MECHANISMS OF GROWTH INHIBITION INDUCED BY METHYLENE-SUBSTITUTED AND RING-SUBSTITUTED DIMS IN BREAST CANCER CELLS

A Dissertation

by

KATHRYN ELISABETH VANDERLAAG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Toxicology

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May 2007

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ABSTRACT

Mechanisms of Growth Inhibition Induced by Methylene-Substituted and Ring-Substituted DIMs in Breast Cancer Cells. (May 2007)

Kathryn Elisabeth Vanderlaag, B.Sc., University of Guelph

Chair of Advisory Committee: Dr. Stephen H. Safe

One in 8 women will be diagnosed with breast cancer in the United States and estrogen receptor (ER) status largely influences the type and subsequent success of treatment employed. Although ER-positive breast cancer can be treated with endocrine therapy, the more invasive ER-negative breast cancer is non-responsive to this therapy and cytotoxic agents are often utilized which are associated with many adverse side effects. Consequently, there is a genuine need to develop more effective, less toxic treatments for invasive breast cancer.

Indole-3-carbinol is a phytochemical found in cruciferous vegetables and one of its major metabolites, 3,3'-diindolylmethane (DIM), exhibits a broad range of anticancer and antitumorigenic activities. ER-negative MDA-MB-231 and MDA-MB-453 breast cancer cell growth was inhibited after treatment with a novel series of methylene-substituted DIMs (C-DIMs), namely 1,1-bis(3'-indolyl)-1-(p-substitutedphenyl) methanes containing trifluoromethyl (DIM-C-pPhCF_3), t-butyl (DIM-C-pPhtBu) and phenyl (DIM-C-pPhC_6H_5) groups. In addition, DIM-C-pPhC_6H_5 (40 mg/kg/d) inhibited tumor growth in nude mice bearing MDA-MB-231 cells as xenografts. Treatment of

breast cancer cells with C-DIMs lead to downregulation of cyclin D1 and induction of non-steroidal anti-inflammatory drug-activated gene 1. Detection of necrosis, caspase-dependent or caspase-independent apoptosis were not observed in breast cancer cells treated with C-DIMs, however autophagic cell death was induced by C-DIMs.

DIM and ring-substituted DIMs have exhibited antitumorigenic activity in tumor murine mammary models. An investigation into the mechanism of cell death induced by DIM and 5,5'-dibromoDIM (5,5'-diBrDIM) in both ER-positive (MCF-7) and ER-negative (MDA-MB-231) breast cancer cells revealed modulation of several key signaling pathways involved in growth control. Both DIM and 5,5'-diBrDIM downregulated cyclin D1, although only 5,5'-diBrDIM induced a depolarization of the mitochondrial membrane. In addition, apoptosis was observed in MCF-7 cells treated with 5,5'-diBrDIM but not MDA-MB-231 cells.

In summary, C-DIMs may represent new mechanism-based agents for treatment of breast cancer through induction of autophagic cell death. The ring-substituted DIMs correspond to a novel class of uncharged mitochondrial poisons that are also highly effective in inhibiting breast cancer cell growth. Results of this research provide evidence for the potential role of two new series of DIM analogs for the treatment of highly aggressive breast cancer.

DEDICATION

To my parents

ACKNOWLEDGEMENTS

Completing my graduate degree would not have been as enjoyable had it not been for my committee. I would first like to express my gratitude to my advisor, Dr. Stephen Safe for his intelligence and enthusiasm for the field of science that was truly inspiring and he was like a second father to me. Dr. Burghardt is one of the kindest people I have ever met who continually provided words of encouragement and always managed to find time to help anyone who asked. Besides being an expert in the art of making dry ice bombs, Dr. Porter always challenged me with regards to my graduate work, which motivated me to learn more throughout my degree. Dr. Phillips always provided a friendly smile in the hall and interesting topics of conversation.

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If I were to choose one aspect of my life that I feel truly fortunate about, it would be my family. When I am having a bad day, I know they will always be there to listen. I am grateful for having supportive parents who have let me pursue whatever I wanted to do, even when it has involved moving to a foreign country. Thanks for making me believe that anything can be accomplished with hard work. My sister, Nikki, has always been a role model and has willingly been there for me when I've needed her. My Aunt Liz has provided motivation for my research and has been supportive both academically and emotionally while I have lived in Texas.

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CHAPTER I

INTRODUCTION

1.1. Cancer

Cancer cells are abnormal cells that can grow uncontrollably and have the ability to invade normal tissues. Cancer encompasses a diverse range of diseases that are named based on their biological origin (prefix) and cell type (suffix). The 4 distinct types of cancer based on the cell origin of the cancer: carcinomas, sarcomas, lymphomas and leukemias (see Figure 1.1). Carcinomas are the most frequent type of cancer and arise from epithelial cells covering internal or external surfaces such as lung,

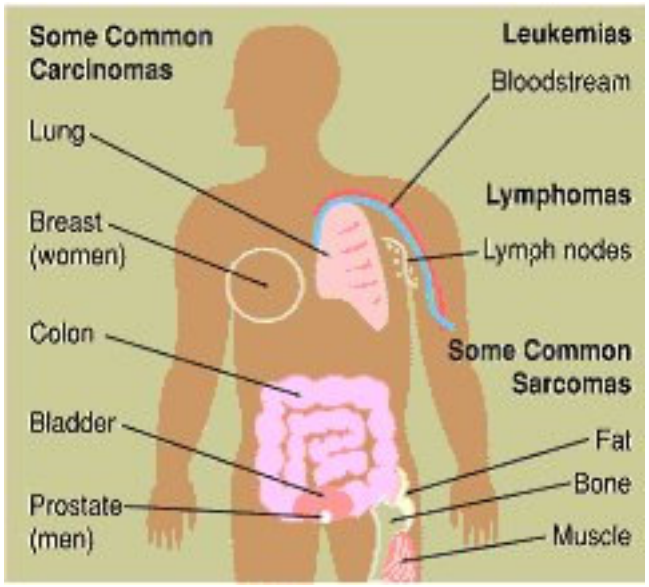


Figure 1.1: Classification of common cancers. There are 4 types of cancer based on type of cell the cancer originated from: carcinomas (epithelial), leukemias (immature blood cells in bone marrow), lymphomas (lymph nodes and immune system) and sarcomas (supportive tissue). Examples of tissues pertaining to each type of cancer are illustrated. [1]

This dissertation follows the style of *Cancer Letters.*

breast or colon. Sarcomas are initiated in the supportive tissue including bone, cartilage, muscle and connective tissue. Tumors originating in lymph nodes or the immune system are classified as lymphomas whereas leukemias are cancers originating from immature blood cells in the bone marrow. Prefixes used to identify the location of the cancer are summarized in Table 1.1. [1]

Table 1.1: Cancer nomenclature. Prefixes based on the location of cancer are summarized in the chart below (adapted from [1]).

Location	Prefix
Gland	Adeno-
Cartilage	Chondro-
Red blood cell	Erythro-
Blood vessels	Hemangio-
Liver	Hepato-
Fat	Lipo-
Lymphocyte	Lympho-
Pigment cell	Melano-
Bone marrow	Myelo-
Muscle	Myo-
Bone	Osteo-

1.1.1. Cancer statistics

Cancer accounted for 22.7% of all deaths in 2003 in the United States, second only to heart disease (see Table 1.2). It is estimated that more than 1.4 million new cases of cancer will be diagnosed in 2006. Cancer incidence varies among sexes with 1 in 2 men and 1 in 3 women expected to develop cancer in their lifetime. It is estimated that more than 1500 Americans die from cancer every day. A comparison of death rates in 1950 compared to 2003 show no change in the cancer mortality rate. The 5-year

survival rate for a variety of cancers has increased significantly in the past few decades due to earlier diagnosis and advances in treatment. However, cancer continues to be a major cause of death in all industrialized countries. [2]

Table 1.2: 2003 US mortality statistics. Causes of death ranked based on number of deaths occurring in 2003. Adapted from [2].

Rank	Cause of Death	No. of Deaths	% of All Deaths
1	Heart diseases	685,089	28.0
2	Cancer	556,902	22.7
3	Cerebrovascular diseases	157,689	6.4
4	Chronic lower respiratory diseases	126,382	5.2
5	Accidents (Unintentional injuries)	109,277	4.5
6	Diabetes mellitus	74,219	3.0
7	Influenza and pneumonia	65,163	2.7
8	Alzheimer disease	63,457	2.6
9	Nephritis	42,453	1.7
10	Septicemia	34,069	1.4

1.1.2. Stages of cancer

Cancer is the result of an accumulation of mutations in genes controlling cellular homeostasis [3]. These include genes that are involved in DNA repair, apoptosis, oncogenes and tumor suppressor genes [3]. An oncogene refers to a gene that when altered, leads to the progression of cancer due to its direct or indirect role in controlling cell growth. In contrast, tumor suppressor genes are normally involved in restricting cell proliferation and mutation or inactivation of tumor suppressor genes can lead to uncontrolled cell growth and cancer progression. In a study of retinoblastoma cases, Knudson determined that the function of a tumor suppressor gene is only lost once both

copies are mutated, which is also true of DNA repair genes [4]. These mutations can be a result of inheritance or environmental exposures.

Genetic damage to homeostatic genes and the resulting development of cancer is a multistage process that can be divided into 3 steps: Initiation, promotion and progression (see Figure 1.2). Initiation is characterized by a genetic mutation occurring as a result of a DNA damaging agent that covalently binds to DNA and is not repaired by cellular DNA repair machinery. At this stage, the cell is not morphologically different from a normal cell. In the next stage of promotion, mutated cells proliferate through epigenetic mechanisms and the expression of genes controlling important cell processes including proliferation, inflammation and differentiation is altered. Mutational activation of oncogenes as well as inactivation of tumor suppressor genes are probable targets of altered gene expression in these early stages of cancer development [5]. Benign tumors can form during the promotion stage but the cells do not yet have invasive or metastatic properties. The final stage of cancer is progression in which cells acquire the ability to invade and metastasize due to the acquisition of additional genetic alterations and progressive genetic instability. Alterations in cell size, shape and organization (dysplasia) occurs at this stage, and ultimately leads to an advanced form of cancer. [3]

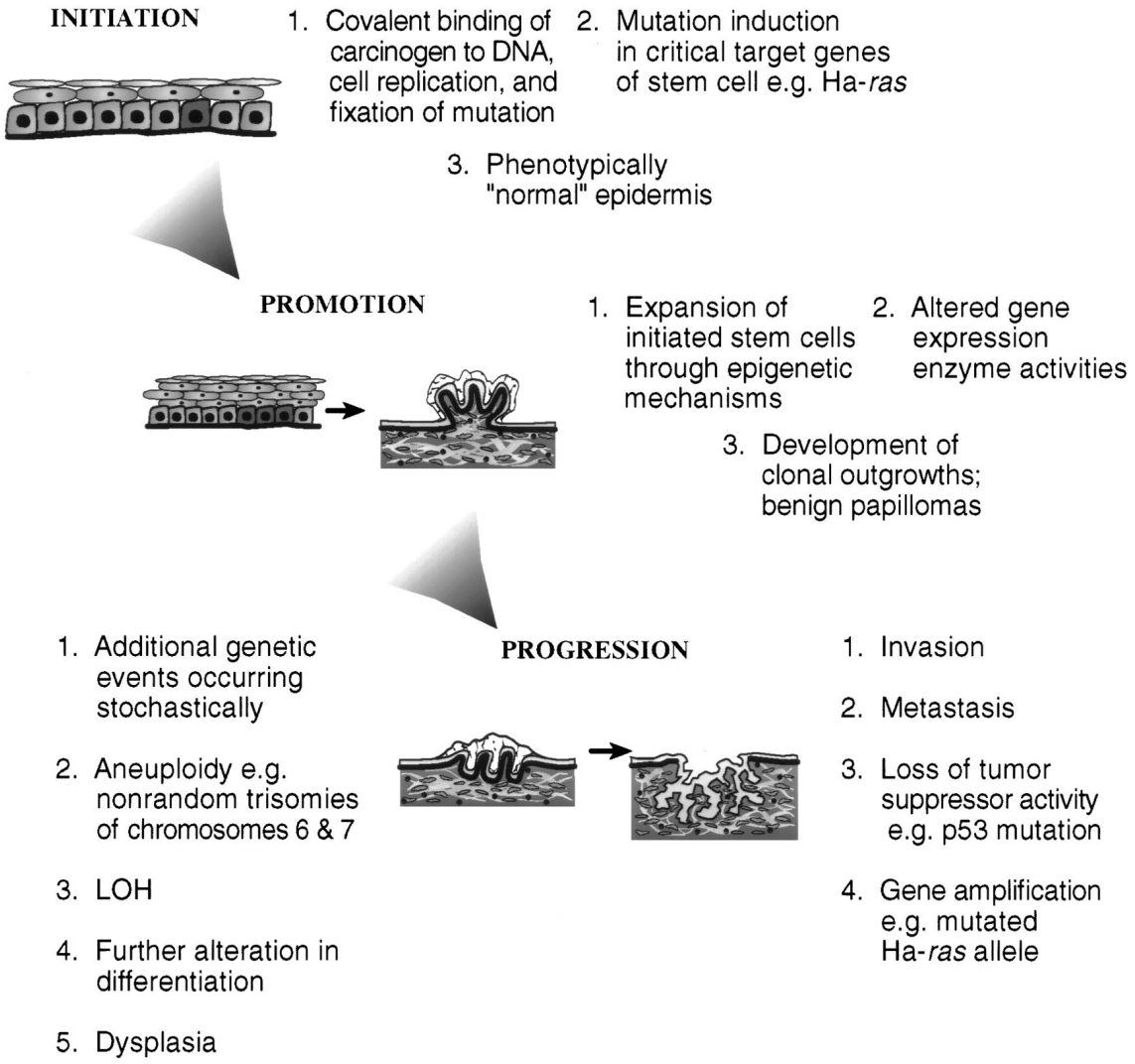


Figure 1.2: Model for skin multistage carcinogenesis. The first stage of carcinogenesis is initiation, where a genetic mutation occurs that is not repaired. In the promotion stage, the mutated cells proliferate and can form benign papillomas. The final stage involves invasion and metastases of the mutated cells. [3]

1.2. Breast Cancer

1.2.1. Rates and statistics

Up to one third of all new cancer cases in women are breast cancer (see Table 1.3) [2]. Breast cancer is the second most common type of cancer diagnosed, next to skin cancer. With the exception of melanoma, all types of cancer listed in Table 1.3 have a lower 5-year survival rate than breast cancer [2], but the high incidence of this disease is cause for concern. Female deaths from breast cancer are second only to lung cancer in cancer deaths, with over 41,000 women dying from breast cancer each year in the US [1].

Table 1.3: 2002 Cancer lifetime probability rates for US women. Adapted from [2].

