Actions of Plant Estrogens on Breast Cancer Cells as Demonstrated by Flow Cytometry

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Abstract

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The activity of plant estrogens, genistein (GEN) and enterolactone (ENL), on mammalian cells is not well understood. Previously, they have been shown to exhibit a biphasic effect, being stimulatory at low doses and inhibitory at high doses. Therefore, consumption of plant estrogens by women with breast cancer has become controversial. Simplified flow cytometry applications to quantify the degree of proliferation and apoptosis were used to measure differentiation and growth inhibitory properties of GEN and/or ENL on two estrogen receptor-positive, MCF-7 and T47D, and two estrogen receptor-negative, MDA-MB-231 and MDA-MB-468, breast cancer cell lines in the presence and absence of 17β-estradiol (E_2). Activity in the presence of 4-hydroxytamoxifen (OHT) was also evaluated. A live cell assay using propidium iodide (PI) and fluorescein diacetate was used for evaluation of live, apoptotic and necrotic populations. The biological activity of ENL and GEN in tissue culture was confirmed by MTT assay. An Annexin V/PI live cell assay confirmed the presence and degree of apoptosis. No effect was seen at lower doses, ie. less than 10 μM and 50 μM in estrogen receptor-positive, ER (+), cells and decreased viability was seen at ENL 10 μM in one estrogen receptor-negative, ER (-), cell line. A significant reduction in viability was seen with treatments of 100 μM of ENL and GEN in both ER (+) cell lines. The effect of

combining E_2 with treatments of GEN 50 and 100 μM was a reduction in viability in one ER (+) cell line. The effect of combining treatments of low doses of ENL and GEN (10 μM) resulted in a reduction in viability in one ER (+) and one ER (-) cell line. Treatment of ER (+) breast cancer cell lines with low dose GEN (10 μM) and high dose ENL and GEN (50 μM) combined with low dose OHT (10 μM) reduced viability in excess of OHT 10 μM alone. High dose OHT (50 μM) significantly reduced viability in all cell lines. However, when high dose OHT was combined with low dose ENL and GEN, there was an effect of rescuing ER (-) cells from OHT-induced apoptosis and necrosis. Although the use of high dose OHT is not typically used to treat ER (-) breast cancer, these results suggest the action of plant estrogens is more varied than previously thought. This work supports the notion that there may be dissimilarity in the actions between ENL and GEN which may warrant separate recommendations regarding dietary consumption, especially in women with breast cancer.

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List of Abbreviations

AF-1: Activation function 1
AF-2: Activation function 2
cAMP: cyclic adenosine monophosphate
ANOVA: Analysis of Variance
Apaf-1: apoptotic-protease activating factor

CAD: caspase-activating DNase
Ca^{++}: calcium

DAID: daidzein
Da: Dalton
DCC: dextran charcoal coated
DCC-FBS: dextran charcoal coated fetal bovine serum
DMEM: Delbecco's modified eagle medium
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
ENL: enterolactone
END: enterodiol
E_2: 17-β-estradiol
ER: estrogen receptor
ERα: estrogen receptor alpha
ERβ: estrogen receptor beta
ER(+): estrogen receptor positive
ER(-): estrogen receptor negative

FACS: Fluorescence Activated Cell Sorter
FADD: Fas-associating protein with death domain
FBS: fetal bovine serum
FDA: fluorescein diacetate
FITC: fluorescein isothiocyanate
FSC: forward side scatter channel

GEN: genistein
GLY: glycitein
g: gram

HPLC: High Performance Liquid Chromatography

ICAD: Inactive caspase-activated DNase
IGF-I: insulin-like growth factor-I
IGF-IR: insulin-like growth factor-I receptor

JAK: Janus kinase
JC-1: 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide

LBD: ligand-binding domain
LL: lower left
LR: lower right

MAPK: mitogen-activated protein kinase
MAT: matairesinol
mins: minutes
ml: milliliter
MTT: 3-[4,5-dimethylthiazol-2-y]-2, 5-diphenyltetrazolium bromide

OHT: 4-hydroxytamoxifen

PBS: phosphate buffered solution
PI: propidium iodide
PI3K: phosphatidylinositol-3′-kinase
PLC: phospholipase
PS: phosphotidylserine

rpm: revolutions per minute
rt: room temperature

SAPK: stress-activated kinase pathway
SECO: secoisolariciresinol
SERMS: selective estrogen receptor modulators
SDG: 4-O-β-D-glucopyranoside
SRC: steroid receptor co-activator
SSC: side scatter channel
STAT: signal transducers and activators of transcription

TAM: tamoxifen
TGF-β: tumour growth factor beta
TNFα/TNFβ: tumour necrosis factor alpha and tumour necrosis factor beta

µg: microgram
µl: microlitre
UL: upper left
UR: upper right

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Thank you to Dr. Nancy Sherwood and the BC Cancer Agency for the tremendous support during the completion of my Master of Science in Biology. The financial support of the CIHR, Health Services Association, and the BC Cancer Agency VIRAD was much appreciated.

Thank you to my committee members. Dr. Perry Howard was always available to answer my questions and to take time to assist me along my journey. I valued the use of the tissue culture area in your lab and made some wonderful friends surrounding the hood during the two years. Thanks also to Dr. Terry Pearson, who allowed me to access the flow cytometer, which provided me with fascinating experimental results. I treasured having the opportunity to work with Nola Erhardt, Lee Haines, Jessa Spencer, Carol Warby, and all the Sherwood lab mates who saved the day on many occasions.

Thank you to Dr. John Wright who always believed in me and showed me so many helpful techniques with my computer that made life so much easier. Your support and friendship have meant the world to me. I will never forget those last crunch moments to try to make the deadlines. These will be memories I will cherish forever.

I would like to apologize to my son, Sean Pendray. The endless hours of work in completing this work were sacrificed from the time he would have preferred to be at the beach or having a vacation. I realize this time is gone now, but I will do what I can to make this up to you. You are still the most important thing in my life.

A special thanks to Mike, my friends and my family in Nova Scotia who gave me support and kind words of encouragement. Your love and support on those 'rainy' days will not be forgotten.

Dedication

To my Nana Shaw for inspiring me to believe I can achieve my dreams, even those that are above and beyond my wildest imagining.

CHAPTER 1

General Introduction

Introduction

Breast cancer is the second most common cancer in the world, and the most common cancer among women (Key *et al.*, 2003). In the UK, breast cancer accounts for 28% of all cancers diagnosed and 17% of all cancer deaths each year in women (Key *et al.*, 2003). Breast cancer is the leading cause of death in women in Canada between the ages of 35 and 44 (Reid, 2005). Age-adjusted breast cancer incidence rates in western countries are about five times higher than rates in less developed countries and in Japan (Key *et al.*, 2003). Plant estrogens, which occur particularly in soybean products, are regularly consumed in Asian countries like Japan, China, Korea and Indonesia (Table 1.1) (Adlercreutz *et al.*, 2004). In a study of Japanese immigrants to Los Angeles County, it was found that when immigration occurred later in life, rates for breast cancer were substantially lower than when migration occurred earlier (Ziegler *et al.*, 1993). Adlercreutz *et al.* (2004) observed that the urinary excretion of plant estrogens of recent Asian immigrants to Hawaii was very low within six months after immigration. In several epidemiological studies, the lowest risk of breast cancer was in subjects who had consumed soy during adolescence and adult life (47% decreased risk) compared with those who were low consumers during both time periods (Adlercreutz *et al.*, 2004).

In the United States, breast cancer rates are higher than in Finland and the excretion of enterolactone (ENL) from consumed plant lignan is considerably lower (Hanf and Gonder, 2005). The difference in plant estrogen excretion is thought to be due to the difference in the diets: Finish women eat much more traditionally prepared wholegrain rye bread containing large quantities of fiber and lignans (Hanf and Gonder, 2005). Lignans are also considered members of the plant estrogen class of natural plant

substances. A similar phenomenon to the Japanese immigrants occurred when Finnish women immigrated to the United States supporting the view that these plant estrogens also may be involved in breast cancer prevention (Adlercreutz *et al.*, 2000).

Isoflavones and lignans have been shown to have estrogenic, anti-estrogenic, anticarcinogenic, antimutagenic, antioxidant, and antiproliferative effects (Hirano *et al.*, 1990). In an animal model, it was found that pure ENL significantly reduced dimethylbenzanthracene-induced mammary cancer in rats (Adlercreutz *et al.*, 2000). The anticancer activities of plant estrogens are not well understood but it is known that they bind to estrogen receptors. They may have an estrogenic effect at low doses but an antiestrogenic effect at high doses (Jones *et al.*, 2002). Therefore, consumption of plant estrogens by women with breast cancer has become controversial. It is important to investigate their role in human health.

Table1.1 Concentrations of plant estrogens in plasma (nM/L) for normal women and men (Morton *et al.* 2003).

	DIAD (nM/L)		**GEN (nM/L)**		**ENL (nM/L)**		**Equol (nM/L)**	
	Japan	**UK**	**Japan**	**UK**	**Japan**	**UK**	**Japan**	**UK**
Women[a]	**246.8**	**12.5**	**501.9**	**27.7**	**22.7**	**18.7**	**57.6**	**2.2**
Men[b]	**282.5**	**17.9**	**492.7**	**33.2**	**32.6**	**24.4**	**99.1**	**0.57**

[a] n = 125
[b] n = 102

Structures of Phytoestrogens

Plant estrogens are a diverse group of compounds that have a phenolic group similar to estrogenic steroids. This phenolic group may play an important role in determining the estrogenic agonist/antagonist properties of these compounds. Plant estrogens have been categorized according to their chemical structures as isoflavonoids, lignans, flavonoids, and stilbenes (Cos *et al.*, 2003).

Isoflavones

The major forms of isoflavonoids are isoflavones and coumestans (Cos *et al.*, 2003). A large number of isoflavones have been identified from plants, with genistein (GEN), daidzein (DAID) and glycitein (GLY) as the principal isoflavones (Figure 1.1.). These molecules are derived from precursors, i.e. genistin, diadzin, and glycetin. GEN is the most prevalent isoflavone and it is derived from the precursor, β-glycoside genistin. GEN is a solid substance that is insoluble in water. Its molecular formula is $C_{15}H_{10}O_5$, and its molecular mass is 270.24 Da (Constantinou and Huberman, 1995).

The pharmacokinetics of GEN in humans is complex and not well understood. Despite the high stability of the β-glycoside genistin during processing, this precursor can be metabolized in the digestive tract by the enzymes of the normal microflora to the corresponding aglycone, GEN (Cos *et al.*, 2003). Some glycoside genistin may be hydrolyzed by hydrochloric acid in the stomach to GEN and some may be hydrolyzed by β-glucosidases in food to GEN. It is absorbed from the small and large intestine and is eventually transported to the liver. Isoflavone glycosides are not absorbed intact across

the enterocyte of healthy adults because hydrolysis of the sugar moiety is required for the absorption of isoflavone glycosides (Cos *et al.*, 2003).

In the liver, GEN undergoes conjugation with glucuronate and sulfate via hepatic phase II enzymes (glucuronosyltransferases and sulfotransferases) (Wei *et al.*, 1995). GEN conjugates may be deconjugated which may be reabsorbed via the enterohepatic circulation. The glucuronate and sulfate conjugates of GEN are excreted in the urine and bile (Wei *et al.*, 1995). It is thought that there is a great degree of individual variation in the absorption and metabolism of ingested GEN. Little is known about its tissue distribution. Setchell *et al.* (2001) demonstrated GEN was efficiently absorbed; it took an average of 5.2 hours after ingestion to reach peak plasma concentrations.

DAID is classified as a phytoestrogen because it is a plant-derived nonsteroidal compound that possesses estrogen-like biological activity. β-glycoside daidzin is the precursor of DAID. DAID is virtually insoluble in water. Its molecular formula is $C_{15}H_{10}O_4$, and its molecular mass is 254.24 Da (Keung *et al.*, 1995).

The pharmacokinetics of DAID in humans is not well understood. In the large intestine, bacterial β-glucosidases hydrolyze β-glycoside daidzin to DAID. Like β-glycoside genistin, β-glycoside daidzin is highly stable during processing. However, the precursor is metabolized in the digestive tract by the enzymes of normal microflora to the corresponding DAID (Cos *et al.*, 2003). Some data are available suggesting DAID may be absorbed from the small intestine and then transported to the systemic circulation by the lymphatics. β-glycoside daidzin is not absorbed intact across the enterocyte of healthy adults because hydrolysis of the sugar moiety is required for absorption (Cos *et al.*,

2003). DAID undergoes conjugation in the liver with glucouronate and sulfate via hepatic phase II enzymes (glucouronosyltransferases and sulfotransferases) (Keung *et al.*, 1996). Setchell *et al.* (2001) demonstrated that DAID took an average of 6.6 hours to reach peak plasma concentrations. The glucuronate and sulfate conjugates of DAID are excreted in the urine and the bile (Keung *et al.*, 1996). DAID is either absorbed and metabolized as above or it can be further metabolized to equol (Figure 1.2.) (Kelly *et al.*, 1993) but this biotransformation has a high individual variability (Cos *et al.*, 2003). Equol is known as an isoflavan rather than an isoflavone as it lacks the carbonyl group. GEN and DAID have been found to have both weak estrogenic and weak anti-estrogenic effects (Wang, 2002).

GLY is a solid substance that is virtually insoluble in water. GLY accounts for only 5-10% of all the soy isoflavones; therefore, it has been the least studied. The precursor of GLY is glycosylated glycitin. The structure of GLY bears a methoxy group which is more difficult for colonic bacteria to remove than a methyl group. The chemical structure of GLY is similar to that of GEN and DAID, and it would be expected to have similar activities. However, except for its probable antioxidant activity, it is difficult to predict activity as small differences in chemical structure can produce great differences in biological activities. What is known is that GLY has estrogenic activity as measured by *in vivo* and *in vitro* assays. *In vivo*, its estrogenic activity may be the highest of the soy isoflavones: three times greater than that of GEN and twelve times greater than that of DAID (Song *et al.*, 1999).

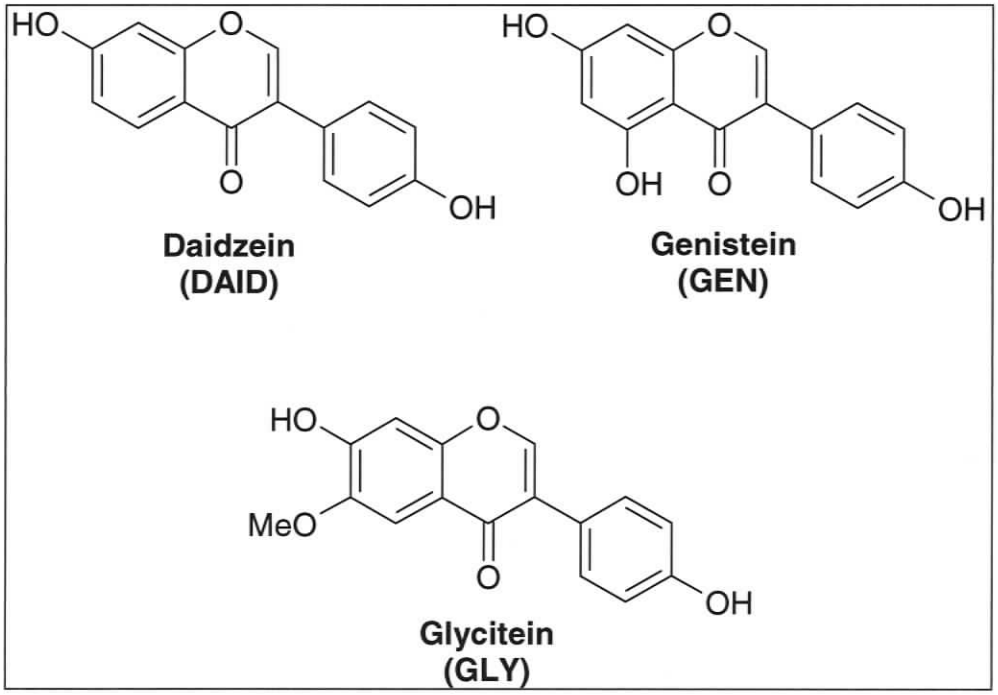

Figure 1.1. Structures of daidzein (DAID), genistein (GEN), and glycitein (GLY). Adapted from (Wang, 2002).

Lignans

Lignan-type plant estrogens are formed from precursors, glycosides of matairesinol (MAT) and secoisolariciresinol (SECO) (Figure 1.3.) (Wang, 2002). SECO and MAT are substrates of the cytochrome P450 system in rat and human livers, although MAT appears to be poorly oxidized by human liver (Niemeyer *et al.*, 2003). In rats both plant lignans are efficiently converted to their corresponding mammalian lignans, enterodiol (END) and ENL, leaving little SECO and MAT for direct absorption (Niemeyer *et al.*, 2003). Because these two compounds are produced in animals as opposed to plants they are termed mammalian lignans to distinguish them from plant lignans. The mammalian lignans differ from plant lignans in the *meta*-position of the aromatic rings.

The precursors are converted to active plant estrogens or mammalian lignan by action of intestinal bacteria although there is extensive variability between individuals and under certain medical conditions, e.g., after application of antibiotic drugs (Wang, 2002). There is little known about the strains of bacteria capable of carrying out the conversion of SECO and MAT to END and ENL (Niemeyer *et al.*, 2003).

Matairesinoside and 4-O-β-D-glucopyranoside (SDG) are also precursors of mammalian lignan, as they are easily hydrolyzed to aglycones by the β-glycosidase, which is widespread in intestinal microorganisms. The main component of flaxseed is 4-O-β-$_D$–glucopyranoside (Wang, 2002). Lariciresinol is also considered a precursor of ENL and END, although the structure of lariciresinol is different from ENL. Pinoresinol found in rye is also a precursor of ENL and END.

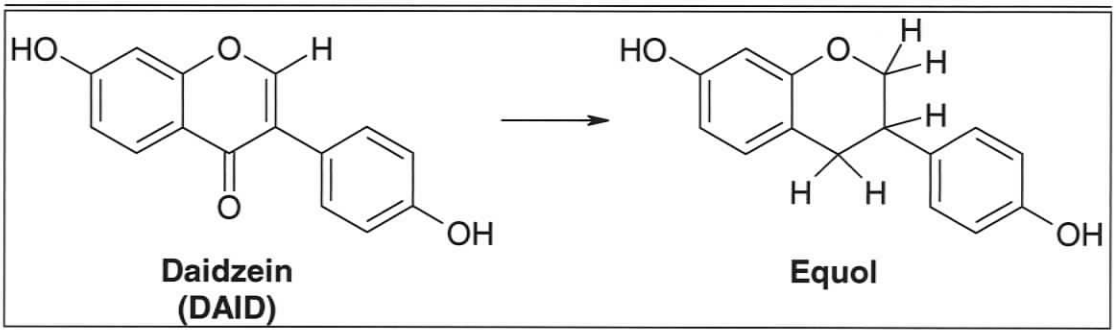

Figure 1.2. Conversion of diadzein to equol. Equol in an isoflavan as it lacks the carbonyl group. Adapted from (Wang, 2002).

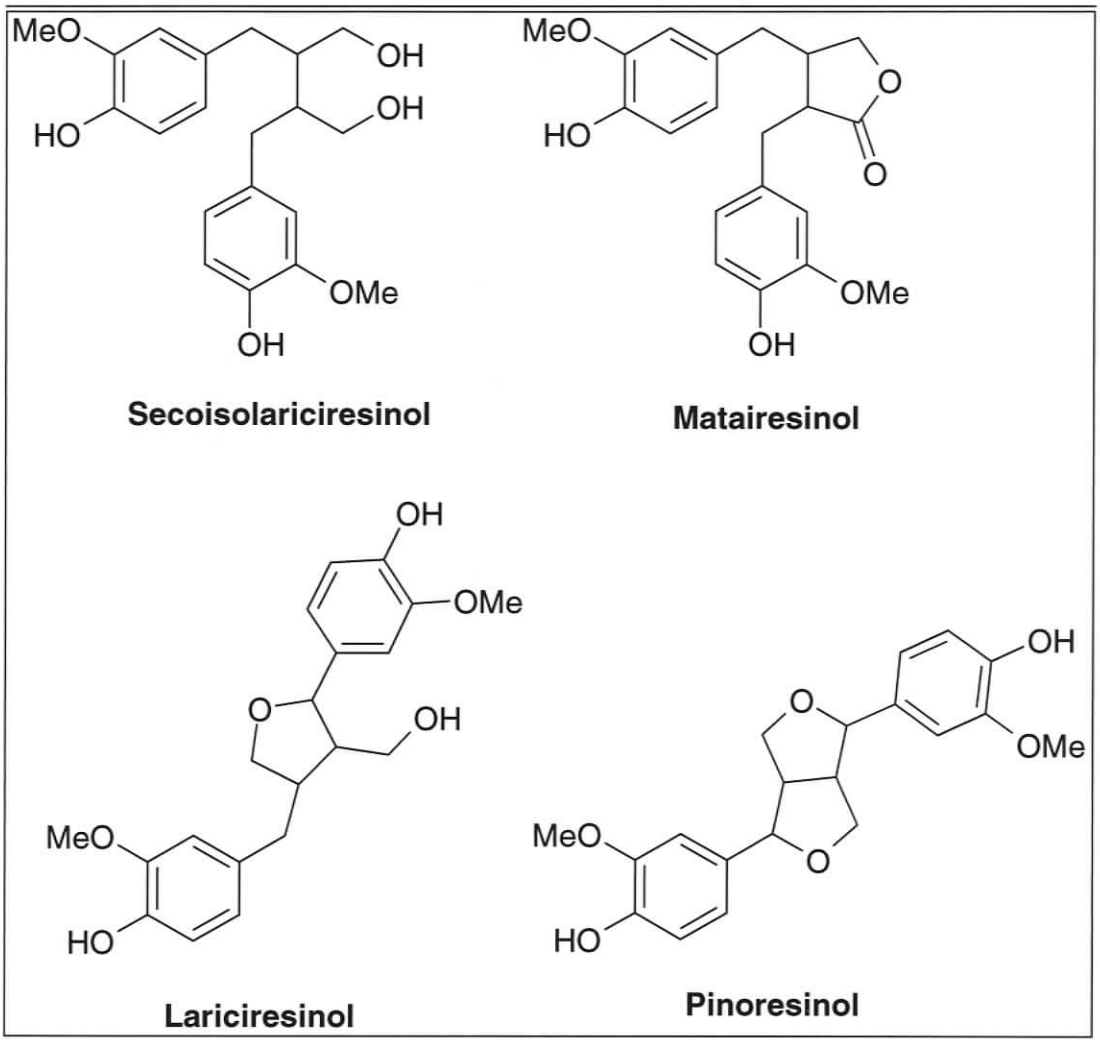

Figure 1.3. Structures of plant lignans. These are the precursors which are converted to mammalian lignans by intestinal bacteria. Adapted from (Wang, 2002).

END and ENL are the two main forms of lignans physiologically active in humans, and hence are also referred to as mammalian lignans (Figure 1.4.) The pharmacokinetics of mammalian lignans, END and ENL, involve a hydrolysis step. In the ileum, the lignans are present as conjugated plant lignans. Most of the plant lignans disappear after formation of a less polar END and ENL, which allows them to be absorbed from the gastrointestinal tract. Then they can enter the enterohepatic circulation where they may be metabolized by enzymes from the liver (Wang, 2002).

Studies with flaxseed have shown that the plasma and urinary levels of ENL and END increase in a dose-dependent manner in humans. After 8 days of supplementation with 25 g raw flaxseed/day, the average plasma concentration of END was 51.75 ± 7.49 nM/L and ENL was 29.35 ± 3.69 nM/L in premenopausal women. Interestingly, no plateau was observed with intake up to 25 mg suggesting increased intake may lead to higher levels of END and ENL in the body. There was no difference in plasma levels of END and ENL with raw versus cooked flaxseed. In this study it was found that total plasma END and ENL levels significantly increased by nine hours after the initial dose and remained high for at least 24 hours. This is in contrast to levels of GEN, which revert to baseline sooner. After several days of dosing, plasma concentrations of lignans were maintained at an elevated level even though the flaxseed was ingested once daily (Nesbitt *et al.*, 1999).

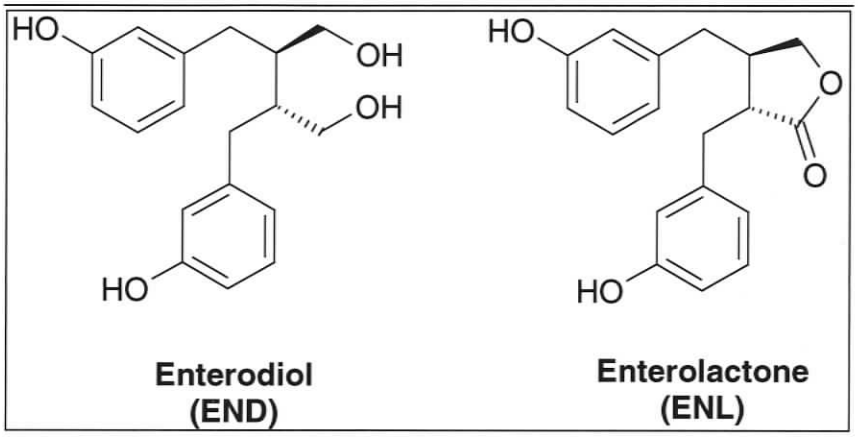

Figure 1.4. Structures of mammalian lignans upon conversion of lignans by intestinal bacteria to enterodiol and enterolactone. Adapted from (Wang, 2002).

Food Sources of Isoflavones

The isoflavone content of soybeans varies considerably depending on over 10,000 varieties of soy beans, the year harvested, geographic location, and processing (Franke *et al.*, 1999). Per serving, soy nuts contain the highest levels of isoflavones of all soy products, ranking higher than tofu, soy milk, and soy concentrate among others (Table 1.2) (Franke *et al.*, 1999). Processed soy products such as soy burgers, soy cheeses and soy yogurts are not as high in isoflavones as they lose a substantial amount of isoflavones due to the processing necessary to produce the soy foods (Franke *et al.*, 1999). Alcohol extraction, a process used in the production of many soy protein concentrates and isolates (used in soy protein powders), results in the removal of up to 90 percent of the isoflavones (Franke *et al.*, 1999). Soy protein concentrates from which meat analogues are prepared have low concentrations of isoflavones if derived by alcohol extraction. Water extraction, another process used in the production of soy protein concentrates, yields only slightly more isoflavone (Cos *et al.*, 2003).

Currently, approximately 15% of all infants in Canada and the United States are fed soy-protein based formulas. The concentration of total isoflavones in soy infant formulas ranges from 32-47 mg/L, compared to ~6 μg/L in human breast milk. Based on typical quantities of formula consumption of 900-1000 ml/d, a 4-month old, soy-formula-fed infant consumes 6-9 mg/kg body wt/day of isoflavones. This dose is 6-11-fold higher than the dose found to have physiological effects in adult humans (Chen and Donovan, 2004). The long-term effects of such concentrated intakes of isoflavones are currently being evaluated.

Table 1.2. *Total genistein (GEN) content of soy foods. Adapted from (Arnot, 1999)*

Foods 100 g serving	GEN mg/100g	Foods 100 g serving	GEN mg/100g
Soybean meal, whole	100	Soy milk	10
Soy flour	94	Tofu yogurt	9
Soy nuts	94	Soy hot dog	8
Soy protein	71	Soy cheddar	4
Miso	52	Soy milk formula	2
Miso paste	38	Soy cheese slices	2
Tofu, firm	31	Tofutti	2
Tofu, soft	22	Soy sauce	1
Soybeans, dry, whole	20		

Enterolactone/enterodiol food sources

The precursors of END and ENL exist in various foodstuffs. Thompson *et al.* (1991) developed an in vitro fermentation method using human fecal microbiota to simulate fermentation in the colon. The END and ENL content of 68 foods were tested using Thompson's method (Table 1.3). Oil seeds produced the highest amounts of END and ENL, with flaxseed meal and flour being the most concentrated sources. Other concentrated sources include dried seaweeds, whole legumes, cereal brans, whole grain cereals, vegetables and fruits (Thompson *et al.*, 1991).

Although phytoestrogens are a diverse group of polyphenolic non-steroidal plant compounds, they may exert mild agonistic and antagonistic effects through the estrogen receptor, depending on the level of endogenous estrogen present and on the tissue being tested. There is a substantial amount of evidence showing that phytoestrogens can inhibit the process of hormone-related cancer development, such as breast cancer. Therefore it is important to examine their estrogenic properties.

Estrogen Receptor

The estrogen receptor (ER) is a member of a superfamily of nuclear receptors that function as ligand-activated transcription factors. The ER mediates the physiological effects of its ligand, the steroid hormone 17-β-estradiol (E_2) (Kong *et al.*, 2003). E_2 diffuses through the plasma and nuclear membrane of cells where it binds to the ER (MacGregor and Jordan, 1998). The hormone activates the receptor protein so that it

Table 1.3. *Enterolactone (ENL) and enterodiol (END) content of foods (Wang, 2002).*

Foods 100 g serving	ENL µg	END µg
Oilseeds		
Flaxseed meal	8517	59024
Flaxseed flour	11818	40861
Rapeseed	975	155
Soybean	693	170
Sunflower	201	195
Peanuts	105	56
Cereal Brans		
Oat bran	265	386
Corn bran	168	480
Wheat bran	269	298
Barley bran	243	140
Rice bran	134	47
Whole cereals		
Triticale	519	405
Wheat	411	79
Purple rice	340	80
Oats	251	89
Brown rice	169	128
Sorghum	199	56
Corn	199	31
Rye	69	91
Barley	41	74
Dried Whole Legumes		
Lentil	789	998
Kidney bean	329	232
Navy bean	353	108
Yellow pea	169	44
Pinto bean	154	47
Fruits		
Pear	112	69
Plum	47	98
Strawberry	41	38
Banana	55	14
Orange	27	12
Cantaloupe	21	16
Apple	34	1

Foods 100g serving	ENL µg	END µg
Vegetables		
Garlic	81	326
Squash	271	110
Asparagus	136	238
Carrot	284	62
Sweet potato	240	55
Broccoli	161	65
Leek	24	174
Green pepper	162	33
Turnip	78	78
Cauliflower	68	77
Beet	109	26
Snow pea	60	62
Iceberg lettuce	58	63
Onion	11	101
String bean	40	56
Potato	33	50
Brussel sprouts	57	18
Boston lettuce	27	47
Cabbage	30	34
Bok Choy	44	14
Mushroom	43	13
Watercress	20	28
Radish	25	10
Celery	17	14
Cucumber	18	11
Fiddle head	14	7
Dried Seaweeds		
Mekuba	167	980
Hijiki	266	387

is able to bind to estrogen response element sequences in the genomic DNA and stimulate transcription of specific genes (O'Malley, 2005).

In the absence of ligand, ER is sequestered in the target cell nuclei within a large inhibitory heat shock protein complex. Upon ligand binding, the receptor undergoes a conformational change that causes the displacement of heat shock proteins (McDonnell and Norris, 2002). The ER-ligand complex forms homo- or heterodimers that bind to a specific motif, the estrogen response elements, on the 5′-regulatory region of target genes (Leung *et al.*, 2004).

ERs possess two separate regions that are required for optimal transcriptional activation (Figure 1.5). An amino-terminal activation function (AF-1) operates independent of ligand binding. A second activation function (AF-2), is located in the ligand-binding domain (LBD) near the carboxy terminus of the molecule. AF-2 activity is dependent upon the binding of an agonistic ligand. In some cells both AFs are required for maximal transcriptional activity, whereas in others only one is required (McDonnell and Norris, 2002)

ERs do not act alone to regulate genes. Regulation of gene expression occurs via a ligand-induced coactivator exchange with corepressors at the C-terminus of the receptor through their ability to associate with AF-1 and AF-2 sites of the receptor (Iwase, 2003). The SRC family of coactivators, including SRC-1, SRC-2 (TIF-2) and SRC-3 (AIB1) boosts the power of transcription in the presence of the ligand-bound receptor, but also directs histone acetylase activity to the gene (O'Malley 2005). Coactivators act as homeostats to sense the environmental signals and coordinate the signals coming from

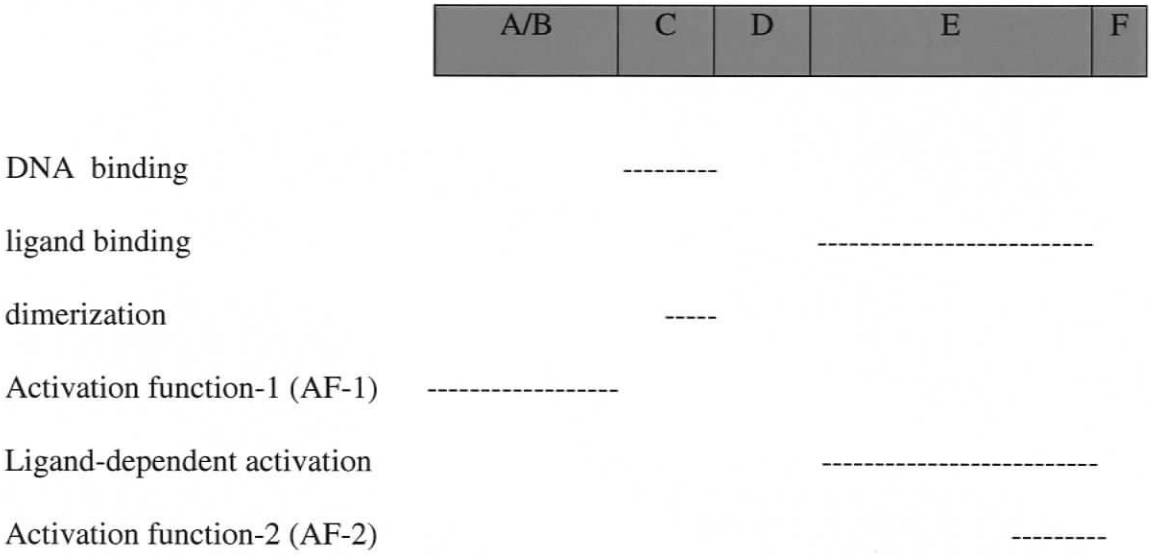

Figure 1.5. Functional domains of the estrogen receptor. A/B, N-terminus; C, DNA binding; D, hinge domain; E, ligand binding domain; F, C-terminus. Adapted from (MacGregor and Jordan, 1998).

the membrane receptors and gene transcription (O'Malley 2005). The coactivators perform this function by receiving phosphorylation signals from the environment via membrane receptors that activate downstream kinases. Coactivator molecules are selectively phosphorylated on their serine/threonine residues, which is controlled by signals from kinase pathways. Depending upon the combination of sites phosphorylated, the coactivator is preferentially directed to bind and activate distinct sets of downstream transcription factors (O'Malley, 2005). This phosphorylation code represents a mechanism by which membrane-initiated signaling pathways can direct limiting quantities of a coactivator toward their own relevant downstream transcriptional activators bound to the target promoter DNA (O'Malley, 2005).

Similar to coactivators, corepressors are components of multi-protein complexes. These complexes appear to repress transcription through histone deacetylation (Iwase, 2003). The ligand may result in the dissociation of the corepressor complex, which then is replaced by a coactivator complex.

In addition to a nuclear site of action, estrogen is proposed to have effects on the membrane receptors that involve cross-talk with other signaling pathways, such as mitogen-activated protein kinase (MAPK), phosphatidylinositol-3′-kinase (PI3K), phospholipase (PLC), cyclic adenosine monophosphate (cAMP), and intracellular Ca^{2+} (Leung *et al.*, 2004). The non-genomic effects occur minutes after treatment. In contrast, several hours are required for the genomic effects, and involve the ER and other estrogen binding factors in the plasma membrane and cytosol (Leung *et al.*, 2004). The neurotransmitter dopamine, cyclin-dependent kinase-7 and epidermal growth factor (EGF) are able to activate the ER independent of the presence of E_2 (Parker, 1998). AF-1

is the target for EGF to stimulate the phosphorylation of serine 118 in the human receptor, which then activates the MAPK pathway. Estrogen is able to activate STAT signaling in a Janus kinase (JAK)-independent manner. Induction of STAT phosphorylation is a nongenomic action of ER involving the LBD, but not the DNA-binding domain or coactivator binding regions. This action is mediated by c-Src, MAPK, and PI3K, possibly via a direct interaction of ER with these proteins (Leung *et al.*, 2004).

