoter I.4 is not involved in the mechanism of action of these compounds since these agents do not suppress aromatase activity after stimulation with dexamethasone. These results open an exciting and promising field of
research in which NS-398 analogs could be developed into the first generation of selective aromatase modulators for the chemoprevention of breast cancer. The disadvantage of the aromatase inhibitors is that they inhibit aromatase activity in a global fashion and this could impact sites where estrogen is required for normal function. Previously we showed that NS-398 can decrease aromatase mRNA expression through promoters I.3 and II and not I.1, suggesting that these compounds show selectivity for aromatase expression and activity through the promoters involved in the process of breast carcinogenesis (7).

Thus, we were able to investigate and propose other possible mechanisms of action for NS-398 in breast cancer that does not involve the direct inhibition of COX-2. These results support the idea of developing NS-398 analogs as selective aromatase modulators for the chemoprevention and treatment of hormone-dependent breast cancer.
Aromatase expression can be stimulated by glucocorticoids and class I cytokines through promoter I.4. The synthetic glucocorticoid dexamethasone (DEX) can also bind to the GR and activate aromatase expression through promoter I.4. PGE$_2$ binds to receptor EP$_1$ and EP$_2$ resulting in the activation of PKC and PKA pathways. Both of these kinases presumably phosphorylate a cAMP-response element binding protein (CREB), resulting in the activation of aromatase expression through promoters I.3 and II. 12-O-Tetradecanoylphorbol-13-acetate (TPA) stimulates phospholipase C (PLC), and forskolin stimulates adenylyl cyclase, resulting in aromatase expression activation. The scheme was adapted from Simpson et al (24).
4.5. Acknowledgements

This study was conducted in collaboration with Bin Su, who synthesized the synthetic NS-398 analogs and assisted in the process of obtaining some of the biological data as well.

I would also like to thank the Tissue Procurement Shared Resource Program of the Ohio State University Comprehensive Cancer Center for supplying the breast tissue specimens.

4.6. References


CHAPTER 5

THE EFFECTS OF PPARγ LIGANDS ON AROMATASE IN BREAST CANCER AND ADIPOSE STROMAL CELLS

5.1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are a member of the orphan nuclear receptor superfamily. There are three subtypes; PPARα, PPARβ/δ and PPARγ. PPARγ is highly expressed in adipocytes and macrophages and is involved in adipocyte differentiation, lipid storage, and glucose homeostasis (1, 2). Ligands for PPARγ include the synthetic thiazolidinediones troglitazone and rosiglitazone, as well as 15d-PGJ2 (15-deoxy-Δ (12,14)-Prostaglandin J2), proposed to be a naturally occurring ligand.

In breast tissue, agonists of PPARγ have been shown to inhibit cell growth (3), inhibit aromatase activity in adipose tissue (4, 5), inhibit estrogen receptor activity (6) and play a role in tumor regression (7). These compounds have been proposed as anticancer therapeutics for breast cancer and this has been tested in clinical trials (8). The role of PPARγ in breast cancer is evident, but the mechanism of action remains unclear.

Troglitazone and the RXR ligand LG10305 inhibited aromatase expression via promoter II of the CYP19 gene in human adipose stromal cells (5). It has also been found
that troglitazone in combination with LG10305 inhibit aromatase expression in cultured breast adipose stromal cells via promoter I.4 (4, 5). Studies have shown that normal breast tissues contain more than 2-fold higher levels of PPARγ protein compared to tumors (9), and the levels decrease even more in the case of metastases (10). Down-regulation of PPARγ expression and lower tissue levels of 15d-PGJ2 have been identified as markers of breast cancer risk.

The roles of nuclear receptor systems for the aromatase activity in aromatase-expressing tissue or cells have not been fully investigated. It is suggested that PPARs are involved in the process of breast cancer development and progression. It has also been implicated that PPARs regulate aromatase at the transcriptional level. This study focuses on the effect of PPARγ agonists and antagonists on aromatase activity and mRNA expression in breast cancer and adipose stromal cells. Although some work has been done on breast adipose stromal cells and other tissues, little work has been done on breast cancer cells. By figuring out the regulatory pathways involved between PPARγ and aromatase, and COX-2, we may help decipher new mechanisms occurring at the molecular level in the breast microenvironment. This data, together with the COX-2-aromatase interrelationship data discussed earlier, could result in possible new ways to prevent and/or treat breast cancer.

5.2. Experimental

5.2.1. Chemicals, Biochemicals and Reagents

Radiolabeled [1β-3H]-androst-4-ene-3,17-dione was obtained from NEN Life Science Products, Boston, MA. Ciglitazone, rosiglitazone, troglitazone, BADGE and
GW9662 were obtained from Cayman Chemical, Ann Arbor, MI. Dexamethasone, forskolin, 12-O-tetradecanoylphorbol-1, 3-acetate (TPA) and 9-cis-retinoic acid were purchased from Sigma, St. Louis, MO. Trypsin, TRIzol, and all enzymes were obtained from Invitrogen, Carlsbad, CA. Radioactive samples were counted on a LS6800 liquid scintillation counter, Beckman, Palo Alto, CA. Mixture 3a70B was obtained from Research Prospect International Corp, Mount Prospect, IL.

5.2.2. Cell Culture

MCF-7 and SK-BR-3 cell lines were obtained from ATCC, Rockville, MD. Cell cultures were maintained in phenol red-free custom media (MEM, Earle’s salts, 1.5x amino acids, 2x non-essential amino acids, L-glutamine, 1.5x vitamins (Life Technologies, Carlsbad, CA)) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 20 mg/l gentamycin. Fetal calf serum was heat inactivated for 30 min in a 56°C water bath before use. Cell cultures were grown at 37°C, in a humidified atmosphere of 5% CO₂ in a Hereaus CO₂ incubator. For all experiments, adipose stromal and SK-BR-3 cells were plated in either 100 mM plates or 60 mM plates, and grown to subconfluency. Before treatment, the media was changed to a defined one containing DMEM/F12 media (Sigma) with 1.0 mg/ml human albumin (OSU Hospital Pharmacy), 5.0 mg/l human transferin and 5.0 mg/l bovine insulin.

Adipose stromal cells were isolated from patient breast tissues obtained through the Tissue Procurement Shared Resource Program of the Ohio State University Comprehensive Cancer Center. Samples (~ 1.0 g each) were obtained from cancer-free female patients undergoing reductive mammoplasty. Samples were aseptically minced and incubated with 1% collagenase for 16 hours at 37°C. The supernatant was recovered
and centrifuged at 1500 x g for 5 min. Lipid accumulated at the top was removed and the supernatant containing epithelial cells and fibroblasts was transferred into a T-75 flask containing DMEM/F12 media supplemented with 10% fetal bovine serum, l-glutamine (5 mM), and gentamicin (0.025%). Under these conditions, any contaminating epithelial component will not plate and will be washed away with regular media changes. Cultures were grown until they reached near confluence and then used in the experiments.

5.2.3. Tritiated Water-Release Assay

Measurement of aromatase enzyme activity was based on the tritium water release assay (11). SK-BR-3 cells in 60 mM plates were treated with 0.1% DMSO (control) and the different compounds for 24 h. In the case of adipose stromal cells, cells were stimulated with 200 nM dexamethasone or 10 nM TPA + 10 μM forskolin for 24 h prior to the addition of the agents. Then, cells were incubated for 6 hours with fresh media along with 50 nM androstenedione including 2 μCi [1β-3H]-androst-4-ene-3,17-dione. Subsequently, the reaction mixture was removed, and proteins were precipitated using 10% trichloroacetic acid at 42°C for 20 min. After a brief centrifugation, the media was extracted three times with an equal amount of chloroform to extract unused substrate and further dextran-treated charcoal. After centrifugation, a 200-μl aliquot containing the product was counted in 5 ml of liquid scintillation mixture. Results were corrected for blanks and for the cell contents of culture flasks, and results were expressed as picomoles of ³H₂O formed per hour incubation time per million live cells (pmol/h/10⁶ cells).
5.2.4. Diphenylamine DNA Assay

To determine the amount of viable cells in each flask, the cells were trypsinized and analyzed using the diphenylamine DNA assay adapted to a 96-well plate (11, 12). DNA standards (0 – 30 µg) were prepared using double-stranded DNA reconstituted in PBS and added in triplicates directly to the wells. A uniform cell suspension was prepared from the 60 mM plates in 30 µl PBS, and 10 µl of the unknown samples were added in triplicates to separate wells. A solution of 0.16% acetaldehyde in water was prepared and mixed at a 1:5 ratio with perchloric acid (20% vol/vol). This solution (60 µl) was added to each well along with 100 µl of a 4% diphenylamine solution in glacial acetic acid. The plates were incubated at 37°C for 24 hours and the OD_{595} was measured using a microplater reader. The DNA concentration was determined by extrapolation to the standard curve and the amount of cells/flask was calculated using the equation: 1 cell = 7 pg DNA.

5.2.5. RNA Extraction

Total RNA was isolated using the TRIzol reagent according to the manufacturer’s protocol. Total RNA pellets were dissolved in DNase, RNase-free water and quantitated using a spectrophotometer. The quality of RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide; the 18S and 28S rRNA bands were visualized under ultraviolet light.

5.2.6. cDNA Synthesis

Isolated total RNA (2 µg) was treated with DNase I, Amplification grade, according to the recommended protocol to eliminate any DNA before reverse transcription. Treated total RNA was denatured at 65°C for 5 min in the presence of 2.5
ng/µl random hexamers and 0.5 mM dNTP mix. The samples were snap-cooled on ice and centrifuged briefly. Complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase according to the recommended protocol. Briefly, the reactions were conducted in the presence of 1X First-Strand Buffer and 20 mM DTT at 42°C for 50 min and consequently inactivated at 70°C for 15 min. The cDNA generated was used as a template in real-time PCR reactions.

5.2.7. Real-time PCR

Real-time PCR was performed using the Opticon™ 2 system from MJ Research, Waltham, MA. For the CYP19 gene the PCR reaction mixture consisted of Taqman® Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 600 nM of each primer (Invitrogen) (Table 5.1), 250 nM Taqman probe, 18S rRNA (Applied Biosystems) and 2.5 µl of each RT sample in a final volume of 25 µl. Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min.