Site	Rate
All sites	**1 in 3**
Breast	**1 in 8**
Lung and bronchus	**1 in 17**
Colon and rectum	**1 in 18**
Uterine corpus	**1 in 38**
Non-Hodgkin lymphoma	**1 in 55**
Ovary	**1 in 68**
Melanoma	**1 in 77**
Pancreas	**1 in 79**
Urinary bladder	**1 in 88**
Uterine cervix	**1 in 135**

1.2.2. Mammary gland development

1.2.2.1. Stages of development

The mammary gland has distinct stages of development and is a unique organ due to its ability to continually change over a lifetime. These stages of mammary development have been summarized in Figure 1.3. The mammary gland develops early in embryogenesis as a derivative of the epidermis [6]. During puberty, ductal branching and elongation occurs and at the distal epithelial-stromal boundary, a club-shaped terminal end bud forms. There are two distinct ductal branching processes: (1) terminal end bud bifurcation, leading to the formation of two smaller ductules or alveolar buds;

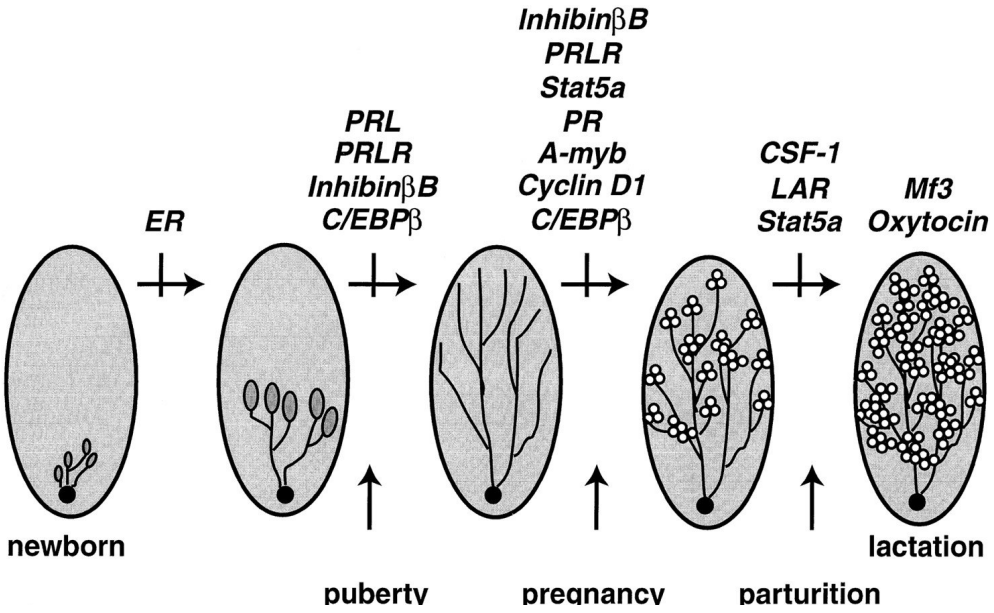


Figure 1.3: Distinct stages of mammary gland development. The mammary gland initially develops during embryogenesis. During puberty, there are events of ductal branching and elongation. Alveolar proliferation occurs during pregnancy. Critical gene products including hormones and hormone receptors are shown. Abbreviations used: ERα (estrogen receptor), PRL (prolactin), PRLR (prolactin receptor), PR (Progesterone receptor), CSF-1 (macrophage specific colonary stimulating factor 1). [9]

(2) sprouting of side branches from mature ducts [7,8]. A group of branches is referred to as a lobule and lobule (Lob) types 1, 2, 3 are defined by the cluster size, with 11, 47 and 80 being the average number of ductules, respectively [7]. During pregnancy, alveolar proliferation occurs to prepare for lactation. The expansion of the mammary epithelium into milk-producing alveoli occurs as a result of hormonal stimulation. Functional differentiation is another morphogenetic change that can occur during parturition and lactation [9]. At this stage, the secretory epithelium dies via apoptosis, the fat cell redifferentiates and the mammary gland is remodeled to its pre-pregnancy state [8].

1.2.2.2. Stromal-epithelial interactions

The mammary gland consists of both stromal and epithelial cells that communicate with each other via the extracellular matrix [8]. This communication is critical for proper patterning and normal mammary gland function and its disruption can lead to the development of breast cancer [6]. There are parallels between the different stages of mammary development and tumor progression including invasion, reinitiation of cell proliferation, resistance to apoptosis and angiogenesis [8]. Epithelial cells possess invasive properties in that they proliferate and invade the stroma. Tumorigenicity is greatest in the mammary epithelium due to its proliferative and invasive abilities but epithelial cells are controlled by stromal cells. The stroma is a heterogenic combination of adipocytes, pre-adipocytes, fibroblasts, blood vessels, inflammatory cells and extracellular matrix that are all subject to regulation in various

stages of mammary development. The lactating mammary gland possesses anti-apoptotic mechanisms to prevent premature involution. Angiogenic remodeling is also a part of the morphogenetic changes that occur in the mammary gland [10].

1.2.2.3. Hormones and mammary gland development

The exact roles of the hormones estrogen and progesterone on mammary proliferation are still somewhat controversial. The potential effects of estrogen on various types of mammary epithelial cells are summarized in Figure 1.4. Normal ductal growth is controlled by estrogen and progesterone that are secreted from the ovaries and their excretion is positively regulated by growth hormone (GH) [7]. Estrogen is thought to play a major role in stimulating growth of both normal and neoplastic mammary epithelium [11,12], however, when estrogen and progesterone are at their peak levels during normal cycling, maximal proliferation is not achieved. Peak growth is achieved during the luteal phase where levels of progesterone and estrogen are significantly lower [8]. There is added complexity regarding the effects of estrogen and progesterone in animal models and cell culture, estrogen stimulates growth of cells in culture and in athymic nude mouse models whereas progesterone has no effect on growth and can be growth inhibitory [13,14].

The estrogen receptor (ER) and progesterone receptor (PR) status is correlated with proliferative capacity. Lob 1 contains the highest level of these ovarian hormone receptors and has the greatest proliferative capacity [7]. Interestingly, nulliparous women have the highest percentage of Lob 1 and also have an enhanced risk of breast

cancer [15]. Another intriguing observation is the lack of correlation between proliferating cells and ER-positive cells, yet there is a positive association between proliferation and nuclear receptor expression [16].

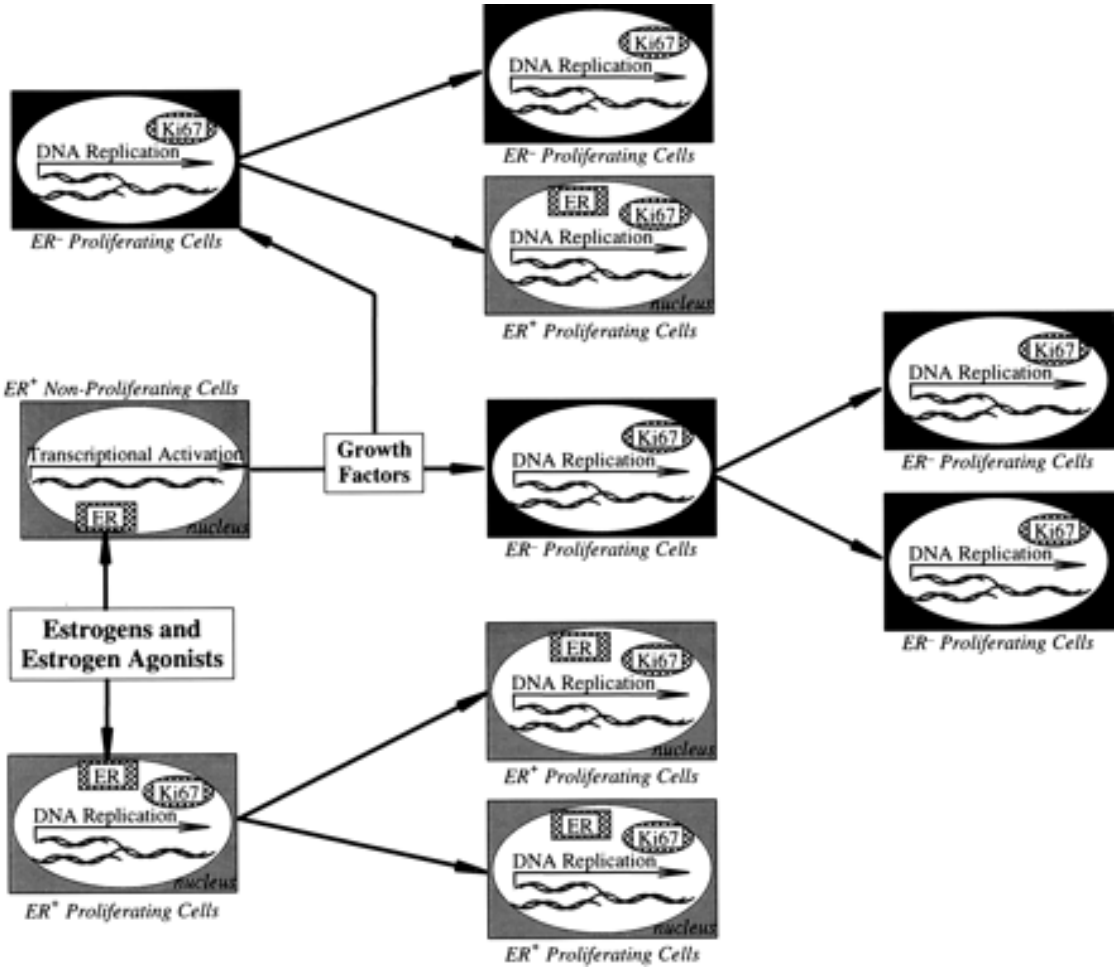


Figure 1.4: Potential pathways of estrogen action on mammary epithelial cells. Estrogen can stimulate ERα positive proliferating cells. The effects of estrogen on ERα positive nonproliferating cells may involve the production of a growth factor that in turn, stimulates growth in ERα negative cells. During neoplastic transformation, ERα negative cells may convert to ERα positive cells. [7]

Several other hormones principally are also involved in mammary gland development. Both activins and inhibins, which are members of the transforming growth factor beta (TGF-β) family are involved in ductal elongation and alveolar development, as shown in Figure 1.3 [6]. The exact role of activins/inhibins in these processes has yet to be elucidated but may involve a role as local mediators of hormone signals [9]. Prolactin signaling is also essential for lobulo-alveolar differentiation during pregnancy [17] and prolactin acts through the JAK-STAT pathway to induce this stage of differentiation [18]. There is also evidence that prolactin may play a role earlier in development during puberty [19]. Oxytocin is another hormone that is necessary for milk ejection and alveolar cell proliferation [9]. Oxytocin is transported through the bloodstream, interacts with receptors on the surface of myoepithelial cells to initiate their contraction and the subsequent release of milk from the alveoli into the ducts [20]. Colony-stimulating factor-1 (CSF-1) is also important for lactation. Deletion mutants of CSF-1 have reduced ductal growth during pregnancy and lack of milk secretion [20].

1.2.3. Characterization of breast cancer

1.2.3.1. Histologic types of breast cancer

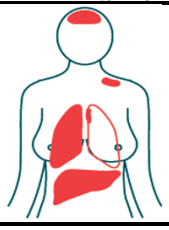
Breast tumors can be classified based on histological examination of their tumor location. In situ refers to a non-invasive form of breast cancer that can be confined to the ducts (ductal carcinoma in situ, DCIS) or lobules (lobular carcinoma in situ, LCIS). DCIS is the most common type of non-invasive breast cancer. If women have DCIS or LCIS, they run a higher risk of developing an invasive form of breast cancer. Once

cancer cells break through the duct wall and spreads into the fatty tissue of the breast, it is classified as invasive ductal carcinoma (IDC) which comprises about 10-15% of invasive breast cancer. Conversely, invasive lobular carcinoma constitutes 85-95% of breast cancer cases and involves cancer cells extending past the lobule wall [5]. Medullary carcinoma is a special type of IDC where there is a distinct boundary between the tumor and normal tissues and accounts for 5% of breast cancer cases. There are also a large number of tumor cells and immune cells at the edges of the tumor. Colloid cancer is a specific type of ductal breast cancer formed by mucus-producing cancer cells. Tubular carcinoma is a special type of infiltrating ductal breast carcinoma. Inflammatory breast cancer is found in 1-3% of breast cancer cases. [3]

1.2.3.2. Breast cancer stages

Breast cancers are initially categorized by tumor size (T), lymph node development (N), and the presence or absence of distant metastases (M). Based on these parameters, they are graded into 4 stages, with a higher stage pertaining to increased invasiveness. A summary of the stages and combination of T, N, and M values is shown in Table 1.4. Stage I is early-stage cancer and refers to the presence of a breast tumor less than 2 cm and no signs of lymph node development or distant metastasis. In the next stage, the tumor is between 2 and 5 cm or there are signs of lymph node development but no signs of metastasis. Inflammatory breast cancer is characterized as IIB as long as it has not spread to other organs. Stage IIIA characterizes tumors more advanced than Stage II tumors in terms of a higher degree of lymph node development where they may

Table 1.4: Summary of characteristics for each stage of breast cancer. Tumor size classes (T): T0=no tumor; T1=≤ 2.0 cm, T2=≥ 2.0 cm ≤ 5.0 cm; T3=≥ 5.0 cm; T4= Any size with direct extension to chest wall or skin. Lymph node development classes (N): N0=No regional lymph node metastasis; N1= metastasis to movable ipsilateral axillary lymph node; N2=Metastasis to ipsilateral axillary lymph node(s) fixed or matted. Distant metastasis classes (M): M0= No distant metastasis; M1=Distant metastasis. Adapted from [2,21]

Stage	5-Year Survival Rate	Stage Subtype	Tumor size (T)	Lymph node development (N)	Distant Metastasis (M)
I 	95-98 %	-	T1	N0	M0
II 	76-88 %	A	T0-T2	N0-N2	M0
		B	T2-T3	N0-N1	M0
III 	49-56 %	A	T0-T3	N1-N2	M0
		B	T0-T3	N0-N2	M0
		C	T4	N0-N2	M0
IV 	15-20 %	-	T4	Any N	M1

be clumped or a tumor larger than 5 cm. In Stage IIIB, the tumor includes the breast skin or extends to the chest wall or involves internal mammary lymph nodes. The fourth and most advanced stage of breast cancer can be generally applied to tumors that have metastasized most commonly to the liver, lung or brain. Each increase in stage is correlated with a worse prognosis which requires more aggressive treatment modalities. [21]

1.2.3.3. ER status of breast cancer

The ERα status of a tumor is a critical prognostic factor and important for determining appropriate treatments. ERα–positive breast cancers are defined as those that positively respond to hormone therapy. Hormone therapy specifically targets estrogen signaling and the subsequent mitogenic effects of estrogen on tumor cells. ERα–negative breast cancers do not respond to hormone therapy and are considered more aggressive with a worse prognosis than their ERα–positive counterparts. Because they do not respond to hormone therapy, the use of cytotoxic drugs is often employed and these compounds have an extensive list of adverse side effects.

ERα-negative nondividing cells constitute the largest group of cells in the mammary gland [22]. Only 30% of cells are ERα-positive at any one time and these cells are rarely dividing [16]. Invasive breast cancer typically arises from an increasingly abnormal series of hyperplasias, atypical hyperplasias and noninvasive carcinomas [23]. There are two models used to describe the origins of ERα-negative tumors: (1) the loss of the ability to synthesize ERα or (2) ERα-positive and ERα-

negative cancers originate from entirely different subpopulations of cells [24,25]. ERα methylation is more prominent in ERα-negative breast cancer and may account for repressing ERα expression [26]. ERα is degraded via hypoxia-induced factor 1 alpha under hypoxic conditions, which is common feature of the tumor microenvironment [27]. The precise etiology of ERα–negative breast cancer is still under investigation.