An additional form of the ER, called ERβ, is similar in sequence to the previously known isoform, now to be called ERα (Figure 1.6). There are differential expression patterns in different tissue types, which suggest that tissues could be differentially activated depending on the receptor. Although the evolutionarily conserved regions between ERα and β are homologous, various nonconserved regions exist. The ligand-binding domains have a high sequence identity (47%) even though the AF-1 region is lacking in the β isoform (Kong *et al.*, 2003). Although the binding of compounds such as E_2 appears nonselective, the action of estrogen is likely to be influenced by ERβ (Manas *et al.*, 2004). In studies of ERα knock-out mice, the females were infertile and did not develop normal uteri and ovaries (MacGregor and Jordan, 1998). Thus, if ERβ were expressed in the ovaries of these knock-out mice, they were not functioning to compensate for the loss of ERα. It was interesting to note that there were very high circulation levels of E_2 in the ERα knockout mice (MacGregor and Jordan, 1998).

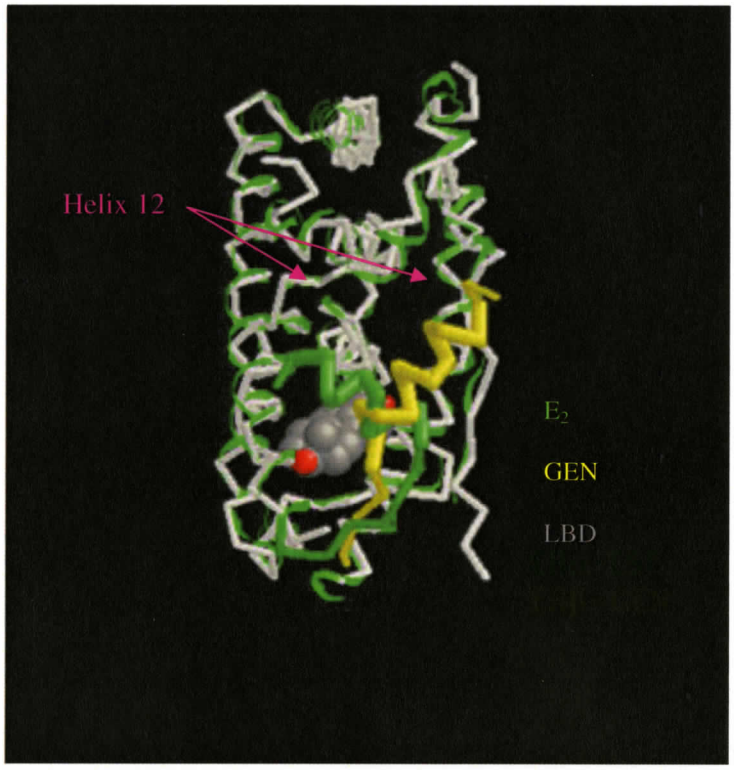

Figure 1.6. Binding of 17β estradiol (green) and GEN (yellow) to ERα and ERβ. The ER LBD (gray) monomer is a three-layered α helical sandwich and the ligand is buried within a hydrophobic cavity. This structure is a representative monomer depicting differences when in complex with 17-β-estadiol and GEN. In the 17β estradiol structure, helix 12 lies across the LBD, but in the GEN structure, helix 12 adopts a quasi-antagonist position, inhibiting the AF-2. Adapted from (Shiau *et al.* 1998)

The function of ERβ in breast cancer is not well understood at present. There appears to be no consistent distribution of ERα and ERβ expression in low versus high grade tumours. Some studies have shown that tumours which co-express ERα and ERβ were node-positive and tended to be of higher grade, whereas others have found that expression of ERβ in more than 10% of cancer cells was associated with better survival (Iwase, 2003). Some differences have been found between expressions of the wild-type ERβ and the variant, ERβ cx, β5 in breast cancer tissue, with the wild type seen in patients with a good prognosis and the variant form seen in patients with a poor prognosis (Iwase, 2003). Regarding the use of ERβ as a potential novel target in the treatment of breast cancer, it is currently unclear whether agonists or antagonists would be useful.

Estrogen and breast cancer

E_2 plays an important role in reproductive physiology and in numerous human disease states, including breast and endometrial cancers, cardiovascular disease, osteoporosis, and Alzheimer's disease (Shang *et al.*, 2000). E_2 is an important hormone involved in regulating the differentiation and proliferation of normal epithelial cells. E_2 is produced mainly by the ovaries and originates from cholesterol. In females, androgens, particularly testosterone, is converted to E_2 in the tissue or is secreted into the bloodstream and a significant amount is converted to estrogen through the action of aromatase enzyme complex. Most circulating E_2 is bound to albumin or to a steroid-binding protein known as sex hormone-binding globulin, a glycoprotein. Only a small

amount is unbound and thus biologically active. Metabolism of E_2 through oxidation or conversion to glucuronide and sulfate conjugates occurs in the liver. The metabolites are excreted in the bile and then reabsorbed into the bloodstream through the enterohepatic circulation. They are finally excreted via the urine (Griffin 2002).

Prolonged exposure to E_2 has been demonstrated to be the most significant risk factor for developing breast cancer (Berardo *et al.*, 1998). This has been confirmed by a number of epidemiological observations. Exogenous administration of E_2 has recently been implicated in increased overall risk of breast cancer. Clinical approaches including oophorectomies, adrenalectomies, and hypophysectomies have historically demonstrated significant protective effect against the development of breast cancer (Song and Santen, 2003)

A number of treatments that involve E_2 or similar molecules have been tried as breast cancer therapy. Treatment philosophies have resulted in a broad range of approaches over the past several decades and include removal of the organs synthesizing E_2, anti-estrogen therapy, and high dose E_2 therapy. The increased risk of breast cancer through extended exposure to estrogen may be partially caused by the mitogenic effect of estrogen on the epithelium, which may result in the accumulation of genetic damage in frequently replicating cells. ER expression appears to be low in normal breast epithelium, with the exception of a short peak during the first week of the menstrual cycle (Nelson et al., 2005). In 60-70% of breast cancers, the ER is overexpressed. Overexpression of the ER is associated with lower risk of recurrence, extended overall survival and improved response to endocrine therapies relative to ER negative tumours.

ER-positive tumours are generally better differentiated, show lower mitotic activity and are less aneuploid than ER-negative tumours (Clarke et al., 1998).

Most research has focused on the mechanisms of E_2-stimulated breast cancer growth and anti-estrogen-induced cancer cell apoptosis. However, Song and Santen (2003) recently demonstrated that high dose estrogen decreased cell growth by significantly increasing apoptosis in ER-positive cell lines. Prior to the use of antiestrogen therapies for the treatment of breast cancer, E_2 administered in high doses for therapy of advanced breast cancer in postmenopausal women was commonly employed, although the mechanism of action was not understood. Observations with the use of high dose E_2 demonstrated that the duration of the post-menopausal period prior to estrogen treatment is one of the crucial factors affecting success of this therapy. The longer the period of post-menopause prior to estrogen treatment, the more effective the treatment. In a review of the 20-year clinical outcomes, it was noted that the patients who had high dose E_2 therapy survived longer than those receiving tamoxifen, with 35% of the estrogen group and 16% of the tamoxifen group alive at 5 years ($p = .03$). Also, the patients who became tamoxifen resistant experienced disease regression after high-dose E_2 treatment, suggesting the mechanism of action of high-dose E_2 therapy differs from that of tamoxifen (Song and Santen, 2003).

Antiestrogens and breast cancer

ERs can also bind a diverse range of synthetic agonists and antagonists. Antiestrogens have been classified into two major groups: type I that have mixed

estrogenic/antiestrogenic actions and type II that have no E_2-like properties (MacGregor and Jordan, 1998). It has been shown that type I antiestrogens behave as ER agonists in some tissues and ER antagonists in others. These compounds with mixed activity are also called selective E_2 receptor modulators (SERMs). The binding of SERMs to ERα promotes a helix 12 conformation that inhibits binding of coactivator (Shiau *et al.*, 1998). The complex occludes the coasctivator recognition groove by mimicking the interactions of the nuclear receptor box peptide with the LBD (Shiau *et al.*, 1998).

Tamoxifen

TAM and its active metabolite, 4-hydroxytamoxifen (OHT), are nonsteroidal SERMs that compete with E_2 for binding to the ER (Figure1.7). The binding of OHT to the ER induces an ER conformation that does not recruit coactivators to target genes and may recruit corepressors. In addition, TAM and OHT may induce programmed cell death of cancer cells. It is thought that the effects of TAM may be mediated through an ER-independent increase in reactive oxygen species, resulting in caspase activation or through an influx of extracellular calcium. TAM may have an effect on levels of proteins related to cell growth including protein kinase C, transforming growth factor β (TGF-β) and c-Myc (Obrero *et al.*, 2002)

The effects of tamoxifen treatment on the cell cycle kinetics of MCF-7 breast cancer cells have been shown to result in cell cycle arrest in G_1 but also in G_2 phase. Cells treated with steroidal and nonsteroidal antiestrogens had a significant decrease in cyclin D1 mRNA, which suggests that G_1 cyclins may be a target of antiestrogens to block entry into the S-phase (MacGregor and Jordan, 1998).

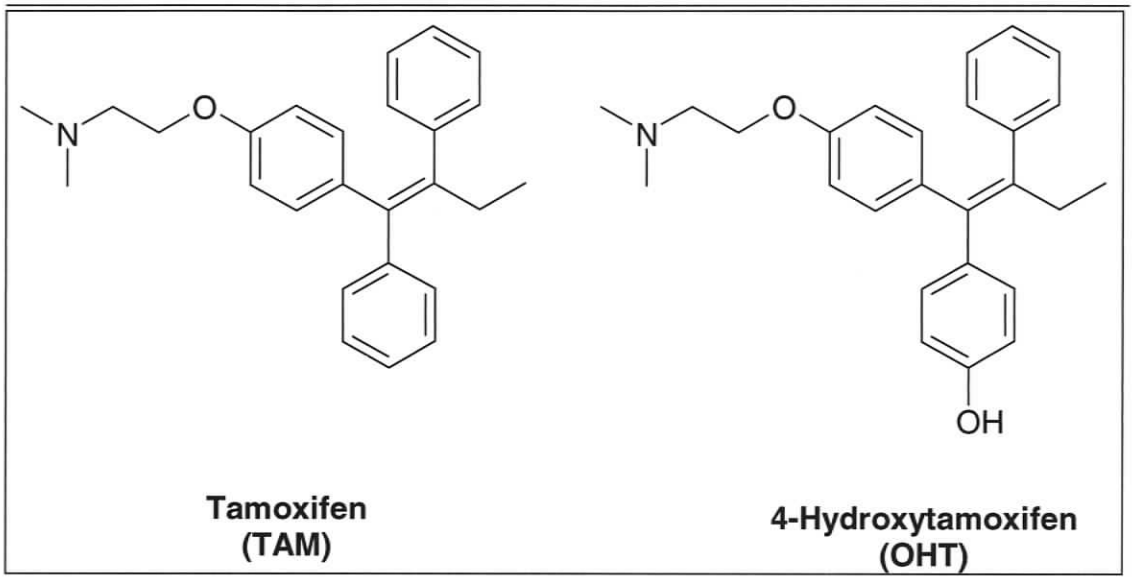

Figure 1.7 Structures of tamoxifen (TAM) and 4-hydroxytamoxifen (OHT). Adapted from (MacGregor and Jordan, 1998).

TAM has partial agonistic properties, and it competes with estrogen for binding to ER's, which in turn bind to estrogen response elements in genes. TAM competitively inhibits estrogen-stimulated growth and but also exhibits partial agonist actions (MacGregor and Jordan, 1998). Therefore, TAM has mixed estrogen/antiestrogen actions (MacGregor and Jordan, 1998). Studies *in vitro* have demonstrated that very low concentrations of TAM can cause mixed estrogen/antiestrogen effects (MacGregor and Jordan, 1998). Low concentrations of TAM can cause a single round of replication in breast cancer cells, but high concentrations of TAM are completely inhibitory (MacGregor and Jordan, 1998). The activity of both AF-1 and Af-2 in the ER have been defined and may affect activity of antiestrogens such as TAM. AF-2 is dependent on the binding of estrogen for full activity and can be blocked by estrogen antagonists. In contrast, AF-1 is unaffected by TAM and seems to be responsible for the agonist activity of this drug (Obero *et al.*, 2002)). The AF-1 domain of ERα has been identified as a target site for the protein kinase signaling cascade, such as the MAPK and PI3K/Akt pathways, acting independently of estrogen activation. Despite an initial benefit in breast cancer patients under hormonal therapy such as TAM, ER-positive tumours often develop a hormone resistant phenotype resulting in more aggressive tumours (St-Laurent *et al.*, 2005).

Plant estrogens may act as E_2 agonists and antagonists. Combining plant estrogens and antiestrogen medication such as TAM may lead to potentiation or inhibition of the antitumourigenic effect. In cell culture studies by Zava *et al.* (Zava and Duwe, 1997), GEN may have partially displaced TAM from E_2 receptors, thus decreasing its inhibitory effect. In contrast, TAM and GEN at very high doses effectively exerted synergistic anti-

proliferative action. The regulation of ER expression in human breast cancer cells is a complex, multifaceted process that varies between different cell types. The understanding of how different E_2 and antiestrogens affect the activity of ER in different cell types may be important in optimizing the development of new antiestrogen therapies that do not promote progression to hormone non-responsive phenotypes.

Effect of plant estrogens on breast cancer

Isoflavones in plants are made via different biosynthetic pathways than those which are used to make E_2 in the human body; the two structures are not closely related chemically. However, both types of molecules have certain structural similarities that enable them to bind with mammalian estrogen receptors (Figure 1.8). Isoflavones are 10^2 to 10^5 times less active than E_2 (Cos *et al.*, 2003). However, they are often present in the human body in higher quantities than endogenously produced E_2 (Cos *et al.*, 2003).

Many factors have been shown to influence the activity of GEN on breast cancer cells *in vitro* and *in vivo*. In the absence of E_2, physiological doses of GEN (<10 μM) have been shown to stimulate growth of ER (+) breast cancer cells but not in ER (-) breast cancer cells. High concentrations of GEN (>10 μM) inhibit the growth and survival of both ER(+) and ER(-) cell lines (Chen and Thompson, 2003). Although there have been several studies on the effects of GEN on breast cancer *in vitro*, few have been conducted on the effect of ENL on breast cancer cell lines. Early studies indicate the stimulatory and inhibitory concentrations of ENL differed according to different methods. Mousavi (1992) showed inhibition of growth in MCF-7 cells with ENL at >10 μM, while Wang and Kurzer (1998) showed inhibition at >50 μM.

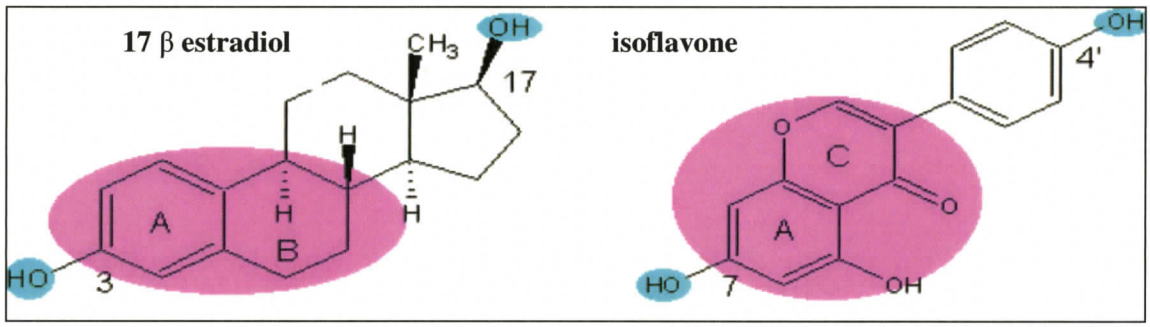

Figure 1.8. Similarities between 17 β estradiol and isoflavones. The A and C rings of the isoflavones are similar to the A and B rings of 17 β estradiol. The actual distance between the two hydroxyl groups on both molecules is nearly identical; these hydroxyl groups are critically located to enable binding to the estrogen receptor. Both molecules have similar polarities and molecular weights. Adapted from Setchell *et al.*, 2001.

In the complex between GEN and the ER ligand binding domain, the AF-2 region is thought to be inhibited (McDonnell and Norris, 2002). It is also thought GEN acts as an agonist through co-activator binding, overcoming the energy barrier (Kong *et al.*, 2001). Whereas E_2 binds to both ERα and ERβ with similar affinity, GEN has selective affinity for the β-subtype, having 10-40 fold greater affinity for ERβ than for ERα (Figure 1.9.) (Harris 2002). Studies of ERβ complexed with GEN have shown that although ERα and ERβ are 58% identical in sequence, there are only two residue substitutions in close proximity to bound agonists: ERα Leu^{384} is replaced by ERβ Met^{336}, and ERα Met^{421} is replaced by ERβ Ile^{373}. So far however, there has not been a suggested mechanism to explain the observed selectivity of GEN to ERβ (Manas *et al.*, 2004).

GEN has also been shown to inhibit several key enzymes in estrogen and androgen biosynthesis pathways, including 5 α-reductase, 17β-hydroxysteroid oxidoreductase, and aromatase (Cos *et al.*, 2003). GEN has exhibited non-hormonal effects *in vitro*, including inhibition of tyrosine kinases, DNA topoisomerases I and II, anti-angiogensis, and antioxidant activity (Cos *et al.*, 2003).

Rationale for present study

Breast cancer is a major health problem in the world. There is a multitude of dietary and natural health products available as supplements for cancer patients. Unfortunately, little is known about their effectiveness, active principles, modes of action, side effects and possible adverse interactions with conventional antitumour drugs.

Many breast cancer patients are consuming soy and flaxseed products in large amounts in hope that their cancer preventative benefits will also arrest their existing breast cancer. Although there have been several studies on the effects of GEN on breast cancer *in vitro*, few have been conducted on the effect of ENL on breast cancer cell lines. Early studies indicate that the stimulatory and inhibitory concentrations of ENL differed according to different methods. Mousavi (1992) showed inhibition of growth in MCF-7 cells with ENL at >10 μM, while Wang (Wang and Kurzer, 1998) showed inhibition at >50 μM. Due to the inconclusive nature of the literature to date regarding phytoestrogens (ENL and GEN) on breast cancer, the consumption of soy products and flaxseed by women with breast cancer has become controversial.

Aim of the present research

The overall aim of the present study was to measure antiproliferative properties of GEN and ENL, alone, in combination, and in the presence of TAM and E_2 in four breast cancer cell lines.

Objectives

My first objective was to examine stability and activity of plant estrogens in tissue culture conditions. The second objective was to evaluate the effect of various doses of GEN or ENL , with and without E_2 in tissue culture. My third objective was to evaluate the effect of plant estrogens on TAM-induced programmed cell death in tissue culture. My fourth objective was to examine the effect of GEN and ENL, alone and in combination in tissue culture.

CHAPTER 2

Stability and Activity of Enterolactone and Genistein in Tissue Culture Conditions

Introduction

The present study was designed to gain insight into the antiproliferative activity of enterolactone (ENL) and genistein (GEN) in human breast cancer cells. The initial objectives were to examine the stability of ENL and GEN in tissue culture conditions and to test their activity in breast cancer cell lines. The stability of ENL and GEN in tissue culture conditions was evaluated using high performance liquid chromatography (HPLC). An HPLC method was selected that was previously used to find the major urinary lignan metabolites in rats (Rickard and Thompson, 2000). Purity was not being evaluated here so multicomponent analysis and the need for prepurification steps were unnecessary. Evaluation of the stability of the solvent, dimethyl sulfoxide (DMSO) and 4-hydroxytamoxifen (OHT), which is the active metabolite of tamoxifen (TAM), were included in these experiments.

Breast cancer cells that grow *in vitro* represent one of the most widely used experimental models in breast cancer research (Clarke *et al.*, 2000). The current understanding of how breast cancer cells respond to estrogenic stimuli is the direct result of *in vitro* studies with human breast cancer cell lines.

Breast cancer cell lines are generally considered in terms of their estrogen receptor (ER) content; either they are ER(+) or ER(-). Two ER(+) or hormone-dependent cell lines, MCF-7 and T47D, and two ER(-), or hormone-independent cell lines, MDA-MB-231 and MDA-MB-468 were chosen for this research based on the suggestion that

ENL and GEN can exert antiproliferative activity dependently and independently of the steroid receptor (Cappelletti *et al.*, 2000).

MCF-7 cells

The MCF-7 cell line is the most widely used and best characterized of all the breast cancer cell lines (Clarke *et al.*, 2000). The estrogen (E_2) dependence for exponential growth *in vitro* and *in vivo* has provided this cell line with a central role in the study of endocrine responsiveness and malignant progression *in vitro* (Clarke *et al.*, 2000). Much of the current understanding of the mechanism of action of E_2 and antiestrogens and their role in regulating proliferation of hormone-dependent breast cancer cells has been derived from work performed using this cellular model (Clarke *et al.*, 2000).

T47D cells

The T47D breast cancer cell line expresses receptors for estrogen, progesterone, androgen, glucocorticoid, and insulin (Clarke *et al.*, 2000). The T47D cells are most notable for their high levels of progesterone receptors and their genetic and phenotypic instability. T47D cells are sensitive to the growth-inhibitory effects of retinoids and antiestrogens (Clarke *et al.*, 2000).

MDA-MB-231 and MDA-MB-468 cells

The MDA-MB-231 cell line is the most widely used ER(-) human breast cancer cell line. Both of these cell lines are highly tumourigenic and can produce lung metastases from mammary fat pad tumours in nude mice (Clarke *et al.*, 2000).

Materials and Methods

Chemicals and reagents

OHT, ENL, GEN, and DMSO were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The MTT cell proliferation and viability assay kit, TACS™ were obtained from R&D Systems, Inc., (catalogue # TA5355 Minneapolis, MN). The OHT stock solutions were prepared in ethanol to increase solubility. The final ethanol concentration in the wells was < 0.3%. ENL and GEN were prepared in DMSO with a final concentration of DMSO of < 0.1% in the wells (Mousavi and Adlercreutz, 1992).

HPLC analysis

This procedure was used to evaluate changes to the stability of ENL, GEN, and OHT during a 24 hour incubation period at 37°C and 5% CO_2 mimicking tissue culture conditions. Each of the 1 ml samples consisted of 1 mM ENL (0.299 mg ENL powder/1 ml DMSO solution) and 1 mM GEN (0.270 mg GEN/1 ml DMSO solution). Two control samples of DMSO were included in this analysis: one freshly prepared sample and one sample that was stored at 37°C and 5% CO_2 for 24 hours. A 1 ml sample of OHT consisting of 1 mM OHT was used as a positive control (0.3875 mg OHT powder/1 ml ethanol solution). The solutions were mixed by vortex for 30 seconds to ensure complete mixing. A 300 µl aliquot was removed and syringe filtered for analysis with a Beckman System Gold Module 125 HPLC system, monitored at 280 nm with System Gold

software (Beckman Instruments Canada Inc., Mississauga, ON). The analysis was performed with the assistance of Carol Warby in the Sherwood laboratory, Biology Department, University of Victoria.

Cell lines

The breast cell lines, MCF-7 (ER(+)), T-47D (ER(+)), MDA-MB-231 (ER(-)), and MDA-MB-468 (ER(-)) were obtained from American Type Culture Collection (Rockville, MD). All tissue culture supplies including Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS), fetal bovine serum (FBS), Delbecco's Modified Eagle Medium-nutrient mixture (DMEM) with and without phenol red, L-glutamine, Liebowitz L-15 medium with and without phenol red, 100 IU/ml streptomycin/ 100 IU/ml penicillin and 0.25% (w/v) trypsin -0.03 % (w/v) ethylenediaminetetraacetic acid (EDTA) 0.02% were obtained from Invitrogen (Burlington, ON).

Tissue Culture Conditions

The ER(+) cells were cultured in T75 flasks with DMEM containing 2 mM L-glutamine with phenol red, supplemented with 10% FBS at 37° C in 5% CO_2. The ER(-) cells were cultured in T75 flasks with Liebovitz L-15 medium with 10% FBS at 37°C in a free gas exchange with atmospheric air. A mixture of CO_2 and air was detrimental to ER(-) cells when using this medium for cultivation (Mousavi and Adlercreutz, 1992).

The medium required changing 2-3 times per week, and the cells were passaged once each week. The cells were subcultured to a T175 flask once they had grown to

approximately 80% confluence. In preparation for the experiments, the cells were removed from the T175 flasks by first removing the culture medium, then twice washing the cell layer with 5 ml PBS. Trypsinization followed with addition of 2-3 ml of 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution. Cells were incubated for approximately 5 min at 37°C or until cells were detached from the surface of the flask and complete growth medium (6-8 ml) was added to arrest the activity of the trypsin/EDTA solution.

The ER(+) cells were gently aspirated and transferred to DMEM without phenol red, supplemented with 10% dextran/charcoal-coated (DCC-FBS). DCC-FBS had been previously stripped, thereby removing steroid activity and estrogenic contaminants. The ER(-) cells were transferred to Liebovitz L-15 medium without phenol red, supplemented with 10% DCC-FBS. The cells were seeded for at least 3 days prior to start of experiments. This was sufficient time to circumvent interference from the steroids present in FBS and estrogenic properties from phenol red (Clarke *et al.*, 2001).

Dextran-coated charcoal FBS procedure

To prepare DCC-FBS, 1 g charcoal and 0.1 g dextran were added to 100 ml distilled water. The mixture was then transferred to 3-50 ml tubes in 33 ml aliquots and was centrifuged at 4000 rpm (2,600 g) for 10 min. Supernatant was discarded and 50 ml of FBS was added to tube #1 containing DCC which was incubated at 4°C for 30 min. The DCC-FBS mixture was centrifuged at 4000 rpm (2,600 g) for 10 mins. The supernatant of the DCC-FBS was transferred to tube #2 and #3, repeating the procedure. The DCC-FBS was sterilized twice with 0.2 micron filters.

Viable cell counting with trypan blue and a hemocytometer

The determination of cell number is a key measurement for setting up each experiment with cancer cell lines as well as monitoring cell responses under experimental conditions (Macleod and Langdon, 2004). The simplest protocol involved the use of a hemocytometer (Improved-Neubauer) and was appropriate as only a small number of samples were to be counted. A hemocytometer (Figure 2.1) is an etched glass chamber that held a coverslip 0.1 mm above the chamber. The counting chamber was precisely etched in a total surface area of 9 mm^2. Calculation of cell number was based on counting the number of cells within a defined area underneath the coverslip.

The breast cancer cell lines were adherent cells that grew in a monolayer. Prior to use in the experiments, the cell cultures were checked to ensure the cells were in late/early plateau phase (80-90% of the surface area was covered) and to confirm the cells were healthy and free of contamination. To remove the cells from the flasks, trypsinization was used. The cell culture medium was removed by pipette and discarded. The cells were washed twice with PBS to remove traces of serum that will inactivate trypsin. To a T75 flask, 2 ml of trypsin were added and the solution was swirled across the monolayer to ensure the trypsin reached all the cells. The flask was returned to the incubator for approximately 5 min or when the cells were

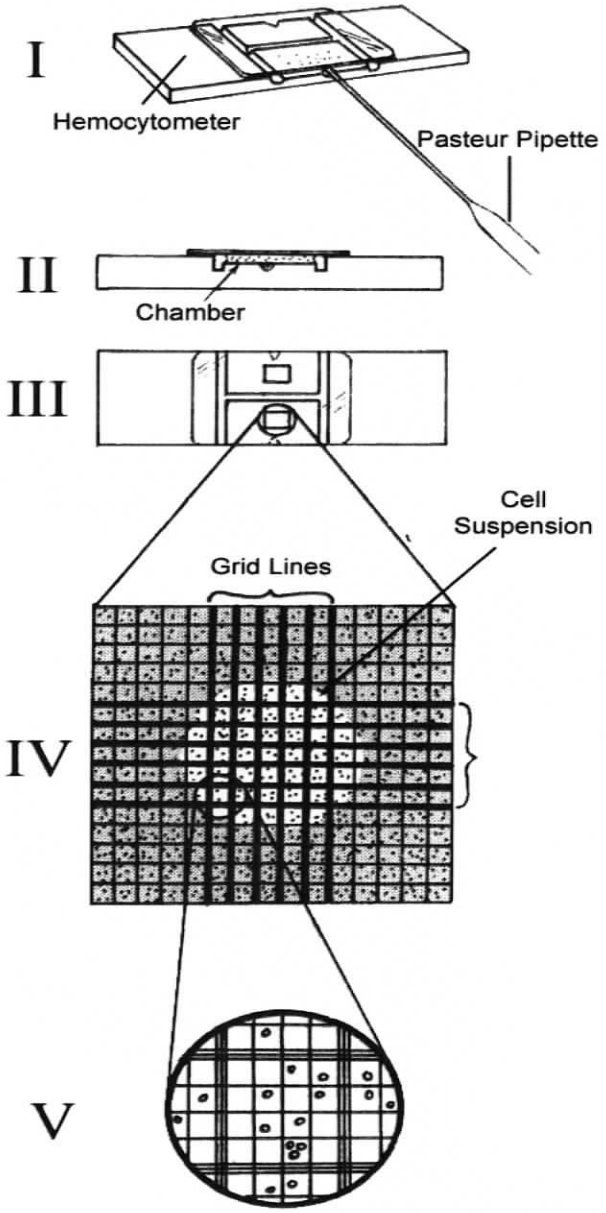

Figure 2.1. Use of a hemocytometer slide for cell counting in tissue culture systems. I. Addition of cell suspension to the slide. II. Side view of a slide in which the position of the cell sample is shown. III. Top view of the slide. IV. Enlarged view of the total area of the grid. V. Magnified view of one of the 25-smaller squares, making up the 1 mm^2 central area. This is subdivided by single grid lines into 16 small squares to aid in counting. Adapted from (Freshney, 1988).

rounded up and detached from the surface of the flask as viewed under a microscope. Once the cells were detached, fresh medium containing serum was then added to inactivate the trypsin in the cell suspension (Macleod and Langdon, 2004).

To ensure the cells were healthy and viable, a 500 µl aliquot of cell suspension was incubated with 500 µl of 0.4% trypan blue. Trypan blue is a dye that does not interact with the cell unless the cell membrane is damaged (Macleod and Langdon, 2004). Healthy undamaged cells excluded the dye, but it was readily absorbed by damaged cells and rendered them clearly visible (blue) under the microscope.

The number of the cells in the suspension was determined by placing the cells in the hemocytometer. The cell concentration was obtained by counting the 4 outer squares. Total cell count in 4 squares x 2500 = number of cells/ml. The accuracy of the procedure depends on the number of cells counted, accurate pipetting of aliquots, and having a single-cell suspension (Macleod and Langdon, 2004).

MTT cell proliferation and viability assay

To predict activity of ENL and GEN in breast cancer, a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell proliferation and viability assay in breast cancer cell lines was used. Cytotoxicity assays such as the MTT cell proliferation and viability assay are widely used especially in the field of new drug development (Macleod and Langdon, 2004). These assays measure the effect of a drug on the growth of a population of cells and the endpoint is absorbance which is representative of an increase or decrease in cell number (Macleod and Langdon, 2004).

This was accomplished in two phases:

1. Determination of optimal cell numbers for analysis and comparison after either stimulation or inhibition of cell proliferation.
2. Evaluation of overall cell viability in experimental samples compared to untreated control samples.

To perform both phases of the MTT assay, a MTT cell proliferation and viability assay kit, R&D Systems TACS^{TM} (Minneapolis, MN) was used. MTT is a yellow water soluble tetrazolium dye that is reduced by live, metabolically active cells to insoluble purple formazan dye crystals. The amount of MTT-formazan produced was determined spectrophotometrically once solubilized in a suitable solvent (MTT detergent). The amount of MTT-formazan produced was proportional to the number of viable cells.

Phase one: Determination of Cell Numbers

Cells were trypsinized, harvested to prepare a cell suspension and counted as described in *viable cell counting with trypan blue and a hemocytometer.* A known number of cells were pelleted by centrifugation at 500 x g for 5 minutes at 2-8 ° C and the supernatant was discarded. The cells were washed by resuspending in 5 ml of PBS and pelleted a second time by centrifugation at 500 x g for 5 min at 2-8 ° C. Twelve culture tubes were assembled (Figure 2.2). The serial dilutions were prepared by adding a 400 µl aliquot of stock (from tube #1) to the media in tube #2. After mixing, a 400 µl aliquot of cells from tube #2 was added to the media in tube #3. These steps were repeated through to tube #12 with the final cell concentration in tube #12 being 2.44 x 10^3. Dilutions from

5×10^6 to 2.44×10^3 cells/ml were sufficient for the four breast cancer cell lines to be used in the experiments.

For the MTT assay procedure, cells were added to a 96-well plate from the serial dilutions prepared in the 12 culture tubes. Cells from each of the culture tubes were plated in triplicate (305-6250 cells/100 µl/well) and incubated for 24 hours in DCC and phenol red-free medium. MTT reagent [10 µl] was added to each well and incubated for 24 hours at 37°C and 5% CO_2. The cells were kept in the dark for at least 2 hours-overnight at 37°C and 5% CO_2 to solublize the crystal formation. The absorbance of the wells at 550 nm was determined with use of an EL_x 808 Ultra Microplate reader (Bio-Tek Instruments Inc., Winooski, VT). The statistical software used for curve-fitting the microplate reader data was KC Junior, Bio-Tek Instruments Inc.

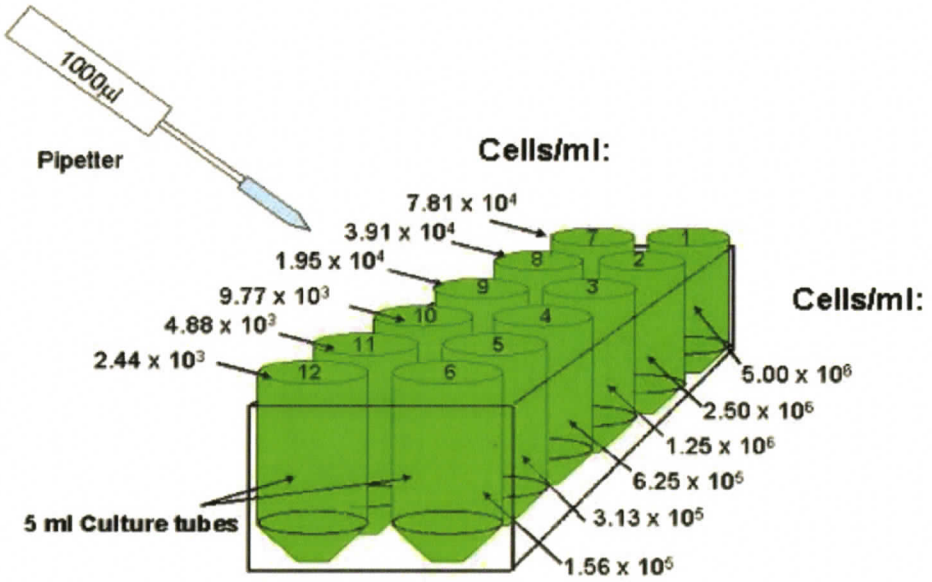

Figure 2.2. Serial dilutions of cells in culture tubes. Dilutions from 5 x 10^6 to 2.44 x 10^3 cells/ml were sufficient for the four breast cancer cell lines to be used in the experiments.

