INVESTIGATION OF THE PLEIOTROPHIC EFFECTS OF A SERIES OF ISOFLAVONOID ANALOGUES IN HORMONE-DEPENDENT AND HORMONE-INDEPENDENT BREAST CANCER CELLS

DISSERTATION

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By

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ABSTRACT

Genistein, the predominant isoflavone, has several interesting properties including and not limited to cell cycle arrest, induction of apoptosis, tyrosine kinase inhibition, and effecting cell signaling transduction pathways. To enhance the anti-cancer activity of genistein, a series of novel isoflavone analogs were synthesized based on the isoflavone skeleton backbone and the side chain of raloxifene.

The synthetic isoflavone analogs were shown to be effective antiproliferative, cytotoxic, and apoptosis agents in both hormone-dependent and hormone-independent breast cancer cell lines. We also determined that the MDA-MB-231 cell line was more sensitive to the cytotoxic and apoptosis inducing ability of the isoflavone analogs. Flow cytometry analysis revealed that selected isoflavone analogs arrested breast cancer cells at the G_1 to S transition with concomitant increase in the endogenous cdk inhibitors (p21 and p27). Of this series, compound **5** showed selectivity in its mechanism of action for the MDA-MB-231 cell line, suggesting that its activity was not strictly mediated through estrogen-dependent pathways.

Potential cell death mediated targets in the apoptosis pathway were furtherd explored. A 48 hour treatment with 5 and 10 μM of compound **5** resulted in a significant increase in Bax expression in both breast cancer cell lines. Additionally, the Bax/Bcl-2 ratio was significantly increased in the MDA-MB-231 breast cancer cell line. Also,

treatment with compound **5** in the MDA-MB-231 breast cancer cell line resulted in decreased expression of activated Akt at Serine-473.

High-throughput analysis revealed an increase in gene expression of the caspase-dependent, TNF/FAS-mediated, and death domain related families following treatment with compound **5** in the MDA-MB-231 breast cancer cell line. Gene expression was validated by real time PCR, western blotting of relevant caspase proteins, and decrease in apoptosis in the presence of the general caspase inhibitor, z-VAD-fmk.

A series of nimesulide analogs were also examined and not found to induce apoptosis in SK-BR-3 cells, which supports their further exploration as novel aromatase suppression modulators in breast cancer cells.

These findings provide important insights into the potential molecular targets of a novel series of synthetic isoflavone analogs, with valuable implications that can impact the overall etiology and treatment of breast cancer.

Dedicated to my parents, Melvin and Sheila P. Davis, who provided a very loving and Christ-centered home where I felt secure and warm each night my head touched the pillow.

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Thank you Creator God for allowing me to write these final words. This journey has truly been a walk of faith and I'm reminded yet again that the race is not given to the swift or strong, but to the one who endures until the end. You heard every supplication and pleaded on my behalf before the throne. There have been many long nights and I thank you for your watchful eye of protection; for petitioning Your angels to keep me from falling and keeping me out of harms way when I didn't realize the danger just ahead. You truly are a gracious and longsuffering God and I praise You for allowing me to see this day!

Thank you for blessing me with two God-fearing and wonderful parents, Melvin and Sheila P. Davis. I love these guys with my whole heart. Both of you are my cheerleaders, my prayer warriors, my confidants, and two of my best friends. Thank you for showing me what it means to work hard, to sacrifice, 'prioritize', and realize that success is within my reach if I just hold on a little longer. The lessons I learned under your instruction have helped to lay the foundation for tackling some of my more difficult challenges in graduate school. God entrusted me to the two of you and I thank Him everyday for choosing us to be together and to experience each other's love while on this earth.

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Nadia Brianna Deneace Williams, Delia Ann Williams, and little Mr. Micah Brill Daniel Williams, my precious nieces and nephew. . .Auntie Dan (as you so affectionally refer to me), I love you so much. . ,sorry for missing the birthdays and baby blessings and other special events, but please know that I think of each of you often. The little I LOVE YOUS, telephone kisses, and sweet messages on my cell phone brightened so many of my days! Thanks Deia for making sure I remain a part of their lives and of course for relaying to me all the little compliments they get from the outfits I send them-lol.

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discuss very important matters. I also want to thank you Che' for being open about your faith and for providing me with countless opportunities to talk about God and His goodness in this setting.

A very special thank you to my advisor, Dr. Robert W. Bruggemeier (Dr. B), who allowed me to complete my last and final laboratory rotation and subsequently accepted my request to join his laboratory group. Thank you Dr. B for providing the necessary tools that allowed me to become the scientist I am today. Your sincerity, kindness, patience, trust and financial support have truly made the difference in my overall success in this program. I especially thank you for your belief in my capabilities within the lab and the capacity for me to develop the necessary skills to independently approach scientific research.

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Last but not least, CHICKEN, SOOGGIE, TURKEY, TO-FU you need no introduction (lol). You, nor my sister are allowed to speak at my wedding because both of you know too much about me;-). Thanks for always putting a smile on my face and being willing to share your scientific expertise with various protocols and procedures in the lab. I feel so honored to have been a witness to your beautiful marriage to Yoko and I know that blessings will continue to be with all four of you + the adorable LEO. You will never know how much your friendship, kindness, advice, generosity, and giving of your personal time have meant to me. I truly count you as a friend for life, that is unless you turn into a CHICKEN (*wink, wink*). A special thanks also to Mrs. Brueggemeier and Jen Whetstone for your warm hospitality, kindness, encouragement, and delightfully prepared food each time the two of you hosted the lab group to your home.

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VITA

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PUBLICATIONS

Research Publications

1. Su B, Landini S, Davis DD, Brueggemeier RW. Synthesis and Biological Evaluation of Selective Aromatase Expression Regulators in Breast Cancer Cells. *J Med Chem* 2007;50:1635-1644.

FIELDS OF STUDY

Major Field: Biochemistry

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3β-HSD – 3β-hydroxysteroid dehydrogenase
4-OHA – 4-hydroxy-4-androstene-3,17-dione
AAD – 7-amino-actinomycin D
AG – aminoglutethimide
AHEI – alternate healthy eating index
Akt – protein kinase B
AMED – alternate Mediterranean diet score
AP-1 – activator protein
ATCC – American type culture collection
ATM – ataxia-telangiectasia gene
BRCA – breast cancer associated gene
CARD – caspase recruitment domain
CDKS – cyclin dependent kinases
CDNA – complementary DNA
CIDE – cell death-inducing DFF 45(DNA fragmentation factor 45)
CKIS – cyclin kinase inhibitors
COMT – catechol-*O*-methyltransferase
COX – cyclooxygenase
CPP32 – caspase 3
CS-FBS – charcoal stripped fetal bovine serum
DCCS – dextran coated charcoal stripped
DHT – dihydroxytestosterone
DMEM – dulbecco's modified eagle's medium
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
DQIR – diet quality index revised
ELISA – Enzyme-Linked ImmunoSorbent Assay
G – grade
ECL – enhanced chemiluminescence
EGFR – epidermal growth factor receptor
ER – estrogen receptor
EROD – ethoxyresorufin *O*-deethylase
E_1 – estrone
E_1S – estrone sulfate
E_2 – estradiol
FBS – fetal bovine serum
FISH – fluorescence *in situ* hybridization

GEN – genistein
HEI – healthy eating index
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNSCC – head and neck squamous cell carcinoma
IAP – inhibitor of apoptosis protein
IC_{50} – 50 % inhibitory concentration
IGR – insulin growth factor receptor
MeO - methoxy
MBC – metastatic breast cancer
M-PER – Mammalian Protein Extraction Reagent
MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB – nuclear factor-kappaB
NGF – nerve growth factor
NSAID – nonsteroidal anti-inflammatory drug
ODC – ornithine decarboxylase
OHCE – 4-hyroxy-catecholestrogen
OH – hydroxy
PARP – Poly (ADP-ribose) polymerase
PBS – phosphate buffered saline
PDGF – platelet-derived growth factors
PE – phycoerythrin
PI3K – Phosphoinositide-3 kinase (PI3K)
PTKS – protein tyrosine kinase inhibitors
PVDF – Polyvinylidene Difluoride
RNA – ribonucleic acid
RT-PCR – reverse transcriptase polymerase chain reaction
SER - Serine
SERM – selective estrogen receptor modulator
ssDNA – single-stranded deoxyribonucleic acid
TAM – tamoxifen
THR – threonine
TNF_{α} – tumor necrosis factor α
TPA – 12-*O*-tetradecanoylphorbol-13-acetate
TRAF – TNF-associated factor
UGT – UDP-glucuronosyl transferase
Z-VAD-FMK - Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone

CHAPTER 1

THE ROLE OF ISOFLAVONES AND THEIR INFLUENCE ON THE BIOLOGICAL MECHANISMS OF BREAST CANCER

1.1. Breast Cancer: Biology and Statistical Implications

It is estimated that in 2007 approximately 559,650 Americans are expected to die from cancer, which equates to more than 1,500 people a day. Breast cancer continues to account for at least 26% of the estimated new cases of cancer in women and 15% estimated deaths this year (1). Breast cancer remains to be a complex and multifaceted disease that involves multiple gene regulatory changes and like other cancers requires an understanding from all treatment and chemoprevention perspectives to tackle this complex phenomenon.

Mammary gland development of the breast is mediated by a series of ductal structures and epithelial cells that represent the building blocks of alveoli formation. A description of the functional anatomy of the human breast is illustrated in (Figure 1.1). Although not entirely understood, it is believed that the basic evolution of breast cancer is a result of the outgrowth of tumorigenesis from an abnormal cell environment. Furthermore, Bagga *et al*., suggest that breast disease originates from epithelial cells of the terminal duct lobular unit in both breast cancer and hyperplastic lesions of proliferative breast disease (2). The role of genetic alterations in the microenvironment

of the breast have been thought to be the predominant contributing factor in the multistep process of epithelial cell transformation in multistep carcinogenesis. Bissel *et al*., suggest that this (genetic model) cannot fully explain the phenomenon whereby tumor cells are able to express diverse phenotypes during the multistage process of tumor development and progression. The consensus suggests that there are concomitant changes that also occur in cells surrounding the epithelial neoplasms which are localized in the microenvironment of the breast which further influence the cancer state (3).

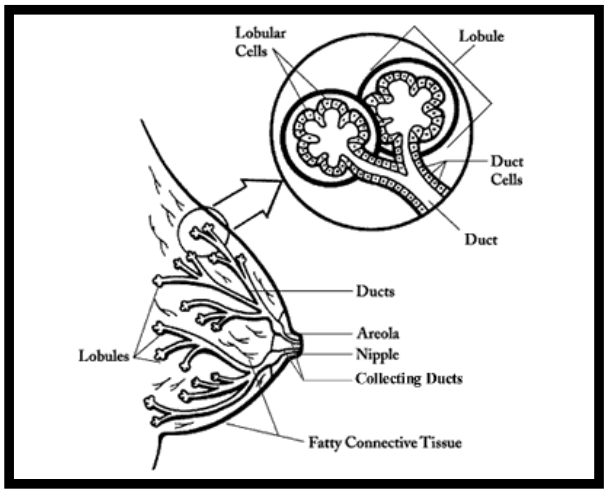


Figure 1.1: Functional Anatomy of the Human Breast. The parenchyma of the breast mammary gland is comprised of approximately 10 to 15 ducts extending from the nipple and traversing through the mammary fat pad ultimately converging in clusters of alveoli. The lobules, which are served by individual ducts are separated and supported by thick connective tissue septa consisting of epithelial cells surrounded by adipose tissue comprised of stroma cells. Adapted from the American Cancer Society, Cancer Reference Information (4).

The treatment strategies for breast cancer are often a combination approach depending on multiple variables and include but are not limited to: hormone dependence, tumor-type, receptor status, etc. Although beyond the scope of this introduction, some common surgical and treatment options include lumpectomy (breast-conserving surgery), mastectomy (removal of the affected breast), radiotherapy (option of lymph node specific radiation/radiation to destroy ovaries), chemotherapy, and hormonal treatment strategies (antiestrogen and aromatase inhibitors-generation and type specific).

1.2 Estrogens and Hormonal Dependence in Breast Cancer

1.2.1 Estrogen receptor-negative breast carcinomas

Estrogens, which belong to the broad class of steroid hormones, play an important role in regulating the differentiation and proliferation in a large number of tissues including bone, brain, cardiovascular system and the breast. Essential for normal mammary gland development, maintenance of hormone balance, and circulatory levels in the reproductive system, the fluctuations in estrogen levels have been linked to transformation of malignant cells. Consequently, it has been suggested that a prolonged estrogen exposure may induce breast cancer (5,6,7).

Hormonal influences on the etiology of breast cancer are thought to be a predominant controlling factor in the evolution of this complex disease. Initial evidence that pointed towards the involvement of estrogen in the increased risk of developing breast cancer was based on treatment studies that reported that women treated with the estrogen diethylstilbestrol increased their likelihood of developing this disease (8). Additionally, studies showed that estrogen stimulated the growth of breast cancer cell

lines in ovariectomized nude mice and in cell culture (9). Besides the role of the ovaries in the direct secretion of estrogens into the bloodstream, the adrenal steroids are a second source of estrogen in adult women. The enzyme aromatase (CYP19) is expressed in several extra-ovarian tissues, particularly the adipose tissue, and is responsible for conversion of the adrenal steroids, such as, dehydroepiandrosterone, its sulfate, and androstenedione (referred to as C19 steroids) into estrogens (10,11,12). As a result, considerable research has focused on the development of antiestrogens and aromatase inhibitors for breast cancer treatment.

The interrelationship between aromatase and its interaction in the microenvironment of hormone dependent breast cancer is shown in (Figure 1.2). Briefly, the involvement of the cyclooxygenase isozymes (constitutive COX-1 isozyme and inducible COX-2 isozyme, which catalyze the local production of PGE_2) in breast cancer is suggested due to a strong linear correlation between CYP19 expression and the sum of COX-1 and COX-2 expression using human breast cancer specimens (13). The beneficial effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on breast cancer have been attributed to their effectiveness as COX inhibitors (14,15,16,17).

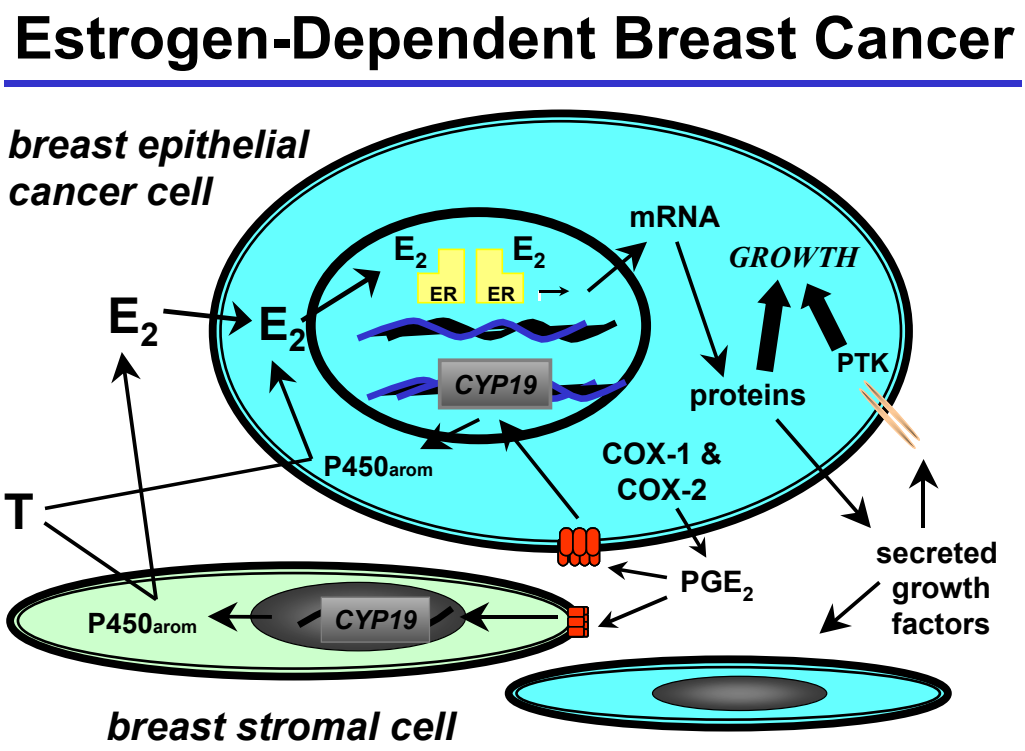


Figure 1.2: Model of autocrine and paracrine pathways of aromatase and COXs in hormone-dependent breast cancer. A dynamic relationship exits between the breast epithelial environment and the stromal cell all of which is influenced by estrogen production which is mediated by the CYP19 gene (aromatase) and the presence of cyclooxygenase isozymes and growth factors. E2, Estradiol; T, testosterone; ER, estrogen receptor; PTK, protein tyrosine kinase. Adapted from Brueggemeier *et al*., (14).

Furthermore, the close mechanistic association shared by aromatase (CYP19) and the COX enzyme eludes to the involvement of autocrine and paracrine effects that may mediate the growth stimulatory behavior in hormone dependent breast cancer development influenced by localized estrogen biosynthesis (14).

In addition to the role of estrogens in the modulation of growth factor genes, Garcia *et al*., report that estrogens are also involved in the increased production of proteases such as pro-cathepsin D which can play a role in the enhancement and invasiveness of tumor cells (18).

1.2.2. Antiestrogens and Aromatase Inhibitors

With approximately 75% of breast cancers falling under the category of hormone-dependence, estrogen depletion has been the primary treatment design strategy for this breast cancer type. Tamoxifen treatment has historically represented an effective strategy for first-line defense in post-menopausal patients with breast cancer; however, cancer relapses and increased risk for other diseases has led to the development of better drug design strategies. Aromatase, is enzyme product of the CYP19 gene and belongs to the larger super family of cytochrome P450 enzyme complexes. Uniquely located in a variety of tissues, including subcutaneous fat, liver, muscle, normal breast tissue, brain and mammary adenocarcinoma, aromatase is crucial to regulating endocrine functions within the body. Aromatase is responsible for the last step in the production of estrogen biosynthesis and thereby represents the predominant source of estrogen in postmenopausal women. As such, the need for effective estrogen depletion agents have been primarily geared towards development of aromatase inhibitors (19,20,21).

There are two types of aromatase inhibitors (type-1 and type-2), type-1 inhibitors are classified as steroidal and irreversible because of their ability to compete with the substrate androstenedione for the enzyme active site and thereby decrease the formation of product (Table 1.1). Type-2 inhibitors (nonsteroidal and reversible) have characteristic chemical features, i.e., a heteroatom, which allow for interactions with the heme iron of the cytochrome P450 and thereby influence steroid hydroxylations (14).

Further grouping of the aromatase inhibitors is within first, second and third generation-classes, which are categorized according to their potency and specificity profile. Aminoglutethidmide is classified as the first generation aromatase inhibitor. Originally thought to serve as an effective anticonvulsant agent in the treatment of epilepsy, aminoglutethidmide was later associated with adverse side effects and consequently no longer used as an effective treatment strategy (22). With improvements in specificity and subsequent selectivity, second-generation aromatase inhibitors were developed and include; (4-OHA) formestane (type-1, steroidal and reversible) and fadrozole (type-2, nonsteroidal and irreversible). When the clinical efficacy of second generation aromatase inhibitors was compared with standard therapy (megastrol acetate as second-line and tamoxifen as first-line therapy) there were no improvements in treatment outcome for these compounds (23,24,25,26,27). Fadrozole was found to be a more effective aromatase inhibitor than AG or 4-OHA, and was subsequently approved for treatment in Japanease people with advanced breast cancer (28,29,30).

Perhaps the more publicized generation of aromatase inhibitors are those compounds belonging to the third-generation of inhibitors. The third-generation inhibitors include the type-1 (steroidal) inhibitor exemestance and the type-2

(nonsteroidal) inhibitors, anastrozole, letrozole and vorozole shown in (Table 1.1). This generation of inhibitors is classified as the most potent and selective group of compounds and thereby shown to have the most significant impact on treatment options in postmenopausal patients. Exemestane (Aromasin®) was shown to effectively inhibit rat ovarian aromatase (ED_{50} of 1.8 and 3.7 mg/kg, respectively) when given subcutaneously or through oral administration and also potently inhibit human placental aromatase with $K_{i_{app}}$ of 26 nM (31,32).

When anastrole (Arimidex®) was compared with tamoxfen in several phase III studies comparing the efficacy of third-generation AIs and tamoxifen as first line therapy in metastatic breast cancer (MBC), anastrozole was approved as first-line therapy for MBC (33,34). Anastrozole is also shown to be just an effective agent *in vitro* with an IC_{50} of 15 nM in human placental microsomes (35). Vorozole, which is also part of the trilogy of trizole derivatives is also a potent inhibitor of aromatase in various *in vitro* systems with reported apparent K*i* values of 1.3 nM in human placental microsomes (36). Lastly, letrozole (Femara®) the last of the third-generation aromatase inhibitors was also compared with tamoxifen for the potential use as a first-line therapy for MBC in a randomized trial. Letrozole in addition to anastrole (Arimidex®) was found to have approval for use as first-line therapy for MBC (37). Furthermore, the inhibitory activity of aromatase in shown *in vitro* with letrozole having a reported IC_{50} value of 11.5 nM in human placental microsomes (38). The *in vivo* evidence supports the use of letrozole as an orally active agent in causing regression of tumors in the 7,12-dimethylbenz(a)-anthracene hormone-dependent rat tumor model, which also corroborates with aromatase inhibition in the patient setting (39).

With the different safety profiles established for aromatase inhibitors over tamoxifen, AIs are fast becoming the new standard of treatment in post menopausal patients with breast cancer. Furthermore, in the first-line setting, large phase III trials have revealed the superiority of third-generation inhibitors over tamoxifen in women with metastatic breast cancer. In conclusion, aromatase inhibitors appear to be emerging as the most widely used agent to treat hormonal responsive breast cancer. Although not appropriate in every type of estrogen or progesterone receptor breast tumor, the lack of cross-resistance among the aromatase inhibitors will allow for more diverse treatment options curtailed to individual patient needs.

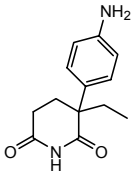
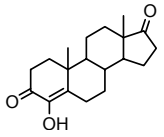
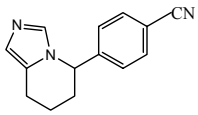
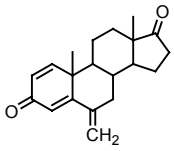
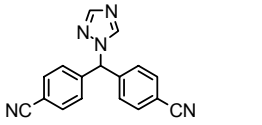
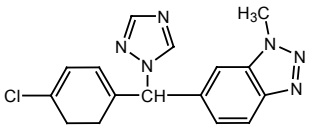
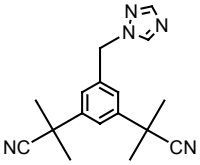
Generation	*Type 1 (Steroidal)*	*Type 2 (Nonsteroidal)*
First	**None**	 **Aminoglutethimide**
Second	 **Formestane**	 **Fadrozole**
Third	 **Exemestane**	 **Letrozole**  **Vorozole**  **Anastrozole**

Table 1.1 Classification and category of common aromatase inhibitors.

1.2.3. Estrogen Biosynthesis

The major pathways leading to E_2 biosynthesis and metabolism in breast tissue are outlined in (Figure 1.3) (40). The metabolic transformations of E_2 in the breast involve the conversion of E_2 to E_1 by 17β-hyroxysteroid dehydrogenase and subsequent conjugation to E_1S by estrogen sulfotransferase. Additionally, the involvement of cytochrome P450 enzymes play a role in oxidative metabolism by the employment of E_2 and E_1 as substrates at various sites on the estrogen molecule (41). The model for estrogen biosynthesis and metabolism in breast tissue (shown in Figure 1.3) showcases four pathways that are relevant to the generation of estrogen metabolites in target cells which are involved in biological properties distinctive of estrogen (40). Also shown is the metabolic process which leads to the complete unreactivity of estrogen through a reaction that is catalyzed by glucuronosyltransferase (UGT) enzymes and recently shown to have a role in cancer risk in estrogen target tissues (42,43,44,45,46,47,48).

ER-negative hormone-independent breast carcinoma is categorized by the lack of estrogen influence on the molecular transformation involved in hormone dependent breast cancer. As the heterogeneity of breast cancer increases, new therapies that target specific genes and proteins actively involved in the pathophysiology of breast cancer are warranted. There has been considerable effort made in targeting hormone dependent breast cancer, which has led to substantial progress in treatment strategies. The development of effective endocrine treatments with aromatase inhibitors in place of or sequentially with tamoxifen are found to reduce the risk of recurrence in postmenopausal women with estrogen receptor (ER)-positive tumors (49,50,51).