1.2.4. Influences on breast cancer development

1.2.4.1. Genetic factors

Inherited genetic susceptibility accounts for approximately 5-10% of breast cancer cases [28] and the genes associated with hereditary breast cancer include breast cancer 1 gene (BRCA1), breast cancer 2 gene (BRCA2), tumor protein p53 (TP53), checkpoint kinase 2 (CHK2) and Ataxia-telangiectasia gene (ATM) [29]. All of these genes are involved with DNA repair and maintenance of genomic integrity. BRCA1 was initially named in 1992 after early-onset familial breast cancer cases were linked to chromosome 17q21 [30]. Families with high incidences of male breast cancer did not have BRCA1 mutations, which spurred the discovery of BRCA2 on chromosome 13q12-13 [31]. BRCA mutations are frequently protein truncations and occur at a frequency of 1 in 250 women [29]. Penetrance is defined as the risk of developing breast or ovarian cancer by the age of 70 and is 80% for women with BRCA1 and BRCA2 mutations [29]. Tumor characteristics of BRCA1 and BRCA2 mutations are summarized in Table 1.5. Both BRCA1 and BRCA2 function in DNA repair by interacting with RAD51, a key DNA repair enzyme which catalyzes homologous recombination. Therefore BRCA

mutations lead to hypersensitivity to agents that crosslink DNA strands [29]. BRCA1 also plays a role in ubiquitylation, a process by which proteins are tagged for degradation. The BRCA1-BARD1 (BRCA1-associated ring domain protein) heterodimer has significant ubiquitin ligase activity [32]. In addition, BRCA1 plays a role in checkpoint control and chromatin remodeling [29]. In summary, BRCA proteins play roles in a number of critical cell processes.

Table 1.5: Key characteristics of BRCA1 and BRCA2-associated breast cancers. [29]

Phenotype	**BRCA1-associated**	**BRCA2-associated**
Morphology	Ductal (75%), atypical medullary (~10%)	Ductal (75%), atypical medullary (<5%)
Grade	High (grade 3)	Medium (grade 2) or high (grade 3)
Estrogen receptor expression	Negative (75%)	Positive (75%)
ErbB2 expression	Negative (95%)	Negative (95%)
p53	Positive (50%)	Positive (40%)
Cyclin D1 expression	Negative (90%)	Positive (60%)
Carcinoma in situ	Rare	Common

Another frequently mutated gene in breast cancer is p53, with 30% of breast tumors having a mutation [33]. p53 is a transcription factor that is involved in cell cycle regulation, DNA maintenance and genomic integrity, repair after DNA damage and apoptosis [33]. p53 mutations commonly precede loss of heterozygosity (LOH) of the wild-type allele [33]. Due to the critical role of p53 in the cell, its mutation can lead to cancer since cell growth continues in spite of damaged DNA and therefore increases the

frequency of other gene mutations. Germline mutations in the p53 gene have been responsible for Li-Fraumeni syndrome [34]. The penetrance for breast cancer in families with Li-Fraumeni syndrome is a minimum of 50% by the age of 50, suggesting that p53 is important in the early stages of breast cancer [33]. The type and frequency of p53 mutations is modulated by BRCA1/BRCA2 status, with a higher tumor incidence of p53 mutations in BRCA1/2 patients [35]. Recently, CHK2 germline mutations have been associated with classical and variant Li-Fraumeni syndrome [28]. CHK2 and p53 closely interact and CHK2 directly phosphorylates the site where p53 binds to Mdm2, thus preventing the Mdm2 inhibition of p53 [36].

The third principal syndrome associated with autosomal dominant inheritance of breast cancer risk is Cowden syndrome [28]. Cowden syndrome is due to PTEN germline mutations [37] and is a rare autosomal dominant condition. Women with Cowden's syndrome have a 30-50% chance of developing breast cancer by the age of 50 [38]. PTEN is a phosphatase that negatively regulates the phosphoinositide 3-kinase signaling pathway [39].

Ataxia-telangiectasia is a rare autosomal recessive disorder, with homozygotes occurring only 3 in 1 million and increases risks for a variety of diseases including an increased incidence of breast cancer [39]. Both in vivo and in vitro studies have shown AT carriers have an extreme sensitivity to radiation and an increased risk of radiation leading to cancer [40]. ATM is the only gene causally linked to ataxia-telangiectasia. Like many other genes implicated in inherited breast cancer, ATM is involved with

DNA repair and is required for DNA double-strand break repair, apoptosis and cell cycle arrest [39]. Although homozygosity is rare, AT heterozygosity is present in 1.5% of the population and may account for 7.5% of breast cancer cases [41].

1.2.4.2. Environmental factors

In addition to genetic factors, there are a variety of factors that can influence the development and severity of breast cancer. Environment plays a much larger role than genetics in terms of cancer susceptibility. In an epidemiology study of twins in Finland, 18% and 77% of the liability in inter-individual variation in the risk of breast cancer were attributable to shared environmental factors and unique environmental factors, respectively [40]. Female gender, country of birth and age are major risk factors for breast cancer [42]. Only 0.6% cases of breast cancer are males. A summary of established risk factors is shown in Table 1.6. There are other more inconclusive risk factors that may contribute to breast cancer risk. Rodents fed a high-fat diet had enhanced mammary tumor development [43]. More specifically, diets rich in linoleic acid have increased breast cancer development [44]. Other fats including oleic acid and fish oil have a protective effect against cancer [44].

Many of the known risk factors of breast cancer are associated with reproductive characteristics including pregnancy-related and menstrual factors. The risk of breast cancer increases the older a woman is for her first pregnancy. The relative risk is 0.5 for mothers aged 20 or younger and increases to 1.4 over the age of 35 [45]. This may be due to changes in breast tissue that result in decreased susceptibility to carcinogenic

agents or hormonal changes that reduce breast cancer risk [46]. Nulliparity is also a risk factor for breast cancer. Women with at least 5 pregnancies have a breast cancer relative risk of 0.5 compared to women who have had no children [47,48].

Females that have begun menstrual cycles at an earlier age and women that reach menopause at a later age have an enhanced breast cancer risk [47,49]. These effects are due to a positive association between the total number of years of menstrual active and breast cancer incidence [49]. Oophorectomies at a young age (before 40) are also associated with a decreased risk of breast cancer [50]. Both human reproductive and menstrual factors and animal studies indicate that hormones play a significant role in breast cancer incidence and epidemiology studies suggest that lifetime estrogen exposure is associated with an increase risk of breast cancer [50,51]. However, the mechanisms of estrogen-induced breast cancer are not that well understood.

A Women's Health Initiative (WHI) study recently showed that combined estrogen and progestin used in postmenopausal women was associated with an increased incidence in invasive breast cancer taking these hormones for a 5-year period [52,53]. In addition, breast cancers diagnosed in women taking estrogen and progestin therapies were a more advanced form of cancer [53]. Other studies suggest estrogen and progestin exposure increase mammographic breast density when compared to control groups or women treated with estrogen alone [54,55]. The biological relevancy of breast density on breast cancer risk is still under investigation. Although the WHI study did not support a relationship between postmenopausal hormone replacement therapy and the

incidence of invasive lobular breast cancers [53], other studies reported an increased incidence in this type of cancer with hormonal replacement therapy [56,57].

Prior to the WHI study, breast cancer mortality was reported to be lower in women using hormone replacement therapy [58-60], and it was suggested that this was due to earlier diagnosis and treatment [61,62]. Differences between studies may be a result of older women and the different treatment regimen in the WHI study compared to the younger women studied in previous reports [63].

Exogenous hormones from oral contraceptives, depot-medroxyprogesterone acetate and estrogen replacement therapy have been studied and a number of epidemiology studies have shown no correlation between oral contraceptive use and breast cancer risk [64-66], however, this conclusion remains controversial [67]. No relationship has been found between the injectable contraceptive, depot-medroxyprogesterone (DMPA) and breast cancer risk [68,69]. Several studies have reported no association between breast cancer risk and hormone replacement therapy [70-72], however an increased risk was observed with the use of hormone replacement therapy for more than 20 years [73,74] or estrogen/progestin therapy [75].

In postmenopausal women, body weight and height have been correlated to breast cancer risk [76]. Heavy menopausal women have higher rates of conversion of androstenedione to estrogen in adipose tissue and lower levels of sex-hormone-binding globulin compared to thinner women [46]. For younger, premenopausal women, there is a higher risk of breast cancer in thinner women but this may be due to easier detection of

tumors in these individuals [76]. Adult weight gain increases the premenopausal risk of breast cancer whereas weight loss may be protective.

There have also been some reports linking diethylstilbestrol (DES) use during pregnancy to a modest but significant increase in breast cancer risk and this does not increase over time [77,78]. The proportion of ERα-positive tumors increases with age, is higher in whites than in blacks and may be higher in postmenopausal than in premenopausal women. It is not known whether the estrogen-receptor status of normal cells affects the likelihood of cancer following exposure to a carcinogenic agent.

The effects of several environmental exposures on breast cancer incidence have also been investigated. Organochlorine compounds are present in the environment due to their use in industry and as pesticides and environemental persistence. High serum levels of organochlorines in breast cancer patients in some studies have been associated with up to 4-fold increased breast cancer risk [79-81]. However, epidemiology studies have not shown a statistically significant link between organochlorine exposure and increased breast cancer incidence [82-84]. Organic solvents are commonly employed in the production of glues, paints and other industrial chemicals. The influence of organic solvent exposure on breast cancer risk have been inconclusive with some occupational studies showing an increased risk [85,86] whereas other studies showed no increased breast cancer risk in women occupationally exposed to high levels of organic solvents [87,88]. Epidemiological studies have also shown a modest increase in breast cancer

risk after metal exposure [89] and some divalent metals including cadmium, copper and cobalt exhibit estrogenic activity in MCF-7 breast cancer cells [90].

Cigarette smoke contains an array of carcinogens and for this reason has been of interest as a risk factor for a variety of cancers. In vitro and in vivo studies have suggested the breast as a target tissue for tobacco smoke carcinogens since they can pass through the alveolar membrane and are transported by lipoproteins to the breast [91-93]. The link between breast cancer and smoking has been extensively studied but has generated inconclusive results, with positive, inverse and null relationships between smoking and breast cancer risk [91]. Critical timing of smoking has also been investigated and this is based on the hypothesis that when the breast is developing, it is most susceptible to carcinogens present in cigarette smoke. The temporal effects of smoking on breast cancer have been investigated and exposures during breast development has also failed to yield any positive associations with increased breast cancer risk [94,95].

A recent meta-analysis of 89 studies comparing alcohol consumption to breast cancer incidence showed 29 studies with odds ratios less than 1 and 60 studies greater than 1, overall supporting a modest, but enhanced risk of breast cancer associated with increased alcohol consumption [96]. There was no significant difference in breast cancer risk seen between consumption of beer, wine or spirits. An increase of one drink per day resulted in a 10-13% increase in breast cancer risk.

Table 1.6: Established risk factors for breast cancer in females. Adapted from [46,97]

Risk Factor	High-Risk Group	Low-Risk Group
HIGHEST RISK (RELATIVE RISK GREATER THAN 4.0)		
Age	Old	Young
Country of birth	North America Northern Europe	Asia, Africa
Mother and sister with history of breast cancer	Yes	No
MODERATE RISK (RELATIVE RISK FROM 2.0 TO 4.0)		
Socioeconomic status	High	Low
Age at first full-term pregnancy	>30 years	<20 years
Oophorectomy premenopausally	No	Yes
History of cancer in one breast	Yes	No
History of benign proliferative lesion	Yes	No
Any first-degree relative with history of breast cancer	Yes	No
Mammographic parenchymal patterns	Dysplastic parenchyma	Normal parenchyma
Radiation to chest	Large doses	Minimal exposure
LOWER RISK (RELATIVE RISK FROM 1.1 TO 1.9)		
Marital status	Never married	Ever married
Place of residence	Urban	Rural
Race >45 years *<40 years*	White Black	Black White
Nulliparity	Yes	No
Age at menopause	Late	Early
Age at menarche	Early	Late
Weight, postmenopausal women	Heavy	Thin
Alcohol consumption	>4 alcoholic beverages/day	No alcohol
History of primary cancer in endometrium or ovary	Yes	No

1.2.5. Current treatments

There are 3 primary surgical options for treatment of breast cancer: (1) lumpectomy surgery and radiation (2) mastectomy plus reconstruction (3) mastectomy alone. Lymph node surgery also may be employed in cases where cancer has spread to the lymph nodes (>T0). There are adjuvant therapies, which increase the efficacy of the primary treatment and therefore reduce the likelihood of cancer reoccurrence and these include radiation, systemic therapy, chemotherapy and monoclonal antibody therapy. Radiation therapy is used to destroy any remaining cancer cells left in the breast, chest wall or lymph nodes after surgery and is most commonly used after lumpectomies. Adjuvant systemic therapy targets cells that may have spread beyond the breast and adjacent tissues. This includes hormone therapy if the tumor is ER-positive and drugs such as tamoxifen, or aromatase inhibitors (e.g. anastrozole) are used. There is also neoadjuvant therapy, which is carried out prior to surgery as a potential method to shrink the tumor so it can be removed surgically without the need for mastectomy. [1,98]

Adjuvant chemotherapy usually involves the use of a genotoxic agent and common genotoxic agents employed in breast cancer are summarized in Table 1.7. Doxorubicin and epirubicin work by two mechanisms: (1) DNA intercalation and subsequent blockage of DNA synthesis and transcription and (2) inhibition of topoisomerase II enzyme. These effects lead to breaks in the genomic DNA. Cyclophosphamide acts as a DNA alkylating agent and other chemotherapeutic agents also interact with DNA by acting as a DNA alkylating agent (cyclophosphamide) or a

pyrimidine analog (gemcitabine). Other drugs interfere with chromosomes and hence halt cell division and induce cell death. In the presence of paclitaxel or docetaxel the chromosomes are unable to move to opposite sides of the dividing cell because microtubules are not broken down. The vinca alkaloids first bind to tubulin monomers inhibiting the formation of microtubules. Without adequate numbers of microtubules, the newly replicated chromosomes can not be separated and cell division is blocked. The aborted cell division process triggers cell death. Capecitabine is metabolized into 5-FU in the cells which interferes with cell division, RNA and protein processing and ultimately results in cell death. [99]

Table 1.7: Summary of commonly used cytotoxic chemotherapeutic breast cancer drugs [1,99]

Brand Name (Generic Name)	Mechanism of Action
Adriamycin (Doxorubicin)	DNA intercalation Topoisomerase II inhibition
Cytoxan (Cyclophosphamide)	DNA alkylating agent
Ellence (Epirubicin)	DNA intercalation Topoisomerase II inhibition
Gemzar (Gemcitabine)	Pyrimidine analog
Navelbine (Vinolrelbin)	Inhibits microtubule formation
Taxol (Paclitaxel)	Prevents microtubule breakdown
Taxotere (Docetaxel)	Prevents microtubule breakdown
Xeloda (Capecitabine)	Interferes with RNA and protein processing

Adjuvant monoclonal antibody therapy is also commonly employed in women who have HER2/Neu overexpressing tumors. HER2/Neu activates the PI3K/Akt pathways which inhibits apoptosis, therefore enhancing cell proliferation. In addition, HER family members have also been associated with secretion of pro-invasive proteins that degrade the basement membrane. Monoclonal antibody therapy inhibits the function of HER2/Neu receptor to favor cell death and therefore reduces the invasive capacity of the cells. [1]

1.2.6. Chemoprevention

Cancer chemoprevention refers to the use of a chemical, either natural or synthetic, that can block cancer in the early stages or hinder cancer progression before the cancer progresses to a malignant stage. To block the early stages of cancer development, chemicals can reduce the likelihood of DNA damage by modulating metabolizing enzymes or by increasing the quantity of antioxidants that detoxify potentially mutagenic compounds. Since cancer also requires cell proliferation to progress, inhibition of cell proliferation is a necessity and this can be accomplished by either reducing inflammation, which can lead to increased cell proliferation or by modulating regulatory components involved in cell growth.