The absorbance data were plotted for each of the four breast cancer cell lines (Figure 2.3). The average cell number that fell within the portion of the curve that provided values between the range of 0.75 and 1.25 was the optimal cell number per experimental sample that allowed the measurement of stimulation and inhibition of cell proliferation.

Phase two: Evaluation of cell proliferation and viability

Proliferation or reduction in viable cells treated with ENL, GEN or OHT was measured with each breast cancer cell line in triplicate and repeated in three separate experiments. Experimental samples of each breast cancer cell line, including controls, were seeded according to the results of the MTT assay in which the number of cells was determined. Cell-based negative controls were plated in triplicate and also repeated in three separate experiments. Once the cells recovered and had reattached, they were treated with 1, 10, 50, and 100 μM of ENL or GEN. The positive control of the experiment, OHT was used in 1, 10 and 100 μM concentrations. From the results in the MTT assay procedure used in phase one, cells were plated at approximately 3125 cells/well and incubated for 24 hours in DCC and phenol-red free medium .

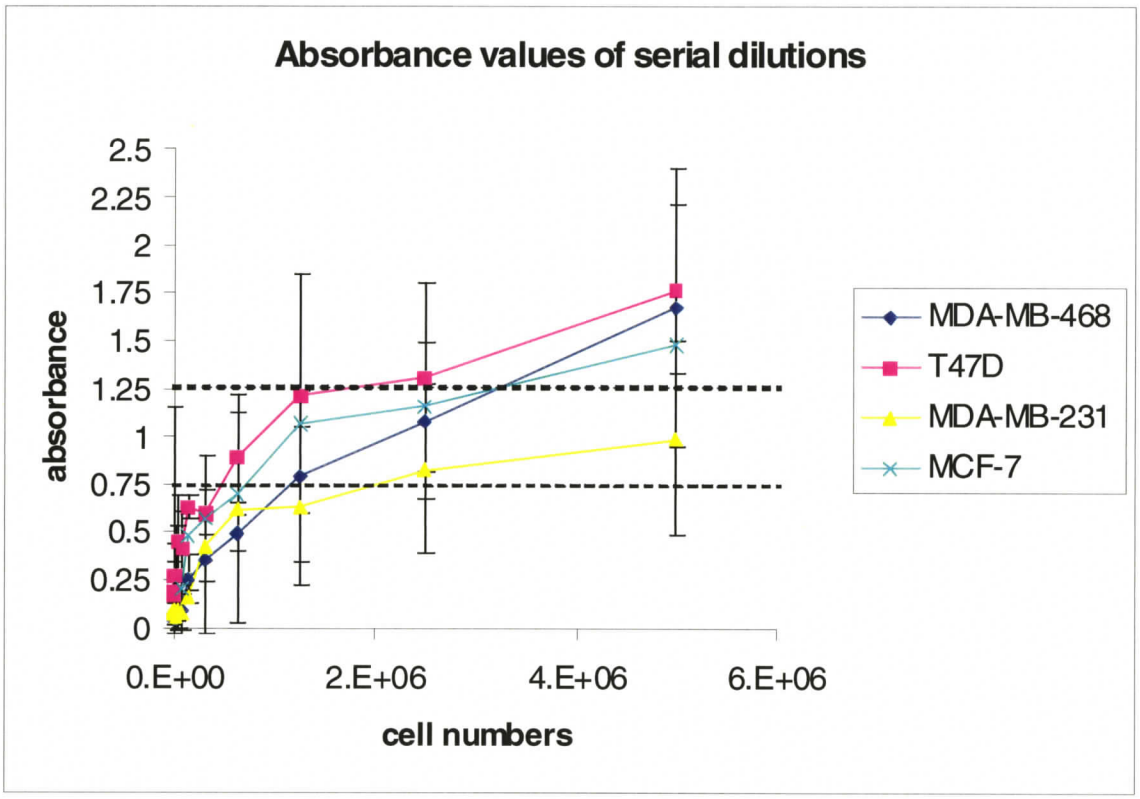

Figure 2.3. Absorbance values of serial dilutions of four breast cancer cell lines obtained from the MTT Assay for determination of optimal cell numbers to be used in subsequent experiments. The portion of the curve for each cell line with absorbance values between the range of 0.75 and 1.25 was the cell number used for the measurement of both stimulation and inhibition of cell proliferation. From this dot plot the optimal cell number for the cell lines was 2.5 x 10^6 cells/ml.

MTT reagent (10 µl) was added to each well and incubated for 24 hours at 37°C and 5% CO_2. When purple precipitate was clearly visible under the microscope, 100 µl of detergent reagent were added to each well. The cells were kept in the dark for at least 2 hours-overnight at 37°C and 5% CO_2 to solublize all the crystals that had formed. The absorbance of the wells at 550 nm was determined with use of an EL_x 808 Ultra Microplate reader, Bio-Tek Instruments Inc. The statistical software used for curve-fitting the microplate reader data was KC Junior, Bio-Tek Instruments Inc. One-way Analysis of Variance (ANOVA) statistical analysis was performed using the absorbance data from the microplate reader. The P value is < 0.001, and was considered highly significant. Tukey-Kramer multiple comparison test was used to compare results generating confidence intervals. A computerized statistical program, GraphPad InStat® version 3.0 was used to perform the analysis. The data plots depicting the results of the cell proliferation and viability experiments were expressed as percentage of the untreated controls for each of the four breast cancer cell lines.

Results

HPLC analysis

There were two HPLC profiles generated for OHT, one freshly prepared sample (top panel) and one sample stored at 37°C and 5% CO_2 (bottom panel) (Figure 2.4).

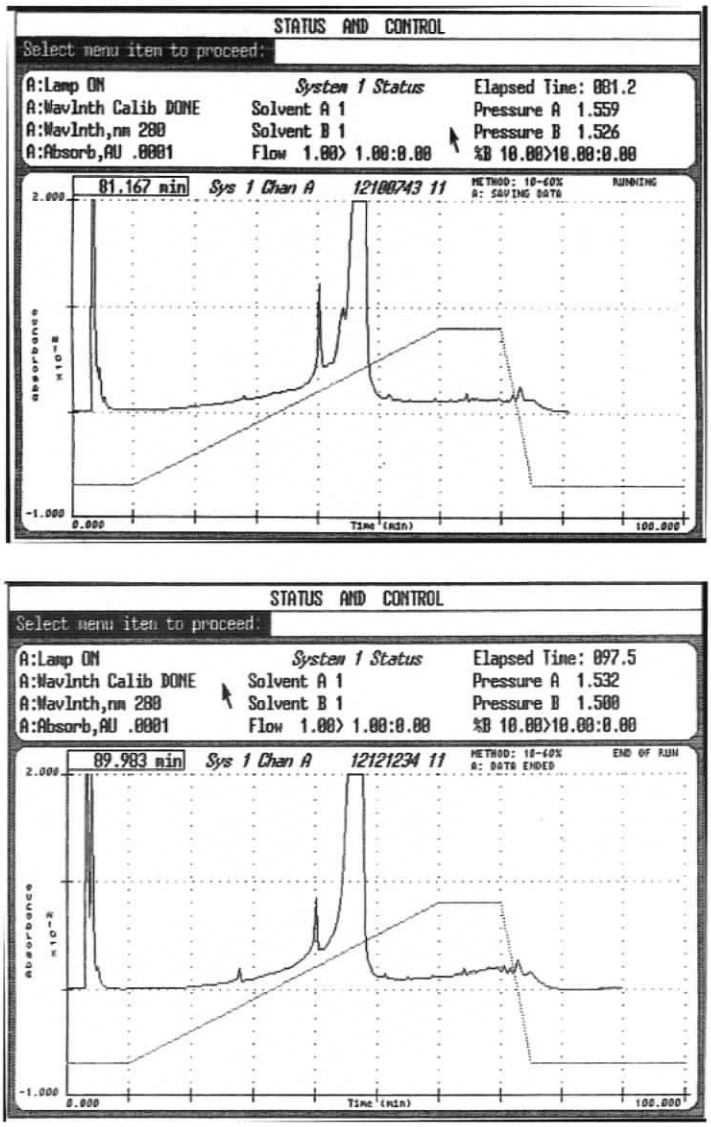

Figure 2.4. HPLC results of two samples of 4-hydroxytamoxifen (OHT); 1 mM of freshly prepared OHT (top panel) and 1 mM of OHT stored at 37°C and 5% CO_2 for 24 hours (bottom panel). Few changes occurred between the two samples indicating OHT was stable during storage for 24 hours in tissue culture conditions.

There were few variations observed between the sample of OHT that was freshly prepared and the sample stored for 24 hours at 37°C and 5% CO_2.

There were two HPLC profiles conducted for DMSO, one freshly prepared sample (top panel) and one sample stored at 37°C and 5% CO_2 (bottom panel) (Figure 2.5). There was an additional peak noted between the freshly prepared DMSO sample and the sample stored for 24 hours at 37°C and 5% CO_2. Figures 2.6 and 2.7 represent the HPLC profiles of GEN and ENL. There was an additional peak noted between the freshly prepared GEN and ENL samples and the samples stored for 24 hours at 37°C and 5% CO_2 in the same position as the stored DMSO control.

Evaluation of cell viability

Evaluation of proliferation or reduction in viable cells treated with GEN, ENL or OHT was conducted with each cell line using a MTT cell proliferation and viability assay. The negative controls were plated cells that remained untreated. The positive controls were cells plated as the controls but treated with 10 or 100 μM OHT. The data plots depicting the results of the cell proliferation and viability experiments were expressed as percentage of the negative controls and provided a curve for each of the four breast cancer cell lines. If the absorbance values of the treated experimental samples were higher than the negative control cells, this indicated an increase in cell number. Alternatively, if the absorbance rates of the treated experimental samples were lower than the negative controls, this indicated a reduction in the rate of proliferation or a reduction in overall cell viability.

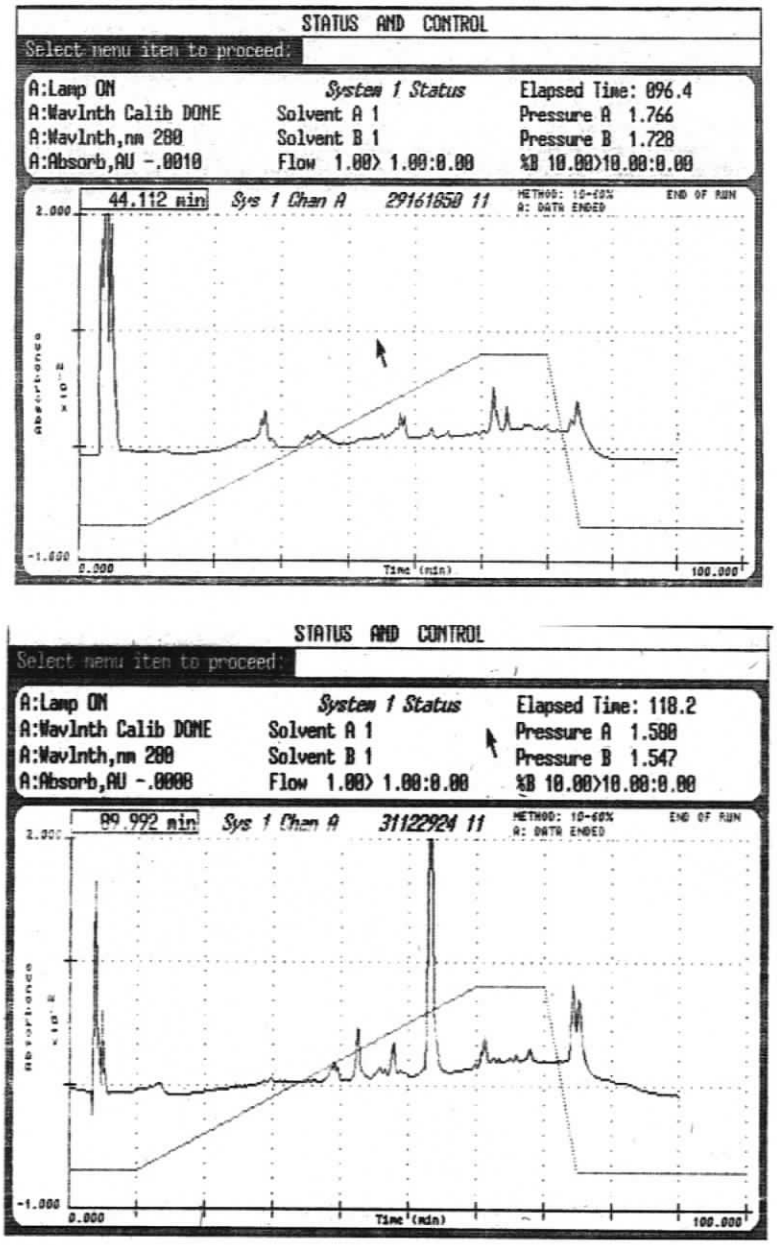

Figure 2.5. HPLC results of two samples of 99% pure DMSO; 400 µl of freshly prepared DMSO (top panel) and 400 µl of DMSO which was stored at 37°C and 5% CO_2 (bottom panel). The stored DMSO sample varied from the freshly prepared sample indicating the compound experienced changes in composition during storage.

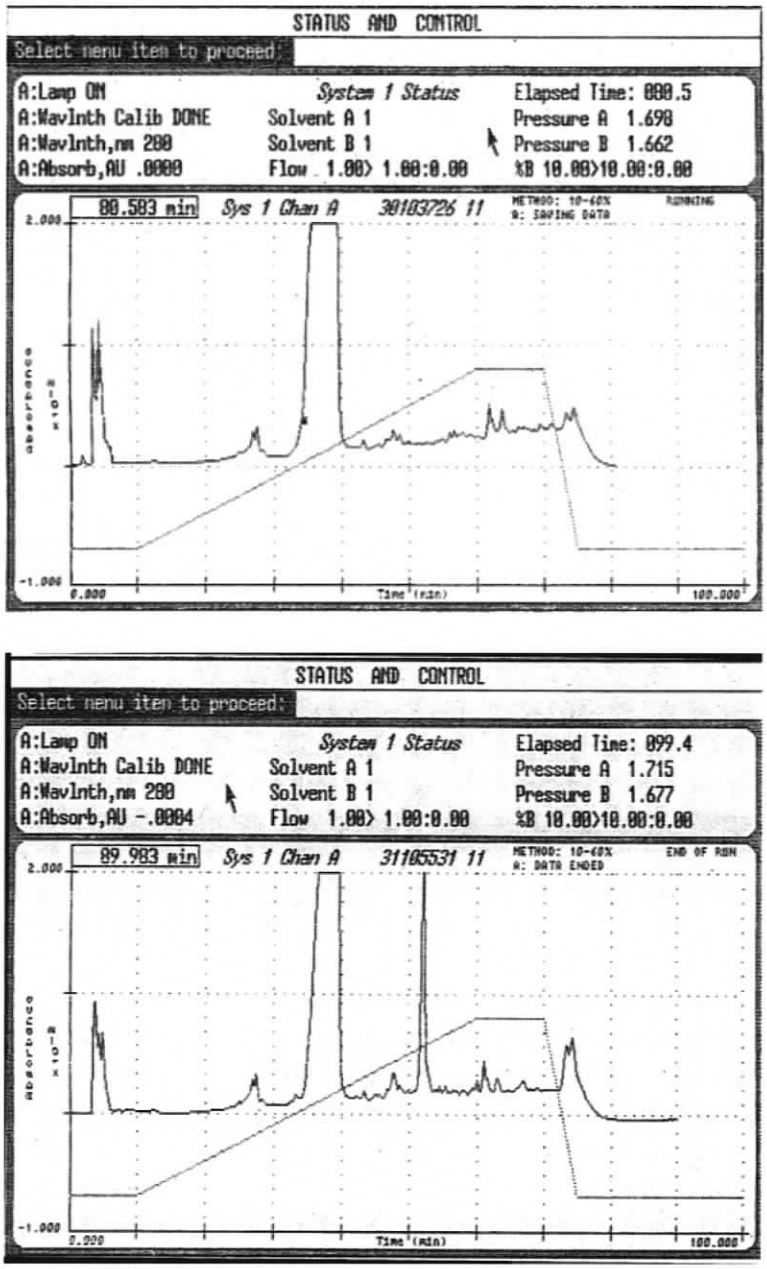

Figure 2.6. HPLC results of two samples of 1 mM enterolactone (ENL). One sample was freshly prepared in DMSO (top panel) and the second sample was prepared in DMSO and stored at 37°C and 5% CO_2 for 24 hours (bottom panel). A second peak occurred in the stored sample which was attributed to changes to the DMSO during storage in tissue culture conditions.

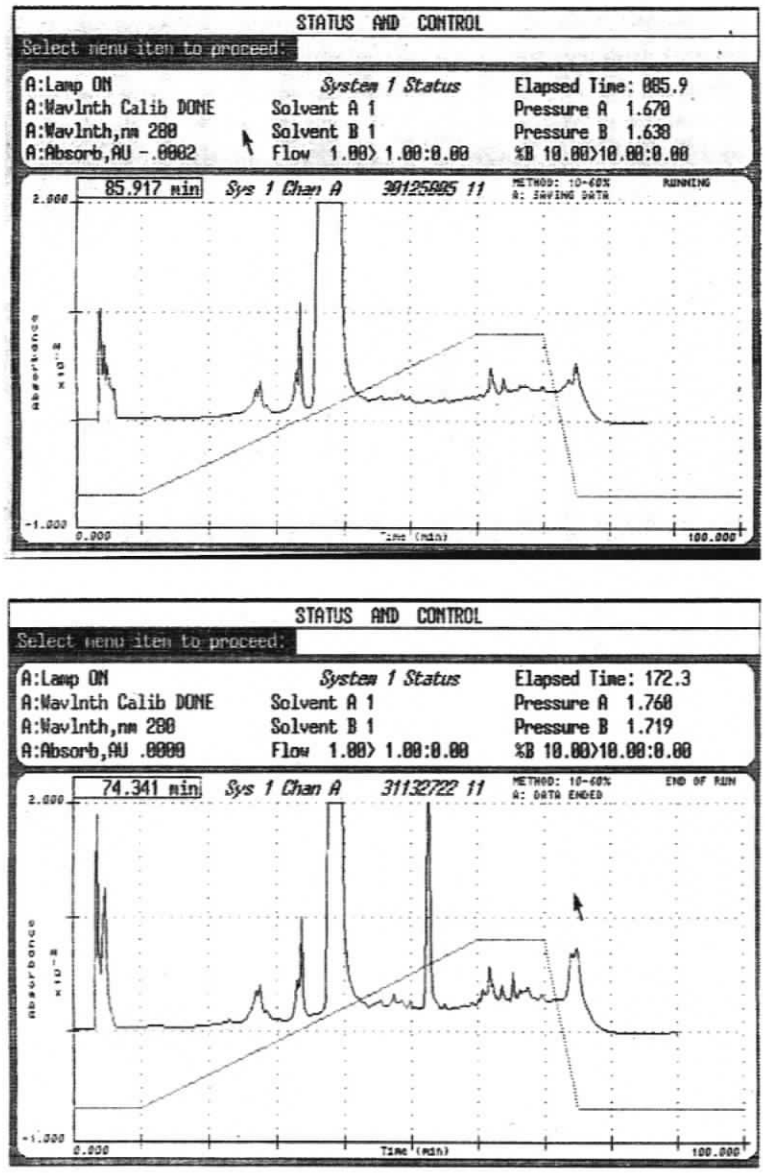

Figure 2.7. HPLC results of two samples of 1 mM genistein (GEN). One sample was freshly prepared in DMSO (top panel) and the second sample was prepared in DMSO and stored at 37°C and 5% CO_2 for 24 hours (bottom panel). The second peak was attributed to degradation of the DMSO during storage in tissue culture conditions.

In Figure 2.8, MCF-7 cells treated with ENL or GEN demonstrated no significant increase or decrease in viability with 1 μM, 10 μM, 50 μM, and 100 μM treatment compared to negative controls. Combinations of ENL and GEN did not significantly increase viability at 10 μM or 50 μM. Doses of 10 μM OHT did not significantly decrease viability but the effect was statistically significant when the dose was increased to 100 μM ($p < 0.001$).

In Figure 2.9, T47D cells treated with ENL or GEN did not demonstrate a significant increase in viability with 1 μM, 10 μM, 50 μM and 100 μM of ENL or GEN compared to negative controls. Combinations of ENL and GEN did not significantly affect viability at doses of 10 μM or 50 μM. A dose of 10 μM of OHT did not reduce viability whereas a dose of 100 μM OHT did reduce viability ($p < 0.001$).

In Figure 2.10, MDA-MB-231 cells did not significantly increase viability at 10, 50 and 100 μM of ENL or GEN compared to negative controls. Combinations of ENL and GEN 10 μM or 50 μM did not result in a statistically significant effect on cell numbers. The effect of treatment with 10 μM OHT did not significantly reduce the number of viable cells. However, the effect of treatment with 100 μM OHT resulted in an antiproliferative effect which was statistically significant ($p < 0.001$).

When MDA-MB-468 cells were treated with 1 μM, 10 μM, 50 μM, and 100μM of ENL or GEN the number of viable cells was not significantly reduced (Figure 2.11). Combination treatments with 10 or 50 μM of ENL and GEN did not result in a significant reduction in the number of viable cells. Treatment with 10 μM OHT did not significantly reduce the

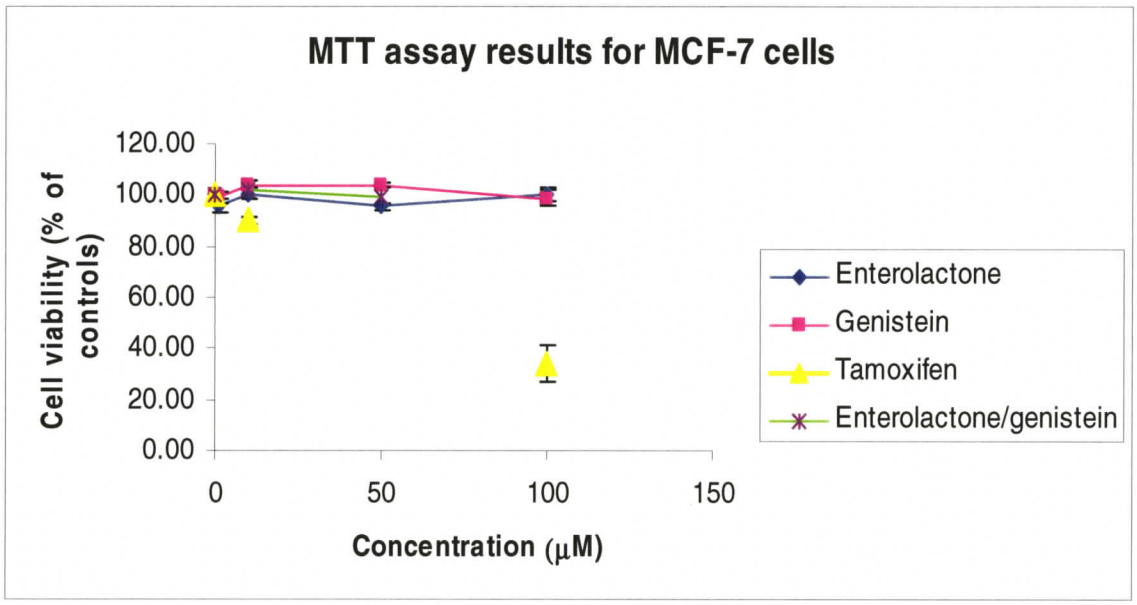

Figure 2.8. MCF-7 cells treated with ENL or GEN were plotted as percentage of untreated controls. The controls were MCF-7 cells which were plated in medium only and values were expressed as percentage of untreated controls. There were no differences in viability with treatments of 1 μM, 10 μM, 50 and 100 μM of ENL or GEN. There were no differences in viability with combinations of ENL and GEN. Doses of 100 μM of OHT reduced viability and the effect was statistically significant ($p < 0.001$).

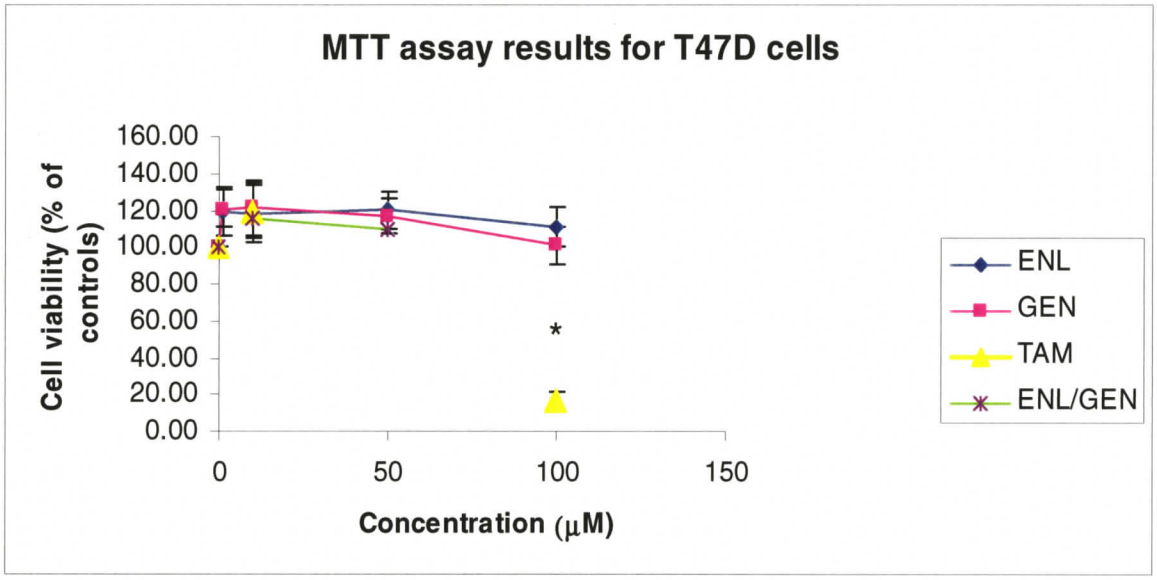

Figure 2.9. T47D cells treated with ENL and GEN demonstrated no significant difference in viability with 1 μM, 10 μM, 50 μM, and 100 μM compared to untreated controls. The controls were T47D cells in medium only and values were expressed as percentage of untreated controls. There were no differences in viability seen with treatments of combinations of 10 μM and 50 μM ENL and GEN compared to untreated controls. There was a statistically significant reduction in viability seen when the dose of OHT was increased to 100 μM ($p < 0.001$).

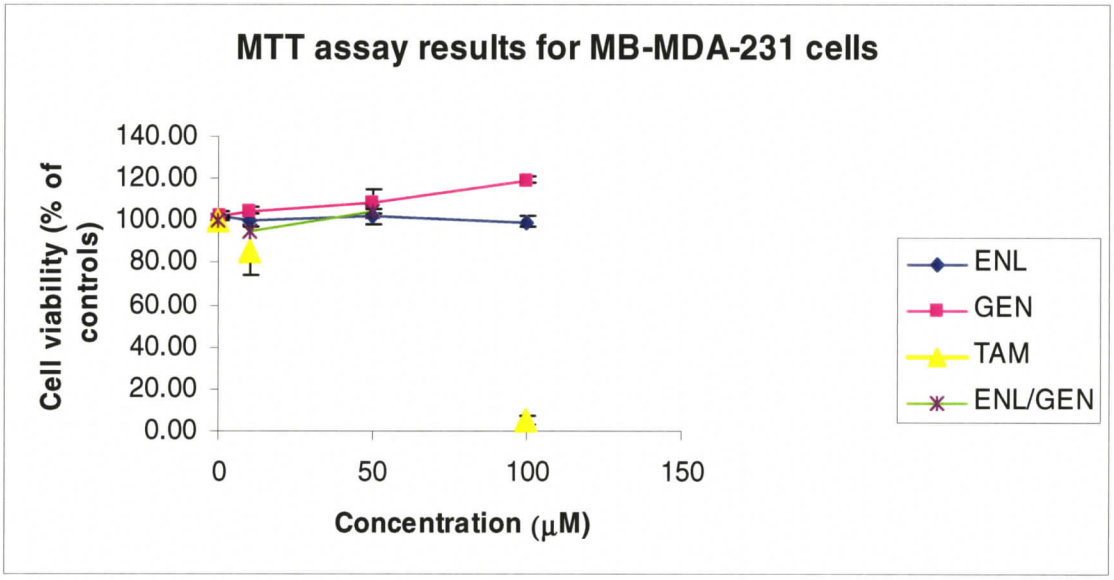

Figure 2.10. There was no significant reduction in viability of MDA-MB-231 cells in response to treatment with 1 μM, 10 μM, 50 μM and 100 μM of ENL or GEN compared to untreated controls. The controls were MDA-MB-231 cells which were plated in medium only and all values were expressed as percentage of untreated controls. In treatments with combinations of ENL and GEN were similar with 10 μM and 50 μM there was no significant difference seen in viability compared to untreated controls. There was a significant reduction in viability with treatment of 100 μM OHT ($p < 0.001$).

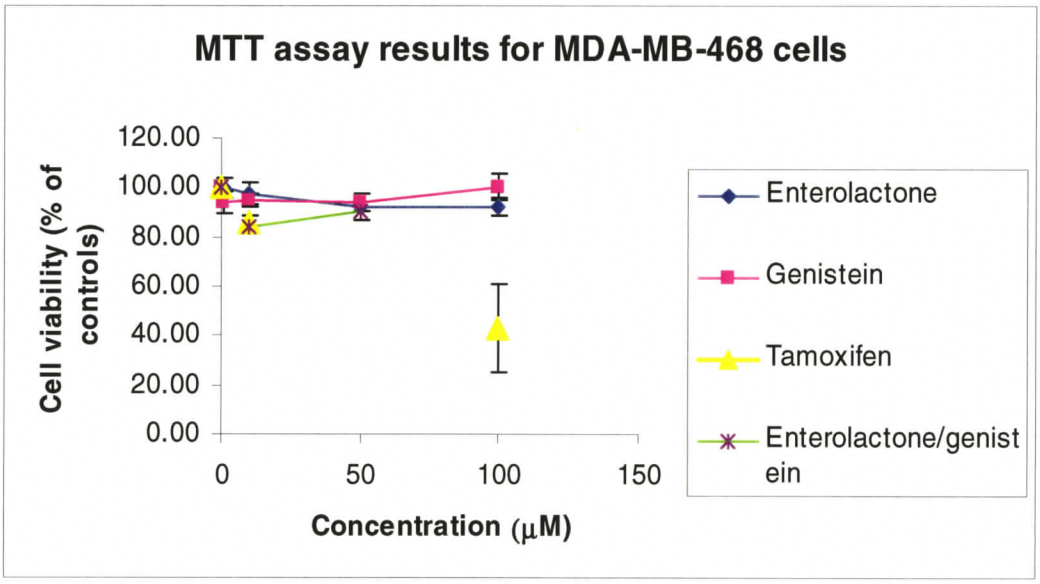

Figure 2.11. MDA-MB-468 cells treated with 1 μM, 10 μM, 50 μM, and 100 μM of GEN or ENL were not significantly reduce viability. The controls were MDA-MB-468 cells which were plated in medium only and all values were expressed as percentage of untreated controls. There was no significant difference in reduction of viability seen with combination treatments with 10 and 50 μM of ENL and GEN. There was a significant reduction in viability with treatments of 100 μM OHT ($p < 0.001$).

number of viable cells but the antiproliferative effect of 100 μM OHT was statistically significant ($p < 0.001$).

Discussion

In the present study, the stability of two plant estrogens, ENL and GEN, in tissue culture conditions was examined by HPLC. The growth regulatory effects of single doses and combinations of doses of the two plant estrogens, ENL and GEN were also evaluated. Negative (untreated) controls were included in all experiments and positive controls were treated with OHT. Ethanol was used as a solvent for OHT to increase solubility whereas DMSO was used as a solvent for ENL and GEN. The role of ethanol on human breast epithelial cell proliferation is poorly understood. An ethanol concentration of 0.3% has no effect (Etique *et al.*, 2004) or enhanced proliferation of ER(+) and ER(-) breast cancer cells (Izevbigie *et al.*, 2002). It is unknown what effect the ethanol may have on the OHT in the current experiments.

Maintenance of breast cancer cell lines

All of the cells were maintained in medium containing phenol red which is used as a pH indicator in cell culture medium. Phenol red is known to be estrogenic, and this activity can stimulate growth and up-regulate the ER (Clarke *et al.*,2001). Serum contains various growth factors and steroid hormones which are well known to stimulate growth of MCF-7 cells (MacGregor and Jordan, 1998). Before the cell response to ENL, GEN or

OHT was measured, the growth factors and steroids were removed by treatment with DCC, as the serum factors can potentially alter antiestrogen responsiveness. DCC treated fetal bovine serum with phenol-red free media was used to maintain the cells in a growth-factor and estrogen-free environment. Although insulin is a potent mitogen for many of these cells, it does not appear to be required for growth *in vitro* in serum-supplemented media for most human breast cancer cell lines. Because insulin can down-regulate ER expression (Clarke *et al.*, 2001) and influence the growth-inhibitory effects of antiestrogens, it was not used in these experiments.

Cell viability was evaluated prior to the use of cells in experiments. Cell viability was assessed using trypan blue dye that did not interact with the cell unless the cell membrane was damaged. A hemocytometer was used as the method to count cells prior to use in experiments. One advantage of using the hemocytometer method was that it allowed for a variation of technique involving the use of trypan blue dye to enable differentiation between dead/damaged cells and the healthy viable cell population (Macleod and Langdon, 2004). Hemocytometer counting was inexpensive and provided the opportunity to visualize the cells. However, the procedure is time-consuming and may be prone to error both in the method of sampling and the size of samples and requires a minimum of 10^5 cells/ml (Freshney, 1988). Most of the errors usually occur during the transferring of cells to the hemocytometer chamber (Freshney, 1988). The cell suspension required thorough mixing prior to transferring the sample to ensure the cells did not settle or adhere in the tip of the pipette. It was necessary to ensure that a single cell suspension was used as large aggregates were slow to enter the chamber and difficult to count.

Electronic cell counting is rapid, convenient and has a low inherent error due to

the high number of cells counted (Freshney, 1988). However, electronic particle counters are expensive and this method was not available for use in these experiments.

Stability OHT, ENL and GE during storage in tissue culture conditions

The HPLC analysis showed that OHT, ENL and GEN were stable whether freshly prepared or stored for 24 hours at 37°C and 5% CO_2. There was an additional peak seen in the DMSO stored for 24 hours at 37°C and 5% CO_2, which represented a small degradation of the DMSO upon storage in tissue culture conditions. Because the samples of GEN and ENL stored for 24 hours at 37°C and 5% CO_2 experienced a similar variation to that of the stored DMSO, the variation of GEN and ENL during storage was concluded to be due to a change in the composition of the DMSO. Ethanol was found to be stable in tissue culture conditions (data not shown).

Counting of cell numbers in experiments

The aim was for the cells to remain in exponential growth throughout the assay (Macleod and Langdon, 2004). If the cells in the control wells (i.e. those not exposed to a drug treatment) were to have become confluent they would have stopped dividing, while the drug-treated cells would have continued to grow. As a result drug sensitivity would be underestimated since the control absorbance would be lower than it should have been (Macleod and Langdon, 2004). The optimum density can range between 2.5×10^3 to 10^6 cells/ml depending on the cell line (Macleod and Langdon, 2004). A straightforward method to determine the optimum density was plating cells in a range of densities and incubating them with MTT. Selection of the density that provided an absorbance value of

0.75-1.25 was the optimum density for plating cells (Macleod and Langdon, 2004). Dilution of cell numbers from 5 x10^6 to 2.5 x 10^3 cells/ml was sufficient to determine the cell number to be used in each experiment with the four breast cancer cell lines.