Estrogen metabolism in the Breast Microenvironment

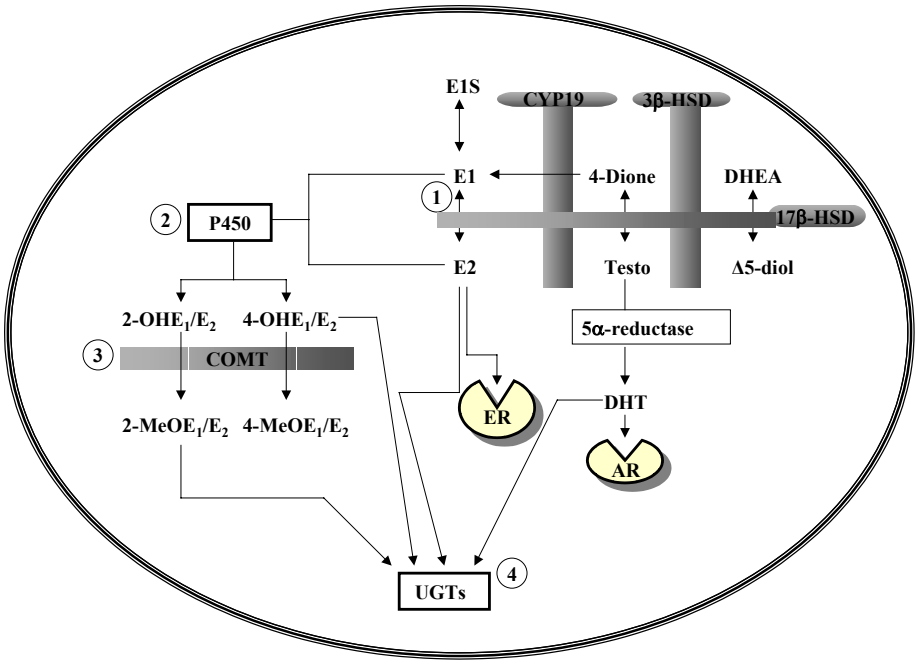


Figure 1.3: Illustration of estrogen metabolism. (1) Conversion of Estradiol (E_2) to estrone (E_1) by 17β-hyroxysteroid dehydrogenase (17β-HSD); Estrogen sulfotranserase is responsible for further conjugation to estrone sulfate (E_1S). (2) Alternate metabolic pathway showing cytochrome P450 enzymes responsible for oxidation of $E_1$and E_2 to 2- and 4-hydroxy-catecholestrogen (OHCE). (3) Catechol estrogens are subsequently inactivated by *O*-methylation, which is catalyzed by catechol-*O*-methyltransferases (COMT). (4) Alternate possible conjugation of parent estrogens E_2 and E_1 by glucuronosyltransferase (UGTs) which ultimately results in estrogen glucuronides and complete estrogen unreactivity. AR, androgen receptor; 3β-HSD, 3β-hydroxysteroid dehydrogenase; CYP19, aromatase; Δ5-diol, androstenediol; DHT, dihydrotestosterone; ER, estrogen receptor; MeO, methoxy; OH, hydroxy; testo, testosterone. Adapted from Lépine *et al*., (40).

Although ER status still remains to be a weak variable for targeting treatment options and strategies, it is still a strong indicator of overall patient responsiveness to endocrine therapy. Support for this is based on the positive correlation between responsiveness to endocrine therapy and a better disease survival in ER-positive breast cancers (52).

1.2.4. Implications for an ER-negative phenotype

The progression of an ER-positive phenotype to an ER-negative phenotype typically is associated with a more aggressively categorized level of breast cancer. Dupont *et al*., further suggest that ER-negative tumors are typically associated with a higher histological grade, and the patients tend to have an overall decreased survival which is correlated with age and lymph node status (53). In postmenopausal women ER receptor status is linked to survival with a higher recurrence rate observed in ER-negative tumor types (54). Poor differentiation of ER-negative tumors has limited the classification of selective cancers, however, there has been a small subset of invasive cancers that have been identified as ER-negative (adenoid cystic carcinoma, secretory carcinoma) with a favorable prognosis and least likely to recur (55,56,57).

The loss in hormone dependence of estrogen has been attributed to the constitutive overexpression of growth-promoting genes that are normally under regulatory control of estrogen. This loss in estrogen regulation is directly correlated to resistance to anti-estrogens and the previously mentioned aggressive phenotype associated with this hormone-independent state (58). Harris *et al*., further suggest that the upregulated growth factor signaling seen in ER-negative tumors may be a result of overexpression of growth factor receptors such as epidermal growth factor receptor (EGFR) and c-erbB2, which may provide an alternate growth stimulus (59,60). The

breast cancer-associated gene 1 (BRCA1), which belongs to the family of tumor suppressors, is the found to be the most frequently mutated gene in familial breast cancers. By the age of 70 years old, individual carriers of the BRACA1 mutation have a 50-80% risk of developing breast cancer and therefore represent a unique group for developing specialized preventive and therapeutic agents for treatment (61). Incidentally, Haas *et al*., report that some ER-negative tumors are indeed shown to have higher BRCA1 germline mutations (62). All of these features that characterize ER-negative tumors, which result from transformation to hormone-independence, unveils their heterogeneous nature and the need to develop therapeutic agents to target specific genes and proteins involved in this pathway development.

Because of the heterogeneity that arises in classifying subtypes of breast cancer, there is a growing trend towards using common expression profiles based on genomic studies to determine individualized patient options. Using affymetrix microarrays, Ivshina *et al*., were able to study the relative expression profiles of 347 primary invasive breast tumors. With the utility of statistical analysis they were able to determine a grade classification of tumors (G1, G2, or G3) using class prediction algorithms. Overall, six of their 264 robust grade-associated markers, could be accurately categorized as G1 and G3 tumors with further identification of two highly discriminate G2 classes (G2a and G2b genetic grades). Additionally, there was found to be a correlation among overall survival in patient outcome relative to tumor class. This unique expression profile based on the determination of a genetic code signature for tumors is likely to improve overall treatment planning and prognosis for better individualized care (63).

Gene expression patterns have also provided improved prognostic tools in assessment of p53 mutation status in patients. Because of the strong correlation between p53 status and subtype of breast cancer that is influenced by so many other downstream signaling events, p53 represents an attractive target for molecular classification. This confounding issue was addressed by using both ER- and ER+ cell lines and experimental data collected in patients to generate a more concise gene list. The final p53-associated list contains 52 genes, with unique patterns of up and down-regulation in two biologically relevant gene clusters. The findings were consistent with previous reports of the unique downstream and indirect effects of mutant p53. Overall, combining genomic studies with biologically-based methods led to identification of unique genes in the signature profiles of p53 status in breast cancer which led to developing a classification system for common p53-subtypes in breast cancer (63,64,65,66). Standarized methods of gene expression is fast becoming a clinical tool for better prognosis in individualized patient care.

1.2.5. Targeting Estrogen-receptor negative breast cancer

The treatment strategies for ER-negative tumors have been uniquely specialized because of the absence of the ability to target the tumors with antiestrogen or aromatase inhibitors, both of which are effects in treated ER+ tumors. In 1988 the committee for Early Breast Cancer Trialists' Collaborative Group, which included 8,000 women with ER-poor breast tumors, explored the benefits of tamoxifen treatment. Their conclusive results were that in each subgroup evaluated (ER-poor/PR-poor tumors; ER-poor/PR-positive tumors; women over the age of 50) there was no evidence of a trend toward a benefit with longer tamoxifen treatment (67). In September 2000, an updated Overview analysis was presented in Oxford in a preliminary forum, which included a study of

12,000 women with ER-negative tumors, half of who were treated with tamoxifen. The results from the preliminary report still did not support a tamoxifen treatment regimen in women with ER-negative breast cancer (68). Another approach in the treatment of ER-negative tumors is by using the MDA-MB-231 human breast cancer cell line as a model of ER-negative breast cancers. Various ER-deleted variants are transfected into an ER-negative cell line in an attempt to recover anti-estrogen responsiveness (69). Additionally, cathepsin D, which is overexpressed in ER-negative tumors and rarely associated with HER2-Neu amplification is also an attractive target because it represents one of the rate-limiting factors involved in the proliferation of micrometastases at distinct sites in MDA-MB-231 breast cancer cells (70).

Overexpression of HER2+ is associated with a higher degree of recurrence and death in breath cancer. Tumors are more likely to be poorly differentiated with a high rate of proliferation, axillary lymph nodes are positive, and expression of estrogen and progesterone receptors is decreased. Trastuzumab (Herceptin®) was found to be an effective strategy for treatment of HER2+ tumors. In patients with metastatic disease, trastuzumab has become an effective treatment without any appreciable toxic effects. And in patients where tumors were detected by fluorescence in situ hybridization (FISH), or in HER2 overexpression, trastuzumab treatment alone was responsible for the tumors having a 34 percent response rate (51). Additionally, phase 3 clinical trials show that chemotherapy with combination of trastuzumab treatment results in an overall better improvement of therapeutic end points including-relative progression, rate of response, length of response and better predictor of survival (7,8).

Lastly, chemotherapy has proved to be an effective treatment in ER-negative tumors and with improvements in this treatment regimen patients with ER-negative tumors stand to benefit greatly. The International Breast Cancer Study Group reports that a three cycle-regimen of cyclophosphamide, methotrexate, and fluorouracil were found to have substantial benefit in those patients with ER-negative tumors with node negative disease treated with tamoxifen. Their conclusions are that ER-negative tumors without hormone receptors are more sensitive and respond better to chemotherapy, which lead to a higher successful rate and pathological response (71,72,73).

1.2.6. Effect of diet prevention on breast cancer

Because of the predominant role of estrogen exposure leading to the development of hormone-dependent breast cancer, it represents one of the strongest risk factors for ER+ tumors. As such, any potential dietary influences that may influence ER+ tumors is overshadowed by the strong presence of hormone dependence within this tumor type. Hormone-independence in ER- tumors and the multifaceted approach that is needed to treat this category of breast cancer has been previously highlighted. Additionally, in ER- tumors other treatment strategies, including diet, may exert a more significant response and influence because of the absence and dependence of the hormone variable. The original premise that diet influenced postmenopausal breast cancer risk and incidences was based on observed differences in lower breast cancer among women of Asian decent and increasing risk of breast cancer among emigrant Japanese women to North America (74,75). Incidentally, the status of the estrogen receptor in normal breast tissue is much lower in Asian women than among Western women (76,77,78). While controlling for estrogen receptor status, Olsen *et al*., investigated the effects of diet including fruit and

vegetable consumption on the incidence of postmenopausal breast cancer. Their data collection was based on information collected on diet and other established risk factors provided by postmenopausal women (n = 23,798; aged 50-64 y) in the cohort "Diet, Cancer and Health." The findings of this study were that diet independently was not associated with decreasing the incidence rate of breast cancer; however, diet did play a preventive role in the incidence of estrogen-receptor negative cancer. Taken together, their study at least point to a plausible role for diet impacting breast cancer when controlled for estrogen receptor status (79).

Fung *et al*., have examined the association of diet and the risk of breast cancer in postmenopausal women by focusing on diet quality indices taken from 71,058 women with follow-up for up to 18 y, from 1984 to 2002. The methods by which they assessed diet quality indices were Healthy Eating Index (HEI), Alternate Healthy Eating Indix (AHEI), Diet Quality Index-Revised (DQIR), Recommended Food Score (RFS), and the alternate Mediterranean Diet Score (aMed), all of which were evaluated by taking into account ER status and risk-references. Each major food component of the diet was evaluated and included: fiber, fruit, vegetables and meats with each subcategory of vegetables (yellow/orange, leafy, cruciferous and other) taken into consideration. The study took into consideration the effect of diet on relative risk of both ER+ and ER- breast cancer. Their results showed that higher score indexes reflected in each instrument used to assess diet was associated with a lower risk of ER- breast cancer. Although they report that a close association of diet with ER+ tumors was not found, this did not negate that diet at an early onset may influence the overall development and progression of ER+ tumors (80).

Whether the incorporation of fruits and vegetables in the diet cause a preventive effect on the onset of breast cancer still remains to be determined with findings supporting either side of the continuum. What is apparent is that ER- tumors respond differently to dietary and other inhibitory factors. With regards to treatment strategies, there still represents a need to develop novel targets to effectively treat hormone-independent breast carcinoma and possibly reduce the severity of treatment options in this disease.

1.3 Chemopreventive Phytochemicals

1.3.1 Isoflavonoids

Isoflavonoids are a class of compounds belonging to a larger umbrella of compounds called the flavonoids. These natural product derivatives are classified with a larger group of chemopreventive phytochemicals in their role as effective agents to negatively influence signal-transduction pathways involved in carcinogenesis (Table 1.4).

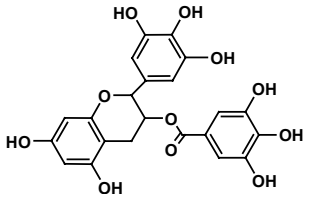
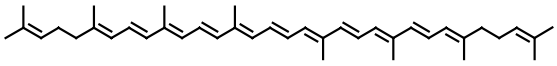
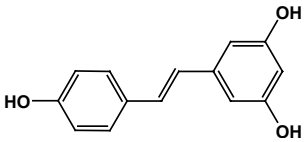
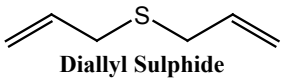
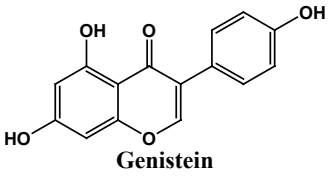
Common Chemopreventive Foods	Dietary Precursor
Green Tea	 Epigallocatechin-3-gallate
Tomatoes	 Lycopene
Grapes	 Resveratrol
Garlic	 Diallyl Sulphide
Soybean	 Genistein

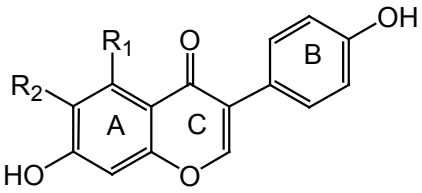
Figure 1.4: Common chemopreventive foods and their dietary precursors.

Because of the multifaceted nature of human cancers and the intricate pathway-specific transformations leading to tumorigenesis, any agent that plays a role in influencing the final carcinogenic transformation is crucial to treatment options. Isoflavones are of particular interest because of the relatively high amount found in soybeans. Its been reported that for every gram of ingested soy protein, at least 3.5 mg of isoflavones are also ingested (81). There are at least three reported soybean isoflavones and include the aglycones genistein, daidzein, and glycitein. Each isoflavone is a derivative of its biological active form, the β-glycoside conjugates (genistin, daidzin, and glycitin), which contains either an acetyl or malonyl group moiety (Table 1.2) (82,81).

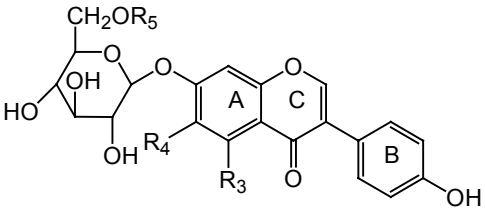
Genistein is the predominant isoflavone in soy and thus deemed responsible for the largely chemopreventive benefits of soy consumption. The half-life of isoflavones is roughly 8 hours followed by an excretion within a 24-hour period (83). Clinical trial data that report on the benefits and drawbacks of dietary isoflavone consumption and relative risk of breast cancer may be influenced by metabolism variations coupled to their short half-life, although this needs to be further examined (84,85,86).

In regards to binding capability, isoflavones display the ability to bind both estrogen receptors ERα and ERβ and are considered to be classified as phytoestrogens because of their weak estrogenic potential (87,88). The estrogenic potential of isoflavones is related to their ability to preferentially bind ERβ and therefore may fall under the category of selective estrogen receptor modulators (SERMS), as detailed in recent reviews (89,90,91). The estrogenic potential of isoflavones is precisely the reason behind the larger debate of the benefits of soy consumption.

Aglycones



R1	R2	Compound
H	H	Daidzein
OH	H	Genistein
H	OCH_3	Glycitein



Glycosides

R3	R4	R5	Compound
H	H	H	Daidzin
OH	H	H	Genistin
H	OCH_3	H	Glycitin
H	H	$COCH_3$	Acetyldaidzin
OH	H	$COCH_3$	Acetylgenistein
H	OCH_3	$COCH_3$	Acetylglycitin
H	H	$COCH_2COOH$	Malonyldaidzin
OH	H	$COCH_2COOH$	Malonylgenistin
H	OCH_3	$COCH_2COOH$	Malonylglycitin

Table 1.2 Isoflavone aglycones and their chemical precursors. Adapted from Teekachunhatean *et al*., (92).

1.3.2 Genistein, the predominant isoflavone

The relationship amongst increased soy protein consumption, genistein and the relative risk of breast cancer is controversial. The question arises as to what is so uniquely responsible for this predominant isoflavone to be cast in such a chemopreventive role as it relates to influencing the etiology of breast cancer. One primary reason attributed to the anticancer potential of genistein is its ability to specifically target signaling molecules involved in abnormal cell growth and proliferation. A concentration dependence influences the antiproliferative role of genistein in breast cancer cells such that inhibition of growth is seen only at high concentrations, while stimulation is observed at lower ones.

Regardless of these opposing roles, genistein's involvement in estrogen dependent and independent mechanisms include modulating the expression of relevant proteins involved in the activation of transcription factors, NF-κB and AP-1. The activation of NF-κB is intricately involved in the phenotypic changes that occur at the stage of carcinogenesis which leads to transformation of the neoplastic cell (93,94,95). Additionally, AP-1 is another transcription factor involved in the proliferation and differentiation associated with malignant tumor cell production (96,97,98,99). Therefore, the ability of genistein to effectively target activation of NF-κB and AP-1 is crucial to the influence of a cascade of divergent signal transduction pathways that lead to biotransformation from the pro-carcinogen to ultimately the neoplastic cell (Figure 1.5). Additionally, genistein has been shown to inhibit tyrosine kinase activity and thereby effecting downstream mitogens (100).

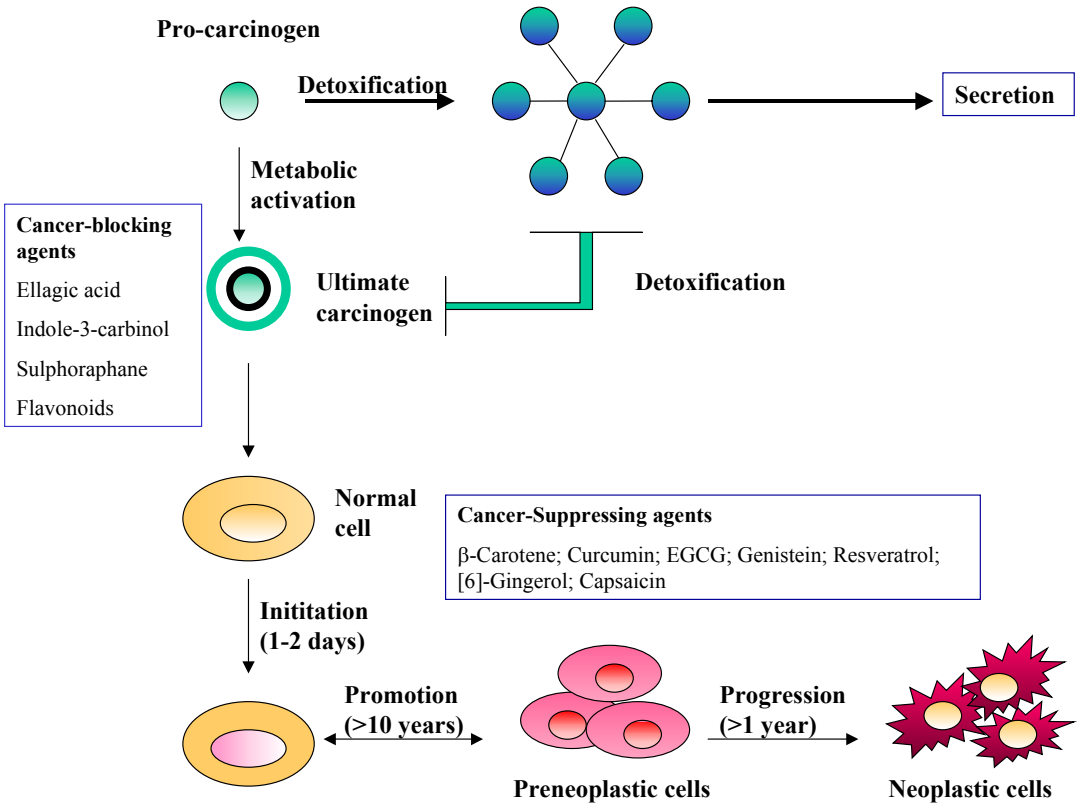


Figure 1.5: Schematic showing the transformation of carcinogenesis from the pro-carcinogen to evolution of the neoplastic cell. Dietary phytochemicals that are involved in blocking stages of carcinogenesis and suppresses molecular progression to a cancerous state are also shown. Adapted from Surh (101).

Also, evidence exists for its role as an effective DNA topoisomearse activity inhibitor, angiogenesis suppressor and finally shown to have inhibitory control of cell cycle regulatory proteins ultimately converging in programmed cell death (102,103,104,105).

1.4. Overview and clinical relevance of two isoflavones

1.4.1 Phenoxodiol, an isoflavone analog

Phenoxodiol (2H-1-benzoypyran-7-0, 3-(hydroxylphenyl) which is an analog of genistein (Figure 1.6) has been evaluated in several human cancer models and discovered to be a potent initiator of apoptosis with broad-range implications for anti-tumor activity.

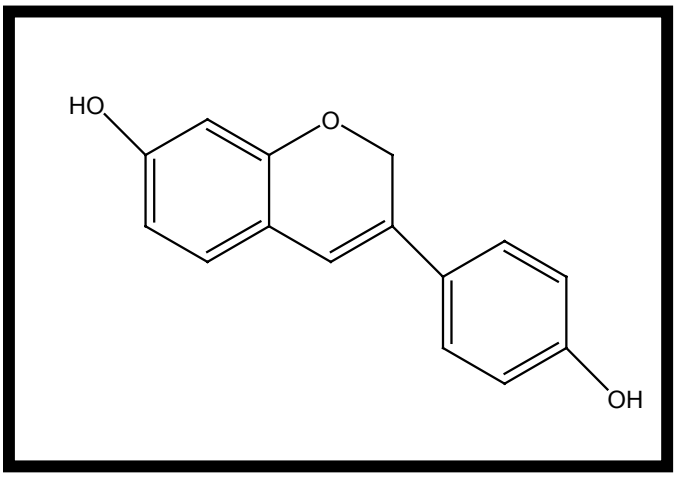


Figure 1.6: Molecular structure of phenoxodiol (2H-1-benzopyran-7-0, 3-(hydroxylphenyl). Adapted from Choueiri *et al*., (106).

Senderowicz *et al*., set out to determine the mechanisms responsible for the antiproliferative behavior of phenoxodiol in a battery of human cell lines derived from

head and neck squamous cell carcinoma (HNSCC, HN12, HN17, and HaCaT). They first determined the ability of phenoxodiol to induce unrestricted cell death indicative of colony formation as tested by the "clonogenic assay". Further investigation revealed a caspase-dependent cell death, which was independent of clonogenic death and further showed the possibility of other cell-death mediated mechanisms that phenoxodiol may be responsible for. When HN12 cells were preincubated with ZVAD, a general caspase inhibitor, flow cytometry analsysis revealed a reduction in Annexin V stained cells, which further eluded to a caspase-dependent mechanism in the presence of phenoxodiol.

To further investigate the antiproliferative effects of phenoxodiol they turned their attention to cell cycle arrest and cyclin-dependent kinase activity. Accumulation of cells occurred at the G_1-S cell cycle at least 12 hours prior to induction of apoptosis and analysis of *in vitro* inhibition of cyclin-dependent kinase (cdk) activity did not reveal a dependence on phenoxodiol activation (although cellular cdk2 activity underwent a reduction). Finally, the presence of phenoxodiol resulted in the accumulation of the endogenous cdk inhibitor, $p21^{WAF1}$, which was shown to result in loss of cellular cdk2 activity (107). Taken together, this isoflavone analog was found to show great promise and clinical relevance for anti-cancer therapy and is currently in phase I trials to to explore its role as a chemo-sensitizer for patients suffering from final-stage, solid turmorigeneis (106).

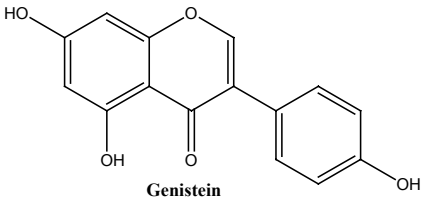
1.4.2 Red Clover-a derived isoflavone supplement

Red clover (*Trifolium pratense*), a member of the plant family Leguminosae is part of the 250 *Trifolium* genus species, was cultivated in Europe and can be traced back to the third and fourth century (108).

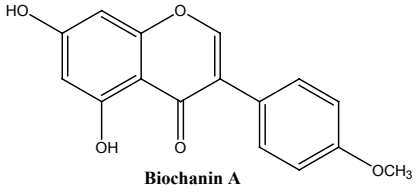
Its main components are genistein, daidzein, biochanin A and formononetin, with genistein being the predominant isoflavone (Fig 1.7). The estrogenic activity associated with red clover is solely based upon its isoflavone content and has thereby been characterized as phytoestrogenic with beneficial and at times contradictory results, as is common for this class of compounds. Clinical studies that have aimed to explore alternative treatments for menopausal disorders have evaluated the substitutionary benefit of red clover as supplements (Promensil®), or as alternative red clover preparations (109). In regards to the effect of hot flushes in postmenopausal women, when compared with a placebo, red clover supplements resulted in the reduction but not the frequency of hot flushes. These results were corroborated by additional randominzed, double-blind, placebo-controlled studies which also report no significant different in menopausal disorders amongst treatment with red clover supplement/preparations and placebo group. However, when the study design was better controlled for isoflavone-containing food intake, there was reported a significant decrease in the frequency of hot flushes in the Dutch population (110).