For the specific exon I promoter regions the PCR reaction mixture consisted of DyNAmo Hot Start SYBR Green qPCR kit (MJ Research), 600 nM of each primer (Table 1.2), and 2.5 µl of each RT sample in a final volume of 20 µl. SYBR Green uses a dye that will bind to double stranded DNA, in this methodology the primers are carefully designed to each of the promoter regions of aromatase exon I. Cycling conditions were 95°C for 15 min, followed by 50 cycles at 94°C for 10 s and 60°C for 25 s and 72°C for 30 s.
Table 5.1: Oligonucleotide primer and probe sequences for real-time PCR. Total CYP19 gene was analyzed using Taqman methodology and a sequence specific fluorogenic probe. Exon I.4 and promoter II specific gene expression were analyzed using SYBR Green methodology. (S) = Sense; (A) = Antisense.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP19</td>
<td>Primer (S)</td>
<td>5’-TGT CTC TTT GTT CTT CAT GCT ATT TCT 3’</td>
</tr>
<tr>
<td></td>
<td>Primer (A)</td>
<td>5’-TCA CCA ATA ACA GTC TGG ATT TCC-3’</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>6FAM 5’-TGC AAA GCA CCC TAA TGT TGA AGA GGC AAT-3’TAMRA</td>
</tr>
<tr>
<td>I.4a</td>
<td>Primer (S)</td>
<td>5’-AAC GTG ACC AAC TGG AGC CTG-3’</td>
</tr>
<tr>
<td></td>
<td>Primer (A)</td>
<td>5’-CAT CAC CAG CAT CGT GCC TG-3’</td>
</tr>
<tr>
<td>PIIa</td>
<td>Primer (S)</td>
<td>5’-CTC TGA AGC AAC AGG AGC TAT AGA T-3’</td>
</tr>
<tr>
<td></td>
<td>Primer (A)</td>
<td>5’-CAT CAC CAG CAT CGT GCC TG-3’</td>
</tr>
</tbody>
</table>

(a) Reference (13)
5.2.8. Statistical Analysis

Statistical and graphical information was determined using GraphPad Prism software (GraphPad Software Incorporated, San Diego, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA). Determination of IC₅₀ values were performed using nonlinear regression analysis. Statistically significant differences were calculated with the two-tailed unpaired Student’s *t*-test and *P* values reported at 99% confidence intervals.

5.3. Results

5.3.1. Effect of PPARγ ligands on aromatase activity in breast cancer cells

The synthetic thiazolidinediones; troglitazone, ciglitazone and rosiglitazone were selected to investigate whether PPARγ agonists inhibit aromatase activity in breast cancer cells (Figure 5.1). Peroxisome proliferator-activated receptors bind to the DNA as obligate heterodimers with the retinoic X receptor. We decided to treat cells with a combination of 100 nM 9-cis-retinoic acid (9-cis-RA) and PPARγ agonists to measure aromatase activity in SK-BR-3 cells. Treatment with the combination of 9-cis-RA with troglitazone and rosiglitazone resulted in a significant suppression of aromatase activity, whereas the combination of 9-cis-RA with ciglitazone resulted in an increase of aromatase activity (Figure 5.2). The fact that only ciglitazone resulted in an increase in aromatase activity and also the evidence that shows that retinoic acids could promote the action of aromatase (14), encouraged us to evaluate the effect of 9-cis-RA and PPARγ
agonists separately. Results showed that 9-cis-retinoic acid promotes aromatase activity and the PPARγ agonists alone, ciglitazone and rosiglitazone, suppress aromatase activity (Figure 5.3).

Compounds GW9662 and BADGE were selected to study the effect of PPARγ antagonists on aromatase activity (Figure 5.4). Treatment with both PPARγ antagonists resulted in a statistically significant suppression of aromatase activity in SK-BR-3 cells (Figure 5.5).

![Chemical structures](image)

Figure 5.1: Chemical structures of 9-cis-retinoic acid and the PPARγ agonists used in this study.
Figure 5.2: Effect of the combination of PPARγ agonists and 9-cis-retinoic acid on aromatase activity in SK-BR-3 breast cancer cells. SK-BR-3 cells were treated with the combination of 100 nM 9-cis-retinoic acid (RA) with 10 µM ciglitazone (Cig), 10 µM troglitazone (Trog), and 25 µM rosiglitazone (Ros). Aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.003 pmol/hr/10$^6$ cells. Each data bar represents the mean results of three independent determinations. *, $P < 0.05$ vs. control by unpaired $t$ test, n = 3.
Figure 5.3: Effect of 9-cis-retinoic acid and PPARγ agonists alone on aromatase activity in SK-BR-3 breast cancer cells. SK-BR-3 cells were treated with 100 nM 9-cis-retinoic acid (RA), 10 μM ciglitazone (Cig), and 25 μM rosiglitazone (Ros). Aromatase activity was measured as described in the experimental section. Values are expressed as picomoles 3H2O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.003 pmol/hr/10^6 cells. Each data bar represents the mean results of three independent determinations. *, P < 0.05 vs. control by unpaired t test, n = 3.
Figure 5.4: Chemical structures of the PPARγ antagonists used in this study.

Figure 5.5: Effect of PPARγ antagonists on aromatase activity in SK-BR-3 breast cancer cells. SK-BR-3 cells were treated with the indicated agents and aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.003 pmol/hr/10^6 cells. Each data bar represents the mean results of three independent determinations. *, $P < 0.05$ vs. control by unpaired $t$ test, n = 3.
5.3.2. Effect of PPARγ ligands on CYP19 mRNA expression

Analysis of CYP19 mRNA transcripts was performed using real-time PCR in order to determine if the effect in aromatase activity by PPARγ ligands was due to a down-regulation of aromatase expression at the transcriptional level. SK-BR-3 cells were treated with the indicated compounds for 24 hours using the combination of 9-cis-retinoic acid with PPARγ agonists (ciglitazone, troglitazone and rosiglitazone). Treatment with the combination of 9-cis-RA with rosiglitazone resulted in a significant decrease in CYP19 gene expression relative to control levels (Figure 5.6). While the combination of 9-cis-RA with troglitazone did not change the levels of CYP19 mRNA significantly, ciglitazone resulted in a significant increase in CYP19 mRNA levels.

MCF-7 cells were treated with the combination of 9-cis-RA with ciglitazone and troglitazone to evaluate the effect of PPARγ agonists on CYP19 mRNA expression in an estrogen-dependent breast cancer cell line (Figure 5.7). The combination of 9-cis-RA with ciglitazone resulted in an increase CYP19 expression, even though it was not statistically significant. The combination of 9-cis-RA with troglitazone resulted in a significant decrease in CYP19 mRNA expression.

SK-BR-3 cells were treated with PPARγ antagonists for 24 hours and CYP19 mRNA expression was evaluated. The compounds, GW9662 and BADGE, resulted in statistically significant decreases in CYP19 mRNA expression (Figure 5.8). These results agree with the previously discussed results on aromatase activity.
Figure 5.6: Real-time RT-PCR analysis of *CYP19* mRNA expression in SK-BR-3 cells. Cells were treated for 24 hours with the combination of 100 nM RA with 10 μM ciglitazone (Cig), 25 μM rosiglitazone (Ros), and [10 μM] troglitazone (Trog) and total RNA was isolated. Results are expressed as means of *CYP19* (normalized to 18S rRNA) ± S.E.M. *, $P < 0.05$ vs. control by unpaired t test, n = 9.
Figure 5.7: Real-time RT-PCR analysis of \textit{CYP19} mRNA expression in MCF-7 cells. Cells were treated for 24 hours with the combination of 100 nM RA with 10 µM ciglitazone (Cig) and 10 µM troglitazone (Trog) and total RNA was isolated. Results are expressed as means of \textit{CYP19} (normalized to 18S rRNA) ± S.E.M. *, $P < 0.05$ vs. control by unpaired $t$ test, $n = 9$. 
Figure 5.8: Real-time RT-PCR analysis of CYP19 mRNA expression in SK-BR-3 cells. Cells were treated for 24 hours with the indicated agents and total RNA was isolated. Results are expressed as means of CYP19 (normalized to 18S rRNA) ± S.E.M. *, $P < 0.05$ vs. control by unpaired $t$ test, $n = 9$. 

![Graph showing relative CYP19 mRNA expression]
5.3.3. Effect of PPARγ ligands on aromatase activity on human breast cells stimulated with TPA + forskolin and dexamethasone

Studies have shown that troglitazone in combination with the RXR ligand LG101305 inhibits aromatase activity mediated by promoter II in human breast adipose stromal cells (5). Here we evaluate the effect of PPARγ ligands on aromatase activity stimulated by inducers of promoters I.3 and II in breast cancer cells. Cells were stimulated for 24 hours with 10 nM TPA + 10 μM forskolin before treating with the agents. The stimulation of SK-BR-3 cells with TPA and forskolin resulted in an approximately 2.2-fold increase in aromatase activity. The treatment of cultured SK-BR-3 cells with PPARγ agonists ciglitazone and rosiglitazone demonstrated that these ligands inhibited TPA + forskolin-stimulated aromatase activity (Figure 5.9). While ciglitazone decreased aromatase activity by approximately 2.2-fold, rosiglitazone resulted in a 1.5-fold decrease.

SK-BR-3 cells were stimulated with TPA and forskolin for 24 hours and then treated with PPARγ antagonists to study their effect on aromatase activity. Our results show that GW9662 and BADGE significantly suppressed aromatase activity by 3.5- and 5.2-fold, respectively, on TPA + forskolin-stimulated SK-BR-3 cells (Figure 5.9).

Adipose tissue is the main site of estrogen biosynthesis in postmenopausal women. We have proved previously that the basal levels of aromatase activity in breast adipose stromal cells can be up-regulated in the presence of the synthetic glucocorticoid dexamethasone. The PPARγ agonists ciglitazone and rosiglitazone failed to suppress
aromatase activity levels, whereas the PPARγ antagonists GW9662 and BADGE suppressed dexamethasone-stimulated aromatase activity in human breast adipose stromal cells (Figure 5.10).

Figure 5.9: Effect of PPARγ ligands on aromatase activity in SK-BR-3 cells stimulated with TPA and forskolin. Cells were stimulated with 10 nM TPA + 10 µM forskolin for 24 hours. Then, cells were further stimulated and treated with the indicated agents for another 24 hours. Aromatase activity was measured as described in the experimental section. Values are expressed as picomoles ³H₂O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.000125 pmol/hr/10⁶ cells. Each data bar represents the mean results of three independent determinations. *, P < 0.05 vs. control by unpaired t test. #, P < 0.05 vs. TPA+forsolin treatment by unpaired t test, n = 3.
Figure 5.10: Effect of PPARγ ligands on aromatase activity in human breast adipose stromal cells stimulated with dexamethasone. Cells were stimulated with 200 nM dexamethasone for 24 hours. Then, cells were further stimulated and treated with the indicated agents for another 24 hours. Aromatase activity was measured as described in the experimental section. Values are expressed as picomoles ³H₂O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.000125 pmol/hr/10⁶ cells. Each data bar represents the mean results of three independent determinations. *, P < 0.05 vs. control by unpaired t test. P < 0.05 vs. DEX treatment by unpaired t test, n = 3.
5.3.4. Effect of PPARγ ligands on *CYP19* mRNA expression on human breast adipose stromal cells stimulated with dexamethasone

Analysis of total *CYP19* mRNA transcripts was performed using real-time PCR in order to determine whether the effects on aromatase activity compare to changes in gene transcription. Administration of dexamethasone (200 nM) resulted in elevated transcript levels of *CYP19* mRNA by approximately 3-fold increase when compared to control in human breast adipose stromal cells (Figure 5.11). The PPARγ agonists, ciglitazone and rosiglitazone demonstrated increases in aromatase expression when measuring the total *CYP19* gene.