Viability or reduction effects of GEN, ENL and OHT

The MTT viability assay was used to determine the preliminary cytotoxicity effects of ENL and GEN at different concentrations on four breast cancer cell lines. From the literature it is known that the most marked effects of drugs are seen during the first 24 hours and that sensitivity plateaus by 72 hours (Macleod and Langdon, 2004). Depending on the mechanism of action of the drug, 24-72 hours would ensure activity of most cytotoxic agents (Macleod and Langdon, 2004). In the present study, there was a variability in cell doubling times among the cell lines, so the accuracy of comparing drug effects on increased proliferation between cell lines was limited. Another drawback of the assay is that it did not distinguish between a cytotoxic (cell kill) and a cytostatic (reduced growth rate) effect (Macleod and Langdon, 2004). Many factors can affect the reduction of MTT. The cells require an adequate energy supply from culture medium, and reduction of the MTT reagent can be inhibited by some cytotoxic drugs (Macleod and Langdon, 2004).

Plant estrogens have anticarcinogenic potential, but they also have significant estrogenic properties (Peeters *et al.*, 2003). A hallmark response to estrogenic stimuli is the proliferation of cells *in vitro* (Mueller *et al.*, 2004). Depending on the concentration of GEN and the presence of estrogen, a biphasic effect of GEN on mammary cells has

been demonstrated (Chen and Thompson, 2003). In the present study, the results of ENL and GEN in the MTT viability assay did not demonstrate a biphasic trend.

In ER(+) breast cancer cell lines, there was no effect seen on reduction of cell viability with treatments of low dose ENL and GEN (1 μM and 10 μM) and higher doses (50 μM and 100 μM). Low dose OHT (1 μM and 10 μM) did not affect cell viability but high dose (100 μM) resulted in a significant decrease in cell numbers ($p < 0.001$).

In the ER(-) breast cancer cell lines, cell viability with treatments of low dose ENL and GEN (1 μM and 10 μM) and higher doses (50 μM and 100 μM) was not decreased. This represents a reverse of the trends seen in ER(+) breast cancer cells. High dose OHT (100 μM) significantly reduced the number of viable cells ($p < 0.001$).

In the present study, the effect of combining GEN and ENL did not vary treatment with single compounds in ER(+) breast cancer cell lines. Although the effect was not significant, there was a trend to a reduced cell number seen in ER(-) breast cancer cell lines.

Conclusion

Breast cancer is generally classified according to the ER content of cells. This classification largely reflects the clinical value of steroid hormone receptor expression for predicting a response to endocrine therapies. Approximately 30% of all breast cancer patients respond to endocrine therapy (Clarke *et al.*, 2000). Therefore, it is important to investigate the potential of other anticancer agents that may work to enhance the

effectiveness of current endocrine therapies employed in the treatment of breast cancer. Breast cancer cells that grow *in vitro* represent one of the most widely used experimental models of breast cancer. Breast cancer cell lines are also classified according to their ER content, which provides a system to evaluate new potential endocrine therapies for their anticancer activity. The cell seeding density of these breast cancer cell lines can have a considerable effect on cellular growth and metabolism. Therefore, it was important to determine the cell numbers for use in the experiments with each breast cancer cell line.

The MTT assay is important as it is useful for determining the cell numbers to be used in subsequent experiments as well as important for determining preliminary cell proliferation or reduction effects of ENL and GEN. Although these effects of treatments with ENL and GEN did not reach statistical significance, the trends were important to continue with a more in-depth evaluation. The MTT assay demonstrated there are similar effects seen with both ENL and GEN, which suggests their mechanisms may also be similar. The MTT cytotoxicity assay was quick and easy and allowed a large number of assays to be carried out at once. This is an important consideration when making comparisons between cell lines, between cytotoxic agents, or when evaluating combinations of drugs (Macleod and Langdon, 2004). No one cytotoxic assay is ideal, and it is advisable to support initial effects with further results obtained from alternative assays (Macleod and Langdon, 2004). Although the MTT results did not reach statistical significance, they indicated a need to evaluate the plant estrogen effect further with other assays.

CHAPTER 3

Effects of Genistein and Enterolactone on Proliferation and Apoptosis in Breast Cancer Cell Lines

Introduction

Increasing attention is being paid to the approaches that might characterize tumour cell populations, i.e. cell proliferation, differentiation and apoptosis. A proper dose of anti-cancer drug can kill cancer cells by activating the apoptotic death program in target cells. In apoptosis, the products of protein and DNA degradation are released in a controlled process that allows them to be taken up and reused by neighboring cells. Apoptosis allows for the elimination of a cell without wasting its components or causing harmful inflammation (Nelson and Cox, 2005).

Importance of Apoptosis

Apoptosis or programmed cell death is a normal and fundamental event, which occurs in a well-defined manner during development and aging of animals. However, sometimes cell suicide is not programmed but occurs in response to biological circumstances that threaten the rest of the organism. Apoptosis is also induced by cytotoxic stresses such as heat, hyperosmolarity, UV light, gamma irradiation and anti-cancer drugs (Nelson and Cox, 2005). Inducers of apoptosis are categorized into three groups: death factors, genotoxic anti-cancer drugs, and growth factor deprivation (Nagata *et al.*, 2003).

Although these triggers initiate a cascade of events that finally results in cell death by apoptosis, they differ in the length of the trigger phase, i.e., the lag time between exposure to the trigger and the time of the first morphological signs of apoptosis (Nagata *et al.*, 2003). The duration of this phase depends on the cell type, type of trigger and

growth conditions of the cell (Nagata *et al.*, 2003). All the different induction mechanisms converge into the activation of a proteolytic cascade. The activation of the proteolytic cascade of caspases is the beginning of the execution phase of the apoptotic process (Nagata *et al.*, 2003), which is short and has been shown to have little variation in duration.

Apoptosis is essentially mediated by this family of proteases that are activated by processing of inactive precursors. Thirteen members of the human caspase family have been identified. Some of the family members are involved in apoptosis and these can be divided into two subgroups. The first group consists of caspase 8, caspase 9, and caspase 10, which contain a long prodomain at the N-terminus; they function as intiatators of the cell death process (Nagata *et al.*, 2003). The second group contains caspase 3, caspase 6, and caspase 7; they have a short prodomain and work as effectors, cleaving various death substrates that ultimately cause the morphological and biochemical changes seen in apoptotic cells (Figure 3.1). The other effector molecule in apoptosis is apoptotic-protease activating factor (Apaf-1), which, together with cytochrome c, recruits pro-caspase 9 in an ATP-dependent manner, and stimulates the processing of pro-caspase 9 to the mature enzyme (Nagata *et al.*, 2003). The mechanisms that trigger apoptosis involve some of the same proteins that regulate cell cycle (Nelson and Cox, 2005). The signal for suicide often comes from outside, through a surface receptor. Fas ligand, a representative death factor, binds to Fas receptor, and causes its trimerization. The trimerized death domain in the Fas cytoplasmic region recruits pro-caspase 8 through a FADD/MORT1 adaptor, and forms a

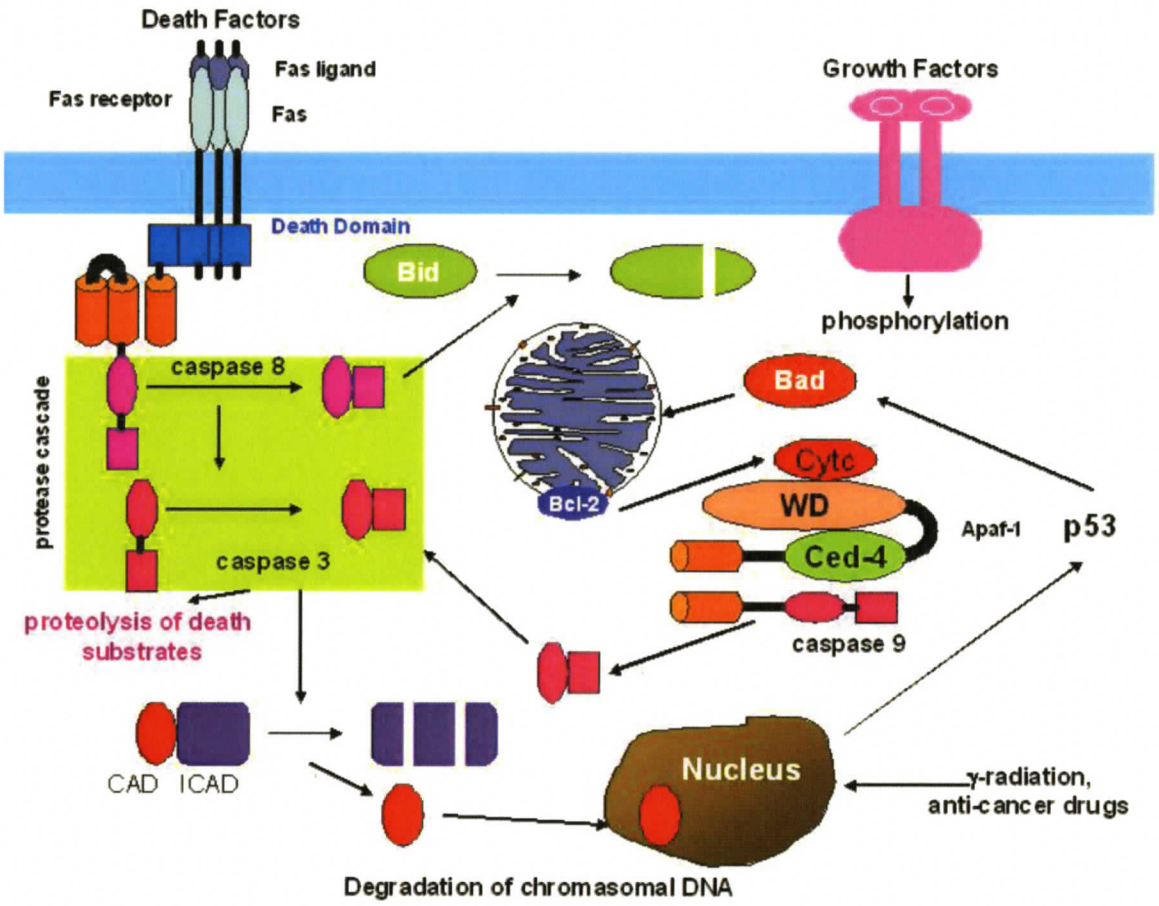

Figure 3.1. Molecular components of the apoptotic cascade. The signal for suicide often comes from outside, through a surface receptor. Fas ligand, binds to Fas receptor, and causes its trimerization. The trimerized death domain in the Fas cytoplasmic region recruits pro-caspase 8. Two routes have been identified to activate caspase 3 by caspase 8. In one route, caspase 8 directly processes pro-caspase 3 in the downstream, and caspase 3 cleaves various cellular proteins including Inactive Caspase-Activated DNase (ICAD). Caspase-Activated DNase (CAD) is released from ICAD, and degrades chromosomal DNA. In another route, caspase 8 cleaves Bid, a pro-apoptotic member of Bcl-2, which translocates to mitochondria to release cytochrome *c* into the cytosol. The cytochrome *c* then activates caspase 9 together with Apaf-1, and caspase 9 in turn activates caspase 3. Genotoxic anti-cancer drugs such as etoposide and γ-radiation generate damage in chromosomal DNA. Adapted from (Nagata *et al.*, 2003).

death-inducing signaling complex (DISC) consisting of Fas receptor, FADD, and pro-caspase 8 (Nagata *et al.*, 2003). Caspase 8 is processed to an active enzyme at the DISC. Caspase 8 can directly activate caspase 9 or can cleave Bid. The truncated Bid then transolocates to mitochondria and stimulates release of cytochrome c, which activates caspase 9 together with Apaf-1. The activated caspase 9 causes processing of pro-caspase 3 to the mature enzyme downstream. Small amounts of cytochrome c released from mitochondria function in a positive feedback loop by binding to insP3 receptors on the endoplasmic reticulum, triggering calcium release (Boehning *et al.*, 2003). This increase in cytosolic calcium stimulates a mass exodus of cytochrome c from mitochondria that induces apoptotic events (Boehning *et al.*, 2003). Caspase 3 activated downstream of the caspase cascade activates a DNase, caspase-activated DNase (CAD). CAD is complexed with an inhibitor of CAD, Inhibitor caspase-activated DNase (ICAD) in proliferating cells but when caspase 3 is activated in apoptotic cells, it cleaves ICAD to release CAD. CAD then causes DNA fragmentation in the nuclei (Nagata *et al.*, 2003).

Methodologies to determine activity of antiestrogens on breast cancer have often been limited to evaluation of fixed cells post treatment, often with relatively few cells processed per experiment. The applications of flow cytometry have been widening to include the monitoring of the effectiveness of antiestrogen therapy, both *in vitro* and *ex vivo.* DNA flow cytometry was instrumental in the study of the effects of prolonged low-dose TAM treatment on ER(-) MDA-MB-231 cells, where it was shown that TAM may have significantly altered cell cycle kinetics and tumourigenicity of these cells, and selected for a new, more aggressive, and rapidly growing clone (Koester *et al.*, 1994).

Thus, it is important to study the trends (reduction of proliferation or increases in differentiation or apoptosis) leading to establishment of a new dynamic balance. Methods of multiparameter flow cytometry are especially suitable for the description of these trends. Flow cytometry has been found to be useful in assessing the following parameters:

1. Proliferation parameters via the detection of the cell cycle stage and staining of DNA-replicating cells.
2. Apoptosis parameters via the detection of DNA fragmentation, phosphtidylserine externalization, mitochrondrial membrane potential detection, morphological changes, intracellular acidification, cytoplasmic dehydration and detection of the activation of caspases.

The rapid processing of large numbers of cells using flow cytometry and the potential to evaluate activity of plant estrogens on live breast cancer cells in vitro would provide much needed information, which would supplement current information available. Also, a comprehensive analysis of the potencies of plant estrogens is lacking.

A flow cytometer identifies different cells by measuring the light that they scatter and the fluorescence that they emit as they flow through a laser beam; the cytometer also has the capacity to sort the cells of a particular type from a mixture (Figure 3.2). The main components of a flow cytometer are:

1. A flow cell in which a liquid stream (sheath fluid) carries and aligns the particles so that they pass in a single cell suspension through the light beam for sensing (Figure 3.3).

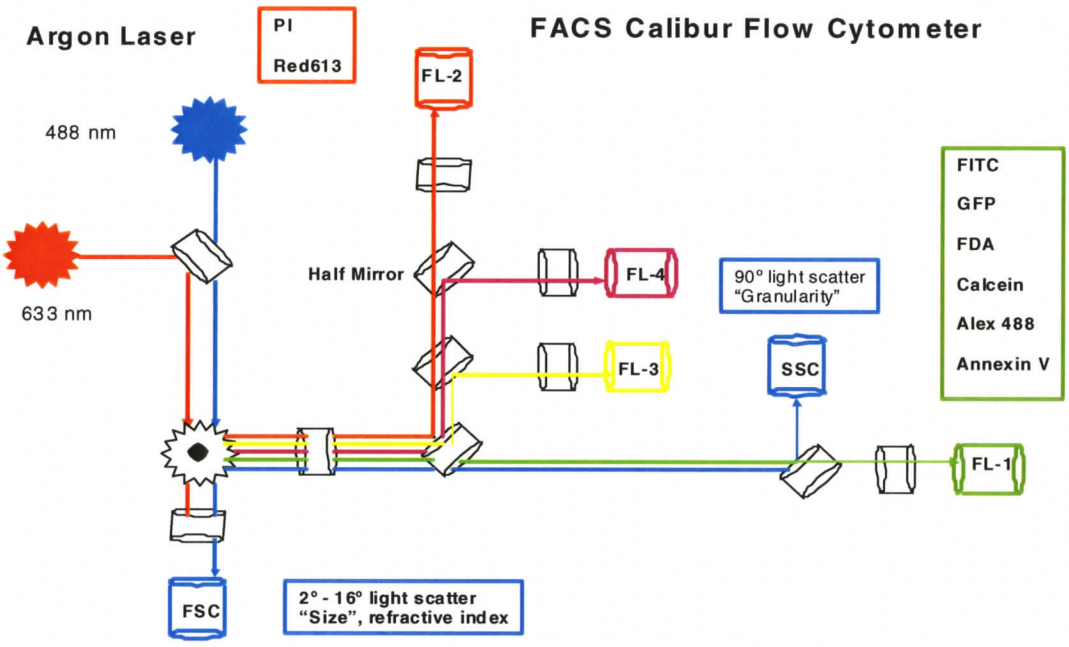

Figure 3.2. Diagram of a flow cytometer. Flow cytometry is a technique allowing simultaneous analysis of multiple cellular parameters, including DNA content. The technique relies on a single-cell suspension being passed within a stream of sheath fluid through an optically focused excitation light source, either a laser or an arc lamp. In flow cytometry analysis, a beam of light (usually laser light) of a single frequency is directed onto a hydrodynamically focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam; one is line with the light beam (forward scatter channel) and several are perpendicular to it (side scatter channel). One or more are fluorescence detectors. (Shapiro, 2004).

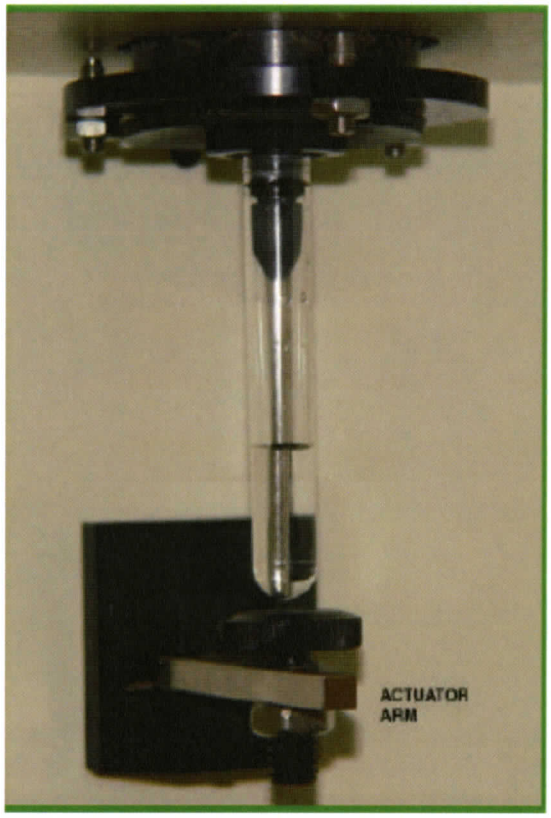

Figure 3.3. Flow cytometer sample uptake port. A sample is connected and actuator arm is in position under the tube for sample uptake. When the actuator arm is moved to the left or right the droplet containment motor is activated which prevents sheath fluid from dripping out of the system.

2. A light source which could include lamps (mercury, xenon); high power water cooled lasers (argon, krypton, dye laser); low power air-cooled lasers: argon (488nm), red-HeNe (633nm), green-HeNe, HeCd (UV), and diode lasers (blue, green, red, violet).

3. A detection and analogue to digital conversion system which can generate forward scatter channel (FSC) and side scatter channel (SSC) as well as fluorescence signals.

4. An amplification system which is linear or logarithmic.

5. A computer for analysis of the signals (MacOS was used a the operating system for a MAC computer with the flow cytometer in Pearson lab at UVic).

Flow cytometry is a rapid technique allowing simultaneous analysis of multiple cellular parameters, including DNA content (Macleod and Langdon, 2004). A single-cell suspension is passed within a stream of sheath fluid through an optically focused excitation light source, either a laser or an arc lamp. A number of detectors are aimed at the point where the stream passes through the light beam; one is aligned with the light beam (FSC) and several detectors are perpendicular to it (SSC); one or more is a fluorescence detector. The most common excitation wavelength used in flow cytometers is 488 nm wavelength light from an argon laser (Macleod and Langdon, 2004). Lasers provide a single wavelength of coherent light, whereas arc lamps produce a mixture of incoherent wavelengths that must be filtered. When a laser light source is used, the amount of light scattered in the forward direction FSC is detected in the forward angle light scatter channel and the light intensity is roughly proportional to the size of the cells

or particles (Macleod and Langdon, 2004). The amount of light scattered to the side at a right angle is a measure of cell granularity and complexity (Figure 3.4). If the cells have been stained with fluorochromes, they will also emit fluorescence intensities at levels that directly correspond to the density of fluorochrome on or within the cell (Macleod and Langdon, 2004). The fluorescence signal emitted by any specific fluorochrome (Table 3.1) is collected through separate channel detectors (photomultipliers) by means of a series of optical filters and mirrors that guide the beam of light. In order to simultaneously measure more than one fluorescence signal from any given cell, multiple channels/detectors are used (Macleod and Langdon, 2004).

DNA dyes (such as propidium iodide) are useful in that they are can be used to quantify DNA within a cell. Fluorescein diacetate (FDA) is currently the most commonly-used fluorescent dye for FACS analysis of cell viability (Shapiro, 2004). FITC is a small organic molecule , and is typically conjugated to proteins via primary amines (i.e. lysines) (Shapiro, 2004). Usually, only a few FITC molecules are conjugated to the protein; higher conjugations may result in solubility problems as well as internal quenching (and reduced brightness). FDA is typically excited by the 488 nm line of an argon laser, and emission is collected at 530 nm (Shapiro, 2004).

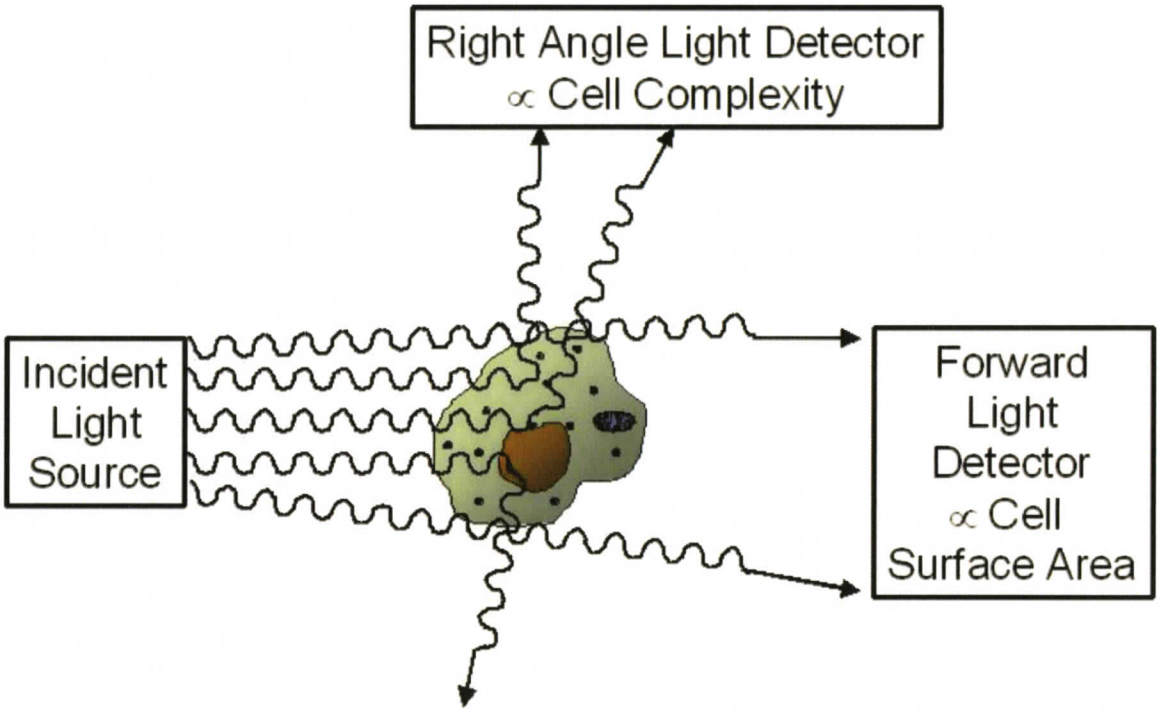

Figure 3.4. Properties of forward scatter (FSC) and side scatter (SSC) in flow cytometry. Forward scatter is diffracted light proportional to cell surface area. FSC is detected along axis of incident light in the forward direction. SSC is reflected and refracted light proportional to cell granularity and complexity. It is detected at 90° to the laser beam. Adapted from (Shapiro, 2004).

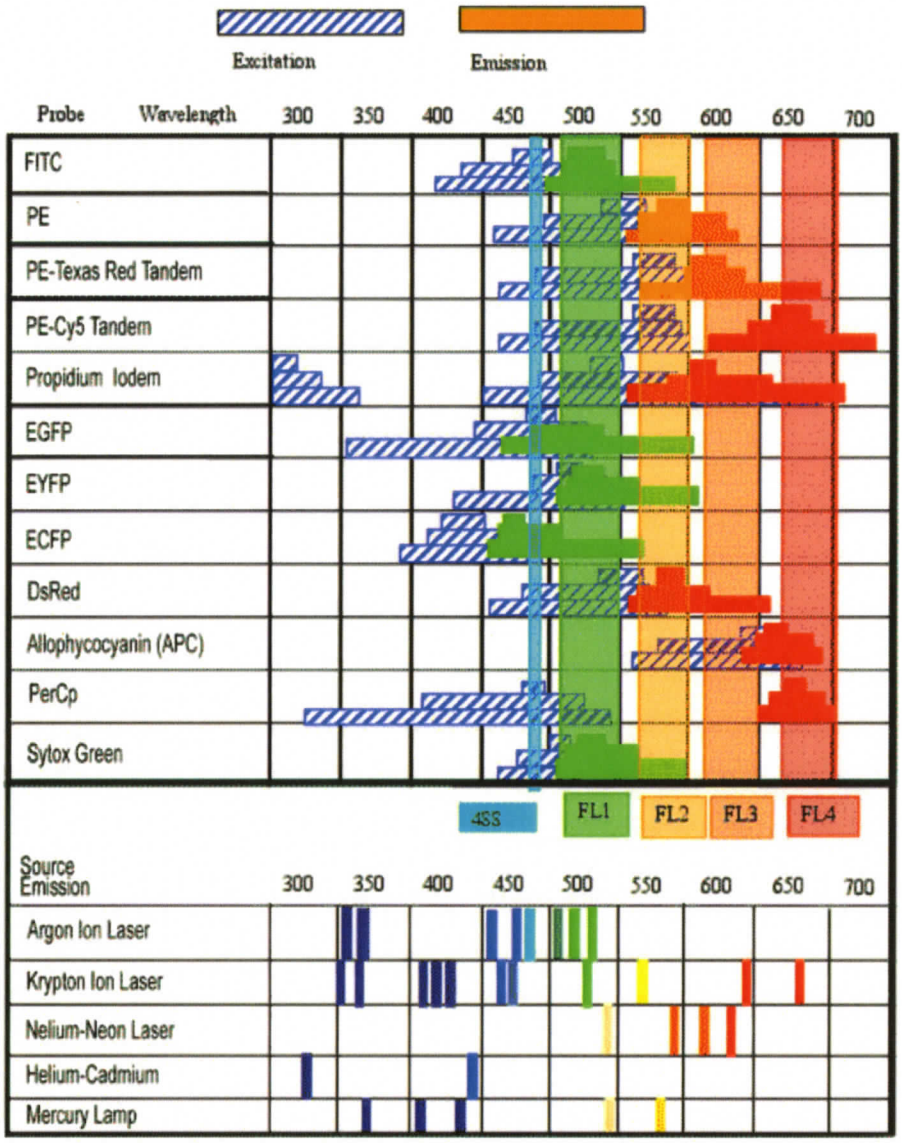

Table 3.1. Fluorescence spectra of commonly used fluorochromes. Excitation spectra are represented by diagonal lines while emission spectra are in solid colours. The bottom section is a summary of the emission wavelengths of various light sources used in flow cytometry.

Protocols for the measurement of apoptotic parameters:

Annexin V/PI assay

Apoptotic cells can be distinguished from viable, dead, or necrotic cells by several methods on the basis of morphological and biochemical characteristics. The activation of the caspase family of proteases results in the breakdown of cellular proteins such as the nuclear matrix, the cytoskeleton, and the poly-ADP-ribose polymerase, either directly or through activation of other cellular proteases such as calpain or proteasomes (Nagata *et al.*, 2003). At this stage of the process, apoptotic cells also display phosphatidylserine (PS) at their outer membrane (van Engeland *et al.*, 1998). Viable cells maintain an asymmetric distribution of different phospholipids between the inner and outer leaflets of the plasma membrane (Lodish *et al.*, 2004). Choline-containing phospholipids, phosphtidylcholine and sphingomyelin, are primarily located in the viable cells' outer leaflet. Aminophospholipids, phosphatidylethanolamine, and PS are found at the cytoplasmic face (Lodish *et al.*, 2004).

The plasma membrane lipid asymmetry in viable cells is maintained by flippases, membrane proteins that facilitate the translocation of lipid molecules from one leaflet to the other (Lodish *et al.*, 2004). The relationship between PS and apoptosis was first identified with leukocytes. In leukocytes during apoptosis, PS is exposed on the outer leaflet of the plasma membrane (van Engeland *et al.*, 1998). This cell-surface PS functions as a tag for specific recognition of macrophages and for phagocytosis of the dying cell (Macleod and Langdon, 2004). Investigations into the exposure of PS at the outer membrane leaflet of cells was facilitated by the finding that annexin V specifically binds to PS in the presence of calcium (Macleod and Langdon, 2004). Using

fluorescently labeled annexin V, van Engeland (1998) demonstrated that apoptotic lymphocytes expose PS at their outer membrane early after onset of the execution phase of apoptosis. PS appears at the outer leaflet of the plasma membrane, the integrity of which has not been compromised as yet. PS exposure seems to last from the beginning of apoptosis until the final stage, when the cell has broken into apoptotic bodies (van Engeland *et al.*, 1998).

Annexin V is an impermeable plasma protein which specifically binds to phosphatidylserine once it is translocated to the outer leaflet of the membrane during apoptosis (Saha *et al.*, 2003). Annexin V is often used to detect cells undergoing apoptosis as Annexin V is not able to penetrate the phospholipid bilayer (van Engeland *et al.*, 1998). Based on the phenomenon that PS is exposed during apoptosis and has the ability to bind annexin V with high affinity, a method was developed to use extrinsically applied hapten (i.e. FITC) labeled annexin V to detect apoptosis (van Engeland *et al.*, 1998). Figure 3.5 is an example of how annexin V/PI separates Jurkat cells (T-cell leukemia, human) into three groups using flow cytometry: live cells, apoptotic cells, and necrotic cells.

Fluorescein diacetate/PI assay

Esterases are universally present in all types of viable cells. Therefore, the disappearance of esterases has been considered an important marker of apoptosis (Saha *et al.*, 2003). FDA, which reacts with esterases, in combination with propidium iodide (PI)

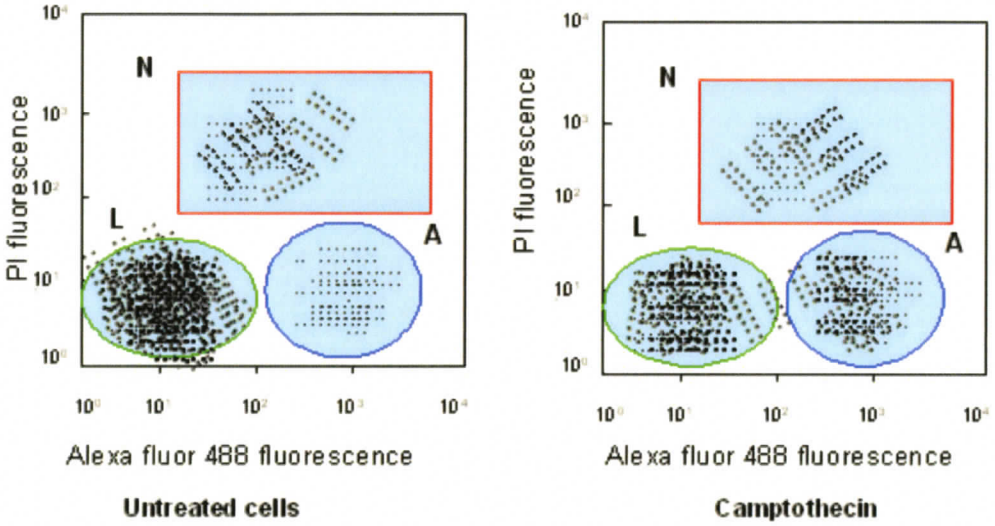

Figure 3.5. Example of Annexin V/PI assay**.** Jurkat cells (human T-cell leukemia), untreated (left panel) or treated with camptothecin 10 μM for four hours (right panel). Cells were then treated with Annexin V and propidium iodide, followed by flow cytometric analysis. Note that the comptothecin-treated sample has a higher percentage of apoptotic cells than the untreated sample. A = apoptotic cells, L = live cells, and N = necrotic cells. (Adapted from Koopman *et al.*, 1994).

provided the basis to develop a double staining flow cytometric assay (Saha *et al.*, 2003). The FDA/PI technique is comparable to the Annexin V-PI technique and can be used accurately in the quantification of apoptosis in fish leucocytes (Saha *et al.*, 2003). The results of Saha *et al.* (2003) suggest that the disappearance of esterases and externalization of PS may be common to many apoptotic pathways. FDA, a non-fluorescent molecule, can penetrate the cell membrane and be hydrolyzed by non-specific esterases in viable cells; green fluorescence is produced and retained in the cytoplasm of the intact cells (Saha *et al.*, 2003). In the case of apoptotic and dead cells, fluorescence is not produced due to the lack of esterases. The FDA leaks away from the cells because of poor membrane integrity (Saha *et al.*, 2003). In addition, an apoptotic cell is unable to metabolize FDA (Saha *et al.*, 2003). PI, a nucleic acid binding dye, cannot penetrate the membrane of viable cells, but can enter cells readily during the cell death process due to a loss of membrane integrity (Saha *et al.*, 2003). Therefore, FDA and PI co-labeling is able to distinguish viable ($FDA^{+}PI^{-}$) and dead cells ($FDA^{-}PI^{+}$) from apoptotic cells, which are FDA and PI negative ($FDA^{-}PI^{-}$).

Protocols for measurement of DNA content and stage of cell cycle SYBR-14/PI assay

Utilization of SYBR-14® , a DNA stain used for detection of DNA in gels, and PI to measure DNA content and analyze the cell cycle stage in living human cells lines was previously developed for sperm quality assessment in 1995 (Nunez *et al.*, 2004a). Recently, the sorting of living DNA-stained breast cancer cells based on DNA content and cell cycle stage has been reported by Nunez *et al.* (Nunez *et al.*, 2004a). The utilization of SYBR-14 for staining DNA of living normal and tumour cells is an example

of a potential innovative method that could help to overcome the restrictions of the traditional DNA dyes and cell cycle protocols. There appears to be some advantages over traditional methods such as elimination of the need to fix cells and the stability of SYBR-14 independent of time (Nunez *et al.*, 2004a).

Materials and Methods

Chemicals and reagents

In addition to the chemicals used in Chapter 2, E_2, PI, and FDA were purchased from Sigma Chemical Co. Annexin V was purchased from Molecular Probes Inc. (Eugene, OR). The JC-1 fluorescent stain and 10x assay buffer were kindly supplied by Lee Haines, Pearson Lab, UVic. The E_2 stock solution was prepared by dissolving E_2 in ethanol and then mixing with medium according to the manufacturer's instructions (20 μg/ml). OHT stock solutions were prepared in ethanol. The final ethanol concentration in the medium was <0.3%. GEN was prepared in DMSO with a final concentration of DMSO at <0.1% in the cells.

Cell lines

All details concerning *cell lines* and *tissue culture conditions* were identical to those used in Chapter 2. The cells were pelleted by centrifugation at 500 x g for 5 minutes at 2-8 ° C and the supernatant was discarded.

Flow Cytometric Analysis

In selected time intervals after experimental treatment, proliferation and cell death were detected simultaneously to obtain cytokinetic data. Multiparametric flow cytometer analysis were the principal methods used, since they allow concurrent detection of several parameters, i.e. specific gene or intracellular molecular expression during individual phases of the cell cycle, together with markers of cell death.