1.2.6.1. Antimutagens

Antimutagens encompass agents that inhibit the initiation stage of cancer. Sulforaphane is a glucosinolate phytochemical found in cruciferous vegetables and is considered an antimutagen. Early studies showed that sulforaphane inhibits Phase I drug

metabolizing enzymes involved in carcinogen activation as well as induce Phase II drug metabolizing enzymes such as glutathione-S-transferase (GST) involved in the detoxification of carcinogens [100]. However, more recently other effects of sulforaphane have been reported. For example, sulforaphane induces cell cycle arrest including a G2/M arrest also disrupts microtubule polymerization in MCF-7 breast cancer cells [101,102]. Other studies have also shown that sulforaphane is an HDAC inhibitor, inhibitor of H. pylori inflammation and induces apoptosis in other cancer cell lines [103-105] .

1.2.6.2. Antiinflammatory compounds

Antiinflammatory drugs such as cyclooxygenase-2 (COX-2) inhibitors inhibit both the promotion and progression of neoplasia. NSAIDs, which inhibit both cyclooxygenase (COX-1) and COX-2 also reduce breast cancer incidence [106]. Cyclooxygenase enzymes are involved in prostaglandin synthesis, which play important roles in blood clotting, wound healing, immune response, bone metabolism, nerve growth and development [107]. Inhibition of COX-2 has been associated with decreased cell proliferation, induction of apoptosis and a reduction in inflammation [108] and therefore is a putative chemotherapeutic target. In addition, COX-2 expression is elevated in some tumors such as colorectal tumors [109].

1.2.6.3. Antioxidants

Tea is the second most popular beverage in the world and recent epidemiological studies have linked an increased consumption of green tea with a decreased incidence of breast cancer [110]. In addition to the epidemiological data, green tea has been extensively investigated by cancer researchers due to its antioxidant properties [111]. Antioxidants have the ability to inhibit both initiation and promotion of cancer due to their ability to act as reactive oxygen species (ROS) scavengers [112]. In vitro and in vivo studies have also shown that green tea extracts, specifically catechins, inhibit breast cancer cell growth and reduce the size and multiplicity of carcinogen-induced rodent tumors [113,114]. Green tea extracts are promising new cancer agents, although more work on the mechanism of growth inhibition still needs to be carried out.

Omega-3 fatty acids also have a protective effect against breast cancer development. Fish oil, which is rich in omega-3 fatty acids, inhibited growth in breast cancer cells and reduced lung metastases in a xenograft model [115] and there is increasing evidence that omega-3 fatty acids also have effects on cell transformation based on data showing that omega-3 fatty acids decreased levels of ras p21, a proto-oncogene. However, case control studies have been unable to show a link between fish consumption and breast cancer incidence in the United States and Norway [116,117].

1.2.6.4. Hormone modulators

Endocrine therapy for breast cancer targets estrogen by either inhibiting the binding of estradiol to ER through the use of selective estrogen receptor modulators (SERMs) such as tamoxifen or by reducing the amount of estrogen produced through the use of aromatase inhibitors. Aromatase inhibitors interfere with the conversion of testosterone to estradiol by blocking the aromatase enzyme necessary for this reaction. Both endocrine therapies are focused on decreasing the mitogenic effects of estrogen on mammary epithelial cells. There have been large prospective clinical trials showing a reduction in breast cancer incidence in women with adjuvant treatment of tamoxifen [47-49,118] and aromatase inhibitors such as anastrozole look even more promising. Premenopausal women with early breast cancer had significantly reduced onset to an invasive form of breast cancer with anastrozole treatment compared to tamoxifen treatment [52].

1.2.6.5. Signal transduction modulators

Signal transduction modulators inhibit the promotion and progression of neoplasia. Vitamin D is known to play a significant role in controlling Ca^{2+} homeostasis and is involved in cell proliferation and hence has been investigated for its chemotherapeutic potential. 1,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3) inhibited tumor growth in the 7,12-dimethylbenz[a]anthracene (DMBA)-induced rat mammary cancer model as well as inhibiting cell proliferation in breast cancer cells [119]. However, there were many side effects after treatment with 1,25$(OH)_2$D3 and these are associated with

its prominent role in calcium homeostasis [119]. Treatment with analogues of vitamin D3 also inhibited mammary tumor growth without the potent hypercalcemic side effects of Vitamin D [120,121]. These compounds may be inhibiting growth by modulating genes involved in proliferation, differentiation and cytokine production via the vitamin D receptor [122]. Synthetic analogs of Vitamin D are promising new agents for breast cancer treatment.

The consumption of cruciferous vegetables has been correlated with a decreased risk of several types of cancer [123-127]. 3,3'-Diindolylmethane (DIM) is formed in the gut as a condensation product of the phytochemical indole-3-carbinol (I3C) [128,129]. Several studies show that cruciferous vegetables (or their extracts), indole-3-carbinol (I3C) and 3,3'-diindolylmethane (DIM) exhibit antitumorigenic activity in *in vivo* rodent models and inhibit growth of cancer cell lines *in vitro* [130-135]. For example, I3C inhibited growth and/or formation of DMBA-induced and spontaneous age-dependent mammary tumors and decreased the incidence of spontaneous endometrial tumors in rodent models [131,133].

1.3. Mechanism-Based Treatment Targets

The ideal anticancer drug specifically targets cancer cells with minimal side effects on other tissues. Two properties that can significantly influence the adverse effects associated with a drug include the mode of cell death and the specificity of the biological target. Early chemotherapeutic agents such as mustard gas primarily affected rapidly dividing cells due to its DNA alkylation properties. Although tumor cells are rapidly dividing, there are a number of other cell types that undergo rapid proliferation and these include epithelial cells lining the intestine and cells of the immune system and cytotoxic drugs can induce many adverse side effects in these rapidly dividing tissues/cells. The form of cell death induced by a drug can also have a critical influence on its side effects since only necrotic cell death elicits an unfavorable immune response.

1.3.1. Types of cell death

Cell death has been an area of scientific interest for decades. Initially, two distinct forms of cell death were described; namely apoptosis and necrosis. Apoptosis is related to a programmed form of cell death and another form of cell death was characterized as necrosis. Currently 11 different forms of cell death have been characterized and these are summarized in Table 1.8 [136]. The differences between these forms of cell death are now less clear since they have overlapping characteristics [137]. Anoikis refers to apoptotic cell death triggered by cell detachment. In addition, forms of cell death referred to as apoptotic necrosis has also been described

Table 1.8: Different types of cell death. The features of 11 different forms of cell death are summarized below. Abbreviations used: WD, Wallerian degeneration; PLT, platelets; TG, transglutaminases; NO, nitric oxide; NCX, sodium calcium exchange channel; IAP, inhibitor of apoptosis proteins [136]

	Necrosis	**Apoptosis**	**Anoikis**	**Caspase-independent apoptosis**	**Autophagy**	**WD**	**Excito toxicity**	**Erythro-poiesis**	**PLT**	**Corni-fication**	**LENS**
Genetic Program	None	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Membrane	Lysed	Intact PS exposure	Intact PS Exposure	Intact PS exposure	Intact PS exposure	Intact	Intact	Intact	Intact	Intact	
Organelles	Lysed	Intact	Intact	Intact	Intact lipid-reassembly	Intact	Intact	Intact	Intact	Crosslinked lipid-reassembly	
Mitos	Blown	Intact	Intact					Lost		Lost	Lost
Nucleus		Chr. condens. DNA fragm.	Chr. condens. DNA fragm.	Chr. condens. DNA fragm.	Chr. condens. DNA fragm.			Lost	Lost	Lost	Lost
Enzymes	None	Caspases	Caspases	Calpains	Lysosomal Beclin1	VPR	Calpains NCX	Calpains		TG 1,3,5	TG
Receptors		Death Rec									
Regulators		Bcl family IAP					NO calcium	GATA2		AP1 calcium	

where apoptotic cells are not recognized by phagocytes and consequently display necrotic features [138]. The form of cell death induced is dependent on the specific agent as is also cell-context dependent. These factors critically influence which signaling pathways are activated or inhibited, ultimately determining the pathway of cell demise. Apoptosis, autophagy and necrosis are the three most relevant forms of cell death in terms of cancer treatments and will be described in detail below.

1.3.1.1. Apoptotic cell death

Apoptosis refers to programmed cell death that is a genetically encoded form of cell suicide and critical during development and tissue homeostasis [139,140]. During development, cells produced in excess undergo apoptosis for organ and tissue sculpting [141]. The morphologic hallmarks of apoptosis include membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation and eventual engulfment by macrophages or neighboring cells [142]. Packing of cell contents into apoptotic bodies and subsequent phagocytosis occurs in the later stages of apoptosis. One of the "eat me" signals on apoptotic cells is phosphatidylserine flipping from the inner surface to the external surface of the cell membrane as an indicator for phagocytic engulfment [142].

1.3.1.1.1. Intrinsic and extrinsic pathways of apoptosis

There are 2 major pathways of apoptosis: the extrinsic pathway, which is mediated by death receptors and the intrinsic pathway, which is mitochondrial-mediated (see Figure 1.5). Death receptors are cell surface receptors belonging to the tumor necrosis factor receptor (TNFR) family that bind ligands which sense abnormal intracellular or extracellular conditions. Ligands of this family include tumor necrosis

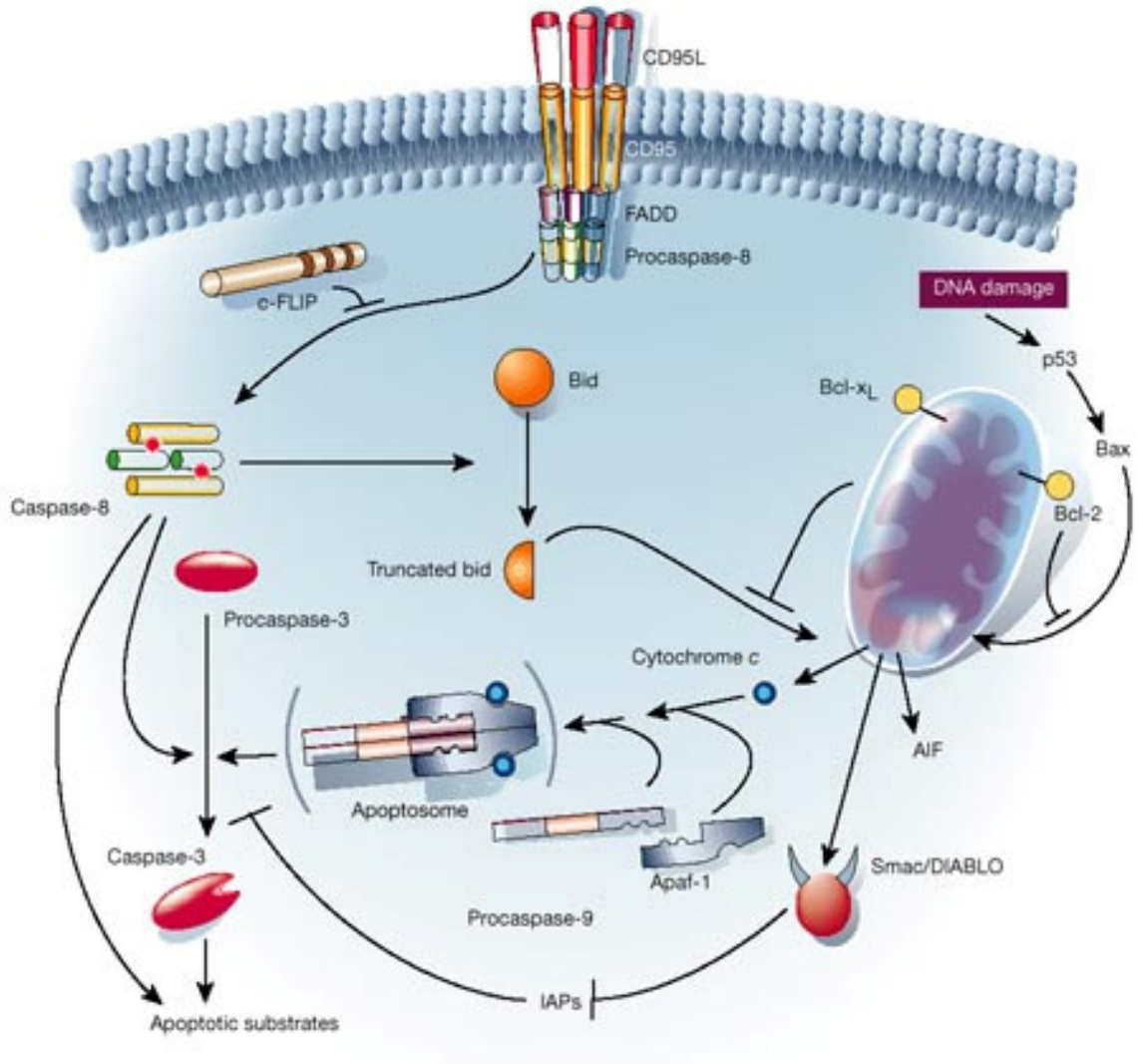


Figure 1.5: Apoptotic death pathways in mammalian cells. There are 2 apoptotic pathways in cells: (1) The extrinsic pathway involving death receptors (e.g. CD95L) that upon ligand binding induce formation of a DISC complex which activates the apical caspase-8 and downstream effector caspase-3 and subsequent cleavage of death substrates. FLIP can inhibit caspase-8 activation. Activated caspase-8 can also cleave Bid, which can induce the mitochondrial-mediated apoptotic pathway. (2) The intrinsic pathway involves the mitochondria and can be activated by various apoptotic stimuli including DNA damage, which can lead to the release of small molecules from the mitochondria including cytochrome c, AIF, Smac/DIABLO. Cytochrome c release triggers the formation of the apoptosome and activation of apical caspase-9, leading to caspase-3 activation, cleavage of death substrates and ultimately apoptosis. Smac/DIABLO induces apoptosis by inhibiting IAP proteins. [143]

factor-alpha (TNF-α), CD-95L/Fas and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TNFRs are ligand-specific in that TNF-α binds to TNFR1, TRAIL binds to DR4 and DR5 and CD-95L bind to the CD-95 receptor. There have also been decoy receptors identified including a soluble CD-95 decoy receptor (TNFR) and three decoy receptors for TRAIL: osteoprotegerin (OPG), decoy receptor 1 (DcR1) and decoy receptor 2 (DcR2). These decoy receptors have mutations to account for their nonfunctional activity. DcR1 lacks a cytosolic region and DcR2 has a truncated nonfunctional form of the death domain. Upon homotrimeric ligand binding, the receptors trimerize and changes occur to the death receptor domain such that the adaptor protein Fas-associated death domain (FADD) and procaspase-8 are recruited, forming the death-inducing signaling complex (DISC). Caspase-8 is autoactivated, which then activates downstream effectors caspases including caspases-3, -6 and -7. In addition, Bid can also be cleaved by caspase-8 to form truncated Bid (tBid), which activates the mitochondrial-mediated pathway of apoptosis. [144]

The intrinsic apoptotic pathway can be activated by various stimuli including DNA damage, growth factor deprivation or oxidative stress [145]. Mitochondria are critical regulators of apoptosis with mitochondrial membrane potential perturbations leading to the production of ROS or mitochondrial membrane permeabilization (MMP) [146]. ROS production can trigger lysosomal membrane permeabilization (LMP) which releases lysosomal enzymes that can cause MMP or lysosomal-mediated autophagic cell death [77,147]. Mitochondria sequester many anti- and pro-apoptotic proteins principally involved in the regulation of apoptosis and these pro-apoptotic proteins can

be released from the mitochondria after MMP. These proteins cause the induction of apoptosis through a multitude of mechanisms.