Aromatase expression is driven through promoters II and I.4 in breast adipose stromal cells. Exon I-specific real-time PCR was performed to determine if PPARγ ligands specifically affect *CYP19* mRNA expression through exons I.4 and promoter II. Aromatase expression specific for promoter II did not change when cells were stimulated with dexamethasone (Figure 5.12A). On the other hand, the levels of exon I.4 transcripts were induced in dexamethasone-stimulated cells (Figure 5.12B). The PPARγ ligands, ciglitazone and rosiglitazone significantly decreased aromatase transcript levels specific for promoter II by approximately 40%, whereas no effect was observed when measuring exon I.4 specific transcripts.

The PPARγ antagonist BADGE significantly decreased the expression of aromatase expression through promoter II (Figure 5.13A) and exon I.4 (Figure 5.13B), suggesting that PKA and PKC-mediated pathways and the glucocorticoid response element may be affected by this agent. On the other hand, compound GW9662 only decreased expression of aromatase through exon I.4 specific transcripts.
Figure 5.11: Real-time RT-PCR analysis of CYP19 mRNA expression in breast adipose stromal cells. Cells were stimulated with 200 nM dexamethasone (DEX or D) for 24 hours. Then, cells were further stimulated and treated with 15 µM ciglitazone (Cig) and 25 µM rosiglitazone (Ros) for 24 hours and total RNA was isolated. Results are expressed as means of CYP19 (normalized to 18S rRNA) ± S.E.M. *, $P < 0.05$ vs. control by unpaired $t$ test. #, $P < 0.05$ vs. DEX treatment by unpaired $t$ test, n = 6-9.
Figure 5.12: Real-time RT-PCR analysis of CYP19 (A) promoter II and (B) exon I.4 mRNA expression in breast adipose stromal cells. Cells were stimulated with 200 nM dexamethasone (DEX or D) for 24 hours. Then, cells were further stimulated and treated with 15 µM ciglitazone (Cig) and 25 µM rosiglitazone (Ros) for 24 hours and total RNA was isolated. Results are expressed as means of CYP19 (normalized to 18S rRNA) ± S.E.M. *, P < 0.05 vs. control by unpaired t test, n =6- 9.
Figure 5.13: Real-time RT-PCR analysis of CYP19 (A) promoter II and (B) exon I.4 mRNA expression in breast adipose stromal cells. Cells were stimulated with 200 nM dexamethasone (DEX or D) for 24 hours. Then, cells were further stimulated and treated with 25 µM GW9662 (GW) and 50 µM BADGE for 24 hours and total RNA was isolated. Results are expressed as means of CYP19 (normalized to 18S rRNA) ± S.E.M. *, P < 0.05 vs. DEX treatment by unpaired t test, n =6-9.
5.4. Discussion

PPARs have been the focus of research in the past decade. One of the members of this family, PPARγ, has been linked to many molecular pathways involved not only in the process of adipogenesis (15) but also in the process of carcinogenesis by regulating the expression of different genes (16). The first report of the use of a ligand for the PPARγ to prevent experimental breast cancer came in 1999 by Suh and co-workers (17). Studies have shown that while COX-2 is induced, PPARγ is inactivated in human breast cancer (10). Independent studies have shown that normal breast tissues contain more than 2-fold higher levels of PPARγ protein compared to tumors (9), and the levels decrease even more in the case of metastases (10).

A small clinical trial for the treatment of refractory breast cancer showed that troglitazone has little apparent clinical value among patients with this type of metastatic cancer (8). This study was discontinued after a short period of time after troglitazone was withdrawn from commercial availability following FDA warnings on hepatic toxicity. The number of patients enrolled and the duration of treatment was limited. Even though the outcome of this clinical trial was not as promising as expected, researchers are still looking for answers in the PPARs.

In vitro studies suggest that activation of PPARγ and RXR down-regulates aromatase expression through suppression of NF-κB-dependent aromatase activation in fibroblast cells (18). It has also been shown that PPARγ agonists and antagonists inhibit cell growth by inhibiting the incorporation of thymidine into DNA of human breast cancer cells (19). Treatment of MCF-7 cells with ciglitazone or 15-deoxy-Δ 12,14-prostaglandin J2 (15d-PGJ2) resulted in a concentration- and time-dependent decrease of
cyclin D1 and ERα proteins, and this was accompanied by decreased cell proliferation (20). Studies have also found that in human non-small cell lung carcinoma cell lines, inhibition of cell growth by PPARγ ligands (ciglitazone, troglitazone, and rosiglitazone) was associated with a significant decrease in EP2 mRNA and protein levels (21).

The specific aim of our studies was to evaluate the effect of PPARγ ligands on aromatase activity in breast cancer cells. To investigate if this effect occurs at the transcriptional level, studies on CYP19 gene expression were also completed. An extensive review of the literature was made to select the best cell lines for our in vitro studies. Elstner et al. reported that the rank order of protein expression of PPARγ in breast cancer cell lines was SK-BR-3 > MCF-7 > MDA-MB-231 (3). Previous studies in our lab have also identified SK-BR-3 cells as the cell line with the highest aromatase/COX-2 ratio (22). For this reason, SK-BR-3 cells were selected as our cell model. The synthetic thiazolidinediones; troglitazone, ciglitazone and rosiglitazone were the PPARγ agonists selected for our studies. GW9662 and BADGE were selected as our PPARγ antagonists in this study.

Aromatase activity was decreased in SK-BR-3 cells by the combination of PPARγ agonists troglitazone and rosiglitazone with RA, whereas the same combination with ciglitazone failed to do so. Studies have shown that retinoic acids promote the action of aromatase by increasing aromatase activity (14). Treatment of SK-BR-3 cells with ciglitazone and 9-cis-retinoic acid alone resulted in a suppression and increase in aromatase activity, respectively.

Aromatase activity was induced by approximately 2.2-fold by the combination of TPA with forskolin in SK-BR-3 cells. Treatment of SK-BR-3 cells with the PPARγ
agonists, ciglitazone and rosiglitazone, resulted in a significant decrease of TPA+forskolin-induced aromatase activity. Aromatase activity was induced by approximately 16-fold by dexamethasone in breast adipose stromal cells. The PPARγ agonists, ciglitazone and rosiglitazone, did not affect aromatase activity on dexamethasone-stimulated breast adipose stromal cells.

In the case of dexamethasone-stimulated breast adipose stromal cells, real-time PCR analysis of the total \( CYP19 \) gene expression on cells demonstrated that changes in mRNA expression were consistent with enzyme activity data. Interestingly, when real-time PCR analysis of exon I-specific promoters for aromatase was studied, both compounds decreased aromatase expression through promoter II and no effect was seen when exon I.4 was analyzed.

The PPARγ antagonists, GW9662 and BADGE, suppressed aromatase activity by approximately 50% in SK-BR-3 cells. The same treatment resulted in a decrease of aromatase mRNA expression in SK-BR-3 cells when the total gene was analyzed by real-time PCR. Aromatase activity was induced by approximately 2.2-fold by the combination of TPA with forskolin in SK-BR-3 cells. Treatment of TPA+forskolin-stimulated SK-BR-3 cells with the PPARγ antagonists, GW9662 and BADGE, resulted in a decrease in aromatase activity. Aromatase activity was induced by dexamethasone in breast adipose stromal cells. The PPARγ antagonists, GW9662 and BADGE, suppressed dexamethasone-stimulated aromatase activity by approximately 1.3- and 3.3-fold respectively. The effect of these two agents on the exon I-specific promoters (I.4 and II) for aromatase was also investigated using real-time PCR in dexamethasone-
stimulated breast adipose stromal cells. Both compounds decrease aromatase expression through exon I.4, whereas only BADGE significantly decreased aromatase expression through promoter II.

Our results show that PPARγ agonists suppress the production of estrogens by inhibiting aromatase activity in both breast cancer and normal tissue. These results are supported by the CYP19 gene expression, suggesting that the effects observed in activity are very likely to start at the transcriptional level. The fact that these agents selectively inhibited aromatase activity and expression in TPA+forskolin-stimulated cells, suggest that PKA and PKC-mediated pathways are likely to be involved in their mechanism of action. On the other hand, the PPARγ agonists studied failed to suppress aromatase activity and expression on dexamethasone-stimulated cells suggesting that these agents do not affect exon I.4-mediated pathways. These results show the potential of aromatase inhibition selectivity in exon I-specific promoter II, which is the one of the main promoters involved in aromatase expression in breast cancer.

The mechanism in which PPARγ antagonists act upon aromatase remains to be elucidated. Our results show a decrease in aromatase expression and activity when cells were treated with compound GW9662 and BADGE. The study of the effect of these agents on TPA+forskolin-stimulated SK-BR-3 cells showed that these agents suppressed aromatase activity levels beyond control levels, suggesting that the aromatase inhibitory properties of these compounds involve other pathways rather than just PKA and PKC-mediated pathways. A study showed that the selective and irreversible PPARγ antagonist GW9662 inhibited cell growth and survival of breast cancer cell lines in a
PPARγ-independent pathway (23). These results recommend further studies to reveal the mechanism of action of PPARγ antagonists on aromatase activity and expression in breast cancer.

The results presented here provide data in support of PPARγ ligands as agents that could inhibit estrogen biosynthesis in the breast adipose and cancer tissue by inhibiting the expression and activity of aromatase. These findings suggest that PPARγ ligands might have therapeutic utility on the treatment of breast cancer in postmenopausal women.

5.5. References


CHAPTER 6

EFFECTS OF SYNTHETIC ISOFLAVONOIDS ON CELL PROLIFERATION
AND AROMATASE ACTIVITY IN BREAST CANCER CELLS

6.1. Introduction

Selective estrogen receptor modulators (SERMs) are nonsteroidal compounds that interact with the estrogen receptor and can exert their effects in a tissue specific manner (1). Hormone-dependent breast cancer tumors contain a large number of estrogen receptors and depend on estrogens for tumor growth. For years, tamoxifen has been the drug of choice to treat this type of cancer by blocking the binding of estrogens to the estrogen receptor. However, in many cases tumors can develop resistance to the drug after long exposure. For this reason the efficacy of pure anti-estrogens on tamoxifen-resistant tumors is of clinical importance. These considerations have encouraged the development and testing of new antiestrogens for the treatment of breast cancer. From these investigations, drugs like ICI 182,780 were synthesized and shown to display pure antagonistic activity not only \textit{in vitro} but also \textit{in vivo} (2, 3).