Two-parameter and mulitcolour flow cytometric analysis was performed using a FACS/Calibur flow cytometer (Becton Dickenson, San Jose, CA) equipped with an argon ion laser operating at 488 nm. This allowed both forward and side scatter analysis. Forward scatter represents diffracted light with detection along the axis of incident light in the forward direction capturing cell surface area. Side scatter reflected and refracted light related to granularity and complexity of the cells. The cells were routed to the generated light signals and specific detectors by the collection optics. For analysis of apoptosis, necrosis, and stage of live cell cycle, percentage of cells in each category was evaluated using Cellquest software.

Annexin V/PI assay

This procedure was used for the evaluation of the effects of treatment with OHT, ENL, GEN and/or E_2 on distribution of live, apoptotic and necrotic cells. The human vascular anticoagulant, annexin V, is a Ca^{++}-dependent phospholipids-binding protein that has a high affinity for PS. Use of a cell-impermeant dead-cell stain in combination

with annexin V-conjugate staining was helpful in distinguishing necrotic cells from apoptotic cells.

Cells were seeded at 5.0 x 10^5 per well and incubated with 10% DCC-FBS-DMEM (phenol red-free) for 3 days. The cells were treated with various doses of GEN, ENL or OHT for 24 hours. Cells were removed from the 24-well plate and washed with cold PBS and supernatant was discarded. The cells were resuspended in Annexin-binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$ prepared in distilled water, pH 7.4) to a density of 1.0 x 10^6 cells/ml. Cell suspensions of 100 µl per assay were aliquoted in separate Eppendorf tubes. Five µl of the annexin V conjugate and 25 µl of 1 mg/ml PI were added to each 100 µl cell suspension. The cells were incubated at room temperature (rt) for 15 mins and 400 µl of Annexin-Binding buffer was added. The cells were mixed gently and placed on ice. The cells were analyzed immediately using flow cytometry.

FDA/PI live-cell assay

This procedure was used for the evaluation of the effects of treatment with OHT, GEN, ENL and/or E_2. Necrotic cells are FDA-negative and PI-positive. Healthy cells are FDA-positive and PI-negative. Because apoptotic cells maintained membrane integrity until late in the death process but were increasingly unable to convert FDA (Erhardt and Sherwood, 2004). Cells were seeded 5.0 x 10^5 per well and incubated with 10% DCC-FBS-DMEM phenol red-free for 3 days. The cells were treated with various doses of GEN, ENL or OHT with or without E_2 for 24 hours. Cells were also treated with combined doses of OHT and 1.0 nM E_2. Cells were removed from the 24-well plate and

washed with PBS. The cells were incubated in 500 µl of 0.05 µg/ml FDA in PBS for 30 minutes at 37° C and 500 µl of 0.4 µg/ml PI in distilled water for 30 minutes at rt. The cells were resuspended in a cold 2% FBS in PBS and maintained on ice until analysis by flow cytometry.

SYBR/PI live-cell assay

This procedure was used to measure the DNA content and effect on cell cycle of breast cancer cell lines treated with OHT, GEN, ENL and/or E_2. Three populations of cells of interest were sorted: SYBR-I-stained living cells, PI-stained dead cells, and moribund cells that are double-stained (Nunez *et al.*, 2004a). In order to define the stage of cell cycle, the microtubule polymerization inhibitor nocodazol was utilized for blocking the cells at the G_2/M stage of cell cycle. Cells were seeded at 5.0×10^5 per well in 24-well culture plates and incubated with 15% DCC-FBS-phenol red-free DMEM for 3 days. Once the cells were removed from the 24-well plate, they were washed with PBS and gently resuspended in 5 µl of 20 mM SYBR-I in 500 µl of PBS for 30 minutes at room temperature and 500 µl of 0.4 µg/ml PI in distilled water for 30 minutes at room temperature. The cells were resuspended in 2% FBS in PBS and maintained on ice until analysis by flow cytometer.

Data Analysis

For each experiment, a minimum of 20,000 events via flow cytometry were evaluated. Samples were measured in triplicate and each experiment was performed three times. The percentage live cells from each assay were calculated from the mean ± of at

least three independent experiments. The data were analyzed by one-way ANOVA followed by Dunnet Multiple Comparisons test with InStat3.0 Software (Graphpad Software, Inc., San Diego, CA). Statistical significance was assessed at $p<0.05$, $p<0.01$ and $p<0.001$. ANOVA assumes that the data were sampled from populations that follow Gaussian distributions. This assumption was tested using the Kolmogorov and Smirnov method.

Results

Comparison of the FDA/PI and Annexin V/PI methods

The two methods were compared using identical treatments in MDA-MB-231 breast cancer cells and analyzing the live, apoptotic, and necrotic populations using both methods concurrently. The treatments were with ENL 10 μM, GEN 10 μM, OHT 50 μM, and OHT 50 μM combined with ENL 10 μM along with untreated samples which were used as control samples. The comparison of the four treatments is seen in Figure 3.6. Statistical analysis showed a highly significant correlation between the FDA/PI and Annexin V/PI methods**.** Dot plot analyses are shown in Figure 3.7. Live, apoptotic and necrotic MDA-MB-231 breast cell populations in ENL and GEN 10 μM were distinguished by the FDA/PI live cell assay method and the Annexin V/PI assay.

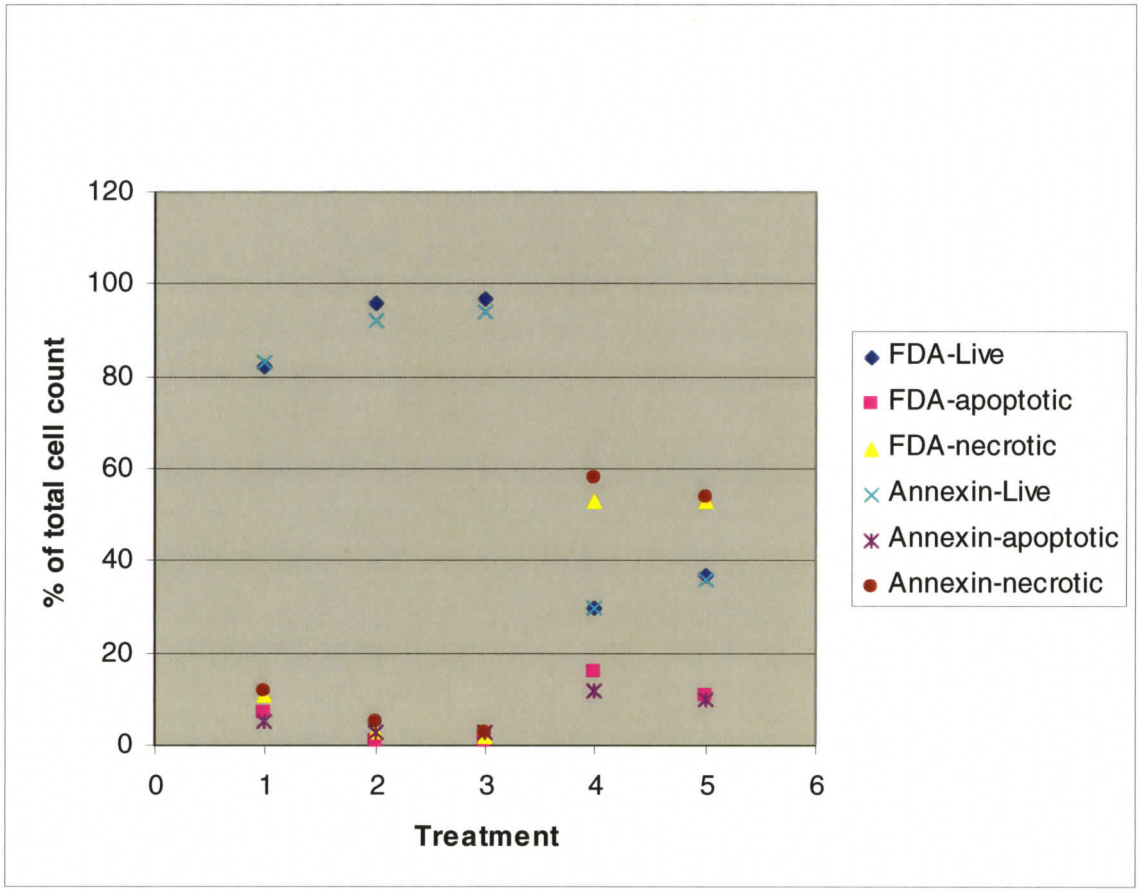

Figure 3.6. Comparison of Annexin V and FDA fluorescent stains in the identification of live, apoptotic, and necrotic cell populations in MDA-MB-231 breast cancer cell lines. Treatments included ENL 10 μM, GEN 10 μM, OHT 50 μM, and ENL 10 μM combined with OHT 50 μM. Two groups of controls of untreated cells were included in the comparison of the two fluorescent stains. There was a highly significant correlation between the Annexin V/PI and FDA/PI methods of identifying live, apoptotic and necrotic cell populations.

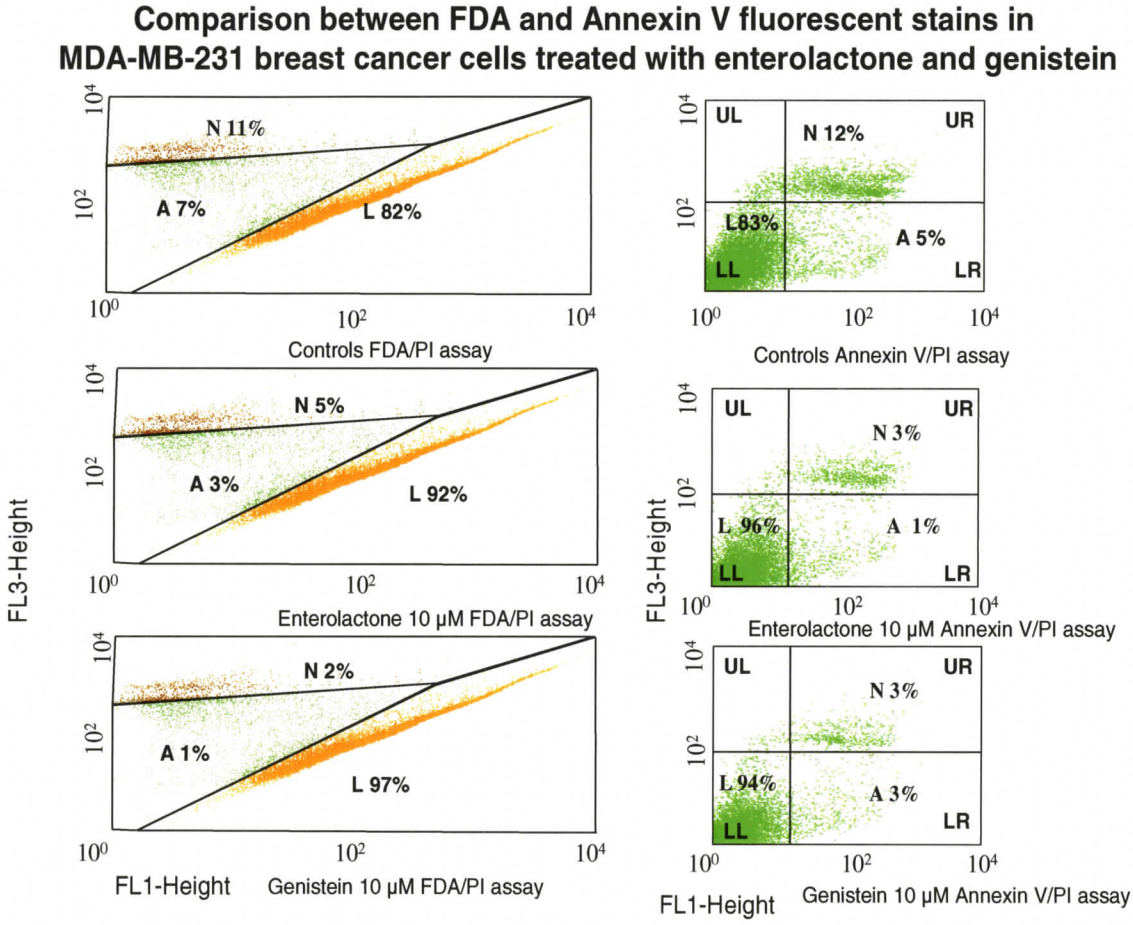

Figure 3.7. FDA/PI live cell assay technique was compared to the Annexin V-PI live cell assay technique in the accurate quantification of apoptosis in breast cancer cells. The two methods were comparable using two sets of identical treatments in MDA-MB-231 breast cancer cells. One set of treatments was ENL 10 µM (top panels) and the second set was GEN 10 µM (bottom panels). The live, apoptotic, and necrotic populations were analyzed using both methods concurrently.

The two methods were compared using two sets of identical treatments in MDA-MB-231 breast cancer cells. The live, apoptotic, and necrotic populations were analyzed using both methods concurrently. In samples treated with ENL 10 μM using the FDA/PI method, 92% of cells were live or FDA^+ but PI^- indicating viable cells, whereas the others were FDA^- cells. Since PI binds with dead cells, FDA^-PI^- cells were apoptotic (3%) cells. The PI^+ regions indicated dead, necrotic cell populations (5%). In the Annexin V/PI method, the cells in the LL quadrant were both Annexin V^- and PI^- (96%), indicating viability. Annexin V^+ cells in the LR quadrant indicate apoptotic cells (1%) and PI^+ cells in the UL and UR quadrant indicate necrotic cells (3%). The samples treated with GEN 10 μM and evaluated using the FDA/PI method showed 97% cells were FDA^+ but PI^- indicating live cells. Necrosis accounted for 2% and apoptotic was 1% of the cell population. In the Annexin V/PI method, the cells in the LL quadrant were live (94%). The PI^+ cells in the UR and UL quadrants (3%) were necrotic cell populations. In the LR quadrants (3%), the cells were experiencing apoptosis.

Usefulness of SYBR method in detecting apoptosis

PI enters apoptotic and necrotic cells with peaks in the appropriate FL-2-height ranges (10^0-10^2), however SYBR-I staining did not indicate peaks (Figure 3.8). Although absorption occurred in FL-1, the lack of peaks present may indicate the dye did not completely enter the DNA. Arrest of cell cycle at the G_2/M stage with nocodazol was tried. Nocodazol was difficult to maintain in solution and caused blockage of the flow cytometric equipment and was not further investigated.

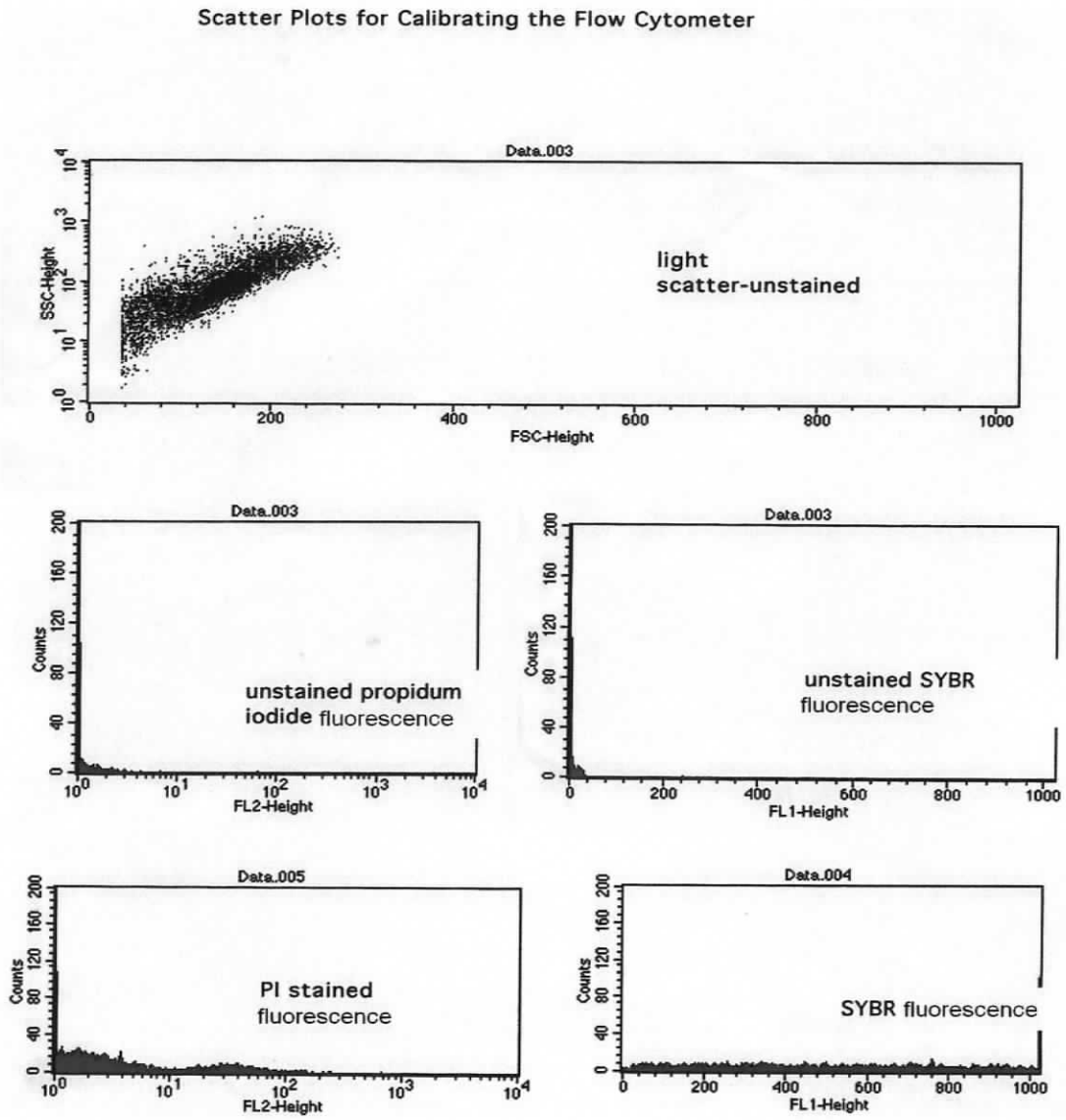

Figure 3.8. The use of SYBR/PI assay for determining DNA content and stage of cell cycle in live MCF-7 cells. Compared to the unstained cells, PI enters apoptotic and necrotic cells with peaks in the appropriate ranges (10^0 - 10^2), however SYBR-I staining did not indicate peaks. Although absorption occurred in FL-1, the lack of peaks present may indicate the dye did not completely enter the DNA.

Fluorescence microscopic observation

MDA-MB-231 cells stained with FDA/PI contained both FDA^+ and PI^+ cells (Figure 3.9). Since FDA^+ and PI^+ reflect live and dead cells respectively, FDA^- PI^- cells were regarded as the apoptotic cells as indicated by the arrow. Cultured cells that were heat killed and then stained with FDA/PI contained only PI^+ cells (Figure 3.10) and did not stain with FDA indicating a lack of live cells present.

Effect of ENL and GEN using FDA/PI method on MCF-7 cells

In MCF-7 breast cancer cells, treatments with 50 μM and 100 μM GEN combined with E_2 significantly decreased viability ($p<0.01$) (Figure 3.11). ENL and GEN alone at 100 μM significantly decreased proliferation ($p <0.05$ and $p<0.01$ respectively). Dot plot results show differences in live, apoptotic and necrotic cell populations between untreated controls and treatments of combined with E_2 1.0nM/50 μM GEN (Figure 3.12). Dot plot results show differences in live, apoptotic and necrotic cell populations between untreated controls and treatments of 100 μM ENL (Figure 3.13).

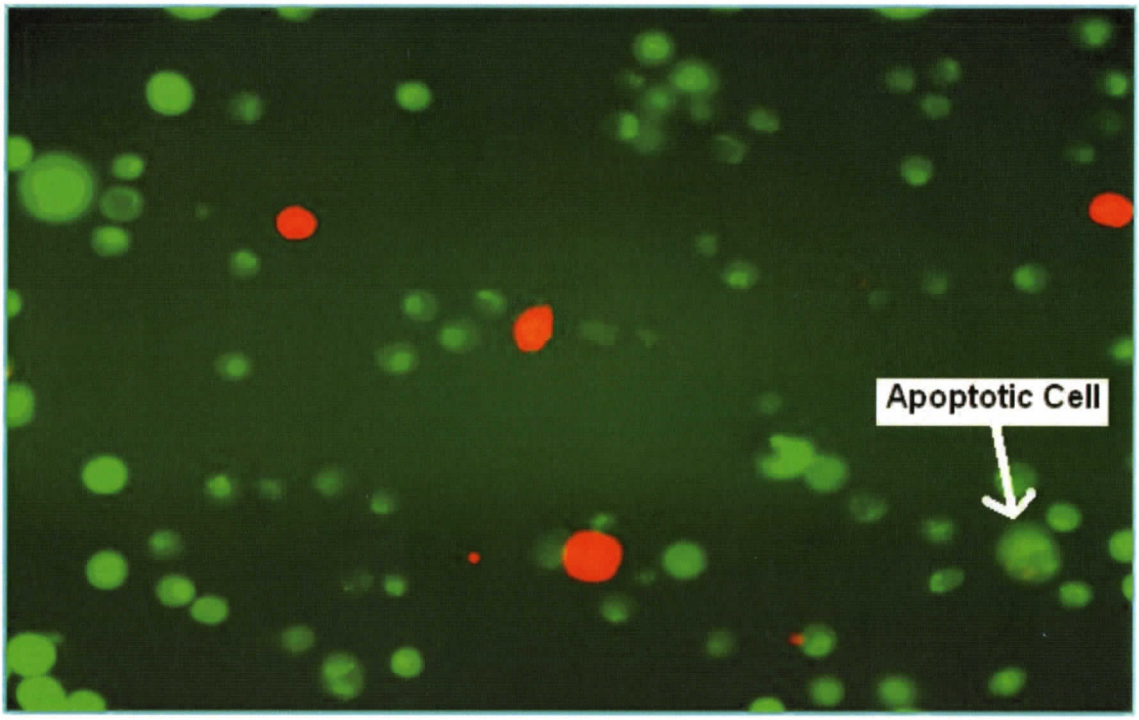

Figure 3.9. Fluorescence microscope image of FDA/PI-stained MDA-MB-231 breast cancer cells. Arrow indicates a doubly stained cell which is apoptotic. Live cells are FDA^{+} / PI^{-} (green), necrotic cells are FDA^{-} / PI^{+} (red) and apoptotic cells are FDA^{-} / PI^{-} (decreasing green and increasing red).

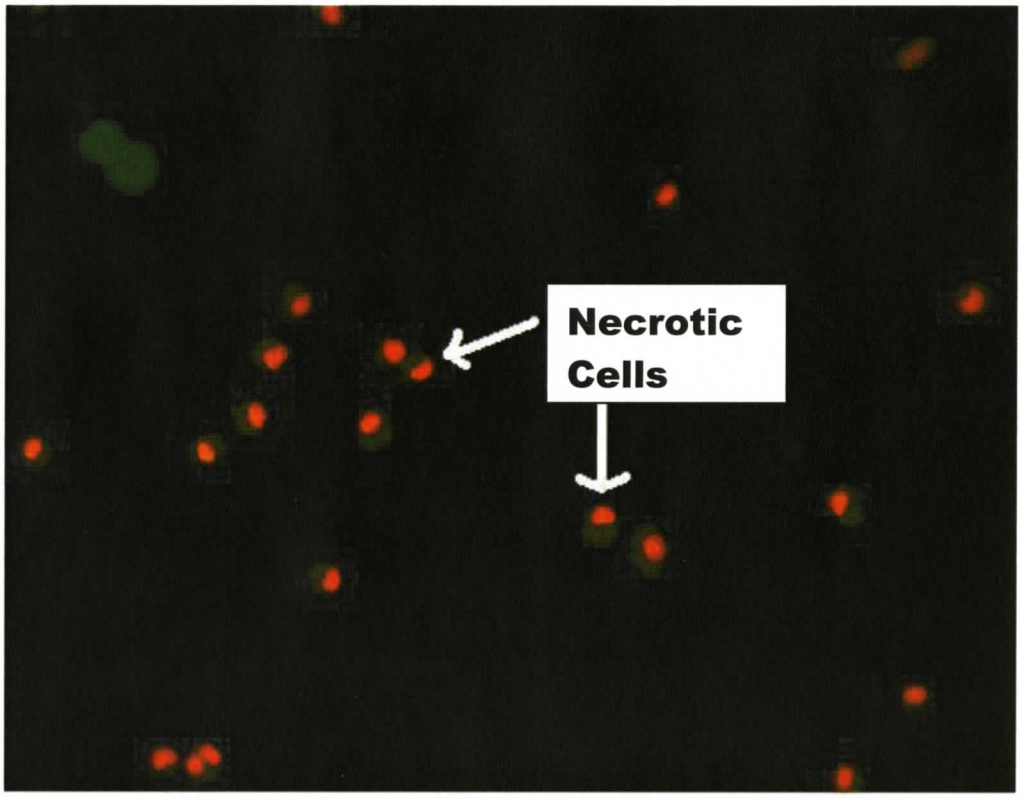

Figure 3.10. MDA-MB-231 breast cancer cells that have been heat-killed and stained with both FDA and PI. The cells are primarily PI-stained (red colour) with no FDA staining indicating these are mostly dead or necrotic cells (as indicated with arrow). This method of heat-killing cells was used in each FDA/PI live cell assay to confirm the necrotic population of cells.

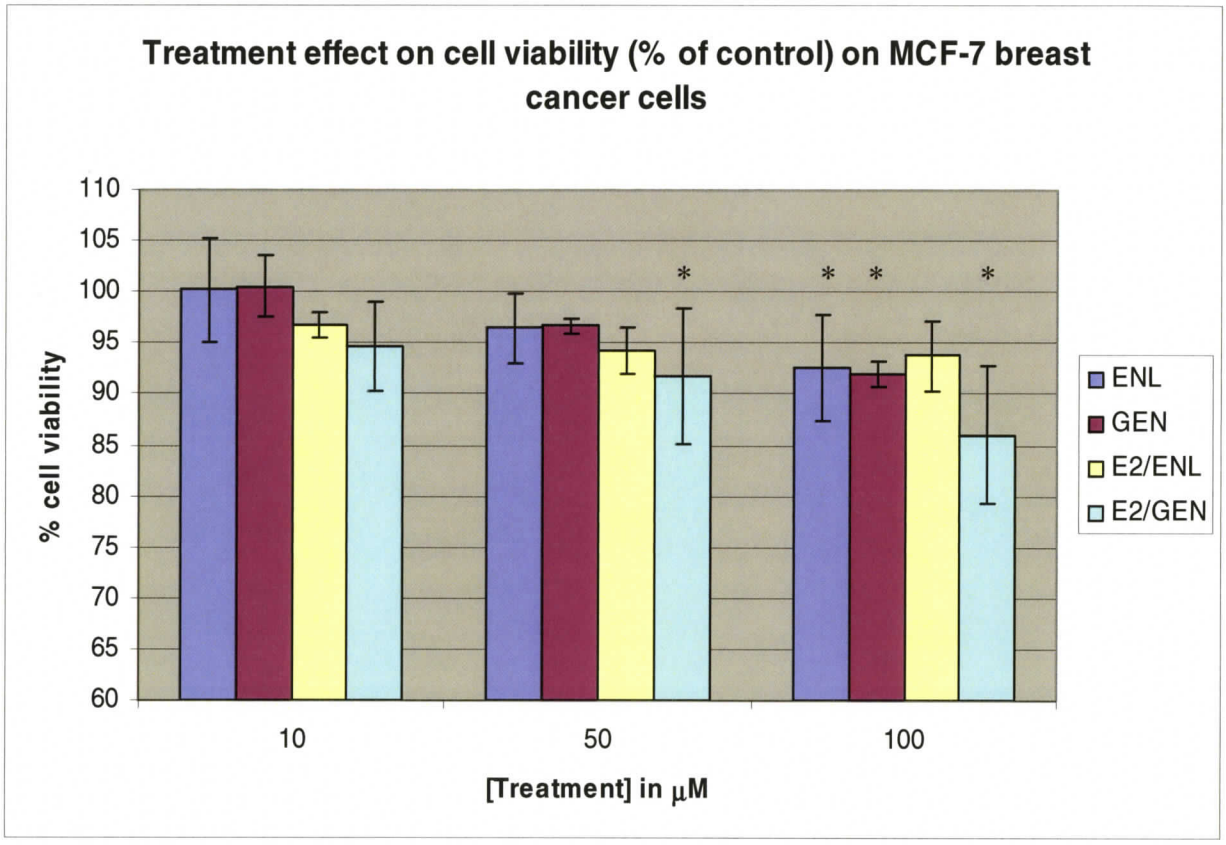

Figure 3.11. The effect of ENL and GEN treatments on MCF-7 breast cancer cells using FDA/PI assay. GEN combined with E_2 at 50 and 100 μM significantly decreased viability ($p<0.01$). ENL and GEN alone at high doses, i.e. 100 μM significantly decreased viability ($p<0.05$ and $p<0.01$ respectivley).

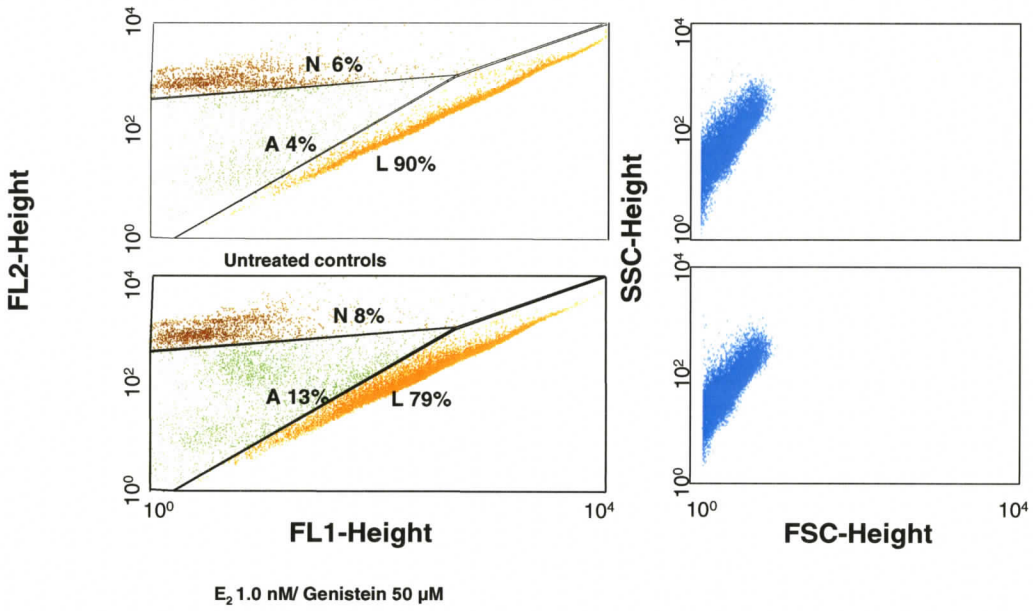

Figure 3.12. FDA/PI live-cell assay dot plot with live (L), apoptotic (A) and necrotic (N) cell populations of untreated controls and treatments with E_2 1.0 nM/genistein 50 µM in MDF-7 breast cancer cells. Treatment with E_2 1.0 nM/ GEN 50 µM significantly decreased viability ($p<0.01$). SSC versus FSC dot plots demonstrated cells were of consistent size and granularity.

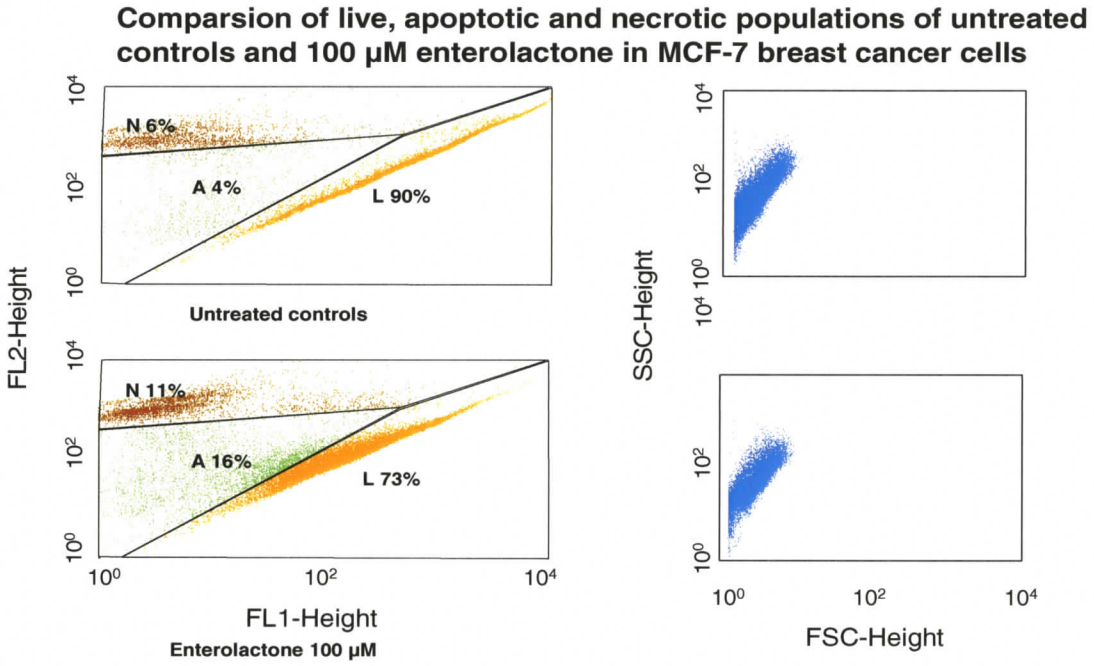

Figure 3.13. Dot plot results with live, apoptotic and necrotic cell populations between untreated controls and treatments of 100 µM ENL. Treatments of 100 µM ENL significantly decreased viability ($p < 0.001$). SSC versus FSC dot plots demonstrated cells are of consistent size and granularity.

In the one-way analysis of variance (ANOVA), the P value was < 0.001 which was considered highly significant. Variation among column means was significantly greater than expected by chance. Dunnett Multiple Comparisons test was conducted on the values. ANOVA assumed that the data were sampled from populations that followed Gaussian distributions. This assumption was tested using the method of Kolmogorov and Smirnov and all groups passed normality testing.

T47D cells

In T47D breast cancer cells, treatment with ENL and GEN 10 μM and 50 μM alone had no effect on viability (Figure 3.14). Treatment with 100 μM of ENL and GEN significantly reduced viability ($p < 0.001$)). The dot plot results show differences in live, apoptotic and necrotic cell populations between untreated controls and treatments of 100 μM ENL (Figure 3.15). Combining ENL or GEN with E_2 did not decrease proliferation.

In the one-way analysis of variance (ANOVA), the P value was < 0.001 which was considered highly significant. Variation among column means was significantly greater than expected by chance. Dunnett Multiple Comparisons test was conducted on the values. ANOVA assumed that the data were sampled from populations that followed Gaussian distributions. This assumption was tested using the method of Kolmogorov and Smirnov and all groups passed normality testing.