Each isoflavone present in red clover is responsible for its medicinal activity.

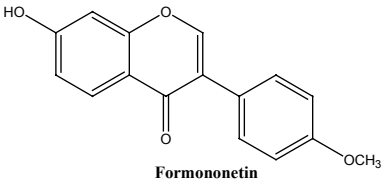
Red clover (*Trifolium pratense*)



Genistein



Biochanin A



Formononetin

Daidzein

Figure 1.7: Photo of red clover (*Trifolium pratense*) courtesy of PDR health. Adapted from PDR health (111).

Whether red-clover derived supplement stand to have any benefical effects on prostate cancer have also been investigated. In a nonrandomized, nonblinded trial study containing a group of 20 men who suffered from prostate cancer, administration of an isoflavone dose of 160 mg/day preeding prostatectomy was analyzed for any benefical effects. Apoptosis was used as a mechanistic marker for treatment comparison and results showed a difference between treatment and absence of treatment among the control tissues. In patients treated with the isoflavone supplement prior to surgery versus the non-treated group, the presence of apoptosis in the treated specimens were much higher (112).

This study further shows support for the claims regarding the protective benefit of isoflavones and their ability to have an effect on signal-transduction pathways leading to the cancerous state. In conclusion, isoflavone analogs and plant derivatives containing isoflavones show great promise as therapeutic agents in the treatment and overall effect on the progression of hormone-dependent states and thereby warrant further exploration to better understand their mechanistic advantages.

1.5. Genistein and Cell-Death Mediated Pathways in MCF-7 and MDA-MB-231 breast cancer cell lines

1.5.1 Mechanistic implications in MCF-7 cells

Much has been reported as it relates to the role of genistein and induction of apoptosis in MCF-7 cells. Loo *et al*., report in 2001 on the effect of genistein on molecular apoptosis markers in both MCF-7 and MDA-MB-231 cell lines. Their findings were that after 6 days of incubation with 50μM genistein, MCF-7 but not MDA-MB-231 cells, showed morphological signs of apoptosis. Furthermore, marginal

proteolytic cleavage of poly-(ADP-ribose)-polymerase and significant DNA fragmentaion were also detected in MCF-7 cells. The conclusions reached by Loo *et al*., suggested that at the concentration of genistein tested, MCF-7 cells but not MDA-MB-231 cells were susceptible to the induction of apoptosis by genistein. They also showed that Bax and Bcl-2 did not play clear predictive roles in the induction of apoptosis (113). The pleotropic effects of genistein on MCF-7 breast cancer cells was reported by Sarkar *et al*., which showed not only the ability of genistein to inhibit growth of MCF-7 breast cancer cells in a dose dependent manner but the involvement of other growth inhibitory effects. The antiproliferation of genistein was found to accompany a reduction in the number of mitotic cells and overexpression of cyclin dependent kinase inhibitor $p21^{WAF1}$ leading to cell cycle arrest. In addition, Sarkar *et al*., report that the telomeric area was significantly reduced in genistein. They studied the involvement of multiple genes involving the apoptotic pathway which revealed inhibition of Akt activity without affecting the steady state levels of Akt protein expression and the down regulation of proapoptotic gene BAD expression. The conclusion reached was that genistein-induced inhibition of cell division is partly mediated by decreased telomere length, reduced mitosis and inhibition of Akt activation, leading to induction of apoptosis (114).

Genistein and the involvement of caspase-relevant cell markers in MCF-7 cells was demonstrated by Sergeev *et al*., which report that genistein induces apoptosis in breast cancer cells via activation of the Ca^{2+}-dependent proapoptotic proteases, μ-calpain, and caspase-12. Their studies show that treatment of MCF-7 breast cancer cells with genistein induced a sustained increase in concentration of intracellular Ca^{2+} resulting from depletion of the endoplasmic reticulum Ca^{2+} stores. Furthermore, they suggest that

Ca^{2+} -dependent proteases are potential targets for genistein in breast cancer cells and that the apoptotic mechanism involved with genstein are attributed to cellular Ca^{2+} regulatory activity (115). As reported, the involvement of cell death mediated mechanisms and additional pleotropic effects of genistein have been chiefly attributed to a hormone-dependent fashion as it relates to breast cancer cells.

1.5.2 Investigation of antiproliferative and cell-death mediated pathways in MDA-MB-231 cells.

The exploration of genistein and its ability to be an effective agent in hormone-independent breast cancer has been evaluated. Sarker *et al*., who reported on the pleotropic effects of genistein on MCF-7 breast cancer cells made an earlier attempt to explore apoptosis in MDA-MB-231 breast cancer cells by genistein. They reported that in MDA-MB-231 cells there was an upregulation of Bax and $p21^{WAF1}$ expression and down-regulation of Bcl-2 and p53 expression in genistein treated cells. Furthermore, DNA ladder formation, CPP32 activation, and PARP cleavage were observed after treatment with genistein, indicative apoptotic cell death. They also showed by flow cytometry that the number of apoptotic cells increased with longer treatment of genistein. The conclusions reached by their group were that genistein treatment at 30μM 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 (116).

The discrepancy in findings of Sarker *et al*., as it relates to the involvement of genistein and expression of apoptosis-related genes in MDA-MB-231 cells was reported by Loo *et al*., who did not find any appreciable role for genistein to induce apoptosis in MDA-MB-231 cells, but a greater involvement of relevant cell-death markers to be

attributed to MCF-7 breast cancer cells. They offer that differences in culture medium, source of purified genistein, and primary antibodies to detect Bax and Bcl-2 may explain the differences in results (113). Furthermore, Yee *et al*. suggested that the MDA-MB-231 cells may be phenotypically different and thus maintained under different conditions which may further lead to discrepancies in reported data (117). Nevertheless, Loo *et al*., did expose cells to similar conditions but did not see any morphological signs of apoptosis although cell proliferation was apparently inhibited (113). However, genistein's role as an effective chemotherapeutic agent in the treatment of hormone-independent breast cancer should not be minimized. Nam *et al*., recently reported on the effectiveness of genistein against human breast cancer cells by evaluating the chemopreventive and cytotoxic effect of genistein in MCF-7 and MDA-MB-231 breast cancer cells. Their results show that cytochrome P450 (CYP) 1A1-mediated ethoxyresorufin *O*-deethylase (EROD) activity was inhibited by genistein in a concentration dependent manner. Futhermore, genistein significantly inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced cyclooxygenase-2 activity and protein expression at the contrations of 10 ($p<0.05$), 25 ($p< 0.05$) and 50μM ($p< 0.01$). Additionally, ornithine decarboxylase (ODC) activity was reduced to 53.8% of the control after 6 h treatment with 50μM genistein in MCF-7 breast cancer cells. Their conclusion was that genistein could be of therapeutic value in preventing human breast cancer (118). A summary of the current understanding of the inhibitory effect of genistein in breast cancer cells is shown in (Figure 1.8).

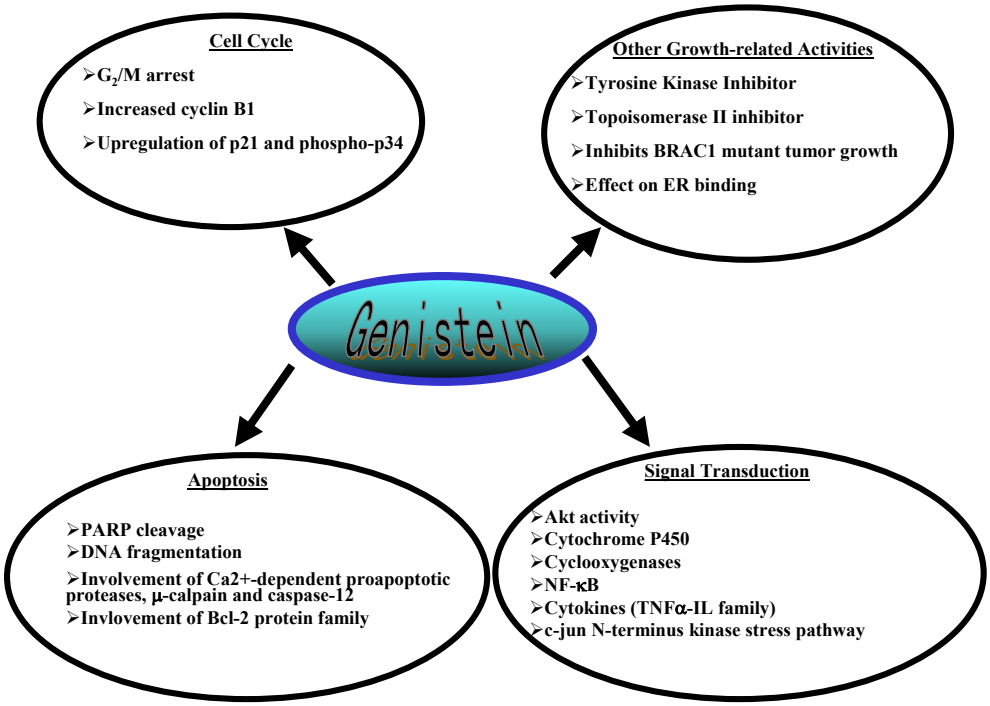


Figure 1.8: Summary of current understanding of the inhibitory effects of genistein in breast cancer cells. This does not represent a complete list of genistein's involvement in signal-transduction pathways, but is representative of the general understanding of genistein and its influence in breast cancer cells.

1.6. Summary

This introduction was designed to first provide the reader with an overview of the complex nature of breast cancer type and treatment variability and the need to explore fundamental variables that may influence the development to the cancerous state. Chemopreventive phytochemicals in the treatment of cancer and other diseases is not a new phenomenon. As such, there are many laboratories that have exhausted this avenue of research and many of their findings are reported in this review. Our research group is interested in the development of novel isoflavone analogs for the treatment of breast cancer.

This introduction further explores the complex issue of hormone-dependence as an important variable for treatment design options. Additionally, the role of diet and isoflavone analogs are shown to more effectively play a role in influencing the overall biology of breast cancer in the absence of hormone contributing factors. Two isoflavones (phenoxodiol-an isoflavone analog) and (red clover-an isoflavone mixture) are reviewed. Phenoxodiol was found to significantly have an impact in cell-death mediated pathways in the treatment of cancer and is currently in phase I trials for use as a chemotherapeutic agent. Red clover has been implicated in improving the quality of symptoms in postmenopausal women and inducing apoptosis in prostate cancer cells. As such, the role for isoflavones as effective agents in improving the overall response to cancer therapeutics is still very prevalent.

Genistein, the predominant isoflavone, was thoroughly explored for its effective use as an agent for reducing risk in breast cancer. Furthermore, its ability to influence signal transduction pathways that are intricately involved in carcinogenesis are evaluated

in both hormone-dependent and hormone-independent cell lines. In conclusion, we hope to provide the reader with a knowledge of the beneficial role that isoflavones have in influencing the overall etiology of breast cancer and other disease states. In regards to the ability of genistein to significantly impact signal transduction in breast cancer, it is still imperative to develop novel isoflavone analogs of genistein as effective agents in the treatment of breast cancer.

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CHAPTER 2

STATEMENT OF THE RESEARCH PROBLEM AND SPECIFIC AIMS

2.1. The Research Problem

Exploring the evolution of breast cancer for plausible treatment options continues to be an important focus of substantial research efforts. A diagnosis of breast cancer continues to be a fatal blow for many women in the face of many treatment advances that have been attributed to more successful outcomes. Additionally, with the hormonal influence of estrogens in mediating the autocrine and paracrine effects of the breast tumor environment, strategically exploring treatment options has become even more intertwine. With the evolution of hormone-independence as an outgrowth of phenotypically resistant tumors to hormone therapy, a need exists for novel strategies to effectively target the signal transduction pathways that lead to this biotransformation in breast cancer.

The relationship established between the consumption of soy in Asian countries and the lower incidence in breast cancer compared to migratory individuals of Asian decent establishes a need for further chemopreventive and therapeutic exploration. Genistein, the predominant isoflavone in soy and characterized as a phytoestrogens, has shared somewhat of a dichotomous relationship in influencing breast cancer growth, although it still has proven as an effective agent against breast cancer cells in the laboratory. The experimental and epidemiological evidence regarding genistein and its

ability to impact signal transduction pathways leading to breast cancer has warranted further implications for developing novel agents using the isoflavonoid scaffold. Genistein, which is often characterized as a weak phytoestrogen, has shown the ability to play a role in ER binding and thereby influence the overall estrogenic tumor environment. Additionally, genistein is shown to be an effective agent in inhibiting proliferation of several hormone-dependent and hormone-independent breast cancer cell lines including MCF-7, T47-D, MD-MBA-231 and SKBR3 by multiple mechanistic approaches.

Furthermore, the ability to increase expression of relevant proteins in signal transduction pathways that ultimately lead to cell-death mediated mechanisms in breast cancer makes the development of isoflavone analogs an even more attractive approach. When reviewing genistein's ability to turn on expression of relevant proteins involved in signal tansduction including and not limited to the NFκB, AP-1, Akt, Cdks cascades, the caspases, and Bcl-2 family, we envisioned that development of isoflavone analogs that would be more potent and selective agents in breast cancer.

The evolution of the breast tumor environment is quite complex and studies show that hormone dependence is an additional complication for strategic treatment design. Additionally, hormone-independence represents another phenotype of breast cancer with more aggressive characterization. There has been considerable progress made in the treatment of hormone-dependent breast cancer and the involvement of antiestrogens and aromatase inhibitors, but there still represents a need to develop small molecules for targeted therapy in breast cancer.

2.2. Specific Aims

1) To evaluate and determine the potential of the library of synthetic isoflavonoids as antiproliferative and cytotoxic agents in MCF-7 and MDA-MD-231 breast cancer cell lines.

2) To explore the role of cell cycle arrest and concomitant induction of relevant cell cycle inhibitory proteins within the library of synthetic isoflavonoids.

3) To determine the influence of synthetic isoflavonoids on signal transduction pathways mediated by cell death.

4) To use high throughput technology to provide the gene expression profiles of relevant protein families involved with the synthetic isoflavonoids mechanism of action.

5) To provide data on molecular targets of a class of nimesulide analogues, which were developed as potential aromatase suppression regulators.

CHAPTER 3

EFFECTS OF SYNTHETIC ISOFLAVONOIDS ON CELL PROLIFERATION, CELL CYTOTOXICITY, AND G_1 ARREST IN BREAST CANCER CELLS

3.1. Introduction

Breast cancer, one of the most common cancers among women and second only to skin carcinoma, is responsible for 1 in 33 (3%) deaths in American women. The American Cancer Society estimates that in 2007, 178,480 new cases of invasive breast cancer will be diagnosed among women, as well as an estimated 62,030 additional cases of *in situ* breast cancer (non-invasive and the earliest form of breast cancer). Furthermore, of the estimated reported cases of cancer related deaths, African-American women have the highest percentage of breast cancer related deaths, 19%, of any racial and ethnic group (1).

Although there appears to be a large disparity among those ethnic groups diagnosed with breast cancer, epidemiological studies have shown that native Japanese and Chinese (and other Asian populations in their homelands) have the lowest rates of breast cancer and historically, an approximate 6-fold difference in breast cancer risk between these population groups is reported (2). Additionally, the incidence rates of

breast cancer are as high as 1 in 8 in the United States and as low as 1 in 30 in Japan, taken together it is suggested that environmental influences, such as diet, may play a predominant role in the onset of this disease (3). Increased soy consumption has been associated with this reported reduction in the risk of breast cancer among Asian populations. Genistein, the predominant isoflavone in soy, has been proposed to be the agent responsible for the lower risk of breast cancer in Asian women (4). Although the mechanism of genistein as a chemopreventive agent are not completely understood, it has been extensively evaluated for its anticarcinogenic activity *in vivo* and its antiproliferation in a host of cell lines (5).

Fioravanti *et al*., report that in MCF-7 breast cancer cells, genistein had an effect on proliferation growth factors in the presence of estradiol. Further inhibition was shown in paracrine stimulation by coculture with estrogen-negative cells, which was followed by decreased tyrosine phosphorylation under the regulatory control of transforming growth factor-α. They further report that the observed growth-inhibitory effects of genistein were accompanied by cell cycle arrest in the late S and G_2M phase of the cell cycle (6).

Also responsible for the growth inhibitory activity of genistein is its role as a topoisomerase II inhibitor, which is generally associated with a G_2M block in cell cycle progression (7). Genistein falls under the class of protein tyrosine kinase inhibitors (PTKs), which are known to play a major role in influencing cell cycle growth, apoptosis and overall oncogenesis transformation in cell malignancy (8,9). Singletary *et al*., showed the ability of genistein to act as an antiproliferative agent in nonneoplastic MCF-10F human breast cells by arresting cells at the G_2M transition. They report an induction in the cell cycle inhibitory protein $p21^{Waf/Cip1}$ as well as inhibition of Cdc2 in a

phosphorylation-dependent manner (10). Genistein has an effect on cell cycle regulatory proteins and cell cycle arrest in breast cancer cells, with the predominant arrest profile at the G_2M phase transition. The mechanism of antiproliferative effects of genistein in hormone-independent breast cancer cells containing mutant p53 have also been linked to a dose-dependent accumulation at the G_2M phase of the cell cycle. Additionally, genistein treatment is associated with an increase in cycle B_1 (p62) but not necessarily coupled with disruption of the $p34^{cdc-2}$/cyclin B_1 complex (11).

The cell cycle is tightly regulated by cyclin dependent kinases (CDKs), that ensure cell division maintains functional balance within the cellular environment. The CDKs remain important candidates for therapeutic tumor suppression because of the disruption of tight regulatory checkpoints that occur following DNA damage to the cell (12). Disruption of CDK complexes is achieved by a family of cell cycle inhibitory proteins called the cyclin kinase inhibitors (CKIs). There are two classes of CKIs, the INK4 family (p15, p16, p18, p19) and the Cip/Kip family (p21, p27, p57), both of which are crucial to disruption of CDK complexes and tightly controlled regulatory processes of the cell cycle (13,14,15,16).

Several isoflavones have affinity for both estrogen receptor subtypes, ERα and ERβ, and are therefore classified as weak phytoestrogens due to their weak estrogenic potential (17,18). Genistein preferentially binds ERβ which lends itself to being categorized as a selective estrogen receptor modulator (SERMs) as detailed in recent reviews (19,20,21). The mixed agonistic/antagonistic activity privy to this class of compounds is responsible for the larger debates for soy consumption and the concentration dependence of the antiproliferative activity of genistein. Development of

novel synthetic isoflavone analogs with greater selectivity and/or potency is needed for targeted therapy in the treatment of breast cancer.

Our laboratory has synthesized a new series of SERMs containing the isoflavone scaffold and the amine-bearing side chain of raloxifene (22). These isoflavone analogs were designed to contain a heteroatom, *i.e.*, sulfur or oxygen, as an isostere of the carbonyl group in raloxifene, which could theoretically serve as a hinge to orientate the direction of the basic side chain in the binding pocket of the ER for SERM functionality. The binding affinities of these compounds have been previously reported and were shown to have mixed affinities for the estrogen receptors (22). In the present study, we evaluated the effect of the synthetic isoflavones on growth inhibition and cytotoxicity in MCF-7 and MDA-MB-231 breast cancer cells. We also determined the ability of the synthetic isoflavones to induce cell cycle arrest and effect the expression of relevant CKIs, $p21^{WAF1/Cip1}$ and $p27^{KIP1}$ that are involved in cell cycle regulatory processes.

3.2. Experimental

3.2.1. Chemicals, Biochemicals, and Reagents

Isoflavonoid analogs were prepared by Y.W. Kim as described (22) 3,(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt was obtained from Promega (Madison, WI). Genistein was obtained from Indofine Chemical Company (Belle Mead, NJ). 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. DMEM-F12 culture media was obtained from Sigma, and the human albumin was obtained from OSU

Hospital Pharmacy. Antibodies (β-actin, $p21^{WAF1/Cip1}$, $p27^{Kip1}$) were purchased from Cell Signaling (Cell Signaling Technology, Inc. Danvers, MA).

3.2.2. Cell lines and culture conditions

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, 1.5 x amino acids, 2 x non-essential amino acids, L-glutamine, and 1.5 x 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 % CO_2 in a Hereaus CO_2 incubator. For all experiments, cells were plated in either 6-well plates or 100 mm petri dishes. Before treatment, the media was changed to 5 % DCCS DMEM/F12 media with 2 mM L-glutamine and 20 μg/ml gentamycin.

3.3.3. Cell Proliferation Assay

Cellular proliferation in the presence or absence of experimental compounds was determined using the CellTiter 96® aqueous non-radioactive cell proliferation assay (23). 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 in the modified MEM media with 10% FBS as described earlier in conditions of cell culture. Prior to drug treatment, the modified MEM media were removed and cells were washed with PBS. Defined media was added and cells maintained for 24 hours. Then culture wells (n=6) were treated with the compounds (synthetic isoflavones ± estradiol) in 400 μl defined media 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.

3.3.4. 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, modified MEM media was removed and cells were washed one time with PBS. Culture wells (n=6) were treated with the compounds (100 μl volume), dissolved in defined media and incubated for 48 hours at 37°C. After incubation, the same protocol as described above was followed and absorbance was measured.

3.3.5. Analysis of Cell Cycle Progression by Flow Cytometry

Cell cycle arrest was determined by flow cytometric analysis of PI-labeled cells. MCF-7 and MDA-MB-231 cells were grown to exponential phase, seeded at a density of 1-5 x 10^6 cells/100-mm dish in B-media. Following overnight attachment, the cells were treated with 50 μM genistein and 5 μM of the synthetic isoflavones in 5 % DCCS DMEM/F12 media for 24 h. Cells were harvested, suspended in 1 ml PBS and fixed in ice-cold 70% ethanol for ≥ 2hr. Following fixation, cells were centrifuged for 5 min at

200 x g, ethanol was thoroughly decanted and tubes were transferred to a 37°C water bath for a 30 minute incubation on a shaker. Following incubation, cells were centrifuged for 10 min at 1500 x g, supernatant was removed and cells were resuspended in 1 ml PI staining solution with RNase A. The samples were kept 30 min at room temperature, protected from light. For flow cytometric analysis, a FACSCalibur flow cytometer (Becton Dickinson, NJ) equipped with a single argon ion laser was used. DNA content of 10,000 cells per analysis was monitored using the FACSCalibur system. A minimum of 10,000 cells per sample was used for analysis performed using CellQuest software. Cell-cycle analysis was performed using ModFit software from Verity Software House.

3.3.6. Preparation of total cell lysates and immunoblot analysis

To prepare the whole-cell extract, MCF-7 and MDA-MB-231 cells (1-5 x 10^6 cells/100-mm dish) were detached following addition of the appropriate amount of M-PER® Reagent, to which Halt™ Protease Inhibitor Cocktail was added to produce a 1X final concentration (Pierce, Rockford, IL). The cellular suspension, which included floating cells was collected and freeze-thawed thrice to insure complete protein extraction. Total protein content was determined using Bio-Rad DC protein assay with bovine serum albumin as the standard. Equal amounts of (50 μg) the denatured proteins were loaded into each lane, separated on 10-12 % SDS polyacrylamide gels, followed by a transfer of the proteins to PVDF membranes for 1.0 hour. Membranes were blocked with 4.0 % ECL advance blocking agent (Amersham Biosciences, Piscataway, NJ), in Tris-buffered saline for 1.0 hour at room temperature and the membranes were reacted with appropriate secondary antibodies overnight. They were then incubated with an ECL anti-mouse IgG or anti-rabbit IgG, horseradish peroxidase-linked species specific whole antibody for 1h

before being developed using the ECL Advance western blotting detection system. Images were acquired using a Lumi-Imager Workstation equipped with a CCD Camera (Boehringer Mannheim, Germany).

3.3.7. Statistics

Statistical and graphical analysis information was determined using GraphPad software and Microsoft Excel (Microsoft Corporation, Redmond, WA). Statistically significant differences were calculated with the two-tailed unpaired Student's *t*-test and *P* values reported at 99% confidence intervals.

3.3. Results

3.3.1. Library and Rationale for synthesis of the isoflavone library

A series of 2, 4', 7-trisubstituted isoflavones were designed based on the rationale shown in Figure 3.1. The amine bearing side chain of raloxifene is crucial for SERM activity. On this premise, the isoflavone scaffold and the basic side chain of raloxifene were utilized in drug design to synthesize a series of isoflavone analogs (Figure 3.2) that contain a sulfur or oxygen as an isostere of the carbonyl group. The isoflavones were synthesized with the idea that the unique heteroatom could possibly serve to orientate the basic side chain to the correct binding pocket region of the estrogen receptor for SERM capability.