The marked difference in the incidence rates of breast cancer in women from Western countries compared to those in nonwestern countries led researchers to examine
dietary sources. Investigators have hypothesized that the Asian diet, which is high in soy, may be the factor to explain the low incidence in breast cancer (4, 5). Soy products are typically rich in flavonoids.

Flavonoids and isoflavonoids exhibit a range of mammalian health-promoting activities that are currently the focus of intense study (6). The primary isoflavone component of soybeans, genistein, has been associated with breast cancer chemoprevention. Genistein and other isoflavones are strikingly similar in chemical structure to mammalian estrogens. Genistein binds to the estrogen receptor in vitro (7). In a different study, animals receiving the highest concentration of dietary genistein developed the lowest number of mammary tumors per rat, suggesting that dietary genistein may reduce the susceptibility to mammary cancer (8).

For the past few years our group has been interested in the isoflavone basic ring system as a core of potential therapeutic agents for the treatment of hormone-dependent breast cancer. Our idea was to develop a new series of SERMs constructed on the isoflavone scaffold using genistein as our model compound.

One key point for the development of our new series of compounds consisted of using the pharmacophore of the estrogen receptor as a starting point. The pharmacophore for the estrogen receptor was first described in 1950 as two hydroxyl groups separated by a hydrophobic spacer (9). The importance of the two hydroxyl groups of estradiol has been extensively investigated (10). The oxygen-oxygen distance in estradiol is 10.9 Å, and to our advantage, genistein contains two phenolic groups separated by
approximately 11 Å. Researchers have stated that the optimal pattern of hydroxylation that is necessary for a flavonoid to have estrogenic activity is at 4’ and 7 positions and an additional hydroxyl group at position 5 (11).

Most of the published SERMs contain the amine-bearing side chains of tamoxifen and raloxifene. It is reported to play a key role for SERM activity by preventing the proper positioning of helix 12 for agonistic activity (12). The nonplanar orientation of this aminoalkoxyaryl side chain is believed to be important for maintaining the observed profile of tissue-specificity (13). Our rationale can be summarized in Figure 6.1.

Figure 6.1: Rationale for the synthesis of the isoflavone library.
On the basis of this rationale, we designed a library of 2,4',7-trisubstituted isoflavones as shown in Figure 6.2. We focused on compounds that contain a sulfur or oxygen as an isostere of the carbonyl group. We envisioned these heteroatoms could serve as a hinge to direct the basic side chain to the proper region in the binding pocket of the estrogen receptor for the SERM profile (14).

![Chemical structures of the target 2,4',7-trisubstituted isoflavones.](image)

\[
R_1 = \text{OH, OMe} \\
R_2 = \text{OH, OBn} \\
R_3 = \text{OH, 2-(1-piperidin-1-yl)ethoxy} \\
X = \text{O, S}
\]

Figure 6.2: Chemical structures of the target 2,4’,7-trisubstituted isoflavones.

Flavones have also been found to inhibit the aromatization of androstenedione and testosterone to estrogens catalyzed by human placental microsomes. The kinetic analyses suggest that flavones may inhibit aromatization by competing with androstenedione for the substrate binding site on the enzyme (15). Our research group is interested in exploiting the isoflavonoid basic structure as a core template for novel aromatase inhibitors. Previous studies showed that substituting a (4'-pyridylmethyl)thio group at the 2-position and a benzyloxy group at the 7-position of the isoflavonoid basic structure (**Compound B**) resulted in a significant inhibition of aromatase activity in human placental microsomes (IC\(_{50} = 0.21 \ \mu M\) (16) (Figure 6.3).
In an effort to further investigate and optimize **compound B**, we decided to explore the 7-position as a potential way to lead to better aromatase inhibitors. We hypothesize that the 7-benzyloxy group plays an important role in the binding process of these synthetic isoflavonoids to the target enzyme. Herein, we study the effect of 2-(4'-pyridylmethyl)thio, 7-alkyl or aryl substituted isoflavones (Figure 6.4) on aromatase activity in a breast cancer cell line.
Figure 6.4: 2-(4'-pyridylmethyl)thio, 7-alkyl or aryl substituted isoflavones.

5a: R = 2-nitrophenylmethoxy
5b: R = 3-nitrophenylmethoxy
5c: R = 4-nitrophenylmethoxy
5d: R = α-naphylmethoxy
5e: R = β-naphylmethoxy
5f: R = phenylethoxy
5g: R = 2-methoxyphenylmethoxy
5h: R = 4-methoxyphenylmethoxy
5i: R = cyclohexylmethoxy
5j: R = 4-fluorophenylmethoxy
5k: R = 4-bromophenylmethoxy
5l: R = 4-chlorophenylmethoxy
5m: R = 4-phenylphenylmethoxy
5n: R = 2-pyridylmethoxy
6.2. Experimental

6.2.1 Chemicals, Biochemicals and Reagents

Flavonoid analogs were prepared by Y.W. Kim as described (14). 3, (4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt was obtained from Promega, Madison, WI. 17β-Estradiol, 4-hydroxytamoxifen, and phenazine methosulfate were obtained from Sigma, St. Louis, MO. MEM culture media (B-media), trypsin-EDTA, gentamycin, glutamine, fetal bovine serum (FBS), transferin, bovine insulin, and phosphate-buffered saline (PBS) were obtained from Invitrogen Corp., Grand Island, NY. DMEM-F12 culture media was obtained from Sigma, and the human albumin was obtained from OSU Hospital Pharmacy.

6.2.2 Cell Culture

MCF-7, MDA-MB-231, and SK-BR-3 cell lines were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). They were maintained in phenol red-free custom media (B-media: MEM, Earle’s salts, 1.5x amino acids, 2x non-essential amino acids, L-glutamine, and 1.5x vitamins), supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 20 µg/ml gentamycin. Fetal calf serum was heat inactivated for 30 min in a 56°C water bath before use. Cell cultures were grown in monolayers at 37°C, in a humidified atmosphere of 5% CO2 in a Hereaus CO2 incubator. Cell cultures were passaged once or twice weekly using trypsin-EDTA to detach the cells from their culture flasks. For all experiments, cells were plated in either 24-well or
96-well plates. Before treatment, the media was changed to a defined one containing
DMEM/F12 media with 1.0 mg/ml human albumin, 5.0 mg/L human transferin and 5.0
mg/L bovine insulin.

6.2.3. Cell Proliferation Assay

Cellular cytotoxicity in the presence or absence of experimental compounds was
determined using the CellTiter 96® aqueous non-radioactive cell proliferation assay
(17). Rapidly growing cells were harvested, counted, and plated at a concentration of 1 x
10^4 cells/well for both MCF-7 and MDA-MB-231 cells in 400-µl total volume/well.
After 24 hours, the culture medium was removed and cells were washed one time with
PBS. At this time cell media was changed to a defined one. After 24 hours, culture wells
(n=6) were treated with the compounds (400 µl volume) every two days for a total of six
days. Twenty-four hours after the last treatment, 3,(4,5-dimethylthiazol-2-yl)-5-(3-
carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and phenazine
methosulfate were prepared in PBS at a final assay concentrations of 333 µg/ml and 25
µM respectively. These solutions were combined and 20 µl of this mixture were added
to each well. After 3 hours of incubation at 37°C, absorbance at 490 nm (reference
wavelength 700 nm) was measured using a SPECTRAMax plate reader.

6.2.4. Cell Cytotoxicity Assay

Cellular cytotoxicity in the presence or absence of experimental compounds was
determined using the CellTiter 96® aqueous non-radioactive cell proliferation assay.
Rapidly growing cells were harvested, counted, and plated at a concentration of 1 x 10^4
cells/well for both MCF-7 and MDA-MB-231 cells in 100-µl total volume/well into 96-
well microtiter plates. After 24 hours, the culture medium was removed and cells were washed one time with PBS. Culture wells (n=6) were treated with the compounds (100 µl volume) and incubated for 48 hours at 37°C. After incubation, the same protocol as described above was followed and absorbance was measured.

6.2.5. Tritiated Water-Release Assay

Measurement of aromatase enzyme activity was based on the tritium water release assay (18). Cells in T-25 flasks were treated with 0.1% DMSO (control), aminoglutethimide, and the synthetic isoflavonoids (5a – 5n) at 1 µM. After 24 hours, the cells were incubated for 6 hours with fresh media along with 50 nM androstenedione including 2 µCi [1β-³H]-androst-4-ene-3,17-dione. Subsequently, the reaction mixture was removed, and proteins were precipitated using 10% trichloroacetic acid at 42°C for 20 min. After a brief centrifugation, the media was extracted three times with an equal amount of chloroform to extract unused substrate and further dextran-treated charcoal. After centrifugation, a 250-µl aliquot containing the product was counted in 5 ml of liquid scintillation mixture. Results were corrected for blanks and for the cell contents of culture flasks, and results were expressed as picomoles of ³H₂O formed per hour incubation time per million live cells (pmol/h/10⁶ cells).

6.2.6. Diphenylamine DNA Assay

To determine the amount of viable cells in each flask, the cells were trypsinized and analyzed using the diphenylamine DNA assay adapted to a 96-well plate (18, 19). DNA standards (0 – 30 µg) were prepared using double-stranded DNA reconstituted in PBS and added in triplicates directly to the wells. A uniform cell suspension was prepared from the T-25 flasks in 100 µl PBS, and 20 µl of the unknown samples were
added in triplicates to separate wells. A solution of 0.16% acetaldehyde in water was prepared and mixed at a 1:5 ratio with perchloric acid (20% vol/vol). This solution (60 μl) was added to each well along with 100 μl of a 4% diphenylamine solution in glacial acetic acid. The plates were incubated at 37°C for 24 hours and the OD_{595} was measured using a microplater reader. The DNA concentration was determined by extrapolation to the standard curve and the amount of cells/flask was calculated using the equation: 1 cell = 7 pg DNA.

6.2.7. Statistical analysis

Statistical and graphical analysis information was determined using GraphPad software (GraphPad Software Incorporated, San Diego, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA). Determination of IC_{50} values were performed using nonlinear regression analysis. Statistically significant differences were calculated with the two-tailed unpaired Student’s t-test and P values reported at 99% confidence intervals.

6.3. Results

6.3.1. Effect of estradiol and antiestrogens on breast cancer cells proliferation

We first studied the effect of the ER agonist estradiol and a selected group of antagonists (4-hydroxytamoxifen, ICI 182,780, and genistein) on breast cancer cell proliferation (Figure 6.5). Two different cell lines were chosen for this study due to their different response to estrogens in cell growth, MCF-7 and MDA-MB-231 cells. The MCF-7 cell line is an estrogen receptor-positive breast cancer cell line (20). It has been shown that MCF-7 cells have unfilled ERs in the nucleus as well as in the cytoplasm.
The MDA-MB-231 cell line is an estrogen-negative breast cancer cell line, and it provides a model for human breast cancer which exhibits an estrogen-independent state (22). MDA-MB-231 cells are considerably more invasive than MCF-7 cells regardless of which medium the cells are cultured in prior to testing (23).