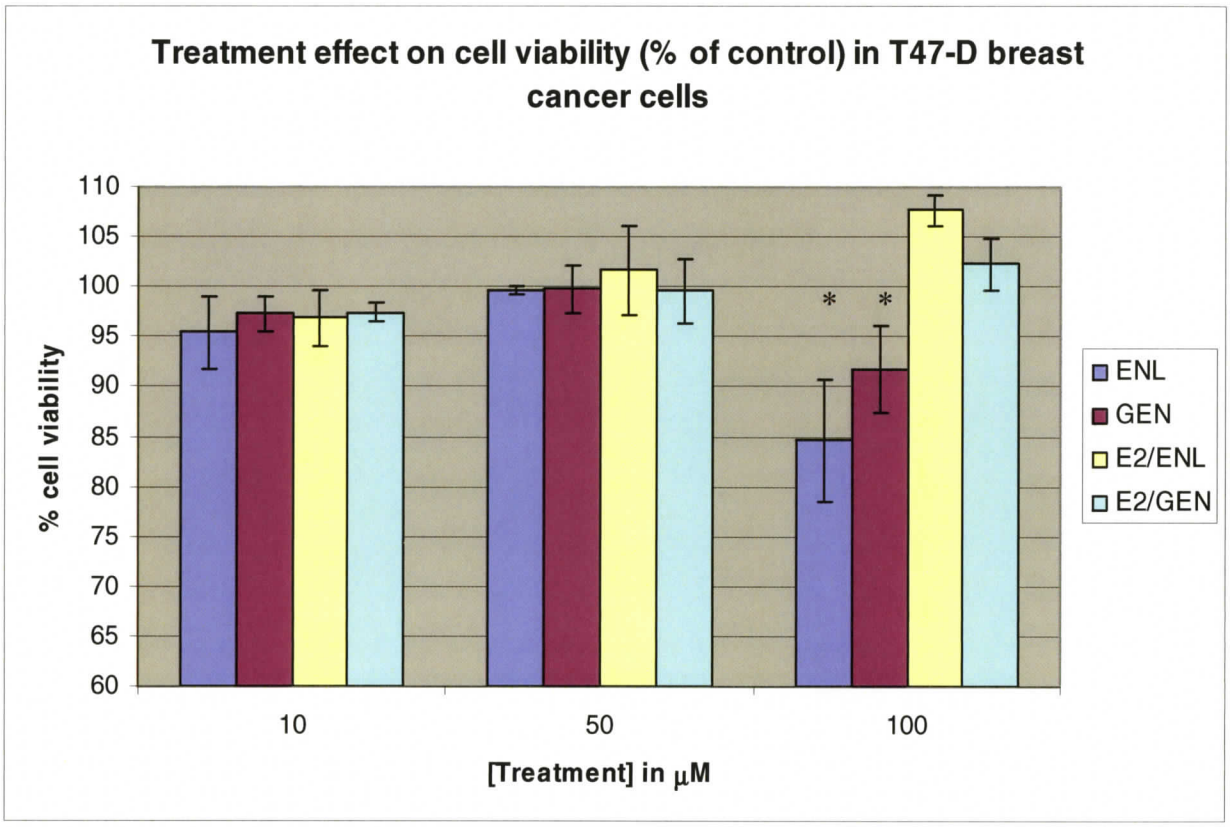

Figure 3.14. The effect of ENL and GEN treatments on T47D breast cancer cells using FDA/PI assay. In T47D breast cancer cells, ENL and GEN alone at doses of 10 μM and 50 μM had no effect on viability. At high doses i.e. 100 μM, ENL and GEN significantly reduced viability. Combining ENL and GEN with E_2 did not affect viability.

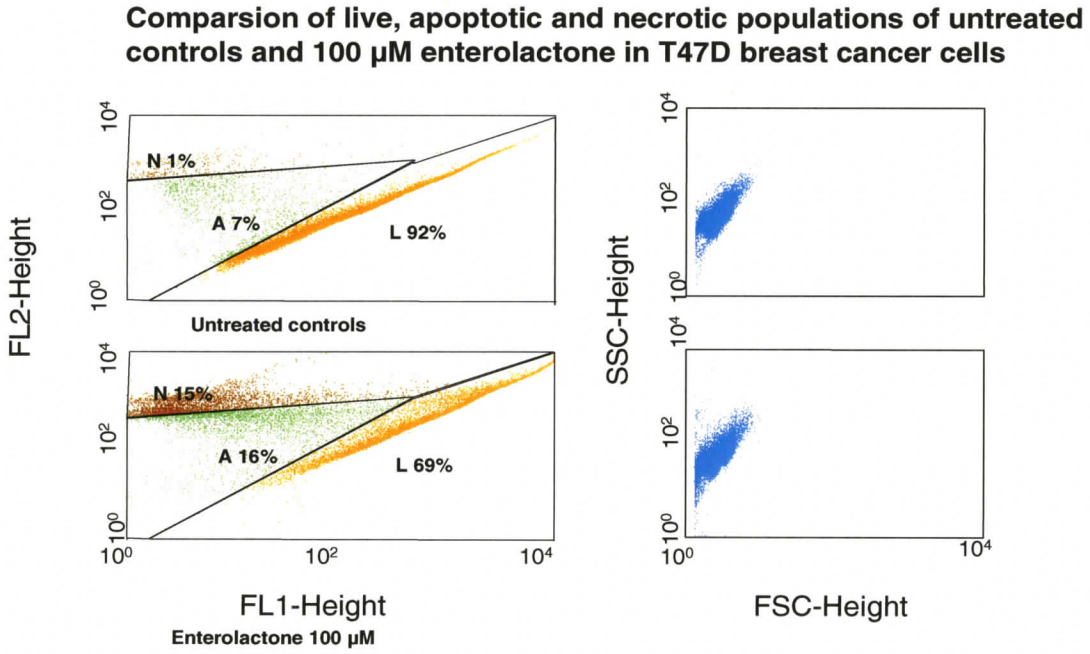

Figure 3.15. FDA/PI live-cell assay dot plot with live (L), apoptotic (A) and necrotic (N) cell populations of untreated controls and treatments of ENL 100 µM in T47D breast cancer cells. ENL significantly reduced viablity ($p < 0.001$) in T47D breast cancer cells. SSC versus FSC dot plots demonstrated cells are of consistent size and granularity.

MDA-MB-231 cells

In MDA-MB-231 breast cancer cells, treatment with ENL 10 μM significantly reduced viability ($p < 0.001$)(Figure 3.16). Dot plot results show differences in live, apoptotic and necrotic cell populations between untreated controls and treatments of 10 μM ENL (Figure 3.17). Treatment with 100 μM GEN combined with E_2 significantly reduced viability ($p < 0.05$).

In the one-way analysis of variance (ANOVA), the P value was < 0.001 which was considered highly significant. Variation among column means was significantly greater than expected by chance. Dunnett Multiple Comparisons test was conducted on the values. ANOVA assumed that the data were sampled from populations that followed Gaussian distributions. This assumption was tested using the method of Kolmogorov and Smirnov and all groups passed normality testing.

MDA-MB-468 cells

In MDA-MB-468 breast cancer cells, ENL or GEN alone did not reduce proliferation (Figure 3.18). Treatments with 100 μM ENL or GEN significantly increased proliferation ($p<0.001$ and $p<0.01$ respectively). Because there was no reduction in proliferation seen at any of the treatment dosages, these cells were not tested in combination with E_2.

In the one-way analysis of variance (ANOVA), the P value was < 0.001 which was considered highly significant. Variation among column means was significantly greater than expected by chance. Dunnett Multiple Comparisons test was conducted on the values.

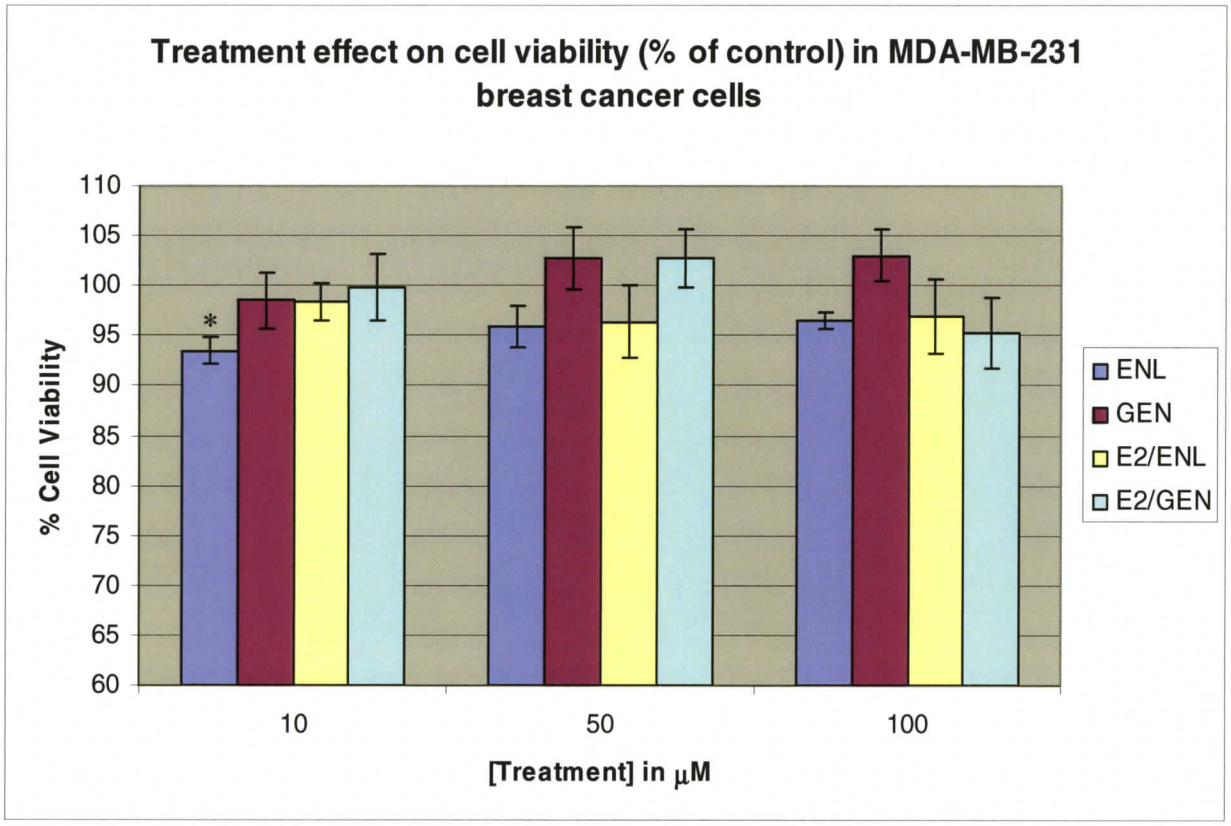

Figure 3.16. The effect of ENL and GEN treatments on MDA-MB-231 breast cancer cells using FDA/PI assay. In MDA-MB-231 breast cancer cells, ENL alone at doses of 10 μM significantly reduced viablity.

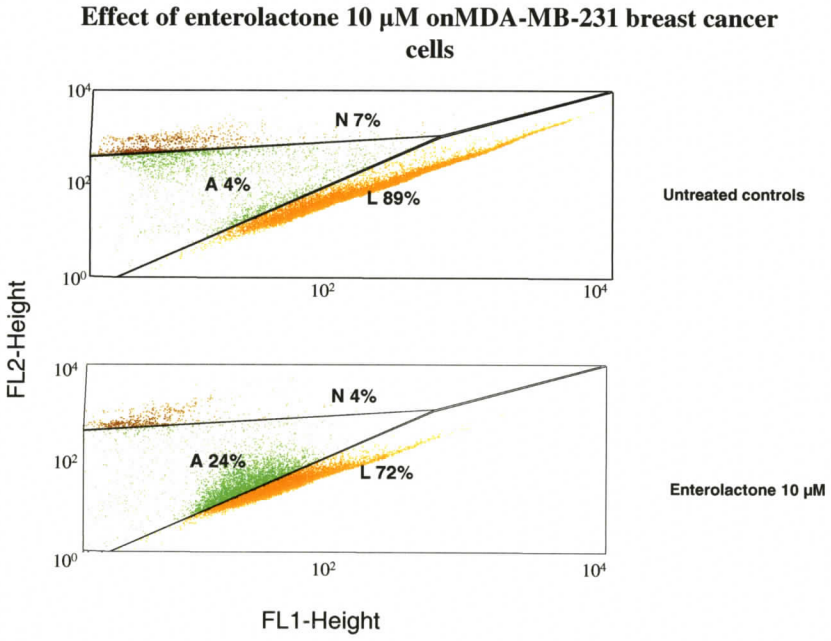

Figure 3.17. FDA/PI dot plot results for MDA-MB-231 breast cancer cells. Live, apoptotic, and necrotic cells are gated and quantified. Treatment with ENL 10 µM significantly decreased viablity ($p < 0.001$).

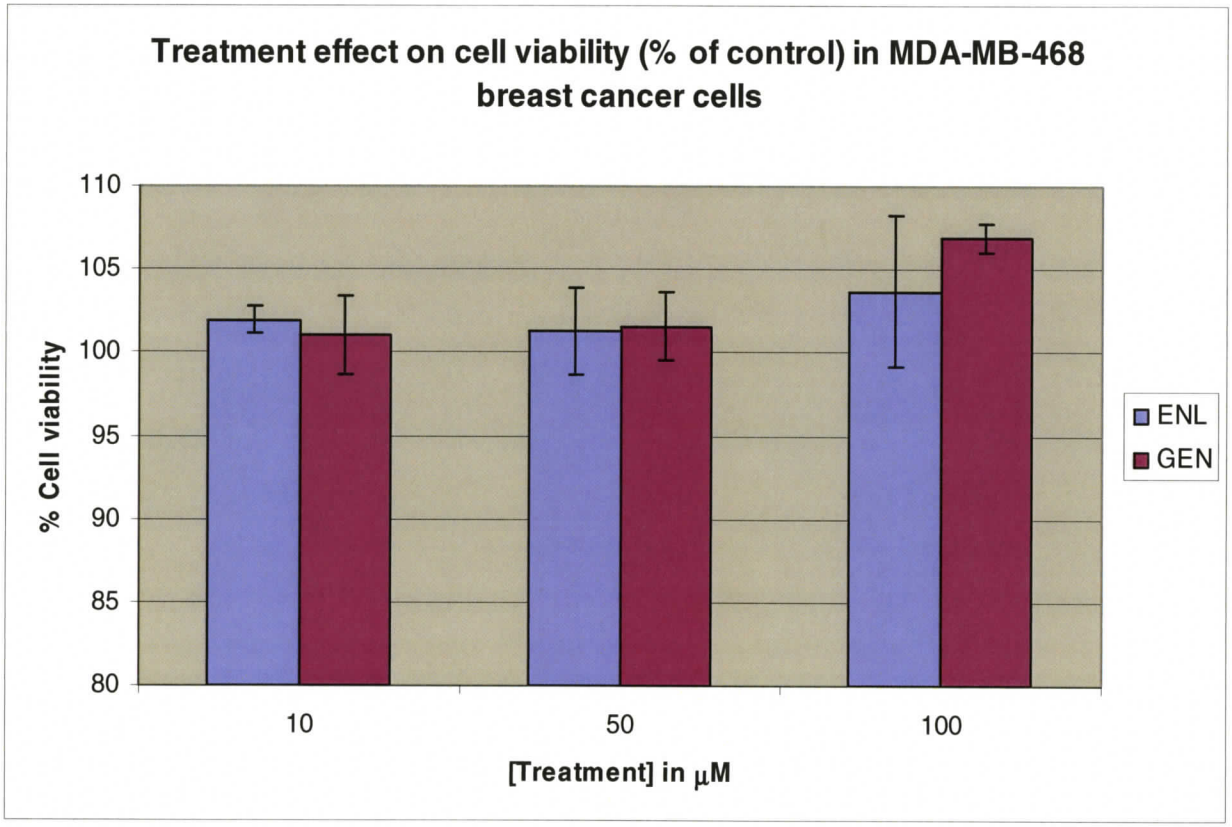

Figure 3.18. The effect of ENL and GEN treatments on MDA-MB-468 breast cancer cells using FDA/PI assay. In MDA-MB-468 breast cancer cells, ENL and GEN alone did not reduce viability. Treatments of ENL or GEN at 100 μM significantly increased viability ($p<0.001$ and $p<0.01$ respectively). Because there was no reduction in viability seen at any of the doses, these cells were not tested in combination with E_2.

Discussion

The FDA/PI assay, which was previously established (Saha *et al.*, 2003) and used here was similar to the Annexin- V/PI method for detecting apoptotic cells. Using this method, FDA^+ cells are viable, PI^+ cells are not, and FDA^-/PI^- cells are regarded as apoptotic. This method is quite accurate, as shown by the significant correlation with the Annexin V/PI method. In addition, the FDA/PI method has several advantages (Saha *et al.*, 2003). The assay is very simple because the reactions can be completed by the addition of FDA and PI directly to the culture medium without repeated washings and centrifugations, which can change the morphology and health of the cells (Saha *et al.*, 2003).

The FDA/PI procedure takes only 60 min and the reagents are far less costly than the Annexin V/PI method. Annexin V failed to measure apoptosis in the HL60 cell line (Saha *et al.*, 2003). In contrast, FDA was reported to be appropriate for detecting apoptosis in all cell-agent combinations (Saha *et al.*, 2003). The FDA/PI method was the principle assay used to determine the effect of all treatments on cell viability, apoptosis and necrosis in all four breast cancer cell lines.

Some variability occurred in the results of treatment with plant estrogen in MCF-7 cells and T47D cells in the FDA/PI assays. A very high dose of plant estrogens was required to significantly reduce viability in the MCF-7 and T47D cells. The presence of 1.0 nM E_2 had the effect of reducing viability of MCF-7 cells when combined with

treatments of 50 or 100 μM . The differences in the antiestrogenic effects of plant estrogens seen between MCF-7 cells and T47D cells suggest differing phenotypic characteristics during tissue culture phase in our lab. According to Clarke *et al.* (2000) these differences can be observed from laboratory to laboratory. However, the differences rarely extend to the pattern of expression of steroid hormone receptors, metastatic potential, or antiestrogen responsiveness. If significant differences are seen, these divergences are almost always the result of an imposed selective pressure (i.e. in vitro growth in the absence of E_2). Many of these variants are unstable and convert to the wild type phenotype whereas other T47D variants are stable for many years (Clarke *et al.*, 2000). One such variant is the $T47D_{CO}$ cells that have lost the ER but have elevated progesterone receptors. These cells grow *in vitro* without E_2 supplementation and are antiestrogen resistant.

It has been well established that in MCF-7 cells, estrogen stimulates cell proliferation probably by shortening the proliferative cycle, and protects against apoptosis by blocking the apoptosis cycle (Song and Santen, 2003). Both effects result in an increased cell pool size. In addition to expression of ER, MCF-7 cells express an E_2-inducible progesterone receptor (Clarke *et al.*, 2000). For these reasons, the use of MCF-7 breast cells was ideal for the study of estrogen and estrogen-like agents such as plant estrogens. Also, it was felt to be important to evaluate the estrogenic and non-estrogenic activities of plant estrogens. Therefore, ER(-) breast cancer cell lines, MDA-MB-231 and MDA-MB-468 were used. MDA-MB-231 cells were useful for this purpose, but MDA-MB-468 showed very little response with the majority of treatments.

The estrogenic properties of the plant estrogens, ENL and GEN have previously been studied (Chen and Wong, 2004) but these have been done in a wide variety of disparate assays (Matsumura *et al.*, 2005). Since both plant estrogens have rarely been tested using similar assays, it has been difficult to make inter-study comparisons and to rank estrogenic potency (Matsumura *et al.*, 2005). The research conducted and many of the conclusions to date on the effect of plant estrogens on breast cancer cells have been on GEN. Since ENL is more prevalent in the North American diet, it is important to add to the existing research available regarding safety. At low doses, GEN in ER(+) breast cancer cell lines appears to mimic E_2 to stimulate cell proliferation through its interaction with ER and to induce E_2 dependent gene expression (Chen and Wong, 2004). Activation of the insulin-like growth factor-I (IGF-I) system plays a critical role in the development and progression of human breast cancer. One research group demonstrated that low concentrations of GEN potentiate the effects of IGF-I on insulin-like growth factor-I receptor (IGF-IR) autophosphorylation as well as tyrosine phosphorylation of IRS-I in MCF-7 cells (Chen and Donovan, 2004). These results suggest that low concentrations of GEN promote human breast cancer cell growth. Therefore, this compound was considered to be a full agonist. Cell cycle analysis following treatment of MCF-7 cells with GEN 1 μM conducted previously (Cappelletti *et al.*, 2000; Chen and Wong, 2004) showed that 1 μM GEN significantly increased S phase and decreased G_0/G_1 phase of MCF-7 cells whereas a dose-dependent growth inhibition was observed between 6 and 20 μM.

In the present study, low doses of ENL and GEN were compared to the activity of high doses of ENL and GEN in four breast cancer cell lines. An increase in proliferation

was not seen with low dose treatments of ENL or GEN. Treatments greater than 10 μM of GEN or ENL reduced viability in MCF-7 cells, and reached statistical significance at 100 μM. Previously, Matsumura *et al.* (2005) found a biphasic effect of GEN on MCF-7 cells, in which GEN at a treatment greater than 10 μM reduced viability. ENL and GEN may inhibit the growth and survival of MCF-7 cells by inhibiting the intrinsic tyrosine kinase activities of growth factor receptors (Matsumura *et al.*, 2005). In that study, combining ENL or GEN with E_2 changed the effect of these plant estrogens on MCF-7 cells. A significant reduction in viability was previously seen with low and high doses of GEN or with high doses of ENL when combined with E_2. Mousavi *et al.* (1992) suggested ENL inhibited E_2. Mueller *et al.* (2004) confirmed that GEN but not ENL has a higher binding affinity for ERβ compared to ERα, but only a slight preference for transactivation of ERβ, compared to ERα which may partially explain the synergestic antiproliferative effect of combining GEN with E_2.

In the present research, there was a significant reduction in viability seen in the ER(-) breast cancer cells, in which MDA-MB-231 cells treated with 10 μM of ENL showed the reduction, whereas GEN-treated cells did not. Dietary flaxseed was found to inhibit the spontaneous metastasis of the ER(-) breast cancer, MDA-MB-435 cells in nude mice (Chen and Thompson, 2003). Furthermore, their data revealed that this inhibition of growth and metastasis of ER(-) human breast cancer was associated with the reduction of expression of epidermal growth factor receptor (EGFR), IGF-I and vascular endothelial growth factor (VEGF) in the primary tumours (Chen and Thompson, 2003). However, the cellular mechanism by which the flaxseed and it components, i.e. ENL, inhibit metastasis has not been investigated to date.

The antiproliferative effect may be due to cell cycle regulation. GEN has been found to increase the expression of $p21^{WAF1}$ and reduce expression of cyclin B1 (Liao *et al.*, 2004). In addition, apoptosis was induced in GEN-treated cancer cells with an increase in the ratio of Bax to Bcl-2.

Conclusion

In conclusion, I have provided evidence that plant estrogens ENL and GEN significantly reduced viability with 100 μM treatments in ER(+) breast cancer cells and ENL with 10 μM treatments in one ER(-) breast cancer cell line. The effect of combining GEN with E_2 enhanced the activity of plant estrogens in one ER(+) and one ER(-) condition. These effects of ENL and GEN may play an important role in the antiproliferation of human ER(+) breast cancer in premenopausal women. Also, ENL and GEN may play a role in the antiproliferation of human ER(-) breast cancer. The effectiveness may be achieved through dietary intakes, which are approximately equivalent to 10 μM treatments *in vitro*. It remains unknown if ENL and GEN are safe for dietary consumption by women in a limited estrogen environment, similar to those found in the circulation of postmenopausal women. Although there were similarities with how ENL and GEN behaved in these assays, there were also some distinct differences. The results suggest the behaviour of ENL and GEN may differ making it difficult to recommend their use in women with breast cancer.

CHAPTER 4

Analysis of Genistein or Enterolactone in Combination with Tamoxifen Including their Role in Apoptotic Cell Rescue

Introduction

Endocrine response pathways in breast cancer have often been used to describe the intracellular pathways used by estrogen (E_2) and the subsequent antagonistic effects that antihormones have on production of E_2 or the estrogen receptor-α (ER α). Tamoxifen (TAM) and its active metabolite, 4-hydroxytamoxifen (OHT) are non-steroidal antihormone selective estrogen receptor modulators (SERMs) that compete with E_2 for binding to ER. The therapeutic effectiveness of TAM in treatment of ER(+) breast cancers and preventing breast cancer in high risk women is thought to arise primarily from its ability to compete with E_2 for binding to the ER (Obero *et al.*, 2002). However, we know that the ER does not work in isolation, and that many other elements, such as growth factor transduction cascades, have been identified that can influence or be influenced by ER signaling (Nicholson *et al.*, 2004). Such elements may have the potential to enhance growth-promoting activities of E_2 in breast cancer. It has been demonstrated that several growth factor-induced protein kinases (eg., erk1/2 MAPK and Akt) (see Figure 4.1) are able to direct by stimulating proliferation and survival signals, and to phosphorlyate regulatory sites on the ER protein, particularly within the AF-1 domain (Nicholson *et al.*, 2004). Studies on tamoxifen-resistant cells reveal an increase in the basal phosphorlyation of ER on serine 118 and 167 residues; these are putative target sites for phosphylation by erk 1/2 MAPK and Akt in which growth is promoted rather than inhibited by TAM (Nicholson *et al.*, 2005). Although TAM initially reduces the levels of total and activated IGF-IR by approximately 80%, it has recently been noted

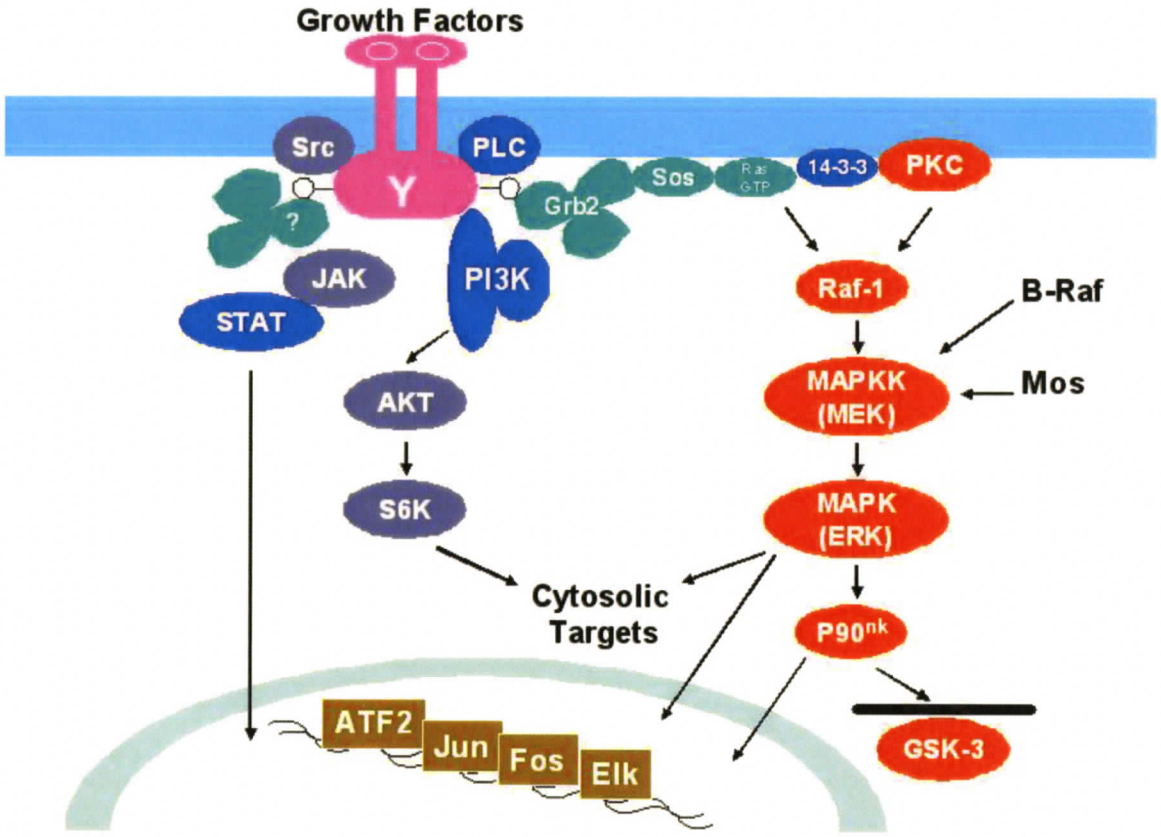

Figure 4.1. Schematic representation of intracellular signaling pathways of the growth receptor. Several MAPK cascades have been identified in mammalian cells, including the extracellular signal-related kinase pathways (ERK1/2, ERK5) and the stress activated kinase pathways (JNK/SAPK, p38 MAPK).On binding, the receptor undergoes autophosphorylation at multiple tyrosine residues. These pathways are linked to many G protein-linked cell surface receptors and receptor tyrosine kinases. Thus, most cytokines, growth factors, hormones, and neurotransmitters can selectively activate these cascades via receptor activation of intracellular second messengers including various SH domain-containing proteins, including PI3-kinase. Activation of the growth receptor also results in phosphorlyation of Shc, which forms a complex with Grb2. Grb2 is tightly associated with the Sos, which activates Ras. This leads to the activation of a cascade of protein kinases including Raf-1 and on or more related kinases, MAP kinases (or Meeks), and the Map kinases (or Erks). The result is alteration in expression of various growth-factor responsive genes and the regulation of cellular proliferation, differentiation, development, cell cycle, and transmission of monogenic signals (Seger and Krebs, 1995). Adapted from (Sigma-Aldrich, 2006)

that IGF-IR level and activation is substantially recovered in TAM-resistant cells (Nicholson *et al.*, 2004). These actions have the potential for TAM to enhance growth-promoting activity of estrogens in breast cancer cells.

ERs also allow expression of genes regulated by other response elements to which ERs do not bind directly, such as the AP-1 site that binds the Jun/Fos transcription factors (Iwase, 2003). The mechanisms by which the ERs increase the activity of Jun/Fos occur via two pathways depending on ER type and ligand; an AF-mediated mechanism for ERα exposed to estrogens and antiestrogens, and an AF-independent mechanism for ERβ (Iwase, 2003). TAM has long been characterized as a beneficial antiestrogen used in treatment for ER(+) breast cancer patients. However, some breast cancer patients, who have ER(+) disease do not respond to TAM. Since the discovery of ERβ, the role of this ER subtype in TAM therapy has been questioned (Power and Thompson, 2003). ERβ has been shown to be higher in patients that are resistant to TAM and Power and Thompson (2003) have suggested that ERβ is a poor prognosticator of responsiveness to hormonal therapy.

The relative expression of coactivators and corepressors can modulate SERMs regulation of ER transcriptional activity. It has been suggested that they could contribute to the tissue-specific ability of mixed antiestrogens to activate or inhibit ER-mediated gene expression (Iwase, 2003).

Apoptosis and TAM

Several conflicting studies have shown that TAM and OHT can actively induce

apoptosis of cancer cells. The mechanisms by which TAM and OHT induce apoptosis have been controversial. Some researchers suggest that high concentrations of both TAM and OHT induce apoptosis. The effects of TAM and OHT might be mediated through an ER-independent increase in reactive oxygen species, resulting in caspase activation or through an influx of extracellular calcium (Obero *et al.*, 2002). The effects of TAM and OHT may be on the level of proteins important in cell growth, ie. protein kinase C, TGFβ, and c-Myc (Obero *et al.*, 2002).

Many compounds induce apoptosis via pathways that involve mitochondria (Figure 4.2). The presence of an apoptotic stimulus triggers a rapid increase in mitochondrial permeability, leading to mitochondrial dysfunction. One of the causes of the mitochondrial permeability transition is the translocation of the proapoptotic Bax protein from the cytosol to the mitochondria, where it forms selective channels in the outer mitochondrial membrane and facilitates the release into the cytosol of cytochrome *c*. In the pathway, the cytosolic cytochrome *c* forms a complex with procaspase 9 and Apaf-1 called the apoptosome, which leads to the ATP-dependent cleavage and activation of pro-caspase 9, the initiator caspase in mitochondrial apoptosis. Activation of pro-caspase 9 results in the activation of the downstream caspases (Nagata *et al.*, 2003). Obero *et al.* (2002) found that TAM and OHT were able to induce two independent pathways of apoptosis. The ER-independent pathways that killed ER-independent cells required a high dose of OHT. In contrast, apoptosis of E- dependent cells was triggered by small amounts of TAM and OHT. The effect was blocked by E_2 demonstrating that binding of TAM and OHT to the ER was required for this pathway of apoptosis (Obero *et al.*, 2002). The ER-independent and ER-dependent pathways both triggered

mitochondrial permeability transition and shared other features of mitochondrial apoptosis, such as translocation of the proapoptotic Bax protein from the cytosol into the mitochondria and the release of cytochrome *c* into the cytosol. The ER-dependent pathway did not result in the cleavage and activation of pro-caspase 9. The ER-dependent mediated pathway resembled the caspase-independent pathway referred to as necrosis-like apoptosis.

There may be highly specific differences in apoptosis in ER(-) and ER(+) breast cancer cells and additional mechanisms to account for the therapeutic efficacy of TAM and OHT (Obero *et al.*, 2002). The existence of an alternative mechanism of action for TAM is supported by the clinical observations that 30% of all ER(+) breast cancer cells respond to TAM and 30% of ER(+) breast cancer cells are not sensitive to TAM (Charlier *et al.*, 1995). The growth of the ER(-) breast cancer cell lines is inhibited by TAM and OHT in a dose-dependent fashion (Charlier *et al.*, 1995).

Effect of enterolactone and genistein on effectiveness of OHT

Genistein (GEN) has been shown to inhibit cancer cell proliferation *in vitro*. This effect has been attributed to a competitive inhibition by occupying ER or to inhibition of several key enzymes, especially tyrosine kinase, which are thought to be involved in cell proliferation and carcinogenesis (Power *et al.*, 2006). A dose-response effect of GEN has been observed when MCF-7 breast cancer cells were treated with increasing concentrations. No effect occurred at concentrations 0.05-50 μM but decreased

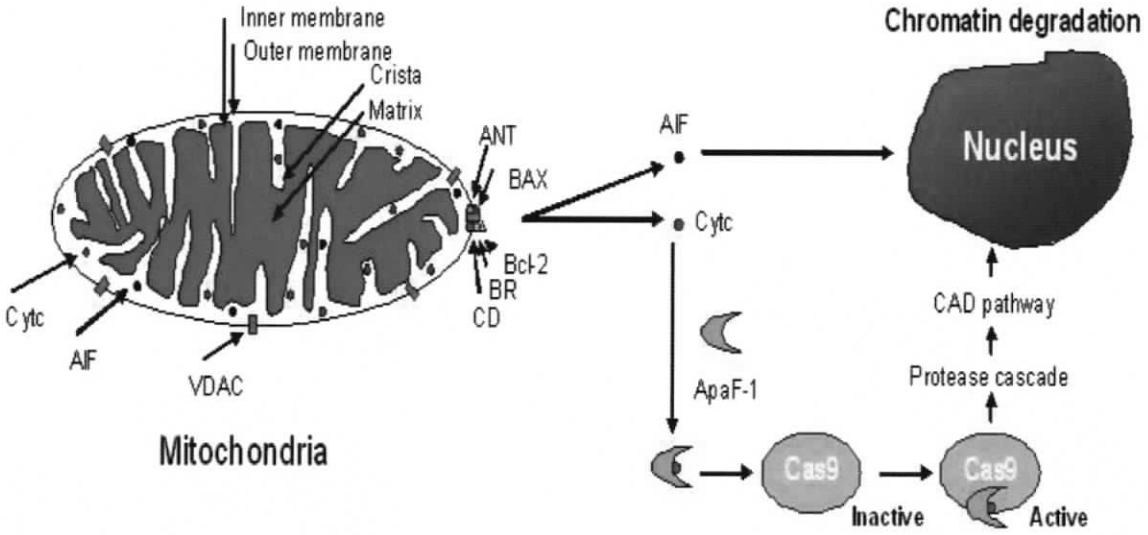

Figure 4.2. Mitochondrial apoptotic pathway. The presence of an apoptosis inducing factor triggers a rapid increase in mitochondrial permeability, leading to mitochondrial dysfunction. One of the causes of the mitochondrial permeability transition is the translocation of the proapoptotic Bax protein from the cytosol to the mitochondria, where it forms selective channels in the outer mitochondrial membrane and facilitates the release into the cytosol of cytochrome *c*. In the pathway, the cytosolic cytochrome *c* forms a complex with procaspase 9 and Apaf-1 called the apoptosome, which leads to the ATP-dependent cleavage and activation of pro-caspase 9, the initiator caspase in mitochondrial apoptosis. Activation of pro-caspase 9 results in the activation of the downstream caspases (Obrero *et al.*, 2002).