MMP is regulated by the family of Bcl-2 proteins, however their role in the release of pro-apoptotic proteins from the mitochondria is a subject of debate and several models have been suggested [143]. One model involves Bcl-2 family members undergoing a conformational change such that they insert into the outer mitochondria to form a channel, but the disagreement lies in whether these channels are large enough to allow for protein transport. Bcl-2 members may also form channels with other proteins such as the voltage-dependent anion-exchange channel (VDAC) located in the outer mitochondrial membrane, however this model must include a significant conformational change of VDAC in order to accommodate the proteins. Another postulation is that Bcl-2 causes the outer mitochondrial membrane to rupture by altering mitochondria physiology such as perturbations in ion exchange or oxidative phosphorylation that would induce membrane fission.

Regardless of the pathway, depolarization of the mitochondrial membrane leads to the release of a number of small molecules including cytochrome c, second mitochondria-derived activator of caspase/direct IAP binding protein (SMAC/Diablo), apoptosis-inducing factor (AIF), endonuclease G (EndoG) and Omi/HtrA2 and these proteins can lead to both caspase-dependent and -independent activation of various cellular pathways (see Table 1.9) [148,149]. Cytochrome c release causes formation of the apoptosome, which include the apical caspase-9 and Apaf-1 and requires ATP for activation [143]. Downstream of caspase-9 is the effector caspase-3, which activates a

pathway leading to the cleavage of a diverse range of substrates. The intrinsic and extrinsic apoptotic pathways converge at activation of caspase-3.

Table 1.9: Summary of mechanism of action for small molecules released from mitochondria [143,147,148]

Mitochondrial protein	Mechanism of action
Smac/DIABLO	Inhibits IAPs, survivin
Omi/HtrA2	Inhibits IAPs
AIF	Nuclear translocation, induce caspase-independent DNA fragmentation and chromatin condensation
Endo G	Induces internucleosomal DNA fragmentation
Cytochrome c	Forms apoptosome and activates apical caspase-9

1.3.1.1.2. Pro- and anti-apoptotic proteins

Apoptosis is carried out by two major families: Bcl-2 proteins and caspases. Bcl-2 was originally discovered as a protein that promoted oncogenesis due to its ability to prevent cell death [150]. There are 3 groups in the Bcl-2 family of proteins that are classified based on the number of Bcl-2 homology (BH) domains. Anti-apoptotic proteins Bcl-2, Bcl-x_L and Mcl-1 contain 4 BH domains. The pro-apoptotic proteins can be divided into 2 groups: BH3-only proteins (Bid, Bax, Bim, Noxa, PUMA) or BH1-3 proteins (Bax, Bak, Bok) [151]. The majority of BH1-4 and BH3-only Bcl-2 proteins reside in the mitochondrial outer membrane. The Bcl-2 family of proteins can form homodimers and perhaps more importantly, heterodimers; heterodimer combinations

vary by cell type [143]. Since anti-apoptotic factors can negate the actions of pro-apoptotic factors, the ratio of pro-apoptotic factors to anti-apoptotic factors is a critical indicator of determining sensitivity to cell death. By contrast, BH3-only family members and pro-apoptotic protein Bax relay death stimuli to the mitochondria. Bid, as mentioned previously, is part of the extrinsic pathway and relays trophic factor deprivation signals [152]. PUMA or Noxa respond to DNA damage by translocating to the mitochondria and interact with Bcl-2/Bcl-xL or Bax/Bak families to cause the release of pro-apoptotic proteins from the mitochondria [152].

Bcl-2 proteins play a critical role in intrinsic apoptotic initiation; however, caspases are fundamental for both the initiation and execution stages of apoptosis. There are 14 mammalian caspases that are synthesized as inactive zymogens. Upon apoptosis induction, caspases can be cleaved to their active form that cleaves Asp-Xxx bonds. There are 3 functional groups of caspases: (1) inflammation caspases (caspase-1, -4, -5, -11, -12, -13, -14); (2) apoptotic initiator caspases that have either a death effector domain (DED) (caspase-8, -10) or caspase activation and recruitment domain (CARD) (caspase-2, -9); (3) apoptotic executioner caspases (caspase-3, -6, -7) that have a short prodomain and are activated by upstream caspases to cleave a variety of cellular substrates. [153] Caspases have a wide variety of targets, ranging from apoptotic and structural proteins to cellular DNA repair and cell cycle proteins. [154]

In addition to the Bcl-2 family of proteins, inhibitors of apoptosis (IAPs) are anti-apoptotic proteins involved in apoptosis regulation. As their name suggests, they can confer protection from death-inducing stimuli [155]. There are currently 8 human IAPs:

NAIP, cIAP1, cIAP2, X-linked inhibitor of apoptosis (XIAP), Ts-XIAP, ML-IAP, apollon, and survivin. c-IAP1 and c-IAP2 are part of a signaling complex recruited to the cytoplasmic domain of Type 2 tumor necrosis factor receptor [156]. Survivin is a unique IAP since its expression is cell-cycle regulated [157]. There are several postulated functions of survivin in apoptosis. It may bind to caspase-3 or caspase-9, but it does not possess the functional domains of other IAPs that directly bind to these caspases. Survivin may also bind to procaspase-9 and thus inhibiting the intrinsic pathway of apoptosis [157]. Survivin may also bind to Smac/DIABLO, therefore hampering the inhibitory function of Smac/DIABLO on IAPs. XIAP is the most characterized IAP and is considered the most potent suppressor of apoptosis. XIAP functions by directly inhibiting several caspases including caspase-3, -7, -9 [158]. Both c-IAP2 and XIAP are regulated by the stress-response transcription factor NF-kB, when it exerts a pro-survival effect [159]. Smac/DIABLO is the regulator of IAPs by displacing caspases from XIAP and most likely other IAPs [156].

1.3.1.2. Autophagic cell death

Autophagy is an evolutionarily conserved mechanism of survival triggered by stress-associated damage or nutrient deprivation and encompasses 3 distinct types of autophagy. Chaperone-mediated autophagy refers to the degradation of cytosolic proteins with a specific pentapeptide consensus motif [160]. Microautophagy is the direct engulfment of cytoplasm at the surface of the degradative organelle. In contrast, macroautophagy is characterized by cytoplasmic materials including organelles that are bound in double membrane vesicles and degraded after fusion with lysosomes.

Autophagic cell death, which is morphologically characterized by the accumulation of autophagic vacuoles in cells lacking hallmarks of apoptosis such as nuclear condensation and cell fragmentation, is a controversial death pathway [161]. In the case of nutrient-deprived cells, autophagy can provide short-term relief by degrading the cytoplasmic contents for both proteins and energy production and therefore has traditionally been considered a mechanism of cell survival [162]. However, excess autophagy may lead to type II autophagic cell death; however, it is not known whether formation of autophagic vacuoles in dying cells are generated for cell survival or cell death [147].

Understanding autophagy was enhanced after identification of autophagy-related genes (Atg) that were shown to be involved in the vacuole targeting (Cvt) pathway and pexophagy (peroxisome degradation) [163,164]. Beclin 1 is essential for autophagosome formation and its overexpression induced autophagy and inhibited tumorigenicity in breast cancer cells [165]. LC3/Atg8 is also critical for autophagosome formation and becomes lipidated during activation of autophagy and serves as a marker protein for autophagic vacuoles [166].

Upon nutrient starvation or growth factor deprivation, nutrient-sensing signaling pathways are activated and initiate autophagy. Autophagy is characterized by the sequestration of cytoplasmic material to form a double-membrane vacuole referred to as an autophagosome (Figure 1.6). Several Atg genes are critical in this initial stage of vacuole formation. The next stage involves a late endosome or lysosome which fuses with the autophagosome to form the autolysosome. The final stage in autophagic cell

death is degradation of the autolysosomal contents via lysosomal degradative enzymes. [167]

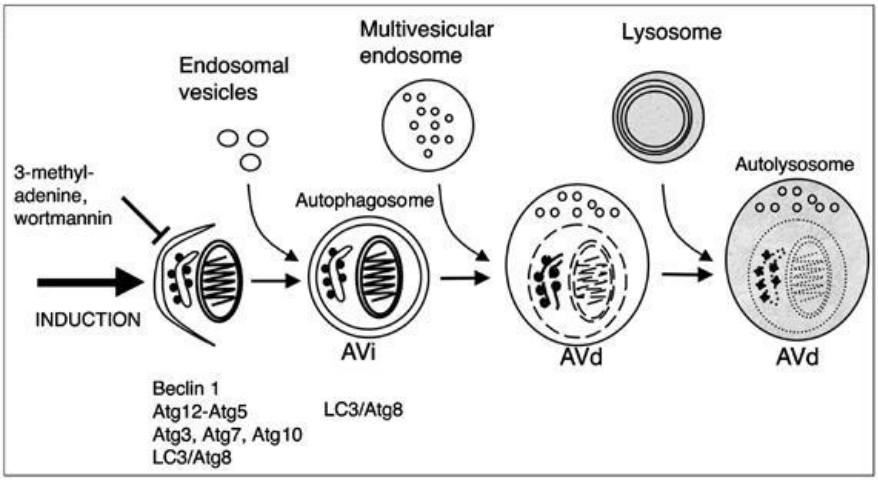


Figure 1.6: Formation and maturation of autophagosomes in mammals. Induction of autophagy involves formation of a double membrane around cytoplasmic materials and/or cytoplasmic organelles and requires Atg genes for this initial stage of autophagosome formation. This induction can be inhibited by Class III PI3K inhibition (3-MA or wortmannin treatment). The autophagosome fuses with a lysosome and the lysosomal enzymes degrade the contents of the autolysosome. Abbreviations used: AVi – Initial autophagic vacuole; AVd – Degradative autophagic vacuoles. [147]

Under growth factor deprivation, autophagy is a critical mechanism for cell survival [147]. Yeast mutants that have defective autophagy genes cannot survive starvation-induced conditions whereas yeast with functional autophagy genes can survive starvation in the short term [168]. The essential role of autophagy genes in cell survival can also be demonstrated in amino acid-deprived mammalian cells [169]. These observations were consistent with autophagy being an unavoidable requirement to prevent apoptosis [169]. Further evidence to support the pro-survival effects of autophagy involved a study utilizing immortalized bone marrow cells from Bax- and Bak-deficient mice that were resistant to apoptotic stimuli [170]. Bone marrow cells

were deprived of the growth factor interleukin 3 (IL3) and were able to maintain ATP production via autophagy; when autophagy genes were inhibited, the cells died. Autophagy, however, can only be considered a short-term mechanism of survival since degradation of all organelles will eventually lead to cell death [147].

Autophagic cell death has been extensively studied in the literature and much remains to be elucidated in terms of the molecular mechanism of autophagic cell death compared to the well-studied process of apoptosis. Cell death could be induced in several cell lines treated with caspase inhibitors and this provided some of the initial evidence for autophagic cell death [171]. Cell death was inhibited via knockdown of caspase-8 or the autophagy proteins Beclin 1 or Atg7. This data suggested that autophagosome formation was important in the mode of cell death induced. The authors also proposed that upstream effectors of autophagy included both RIP and JNK; no evidence has supported the importance of these pathways in autophagy, which could suggest different signaling pathways are involved for autophagy and autophagic cell death [147].

Embryonic fibroblasts from Bax^{-} Bak^{-} mice treated with etoposide or staurosporine also showed signs of autophagic cell death [172]. Etoposide or staurosporine induced formation of autophagic vacuoles and the cell death could be reversed through knockdown of autophagy genes Atg5 and Beclin 1. This data supports autophagic cell death as a viable mechanism of cell demise.

1.3.1.3. Necrotic cell death

Necrosis has traditionally been considered an accidental and uncontrolled form of cell death but more recent findings suggest necrosis is as programmed as apoptosis. Necrosis is morphologically characterized by cytoplasmic swelling, plasma membrane damage and organelle breakdown, eventually leading to cell explosion [173]. Release of the cellular contents elicits an immune response, which does not occur in apoptosis. Recent experiments have suggested that necrosis may be a backup form of cell death when caspases are blocked or caspase-dependent pathways cannot be properly activated [140].

There are a variety of intercellular mediators and their receptors and other responses can induce either apoptosis or necrosis, depending on the cell type and intensity of stimulus (Figure 1.7) [174]. One of the major crossroads for deciding between apoptosis and necrosis is the adaptor protein FADD. As already mentioned, FADD is recruited upon ligand binding to Fas and TRAIL-R death receptors and initiates activation of apoptotic pathways, NF-κB and MAPKs. When caspase activation is inhibited, necrosis ensues. The deciding factor between apoptosis versus necrosis via Fas/TNFR/TRAIL-R is receptor-interacting protein 1 (RIP1) [175]. RIP1 has been involved in NF-kB activation and induction of cell death [176,177]. Toll-like receptor (TLR)4 and TLR3 activation can also cause necrosis [140]. In addition, stimulation of glutamate receptors with excitotoxins in certain cell types lead to necrosis [174].

There are several important mediators of necrosis including both calcium and ROS. Physiologic levels of ROS can regulate transcription, serve as signaling molecules,

and defend against pathogen infection [178]. ROS are primarily produced in the mitochondria due to electron production in the electron transport chain and there are buffers and a number of antioxidants in specific subcellular locations to minimize ROS-induced cellular damage; however, when ROS are in relative excess of their antioxidant mechanisms, cellular damage can occur due to oxidative stress, damage of intracellular molecules and organelles, and ultimately necrosis [140,179].

Calcium can also have a profound effect on cellular function due to its central role as a stimulator of oxidative phosphorylation and nitric oxide synthase (NOS) production. Both increase production of ROS through enhanced electron flow and nitric oxide production, respectively. Elevated levels of calcium can produce both apoptosis and necrosis and the outcome is based on calcium concentration with low to moderate calcium (200–400 nM) leading to apoptosis, whereas higher concentrations of calcium (>1 μM) are associated with necrosis [26]. In addition to ROS production, calcium also regulates a wide variety of enzymes that can also enhance necrosis including phospholipases and proteases.

Phospholipases are responsible for fatty acid liberation from phospholipids. Cytosolic phospholipase A2 (cPLA2) hydrolyzes arachidonic acid-containing phospholipids and its translocation is mediated by calcium [180]. The liberation of arachidonic leads to the production of cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 monooxygeneases. LOXs are involved not only with inflammation but also with lipid mobilization and peroxidation, which can lead to the disruption of

organelles and plasma membranes and ultimately trigger cellular necrosis via damage to protein structure and lipid bilayers [174].