Exposing the hormone-dependent breast cancer cell line MCF-7 to estradiol at a 10 nM concentration resulted in an increased cell proliferation by 140% of control (Figure 6.6A). The activated form of tamoxifen, namely 4-hydroxytamoxifen, significantly decreased cell proliferation by approximately 20%. When culturing these cells with 4-hydroxytamoxifen and 10 nM estradiol, cell proliferation was nearly 100% of the control levels and approximately 40% less than estradiol alone. The pure ER antagonist ICI 182,780 also resulted in approximately 30% cell growth inhibition. The isoflavone genistein also showed antiproliferative properties, with a more evident effect obtained at 10 μM.

As expected, none of the agents showed any marked antiproliferative effects on the hormone-independent breast cancer cell line MDA-MB-231 (Figure 6.6B). This cell line does not respond or depend on estrogens for growth. Genistein at a 10 μM concentration showed a 10% inhibition in cell proliferation. Although genistein can bind to the ER receptor and exert a biological response, other mechanisms such as apoptosis, inhibition of protein kinases and inhibition of mammalian DNA topoisomerase II, have been found to be involved in the inhibition of cell growth by this agent (24-26).
Figure 6.5: Structures of β-estradiol and the selected antiestrogens used in this study.
Figure 6.6: Effect of estradiol and antiestrogens on cell proliferation. (A) MCF-7 and (B) MDA-MB-231 cells were treated with each of the agents at the indicated concentrations and cell viability was measured as described in the experimental section. The results were normalized against a control treatment with vehicle (DMSO). *, $P < 0.0001$ vs. control by unpaired $t$ test, $n = 6$. 

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6.3.2. Effect of synthetic isoflavonoids on breast cancer cells proliferation

A series of synthetic isoflavonoids was synthesized and tested *in vitro* (Figure 6.7). *In vitro* testing activity was determined by measuring the ability of a compound to inhibit cell proliferation of human breast cancer cells (MCF-7 and MDA-MB-231). Cell cultures were exposed to the compounds in the absence and presence of exogenous estradiol.

A 1 µM screening assay with a small group of compounds was performed to determine if the assay conditions were optimal (Figure 6.8). For comparison, 4-hydroxytamoxifen, genistein and estradiol yielded the expected results. Compound **YWK-174** was used to test the importance of the side chain at position 2 as a requirement for the anti-proliferative properties of this series of isoflavonoids. Compound **YWK-174** failed to inhibit cell proliferation of the hormone-dependent MCF-7 cell line. Compound **15c** was the only compound that significantly inhibited cell proliferation in both MCF-7 and MDA-MB-231 cells. These results suggest that compound **15c** might be affecting not only estrogen receptor mediated pathways but also hormone-independent pathways. Compound **15a** resulted in a non-statistically significant decrease in cell proliferation. From this screening data, we could infer that the methoxy at the 4’ position might be the key element to achieve cell cytotoxicity.

Intrinsic estrogen antagonist activity was determined *in vitro* by measuring the ability of these compounds to inhibit estrogen-induced proliferation of human MCF-7 breast cancer cells (Figure 6.9). Under the conditions of this experiment, whereby the cells were stimulated with 10 nM estradiol, compounds **YWK-174**, **16a**, **15a** and **15b**
showed no significant inhibition of cell proliferation. At a 1 µM concentration, compound **15c** reduced the stimulatory effect of estradiol suggesting that a possible hormone-dependent mechanism of action might be involved.

At a higher concentration of 5 µM (Figure 6.10), compound **15c** reduced the stimulatory effect of estradiol suppressing cell proliferation by approximately 90%. The potency of compound **15c** suggests that this compound might be acting through estrogen receptor independent pathways. While compounds **16a, 15a, and 15b** were not active, compounds **15d** and **16b** were able to suppress the stimulatory action of estradiol on cell proliferation. Exogenous estradiol was not able to counteract the effect of these compounds. Compounds sharing the 7-phenylmethoxy substitution - **11g, 14a and 14b** - resulted in a marked decrease in cell proliferation. Compound **11h**, on the other hand, did not show such pronounced effects even though it contains the 7-phenylmethoxy substitution.
Figure 6.7: Chemical structures of the target 2,4',7-trisubstituted isoflavones used in this study.
Figure 6.8: Effect of synthetic isoflavones on cell proliferation. (A) MCF-7 and (B) MDA-MB-231 cells were treated with each of the agents at the indicated concentrations and cell viability was measured as described in the experimental section. The results were normalized against a control treatment with vehicle (DMSO). * $P < 0.0001$ vs. control by unpaired $t$ test, $n = 6$. 
Figure 6.9: Effect of synthetic isoflavones on cell proliferation in the presence and absence of estradiol. MCF-7 cells were treated with 1 µM of each of the agents alone and/or in the presence of 10 nM estradiol and cell viability was measured as described in the experimental section. The results were normalized against a control treatment with vehicle (DMSO). *, $P < 0.0001$ vs. control by unpaired $t$ test, $n = 6$. 
Figure 6.10: Effect of synthetic isoflavones on cell proliferation in the presence and absence of estradiol. The compounds were divided in two groups; (A) and (B). MCF-7 cells were treated with 5 μM of each of the agents alone and/or in the presence of 10 nM estradiol and cell viability was measured as described in the experimental section. The results were normalized against a control treatment with vehicle (DMSO). *, $P < 0.0001$ vs. control by unpaired $t$ test, $n = 6$. 
6.3.3. Effect of synthetic isoflavonoids on breast cancer cells cytotoxicity

Our previous results showed that the mechanism of action of these agents involves other pathways that are not associated with hormone-dependent mechanisms. We exposed both MCF-7 and MDA-MB-231 cells for 48 hours to the synthetic isoflavonoids to study the effect of the agents on cell cytotoxicity.

In MCF-7 cells, estradiol and 4-hydroxytamoxifen failed to exhibit their stimulatory and inhibitory properties, respectively. This was expected since the time of exposure is too short to obtain any effects. Compounds containing the 7-phenylmethoxy substitution, 11g, 14a, 11h, and 14b, resulted in cell cytotoxicity. Compound 16b also resulted in cell cytotoxicity after 48 hours (Figure 6.11A).

The hormone-independent cell line MDA-MB-231 seems to be more sensitive to the synthetic isoflavonoids. Compounds containing the 7-phenylmethoxy substitution, 11g, 14a, 11h, and 14b, resulted in cell cytotoxicity. In addition, compounds 15c, 15a and 16b also showed cytotoxic activities (Figure 6.11B).
Figure 6.11: Effect of synthetic isoflavones on cell cytotoxicity. (A) MCF-7 and (B) MDA-MB-231 cells were treated with each of the agents at the indicated concentrations and cell viability was measured as described in the experimental section. The results were normalized against a control treatment with vehicle (DMSO). *, $P < 0.0001 \text{ vs. control by unpaired } t \text{ test, } n = 6.
6.3.4. Dose-dependent study of synthetic isoflavonoids in breast cancer cells proliferation

The compounds were evaluated for cell proliferation inhibition in a dose-dependent manner. The concentrations used for this study ranged from 0.001 to 20 µM. Compounds YWK-174, 15a, 15b, and 11h showed no significant activity even at the highest concentration tested. Cell proliferation was inhibited in a dose-dependent manner by 4-hydroxytamoxifen and compounds 16a, 15c, 14a, 11g, 15d, 14b, and 16b. Figure 6.12 shows the dose-dependent curves obtained for the synthetic isoflavonoids containing the sulfur-linked side chain. The trend in potency was found to follow this order; 15c > 14a > 11g > 16a. Figure 6.13 shows the dose-dependent curves obtained for the synthetic isoflavonoids containing the ether-linked side chain. The trend in potency was found to follow this order; 14b > 15d > 16b. The IC₅₀ values for each compound were calculated using a non-linear regression curve (Table 6.1).
Figure 6.12: IC₅₀ study of 4-hydroxytamoxifen and the synthetic isoflavones containing the sulfur-link in cell proliferation. MCF-7 cells were treated with agents 16a (■), 15c (▲), 14a (♦), 11g (*), or 4-OHT (○), and cell proliferation was measured as described in the experimental section. The results were normalized against a control treatment with vehicle. Each data represents the mean results of six independent determinations.
Figure 6.13: IC₅₀ study of 4-hydroxytamoxifen and the synthetic isoflavones containing the ether-link in cell proliferation. MCF-7 cells were treated with agents 15d (■), 14b (▲), 16b (٭), or 4-OHT (○), and cell proliferation was measured as described in the experimental section. The results were normalized against a control treatment with vehicle. Each data represents the mean results of six independent determinations.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16a</td>
<td>11.1 ± 5.0</td>
</tr>
<tr>
<td>15c</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>14a</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>11g</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>15d</td>
<td>6.3 ± 1.0</td>
</tr>
<tr>
<td>14b</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>16b</td>
<td>8.2 ± 2.0</td>
</tr>
<tr>
<td>4-OHT</td>
<td>4.2 ± 2.0</td>
</tr>
</tbody>
</table>

Table 6.1: IC₅₀ values for cell proliferation inhibition for the synthetic isoflavonoids.
6.3.5. Effect of 2-(4'-pyridylmethyl)thio, 7-alkyl or aryl substituted isoflavones on aromatase activity in breast cancer cells

A group of 2-(4'-pyridylmethyl)thio, 7-alkyl or aryl substituted isoflavones (Figure 6.14) was synthesized as potential aromatase inhibitors. A 1 µM screening study for aromatase inhibition was performed in SK-BR-3 breast cancer cells (Figure 6.15). The nonsteroidal aromatase inhibitor aminoglutethimide (AG) at a 10 µM concentration served as a positive control. Treatment of cells with aminoglutethimide resulted in approximately 65% inhibition of aromatase activity. Our results showed that halogen substituted compounds 5j, 5k, 5l, 2-nitro and 3-nitro compounds 5a and 5b, and α-naphthyl compound 5d had no effect on aromatase activity. The remaining compounds in this series – 5c, 5e, 5f, 5g, 5h, 5i, 5m, and 5n – displayed statistically significant suppression of aromatase enzyme activity.
Figure 6.14: Chemical structures of 2-(4'-pyridylmethyl)thio, 7-alkyl or aryl substituted isoflavones used in this study.
Figure 6.15: Suppression of aromatase activity in SK-BR-3 breast cancer cells. SK-BR-3 cells were treated with 10 μM aminoglutethimide (AG) and 1 μM of compounds 5a-5n. Aromatase activity was measured as described in the experimental section. Values are expressed as picomoles $^3$H$_2$O formed per hour incubation time per million live cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.003 pmol/hr/10$^6$ cells. Each data bar represents the mean results of three independent determinations. *, $P < 0.01$ vs. control by unpaired $t$ test, n = 3.
6.4. Discussion

The cell growth inhibitory activities of SERMs, the natural isoflavonoid genistein, and a group of synthetic isoflavones were studied in both the hormone-dependent cell line MCF-7 and the hormone-independent cell line MDA-MB-231. The addition of exogenous estradiol to MCF-7 cells resulted in a significant stimulation of cell growth. This agrees with the reported data that shows that the hormone-dependent cell line MCF-7 responds positively to the addition of estrogens. When MCF-7 cells were treated with 4-hydroxytamoxifen, cell growth was inhibited significantly. The addition of 4-hydroxytamoxifen to cells MCF-7 cells stimulated with estradiol reduced the stimulatory effect of estradiol.