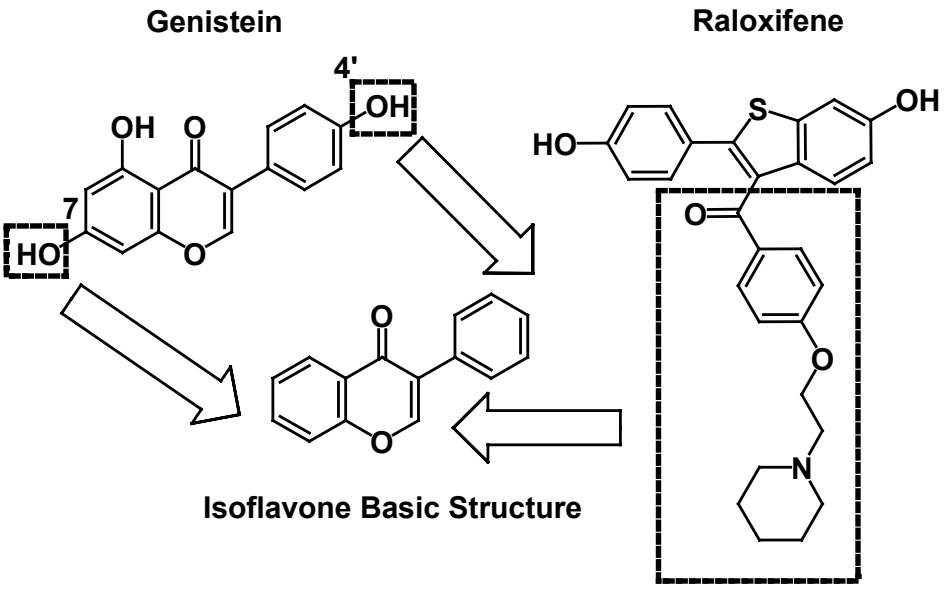


Figure 3.1: Rational for design of synthesized isoflavones.

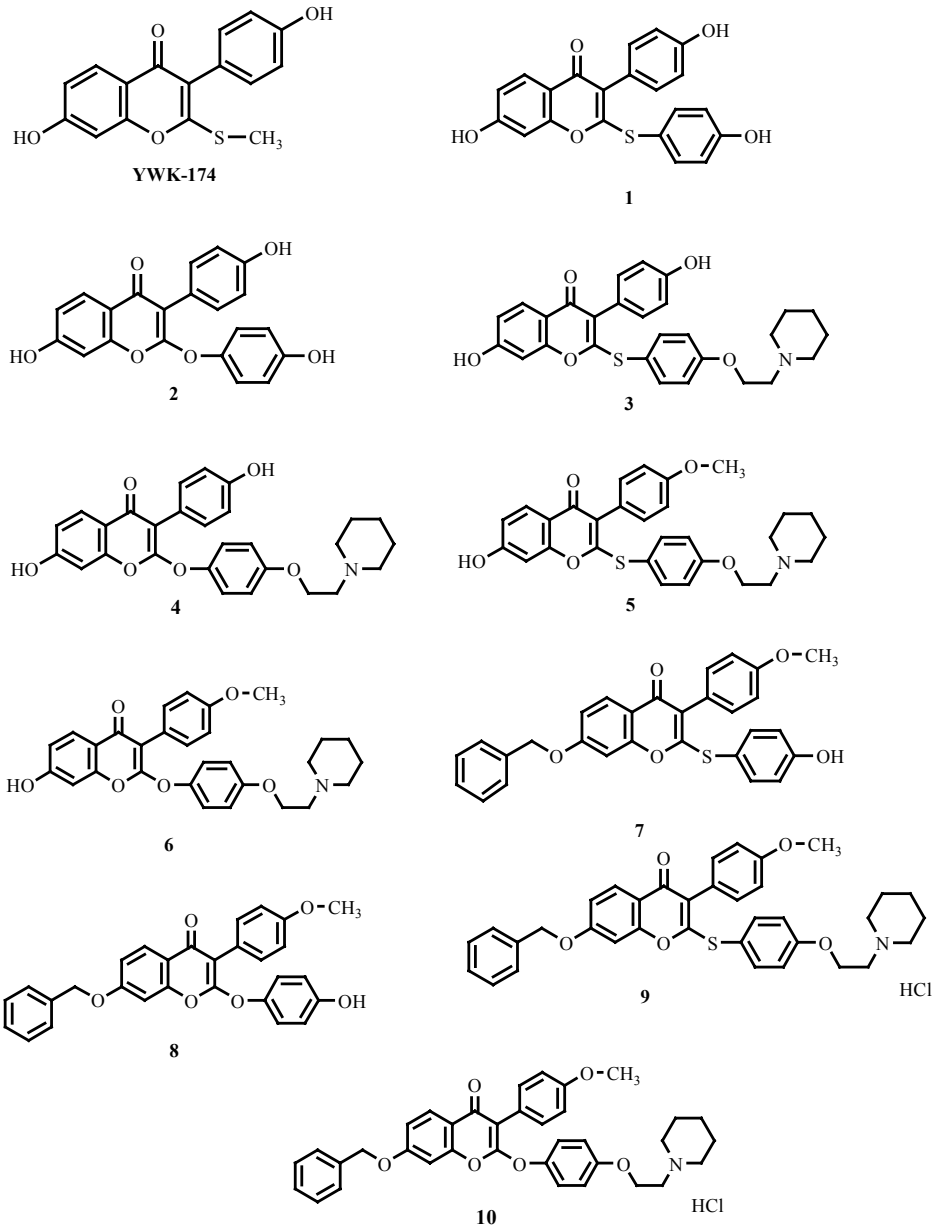


Figure 3.2: Library of 2, 4', 7-trisubstituted isoflavones used for subsequent study.

3.3.2. Inhibition of cell proliferation

Potential antiproliferative ability of these synthetic analogs was determined in both hormone-responsive and hormone-nonresponsive human breast cancer cells. The cells were treated alone with the compounds or treated with both the compounds and estradiol for a period of six days to test their ability to inhibit estrogen-induced proliferation in human MCF-7 breast cancer cells (Figure 3.3). As expected, in the presence of estradiol, MCF-7 cells resulted in significant growth stimulation. Conversely, the addition of 4-hydroxytamoxifen resulted in cell growth inhibition. When exposed to genistein, we observed a growth inhibition in proliferation only at higher concentrations of 10 μM.

Analysis of proliferation in MCF-7 cells revealed that the pattern of inhibition of each compounds was distinctive within the library of the synthetic isoflavones. Compounds **1, 2, 6,** and displayed antiproliferative activity alone but not in the presence of 10 nM E_2, whereas compounds **3** and **4** did not show any potential for antiproliferation in the breast cancer cell line. Compound **5** reduced the stimulatory effect of estradiol and suppressed cell proliferation by approximately 90%, and overcame the additional proliferative effect of estradiol. The potency of compound **5** in the presence of estradiol in MCF-7 cells suggests that its mechanism of action is independent of estrogen receptor status. Compounds **7, 9,** and **10**, which share the 7-phenylmethoxy substitution each resulted in a marked decrease in cell proliferation even in the presence of estradiol addition.

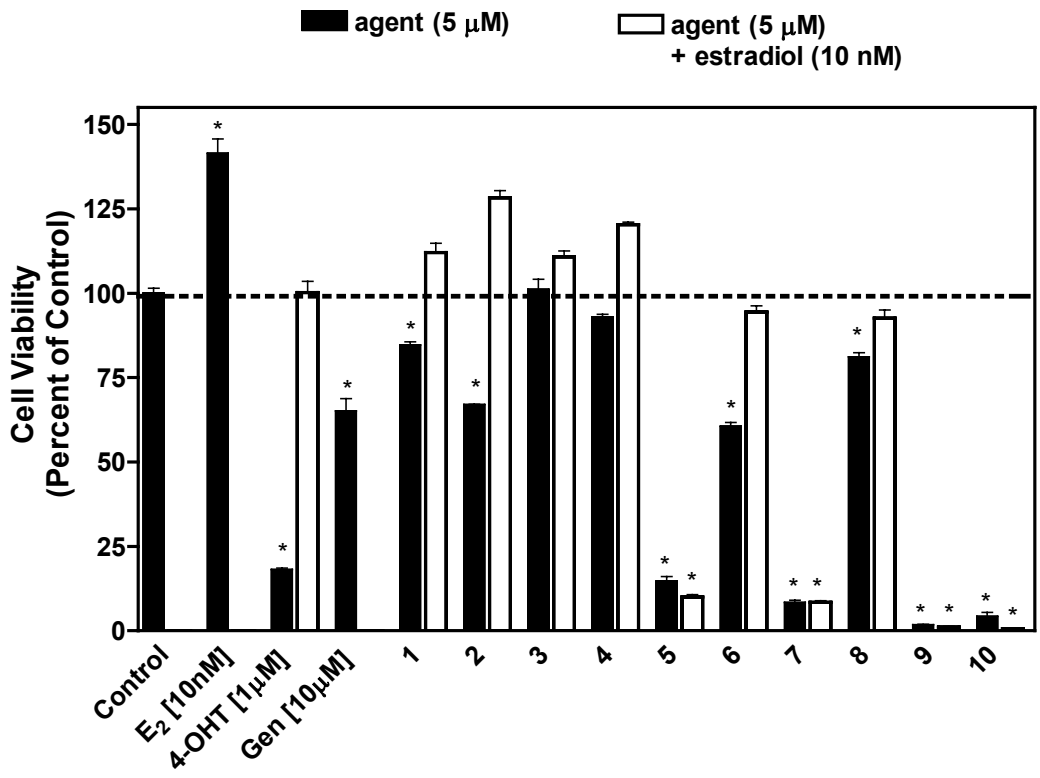


Figure 3.3: Evaluation of synthetic isoflavones on proliferation in the presence and absence of estradiol in MCF-7 cells. MCF-7 cells were treated with 5 μM of each of the agents alone, or in the presence of 10 nM estradiol. Cell viability was measured as described in the experimental section. Estradiol and 4-hydroxy testosterone were used as controls. The results were normalized against a control treatment with vehicle (DMSO). *, $P < 0.0001$ *vs*. control by unpaired *t* test, n = 6.

3.3.3. Cell Cytotoxicity

The antiproliferative results in MDA-MB-231 cells suggest other mechanisms of action of these compounds that may be independent of estrogen receptor status; therefore we tested for additional targets of activity. Using both MCF-7 and MDA-MB-231 breast cancer cell lines, we treated the cell lines for 48 hours with our synthetic isoflavonoids and tested for cell cytotoxicity following duration of treatment. Results analysis showed that compounds **7, 8, 9,** and **10**, all of which contain the 7-phenylmethoxy substitution, resulted in significant cell cytoxicity in the MCF-7 cell line. Compound **2** also resulted in cell cytotoxicity in the MCF-7 cells following the 48 hour exposure (Figure 3.4). Estradiol and 4-hydoxytamoxifen did not result in any appreciable cell cytotoxicity, which isn't surprising following this short exposure time. The synthetic isoflavones showed a greater degree of cell cytotoxicity in the hormone-independent cell line MDA-MB-231. Compounds **7, 8, 9,** and **10**, each containing the 7-phenylmethoxy substitution resulted in significant cell cytotoxicity in the MDA-MB-231 cells. Additionally, compounds **2, 3,** and **5** also showed the ability to induce cell cytotoxicity. Taken together, the cytotoxicity results further substantiate the idea that these synthetic isoflavonoids are able to act independently of estrogen receptor status.

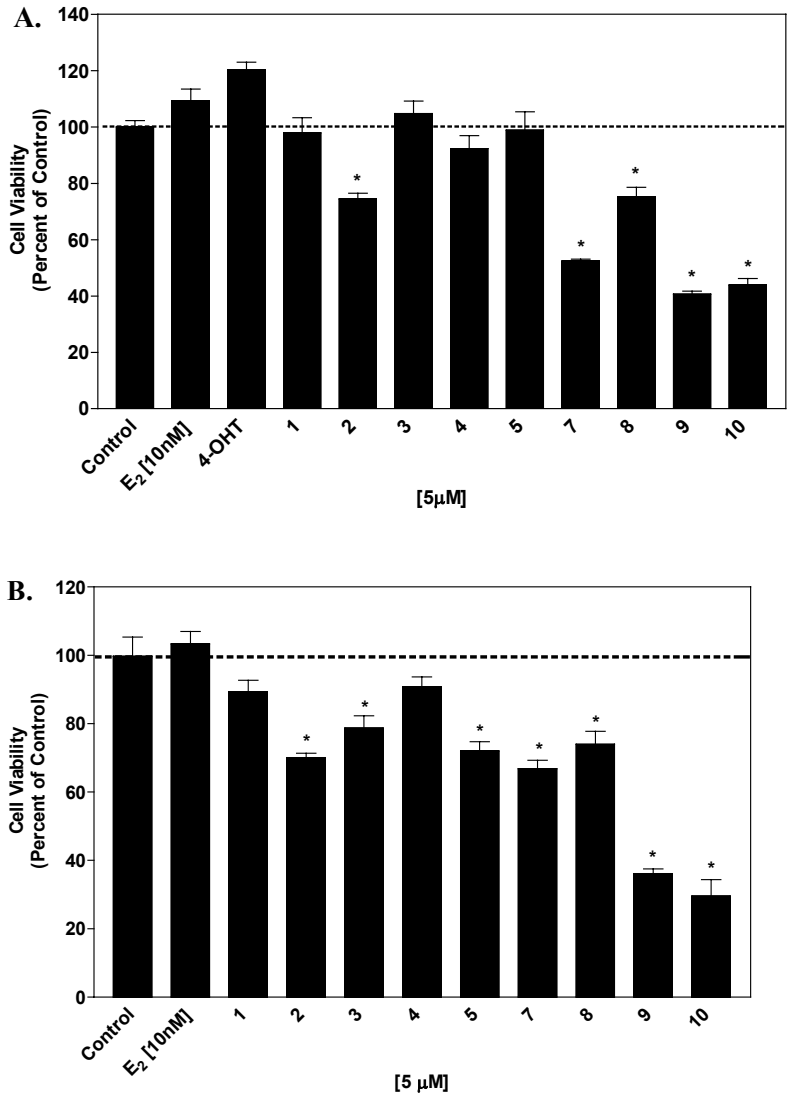
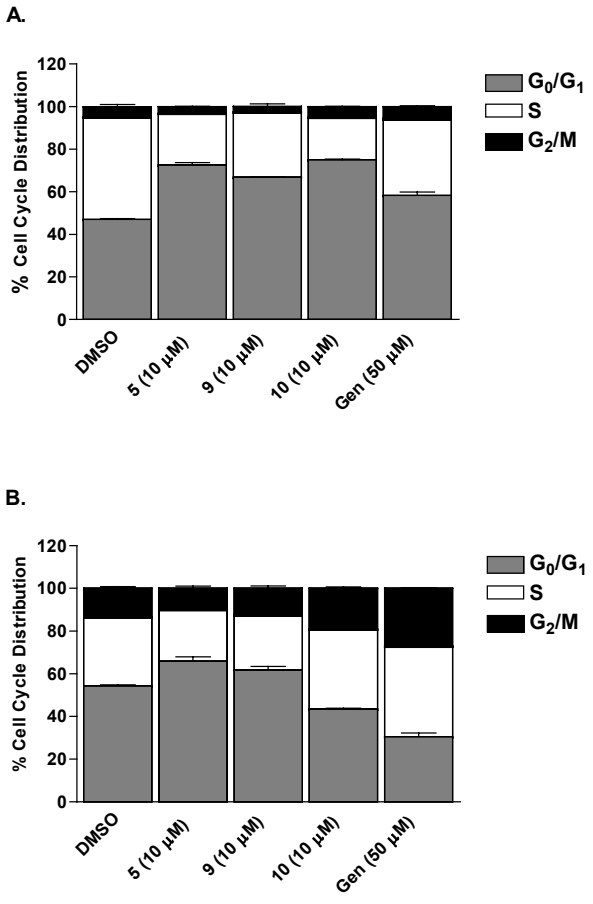


Figure 3.4: Assessing cytotoxicity of the synthetic isoflavones in MCF-7 cells and MDA-MB-231 cells. MCF-7 cells (A) and MDA-MB-231 cells (B) were treated with each of the agents at the indicated concentrations and cell viability was measured as described in the experimental section. The results were normalized against a control treatment with vehicle (DMSO). *, $P < 0.0001$ *vs*. control by unpaired *t* test, n = 6.

3.3.4. Cell cycle arrest in the G1 phase by synthetic isoflavones

The antiproliferative and cytotoxicity profiles from the synthetic isoflavones led us to further investigate other mechanisms of action through which these compounds exert their effects. The cell cycle was examined because of the ability of genistein to arrest cells at the G_2M phase transition in both MCF-7 and MDA-MB-231 breast cancer cells with concomitant induction of the CDK inhibitor, $p21^{WAF1/Cip1}$ (24). Flow cytometric analysis was used to measure the fluorescence of propidium iodide (PI) binding to nucleic acid base pairs (A:T and G:C) to determine the percentage of arrest cells in each phase of the cell cycle. The cells were exposed for 24 hours to 10 μM of compound **5, 9,** and **10**, of which selection was based on cytotoxicity profiles observed in both MCF-7 and MDA-MB-231 breast cancer cells. Cells were also exposed to 50 μM of genistein. Figures 3.5 A and B show that each of the isoflavone analogs exhibit an arrest profile different from that of genistein. As expected, genistein showed an arrest at the G_2M phase transition in the MDA-MB-231 cell line. There wasn't an apparent arrest at the G_2M phase transition by genistein in the MCF-7 cell line, which may be explained by different media preparations and cell staining. However, with the exception of compound **10**, which showed a similar G_2M arrest profile similar to genistein in MDA-MB-231 cells, compounds **5** and **9** showed an arrest at the G_1 phase of the cell cycle in both breast cancer cell lines. Additionally, compound **5** exhibited similar G_1 arrest profiles in both cell lines, MCF-7 and MDA-MB-231. Taken together, compound **5**, which showed selectivity in cytotoxicity for MDA-MB-231 breast cancer cells, not only

showed the ability to arrest cells at the G1 phase transition of the cell cycle but also appears to be more active in MDA-MB-231 cells. Therefore, our synthetic isoflavones



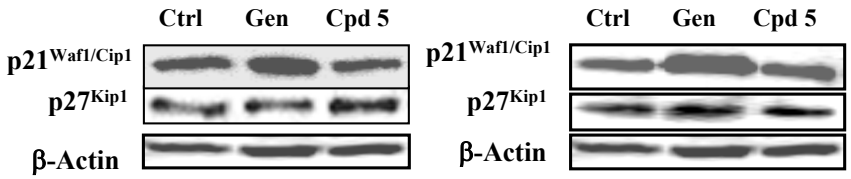
3.5: Flow Cytometric Analysis. Cell cycle arrest as determined by prodidium iodide staining following a 24h treatment in MCF-7 (A) and MDA-MB-231 breast cancer cells (B). Cells were treated with 10 μM of each of the isoflavone analogs and 50 μM of genistein for 24h. Control cells were maintained in vehicle, 0.1% DMSO, for the same time period. Results are presented as percent control of cell cycle distribution. Each plot is representative of three similar experiments.

demonstrate mechanisms of action different from genistein and at concentrations as low as 10 μM are able to arrest proliferating cells in both MCF-7 and MDA-MB-231 breast cancer cell lines.

3.3.5. Involvement of cell cycle inhibitory proteins, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ in breast cancer cells

CDK inhibitors and cyclins play essential roles in the tight regulation of cell cycle progression (25); therefore, the expression of CDK inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ were examined. Based on the cell cycle arrest profiles of each of the isoflavone analogs, compound **5** showed a higher percentage of cells in the G_1 phase in both MCF-7 and MDA-MB-231 cell lines and showed selectivity for cytotoxicity in the MDA-MB-231 cell line. Both CDK inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ are involved in the arrest of cells and therefore block progression of the G_1 to S transition. In addition, a loss of CDK functional inhibitors in human cancers correlates to uncontrolled cell proliferation due to an increase in the levels of the CDK cyclin complex (26). Therefore, western blot analysis was utilized to determine if compound **5** induced expression of the CDK inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. Treatment with 5 μM of compound **5** in the MDA-MB-231 breast cancer cell line resulted in an increase of $p21^{Waf1/Cip1}$ and p27Kip1 in the MDA-MB-231 breast cancer cell line (Figure 3.6). No increase in the CDK inhibitors was observed in the MCF-7 cells after drug treatment. Quantification of bands relative to β-actin to confirm equal protein loading is shown in Figure 3.6 B.

A.



B.

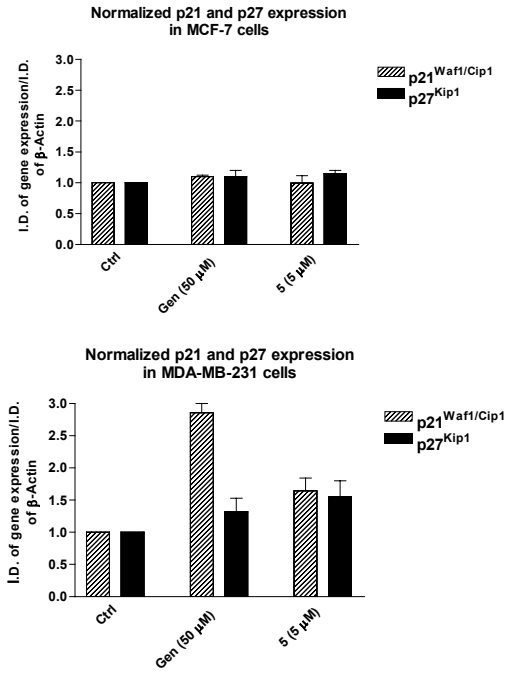


Figure 3.6: Western Blots of relevant cell cycle inhibitory proteins in MCF-7 and MDA-MB-231 breast cancer cell lines. (A) Cell lysates were prepared from each of the following samples: control (0.1 % DMSO), 50 μM genistein treatment and compound 5 at 5 μM, western blotting was performed with anti $p21^{WAF1/Kip1}$, $p27^{Cip1}$ and actin antibodies. (B) Densitometric quantitation of immunoblot representative of three independent experiments.

3.4. Discussion

The design strategy for the synthetic 2, 4', 7-trisubstitued isoflavones was to incorporate onto the isoflavone backbone the basic side chain of raloxifene, a representative SERM, to develop more potent antiestrogenic activity than genistein in hormone dependent breast cancer cells. Although low affinities for both receptor subtypes were reported for the synthetic isoflavones (27), these synthetic isoflavones demonstrated antiproliferative activity in both MCF-7 and MDA-MB-231 breast cancer cell lines. In MCF-7 cells, the antiproliferative potential of the compounds was also measured in the presence of estradiol to determine whether the compounds could block the stimulatory effect of estradiol. Compounds **2** and **6** resulted in inhibition of proliferation in the absence of estradiol, but were not able to inhibit proliferation in the presence of estradiol. Compounds **5, 7, 9,** and **10** exhibited the greatest degree of antiproliferative activity, even in the presence of estradiol, which suggests that their antiproliferative activities may result from estrogen-receptor independent pathways (Figure 3.3).

To investigate other possible mechanisms of action of the synthetic isoflavones, compounds **7, 8, 9,** and **10** bearing the bulky 7-phenylmethoxy substituent resulted in significant cell cytotoxicity in both the MCF-7 and MDA-MB-231 cell line. Compound **2** also showed cell cytotoxicity in the MCF-7 cells. Despite the ability of the compounds to exhibit similar profiles of cytotoxicity in both cell lines, the MDA-MB-231 cells showed the greater degree of sensitivity to the synthetic isoflavones treatment. Compounds **2, 3,** and **5** showed the ability to induce cell cytotoxicity in the MDA-MB-

231 cell line, while compound **5** showed selectivity for the MDA-MB-231 breast cancer cell line (Figure 3.4).

The cytotoxicity for compounds **9** and **10** and the selectivity of compound **5** in the MDA-MB-231 cells suggested that these effects were mediated through arrest of cell cycle progression. Treatment of both hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231) breast cancer cell lines with 10 μM of the synthetic isoflavone analogs results in G_1 arrest (Figure 3.5). This data suggests that one of the mechanisms by which these synthetic isoflavones may exhibit their antiproliferative effect in breast cancer cells is through inhibition of cell cycle progression. Additionally, consistent with other reports, genistein treatment at 50 μM resulted in G_2M arrest in both breast cancer cell lines, with a more significant phase arrest in the MDA-MB-231 cells.

Because compound **5** exhibited similar G_1 arrest profiles in both breast cancer cell lines tested, the role of cyclin dependent kinase inhibitors in promoting cell cycle arrest was further investigated. As shown in Fig. 3.6 A, following treatment with compound **5**, $p27^{Kip1}$ levels increased in MDA-MB-231 cells, but to a much lesser extent in MCF-7 cells. Increased levels of $p21^{WAF1/Cip1}$ following treatment with compound **5** was only observed in the MDA-MB-231 cells. Protein expression of $p27^{Kip1}$ following genistein treatment was not affected, however, $p21^{WAF1/Cip1}$ protein levels were significantly higher with genistein treatment in the MDA-MB-231 cell line. This finding is consistent with other reports where genistein induced cell cycle arrest at the G_2M phase with concomitant increase of relevant CKIs in both cell lines, but to a greater extent in MDA-MB-231 cells (28).

In conclusion, the results of the present study indicate that this library of synthetic isoflavones have the ability to inhibit proliferation in both MDA-MB-231 breast cancer cells and in MCF-7 cells. Several compounds were shown to inhibit proliferation even in the presence of the stimulatory activity of estradiol. Additionally, the synthetic isoflavones were effective as cytotoxic agents in both cell lines tested. Also, compounds with the more bulky substituents were more effective as cytotoxic agents. Additionally, the MDA-MB-231 breast cancer cell line was more sensitive to the cytotoxicity induced by treatment with the compounds. Based on the cytoxicity profile, compounds **5, 9,** and **10** were selected and determined to arrest cells in the G_1 phase transition of the cell cycle. Compound **5,** with similar arrest profiles in both cell lines was also shown to lead to increased expression of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$, both negative cell-cycle regulators that act as cyclin-dependent kinase inhibitors.