Proliferation was shown at higher concentrations (Power *et al.*, 2003). This effect of GEN on MCF-7 breast cancer cells was confirmed by this research in Chapter 3.

The combined GEN or enterolactone (ENL) and 4-hydroxytamoxifen (OHT) treatment was of particular interest because a synergistic effect on dyplastic cell growth inhibition and an additive reduction in viability on malignant cells has been seen in some preliminary studies (Power *et al.*, 2006). In studies conducted in mice, it was shown that dietary lignans could inhibit the growth of MCF-7 xenografts, and enhance rather than antagonize the anticancer effect of TAM (Chen *et al.*, 2004). However, others have reported different results with low GEN concentration; growth stimulation by GEN at a dose of 1.0 nM was inhibited by 10 x 10^{-3} TAM (Zava and Duwe, 1995). The additive and synergistic effect of ENL and GEN with TAM could have a clinical application. Their use might be of particular interest in cases of mammary dysplasia (fibrocystic disease) since there are no other therapeutics that have been found to be effective in severe forms of the disease (Power *et al.*, 2006).

Materials and Methods

All details concerning *cell lines*, *chemicals and reagents, tissue culture conditions, flow cytometric analysis, Annexin V live-cell assay, FDA/PI live-cell assay, and data analysis* were identical to those used in chapter 3.

JC-1 Mitrochondrial membrane potential detection assay

When the redox potential of cellular mitochondria is inhibited through injury, the

transmembrane potential of the mitochondria is likely inhibited (Nunez *et al.*, 2004b). Mitochondrial transition pores, induced when the mitochondrial transmembrane potential is inhibited, function as the portals through which apoptotic mediators, such as cytochrome c, exit the mitochondria (Nunez *et al.*, 2004b) thus activating the caspase cascade. Therefore, quantification of mitochondria membrane potential is a measurement of ongoing apoptosis (Nunez *et al.*, 2004b). JC-1 (5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide), is a dye which fluoresces green in monomeric form upon initial entry into the cytoplasm (Nunez *et al.*, 2004b). Oxidation within the mitochondria causes the formation of J-aggregates; these fluoresce red-orange and are concentrated within the mitochondria (Nunez *et al.*, 2004b). Thus, JC-1 fluorescence in the red-orange range can be used to determine the amount of mitochondrial activity and can be used to signal the loss of mitochrondrial membrane potential occurring in a cell population.

In healthy non-apoptotic cells, the dye stains the mitochondria bright red. The negative charge established by the intact mitrochondrial membrane potential allows the lipophilic dye, bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates. When the critical concentration is exceeded, J-aggregates form, becoming fluorescent red. However, in apoptotic cells, the mitochondrial potential collapses, and the JC-1 remains in the cytoplasm in a green fluorescent monomeric form. Apoptotic cells show primarily green fluorescence.

This procedure was used for the quantification and evaluation of the population of healthy, live cells untreated versus treated with OHT. Cells were seeded 1 x 10^6 per well and incubated with 10% DCC-FBS-DMEM phenol red-free for 3 days. The cells were

treated with 50 μM OHT for 24 hours. Cells were centrifuged for 5 min at 400 x g at rt. The supernatant was discarded. The cells were resuspended in 500 μl of 1 x JC-1 reagent solution and incubated in standard growth conditions (37°C and 5%CO_2 in a humidified incubator) for 15 min. After centrifuging for 5 min at 400 x g at rt, the supernatant was discarded. Cells were resuspended in 2 ml of 1x assay buffer (supplied with kit), centrifuged for 5 min at 400 x g, and supernatant discarded. This step was repeated with 2 ml of cell culture medium instead of 2 ml of 1x assay buffer. The cells were resuspended in 1x assay buffer and analyzed immediately by flow cytometry.

Results

Comparison of the FDA/PI and Annexin V/PI method with OHT

The two methods were compared using identical treatments in MDA-MB-231 breast cancer cells and analyzing the live, apoptotic, and necrotic populations using both methods concurrently. The treatment was OHT 50 μM along with untreated samples which were used as control samples. In the evaluation of live, apoptotic and necrotic MDA-MB-231 breast cell populations in untreated control samples by the FDA/PI assay method, 82% cells were FDA^+ PI^- indicating viable cells, whereas the others were FDA^- cells (Figure 4.3). Since PI binds with dead cells, FDA^-PI^- cells (7%) were apoptotic cells. The PI^+ region indicates dead, necrotic cell populations (11%). With the Annexin V/PI method, 5 % were Annexin V^+ and 12 % of the cells were PI^+. Other cells were both Annexin V^- and PI^- (83%) indicating viability. Since dead cells are PI^+, those

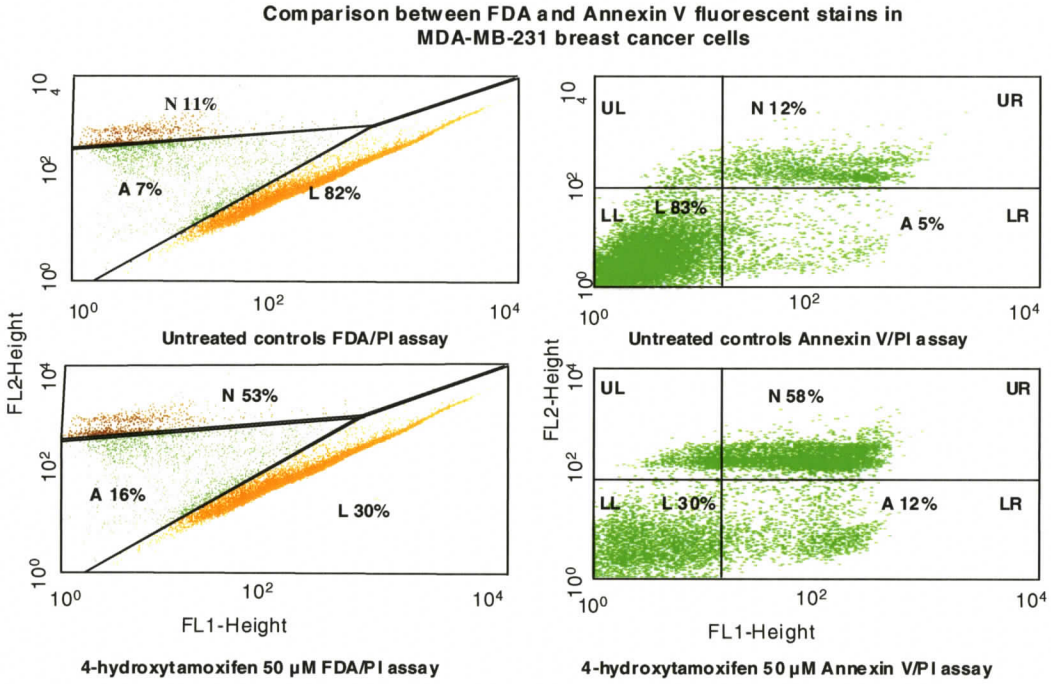

Figure 4.3. FDA/PI technique compared to the Annexin V-PI technique in the accurate quantification of apoptosis in breast cancer cells. The evaluation of live (L), apoptotic (A), and necrotic (N) populations in MDA-MB-231 breast cells is shown and the results are comparable. In untreated control samples using the FDA/PI method, 82% cells were live, 7% were apoptotic, and 11% were dead, necrotic cells. In the Annexin V/PI method, the cells in the LL quadrant (83%) were live. The Annexin V^+ cells in the LR quadrant (5%) were apoptotic and PI^+ cells in the UL and UR quadrants (12%) were necrotic. The samples treated with 50 µM OHT and evaluated using the FDA/PI method showed 30% of the cells were live cells. FDA^-PI^- cells (16%) were apoptotic cells. The PI^+ (53%) were necrotic cell populations. With the Annexin V/PI method, the cells in the LR quadrant (12%) were apoptotic and the cells in the UL and UR quadrants (58%) were necrotic. Other cells were both Annexin V^- and PI^- (30%) indicating they were live.

Annexin V $^{+}$ but PI $^{-}$ cells are apoptotic. In the sample treated with 50 μM OHT and analyzed using the FDA/PI assay method, 30% of the cells were FDA $^{+}$ PI $^{-}$ indicating viable cells, whereas the others were FDA $^{-}$ cells. FDA $^{-}$ PI $^{-}$ cells (16%) were apoptotic cells. The PI $^{+}$ (53%) were necrotic cell populations. With the Annexin V/PI method, 12% were Annexin V $^{+}$ and 58 % cells PI $^{+}$. Other cells were both Annexin V $^{-}$ and PI $^{-}$ (30%) indicating viability. Statistical analysis conducted in chapter 3 showed a highly significant correlation between the FDA/PI and Annexin V/PI methods.

Effect of combinations of OHT with ENL or GEN using FDA/PI live assay

MCF-7 cells

In MCF-7 breast cancer cells, treatments with OHT 10 μM alone did not significantly reduce viability compared to untreated controls (Figure 4.4). Combining low dose OHT (10 μM) with ENL at high doses (50 μM significantly reduced proliferation compared to untreated controls) ($p<0.01$). Combining low dose OHT (10 μM) with GEN at low and high doses (10 and 50 μM) significantly reduced proliferation compared to untreated controls ($p<0.001$) (Figure 4.5). GEN at high doses 50 μM combined with OHT 10 μM significantly reduced proliferation compared to treatment with OHT 10 μM alone ($p<0.05$). Low dose ENL (10 μM) combined with E_2 and OHT 10 μM significantly reduced proliferation compared to untreated controls ($p < 0.01$). High dose GEN (50 μM) combined with E_2 and OHT 10 μM significantly reduced proliferation ($p<0.05$). Low dose GEN (10 μM) in combination with OHT 10 μM

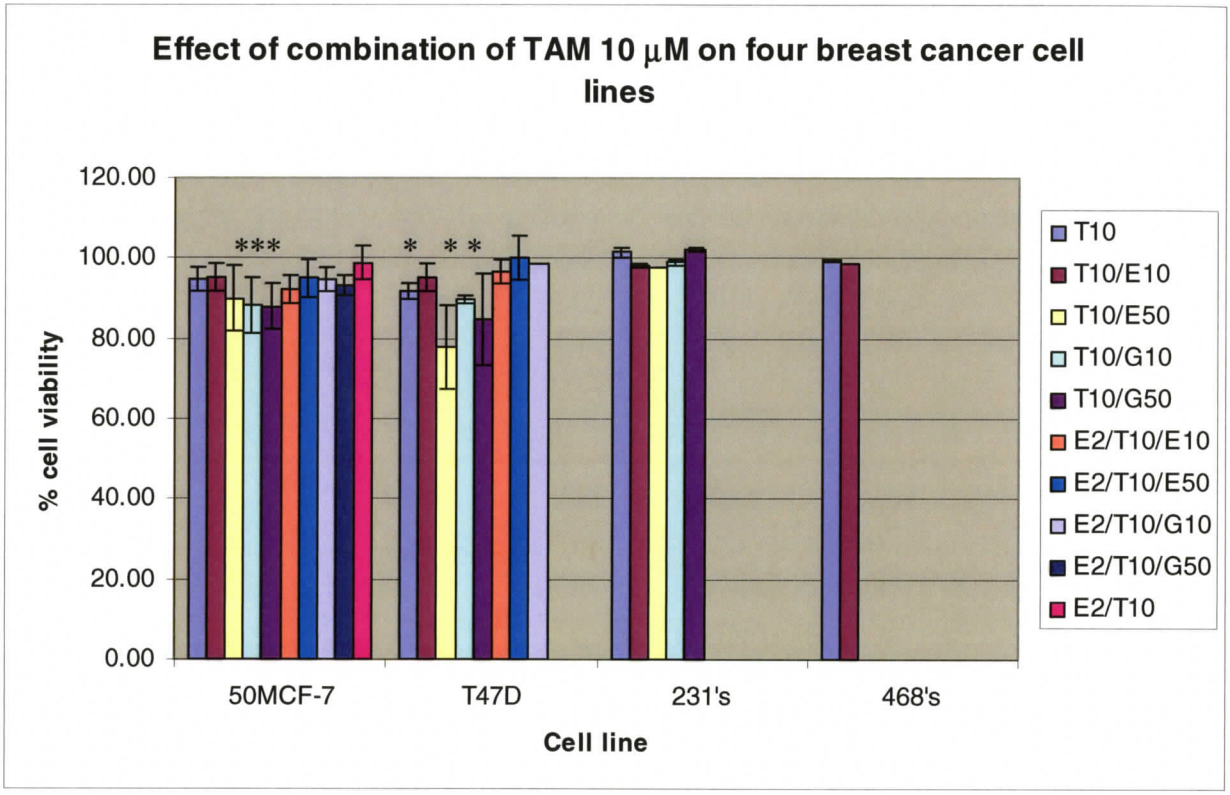

Figure 4.4. FDA/PI live assay results of the effect of OHT (T)10 μM and combinations of OHT 10 μM with ENL(E) or GEN (G) on MCF-7, T47D, MDA-MB-231, and MDA-MB-468 breast cancer cell lines. There was a reduction of viability when treatment of OHT 10 μM was combined with ENL 50 μM and GEN 10 and 50 μM in MCF-7 breast cells. In T47D breast cells, there was a reduction of viability when treated with OHT 10 μM and when OHT 10 μM was combined with ENL 50 μM and GEN 50 μM.

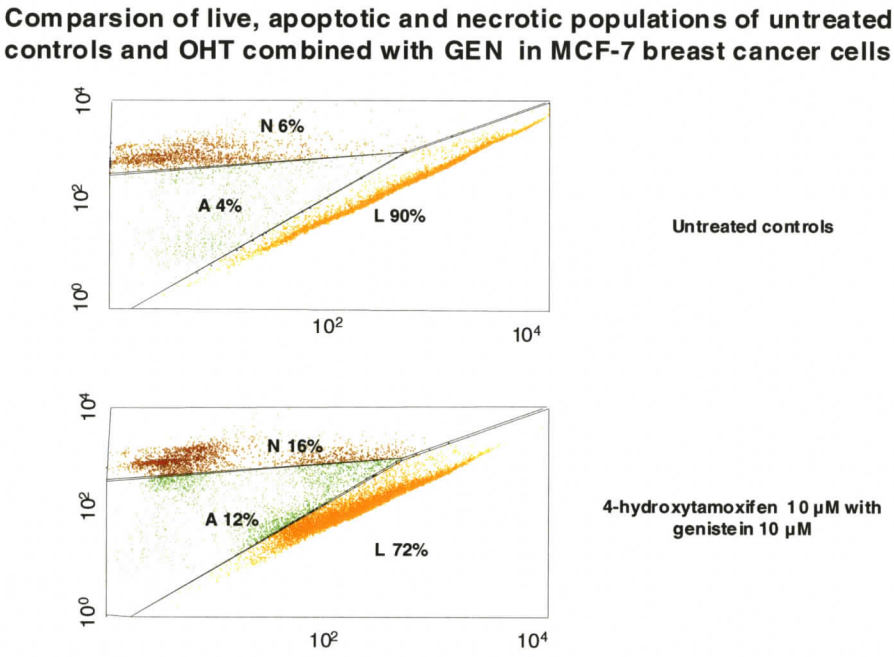

Figure 4.5. FDA/PI live-cell assay dot plot results show differences in live (L), apoptotic (A) and necrotic (N) cell populations of untreated controls and treatments of GEN 10 µM combined with OHT 10 µM in MCF-7 breast cancer cells. Low dose GEN (10 µM) combined with OHT 10 µM significantly reduced viability compared to untreated controls ($p<0.001$).

significantly reduced proliferation greater than low dose ENL 10 (μM)($p<0.05$). Low dose GEN 10 μM combined with OHT 10 μM reduced proliferation to a significantly greater extent than GEN 10 μM with E_2 in combination with OHT 10 μM ($p< 0.05$). In MCF-7 breast cancer cells, treatments with OHT 50 μM alone significantly reduced proliferation compared to untreated controls ($p <0.001$) (Figure 4.6). Treatments of OHT 50 μM combined with ENL or GEN, 10 and 50 μM significantly reduced proliferation compared to untreated controls and did not significantly rescue apoptotic and necrotic cells ($p<0.001$). Combinations of OHT 50 μM and ENL 50 μM significantly decreased proliferation compared to control ($p<0.001$) but had the effect of rescuing MCF-7 cells from OHT-induced apoptosis and necrosis compared to cells treated with OHT 50 μM alone ($p<0.05$) (Figure 4.7).

In the One-way Analysis of Variance (ANOVA) in the OHT 10and 50 μM experiments, the P value is < 0.001 which was considered highly significant. Dunnett Multiple Comparisons test was conducted on the values. The Kolmogorov and Smirnov method confirmed that all groups passed normality testing.

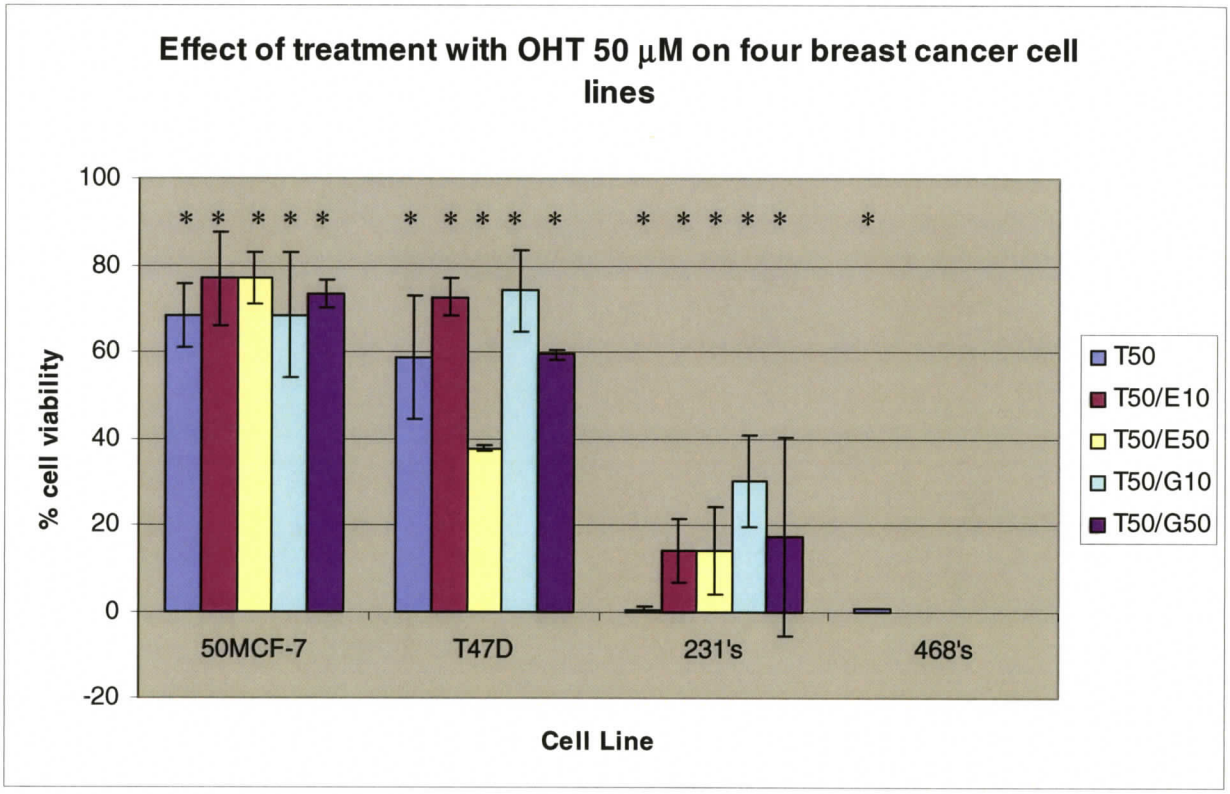

Figure 4.6. FDA/PI live assay results of the effect of OHT (T) 50 μM and combinations of OHT 50 μM with ENL or GEN on MCF-7, T47D, and MDA-MB-231 breast cancer cell lines. Although there was a significant reduction seen in viability in treatments with OHT 50 μM alone as well as when OHT 50 μM was combined with ENL or GEN, 10 μM and 50 μM in all cell lines tested, there was rescue of apoptotic and necrotic cells seen when low dose ENL (10 μM) or GEN (10 μM) were combined with OHT 50 μM.

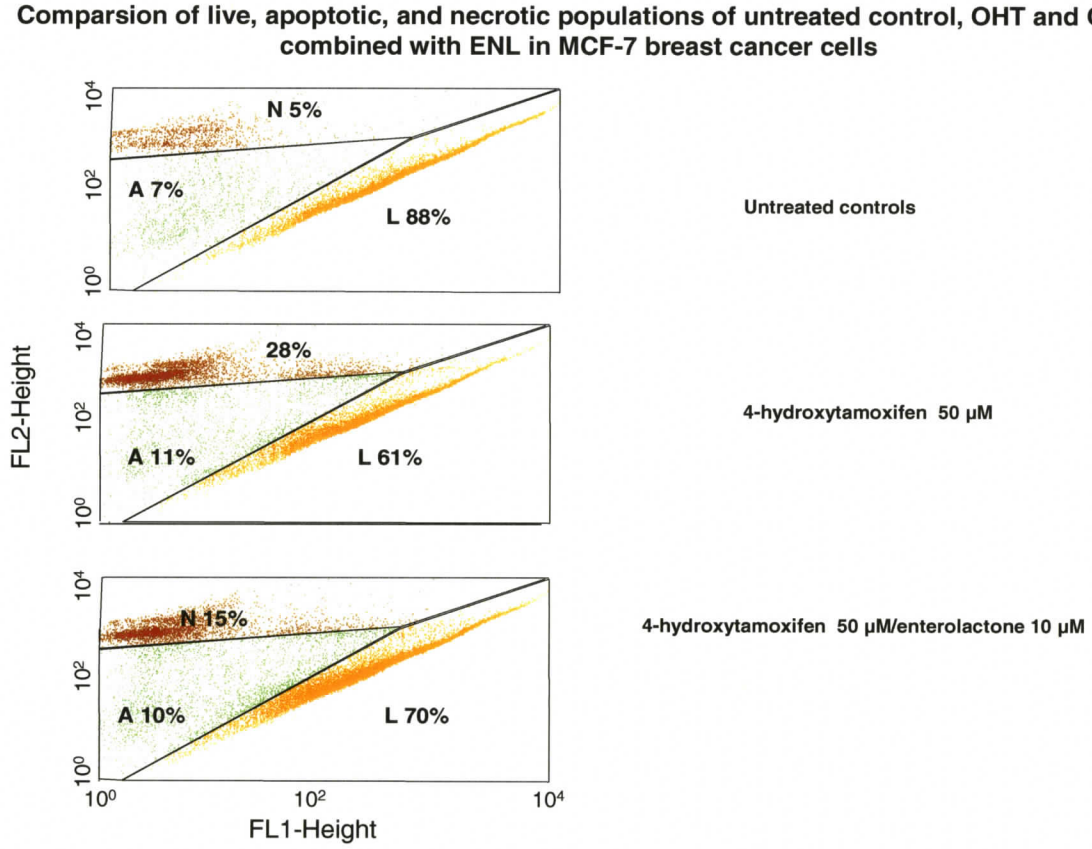

Figure 4.7. FDA/PI live-cell assay dot plot with live (L), apoptotic (A) and necrotic (N) cell populations of untreated controls and treatments of OHT, and combinations of OHT 50 µM and ENL 10 µM in MCF-7 breast cancer cells. Treatments of OHT 50 µM significantly decreased viability ($p<0.001$). Combinations of OHT 50 µM and ENL 10 µM significantly decreased viability compared to control ($p<0.001$). However, ENL 10 µM when added with OHT 50 µM had the effect of rescuing cells from OHT-induced apoptosis and necrosis ($P<0.05$).

T47D cells

In T47D breast cancer cells, treatments with OHT 10 μM alone significantly reduced proliferation compared to untreated controls (Figure 4.4). Combining low dose OHT (10 μM) with high dose ENL or GEN (50 μM) ($p<0.01$) significantly reduced proliferation compared to untreated controls.

In T47D breast cancer cells, treatments with OHT 50 μM alone significantly reduced proliferation compared to untreated controls ($p <0.001$) (Figure 4.6). Treatments of OHT 50 μM combined with ENL or GEN, 10 and 50 μM significantly reduced proliferation compared to untreated controls ($p<0.001$). Also, ENL or GEN 10 μM in combination with OHT 50 μM significantly rescued apoptotic and necrotic cells ($p<0.05$ and $p<0.01$ respectively) compared to treatment with OHT 50 μM alone (Figure 4.8). ENL 50 μM combined with OHT 50 μM reduced proliferation to a greater degree than OHT 50 μM alone ($p<0.01$).

In the One-way Analysis of Variance (ANOVA) in the OHT 10 and 50 μM experiments, the P value was < 0.001 which is considered highly significant. Variation among column means was significantly greater than expected by chance. Dunnett Multiple Comparisons test was conducted on the values. The Kolmogorov and Smirnov method confirmed that all groups passed normality testing.

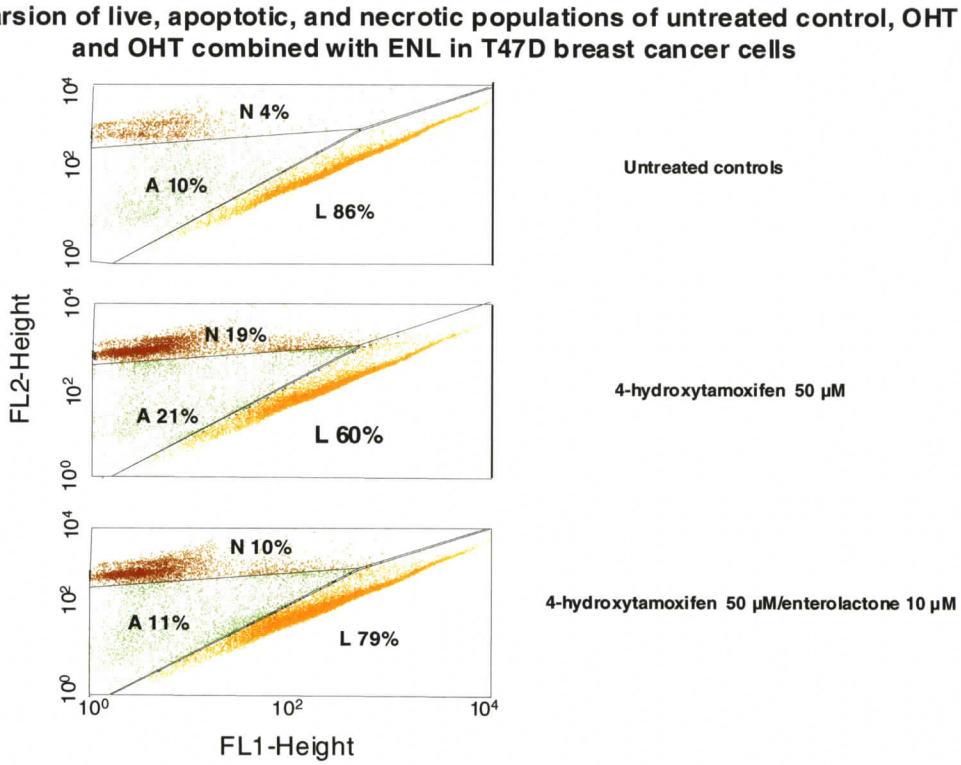

Figure 4.8. FDA/PI live-cell assay dot plot with live (L), apoptotic (A) and necrotic (N) cell populations of untreated control, treatment of OHT, and a combination of OHT 50 µM and ENL 10 µM in MDA-MB-231 breast cancer cells. Treatments of OHT 50 µM significantly decreased viability ($p<0.001$). Combinations of OHT 50 µM and ENL 10 µM did not significantly decrease viability compared to control. When ENL 10 µM was added to OHT 50 µM, it had the effect of rescuing cells from OHT-induced apoptosis and necrosis ($P<0.001$).

MDA-MB-231 and MDA-MB-468 cells

In MDA-MB-231 breast cancer cells, treatment with OHT 10 μM did not significantly reduce proliferation compared to untreated controls (Figure 4.4). Because the effect of OHT 10 μM alone or when combined with ENL or GEN was not different than untreated controls in MDA-MB-231 cells, only preliminary evaluations were conducted with MDA-MB-468 cells. The preliminary findings were similar to those in MDA-MB-231 cells.

Large dose OHT (50 μM) significantly reduced proliferation in MDA-MB-231 breast cancer cells ($p<0.001$)(Figure 4.6). ENL 10 and 50 μM in combination with OHT 50 μM significantly reduced proliferation compared to untreated controls ($p<0.001$). However, GEN 10 or ENL 10 μM in combination with OHT 50 μM significantly rescued apoptotic and necrotic cells ($p<0.001$ and $p<0.05$ respectively) compared to treatment with OHT 50 μM alone (Figure 4.9). The Annexin V live-cell assay also confirmed this trend (Figure 4.10).

In the One-way Analysis of Variance (ANOVA) for the OHT 10 and 50 μM experiments in MDA-MB-231 cells, the P value was < 0.001 which was considered highly significant. Dunnett Multiple Comparisons test was conducted on the values. The Kolmogorov and Smirnov method confirmed that all groups passed normality testing.

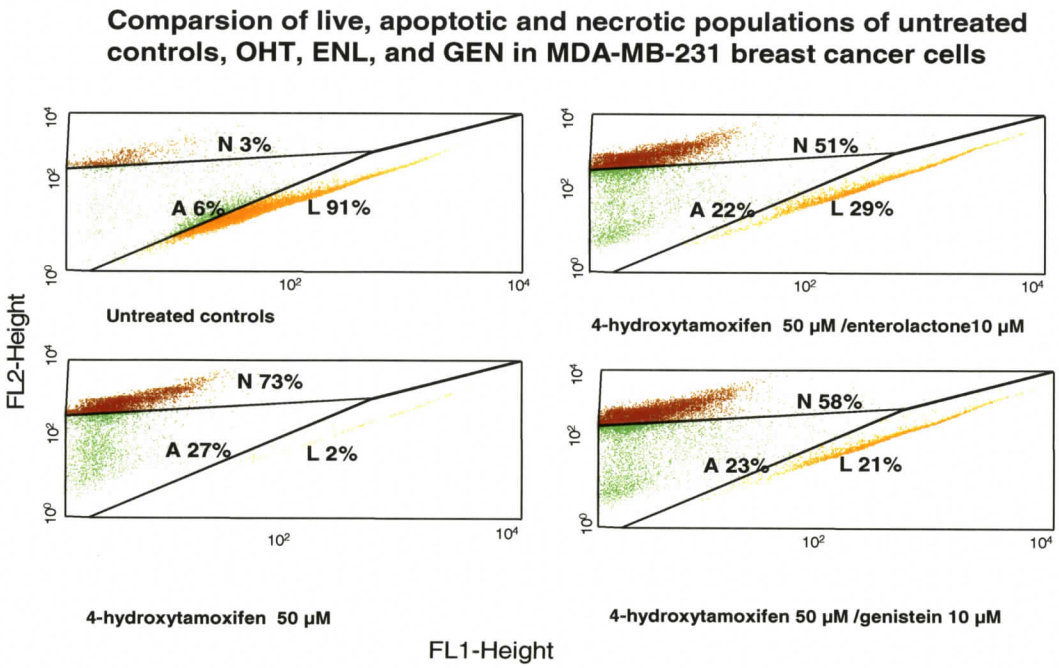

Figure 4.9. FDA/PI live-cell assay dot plot with live (L), apoptotic (A) and necrotic (N) cell populations of untreated controls and treatments of OHT, and combinations of OHT 50 µM and ENL 10 µM or GEN 10 µM in MDA-MB-231 breast cancer cells. Treatments of OHT 50 µM significantly decreased viability ($p<0.001$). Combinations of OHT 50 µM and ENL 10 µM or GEN 10 µM significantly decreased viability ($p<0.001$). However, ENL 10 µM and GEN 10 µM when added to OHT 50 µM rescued cells from OHT-induced apoptosis and necrosis ($P<0.001$).

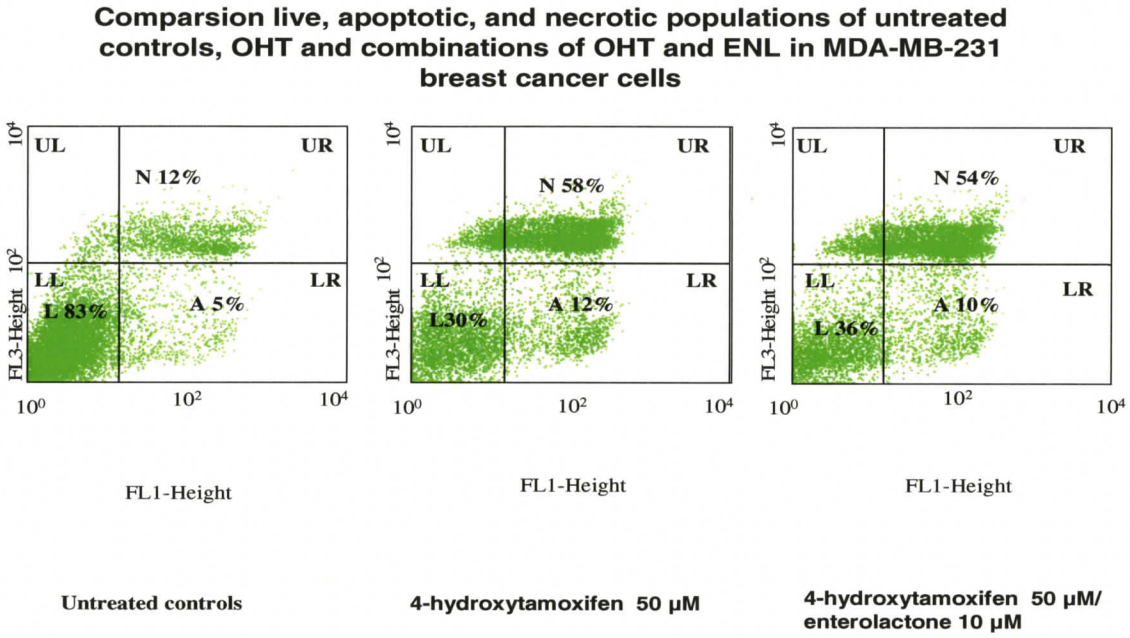

Figure 4.10. Annexin V assay dot plot with live (L), apoptotic (A) and necrotic (N) cell populations of untreated controls and treatments of OHT, and combinations of OHT 50 µM and ENL 10 µM in MDA-MB-231 breast cancer cells. Treatments of OHT 50 µM significantly decreased viability ($p<0.001$). A combination of OHT 50 µM and ENL 10 µM significantly decreased viability compared to untreated control, but there was significant rescue of cells from OHT-induced apoptosis and necrosis when ENL 10 µM was added to OHT 50 µM ($P<0.001$).

In the One-way Analysis of Variance (ANOVA) for the OHT 10 and 50 μM experiments in MDA-MB-468 cells, the P value was < 0.001 which was considered highly significant. Dunnett Multiple Comparisons test was conducted on the values. The Kolmogorov and Smirnov method confirmed that all groups passed normality testing.

Usefulness of JC-1 method in detecting apoptosis

In healthy non-apoptotic cells, JC-1 stains the live cells to a red fluorescence (FL2) but this rapidly changes to green fluorescence (FL1) in the apoptotic cells treated with 50 μM OHT (In Figure 4.11). This assay required a multistep analysis and it was difficult to accurately separate the regions of live and apoptotic cells as it was not possible to determine the necrotic populations of cells. This assay was useful to confirm shifts in live to apoptotic cells seen in the FDA/PI protoccol. Due to these challenges the protocol was not used in further experiments.