Calpains are calcium-dependent proteases that have a diverse range of targets including cytoskeletal proteins, membrane proteins, adhesion molecules, ion transporters, kinases, phosphatases, and phospholipases [179]. Elevated levels of calcium can lead to overactivation of calpain. One of the functions of calpains is the cleavage of the Na^{+}/Ca^{2+} exchanger which is essential for maintaining normal intracellular calcium levels and its cleavage leads to sustained elevated calcium. Calpains can also disrupt lysosomal membranes and release of cathepsins, which are involved in the cell death pathway. Ceramide is also critically involved in necrosis due to its role in transmitting the death signals of RIP1 [140].

In contrast to apoptotic cells, necrotic cells are phagocytosed after loss of membrane integrity, which is a delayed and less effective "clean-up" process compared to apoptosis [181]. These conditions lend itself to an inflammatory response since it allows dying cells to activate pro-inflammatory and immunostimulatory responses, which involves a diverse range of cells involved in the immune response including neutrophils, macrophages and dendritic cells. By comparison, apoptotic cells stimulate antigen-presenting cells (APC) to release cytokines that inhibit the immune response.

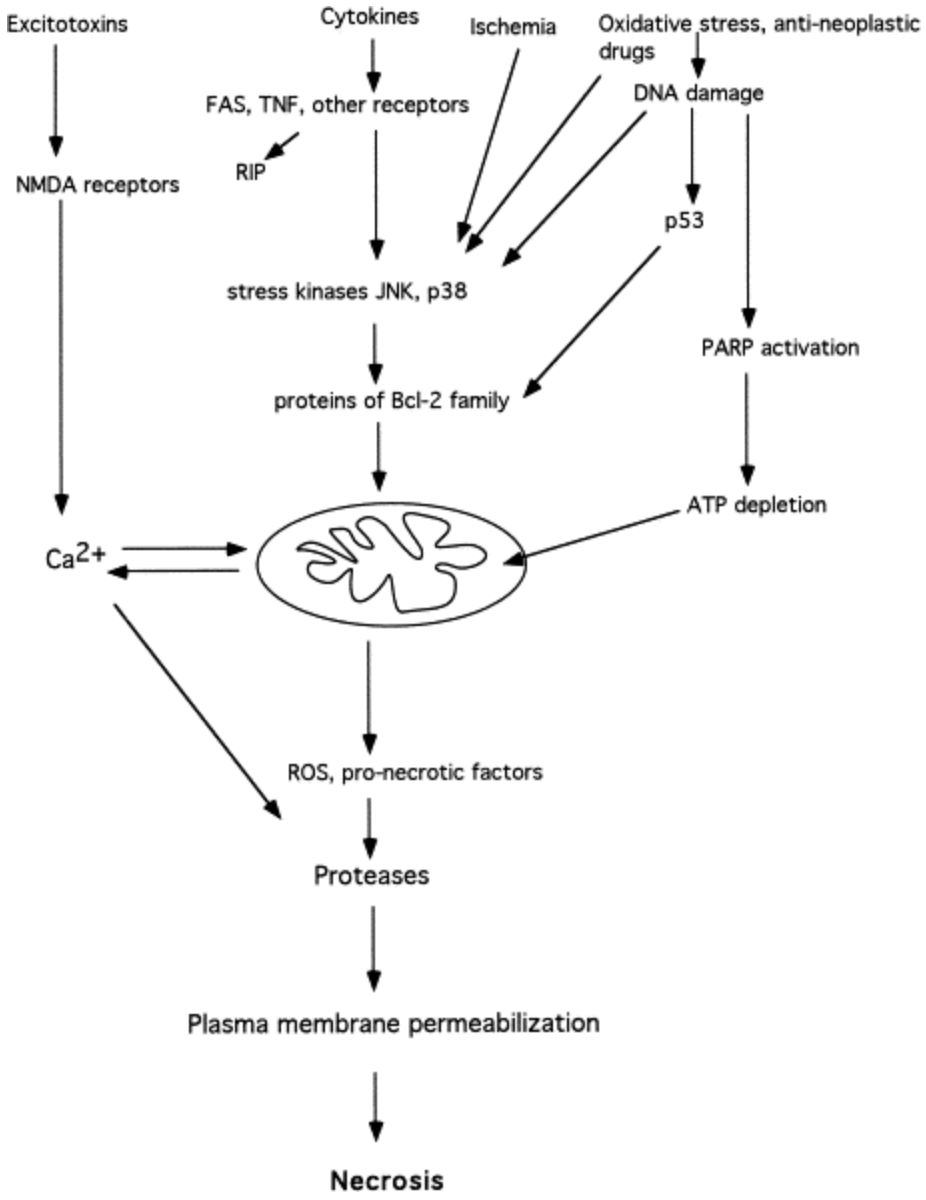


Figure 1.7: Molecular model of necrosis. Necrosis can be triggered by DNA damage directly (e.g. radiation, chemotherapeutic drugs) or indirectly through oxidative stress (e.g. ischemia) or by several receptors such as FAS, TNF receptors, excitoreceptors. Calcium is among the second messengers participating in receptor-mediated necrosis. The last stage of necrotic destruction is the activation of proteases, which leads to plasma membrane permeabilization and necrosis. [174]

1.3.2. Functional capabilities of cancer

To discover drug targets more specific to tumors, it is essential to first understand what distinguishes tumor cells from normal cells and then exploit these differences for developing more effective treatments. Understanding cancer is highly complex and recent advances have provided more information but have also added to the complexity.

Cancer is a result of acquired genetic mutations that lead to the transformation of a normal cell to a highly malignant cell; Hanahan and Weinberg have described six categories of genetic changes that occur in cancer cells [182]. These categories relate

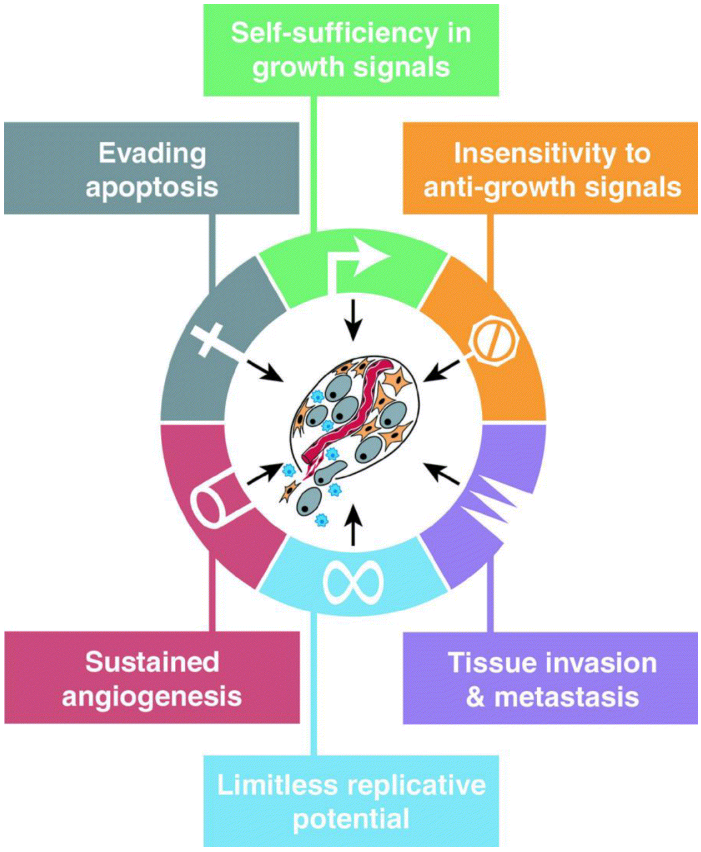


Figure 1.8: Acquired capabilities of cancer. Six hallmarks of cancer progression. [182]

to various cellular mechanisms that are in place to prevent cancer development (Figure 1.8). The categories include: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastases. These events do not necessarily occur in any particular order, as the order varies between cancer types and subtypes [182].

1.3.2.1. Self-sufficiency in growth signals

Cells are dependent on growth signals to proliferate and these signals may be in the form of diffusible growth factors, extracellular matrix components or cell-to-cell adhesion/interaction molecules [182]. These molecules are often secreted from one cell to induce growth of another cell. If cells can become self-sufficient in their growth signals and independent of neighboring cells, they have a greater ability to grow uncontrollably. There are 3 growth signal targets that can be altered and lead to self sufficiency: (1) extracellular growth signals; (2) transcellular transducers (3) and intracellular signaling pathways that translate signals [182].

1.3.2.1.1. Overview of receptor tyrosine kinase receptors

One of the most common mechanisms of self-sufficiency is deregulation of receptor tyrosine kinases that are often involved in transmitting growth signals. Receptor tyrosine kinases constitute a family of 20 receptors that have an extracellular growth factor binding domain, a transmembrane region and an intracellular tyrosine kinase catalytic domain (see Figure 1.9). The intracellular portion of these receptors also contains a juxtamembrane region and carboxy-terminal tail which have regulatory sub-

domains that can influence substrate binding and phosphorylation [183]. The function of receptor tyrosine kinases is to catalyze the transfer of the γ-phosphate of ATP to tyrosine residues on its own chain and other substrates. This results in to second messenger binding and intracellular signal transduction that leads to growth-promoting effects.

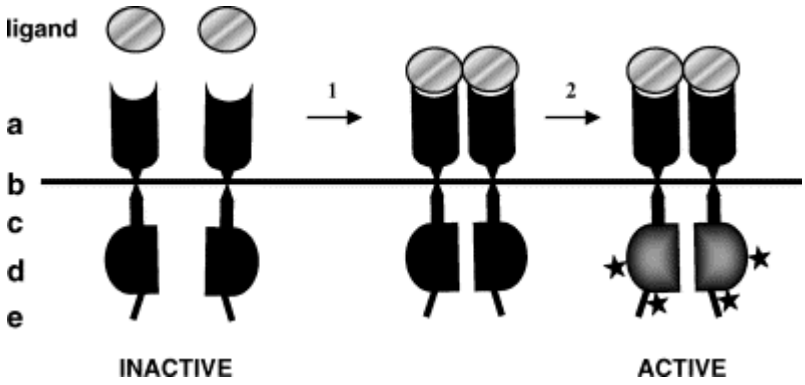


Figure 1.9: Schematic representation of receptor tyrosine kinases and their activation. (a) extracellular ligand-binding domain; (b) unique transmembrane domain; (c) juxtamembrane region; (d) kinase domain; (e) C-terminus. Receptor tyrosine kinases are initially activated upon ligand binding (1), which leads to the catalytic domains becoming closer in proximity and transphosphorylation (2), leading to the fully activated state of the receptor. [183]

Adaptor and scaffolding proteins play a critical role in transmitting signals from receptor tyrosine kinases. They recruit proteins to a specific location and also put together networks of protein to influence signaling pathways [183]. The traditional pathways activated through receptor tyrosine kinases are the phosphoinositide-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Recently, there has also been a link to the signal transducers and activators of transcription (STAT) proteins, where receptor tyrosine kinases directly phosphorylate and induce nuclear translocation of these proteins. Constitutive activation of kinases in cancer cells can occur due to

overexpression of receptor tyrosine kinases and hence an increased concentration of active dimers [184].

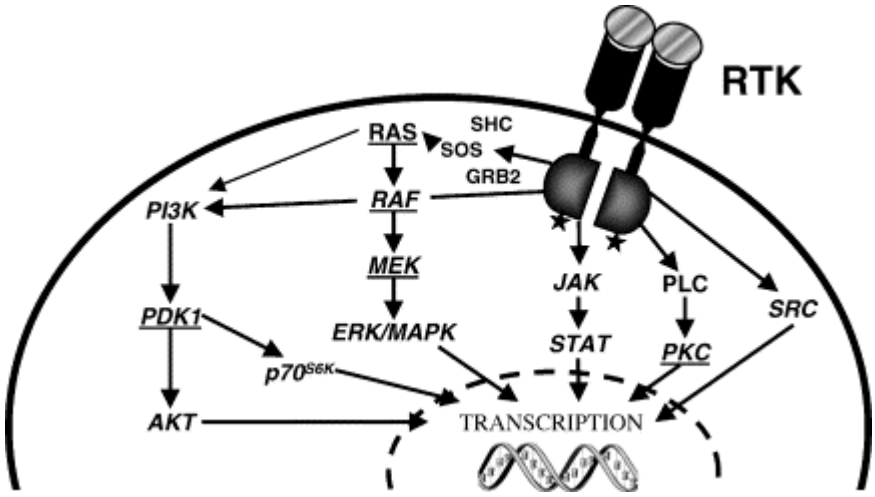


Figure 1.10: Signaling pathways associated with receptor tyrosine kinases. The main pathways are shown and the kinases names are italicized. Abbreviations used: PI3K, phosphatidylinositol 3-kinase; Pdk1, phosphoinositide-dependent protein kinase-1; Akt, oncogenic kinase; $p70^{S6K}$, ribosomal S6 kinase; Shc, src homology collagen; Sos, son of sevenless; Grb2 growth factor receptor-bound protein 2; Erk/MAPK, extracellular signal-regulated kinase/mitogen-activated protein kinase; Jak, janus kinase; Stat, signal transducer and activator of transcription; PLC, phospholipase C; PKC, protein kinase C; Src, oncogenc of chicken Rous sarcom virus. [183]

1.3.2.1.2. Receptor deregulation in breast cancer

Three common receptor tyrosine kinase families associated with breast cancer include epidermal growth factors (EGF), fibroblast growth factors (FGF) and insulin-like growth factors (IGF). The EGF family consists of 3 families of ligands, the first being EGF, transforming growth factor α (TGF-α) and amphiregulin and these ligands bind specifically to EGF receptor (EGFR/ErbB1). Betacellulin, heparin-binding EGF (HB-EGF) and epiregulin form another group of EGF ligands that bind both ErbB1 and EGFR4/ErbB4 receptors. The final group of EGF ligands is the neuregulins (NRGs),

where NRG1 and NRG2 bind EGFR3/ErbB3 and ErbB4 and NRG3 and NRG4 bind only ErbB4. There are no ligands that bind ErbB2 (HER-2/Neu), but it is the preferred heterodimerization partner for other family members [185]. Activated ErbBs preferentially stimulate certain signaling pathways. [186]

Both in vitro and in vivo studies have demonstrated the effects of EGF ligands on mammary epithelial cells. EGF induces DNA synthesis and promotes growth in vitro. EGF, TGF-α and amphiregulin are essential for ductal outgrowth and genetic loss of these ligands in murine models severely affects ductal outgrowth. With the exception of ErbB4, overexpression of EGF receptors has been reported in breast cancer cases [187,188]. Overexpression of ErbB2 both in vitro and in vivo using transgenic mouse mammary glands lead to transformation, tumorigenicity and metastasis [189,190]. A strong relationship has been established between the EGF family and breast cancer in animal models and in humans. ErbB2 is overexpressed in 20-30% of breast cancers and is also associated with a poor prognosis. Poor breast cancer prognosis is also associated with EGFR expression [191].