Thus, a series of synthetic isoflavones produced antiproliferative activity and cytotoxicity in both hormone-dependent and hormone-independent breast cancer cells and are capable of blocking cell cycle progression. Although genistein is effective as a chemotherapeutic agent mediating the progression of relevant proteins leading to breast cancer progression, relatively high concentrations are needed for activity. These synthetic isoflavones display inhibitory activity of breast cancer cells with concentrations as low as 5 μM. Further investigation of the potential molecular targets of these compounds is warranted and may provide a better assessment of the signal transduction pathways involved. Lastly, a better understanding of the molecular complexes that mediate the endogenous cdk inhibitory complexes will lead to better assessment of the role of these compounds in G_1 arrest. These results encourage further development of the

synthetic isoflavones as potential agents for breast cancer prevention and treatment for hormone-dependent and hormone-independent breast cancer.

3.5. Acknowledgements

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CHAPTER 4

DOSE DEPENDENT EFFECTS OF A SELECTED ISOFLAVONE ANALOG ON MOLECULAR TARGETS IN BREAST CANCER CELLS

4.1. Introduction

The benefits of soy consumption as it relates to reducing the risk of breast cancer remains controversial due to the estrogenic potential of genistein, the predominant isoflavone in soy. Genistein is classified as a phytoestrogen due to the structural and functional similarities to estrogen. The potential concern of increased soy in the diet and breast cancer risk relates to the potential for genistein to compete with endogenous estrogens for the estrogen receptor (ER); however, this ER binding of isoflavones also competes with the binding of more potent endogenous estrogens to diminish estrogenicity (1,2,3,4).

Genistein has a biphasic effect on cell growth in breast cancer cells. Lower doses of genistein (<10 μM) increase the proliferation of MCF-7 cells, whereas higher doses (>10 μM) are associated with cell growth inhibition. Furthermore, this dose dependent activity of genistein is believed to be influenced by both estrogen-dependent and estrogen-independent mechanisms (5). Overall, the ability of genistein to show predominant estrogen agonist or antagonistic activity depends largely on dose and cell

type. The hormone-independent mechanisms that genistein is commonly associated with involving *in vitro* and *in vivo* treatment of breast cancer include induction of apoptosis related genes, regulation of the cell cycle, and inhibition of the activation of nuclear factor-κB (NF-κB) and Akt signaling pathways, as well as modulating genes associated with these signaling pathways (6,7,8,9). The *in vivo* evidence of the role of soy and isoflavones is inconsistent, but overall the evidence shows that introduction of soy protein for other protein (generally casein) only slightly (25-50%) reduces tumor incidence and/or multiplicity. In contrast, genistein and soy protein introduced in ovariectomized athymic mice implanted with MCF-7 cells was shown to stimulate tumor growth, but not to the extent of estrogen (10).

Arguments in favor or against the use of genistein and soy protein *in vitro* and *in vivo* strongly point to other variables that influence estrogenic activity, which include and are not limited to concentration dependence and cellular environment. The inhibitory action of genistein in human breast cancer cells is complex and only partially influenced by estrogen dependent pathways, as such multiple mechanistic targets are involved in mediating genistein's activity.

We sought to determine if dose dependence played a role in influencing the molecular targets of the 2, 4', 7-trisubstituted isoflavones synthesized in our lab. Previous screening results in MDA-MB-231 and MCF-7 breast cancer cells showed that several compounds within the library of synthetic isoflavones were effective antiproliferative agents at 5 μM concentration in the absence and presence of the addition of estradiol (11). To further elucidate the mechanism behind the inhibitory effects of the synthetic isoflavones, the agents were examined for cytotoxicity at 5 μM in both breast

cancer cell lines. Several synthetic isoflavones were effective cytotoxic agents in both cell lines, but compound 5 showed selective cytotoxcity in the MDA-MB-231 cell line over MCF-7 cells. Overall, the MDA-MB-231 cell line was more sensitive to cytotoxicity induction by the synthetic isoflavones.

The role of concentration dependence and cell type influencing the estrogenic potential of genistein has already been discussed and proven to be a crucial factor in weighing the benefits of soy consumption and breast cancer. The goal of this chapter is to further explore the molecular targets of compound 5, and determine whether dose dependence plays a predominant or lesser role in its mechanistic profile.

4.2. Experimental

4.2.1. Chemicals, Biochemicals and Reagents

Isoflavonoid analogs were prepared by Y.W. Kim as described (12). The 3,(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt was obtained from Promega (Madison, WI). Genistein was obtained from Indofine Chemical Company (Belle Mead, NJ). 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. DMEM-F12 culture media was obtained from Sigma, and the human albumin was obtained from OSU Hospital Pharmacy. Antibodies (β-actin, Bax, Bcl-2, pAkt, Akt) were purchased from Cell Signaling (Cell Signaling Technology, Inc. Danvers, MA).

4.2.2. Cell Culture and Cell lines

MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). The cells were maintained in phenol red-free custom media (B-media: MEM, Earle's salts, 1.5 x amino acids, 2 x non-essential amino acids, L-glutamine, and 1.5 x 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 % CO_2 in a Hereaus CO_2 incubator. For all experiments, cells were plated in either 6-well plates or 100 mm petri dishes. Before treatment, the media was changed to 5 % DCCS DMEM/F12 media with 2 mM L-glutamine and 20 μg/ml gentamycin.

4.2.3. Cell Proliferation

Cellular proliferation in the presence or absence of experimental compounds was determined using the CellTiter 96® aqueous non-radioactive cell proliferation assay (13). 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 in the modified MEM media with 5 % CS-FBS as described earlier in conditions of cell culture. Prior to drug treatment, the modified MEM media were removed and cells were washed with PBS. Culture wells (n=6) were treated with compound 5 and DMSO in 400 μl MEM media with 5 % CS-FBS for seventy-two hours. Following treatment 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 final assay concentrations of 333 μg/ml and 25 μM respectively. These solutions were combined and

20 μl of this mixture was 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.

4.2.4. Preparation of total cell lysates and immunoblot analysis

To prepare the whole-cell extract, MCF-7 and MDA-MB-231 cells (1-5 x 10^6 cells/100-mm dish) were detached following addition of the appropriate amount of M-PER® Reagent, to which Halt™ Protease Inhibitor Cocktail was added to produce a 1X final concentration (Pierce, Rockford, IL). The cellular suspension, which included floating cells was collected and freeze-thawed thrice to insure complete protein extraction. Total protein content was determined using Bio-Rad DC protein assay with bovine serum albumin as the standard. Equal amounts of (50 μg) the denatured proteins were loaded into each lane, separated on 10-12 % SDS polyacrylamide gels, followed by a transfer of the proteins to PVDF membranes for 1.0 hour. Membranes were blocked with 4.0 % ECL advance blocking agent (Amersham Biosciences, Piscataway, NJ), in Tris-buffered saline for 1.0 hour at room temperature and the membranes were reacted with appropriate secondary antibodies overnight. They were then incubated with an ECL anti-mouse IgG or anti-rabbit IgG, horseradish peroxidase-linked species-specific whole antibody for 1h before being developed using the ECL Advance western blotting detection system. Images were acquired using a Lumi-Imager Workstation equipped with a CCD Camera (Boehringer Mannheim, Germany).

4.2.5. Statistics

Statistical and graphical analysis information was determined using GraphPad software and Microsoft Excel (Microsoft Corporation, Redmond, WA). Statistically significant differences were calculated with the two-tailed unpaired Student's *t*-test and *P* values reported at 95 % confidence intervals.

4.3. Results

4.3.1. Library of isoflavone analogs

The rationale for design of the synthetic isoflavones was previously described. The library containing synthetic isoflavones used in subsequent studies described in the experimental section is shown in Figure 4.1. Modifications were made to the isoflavone skeleton by adding the basic side chain of raloxifene. The isoflavones were synthesized as potential SERMs, but later were shown to exhibit low binding affinities (12). Therefore, mechanistic studies to explore additional molecular targets of the synthetic isoflavones in breast cancer cells were initiated.

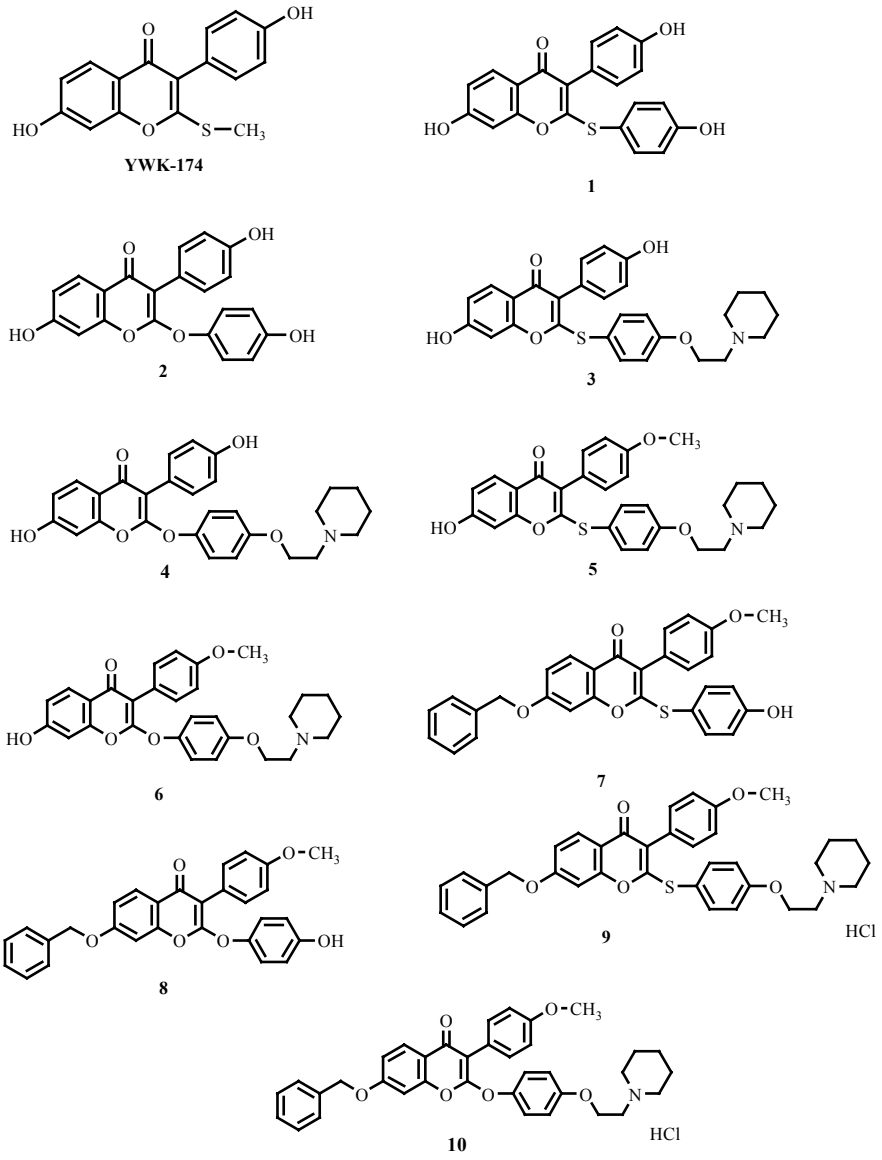

Figure 4.1. Library of 2, 4', 7-trisubstituted isoflavones used in subsequent studies.

4.3.2. Evaluation of dose response on proliferation in MDA-MB-231 cells

The library of synthetic isoflavones (Fig. 4.1) were previously evaluated for antiproliferative activity in the presence and absence of estradiol in the MCF-7 breast cancer cell line. Several compounds inhibited proliferation in MCF-7 cells at 5 μM, and were not affected by the stimulatory effect of exposure to estradiol. Additionally, genistein only resulted in significant growth inhibition at higher concentration. Compound **5** was not affected by the stimulatory effect of estradiol and resulted in a 90% reduction in cell proliferation at 5 μM in the MCF-7 breast cancer cell line. The activity and potency of compound **5** and its ability as an effective antiproliferative agent in the presence of estradiol in the MCF-7 cell line suggested other mechanisms of action that are independent of estrogen receptor status (11).

To further explore the antiproliferative potential of compound **5** in a hormone-independent environment, we turned our attention to the MDA-MB-231 breast cancer cell line. The MDA-MB-231 cell line was exposed to increasing doses of compound **5** for seventy-two hours and proliferation was determined as outlined in the experimental methodology. The highest concentration tested, 10 μM, resulted in a significant decrease in cell viability in the MDA-MB-231 breast cancer cell line (Fig 4.2). Taken together, compound **5** is as effective an antiproliferative agent in MDA-MB-231 breast cancer cells as well as MCF-7 cells, and thereby this agent is able to influence both the hormone-dependent as well as the hormone-independent breast cancer microenvironment.

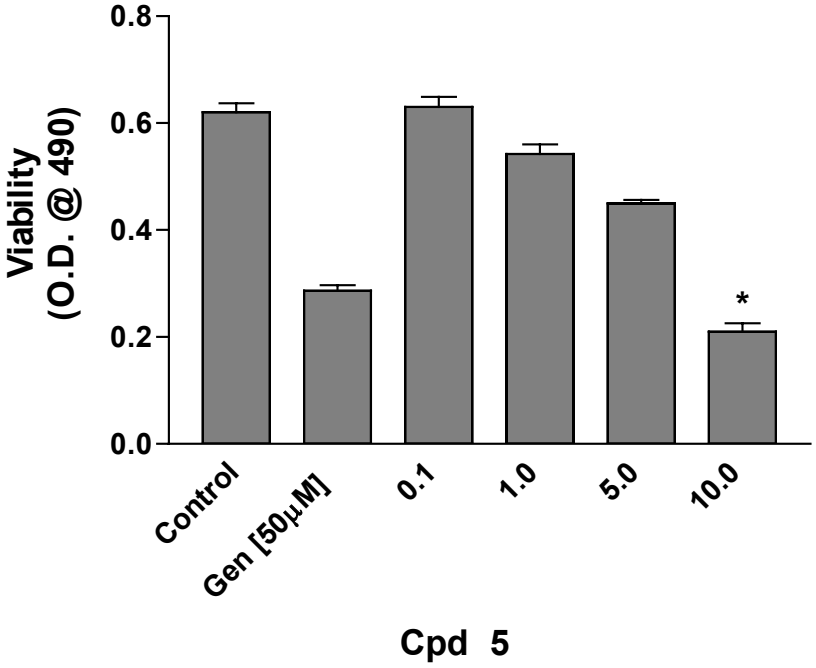

Figure 4.2: Effect of compound 5 on cell proliferation. MDA-MB-231 cells were treated with: 50 μM genistein, control (0.1 % DMSO), and increasing log doses of compound 5 as described in the experimental section. The results were evaluated against a control treatment with vehicle (DMSO), * $P < 0.05$ *vs* control, by unpaired *t* test, n = 6.

4.3.3. Effect of compound 5 on Bax and Bcl-2 protein expression in breast cancer cells

Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) are two important proteins involved in the initiation of apoptosis signals within the cellular network. The involvement of Bcl-2 in apoptosis in drug-induced modulation of its expression is not a strong influence on apoptosis, rather the ratio of Bax:Bcl-2 is a more crucial determinant for cell survival (14,15,16,17). Bax and Bcl-2 share a highly homologous sequence, which results in the formation of heterodimers that allow for antagonism of the anti-apoptotic function of Bcl-2 by the Bax protein. Sarkar *et al*., found that the addition of 15 or 30 μM of genistein incubated for 25-72 h in MDA-MB-231 cells resulted in slight down regulation of Bcl-2 protein. In contrast, concentrations of 5, 15 or 30 μM of genistein treatment for 24 hours in MDA-MB-231 cells were effective in increasing expression of Bax protein (18). Overall, genistein was effective in increasing the expression of Bax and down-regulation of Bcl-2, which may be a possible mechanism responsible for its apoptosis inducing ability in breast cancer cells.

We tested for the ability of compound 5, our synthetic isoflavone of genistein, for its ability to modulate expression of both Bax and Bcl-2 proteins following treatment in MDA-MB-231 and MCF-7 breast cancer cell lines. Our results show that Bax expression was significantly up-regulated following a 48h treatment with increasing concentrations of compound 5 in both breast cancer cell lines. No detectable change in Bcl-2 expression following treatment with compound 5 in MCF-7 cells was observed; however, a dose dependent decrease in Bcl-2 protein expression was observed in the MDA-MB-231 breast cancer cell line. Furthermore, the Bax/Bcl-2 ratio was 3-fold higher following treatment with compound 5 in the MDA-MB-231 cell line. Our results directly

corroborate with the notion that the ratio of Bax to Bcl-2 protein levels are important variables for determining the potential for cells to undergo apoptosis and therefore may be one of the mechanisms involved in the ability of compound 5 to induce apoptosis.

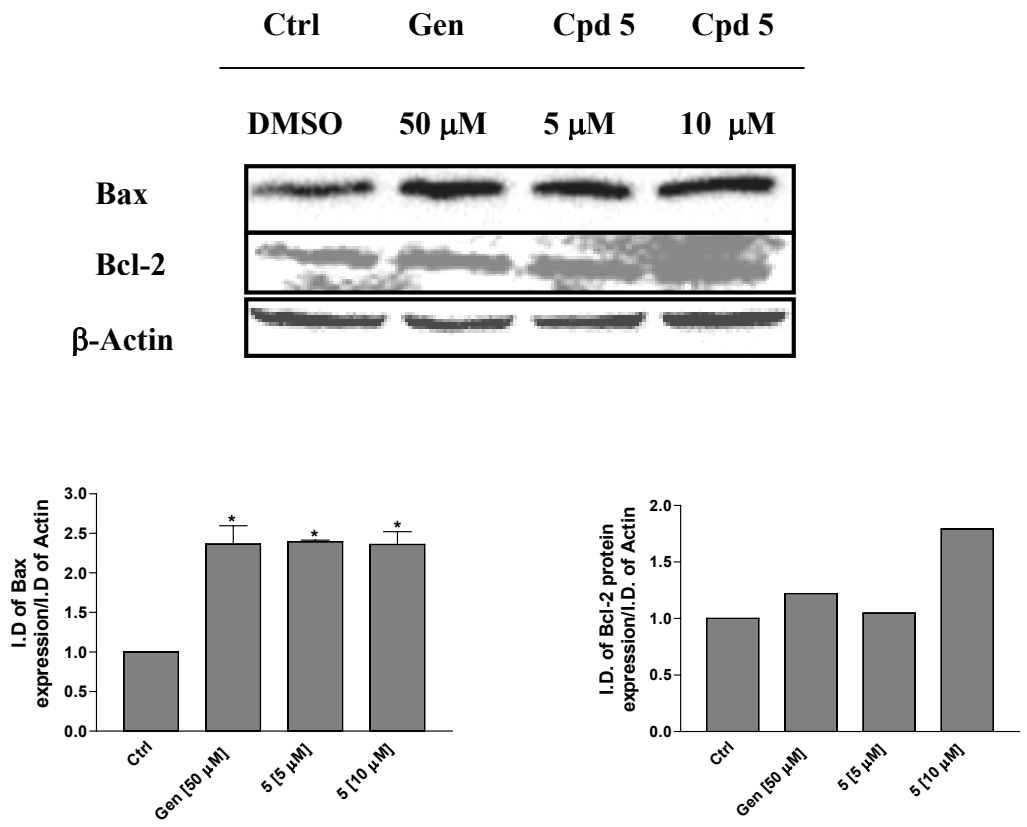

Figure 4.3: Western Blots of Bax and Bcl-2 proteins in MCF-7 breast cancer cells. (A) Western blot and densitometeric analysis of Bax and Bcl-2 in MCF-7 cells. Cell lysates were prepared from each of the following samples following a 48 hour treatment as outlined in experimental methods: control (0.1 % DMSO), 50 μM genistein, and treatment with compound **5** at 5 μM and 10 μM. * $P < 0.05$ *vs* control, by unpaired *t* test, n = 3.

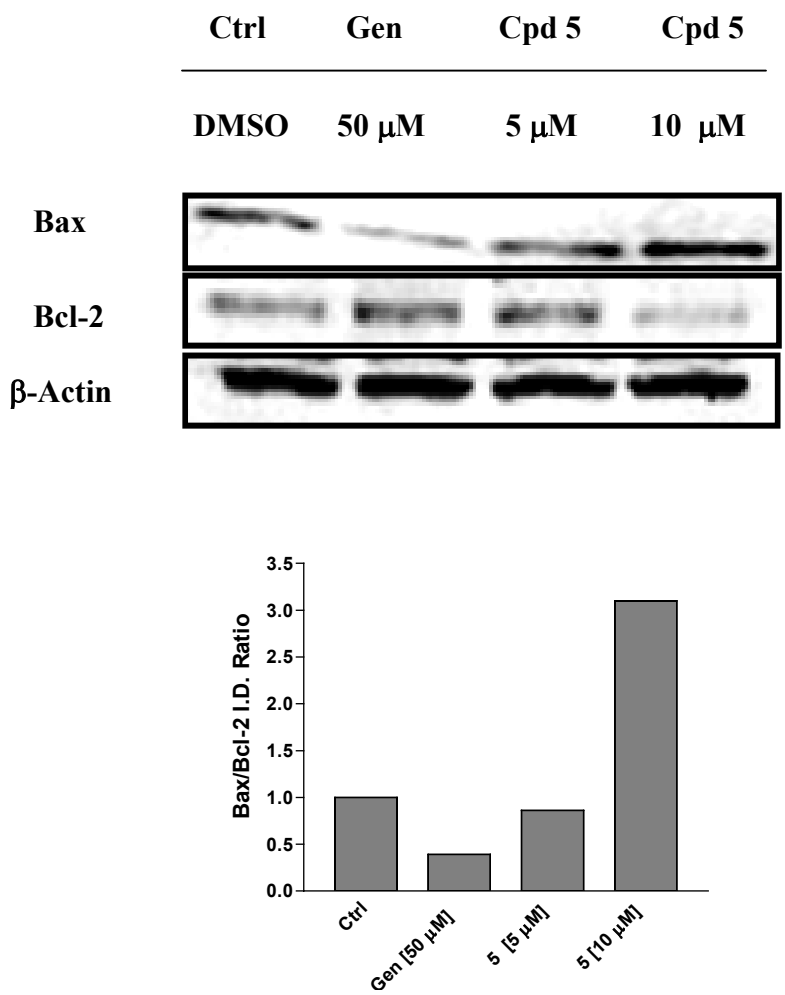

Figure 4.4: Western Blots of Bax and Bcl-2 proteins in MDA-MB-231 breast cancer cells. (A) Western blot and densitometeric analysis of Bax:Bcl-2 ratio in MDA-MB-231 cells. Cell lysates were prepared from each of the following samples following a 48 hour treatment as outlined in experimental methods: control (0.1 % DMSO), 50 μM genistein, and treatment with compound **5** at 5 μM and 10 μM.

4.3.4. Evaluation of PI3 kinase/Akt expression in breast cancer cells

The PI3/Akt pathway, which involves the activation of phosphorylated Akt (P-Akt), remains to be an attractive molecular target because of its involvement in breast cancer development. Constitutive Akt has been linked to increasing proliferation and decreasing apoptosis, both of which lead to malignant transformation, which is a hallmark of tumorigenic potential. Additionally, Akt is involved in modulating the expression of receptors within the human epidermal growth factor family (HER)-2, which represents an important molecular signature in breast cancers (19). Therefore, inhibitors of the Akt signaling pathway, which lead to inhibiting the expression of the activated protein at Thr 308 or Ser 473 by PI3K, still remain an important area of pursuit.

Gong *et al*., reported that the expression of total Akt and phosphorylated Akt at Ser 473 were both downregulated in genistein-treated cells. The authors also showed that the inactivation of NF-κB by genistein in MDA-MB-231 cells was partially mediated via the involvement of the Akt pathway (20). The effect of estrogenic compounds, including genistein, on modulating expression of Akt has been reported to be influenced by the ER status of the cell (21). In order to investigate such mechanisms, we evaluated the effect of compound **5** on Akt activation in both MCF-7 and MDA-MB-231 breast cancer cells. Our results show that treatment with compound **5** in MCF-7 cells shows an inhibition of expression in phosphorylated Akt at Ser 473, but conclusions in modulating expression cannot be reached due to the inability of expression of activated Akt within the MCF-7 breast cancer cell line (Fig 4.5). The same treatment with compound **5** for 48 hours in the

MDA-MB-231 cell line reduced the expression of phosphorylated Akt at residue Ser473 in MDA-MB-231 breast cancer cells (Fig 4.6). Although we observed a slight increase in pSer473 at 10 μM, this increase may possibly reflect a compensatory survival response at higher concentrations. Overall, compound **5** was found to be a potent inhibitor of p-Akt signaling in MDA-MB-231 breast cancer cells.