Discussion

Antiestrogen therapy does not eliminate the malignant stem cell but rather slows its proliferation and promotes apoptosis of progenitors and their progeny (McKinnell *et al.*, 2000). TAM (OHT) is an adjuvant drug for not only ER(+) but also ER(-) breast carcinomas. OHT can block the breast or non-breast cancer metastatic cascade by inhibition of cell adhesion and invasion, and alteration of cell migration (Chen and Thompson, 2003). Although OHT is employed as an estrogen antagonist, under some circumstances OHT demonstrates estrogenic activity (McKinnell *et al.*, 2000). From

Evaluation of live and apoptotic MDA-MB-231 breast cancer cells using JC-1 mitochondrial membrane potential detection assay

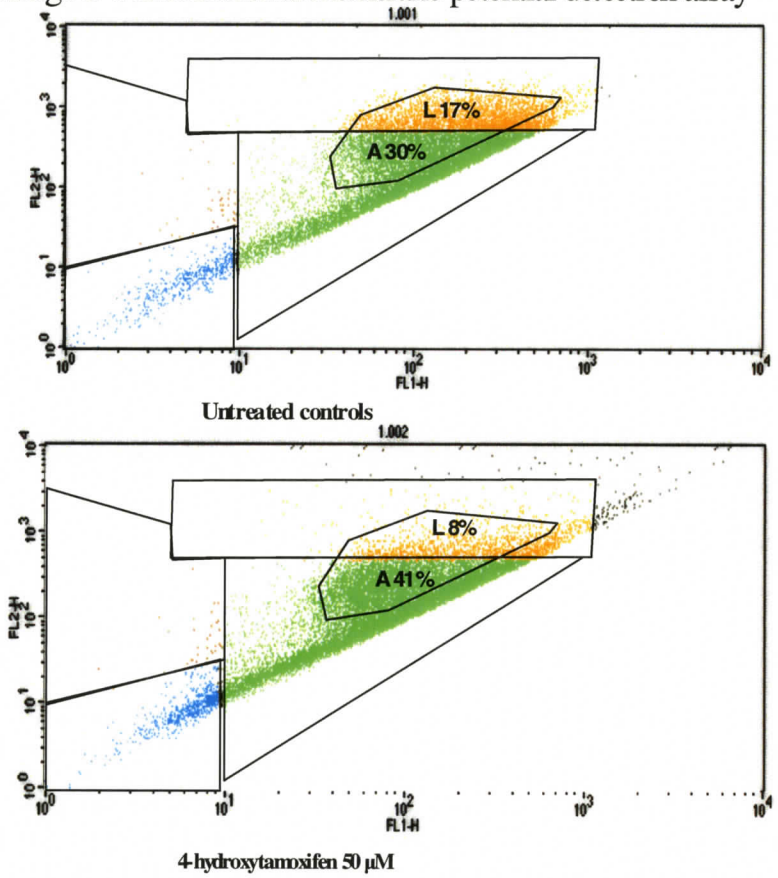

Figure 4.11. Live (L) and apoptotic (A) populations of MDA-MB-231 cells analyzed using JC-1 mitrochondrial membrane potential detection in an untreated control and following treatment with 50 µM OHT. In the top panel, there are healthy non-apoptotic cells present which were stained bright red. The critical concentration was exceeded and J-aggregates formed, which became fluorescent red (FL-2). In the bottom panel, cells treated with OHT 50 µM experienced mitochondrial potential collapse, which caused the JC-1 to remain in the cytoplasm in a green fluorescent (FL-1) monomeric form. Apoptotic cells are indicated with green fluorescence.

previous research, low doses (0.1 μM-10.0 μM) of OHT resulted in an increase in live cells and a reduction in apoptosis and necrosis compared to controls. OHT can cause a brief tumour flare or transient increase in proliferation of breast cancer cells.

The results of these experiments provide evidence to support the notion that ENL and GEN may have an additive effect in ER(+) breast cancer when combined with physiologically relevant dosages of OHT. High dose ENL significantly enhanced the antiproliferative effect of low dose OHT. GEN had a similar synergistic effect at both high and low doses. It is important to mention that low dose ENL, although it did not result in a synergistic effect, had no effect on the antiproliferative effect of low dose OHT. There was no evidence of a synergistic effect of combining ENL or GEN in the presence of E_2 to enhance the antiproliferative actions of low dose OHT. There was no effect seen with low dose OHT or with a combination of ENL or GEN with low dose OHT in ER(-) breast cancer cell lines.

Low concentrations of OHT can cause proliferation in breast cancer cells, but high concentrations are completely inhibitory. At doses of 50 μM and 100 μM, OHT is toxic to breast cancer cells. This closely mimics what has been seen in previous OHT research. The results of the experiments combining high dose OHT with ENL or GEN were less supportive of the notion of an overall synergistic effect. Combining low and high dose ENL and GEN with high dose OHT resulted in a rescuing effect of apoptotic and necrotic cells in T47D and MDA-MB-231 breast cancer cells. In these cell lines, low dose ENL and GEN significantly rescued cells that would have normally experienced apoptosis and necrosis from the treatment of high dose OHT.

The present evidence is among the first to support ENL and GEN acting via different mechanisms than ER α- mediated pathways. A measure of antiestrogen activity is the efficacy compared to a known antiestrogen, such as OHT. However, the present works suggests that the mechanism of ENL and GEN actions may vary widely from those of OHT. GEN has been shown to increase ERβ protein levels and cell proliferation (Power and Thompson, 2003). It is possible that the actions of GEN may be mediated via ERβ pathways which have not been elucidated to date. It is currently unknown whether ENL is able to increase ERβ protein levels. The ERα/ERβ ratio cannot be excluded as playing a role in breast cancer response to therapy and should be further investigated.

It is now widely documented that the inappropriate activation of growth factor signaling cascades, either through enhanced supply of growth factors or via upregulation and increased activation of their target growth factor receptors or their recruited downstream elements, can readily promote antihormone failure of OHT in breast cancer (Nicholson *et al.*, 2005). Previously, Wang and Kurzer (1998) suggested that GEN and ENL may inhibit tyrosine kinase pathways. ENL and GEN may increase phosphorylation of IGF-I or possibly enhance an increase of OHT to phosphorylate Erk 1/2 MAPK and Akt pathways. ENL and GEN may be blocking the anti IGF-I activity of OHT which may be enhancing a shift towards OHT- resistant cells. It is estimated that a four-fold increase in motility and invasion is apparent in OHT-resistant cells (Nicholson *et al.*, 2004).

Resolution of the role of ER in ENL and GEN-induced apoptosis is complicated by the fact that available ER(+) and ER(-) breast cell lines are derived from independent tumours (Obero *et al.*, 2002). These cell lines therefore differ in many respects other than

ER content. The differences in cell lines with respect to ER content, SERM-induced ER regulation, and cell proliferation stress the need to view breast cancer as a heterogeneous disease (Power and Thompson, 2003). Treatment by different SERMs may be more effective if the breast cancer is first characterized more vigorously for components within the cell, ie. ERα, ERβ, coactivators, and corepressors, that will induce a favourable effect by a specific SERM (Power and Thompson, 2003). Although this study was conducted in cell culture, it is a first step in understanding mechanisms in clinical situations.

In the present study, cells were treated with combinations of low and high concentrations of GEN and ENL as well as low and high concentrations of E_2 and OHT. High concentrations of GEN and ENL (50-100 μM) were included in the experiments to avoid the agonistic activity of lower concentrations in ER(+) cell lines. Such concentrations are approximately 6 to 30 times higher than GEN blood levels achieved by subjects with a high dietary soy intake (Cappelletti *et al.*, 2000). However, as with E_2, GEN is much more concentrated in breast fluid than in serum (Zava and Duwe, 1997).

Conclusion

The synergistic inhibitory effect of ENL and GEN with low dose OHT in ER(+) breast cancer cells supports the idea these isoflavonoids might play an important role as an adjuvant or an alternative treatment to TAM (OHT) for women with breast cancer. The observations of ENL and GEN with high dose OHT in ER(+) and ER(-) breast cancer cells lend support to the notion that actions of SERMs are hugely variable. Given the results seen in these experiments, it is important to increase the number and variety *of*

in vivo studies conducted to test the possible use of ENL and GEN as therapeutic agents in the treatment of breast cancer.

CHAPTER 5

Effects of Combinations of Genistein and Enterolactone on some Breast Cancer Cell Lines

Introduction

From epidemiological studies it has been suggested the differences in incidence of breast cancer depend on environmental causes, particularly those of diet and lifestyle. Over 200 case-control and cohort studies were reviewed finding significant relationships between the incidence of breast cancer and vegetable and/or fruit consumption (World Research Fund, 1997). Dietary factors including low consumption of fruit, vegetables, seeds and legumes, particularly soy, have been implicated in the increasing incidence of breast cancer (Key *et al.*, 2003).

The plant estrogens, enterolactone (ENL) and genistein (GEN) are components that are being investigated for their role in chemoprevention and as potential cancer therapeutics. Analysis of serum or urine in humans have revealed the presence of multiple plant estrogens suggesting that plant estrogens act cumulatively (Ying, 2005). However, the studies on the combinational effects of plant estrogens on cell growth and their estrogenic activity on ER have been rather limited or controversial. Ying (2005} examined the growth regulatory effects of two plant estrogens, diadzein (DAID), and biochanin A in the model system for estrogen response, the MCF-7 cells. In agreement with previous studies, the inhibition of growth in MCF-7 breast cancer cells was dose-dependent. Growth stimulation was observed at low concentrations of plant estrogens separately and growth inhibitory actions were observed at high concentrations. The combined regiment of DAID, and biochanin A was found to have a greater inhibitory effect than any single compound (Ying, 2005). Recently, Power *et al.* (2006) examined the effect of ENL and GEN, alone and in combination on the growth of MCF-7 tumours in ovarectomized nude mice. In the ENL-treated mice palpable tumours regressed

significantly (91%). When ENL and GEN were combined, a significant regression of palpable tumour by 87% was seen (Power *et al.*, 2006). No growth-promotion effects were observed. In the present study, the individual and combined growth regulatory effects of ENL and GEN were evaluated using four breast cancer cell lines.

Materials and Methods

All details concerning *cell lines*, *chemicals and reagents, tissue culture conditions, flow cytometric analysis, Annexin V live-cell assay, FDA/PI live-cell assay, and data analysis* were identical to those used in chapter 3.

Results

Effect on viability of combinations of ENL and GEN using FDA/PI live assay

MCF-7 cells

In MCF-7 breast cancer cells, treatments with ENL 10 μM or GEN 10 μM alone did not significantly reduce proliferation compared to untreated controls. Combining ENL 10 μM with GEN 10 μM significantly reduced proliferation compared to untreated controls ($p<0.001$) (Figure 5.1). The difference between ENL 10 μM or GEN 10 μM alone and the two plant estrogens combined was significant ($p<0.001$).

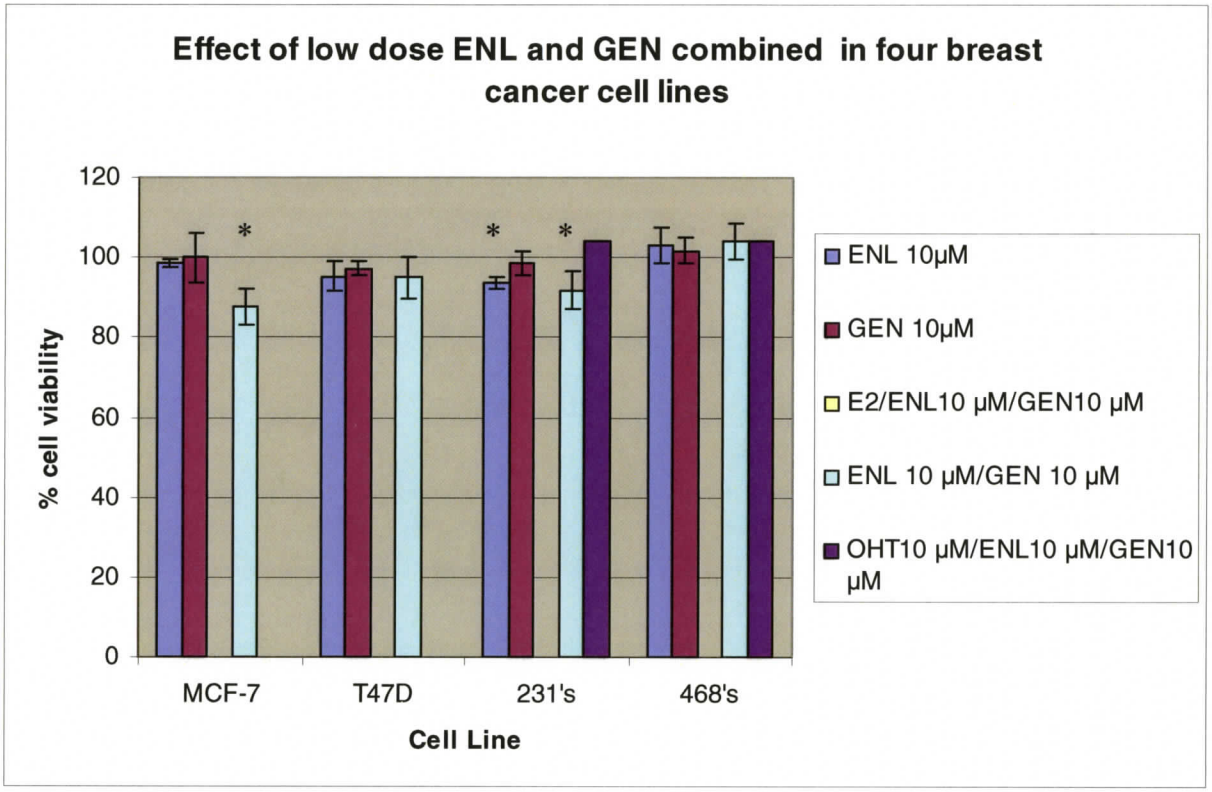

Figure 5.1. Effect of combining low dose ENL and GEN on four breast cancer cell lines. When ENL 10 μM was combined with GEN 10 μM , there was a significant reduction in viability in MCF-7 and MDA-MB-231 breast cancer cell lines ($p<0.001$

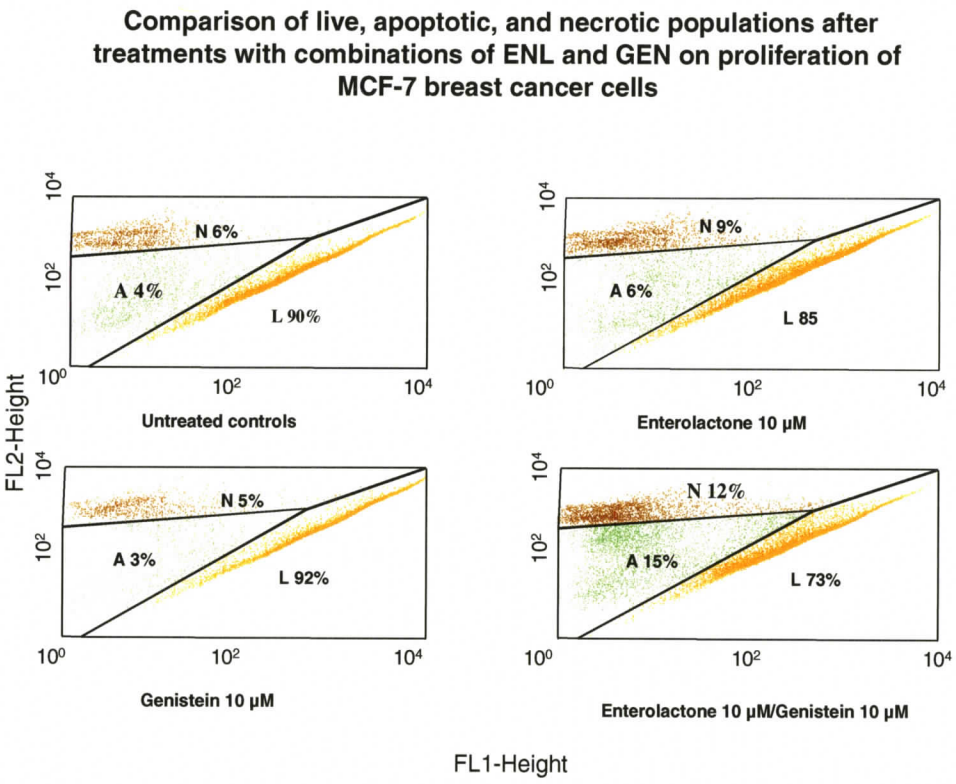

Figure 5.2. FDA/PI live-cell assay dot plot with live (L), apoptotic (A) and necrotic (N) cell populations of untreated controls, treatments of ENL 10 µM or GEN 10 µM; and combinations of ENL 10 µM and GEN 10 µM in MCF-7 breast cancer cells. Treatments of ENL 10 µM or GEN 10 µM did not significantly decrease viability However, combinations of ENL 10 µM and GEN 10 µM significantly decreased viability ($p<0.001$).

Treatments with ENL 50 μM or GEN 50 μM alone did not significantly reduce proliferation compared to untreated controls (Figure 5.2). Combining ENL 50 μM with GEN 50 μM significantly reduced proliferation of MCF-7 cells compared to untreated controls. The difference between ENL 50 μM or GEN 50 μM alone and the two plant estrogens combined was significant ($p<0.001$).

In the One-way Analysis of Variance (ANOVA) in the ENL and GEN combination experiments, the P value is < 0.001 which is considered highly significant. Dunnett Multiple Comparisons test was conducted on the values. The Kolmogorov and Smirnov method confirmed that all groups passed normality testing.

T47D cells

In T47 breast cancer cells, treatments with ENL 10 μM or GEN 10 μM alone did not reduce proliferation compared to untreated controls (Figure 5.1). Combining low dose ENL (10 μM) with low dose GEN (10 μM) did not reduce proliferation compared to untreated controls.

Treatments with ENL 50 μM or GEN 50 μM alone did not reduce proliferation compared to untreated controls. Combining high dose ENL (50 μM) with high dose GEN (50 μM) did not reduce proliferation compared to untreated controls (Figure 5.3).

In the One-way Analysis of Variance (ANOVA) in the ENL and GEN combination experiments, the P value is >0.05 which is considered not significant.

Dunnett Multiple Comparisons test was conducted on the values. The Kolmogorov and Smirnov method confirmed that all groups passed normality testing.

MDA-MB-231 cells

In MDA-MB-231 breast cancer cells, treatment with GEN 10 μM alone did not reduce proliferation compared to untreated controls (Figure 5.1). Treatment with 10 μM ENL significantly reduced viability compared to untreated controls ($p<0.001$). Combining ENL 10 μM with GEN 10 μM significantly reduced proliferation compared to untreated controls ($p<0.001$) (Figures 5.1 and 5.4). An increase of apoptotic cells was seen with the combination (30%) compared with the untreated cells (6%). The difference between GEN 10 μM alone and the two plant estrogens combined was significant ($p<0.001$).

Treatment with ENL 50 μM or GEN 50 μM did not reduce proliferation compared to untreated controls (Figure 5.3). Combining ENL 50 μM with GEN 50 μM did not reduce proliferation compared to untreated controls. The difference between GEN 50 μM alone and the two plant estrogens combined was significant ($p<0.001$). The addition of OHT to ENL 10 μM combined with GEN 10 μM did not result in a growth inhibitory effect.

In the One-way Analysis of Variance (ANOVA) in the ENL 10 μM and GEN 10 μM combination experiments, the P value was < 0.001 which was considered highly significant. Dunnett Multiple Comparisons test was conducted on the values. The Kolmogorov and Smirnov method confirmed that all groups passed normality testing.

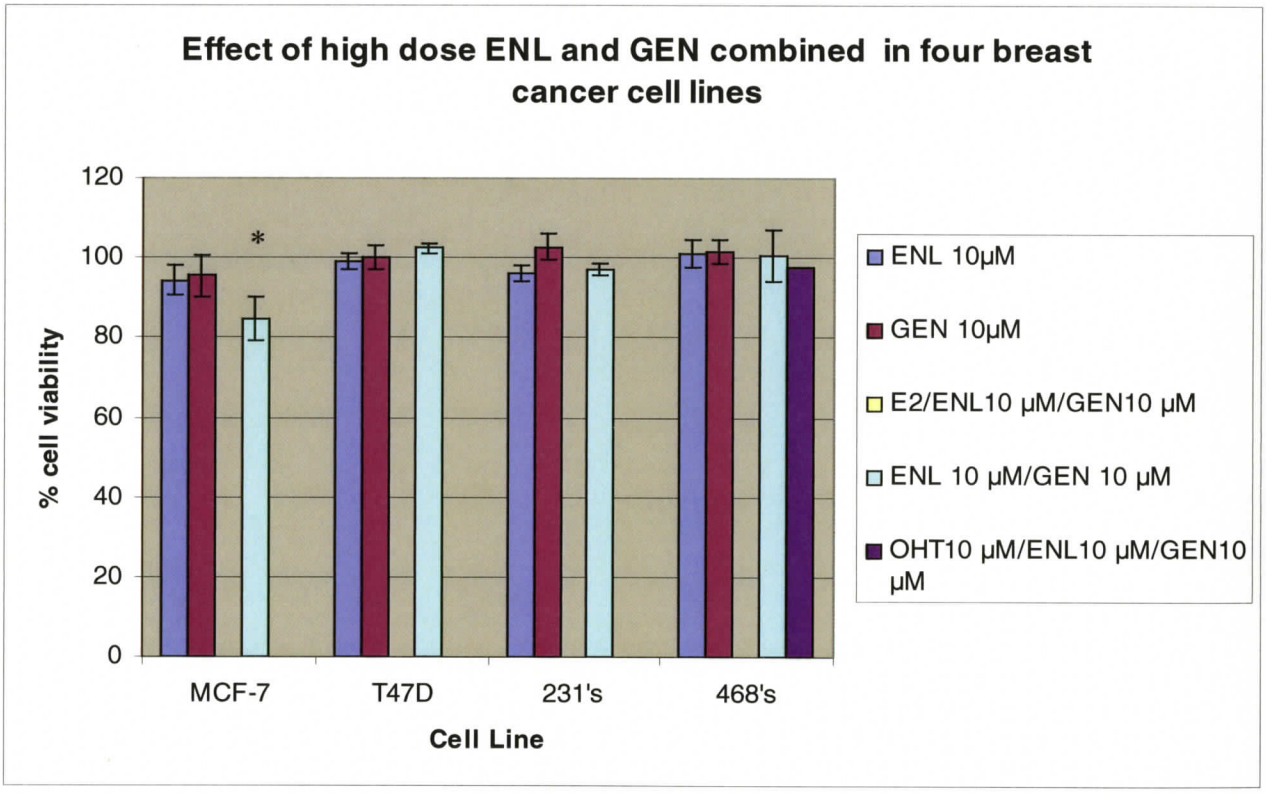

Figure 5.3. Effect of combining high dose ENL and GEN in four breast cancer cell lines. When ENL 50 μM was combined with GEN 50 μM, there was a significant reduction in viability in MCF-7 breast cancer cell lines ($p<0.001$).

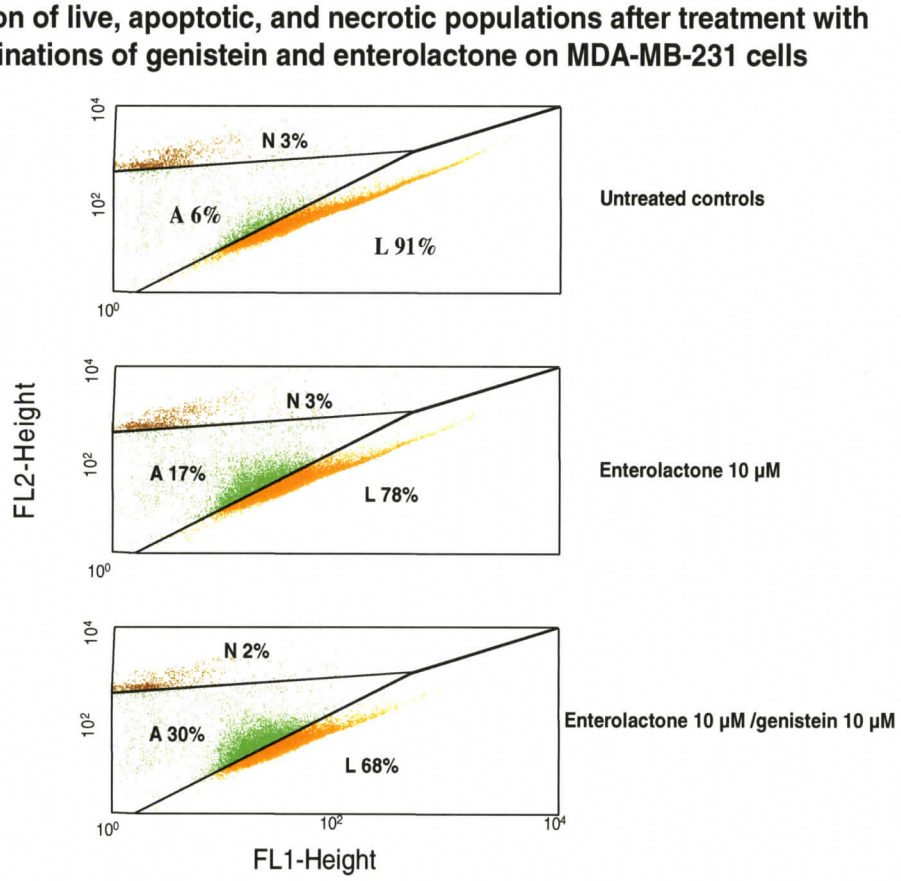

Figure 5.4. FDA/PI live-cell assay dot plot showing live (L), apoptotic (A) and necrotic (N) cell populations of untreated controls, treatment of ENL 10 µM, and a combination of ENL 10 µM and GEN 10 µM in MDA-MB-231 breast cancer cells. The treatment with a combination of ENL 10 µM and GEN 10 µM significantly decreased viability ($p<0.001$).

MDA-MB-468 cells

In MDA-MB-468 breast cancer cells, treatments with ENL 10 μM or GEN 10 μM alone did not reduce viability compared to untreated controls (Figure 5.1). Combining low dose of ENL (10 μM) with low dose GEN (10 μM) did not reduce proliferation compared to untreated controls.

Treatments with ENL 50 μM or GEN 50 μM alone did not reduce proliferation compared to untreated controls (Figure 5.3). Combining high dose ENL (50 μM) with high dose GEN (50 μM) did not reduce proliferation compared to untreated controls. The addition of OHT to ENL 10 μM combined with GEN 10 μM did not result in a growth inhibitory effect.

In the One-way Analysis of Variance (ANOVA) in the ENL and GEN combination experiments, the P value was >0.05 which was considered not significant. Dunnett Multiple Comparisons test was conducted on the values. The Kolmogorov and Smirnov method confirmed that all groups passed normality testing.

Discussion

In the present study, the growth regulatory effects of two plant estrogens, ENL and GEN were examined in MCF-7, T47D, MDA-MB-231 and MDA-MB-468 breast cancer cells. In agreement with previous results from Chapter 3, ENL or GEN at low

doses had no effect on MCF-7 and T47D cells. As previously seen, ENL had growth inhibitory effects on MDA-MB-231 cells. ENL 50 μM or GEN 50 μM had no effect on the viability of four cell lines.

When ENL and GEN were combined at low doses, synergistic growth inhibitory effects were seen in MCF-7 and MDA-MB-231 breast cells. When ENL and GEN were combined at high doses, a synergistic growth inhibitory effect was seen in MCF-7 breast cells. Since high dose ENL or GEN alone did not result in growth inhibition in MCF-7 or MDA-MB-231 cells, the effect of combining low dose ENL and GEN cannot be attributed to simply an increase in dose overall. These results strongly suggest that ENL and GEN may mediate growth inhibition differently and possibly through non-classical ER pathways. Instead, there may be a novel target site involved.

Conclusion

At present, whole foods ie. vegetables, seeds, nuts and beans, show much stronger chemopreventative associations than the isoflavones and lignans that they contain. Secondly, there may be the potential for combinations of plant estrogens to be used as an adjuvant therapy in the treatment for breast cancer and this work substantiates the need for further investigation.

CHAPTER 6

Summary and Future Directions

Summary

There has been a concern surrounding the safety of ENL and GEN use in breast cancer patients, as these compounds may cause elevated cell proliferation leading to estrogen-dependent tumour promotion (Mueller *et al.*, 2004). Menopausal women are being encouraged to consume potent isoflavone-containing supplements and to include soy-based foods in their regular diets. The present study was designed to gain insight into the antiproliferative activity of ENL and GEN using human breast cancer cell lines. This was achieved by comparing the action of ENL and GEN to the antiestrogenic actions of OHT in flow cytometric assays.

A hallmark response to estrogenic stimuli is the proliferation of cells *in vitro* and *in vivo* (Mueller *et al.*, 2004). Depending on concentration and presence of estrogen, a biphasic effect of GEN on mammary cells has been demonstrated (Chen and Thompson, 2003). At doses corresponding to plasma concentrations achieved with a high soy intake (1.0 -10 μM), GEN in the absence of E_2 stimulates cellular proliferation in ER(+) breast cancer cells (Wang and Kurzer, 1998). Although there was a trend showing increased proliferation, this research did not find ENL and GEN at 10 μM had a growth stimulatory effect on ER(+) breast cancer cells. In Figure 3.16 ENL at 10 μM had a growth inhibitory effect in MDA-MB-231 (ER(-) cells. In previous research, physiological doses of GEN, in the presence of E_2 may behave like a competitive inhibitor for the ER and slightly inhibit cellular proliferation (Wang and Kurzer, 1998). This research supports the notion that in the presence of E_2, effectiveness of GEN at 10 μM in inhibiting

proliferation was enhanced in one ER(+) but not in the other ER(+) or in the ER(-) breast cancer cells.

It has been suggested that pharmacological doses (<10 μM) of GEN markedly inhibit cellular proliferation in ER(+) breast cancer cells (Wang and Kurzer, 1998) most likely by inhibiting the intrinsic tyrosine kinase activities of growth factor receptors (Power *et al.*, 2006)). In the present research, a large dose of ENL or GEN (100 μM) was effective in reducing proliferation in both ER(+) lines but not in ER(-) breast cancer cells. The dose of 100 μM significantly increased proliferation in MDA-MB-468 cells (Figures 3.11, 3.14, 3.16, and 3.18).

There has been controversy surrounding the safety of plant estrogen use with TAM (OHT) in breast cancer. Some research has demonstrated a potential synergistic effect whereas others have shown that plant estrogens block the effectiveness of TAM (OHT). In the present research, low dose of GEN (10 μM) and high doses (50 μM) of ENL and GEN combined with low dose OHT (TAM metabolite) (10 μM) in ER(+) were synergistic suggesting a potential benefit of the addition of plant estrogens to TAM (OHT) in the treatment of breast cancer (Figure 4.5). In the present research, high dose OHT (50 μM) was effective in growth inhibition of ER(-) breast cells. However, when this was combined with low and high dose ENL or GEN, there was an effect of rescuing ER(-) breast cancer cells tested (Figure 4.7). Although the use of high dose TAM (OHT) is not typically used to treat breast cancer currently, the present research suggests the actions of plant estrogens are more varied than simply competition for ER binding. The effect of combining low doses of ENL and GEN had not been previously evaluated *in vitro*. Therefore, the results of the present research are particularly interesting. A

significant decrease in proliferation was seen in the(OHT) MCF-7 and MDA-MB-231 cell lines. Since doses of 50 μM did not inhibit growth, the effect of increased dose may not be a factor. The present research suggests both similarities and differences in the effect of ENL and GEN *in vitro*.

Dietary Recommendations

There is a potential for ENL and GEN to play a role in the management of certain types of human ER(-) breast cancer (Table 6.1). Currently, ER(-) breast cancer has a poor response overall to chemotherapy and radiation treatment. The effective dose of plant estrogens may be achieved through dietary intakes, which are approximately equivalent to 10 μM *in vitro*.

Although TAM (OHT) is not always chosen as an adjuvant therapy for the management of ER(+) breast cancer in premenopausal women, the potential synergism of the addition of low dose ENL and GEN to the regimen may increase the overall effectiveness of TAM (OHT) therapy in this group. However, it remains unknown if ENL and GEN are safe for dietary consumption by women with ER(+) breast cancer who have a limited estrogen environment, similar to those found in the circulation of postmenopausal women. Although there were some predicted similarities and differences with how ENL and GEN behaved in the assays, there were also some unpredicted differences, such as the results seen with ENL or GEN combined with low and high dose TAM (OHT). These results suggest the mechanism of action of ENL and GEN may differ which makes it very difficult to give general recommendations on the use of plant estrogens as a whole group in women with breast cancer.

Table 6.1 Comparison of normal breast tissue with ER(+) and ER(-) breast cancer.

Normal Breast Tissue	**ER (+) Breast Cancer**	**ER(-) Breast Cancer**
1 in 9 women in North America at risk	60-70% of tumours	30-40% of tumours high risk of recurrence low survival
ER expression low except for short peak during 1st week of menstrual cycle	↑ER expression (may have ↓ERβ)	↓ER expression (may have ↑ERβ)
E_2 mediated via ERα but ERβ may counteract	E_2 stimulates proliferation	Proliferation independent on E_2
Cells well differentiated	Cells less differentiated	Cells poorly differentiated
Low mitotic index	Increased mitotic index	High mitotic index
Prolonged exposure to E_2 and use of hormone replacement therapy may ↑ risk	Caused by accumulation of genetic damage Hormone replacement therapy ↑'s risk	Caused by further accumulation of genetic damage.
AF-1 and 2 act synergistically when ER activated by E_2	AF-2 predominance	AF-1 predominance
Type I antiestrogens, ie. TAM may prevent breast cancer	Type I antiestrogens, ie. TAM improve survival	Type I antiestrogens, ie. TAM may stimulate proliferation
	TAM effective in 50% of tumours	TAM has not shown benefit
	TAM effective for 5 years only	Sometimes seen following use of TAM
Plant estrogens (PE), ie. GEN and ENL may prevent breast cancer, reduce symptoms of menopause such as hot flushes, bone loss, and heart disease	PE, ie. GEN and ENL have biphasic effect *in vitro*: ↓dose PE, ↑proliferation ↑dose PE, ↓proliferation	PE, ie. GEN and ENL have biphasic effect that is reverse of effect in ER(+) breast cancer cells *in vitro*: ↓dose PE, ↑proliferation ↑dose PE, ↑proliferation
	PE may influence TAM effectiveness *in vitro*: ↓dose PE, ↓effectiveness of TAM ↑dose PE, ↑effectiveness of TAM	No effect on action of TAM *in vitro*
	↑ responsiveness to effects of radiation and chemotherapy treatments	↓ responsiveness to effects of radiation and chemotherapy treatments

Future Directions

The molecular mechanisms of action of these compounds, ENL and GEN have not been determined. Therefore there are several directions of research I have identified:

- Elucidate the molecular mechanism of apoptosis seen in experiments with plant estrogens. This approach may increase the potential to develop a novel adjuvant cancer therapy for ER(+) and ER(-) breast cancer.
- Evaluate the effectiveness of plant estrogens to inhibit the formation of aromatase and ensure the safety of plant estrogen use in patients who are currently using aromatase inhibitor adjuvant therapies to treat breast cancer.
- Evaluate the effectiveness of plant estrogens with drug-resistant breast cancer cell lines, ie. MCF-7 tamoxifen-resistant breast cancer cells and compare to effectiveness of high dose E_2.
- Investigate the effect of plant estrogens on transduction pathway growth factors, ie. autophosphorylation and activation of ERK1/2 MAPK and Akt downstream of EGFR and TGFα using flow cytometry. Do an extensive profiling of EGFR, its important tumour ligand TGFα, and its downstream ERK1/2 MAPK signaling in breast tumour specimens. Past research indicates this concept may prove relevant to clinical disease (Nicholson *et al.*, 2005).
- Express ERα or ERβ in COS cells. Then determine the effect of ENL or GEN at two doses, alone and combined on proliferation of COS cells or on a reporter construct that is activated by ER.

- Investigate a broad panel of tissue-specific markers to determine potential tissue specificity in activity of plant estrogens.
- Test *in vivo* the antiproliferative properties identified in the present research.

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