The IGF family of ligands is another family of growth factors associated with breast cancer progression due to their role as endocrine, paracrine and autocrine regulators of biological processes. There are two IGF ligands, IGF-I and IGF-II which induce proliferation in vitro. In vitro studies show that IGF1R is critical in breast cancer as reduction in IGF1R by siRNA or dominant negative mutants reduces cell growth and transformation in MCF-7 cells [192]. The C-terminal region of IGF1R is critical for tumorigenesis in vitro and in vivo [192]. Other in vivo experiments have demonstrated

the role of IGF-I in terminal end bud formation and ductal growth and like EGF, IGF-I induces DNA synthesis. IGF-II is important during pregnancy since this growth factor is downstream of prolactin, which is essential for alveolar differentiation. IGF1R gene transcription can be stimulated by hormones including growth hormone (GH), follicle stimulating hormone (FSH), leutinizing hormone (LH), thyroid hormone, glucocorticoids and estrogens [193] as well as other growth factors including FGF and PDGF and oncoproteins such as c-myb. PI3K is a major kinase pathway implicated in survival and is activated by IGF1R. Downstream effectors of this pathway lead to such pro-survival events as sequestration of the pro-apoptotic protein BAD as well as induction of cyclin D1. Other survival pathways induced by IGFRs include the Ras/Raf/MAPK pathway. In addition, circulating levels of IGF-I and IGFBP3 (IGF binding protein 3) have been correlated with breast cancer in premenopausal women under the age of 50 [194]. IGF1R expression is seen in many cancer cell lines [195,196] and its overexpression is associated with differentiation, transformation and apoptosis [197].

IGF/ER crosstalk has also been shown by a number of studies where E2 and IGF-I synergistically stimulates proliferation and the extent of IGF1R stimulation is positively correlated with ER expression [198]. Further evidence of the interaction between ER and the IGF family proteins in breast cancer is that antiestrogens inhibit IGF signaling in ER positive breast cancer cell lines.

The FGF family encompasses 22 ligands that bind and activate cell surface receptors (FgfRs) and can act as mitogens, motogens and angiogenic agents [199]. Four

functional FGF receptors (FgfR1-FgfR4) are involved in tyrosine kinase activity in mammals [200]. The role of these growth factors and their receptors in breast cancer is still under investigation, although there are strong links shown in murine models. Mouse mammary tumor virus (MMTV) predisposes mice to breast cancer by retroviral replications, which leads to insertional mutations in the host DNA. Since the provirus remains at the mutation sites, genes can be identified that induced tumor formation [201]. Protooncogenes activated by the MMTV include Fgf3, Fgf4 and Fgf8 [202-204]. Activation of the FgfR1 or Fgf3 expression led to mammary hyperplasia and the formation of invasive lesions in mouse models [205,206]. The data in humans is less convincing and this may be due to issues commonly related to human studies including small sample size and high variability among samples [201]. However, some evidence that supports the role of the Fgf pathway in breast cancer is that both FGFR1 and FGFR4 are amplified in a significant percentage of breast cancer cases [207-209].

1.3.2.2. Insensitivity to anti-growth signals

Cell growth is controlled by a balance of growth stimulatory and growth inhibitory pathways, both involving signal transmission via transmembrane receptors. Growth inhibitory pathways respond to soluble factors, those immobilized in the extracellular matrix and on the surface of neighboring cells. There are 2 major mechanisms that inhibit cell proliferation and these include: (1) forcing cells into a quiescent stage (G_0) of the cell cycle and (2) inducing cells into terminal differentiation [197].

1.3.2.2.1. Overview of cell cycle

The division of cells is tightly regulated by the cell cycle and there are several phases of the cell cycle (see Figure 1.11) including G_0, G_1, S, G_2 and M, each with its own distinct function. Cells in G_0 are in a non-cycling, quiescent state. During G_1 phase of the cell cycle, RNA and protein are synthesized; and DNA is replicated in the S-phase. G_2 is another resting phase as the cell prepares for 'M', which is the phase of mitosis or cell division. [210]

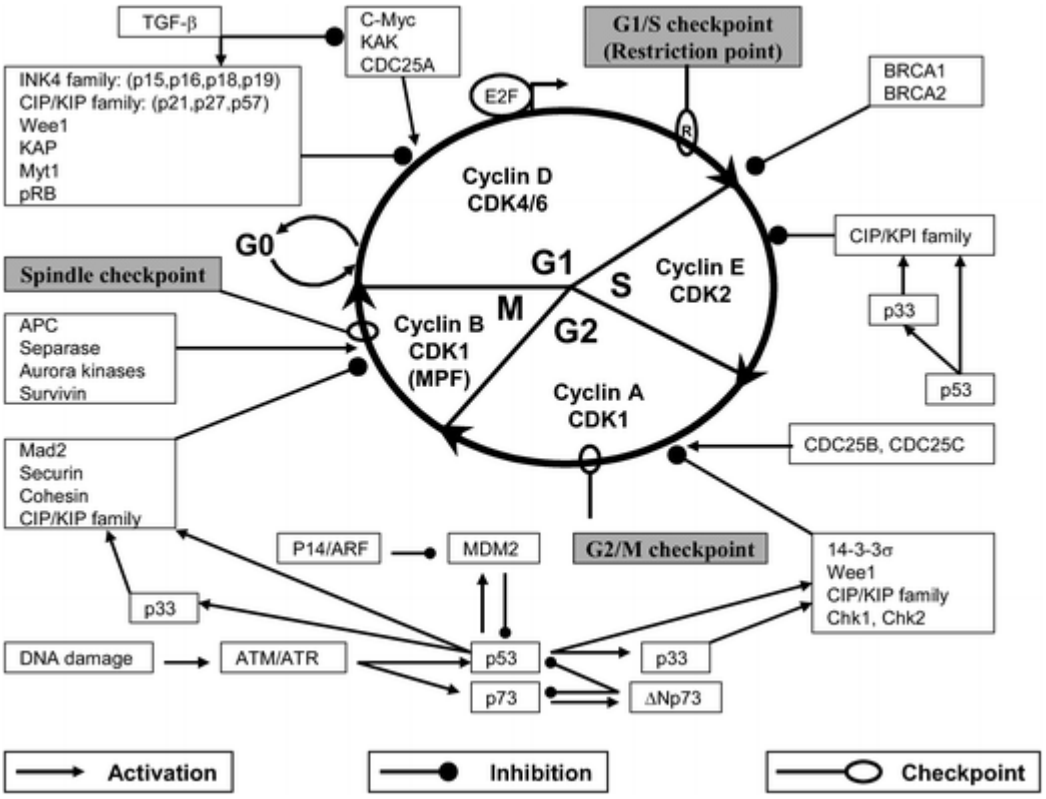


Figure 1.11: Negative and positive regulation of the cell cycle. CDK/cycline complexes driving each phase of the cell cycle are depicted. Inhibitors and promoters of the different phasesa and checkpoints monitoring the proper completion of every phase of the cell cycle are indicated. [211]

Throughout the cell cycle, there are several checkpoints for "quality control". If the checkpoint is failed, cells can be arrested and blocked from proceeding through the cell cycle until the cell meets the standards of the checkpoint. The most critical of these checkpoints is in G_1 which is the major point of decision and therefore is appropriately termed the restriction/start point of the cell cycle. This checkpoint is necessary for DNA integrity and to ensure that any damaged DNA is repaired prior to DNA replication. In addition to the G_1 checkpoint, there is also another one after DNA replication (S phase) to confirm that all DNA has been replicated. A checkpoint also occurs during mitosis to ensure that kinetochores are paired and that other stages of mitosis are carried out without errors prior to cell division. [212,213]

The cell cycle is regulated by the presence of specific protein dimers that facilitate progression through the cell cycle. Cyclins provide the regulatory subunit and cyclin-dependent kinases (cdks) provide the catalytic activity of the dimers [214]. D cyclins are synthesized and activated in response to growth factors and form dimers with cdk4, cdk6, which are required for the latter part of the G1 phase [215]. Cyclin E forms a dimer with cdk2, which is required for S phase entry [216]. Cyclin A/cdk2 and Cyclin A/cdc2 regulate progression through S phase and entry into mitosis, respectively [217]. Cyclin B/cdc2 is also involved in mitosis [218].

The critical nature of the cell cycle in maintaining cellular integrity demands tight regulation of all aspects of the cycle including cyclin/cdk complexes. There are three families of proteins that inhibit the action of these complexes: Retinoblastoma protein (Rb), inhibitors of cdk4 (INK4) family and the KIP family.

Rb activity is controlled by phosphorylation. Typically, Rb is phosphorylated by cyclin D/cdk4,6. In its nonphosphorylated state in G_1, Rb binds E2F family transcription factors, which represses transcription of genes including those critical for S phase. Therefore, S phase cannot be initiated until Rb is phosphorylated and released from E2F. Rb consequently represents one mechanism of regulating progression through G_1. [219,220]

The INK4 family includes $p15^{INK4a}$, $p16^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. All members of the INK4 family specifically bind to cdk4, cdk6 and consequently inhibit the activity of cyclin D/cdk complexes [221]. p15 has the additional function of being involved in transforming growth factor β (TGF-β) mediated cell cycle arrest [222].

The KIP family of proteins include $p21^{WAF1/Cip1}$, $p27^{KIP1}$ and $p57^{KIP2}$. These proteins have a less restricted range of cdk substrates compared to their INK4 counterparts, and these include all G_1 and S phase cdk enzyme complexes. These proteins bind to cdks and prevent their phosphorylation by CAKs, a process required for activation of the cyclin/cdk complexes. In addition, the catalytic activities of the cyclin/cdk complexes are inhibited by KIP proteins. p21 is activated by p53, a gene that is dramatically increased in response to stress stimuli including DNA damage, oncogene activation or hypoxia [223]. In addition, p15, p27 responds to TGF-β and other extrinsic growth signals [224].

1.3.2.2.2. Terminal differentiation

The decision to divide or withdraw from the cell cycle and undergo differentiation occurs during G_1 phase of the cell cycle [225]. Differentiation refers to a complex, multi-step process of cell specialization where a genetic program leads to the expression of lineage-specific genes that dictate biological function [226]. The final step of differentiation is terminal differentiation, which leads to an irreversible loss of proliferative capacity. A multi-step model of differentiation is summarized in Table 1.10 and includes both reversible and irreversible steps of differentiation. The initial stage of predifferentiation quiescence occurs in the G_1 phase of the cell cycle, which leads to the induction of factors that induce the expression of lineage-specific differentiation genes and their consequent biological functions. These genes cause a progressive repression of growth factor responsiveness. Under some conditions, cells may enter an irreversible state of growth arrest, designated terminal differentiation. The decision to undergo terminal differentiation has been linked to an array of genes and is cell-context specific. Repression of P2P gene expression is associated with loss of replicative potential in adipocytes due to its role in encoding proteins that influence RNA expression [226,227]. Other cells such as melanoma cells undergo terminal differentiation after induction of the p21 gene [228].

Table 1.10: Multi-step model of cell differentiation. R=reversible; IR=irreversible [226]

Step 1	Growth and proliferation of determined cells
Step 2	Induction of predifferentiation quiescence (R)
Step 3	Expression of lineage-specific transacting factors (R)
Step 4	Induction of lineage-specific differentiation genes (R)
Step 5	Expression of lineage-specific biological functions (R)
Step 6	Progressive repression of growth factor responsiveness (R)
Step 7	Decision whether or not to undergo terminal differentiation (R/IR)
Step 8	Activation of molecular mechanisms for terminal differentiation (R/IR)
Step 9	Terminal differentiation (IR)

Terminal differentiation is a process for achieving replicative senescence but in many cancer cells, there is a myriad of defects that prevent this final stage of differentiation from occurring. Under physiological conditions, many cancer cells lack the ability to be growth arrested. Cancer cells can be induced to quiescence but have defects with respect to expression of the appropriate combination of lineage-specific genes and lineage-specific biological functions. In addition, cancer cells are typified by their aberrant growth characteristics including both dysplasia and metaplasia since they cannot correctly differentiate. Many cancer cells also lack the ability to be terminally differentiated and hence retain their immortality. [226,229]

1.3.2.2.3. Cell cycle deregulation in breast cancer

The TGF-β family includes activin, inhibin and bone morphogenic protein (BMP) and these factors exert a diverse a range of cellular effects including growth suppression, differentiation, stromal production and immune modulation [230]. TGF-β is of particular interest to cancer researchers due to its tumor suppressive capabilities. TGF-β inhibits growth of mammary epithelial cells and overexpression in mice lead to

mammary hypoplasia and inhibition of tumorigenesis [231,232]. The growth inhibitory effects of TGF-β can be attributed to its influence on cell cycle progression (see Figure 1.12). TGF-β can reversibly arrest cells in G_1 or irreversibly cause terminal differentiation [233] [234].

Cyclins are commonly activated in breast cancer cells. For example, cyclin D1 is overexpressed in a variety of cancers including 60% of breast carcinomas [235] and cyclin E is also overexpressed in breast cancer patients and is a powerful prognostic indicator of breast cancer stage [236]. Cyclin E is associated with chromosome instability and therefore promotes tumorigenesis [237].

p53 is a key player in cell cycle control and may mediate arrest of cells at G_1 near the border of S-phase or in G_2 before mitosis due to its interaction with p21 or GADD45 and 14-3-3σ, respectively [238]. p53 mutations are detected in close to 75% of breast carcinomas [239]. p53 breast carcinomas are typically ER-negative and progesterone-receptor negative and are associated with a high proliferation rate, high histologic grade, aneuploidy and a poor prognosis [238]. p53 expression is also commonly found in ErbB2 overexpressing breast tumors [219].