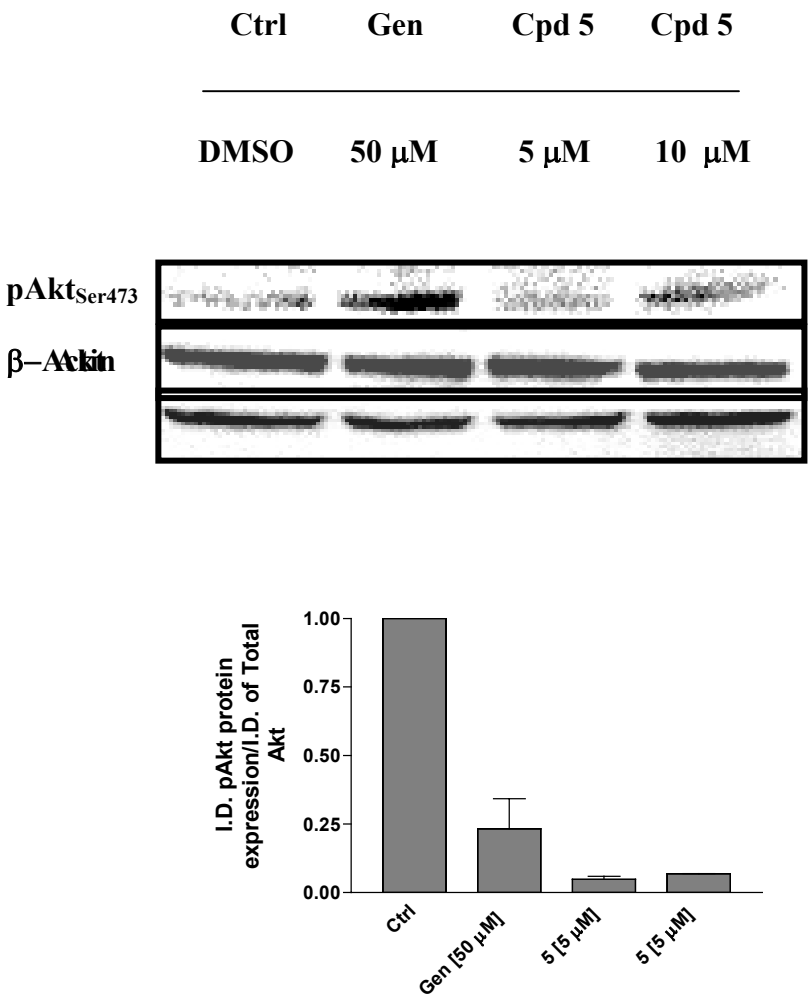

Figure 4.5: Western Blots of Akt and pAkt 473 proteins in MCF-7 breast cancer cells. (A) Western blot and densitometeric analysis in MCF-7 cells. Cell lysates were prepared from each of the following samples following a 48 hour treatment as outlined in

experimental methods: control (0.1 % DMSO), 50 μM genistein, and treatment with compound **5** at 5 μM and 10 μM.

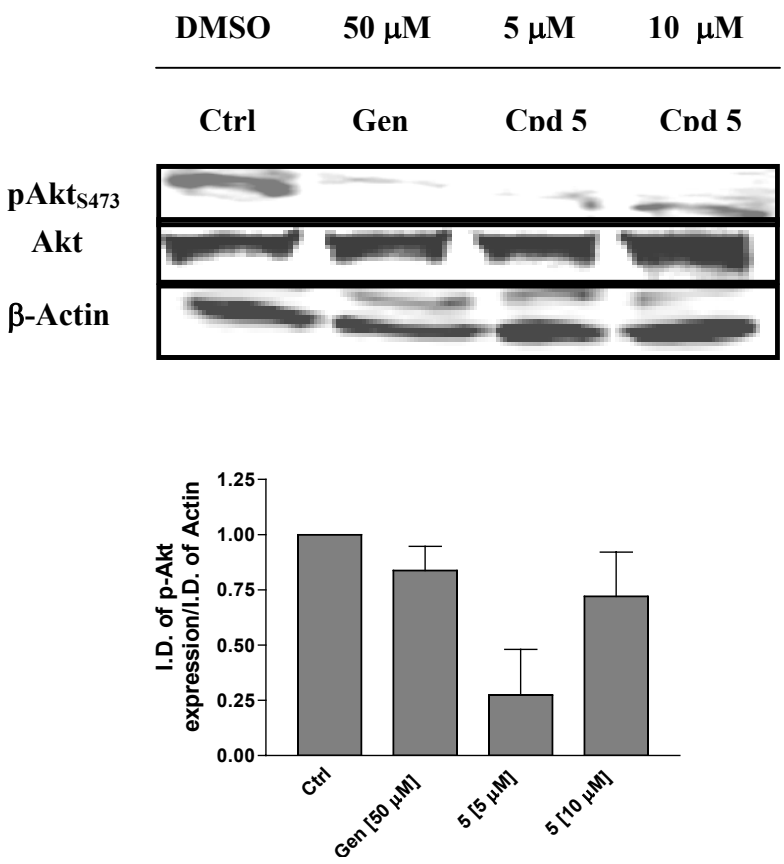

Figure 4.6: Western Blots of Akt and pAkt 473 proteins in MDA-MB-231 breast cancer cells. (A) Western blot and densitometeric analysis in MDA-MB-231 cells. Cell lysates were prepared from each of the following samples following a 48 hour treatment as outlined in experimental methods: control (0.1 % DMSO), 50 μM genistein, and treatment with compound **5** at 5 μM and 10 μM.

4.4 Discussion

Previous studies showed the ability of compound **5** to be antiproliferative in MCF-7 breast cancer cells and have the capability to overcome the stimulatory effect of estradiol. Additionally, compound **5** showed selectivity for cytotoxicity in the MDA-MB-231 breast cancer cell line. Other mechanisms besides those mediated by the estrogen receptor were examined to determine additional molecular targets of the synthetic isoflavone analog. The effect of compound **5** on cell proliferation was determined in the MDA-MB-231 breast cancer cell line. As predicted, compound **5** inhibited cell proliferation in the MDA-MB-231 breast cancer cell line in a dose dependent fashion. These results indicate that the selected isoflavone analog is an effective antiproliferative agent in both hormone-dependent and hormone-independent human breast cancer cells.

The molecular targets which are commonly associated with genistein induction of apoptosis-mediated pathways (18,20) were also examined. The dose-dependent effects of compound **5** on modulating levels of Bcl-2 (anti-apoptotic protein) and Bax (pro-apoptotoic aprotein) expression in MCF-7 and MDA-MB-231 cells were determined by western blot analysis. The levels of Bcl-2 expression in MCF-7 cells were slightly up-regulated with addition of 5 or 10 μM of compound **5** when exposed for 48 h. A different profile of Bcl-2 expression was observed in the MDA-MB-231 cell line, where expression of Bcl-2 was effectively decreased with increasing concentrations of compound **5**.

In contrast, the expression of Bax protein was significantly increased in both the MCF-7 and MDA-MB-231 cell line following treatment with compound **5**. The ratios of

Bax to Bcl-2 protein expression, which is indicative of apoptosis potential, showed a 3-fold increase in expression following treatment with compound **5** in MDA-MB-231 breast cancer cells.

Because of the importance of PI3 kinase and Akt in apoptosis inhibition, we investigated the involvement of compound **5** on activated Akt in MCF-7 and MDA-MB-231 cells by western blot analysis. We found that the expression of pAkt Ser473 was undetectable in MCF-7 cells, so we were unable to determine the influence of compound **5** in reducing the expression of pAkt Ser473. In contrast, MDA-MB-231 cells express pAkt Ser473 and we determined that compound **5** down-regulated the expression of pAkt Ser473 in MDA-MB-231 breast cancer cells.

In conclusion, we present evidence which strongly supports the antiproliferative and pro-apoptotic effects of compound **5**, a synthetic isoflavone of genistein, in MCF-7 and more profoundly in MDA-MB-231 breast cancer cells. This effect is partially mediated by increase in the Bax:Bcl-2 ratio and inhibition of phosphorylated Akt (pAkt Ser473), which provides the initiative to further explore the molecular targets of compound **5** in apoptosis mediated pathways.

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CHAPTER 5

GENE EXPRESSION STUDIES OF THE SYNTHETIC ISOFLAVONOID (COMPOUND 5) ON MOLECULAR TARGETS IN A HORMONE-INDEPEDNENT BREAST CANCER CELL LINE

5.1. Introduction

Breast cancer, one of the most common and often fatal cancers among women, is responsible for 1 in 33 (3%) deaths in American women. The American Cancer Society estimates that in 2007 about 178,480 new cases of invasive breast cancer will be diagnosed among women in the United States. Additionally, 62,030 new cases of *in situ* breast cancer (non-invasive and the earliest form of breast cancer) will also be diagnosed this year (1). Although there is a large disparity among ethnic groups diagnosed with breast cancer, epidemiological studies have shown that native Japanese and Chinese (and other Asian populations in their homelands) have the lowest rates of breast cancer and historically an approximate 6-fold difference in breast cancer risk between these population groups is reported (2).

Genistein, increased soy consumption, and breast cancer have shared an intimate, although at times contradictory, relationship in the cancer field for a number of years.

Genistein, diadzein, and glycitein represent the three soybean isoflavone aglycones, with genistein being the predominant isoflavone and having the highest circulating levels in plasma following dietary consumption. Barnes *et al*., report that as early as the 1990s the causative chemopreventive effects of genistein has been aggressively explored (3). Furthermore, in 2006 the impact of isoflavones on breast tissue at the cellular level in women at high risk for breast cancer was discussed nationally and found to still warrant further study (4). Biphasic effects of genistein on breast cancer cell proliferation are observed, e.g., concentrations lower than 10μM stimulate cell proliferation whereas higher concentrations inhibit cell proliferation (5). Genistein exhibits greater affinity for ERβ and ERα, and this preference may also have implications for breast cancer risk since the binding of certain ligands to ERβ inhibits mammary cancer cell growth (6,7).

Genistein has been identified as a protein tyrosine kinase (PTK) inhibitor, which has implications in terms of oncogenesis, cell proliferation and apoptosis (8). Markovits *et al*., further report that genistein can also inhibit decantenation of DNA by inhibiting topoisomearse II activity and induce DNA damage by generating DNA double-strand breaks (DSBs) through stabilizing the covalent topoisomerae II-DNA cleavage complex (9). Genistein has also been shown to play a role in cell cycle arrest at the G(2)M phase transition with concomitant induction of the CDK inhibitor $p21^{WAF1/CIP1}$, cyclin B1, and phospho-p34 in both MCF-7 and MDA-MB-231 breast cancer cells (10,11,12). Furthermore, genistein has been reported to show a concentration dependent effect of apoptosis induction through decreased expression of Bcl-2 and upregulation of Bax proteins (13).

Dampier *et al*., also showed that genistein inhibited PMA-induced AP1 activity, expression of c-FOS, and ERK activity in certain human mammary cell lines (14). Genistein treatment has also been shown to abrogate NF-κB DNA binding in human hepatocarcinoma cells stimulated with hepatocyte growth factor (15). The mechanistic implications of genistein have also been extensively studied in hormone and non-hormone related diseases of the prostate. Sarkar *et al*., have shown that genistein inhibits the H_2O_2-or TNFα-induced activation of NF-κB in both the androgen-sensitive (LNCaP) and insensitive (PC3) human prostate cancer cell lines at the apoptogenic concentration by reducing phosphorylation of IκBα and the nuclear translocation of NF-κB. Furthermore, genistein inactivation of NF-κB is linked to the downregulation of AKT in the prostate and mammary cancer cells. Sarker's group further eludes to a plausible novel mechanism responsible for the pro-apoptotic activity of genistein attributed to the inhibition of the crosstalk between AKT and NF-κB based upon the revelation that genistein treatment completely blocked AKT transfection which led to the activation of NF-κB (16,17,18).

Our laboratory has explored the 4H-1-benzopyran-4-one ring system as a scaffold for therapeutic agents in the treatment of breast cancer (19). The synthetic compounds (Figure 5.1) exhibit low affinities for estrogen receptors (ERs) and display selectivity for ERα over ERβ. The isoflavones demonstrated antiproliferative and cytotoxic activities in MCF-7 and MDA-MB-231 breast cancer cell lines (20). This manuscript describes further mechanistic investigations of one of the more effective agents, 7-hydroxy-3-(4-methoxyphenyl)-2-[4-[2-(piperidin-1-yl)ethoxy]phenylthio]-4*H*-1-benzopyran-4-one

(compound **5)**, utilizing pathway-specific gene expression analysis, flow cytometry and immunohistochemistry.

5.2. Experimental

5.2.1. Chemicals, Biochemicals and Reagents

Isoflavonoid analogs were synthesized in our laboratory (21). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt was obtained from Promega (Madison, WI). Genistein was obtained from Indofine Chemical Company (Belle Mead, NJ). 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. DMEM-F12 culture media was obtained from Sigma, and the human albumin was obtained from OSU Hospital Pharmacy. Antibodies for β-actin, caspase-7, caspase-3, Akt, and pAkt were purchased from Cell Signaling (Cell Signaling Technology, Inc. Danvers, MA). The general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-FMK) was obtained from BD Pharmingen^{TM} (United States).

5.2.2. Cell culture and Treatment

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, 1.5 X amino acids, 2 X 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 % CO_2 in a Hereaus CO_2 incubator. For all experiments, cells were plated in either 6-well plates or 100 mm petri dishes. Before treatment, the media was changed to 5 % Dextran coated charcoal stripped serum (DCCS) DMEM/F12 media with 2 mM L-glutamine and 20 μg/ml gentamycin.

5.2.3. Analysis of apoptosis

Apoptosis was determined by selective denaturation of DNA in apoptotic cells by formamide and detection of denatured DNA with a monoclonal antibody to single-stranded DNA using an ELISA kit (CHEMICON, Temecula, CA) (22). Cells were plated in a 96-well flat bottom plate from 0.5 x 10^4 to 1 x 10^4 cells/well in B-media. Cells were allowed to adhere to wells overnight. Following incubation, compounds were made up in defined media and a 10 μM screen was performed in each cell line with respective compounds in triplicate for 48 hours. After 48 hours, the plate was centrifuged at 200 x g for 5 min, media was removed followed by the addition of 200 μl of fixative. The plate was incubated for 30 min at 37°C, at which point the fixative was removed and the plate dried for 1-2 hours at room temperature. Fifty microliters of formamide was added to each well following a brief incubation at room temperature for 10 min. The DNA in apoptotic cells was denatured by heating the plate for 10 min, then briefly cooling the plate for 5 min at 4°C following removal of formamide. The plate was rinsed three times with 200 μl of PBS following one hour incubation at 37°C with 200 μl of 3% blocking agent. After removal of the blocking agent, 100 μl of antibody mixture were added to each well for 30 min at room temperature. The plate was washed three times with 1X wash buffer using 250 μl of wash buffer/well followed by the addition of 200 μl of ABTS

solution added to each well for 15-60 minute incubation. The reaction was stopped by the addition of 100 µl of a stop solution and absorbance was measured at 405 nm on a SpectroMax platereader.

5.2.4 Microarray Analysis

MCF-7 and MDA-MB-231 cells were treated with vehicle alone (control) or with 5 µM of compound 1A for 24 hours. Total RNA was isolated with TRIzol® Plus RNA Purification Kit (Invitrogen Corporation© Carlsbad, CA.), reverse transcribed into biotinylated cDNA, hybridized to a GEArray Q Series Human Apoptosis Gene Array containing cDNA fragments of key genes involved in the regulation and mediation of apoptosis, or programmed cell death and detected by chemiluminescence per the manufacturer's (SuperArray, Frederick, MD) instructions.

5.2.5 RNA Isolation and RT-PCR

RNA samples were treated with TURBO DNA-free kit to remove possible DNA contamination from the samples as follow: 20 µL of RNA solution, 2 µL of 10X TURBO DNase buffer and 1 µL of TURBO DNase (2 U/µL) was incubated at 37ºC for 20 minutes. The reaction was extended for another 20 minutes after adding 1 µL of TURBO DNase in the reaction mixture. The reaction was terminated by adding 2.2 µL of DNase Inactivation reagent. The mixture was spam at 10,000 X g for 1.5 min, and then supernatant was transferred to a new tube. After RNA concentration was measured 1 µg of RNA was subjected for a reverse transcription reaction. RT reaction was performed by using RT2 PCR Array First Strand Kit. The RT reaction was carried out at 37ºC for 60 minutes followed by a 95ºC incubation for 5 minutes for inactivation. Ninety-one

microliters of ddH2O was added to each 20 μL of cDNA synthesis reaction. RT2 Profiler PCR array system, cat #APHS-012D (Superarray, Frederick, MD), was used for this analysis.

5.2.5. Flow cytometry

MDA-MB-231 cells were grown to exponential phase, seeded at a density of 1-5 x 10^6 cells/100-mm dish in B-media and allowed to attached overnight. The following drug treatments were dissolved in 5% DCCS DMEM/F12 media: vehicle control (0.1% DMSO), 5 μM of compound 1A in presence or absence of Z-VAD-FMK which was added to cells 2 hours before synthetic isoflavone treatment, with a final concentration of 60 μM. After exposure to drug treatment, MDA-MB-231 cells were harvested, washed with cold PBS and resuspended in 1X Binding Buffer at a concentration of 1 X 10^6 cells/ml. One hundred microliters of the solution (1 X 10^5 cells) was transferred to a 5 ml culture tube followed by addition of 5 μl of Annexin V-PE and 5 μl of 7-AAD. The cells were then gently vortexed and incubated for 15 min at RT (25ºC) in the dark. Four hundred microliters of 1 X binding buffer was added to each tube and flow cytometry was performed within one hour. For flow cytometric analysis, a FACSCalibur flow cytometer (Becton Dickinson, NJ) equipped with a single argon ion laser was used. DNA content of 10,000 cells per analysis was monitored using the FACSCalibur system.

5.2.6. Preparation of total cell lysates and immunoblot analysis

To prepare the whole-cell extract, MDA-MB-231 cells (1-5 x 10^6 cells/100-mm dish) were detached following addition of the appropriate amount of M-PER® Reagent, to which Halt™ Protease Inhibitor Cocktail was added to produce a 1 X final

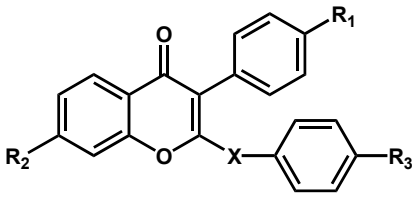
concentration (Pierce, Rockford, IL). The cellular suspension, which included floating cells was collected and freeze-thawed thrice to insure complete protein extraction. Total protein content was determined using Bio-Rad DC protein assay with bovine serum albumin as the standard. Equal amounts of (50 μg) the denatured proteins were loaded into each lane, separated on 10-12 % SDS polyacrylamide gels, followed by a transfer of the proteins to PVDF membranes for 1.0 hour. Membranes were blocked with 4.0 % ECL advance blocking agent (Amersham Biosciences, Piscataway, NJ), in Tris-buffered saline for 1.0 hour at room temperature and the membranes were reacted with appropriate secondary antibodies overnight. They were then incubated with an ECL anti-mouse IgG or anti-rabbit IgG, horseradish peroxidase-linked species specific whole antibody for 1h before being developed using the ECL Advance western blotting dectection system. Images were acquired using a Lumi-Imager Workstation equipped with a CCD Camera (Boehringer Mannheim, Germany).

5.3. Results

5.3.1. Measurement of Apoptosis

The library of isoflavones (Figure 5.1) were examined in MCF-7 and MDA-MB-231 breast cancer cell lines and shown to be antiproliferative and cytotoxic (20). The isoflavone derivatives were further tested to determine if apoptosis-mediated cell death is observed in the MCF-7 and MDA-MB-231 breast cancer cell lines. The apoptotic inducing ability of synthetic isoflavones at 10 μM was evaluated by the ssDNA ELISA over a 48 hour treatment in both MCF-7 and MDA-MB-231 breast cancer cell lines. The ELISA assay is able to differentiate between apoptotic and necrotic cells due to the

absence of immunoreactivity in necrotic cells. The apoptosis data reported is reflective of only those compounds that demonstrated apoptosis induction in one or both of the breast cancer cell lines. Several compounds showed a 1 to 2-fold increase in absorbance compared negative control and have ability to induce apoptosis (Figure 5.2). Compounds



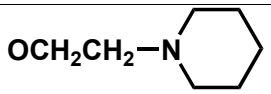
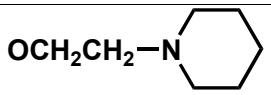
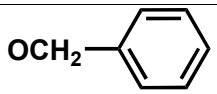
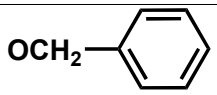
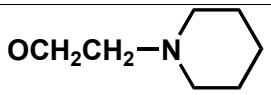
Compound	X	R_1	R_2	R_3
Genistein		OH	OH	
1	S	OH	OH	OH
2	O	OH	OH	OH
3	S	OH	OH	
4	S	OCH_3	OH	
*5	O	OCH_3		OH
6	S	OCH_3		

Figure 5.1: Library of 2, 4', 7-trisubstituted isoflavone analogs and their chemical structures.

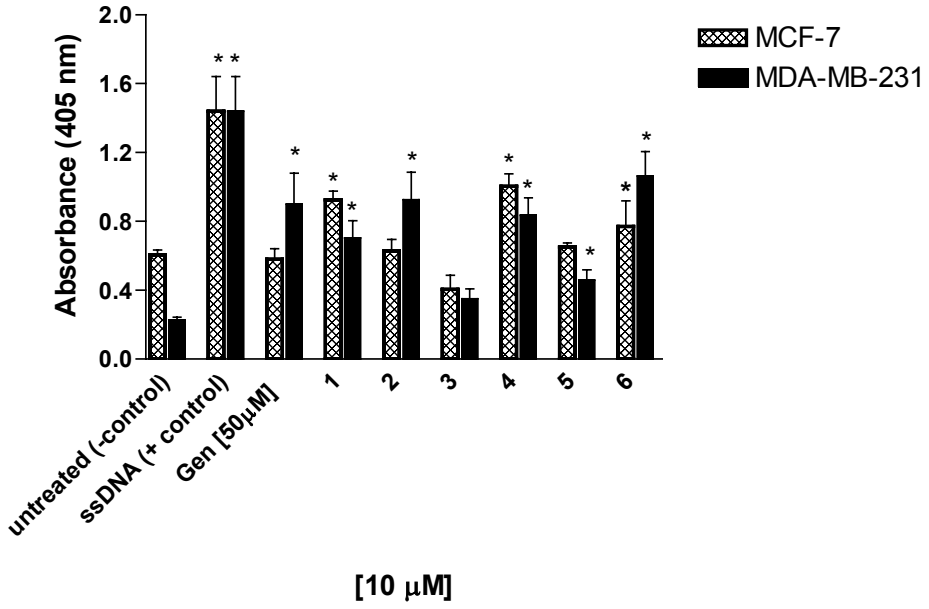


Figure 5.2: Apoptosis induced by 2, 4'-7 trisubstituted isoflavones. MCF-7 cells (A) and MDA-MB-231 cells (B) were treated with 10 μM of each agent for 48 hours and analyzed by ELISA for detection of ssDNA as described in the experimental section. Genistein at 50 μM and ssDNA were used as controls. Results that showed significant difference in absorbance compared to the negative control were characterized as inducing apoptosis. *, $P < 0.01$ *vs*. control by unpaired *t* test, n = 3.

1, 4, and **6** showed the ability to induce apoptosis in MCF-7 cells and compounds **1, 2, 4-6** induced apoptosis in MDA-MB-231 cells at 10 μM. In each case, the estrogen-independent MDA-MB-231 cell line was more sensitive to the effects of these agents. Furthermore, compound **5,** which showed selectivity in the cytotoxicty screening results for MDA-MB-231 cells over MCF-7 cells (23), only displayed apoptosis induction in MDA-MB-231 cells.