The pure antiestrogen ICI 182,780 significantly inhibited the growth of the estrogen-receptor positive MCF-7 cells to a greater extent than 4-hydroxytamoxifen. These results agree with results reported in the literature that states that the maximum growth inhibitory effect of ICI 182,780 exceeds that of 4-hydroxytamoxifen (27, 28). A possible explanation could be the fact that ICI 182,780 is a pure antagonist whereas 4-hydroxytamoxifen has mixed agonist/antagonist effects.

When studying the effect of the natural isoflavone genistein, we found that cell growth was significantly inhibited with a greater effect at a higher concentration [10 \( \mu \text{M} \)]. The reason for using a higher concentration relies on the fact that genistein at low concentrations has been demonstrated to act as an estrogen agonist and promote the growth of estrogen-dependent human breast cancer cells (MCF-7) in vitro (29, 30).

As expected in the estrogen receptor negative cell line MDA-MB-231, cell growth was not significantly affected by the addition of estradiol, 4-hydroxytamoxifen in
the absence or in the presence of estradiol, or the pure antiestrogen ICI 182,780. In previous studies, others have found that ICI 182,780 does not inhibit the growth of estrogen receptor negative cell lines in the absence of estradiol (31). Genistein, at a concentration of 1 µM, showed no effect on cell proliferation. When a higher concentration of genistein was used (10 µM), a significant inhibition of 10% cell proliferation was observed. Genistein has been found to inhibit cell growth in both estrogen receptor positive cell lines (MCF-7) as well as estrogen receptor negative cell lines (MDA-MB-468) suggesting that isoflavones can act via an ER-independent pathways (32). Tyrosine-specific protein kinase activity is known to be associated with oncogene products. It is possible that tyrosine phosphorylation plays an important role for MDA-MD-231 cells proliferation and cell transformation. Others have shown that genistein inhibits the tyrosine kinase activity of the epidermal growth factor receptor in intact cells (26).

Our hypothesis was that synthetic isoflavones possessing the amine bearing side chain of raloxifene with hydroxyl groups at positions 4’ and 7 could act as SERMs in hormone-dependent breast cancer cells. In the 1 µM screening of the compounds, only 15c significantly inhibited cell proliferation. To our disappointment, compounds 15a and 15b did not significantly inhibit cell proliferation. These preliminary experiments suggest that the key element for this effect is the presence of a methoxy group at the 4’ position, since it is the only difference between compounds 15c and 15a. When studying the effect of this first group of compounds on the estrogen receptor negative breast cancer cell line MDA-MB-231, 15c showed antiproliferative activities. These results suggest a non-ER mediated mechanism of action for compound 15c in cancer cells.
The compounds were screened in the absence and in the presence of estradiol to evaluate whether they could inhibit estradiol-induced cell proliferation on MCF-7 cells (Figure 6.10). At a higher concentration (5 µM), compounds 15c, 15d, and 16b were able to inhibit cell proliferation in the absence of estradiol, but the addition of exogenous estradiol was able to restore cell proliferation only on compounds 15d and 16b. This suggests that at high concentrations, 15c is acting through estrogen-independent pathways. The highly protected, bulky compounds 14a and 14b resulted in high antiproliferative activity. The fact that their inhibitory potencies are not affected by the addition of estradiol suggests that the antiproliferative activities of these compounds may not be mediated by estrogen-receptor dependent pathways. While compound 11g was also very potent, its ether-linked analog 11h was not.

Cell cytotoxicity assays were conducted to test for compounds that may result in direct toxicity. Compounds containing the bulky 7-phenylmethoxy substituent, resulted in cell toxicity not only in MCF-7 cells but also in MDA-MB-231 cells. This experiment rules out the possibility of cell proliferation inhibition through hormone-dependent pathways. Compound 16b also resulted in toxicity in MCF-7 cells, suggesting that in the mechanism of action of this compound, not only estrogen receptor dependent mechanisms may be involved but also estrogen receptor independent.

Dose-dependent studies revealed that compound 15c is the most potent agent of this group of synthetic isoflavones with an IC₅₀ of 0.04 µM. The list in potency continued with 15d > 16b > 16a. The trend in potency for the series of compounds containing the bulky 7-phenylmethoxy substituent, follow this order; 14a > 11g > 14b.
The results obtained for the estrogen receptor binding assay reported by Serena Landini (33), suggest that compound 15c only exhibits very weak affinity for the ERα subtype, whereas compounds 15b, 15d, 16a and 16b show binding affinities for both ER subtypes (Table 6.2). In this series of compounds, the oxygen-linkage seems to be a more favorable hinge than sulfur to increase binding to the estrogen receptors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>X</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>RBA (ERα)</th>
<th>RBA (ERβ)</th>
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<tbody>
<tr>
<td>15b</td>
<td>O</td>
<td>OH</td>
<td>OH</td>
<td>2-(1-piperidin-1-yl)ethoxy</td>
<td>14.7</td>
<td>1.66</td>
</tr>
<tr>
<td>15c</td>
<td>S</td>
<td>OMe</td>
<td>OH</td>
<td>2-(1-piperidin-1-yl)ethoxy</td>
<td>0.02</td>
<td>---</td>
</tr>
<tr>
<td>15d</td>
<td>O</td>
<td>OMe</td>
<td>OH</td>
<td>2-(1-piperidin-1-yl)ethoxy</td>
<td>3.1</td>
<td>0.87</td>
</tr>
<tr>
<td>16a</td>
<td>S</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>0.01</td>
<td>0.19</td>
</tr>
<tr>
<td>16b</td>
<td>O</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>0.34</td>
<td>0.66</td>
</tr>
<tr>
<td>Gen</td>
<td></td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>1.26</td>
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<td>E₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 6.2: Relative binding affinities (RBA) of a selected group of synthetic isoflavones, genistein, and estradiol for ERα and ERβ (33).

Relative binding affinities close to the one for genistein were expected. In 1997, a study showed that genistein possessed a higher affinity for ERβ than that for ERα (34).
The binding affinities for our series of compounds were not as potent as those for genistein. The carbonyl group in the aminoalkoxyaryl side chain of raloxifene is required for ER binding and selectively affect its interactions with the estrogen receptor (35). Our results may indicate that both sulfur-linked and oxygen-linked side chains may significantly alter the orientation of the basic side chain, which may not be well tolerated by the ERs.

These results support the idea of hormone-independent pathways as targets for the mechanism of action of compound 15c. Since genistein has been associated with apoptosis at high concentrations, studies are underway to test the possibility of these compounds as apoptosis inducing agents. The high potency of compound 15c in inhibiting cell proliferation has led our lab efforts into its mechanism of action.

Isoflavones possessing the 7-benzyloxy group could play an important role in the binding process of these compounds to the aromatase enzyme. Our results show that introducing a halogen group to the 7-benzyloxy functionality (5j, 5k, 5l), resulted in a complete loss of activity in SK-BR-3 cells. When studying the effect of introducing a nitro group to the 7-benzyloxy functionality, compound 5c with the 4-nitro substituent suppressed activity by approximately 30%, whereas the 2-nitro (5a) and 3-nitro (5b) failed to inhibit aromatase activity.

Expansion of the aromatic system of the benzyloxy analog to α-napthyl (5d) resulted in a loss of activity whereas the β-napthyl (5e) resulted in a ~ 55% activity inhibition. Introducing a methoxy at positions 2 (5g) or 4 (5h) resulted in active compounds as well. Our results demonstrated that extending the hydrocarbon chain to phenylethoxy (5f) resulted in activity suppression. Introduction of an additional phenyl
ring generated the biphenyl analog (5m), which was one of the most potent compounds in this series. Although the cyclohexyl analog (5i) demonstrated activity suppression activity, it was not as potent as the inhibitory activity of compounds exhibiting the phenyl, thus illustrating the importance of unsaturation in the 7-substituent to enhance aromatase inhibitory activity.

These results proved that some of these bulky isoflavonoids can pass the cell membrane and inhibit aromatase activity. In fact, large lipophilic compounds like 5e and 5m can pass cell membrane efficiently and exhibit good aromatase inhibitory activity in cells. This may be due to the hydrophobic effect of the 7-position bulky group. This study has shown that contrary to previous beliefs suggesting that the isoflavone nucleus was inappropriate for the development of aromatase inhibitors; synthetic isoflavonoids can suppress aromatase activity in vitro.

6.5. Acknowledgments

The first study involving the 2,4’,7-trisubstituted isoflavones was conducted in collaboration with Dr. Young W. Kim, who synthesized the synthetic isoflavonoids. Serena Landini studied the binding of these agents to the ERs. The synthesis of the synthetic isoflavonoids and the ER binding data has been reported (33, 36). The second study involving the 2-(4’-pyridylmethyl)thio, 7-alkyl or aryl substituted isoflavones was conducted in collaboration with Bin Su, who synthesized the synthetic isoflavonoids.
6.6. References


CHAPTER 7

EFFECTS OF AZINOMYCIN PARTIAL STRUCTURES ON BREAST CANCER CELLS PROLIFERATION

7.1. Introduction

Azinomycins A and B are naturally occurring antibiotics discovered in the culture broth of strain S42227 (Figure 7.1). This strain was first classified in the genus of *Streptomyces* and then identified as *Streptomyces griseofuscus* (1). Azinomycins A and B showed antibacterial activity against Gram-positive and Gram-negative bacteria, but were inactive against yeast and fungi. These natural products showed *in vitro* cytotoxicity against the lymphoma cell line L5178Y, with an IC$_{50}$ of 0.07 and 0.11 µg/ml for azinomycins A and B respectively (1).

Figure 7.1: Structures of azinomycin A and azinomycin B.

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Azinomycins A and B not only showed biological activities in vitro but also in vivo. Azinomycin B was shown to be effective in a range of 2 to 32 µg/kg almost dose dependently on P388 leukemia cells (2). Azinomycin A also showed a significant increase in survival but to a lesser extent than azinomycin B. Similar results were obtained for B-16 melanoma and Ehrlich carcinoma. Azinomycin A was more cytotoxic than azinomycin B in vitro but azinomycin B was shown to be more potent with respect to antitumor activity in vivo. A clinical study of carzinophilin, a compound that many propose is the same as azinomycin B, showed that this compound was effective in humans for the treatment of malignant neoplasm of the connective tissue (3).

These agents are capable of inducing DNA interstrand cross-links. The mechanism of action of the azinomycins was first proposed in 1966, when carzinophilin markedly inhibited DNA synthesis in an E. coli strain sensitive to azinomycin B (4). In 1977, Lown and co-workers found that DNA inhibition was due to covalent links between the complementary strands of a variety of DNAs and also to an extensive alkylation in DNA (5). These two processes were determined to be pH dependent, with more DNA damage occurring at lower pH. This also helped to explain the good selectivity of these natural products, since tumor cells are characterized by having a lower pH than normal cells (6). It is well established that azinomycin B shows preference for guanine residues. Studies have shown that the initial alkylation of the adenosine involves the aziridine C10 position followed by alkylation of the guanosine by the epoxide C21 to result in covalent cross-link formation (7, 8) (Figure 7.2). Armstrong and co-workers showed the azinomycin B causes interstrand cross-links in the major
groove of duplex DNA by initial alkylation at guanine and subsequent reaction at a second purine residue two bases along on the complimentary strand of the DNA duplex (9).
Figure 7.2: Proposed mechanism for DNA cross-linking by azinomycin B. This figure was adapted from Alcaro and Coleman (8).
The structures of the azinomycins possess an almost unique motif in the form of the 1-azabicyclo[3.1.0]hex-2-ylidene dehydroamino acid fragment. Structure-activity relationship studies have shown that the 1-azabicyclo[3.1.0]hexane ring system was found to be essential for antitumor activity (10). Consistent with the mechanism of action, compounds containing the epoxide and the 1-azabicyclo[3.1.0]hexane ring system display cytotoxic activity. Structures based only upon the bicyclic aziridine subunit possess no activity (11). The naphthalene portion of the molecule intercalates within the DNA duplex (12), while the epoxy amide alkylates but does not induce interstrand cross-links in duplex DNA. Efforts are being made in the chemical synthesis of the azinomycins due to the poor availability from natural sources, the unprecedented functionalities of the molecule, and most importantly due to the need of novel chemotherapeutic agents. While some groups have been working on the chemical synthesis of azinomycin analogs to be evaluated for cytotoxic activity (6, 13), Coleman and co-workers were the first group to describe the total synthesis of azinomycin A (14).

Although the basic mechanism of DNA interstrand cross-links formation by the azinomycins is well established, very little is known about which functionalities of the molecule are involved in binding to the DNA helix or may play a role in the alkylation process.

7.2. Experimental

7.2.1. Chemicals, Biochemicals and Reagents

3,(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt was obtained from Promega, Madison, WI and phenazine
methosulfate from Sigma, St. Louis, MO. MEM culture media (B-media), trypsin-EDTA, gentamycin, glutamine, fetal bovine serum (FBS), transferin, bovine insulin, and phosphate-buffered saline (PBS) were obtained from Invitrogen Corp., Grand Island, NY. DMEM-F12 culture media was obtained from Sigma, and the human albumin was obtained from OSU Hospital Pharmacy.

7.2.2. Cell Culture

MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). They were maintained in phenol red-free custom media (B-media: MEM, Earle’s salts, 1.5x amino acids, 2x non-essential amino acids, L-glutamine, and 1.5x vitamins), supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 20 µg/ml gentamycin. Cell cultures were grown in monolayers at 37°C, in a humidified atmosphere of 5% CO₂ in a Hereaus CO₂ incubator. Cell cultures were passaged once or twice weekly using trypsin-EDTA to detach the cells from their culture flasks. Before treatment, the media was changed to a defined one containing DMEM/F12 media with 1.0 mg/ml human albumin, 5.0 mg/L human transferin and 5.0 mg/L bovine insulin.

7.2.3. MTS-Microculture Tetrazolium Assay

Cellular cytotoxicity in the presence or absence of experimental compounds was determined using the CellTiter 96® aqueous non-radioactive cell proliferation assay (15). Rapidly growing cells were harvested, counted, and plated at the appropriate concentrations (1 x 10^4 and 0.5 x 10^4 cell/well for MCF-7 and MDA-MB-231 cells respectively) in 100-µl total volume/well into 96-well microtiter plates. After 24 hours,
the culture medium was removed and cells were washed one time with PBS. Culture wells (n=6) were treated with the compounds (100 µl volume) and incubated for 48 hours at 37°C. After incubation, 3,(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and phenazine methosulfate were prepared in PBS at a final assay concentrations of 333 µg/ml and 25 µM respectively. These solutions were combined and 20 µl of this mixture were added to each well. After 3 hours of incubation at 37°C, absorbance at 490 nm (reference wavelength 700 nm) was measured using a SPECTRAmax plate reader.

7.2.4. Statistical analysis

Statistical and graphical analysis information was determined using GraphPad software (GraphPad Software Incorporated, San Diego, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA). Determination of IC50 values were performed using nonlinear regression analysis. Statistically significant differences were calculated with the two-tailed unpaired Student’s t-test and P values reported at 99% confidence intervals.

7.3. Results

7.3.1. Role of the Azinomycin Naphthoate, Carboxamide and Epoxide in Cell Proliferation

To provide information on the structure-activity relationship of the left-half side of the azinomycins, partial substructures of the azinomycins were synthesized and studied for their effect on cell proliferation (Figure 7.3). These structures were carefully
designed to study the importance of each of the following moieties of the azinomycins — naphthoate, carboxamide and epoxide — in the mechanism of action of these natural products.

The left half side of azinomycin B, compound 1, was a better compound at inhibiting cell proliferation. This compound shares three of the principal moieties of the azinomycins; the naphthoate, the carboxamide and the epoxide. At a concentration of 5 µM, compound 1 inhibited about 80% and 95% cell proliferation in MCF-7 (Figure 7.4A) and MDA-MB-231 (Figure 7.4B) cells respectively.

To test if the naphthoate moiety plays an essential role in cell cytotoxicity, compounds 7 and 8 were synthesized. Both compounds, devoid of the naphthoate moiety, showed no inhibition of cell proliferation on either cell line. Compounds 3 and 5, both devoid of the epoxide, showed no cytotoxic activity. When replacing the carboxamide in compound 1 with a benzyl ester (compound 4), cell cytotoxicity was completely abolished. Next a study was performed to determine if a primary amide was essential for cell cytotoxicity. Compound 6, with the dimethylamide instead of the amide, showed to be very active reasonable active at inhibiting cell proliferation at a concentration of 5 µM (Figure 7.4).

Compound 2, an intermediate for the total synthesis of azinomycin B, showed some significant cytotoxic activity against both cell lines as well. The hormone-independent breast cancer cell line MDA-MB-231 appears to be more sensitive to the effect of this type of agents than the hormone-dependent breast cancer cell line MCF-7. At a 1 µM concentration, only compound 1 showed cell antiproliferative properties in
MCF-7 cells (Figure 7.5A). The 1 µM concentration screening when tested in MDA-MB-231 cells, showed that not only compound 1 was cytotoxic but also compound 6 (Figure 7.5B).

Figure 7.3: Structures of the azinomycin partial molecules used to study the effect of the naphthoate, central amide and epoxide moieties in cell proliferation.
Figure 7.4: Effect of the azinomycin partial structures on human breast cancer cell lines ([5μM] Screening Assay). (A) MCF-7 and (B) MDA-MB-231 cells were treated with each of the agents at a concentration of 5 μM and cell viability was measured as described in the experimental section. The results were normalized against a control treatment with vehicle (DMSO). *, $P < 0.0001$ vs. control by unpaired $t$ test, $n = 6$. 
Figure 7.5: Effect of the azinomycin partial structures on human breast cancer cell lines ([1µM] Screening Assay). (A) MCF-7 and (B) MDA-MB-231 cells were treated with each of the agents at a concentration of 1 µM and cell viability was measured as described in the experimental section. The results were normalized against a control treatment with vehicle (DMSO). *, $P < 0.0001$ vs. control by unpaired $t$ test, n = 6.
7.3.2. Dose-Dependent Studies of the Azinomycin Partial Structures in Cell Proliferation

The compounds were evaluated for cell cytotoxicity in a dose-dependent manner. The concentrations used for this study ranged from 0.05 to 50 µM. Compounds 3, 5, 7, and 8 showed no activity even at the highest concentration tested.

Cell proliferation was inhibited in a dose-dependent manner for compounds 1, 2, 4, and 6 (Figure 7.6). All four compounds share the naphthoate and the epoxide moieties of the azinomycins. Compound 4, the only one in this group lacking the carboxamide moiety, showed cytotoxic properties only at very high concentrations. While the order of potency for MCF-7 shows that compounds 2 and 6 are essentially equally potent; MDA-MB-231 cells show a clear trend. The trend in potency was found to follow this order; 1 > 6 > 2 > 4. Again, compounds sharing the carboxamide moiety proved to be better inhibitors of cell proliferation. The IC_{50} values for each compound were calculated using a non-linear regression curve (Table 7.1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>MCF IC_{50} (µM)</th>
<th>MDA-MB-231 IC_{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.2 ± 1.3</td>
<td>0.60 ± 0.08</td>
</tr>
<tr>
<td>2</td>
<td>3.8 ± 2.9</td>
<td>4.0 ± 2.5</td>
</tr>
<tr>
<td>4</td>
<td>27 ± 3.0</td>
<td>10.1 ± 3.8</td>
</tr>
<tr>
<td>6</td>
<td>3.9 ± 1.9</td>
<td>1.6 ± 0.7</td>
</tr>
</tbody>
</table>

Table 7.1: IC_{50} values for the azinomycin partial structures on MCF-7 and MDA-MB-231 cells.
Figure 7.6: IC₅₀ study for the azinomycin partial structures in cell proliferation. (A) MCF-7 and (B) MDA-MB-231 cells were treated with agents 1 (■), 2 (♦), 4 (□), or 6 (○), and cell proliferation was measured as described in the experimental section. The results were normalized against a control treatment with vehicle. Each data represents the mean results of six independent determinations.
7.3.3. Role of the Epoxide Stereochemistry of Azinomycin Substructures in Cell Proliferation

The orientation of azinomycin B on the duplex DNA to result in alkylation has also been studied in the last few years. The stereochemistry of azinomycin B, (2S, 3S), raises the question of whether a specific stereochemistry is essential for this agent to produce any cytotoxic effects. A small group of stereoisomers, all compounds sharing the three essential moieties -- naphthoate, carboxamide and epoxide -- of the left half part of the azinomycin molecule, was synthesized (Figure 7.7).