5.3.2. Expression Arrays

The synthetic isoflavone compound **5**, was examined in the GEArray Q Series Human Apoptosis Gene Array (HS-002) to determine gene expression profiles within the family of apoptosis related genes. The array houses cDNA fragments from 96 key genes involved in the regulation and mediation of apoptosis, or programmed cell death. The genes on the array are functionally grouped by structural related features, and include the TNF ligands/receptors; bcl-2 family; caspases; IAP; TRAF; CARD families; death domain/effector domain; CIDE domain family members, in addition genes involved in the p53 and ATM pathways. Gene expression profiling with microarrays was carried out using total RNA from treated MCF-7 and MDA-MB-231 cells as described in materials and methods. Data analysis was performed with GEArray Expression Analysis Suite software according to manufacturer's instructions. Following background correction and normalization, apoptotic-related genes were identified that exhibited a fold increase above a threshold value of 1.5. The same drug treatment in the MCF-7 cell line did not exhibit significant changes in gene expression between control and treated array (not shown). Table 5.1 details the genes with differential expression as a result of exposure to compound 5 in MDA-MB-231 cells. We identified at least 70 (73%) apoptotic related

GenBank	Symbol	Description	Fold Change
NM_033292	CASP1	Caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	2.0
NM_003723	CASP13	Caspase 13, apoptosis-related cysteine protease	1.9
NM_012114	CASP14	Caspase 14, apoptosis-related cysteine protease	1.7
NM_032982	CASP2	Caspase 2, apoptosis-related cysteine protease (neural precursor cell expressed, developmentally down-regulated 2)	2.4
NM_004346	CASP3	Caspase 3, apoptosis-related cysteine protease	5.1
NM_001225	CASP4	Caspase 4, apoptosis-related cysteine protease	11.2
NM_004347	CASP5	Caspase 5, apoptosis-related cysteine protease	2.9
NM_032002	CASP6	Caspase 6, apoptosis-related cysteine protease	1.7
NM _001228	CASP8	Caspase 8, apoptosis-related cysteine protease	3.8
NM_001229	CASP9	Caspase 9, apoptosis-related cysteine protease	2.2
NM_001160	APAF1	Apoptotic protease activating factor	1.9
NM_000051	ATM	Ataxia telangiectasia mutated (includes complementation groups A, C and D)	1.8
NM_003879	CFLAR	CASP8 and FADD-like apoptosis regulater	1.7
NM_003805	CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	1.7
NM_014326	DAPK2	Death-associated protein kinase 2	2.4
NM_001924	GADD45A	Growth arrest and DNA-damage-inducible, alpha	1.6
NM_003824	FADD	Fas (TNFRSF6)-associated via death domain	1.5
NM_000639	FASLG (TNFSF6)	Tumor necrosis factor (ligand) superfamily, member 6	1.6
NM_002392	MDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	1.8
NM_000594	TNF	Tumor necrosis factor (TNF superfamily, member 2)	1.7
NM_001065	TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	1.6
NM_000639	TNFSF7	Tumor necrosis factor (ligand) superfamily, member 7 (Fas ligand)	2.3
NM_001250	TNFRSF8	Tumor necrosis factor receptor superfamily, member 8	1.7
NM_001243	TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	1.7
NM_001561	TNFRSF11	Tumor necrosis factor receptor superfamily, member 11	1.7
NM_000074	TNFSF5	Tumor necrosis factor (ligand) superfamily, member 5 (hyper-IgM syndrome)	1.9
NM_003811	TNFSF9	Tumor necrosis factor (ligand) superfamily, member 9	0.5
NM_	TNFSF10	Tumor necrosis factor (ligand) superfamily, Member 10	0.5

Table 5.1: Genes with Differential Expression as a Result of Exposure to Synthetic Isoflavone. For determination of gene expression patterns, fold-changes were based upon normalization to relevant house-keeping genes and baseline values relative to a control (DMSO) array. Fold-changes with values of 1.5 or greater were considered to be over-expressed and those values below 1.5 were considered to be underexpressed.

genes that were overexpressed in the MDA-MB-231 cells following treatment. Within the functional grouping of the apoptosis related genes, those uniquely expressed were members of the caspase-family, the TNF ligand/receptor family, and the death domain family. Table 5.1 also illustrates the molecular functionality and unique GenBank symbol related to each gene described. The dominant gene expression pattern was expressed in the caspase, apoptosis-related cysteine protease family, and the TNF-family of genes.

The Fas ligand (Fas-L), belongs to the TNF family and can be found as a 40-kDa membrane-bound or a 26-kDa soluble cytokine (24). A series of death-associated adaptor molecules, including FADD (Fas-associated death domain-containing protein) is recruited to the Fas receptor and is responsible for the involvement of the proteolytic cascade that initiates activation of other caspases such as caspase-7, caspase-3, and –6 (25,26). Table 5.1 shows key initiator and effector caspases with relative fold increase values as follows CASP3 (5.1), CASP6 (1.7), CASP8 (3.8) and CASP9 (2.2). Additionally, the elevation of the FADD domain (1.5), GADD45A (1.6), DAPK2 (2.4), and the FASLG (1.6) was observed, each exhibiting differential fold change values relative to control array. Therefore, genes involved in Fas mediated caspase-dependent cell death appear to be the most prominent following treatment with compound **5** in MDA-MB-231 cells and represent a likely target for our compounds.

5.3.3. Real-Time PCR Confirmation of Differential Gene Expression

Gene confirmation was performed using the RT^2 Profiler PCR Array-APHS-012. There is expression of 84 key genes involved in apoptosis present on the array and include the TNF ligands and their receptors; members of the bcl-2, caspase, IAP, TRAF,

CARD, death domain, death effector domain, and CIDE families; as well as genes involved in the p53 and ATM pathways. The array layout contains replicates of the 96 primer sets in a 96-well plate format. Total RNA was prepared as outlined in materials and methods. Genes relevant in the caspase and TNF family were considered to be up-regulated based on a fold change of 1 or greater in MDA-MB-231 cells. Treated MCF-7 cells did not display significant fold changes in either apoptosis family of functional genes. Table 5.2 lists the relevant genes along with fold changes related to the caspase and TNF family. Although the magnitude of differential expression between array and RT-PCR fold changes is smaller, we found consistency amongst the fold difference between the two measurement parameters.

The inherent pitfalls that exist between microarray and qPCR have been extensively reviewed (25,26,27,28,29,30). Morey *et al*., have explored factors influencing correlation between microarrays and RT-PCR and their study reveals that validation of gene expression by RT-PCR is heavily influenced by normalization procedures, which are vastly different for the two methods. Furthermore, they report a threshold of reliability for oligonucleotide arrays and RT-PCR, which corresponds to a fold change of 1.4 with a p-value of 0.0001, or less, which led to a significant reported value of 0.80 for array and qPCR data. Also, values below this reported threshold of 0.80 should not be completely overlooked but rather examined closer. In conclusion, Morey *et al*., suggest that until a universal understanding of normalization is reached, a standard tool for validation of microarray results will be based on individual assessments (31).

GenBank	Symbol	Microarray Fold-Change	RT(PCR) Fold-Change
CASP3	NM_004346	5.1	1.01
CASP9	NM_032002	2.2	1.83
TNF	NM_000594	1.7	1.24
TNSF7	NM_000639	2.3	1.83
TNFSF10	NM_	0.5	-1.10
FADD	NM_003824	1.5	1.04
GADD45A	NM_001924	1.31	1.6

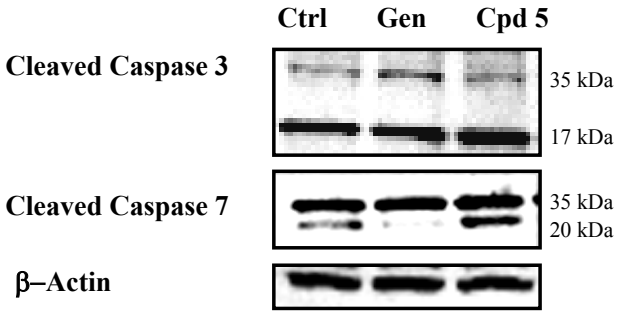
Table 5.2: Reverse Transcription-RT(PCR) Validation of Differential Expression of Selected Genes. NOTE: Gene confirmation was performed using the RT^2 Profiler PCR Array-APHS-012. Reported are the genes considered up-regulated corresponding to a fold change of 1 or greater within the caspase and TNF superfamily of proteins.

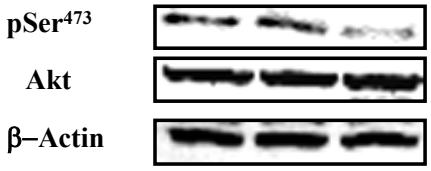
5.3.4. Immunoblots of Relevant Protein Markers

Because our gene expression data pointed to the involvement of the caspase-dependent pathway resulting from treatment with compound **5**, we turned our attention to immunoblotting of relevant caspase proteins. The extrinsic pathway of cell-mediated apoptosis, which is independent of mitochondria and is induced by death receptor-protein complexes, leads to involvement of these caspases. Kaufmann *et al*., have categorized the caspases as initiator (caspase-8, caspase-9, -10, and –12), which cleave other caspases, or executioner caspases (caspase-3, -6, and –7), which cleave various cellular proteins (32). The executioner caspases are involved in cleavage of a broad spectrum of cellular proteins, including poly(ADP-ribose) polymerase, thus leading to cell death (33,34). Treatment of MDA-MB-231 cells for 48 hours with 50 μM genistein and 5 μM of our synthetic isoflavone, compound 5, resulted in the cleavage of caspase-7 and caspase-3 when compared to cells that were not treated with our compound (Figures 5A and 5B). Genistein treatment did not result in significant cleavage of either caspase. The membranes were also probed for β-actin as a loading control. Thus, these data further suggest that caspase-3 and caspase-7 activation is involved in the synthetic isoflavone-induction of apoptosis of MDA-MB-231 cells.

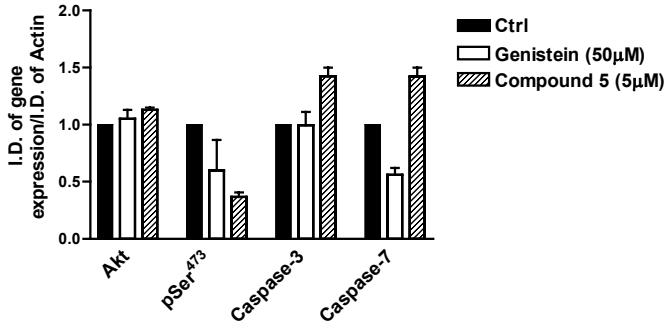
The PI3K/Akt pathway, which is intricately involved in signal transduction, is also an attractive target for chemoprevention. Akt, which is a serine/threonine kinase is regulated by growth factors, and is subsequently activated by a wide variety of growth stimuli including EGF, PDGF, IGR, and NGF (35). The two key sites of phosphorylation on Akt are at Thr308 and Ser473, which occurs by PI3K (36). Phosphorylated Akt (p-Akt) represents an attractive molecular cancer target and convergence point in signaling

A.





B.



5.3: Immunohistochemistry of Relevant Protein Markers in MDA-MB-231 cells. Cell lysates were prepared from each of the following samples as outlined in Materials and Methods: control (0.1 % DMSO), 50 μM genistein treatment, 5 μM synthetic isoflavone. Immunoblotting was performed with anti-caspase-3, anti-caspase-7, β-Actin, total-Akt, pSer473 antibodies. B. Densitometeric quantitation of autoradiagram of two independent experiments.

pathways involved in breast cancer and resistance to chemotherapeutic therapies. Compound **5** was further examined for its effects on phosphorylation of Akt, specifically, phospho-Akt^{S473} in MDA-MB-231 cells. MDA-MB-231 cells, which express high levels of p-Akt, were treated for 48 hours with DMSO, compound **5** (5 μM) and genistein (50 μM). Both the isoflavone analog and genistein suppressed phosphorylation of Akt at serine 473. This data show that our isoflavone analog is capable of inhibiting Akt kinase activity.

5.3.5. General caspase inhibitor (z-VAD-fmk) Blocks Isoflavone-analog Induced Apoptosis in MDA-MB-231 breast cancer cells

Flow cytometry analysis was used as an independent variable to further determine whether compound **5** induces apoptosis in a caspase-dependent fashion. MDA-MB-231 cells were exposed to compound **5** in the presence (and absence) of z-VAD-fmk, and apoptosis assessed by another independent methodology. Annexin V-PE is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. As shown in Figure 5.4B, compound **5** induced apoptosis as measured by Annexin V-PE staining (top right and bottom quadrants), confirming the results obtained by array analysis, RT-PCR, and immunoblotting of executioner caspases. Moreover, MDA-MB-231 cells exposed to our isoflavone analog also showed loss of membrane asymmetry in the early phases of apoptosis as measured by increased 7-AAD staining (top right quadrant).

When MDA-MB-231 cells were preincubated with z-VAD-fmk, a significant decrease by 10 % in Annexiv V-PE positive cells was observed (top and bottom quadrants) as shown in Figure 5.4 C.

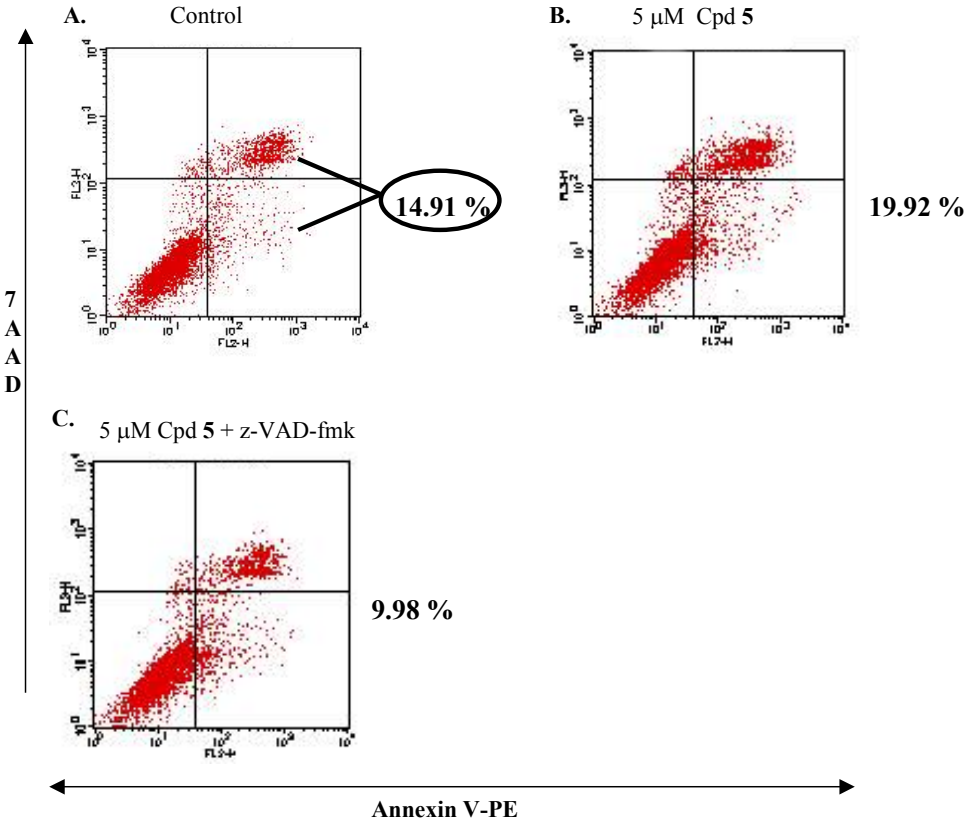


Figure 5.4: Detection of apoptosis by Annexin V-PE assay in presence and absence of z-VAD-fmk. MDA-MB-231 cells were exposed to 5 μM of compound **5** for 24 hours in the presence and absence of 60 μM of our general caspase inhibitor, z-VAD-fmk. Staining for Annexin V-PE and 7-AAD was described in Materials and Methods. Representative of two independent experiments that show similar results. Single arrow, Annexiv V-PE positive quadrant (bottom right quadrant); double arrow, Annexin V-PE, 7-AAD positive quandrant (top right quadrant). Figure 5.4 B shows induction of apoptosis following isoflavone treatment by increased staining of Annexin V-PE relative to control (0.1% DMSO), panel A. Figure 5.4 C shows that preincubation with ZVAD resulted in a significant decrease in Annexin V-PE positive cells.

5.4. Discussion

Substantial progress in the field of carcinogenesis has identified biochemical events that support the use of dietary flavonoids as well as analogs in the treatment strategies involving stages of cancer progression. In this report, we examined a series of synthetic isoflavones exhibiting effective antiproliferative and cytotoxic activity in the presence of MCF-7 and MDA-MB-231 breast cancer cells (22). Within the series, several compounds showed promise as effective cytotoxic agents in the presence of estradiol in the MCF-7 breast cancer cell line. In addition, one analog, compound **5,** showed selectivity in cytotoxcity in the MDA-MB-231 breast cancer cell line, suggesting that its mechanism of activity was independent of any antihormonal effects.

The ability of anticancer drugs to induce apoptosis and the molecular mechanisms underlying their activity is still poorly understood, but still represents an attractive target in drug design and treatment strategies. Examination of the synthetic isoflavones for the involvement of cell-death mediated apoptosis in MCF-7 and MDA-MB-231 breast cancer cells showed that compounds **1, 4,** and **6** induced apoptosis in both MCF-7 and MDA-MB-231 cells. Compounds **2** and **5** also induced apoptosis in the MDA-MB-231 cell line. Furthermore, compound **5**, showed selectivity of apoptosis in the MDA-MB-231 cells, which further substantiated the previous observations of cytotoxicity only in MDA-MB-231 cells.

Genistein-induced apoptosis in cancer cells involves the upregulation of Bax protein and $p21^{WAF1}$ and downregulation of NF-κB, Bcl-2, Bcl-x_L, and surviven (36,37,38). Increased expression of the Bax/Bcl-2 protein ratio represents a gateway to apoptosis-mediated cell death and involvement of a family of proteins from multiple

pathways. In the present study, pathway-specific gene expression arrays were used to investigate possible apoptotic pathways altered by compound **5** in MDA-MB-231 cells. DNA microarrays provide an invaluable and efficient tool for comparison of differential expression among a large cohort of genes in a single reaction. Among the 96 genes on the cDNA microarray, 70 genes were differentially expressed as a result of treatment with compound **5** in the MDA-MB-231 cell line. Furthermore, five out of the eleven categories, which contained genes in the caspase family, TNF ligand and receptor family, and genes within the death domain and death effector family, were involved in apoptosis cell death following treatment with compound **5**. The same treatment with compound **5** in MCF-7 cells did not show significant changes in gene expression between control and treated array. Validation of the gene expression results from the cDNA microarray were observed using real time-PCR. Western blot analysis of the relevant executioner caspases (caspase-3 and capase-7) further confirmed the microarary results of the involvement of the caspase family of proteins. To determine whether compound **5** could potentially serve as an inhibitor of the Akt family, a signaling intermediate in receptors commonly overexpressed in breast cancer (38), western blot analysis of pAkt in MDA-MB-231 cells was performed. Following treatment with compound **5** in MDA-MB-231 cells, expression of $pAkt^{S473}$ was effectively inhibited. Furthermore, flow cytometry results substantiated the involvement of relevant caspases by showing that the presence of z-VAD-fmk, the pan caspase inhibitor, effectively blocked apoptosis following drug treatment with compound **5** in MDA-MB-231 cells.

In summary, we provide mechanistic evidence that compound **5,** a novel synthetic isoflavone derivative of genistein, is capable of inducing apoptosis in MDA-MB-231

breast cancer cells. This observed cell-death mediated process occurs through enhanced expression of relevant genes in the caspase family, TNF superfamily, as well as genes in the death domain and effector family. The schematic (Figure 5.6) illustrates the key proteins involved in apoptosis induction following treatment with compound **5** in MDA-MB-231 breast cancer cells. Furthermore, treatment with compound **5** under the same conditions in MCF-7 cells did not yield any appreciable results. Because hormone-independent breast cancer represents an aggressive form of breast cancer and cause of increased morbidity for women, identification of novel agents that can be explored in treatment strategies is needed. In conclusion, compound **5** represents an attractive lead for further exploration as potential therapeutic agent, either alone or in combination with existing chemotherapeutic agents, for hormone independent breast cancer.

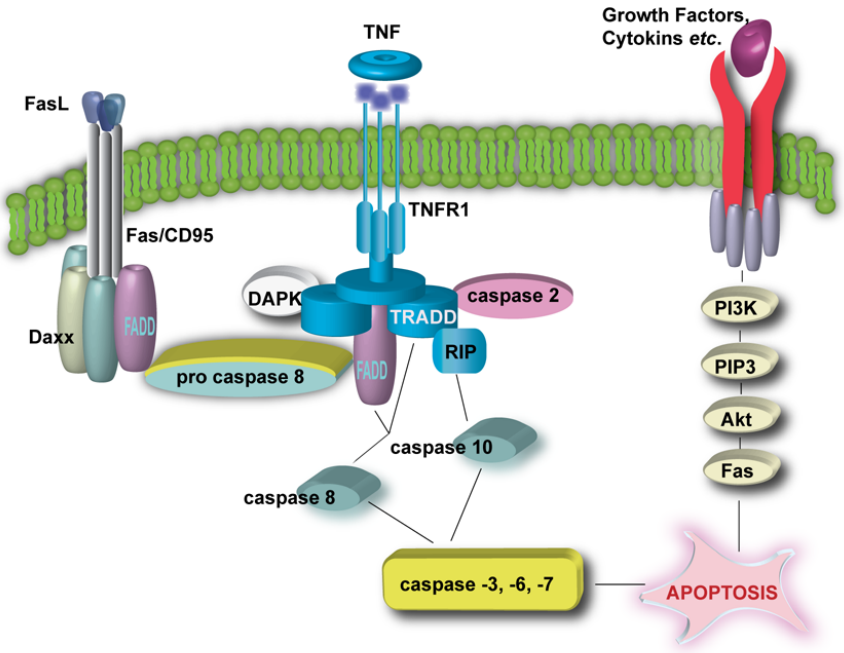


Figure 5.6: The extrinsic and intrinsic pathways of Fas-mediated apoptosis.

5.5 Acknowledgements

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CHAPTER 6

INVESTIGATION OF THE EFFECTS OF NIMESULIDE ANALOGS ON CELL CYTOTOXICITY, APOPTOSIS, AND THEIR POTENTIAL AS SELECTIVE AROMATASE EXPRESSION REGULATORS IN BREAST CANCER CELLS

6.1. Introduction

Options for breast cancer treatment now include aromatase inhibitors, which are now widely used in the adjuvant and metastatic setting and further being investigated as potential agents to prevent breast cancer (1). The aromatase inhibitors commonly prescribed include anastrozole, letrozole, and exemestane, and these drugs are fast becoming safer alternatives to tamoxifen therapy in the treatment of hormone-dependent breast cancer (2,3,4). The biochemistry of the cytochrome P450 enzyme complex called "aromatase" involves the biosynthesis of estradiol and estrone from the estrogen precursors dihydroepiandrostenedione and testosterone, respectively. Furthermore, the tissue-specific regulation of the aromatase gene (CYP19) involves regulatory control by alternative promoters, which are influenced by the breast tumor microenvironment. This influence by breast tumor factors involves the switching from promoter I.4 to c-AMP dependent promoter I.3 and promoter II in breast cancer and the adipose stromal cell environment (5,6,7). The cyclooxygenase isozymes (constitutive COX-1 isozyme and

inducible COX-2 isozyme) are responsible for local production of prostaglandin E_2 (PGE_2) involved in increased cellular cAMP levels and mediating estrogen biosynthesis and estrogen dependent beast cancer. This biochemical interplay shared by COX, PGE_2, and estrogen biosynthesis may be responsible for the beneficial observations of nonsteroidal anti-inflammatory drugs (NSAIDs) use in the treatment of breast cancer (8).

Previously, our lab unraveled a significant relationship between aromatase and COX enzyme systems by showing that NSAIDS, COX-1 and COX-2 selective inhibitors were responsible for a dose-dependent decrease in aromatase activity in breast cancer tissues (9,10). We demonstrated that the COX-2 inhibitors, N-(2-phenoxy-4-nitrophenyl) methanesulfonamide (nimesulide), and N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide (NS-398), acted through suppression of aromatase transcription to subsequently suppress aromatase activity in breast cancer cells (9,11,12). Further studies showed that derivatives of N-(2-phenoxy-4-nitrophenyl) methanesulfonamide result in aromatase activity suppression at similar rates as the parent compound, whereas no COX-inhibition was observed with the N-methyl and 2-methyl derivatives of N-(2-phenoxy-4-nitrophenyl) methanesulfonamides (13).

In an effort to better target hormone-dependent breast cancer, estrogen depletion through regulation of aromatase expression in breast tissue has fast become a more effective strategy in drug design. We have previously shown the ability of nimesulide and NS-398 to suppress aromatase activity by suppressing aromatase transcription (9). In the present study, we describe the biological effects of a series of novel sulfonanilide compounds based on nimesulide as selective aromatase expression regulators.

6.2. Experimental Section

6.2.1. Chemicals, Biochemicals and Reagents

Nimesulide analogues were prepared by Bin Su as described (14). Chemicals were commercially available and used as received without further purification unless otherwise noted. Moisture-sensitive reactions were carried out under a dry argon atmosphere in flame-dried glassware. Solvents were distilled before use under argon. Thin-layer chromatography was performed on precoated silica gel F254 (Whatman). Silica gel column chromatography was performed using silica gel 60A (Merck, 230-400 mesh). High-resolution electrospray ionization mass spectra were obtained on the Micromass QTOF Electrospray mass spectrometer at The Ohio State Chemical Instrumentation Center. All the NMR spectra were recorded on a Brunker DPX 250 and DRX 400 MHz in either DMSO-d_6 or $CDCl_3$. Chemical shifts (δ) for ^1H NMR spectra are reported in parts per million to residual solvent protons. Chemical shifts (δ) for ^{13}C NMR spectra are reported in parts per million relative to residual solvent carbons.

6.2.2 Cell Culture

JAR and SK-BR-3 cells were obtained from ATCC (Rockville, MD). SK-BR-3 cells were maintained in phenol red-free custom media (MEM, Earle's salts, 1.5x amino acids, 2x nonessential amino acids, L-glutamine, 1.5x vitamins, Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 20 mg/L gentamycin. JAR cells were maintained in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 10% FBS. Fetal bovine serum was heat-inactivated for 30 min in a 56ºC water bath before use. Cell cultures were grown at 37ºC, in a humidified

atmosphere of 5% CO_2 in a Hereaus CO_2 incubator. For all experiments, cells were plated in six-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 transferrin, and 5.0 mg/L bovine insulin.