In a 5 µM screening on both MCF-7 and MDA-MB-231 cells, virtually all stereoisomers tested inhibited cell proliferation equally. The sensitivity of MDA-MB-231 cells to these agents makes the process of drawing any conclusion fairly hard (Figure 7.8). When screening these compounds at a lower concentration (1 µM) on MCF-7 cells, only compound 62 (2R,3R) and its enantiomer 63 (2S,3S) significantly inhibited cell proliferation but only at 20-25% while all compounds resulted in cytotoxicity on MDA-MB-231 cells.

Compound 52, the same structure of azinomycin B but lacking the bicyclic aziridine, showed cytotoxic activity in the 5 µM screening assay but surprisingly failed to do so in the 1 µM screening assay on MCF-7 cells (Figure 7.9) even though the stereochemistry is the same one as azinomycin B.
Figure 7.7: Structures of the left-half side of azinomycin used to study the importance of stereochemistry on cell proliferation.
Figure 7.8: Effect of the stereochemistry of the azinomycin partial structures on cell proliferation ([5 μM] Screening Assay). (A) MCF-7 and (B) MDA-MB-231 cells were treated with each of the agents at a concentration of 5 μM and cell viability was measured as described in the experimental section. The results were normalized against a control treatment with vehicle (DMSO). *, $P < 0.0001$ vs. control by unpaired $t$ test, $n = 6$. 

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Figure 7.9: Effect of the stereochemistry of the azinomycin partial structures on cell proliferation ([1 μM] Screening Assay). (A) MCF-7 and (B) MDA-MB-231 cells were treated with each of the agents at a concentration of 1 μM and cell viability was measured as described in the experimental section. The results were normalized against a control treatment with vehicle (DMSO). *, $P < 0.0001$ vs. control by unpaired $t$ test, $n = 6$. 

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7.3.4. Dose-dependent Study of the Epoxide Stereochemistry Substructures in Cell Proliferation

To examine the effect of changing the natural stereochemistry of the azinomycins, dose-dependent studies were conducted on each of the stereoisomers mentioned above. The concentrations used for this study ranged from 0.5 to 30 µM for MCF-7 cells and 0.005 to 10 µM in MDA-MB-231 cells (Figure 7.10).

All stereoisomers resulted in a dose-dependent inhibition of cell proliferation with very little variation in cell cytotoxicity. The IC$_{50}$ values for each compound were calculated using a non-linear regression curve (Table 7.2).

<table>
<thead>
<tr>
<th>Compound</th>
<th>MCF $IC_{50}$ (µM)</th>
<th>MDA-MB-231 $IC_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>4.1 ± 0.3</td>
<td>0.284 ± 0.05</td>
</tr>
<tr>
<td>61</td>
<td>2.8 ± 0.2</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>62</td>
<td>2.1 ± 0.7</td>
<td>0.16 ± 0.07</td>
</tr>
<tr>
<td>63</td>
<td>1.9 ± 0.6</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>64</td>
<td>2.6 ± 1.0</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

Table 7.2: IC$_{50}$ values for the azinomycin partial structures stereochemistry on MCF-7 and MDA-MB-231 cells.
Figure 7.10: IC\textsubscript{50} study for the azinomycin partial structures with different stereochemistry in cell proliferation. (A) MCF-7 and (B) MDA-MB-231 cells were treated with agents 52 (■), 61 (●), 62 (□), 63 (○), or 64 (◊), and cell proliferation was measured as described in the experimental section. The results were normalized against a control treatment with vehicle. Each data represents the mean results of six independent determinations.
7.4. Discussion

Although azinomycin B has been discovered for years, the exact mechanism in which it intercalates DNA resulting in cell cytotoxicity is still not well understood. Different groups have been dedicating time and effort to fully understand this process and partial structures have been synthesized and evaluated for their effect on DNA alkylation and antitumor properties. Here we provide information on the critical role that the naphthoate, carboxamide, and epoxide play in cell cytotoxicity.

Compound 1 is by far the most active substructure of the series of compounds tested in this study. When the native carboxamide of the azinomycins (1) was replaced by a benzyl ester (4), cell cytotoxicity was abolished. This implies that the carboxamide moiety is important in cell cytotoxic activity. We also found that the naphthoate moiety is important in cell cytotoxic activity. Compound 8, which is basically compound 1 without the naphthoate moiety, showed no cytotoxic effects on either cell line. In each case the estrogen-independent cell line MDA-MB-231 was more sensitive to the effects of these agents. These results strongly agree with the DNA alkylating ability of these substructures indicating that alkylation of DNA is a critical biological event in the mechanism of action of these agents and presumably for azinomycin B (16).

When studying the effect of the lack of the reactive epoxide moiety, we found that both compounds 3 and 5 failed to inhibit cell proliferation. Even when replacing the epoxide with an isosteric cyclopropane ring and keeping the naphthoate and the amide (compound 3), cell cytotoxicity was completely abolished. These results agree with previous results obtain by Shipman and coworkers (17).
These results indicate the important role that the azinomycin naphthoate, carboxamide, and epoxide play in increasing cell cytotoxicity. While the amide plays an important role in cell antiproliferative activity, the absence of the naphthoate moiety affects the potency of these agents the most. The naphthoate moiety may play an essential role in the process of DNA binding and alkylation.

Interestingly, even though the two compounds with the highest similarity to azinomycin B (intermediate 2 and 52) showed cytotoxic activities in both cell lines, they failed to exceed the antiproliferative properties of compound 1. This might suggest that in the process of DNA alkylation, molecule size could play an important role. These compounds lack the bicyclic aziridine, which is essential in the process of DNA alkylation. Perhaps the right half part of the molecule of these compounds lacking the reactive aziridine is interfering with the process of DNA binding.

Chiral recognition is not to be an important factor in the cytotoxic activity effects of these compounds. All stereoisomers — 61, 62, 63, and 64 — resulted in similar IC₅₀ values, suggesting that apparently chirality is not important at this level of molecule complexity. When only the left half part of the azinomycin molecule is present, DNA alkylation occurs but not induction of interstrand cross-links in duplex DNA (18).

These studies provide important information about the structure-activity relationships in the left half of the azinomycins, showing that the naphthoate, the N16 amide nitrogen, and the epoxide are important for the antitumor properties of these natural products. These compounds are very unique molecules and represent a promising class of cancer chemotherapeutic agents.
7.5. Acknowledgments

This study was conducted in collaboration with Dr. Robert S. Coleman in the Department of Chemistry, as part of a requirement of the Chemistry-Biology Interface Training Program. The compounds were synthesized and supplied by his laboratory team. The synthesis of some of these agents and a portion of the biological data has been published (16).

7.6. References


CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

A correlation between aromatase, cyclooxygenase-2, and the nuclear receptor PPARγ has been identified. Cyclooxygenase inhibitors suppress aromatase enzyme activity in breast cancer cells. Changes in transcript levels were associated with comparable changes in aromatase activity in breast cancer cells, suggesting that the effect of COX inhibitors on aromatase starts at the transcriptional level. It is likely that prostaglandin E2 produced via cyclooxygenases may act locally in an autocrine fashion to increase the local biosynthesis of estrogen by the aromatase enzyme in hormone-dependent breast cancer development and lead to growth stimulation.

The selective COX-2 inhibitor, NS-398, proved to be unquestionably the best agent at inhibiting aromatase in both breast cancer and adipose stromal cells. The potent inhibitory activity of this compound suggests that COX-2 independent mechanisms may be involved in its mechanism of action. NS-398 and its analogs suppress aromatase activity in a tissue-selective manner through promoters II and I.3, suggesting that PKA and PKC-mediated pathways are being affected within the cell. Future studies should focus on determining if other promoters may be affected by cyclooxygenase inhibitors.
and NS-398 analogs. A better proof of selectivity would help to support the idea of developing NS-398 analogs as selective aromatase modulators for the chemoprevention and treatment of hormone-dependent breast cancer.

PPARγ agonists suppress the production of estrogens by inhibiting aromatase activity in both breast cancer and normal tissue. These results are supported by the suppression of CYP19 gene expression, suggesting that the effects observed in activity start at the transcriptional level. PPARγ agonists suppress aromatase activity on cells stimulated through promoters I.3 and II, suggesting that PKA and PKC-mediated pathways are likely to be involved in their mechanism of action. PPARγ agonists do not suppress aromatase activity and expression through exons I.1 and I.4-mediated pathways. PPARγ is a key player in cell signaling pathways, as a result, future studies should carefully investigate possible genes that could be affected by activating or inhibiting this nuclear receptor. Selectivity is always a major concern when looking for treatments for breast cancer.

PPARγ antagonists inhibit aromatase expression and activity in breast cancer and adipose stromal cells. However, the inhibitory mechanism does not exhibit any selectivity suggesting that multiple pathways within the cell may be affected. Further studies to reveal the mechanism of action of PPARγ antagonists on aromatase activity and expression in breast cancer are recommended.

Breast cancer cell proliferation was inhibited by 2,4',7-trisubstituted isoflavones in hormone-dependent and hormone-independent ways. The synthetic isoflavonoid 7-hydroxy-3-(4-methoxyphenyl)-2-[4-[2-(piperidin-1-yl)ethoxy]phenylthio]-4H-1-benzopyran-4-one (15c), inhibited cell proliferation in a dose-dependent manner
effectively in human breast cancer cells. This suggests that compound **15c**, as well as other synthetic isoflavones, could be activating apoptotic pathways. Further studies are underway to study the possibility of apoptosis inducing properties of these isoflavones especially **15c**. This study has shown that contrary to previous beliefs suggesting that the isoflavone nucleus was inappropriate for the development of aromatase inhibitors; synthetic isoflavonoids can suppress aromatase activity *in vitro*.

Based on our results, we propose the following model to explain the interrelationship between aromatase, COX-2 and PPARγ (Figure 8.1). Higher levels of COX enzymes expression and increased activity would result in higher levels of PGE2. Elevated PGE2 levels increase intracellular cAMP and result in increased aromatase expression via promoters I.3 and II. Higher levels of aromatase would lead to higher levels of estrogens, resulting in increased growth and development of the tumor by both paracrine and autocrine actions.

The results presented here provide data in support of PPARγ ligands as agents that could inhibit estrogen biosynthesis in the breast adipose and cancer tissue by inhibiting the expression and activity of aromatase. The combination of an aromatase inhibitor with a COX-2 inhibitor may increase efficacy beyond the present treatments for postmenopausal hormone-dependent breast cancer. Targeting of COX-2 and PPARγ could also inhibit the growth of human breast cancer cells by inhibiting aromatase expression, making this combination a promising molecular target for the chemoprevention or treatment of breast cancer.
Figure 8.1: Interrelationship between aromatase, COX-2 and PPARγ in human breast cancer.


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