6.2.3 Tritiated Water-Release Assay in JAR and SK-BR-3 Cell Lines

Measurement of aromatase enzyme activity was based on the tritium water release assay. Cells in six-well plates were treated with 0.1 % DMSO (control) and inhibitors at the indicated concentrations. After 24 h, the cells were incubated 3 h (SK-BR-3 cells) or 1 h (JAR cells) with fresh media along with 100 nM [1β-^{3}H]-androst-4-ene-3, 17-dione (1 μCi). Subsequently, the reaction mixture was removed, and proteins were precipitated using 10 % trichloroacetic acid at 42 ºC for 20 min. After a brief centrifugation, the media was extracted three times with an equal amount of chloroform to remove remaining substrate and further treated with 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 ^3H_2O formed per hour incubation time per million live cells (pmol/h/10^6 cells). To determine the amount of cells in each flask, the cells were lysed and analyzed using the diphenylamine DNA assay adapted to a 96-well plate. IC_{50} sigmoidal dose-response data were analyzed with Microsoft Excel and the Graphpad Prism (Version 3.0) program.

6.2.4. Cell Viability Analysis

The effect of nimesulide derivatives on SK-BR-3 cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide assay in six replicates. Cells were grown in custom media in 96-well, flat-bottomed plates for 24 h and were exposed to various concentrations of nimesulide derivatives dissolved in DMSO (final concentration 0.1 %) in defined media for different time intervals. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. The medium was removed and replaced by 200 μL of 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide in fresh media, and cells were incubated in the CO_2 incubator at 37 ºC for 2 h. Supernatants were removed from the wells, and the reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide dye was solubilized in 200 μL/well DMSO. Absorbance at 570 nm was determined on a plate reader.

6.2.5 Analysis of Apoptosis

Apoptosis was determined by selective denaturation of DNA in apoptotic cells by formamide and detection of denatured DNA with a monoclonal antibody to single-stranded DNA using an ELISA kit (CHEMICON, Temecula, CA). Cells were plated in a 96-well flat bottom plate from 0.5 x 10^4 to 1 x 10^4 cells/well in B-media. Cells were allowed to adhere to wells overnight. Following incubation, compounds were made up in defined media and a 5 μM screen was performed in the SK-BR-3 cell line with respective compounds in triplicate for 24 hours. Following treatment, the plate was centrifuged at 200*g* for 5 min, media was removed followed by the addition of 200 μl of fixative. The plate was incubated for 30 min at 37 °C, at which point the fixative was removed and the

plate dried for 1-2 hours at room temperature. Fifty microliters of formamide was added to each well following a brief incubation at room temperature for 10 min. The DNA in apoptotic cells was denatured by heating the plate for 10 min, then briefly cooling the plate for 5 min at 4°C following removal of formamide. The plate was rinsed three times with 200 µl of PBS following one hour incubation at 37 °C with 200 µl of 3 % blocking agent. After removal of the blocking agent, 100 µl of antibody mixture were added to each well for 30 min at room temperature. The plate was washed three times with 1X wash buffer using 250 µl of wash buffer/well followed by the addition of 200 µl of ABTS solution added to each well for 15-60 minute incubation. The reaction was stopped by the addition of 100 µl of a stop solution and absorbance was measured at 405 nm on a SpectroMax 340 UV PlateReader.

6.3. Results

6.3.1. Chemistry of Nimesulide Analogs

The chemical structures of the COX-2 inhibitors N-(2-phenoxy-4-nitrophenyl) methanesulfonamide (nimesulide), and N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide (NS-398) are shown (Figure 6.1). From the library of 42 nimesulide analogues synthesized, evaluations on ability to suppress aromatase activity were performed in both SK-BR-3 breast cancer cells and JAR choriocarcinoma placental cells. The SK-BR-3 breast cancer cell line produces aromatase expression from promoter II and I.3, which is mediated by cAMP, and the JAR cell line produces aromatase expression from promoter I.1, which is mediated by protein kinase C. The nimesulide analogues that

effectively suppressed aromatase activity was selected upon their ability to differentiately suppress aromatase activity only in breast cancer cells and not in placental cells.

6.3.2. Evaluation of aromatase activity in SK-BR-3 cells

The synthesized compounds were first screened for their ability to suppress aromatase activity in JAR cells. Most of the compounds screened suppressed aromatase activity in JAR cells at 15 μM (14), which may be explained by the involvement of multiple signaling pathways leading to the decrease in aromatase activity. In contrast, when the compounds were screened in SK-BR-3 cells, 2.5 μM treatment resulted in significant decrease in aromatase activity (14). Compounds that showed selectivity for

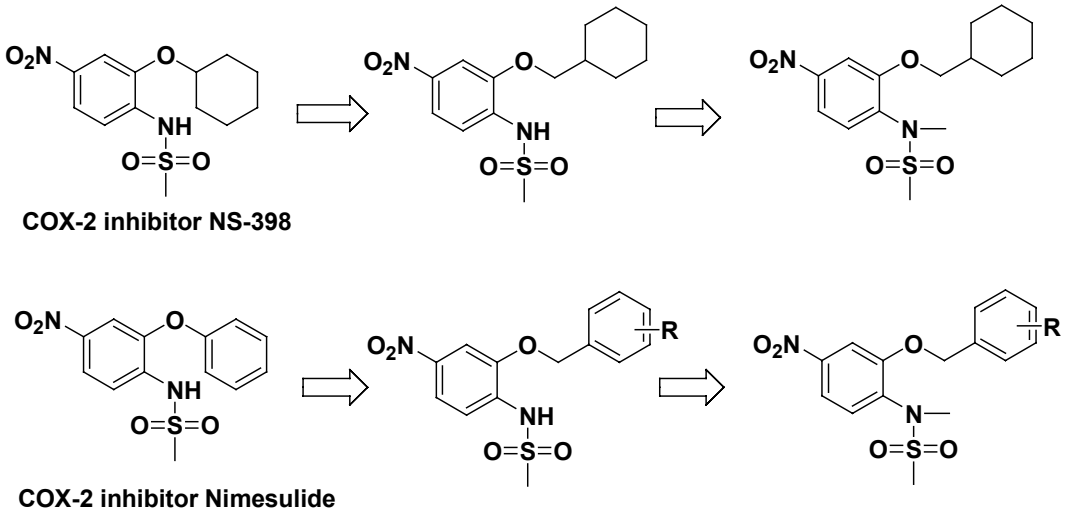

Figure 6.1: Chemical structures of nimesulide, NS-398 and the target compounds

decreasing aromatase activity in SK-BR-3 cells and not in JAR cells (**1f, 2c, 3c, 4c, 5c,** and **8c**) were selected and further investigated for their potential as selective aromatase expression regulators (Fig. 6.2).

Dose-response studies were performed on the selected compounds for their ability to suppress aromatase activity in SK-BR-3 cells. All six compounds showed a dose-response relationship of suppression of aromatase activity (Fig. 6.3), with the IC_{50} values shown in Table 6.1. The conclusions from the dose-response studies are that increased suppression of aromatase activity results from a one-carbon extension at the 2-position of nimesulide, which also favors a more bulky substituent (14). Additionally, real time PCR confirmed that all six compounds at a 5 μM treatment in SK-BR-3 cells significantly decreased expression of the CYP19 gene (14).

6.3.3 Effects of cytotoxicity in SK-BR-3 cells

The compounds were also tested for their ability to induce cytotoxicity in SK-BR-3 breast cancer cells. Three different concentrations of 10, 20, or 30 μM of each of the six compounds, including DMSO (0.1 %) as the negative control, were incubated in the SK-BR-3 cell line for different time intervals. Viability following drug treatment was assessed as described in the experimental methods section. All six compounds screened did not result in significant cell cytotoxicity at the indicated concentrations tested in the SK-BR-3 cell line (Fig. 6.4) (14).

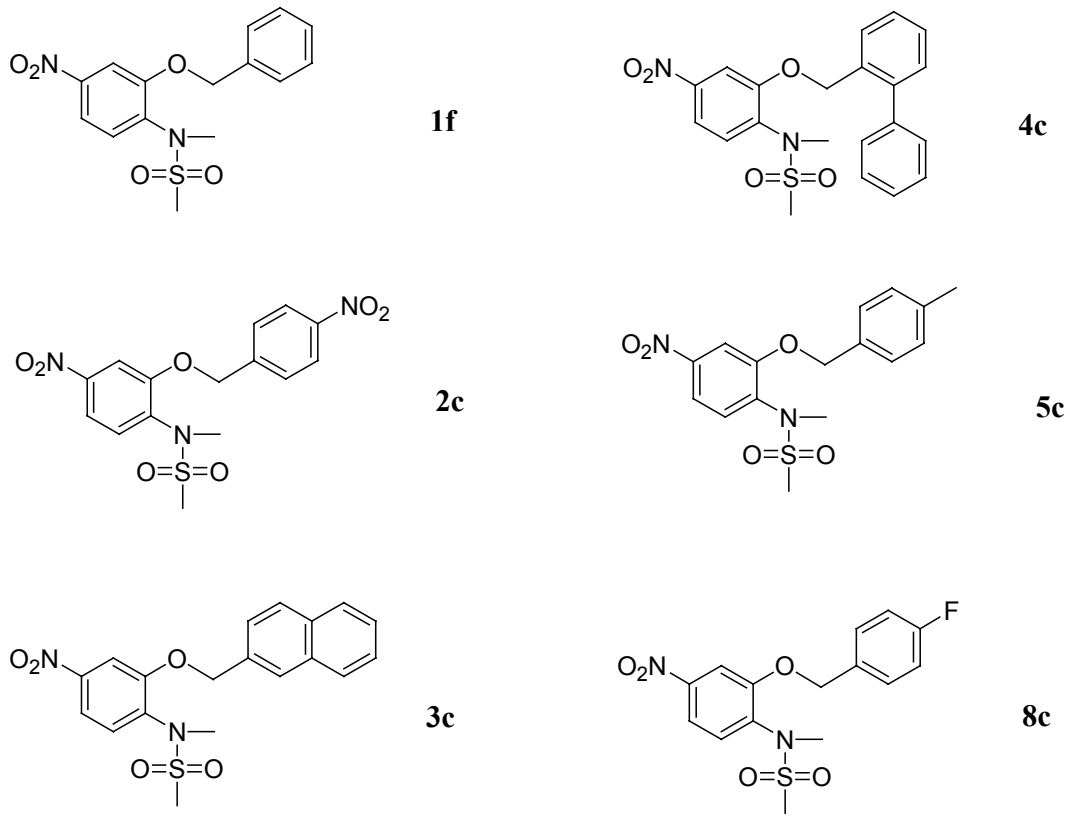

Figure 6.2: Six compounds selected as potential selective aromatase regulators.

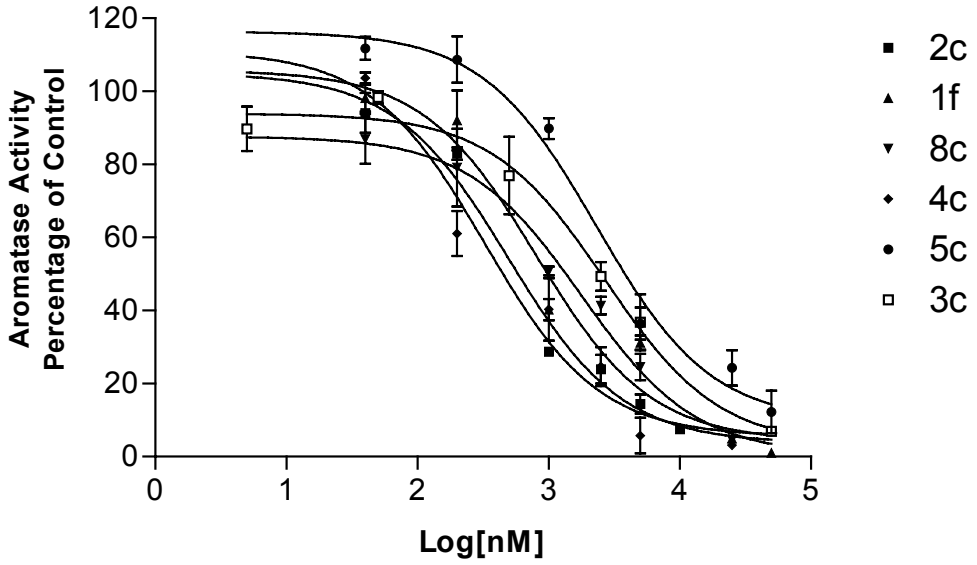

Figure 6.3: Dose-response suppression of aromatase activity in SK-BR-3 cells by novel sulfonanilide. SK-BR-3 cells were treated with 2c (■), 1f (▲), 8c (▼), 4c (♦), 5c (●) and 3c (□) and aromatase activity was measured as described in the experimental section. The results were normalized against a control treatment with vehicle, with the value of 100% is equal to 0.03 pmol/hr/10^6 cells. Each data point represents the mean results of three independent determinations, and the data were statistically analyzed by a nonlinear regression analysis method (14).

Compd	Chemical Description	IC_{50} (μM) [a]
Nimesulide	N-(2-phenoxy-4-nitrophenyl)-methanesulfonamide	27.0 ± 4.70 [b]
1f	N-methyl-N-(2-benzyloxy-4-nitrophenyl) methanesulfonamide	0.81 ± 0.29
2c	N-methyl-N-[2-(4'-nitro benzyloxy)-4-nitrophenyl] methanesulfonamide	0.49 ± 0.14
3c	N-methyl-N-[2-(β-naphthylmethoxy) 4-nitrophenyl] methanesulfonamide	2.68 ± 0.91
4c	N-methyl-N-[2-(2'- phenyl benzyloxy)-4-nitrophenyl] methanesulfonamide	0.33 ± 0.15
5c	N-methyl-N-[2-(4'-methyl benzyloxy)-4-nitrophenyl] methanesulfonamide	2.33 ± 0.66
8c	N-methyl-N-[2-(4'- fluoro benzyloxy)-4-nitrophenyl] methanesulfonamide	1.78 ± 0.63

Table 6.1: Suppression of Aromatase Activity in SK-BR-3 Breast Cancer Cells.
[a] IC_{50} values were calculated by a nonlinear regression analysis (GraphPad Prism). Each dose-response curve contained 6 concentrations, each in triplicate.
[b] See reference (9).

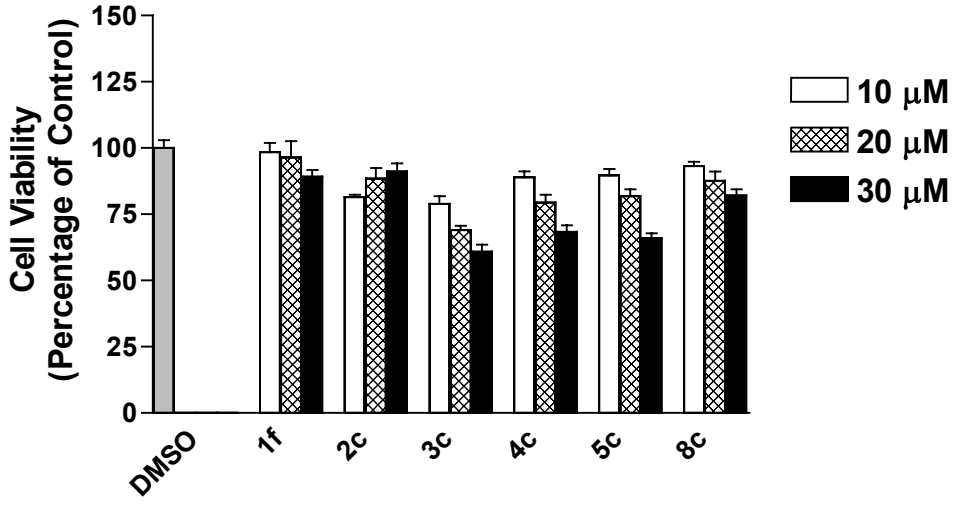

Figure 6.4: Cell cytotoxicity in SK-BR-3 cells treated with novel sulfonanilide. SK-BR-3 cells were treated with indicated compounds at different concentrations and cell viability was measured by MTT assay as described in the experimental section.

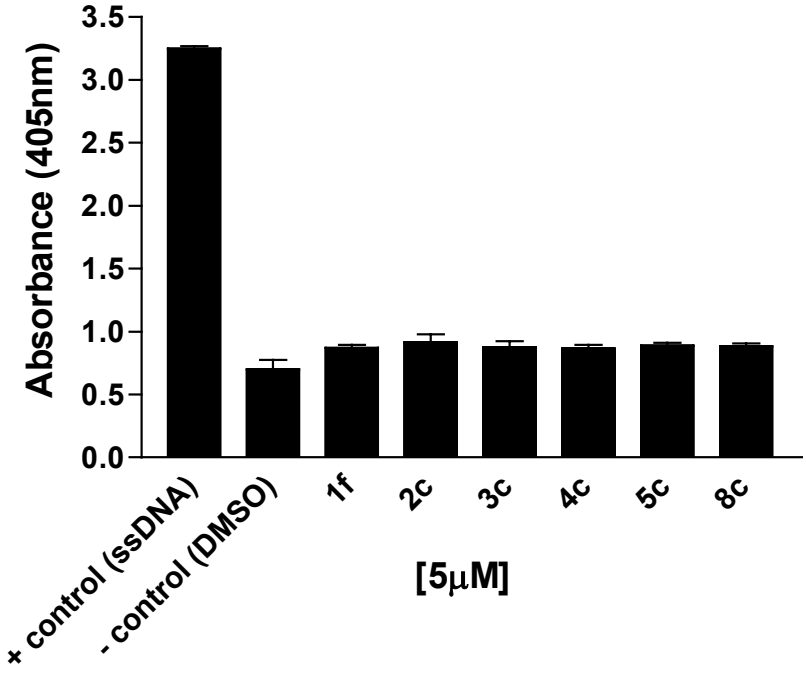

Figure 6.5: Cell apoptosis in SK-BR-3 cells treated with novel sulfonanilide. SK-BR-3 cells were treated with indicated compounds at different concentrations and apoptotic cells were measured as described in the experimental section.

6.3.4 Analysis of apoptosis in SK-BR-3 cells

The six compounds selected did not result in significant cell cytotoxicity when screened in SK-BR-3 cells. To address the question of whether programmed cell-death is involved in their mechanism of aromatase suppression we turned our attention to screening the compounds for apoptosis. Each of the six compounds were screened at 5 μM in the SK-BR-3 cell line, which is an effective concentration for suppressing aromatase, and did not induce apoptosis (Fig 6.5). Therefore, the cytotoxicity and apoptosis results indicate that these compounds, at low micromolar concentrations, are ideal candidates for further investigations as selective aromatase suppression regulators in breast cancer cells.

6.4. Discussion

In closing, our results show that from the library of nimesulide analogs synthesized, several compounds within the series show selectivity for decreasing aromatase activity in SK-BR-3 cells. This modulation in aromatase activity was also achieved at low micromolar concentrations at the enzyme gene expression level, which means this suppression occurs at the transcriptional level. Furthermore, compared to nimesulide, these compounds were 10- to 80- fold more active and had no affect on choriocarcinoma placental aromatase activity in JAR cells.

Although there were a limited number of effective compounds that showed selectivity for aromatase suppression, several potential lead compounds were developed and showed selectivity as aromatase expression regulators. Furthermore, our initial findings provide evidence for specific molecular targets that these compounds are acting

upon. This study has provided a more conclusive understanding for lead optimization that will influence our approach to developing novel selective aromatase suppression regulators for treatment of hormone dependent breast cancer.

6.5. Acknowledgements

This study was conducted in collaboration with Bin Su, who is responsible for the synthesis of the nimesulide analog library. Serena Landini and Bin Su are both responsible for contributing to part of the biological data.

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CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

A class of synthetic isoflavonoid analogs was examined as effective antiproliferative, cytotoxic, and apoptosis agents in both hormone-dependent and hormone-independent breast cancer cells. Inhibition of cell cycle progression at the G_1 to S transition and accumulation of the endogenous cdk inhibitors (p21 and p27) are mechanisms responsible for the antiproliferative activities of these compounds in breast cancer cells.

The synthetic isoflavonoid 7-hydroxy-3-(4-methoxyphenyl)-2-[4-[2-(piperidin-1-yl)ethoxy]phenylthio]-4*H*-1-benzopyran-4-one (compound **5**) showed selectivity in a dose-dependent manner for cytotoxicity as well as apoptosis in the MDA-MB-231 breast cancer cell line. The mechanistic profile of this synthetic isoflavonoid (compound **5**) suggests that estrogen-independent mechanisms may be another factor involved in its mechanism of action. Induction of apoptosis involves multiple pathways and depends on varied stimuli, so potential molecular targets of compound **5** in breast cancer cells were examined .

Apoptosis is a tightly regulated process involving antiapoptotic and apopototic effector molecules, including members within the Bcl-2 family. Treatment with compound **5** in both MCF-7 and MDA-MB-231 breast cancer cells resulted in a

significant increase in expression of the Bax protein. Additionally, the Bax/Bcl-2 ratio, which is a critical determinant of apoptosis, was significantly increased following treatment with compound **5** in MDA-MB-231 breast cancer cells. The intricate balance between cell survival and apoptosis was furthered disrupted by showing that the synthetic isoflavonoid (compound **5**) inhibited the Akt signaling pathway, by decreasing expression of activated Akt at Serine-473.

To further identify the molecular targets involved the cell-death mediated activity of compound **5** in the MDA-MB-231 breast cancer cell line, high throughput microarray technology was utilized. Gene expression profiles revealed a caspase-dependent cell death, involving the Fas signaling pathway through recruitment and interaction of death domains within the MDA-MB-231 breast cancer cell line. Using RT-PCR and western blotting as confirmation techniques, the involvement of relevant caspases and members of the TNF superfamily were confirmed.

The results suggest the following proposed model (Figure 7.1) to explain the mechanisms of action of our synthetic isoflavonoid analog (compound **5**). Treatment with compound **5** in MCF-7 and MDA-MB-231 breast cancer cells results in dose dependent antiproliferative and cytoxic activities. The increase in Bax/Bcl-2 ratio mediates and induces the apoptotic mechanisms within the cell. This may influence the concomitant expression of the caspase-dependent and Fas mediated pathways resulted from gene expression analysis. The reduction in compound **5**-induced apoptosis in MDA-MB-231 cells by the pan caspase inhibitor z-VAD-fmk and subsequent western blot analysis of relevant caspase proteins further confirmed our proposed mechanistic model.

Future studies should focus on determining the exact role of the adaptor protein, FADD, that connects Fas receptor to the cascade. By determining whether overexpression of FADD by stable transformation sensitizes breast cancer cells to cell death and cytotoxicity following treatment with isoflavonoid analogs, the extent of involvement of the Fas/FADD pathway can be determined. Furthermore, the combination of synthetic isoflavonoids and chemotherapeutic and other anti-cancer agents used in breast cancer treatment options should be explored. Finally, this study has explored the molecular targets involved in cell-death mediated pathways of the synthetic isflavonoids; however, other cell signaling transduction pathways need to be further evaluated.

In conclusion, evidence has been provided for structurally-modified derivatives of genistein (2, 4', 7-trisubstituted isoflavones) as promising agents for targeting cell-death and cell-maintainence mediated pathways in breast cancer cells. The results of this study will further aid in providing a molecular template for representative molecular targets of synthetic isoflavonoid derivatives.

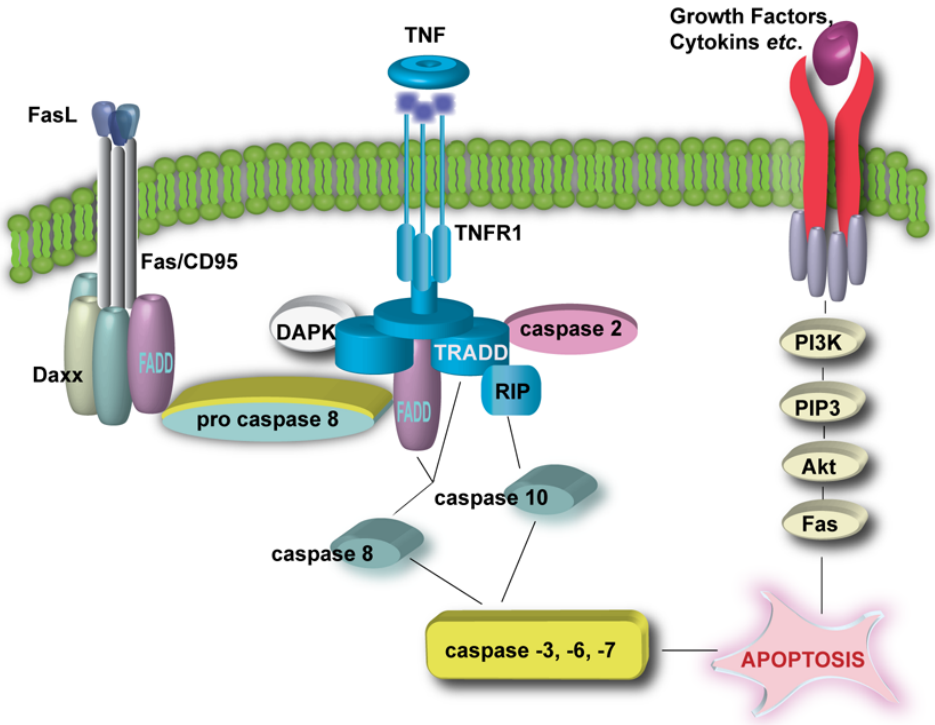

Figure 7.1: Mechanistic targets identified through action of the synthetic isoflavonid derivative in human breast cancer cells.

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