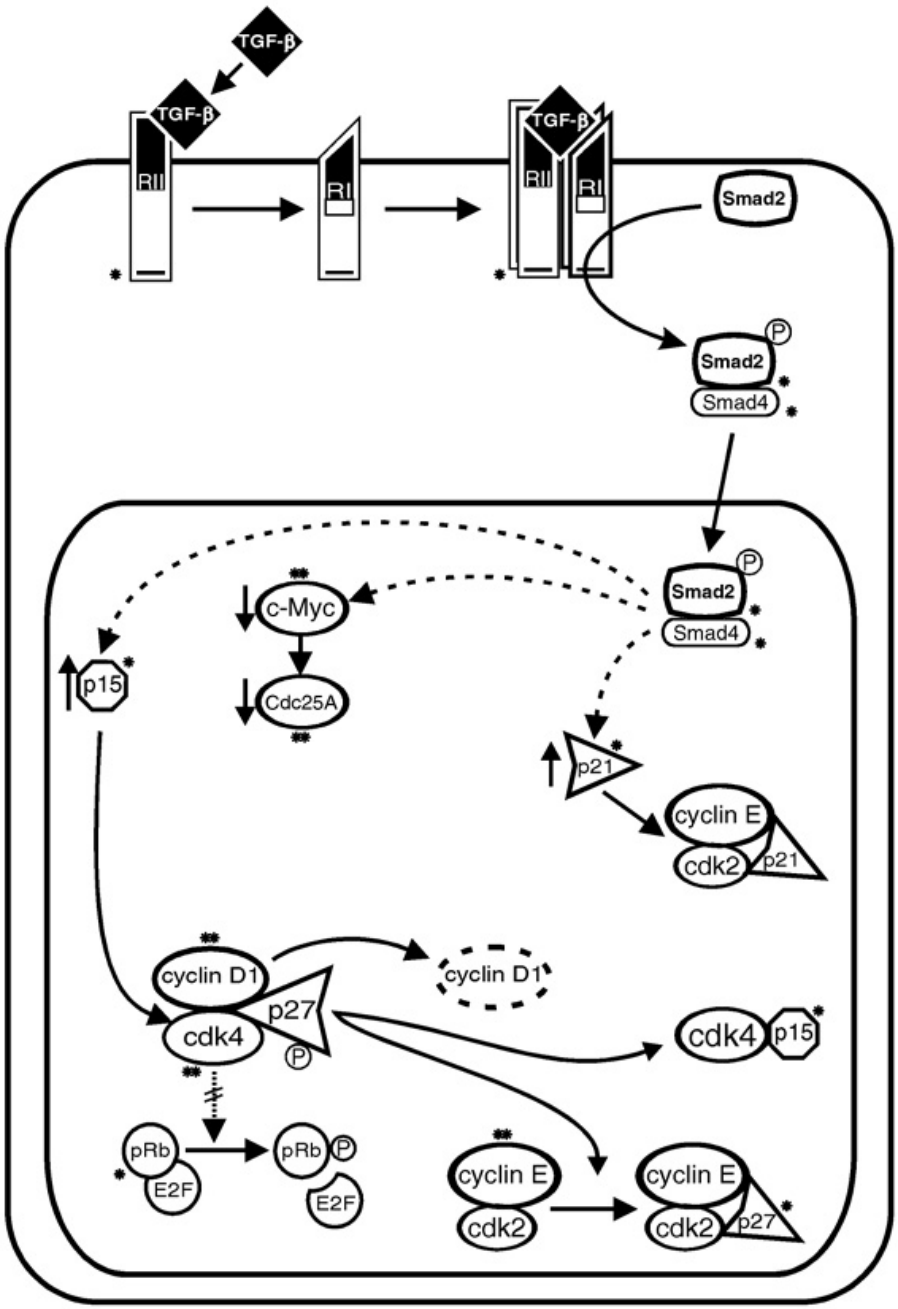


Figure 1.12: Mechanisms of cell cycle arrest induced by TGF-β. TGF-β receptor activation leads to Smad2 phosphorylation, which binds to Smad4. The Smad2-Smad4 complex translocates to the nucleus and modulates transcription. The effects of p15 and p21 may be indirect effect of Smad2-Smad4 action (dotted lines). TGF-β inhibits G1 cyclin-cyclin-dependent kinases (cdks) by increasing p15 and p27 binding to their respective cyclins, thereby inhibiting retinoblastoma protein (pRb) phosphorylation. [240]

1.3.2.3. Evading apoptosis

The breast is a unique tissue since it continues to develop after birth during both puberty and pregnancy [70]. The Bcl-2 family of proteins regulates apoptosis and changes in these protein levels are partially influenced by circulating hormone levels of estrogen and progesterone [70]. Normal breast epithelium goes through fluctuations in apoptosis and Bcl-2 expression during the menstrual cycle [241]. Tissue homeostasis and development in the breast is achieved by a balance between proliferation, differentiation and apoptosis [70]. Apoptosis is a critical programmed cell death pathway essential for inhibiting carcinogenesis since it potentiates the accumulation of genetic mutations and as a result increases the likelihood of breast cancer [242]. In normal breast tissue around invasive breast cancer, there is a comparatively higher rate of proliferation compared to apoptosis [242] (Figure 1.13). Ductal carcinoma in situ and invasive breast cancer both have increased rates of apoptosis compared to normal cells [243,244]. A comparison of human ductal carcinoma in situ and adjacent invasional cancer showed that spontaneous apoptosis is lost concurrently with invasive transformation [245].

Regulators of apoptosis can be divided into sensors and effectors. Sensors of apoptosis are transmembrane receptors that monitor and detect abnormal conditions both intracellularly and extracellularly. Intracellular sensors detect cellular abnormalities such as DNA damage and hypoxia and respond by activating death pathways [246].

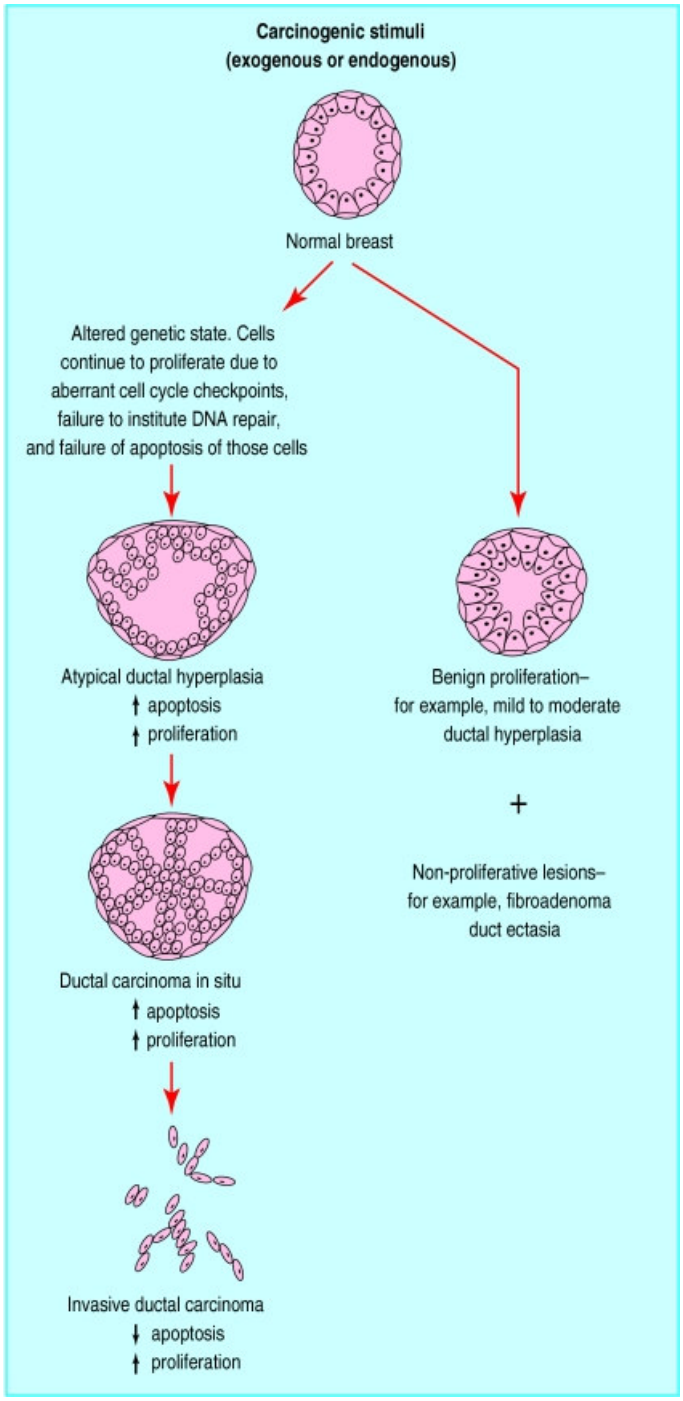


Figure 1.13: Phenotypic changes in stages of breast cancer development. The rate of apoptosis and proliferation is increased in both atypical ductal hyperplasia and ductal carcinoma in situ. As the breast tumor cells become more invasive and are classified as invasive ductal carcinoma, the cells acquire the ability to evade apoptosis. [70]

p53 is an example of an intracellular sensor that activates apoptosis arising from DNA damage due to its role in enhancing expression of the proapoptotic protein Bax. Sensors regulate caspases, which function as effectors of apoptotic death in response to either extracellular or intracellular sensor activation. Bcl-2 family members also are critical effectors in inducing the apoptosis cascade. [182]

1.3.2.3.1. Deregulation of apoptotic proteins in breast cancer

Sensors of apoptosis are frequently mutated in breast cancer patients. p53 is a intracellular DNA damage sensor and as mentioned previously is frequently mutated in breast cancer patients [247]. FasL and its receptor, Fas, are extracellular sensors involved in the initiation of apoptotic death pathways. Non-transformed breast cell lines have a high expression of Fas whereas a number of breast cancer cell lines exhibit low Fas mRNA and protein expression [248]. Fas-positive breast tumors have a higher survival rate than Fas-negative tumors [249]. In addition, when the ratio of FasL to Fas mRNA is greater than 1, there is a significant association with shorter survival and higher mortality rates in breast cancer patients [250].

Bcl-2 expression has a paradoxical role in breast cancer. Bcl-2 is overexpressed in approximately 80% of human breast cancer cases [251]. Hormone receptor positivity and low histologic grade tumors have also been correlated with the expression of the anti-apoptotic protein Bcl-2 [252] and a positive association between Bcl-2 expression and prognosis and survival rate has been reported [253]. Conversely, other studies showed loss of Bcl-2 expression was strongly correlated with increased apoptotic and necrotic cell death, high proliferation rates and high tumor grade [21]. The role of Bcl-2

in mediating anti-apoptotic effects is widespread. Moreover, other Bcl-2 family members have also been linked to increased breast cancer incidence. Bax is a pro-apoptotic member of the Bcl-2 protein family. In breast cancer cells and malignant breast tissue, low expression of Bax has been reported [254] and its low expression has been associated with a poor response to chemotherapy in metastatic breast cancer [251].

Caspases play a critical role in acting as effectors of apoptosis and in invasive forms of breast cancer, increased expression of caspase-3, -6 and -8 and their inhibitors have been reported [255]. This group of caspases has also been linked to high levels of apoptosis and histological grade of breast cancer [256]. Enhanced levels of caspases in tumors suggest that either caspases have a different role in cancer cells or caspases may be inactive due to overexpression of caspase-inhibitor IAPs [257]. IAPs are significantly involved in apoptosis regulation and the IAP, survivin, functions as a caspase inhibitor. Survivin is also overexpressed in a variety of human neoplasms, suggesting that reactivation of the survivin gene frequently occurs in cancers. High survivin expression in neoplasms correlates with more aggressive behavior, decreased response to endocrine agents but enhanced response to chemotherapeutic agents, and shortened survival times compared to cancers that are survivin-negative [255,258].

1.3.2.4. Limitless replicative potential

Although having the ability to evade apoptosis, self-sufficiency in growth signals and insensitivity to antigrowth signals would seem to be sufficient for cancer cells, there is evidence that additional steps are required for cancer progression [197]. One of these steps involves elements that control the stability of the genetic material of the cell that are critical in the pathogenesis of cancer. Cells have a limited replicative potential in place for this purpose [259,260].

1.3.2.4.1. Overview of senescence

The progression of cancer requires an accumulation of genetic mutations. The probabilistic time lapse between acquisition of a first and second genetic mutation is at least 20-30 cell divisions. Since mutations are often recessive it is likely that even more replication events are necessary to achieve loss-of-heterozygosity [210]. Since cells have a finite number of cell divisions, this should limit the progression of a pre-malignant cell into a tumor [261].

Senescent cells are metabolically active but do not proliferate and therefore senescent stromal cells provide an environment of nutrition and growth support for adjacent to premalignant epithelial cells, ultimately instigating epithelial neoplasia by allowing epithelial cells to survive, migrate and divide [261]. Senescent cells secrete factors that are both mitogenic and antimitogenic in tumors and this can lead to changes in the cell function such as secretory pathways, expression of proteases, extracellular matrix components and inflammatory cytokines [210,262,263].

The ends of human chromosomes contain TTAGGG repeat DNA sequences called telomeres that provide both genetic stability and a resource of potentially dispensable DNA [264]. The ends of telomeres have a protective cap called the t-loop that prevents telomeres from being recognized as damaged DNA and consequently this minimizes degradation, recombination and end-joining reactions [210]. DNA polymerase cannot replicate the end sequence during lagging strand synthesis and there are also processing events that ultimately lead to the loss of telomeric repeats after each cell division and this eventually leads to replicative senescence [210]. Malignant tumors therefore must have a method of bypassing this proliferative limitation.

Telomerase is an enzyme that adds telomeric repeats onto the 3' end of chromosomes. It has a protein component that is a human telomerase reverse transcriptase catalytic subunit (hTERT) as well as an RNA component (hTR or HTERC) which acts as a template to synthesize telomeric DNA directly onto the ends of chromosomes [210,265,266]. Telomerase is an ideal therapeutic target because with the exception of germline and somatic stem cells, it is present at undetectable levels in cells but can be highly expressed in proliferating cells. Telomerase expression in telomerase-negative cells can lead to infinite growth and provides direct evidence for this enzyme in pathways that evade replicative senescence (see Figure 1.14) [267].

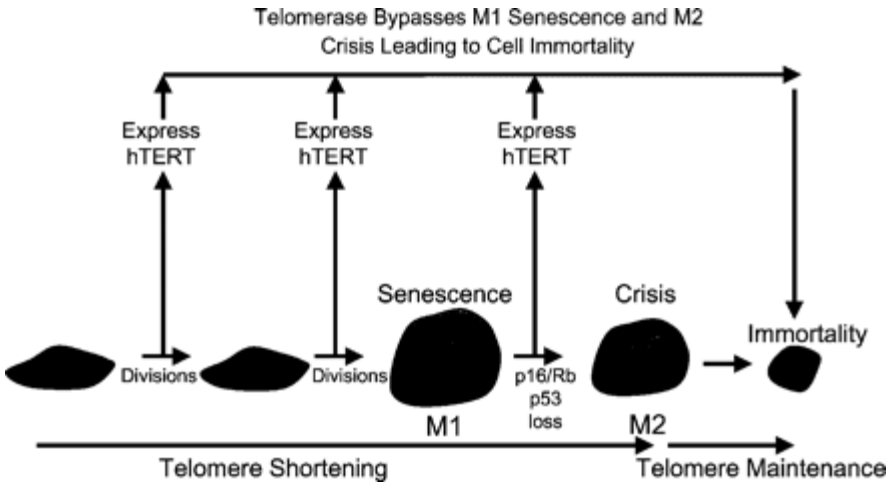


Figure 1.14: Role of telomerase in replicative senescence. Telomeres play an important role in both stages of replicative senescence: M1 (senescence) and M2 (crisis). Expression of telomerase either before or after senescence (M1) can lead to cell immortality. [210]

The initiation steps of replicative (M1) senescence are still uncertain and 3 models have been proposed: (1) telomere position effect (TPE); (2) DNA damage signaling; (3) loss of 3' G-rich telomere single-strand overhangs [210]. In the TPE model, it assumes that genes near telomeres are often silenced and as telomeres shorten, these genes are de-repressed and can potentially reactivate previously silenced genes. There also may be a DNA damage signal induced from "too-short" of a telomere. An alternative hypothesis involves loss of 3' G-rich single-strand overhang since 60-85% of these overhangs are eroded in cancer cases. The sequence of DNA damage response and shortening of the overhang has yet to be determined [210].

1.3.2.4.2. Telomerase expression in breast cancer

Telomerase is overexpressed in approximately 95% of breast cancer cases [226]. Others studies have reported a significant correlation between mean telomerase levels and severity of histopathological change with 14% staining positive in benign breast

disease compared to 94% in invasive breast cancer cases. A significant association between telomerase-positive breast cancer and lymphovascular invasion has also been described and this is a critical step in breast cancer progression. Altogether this data illustrates the critical role of telomerase in breast cancer.

1.3.2.5. Sustained angiogenesis

Tumors have an absolute requirement for a sustained supply of new blood vessels that can provide oxygen and nutrients and aid in metastasis [268]. Angiogenesis refers to the process of new capillaries growing from existing blood vessels that involves basement membrane degradation via enzyme, endothelial cell (EC) migration, proliferation and formation of tubes [269]. This process is regulated by both pro-angiogenic and anti-angiogenic factors. Identified stimulators of angiogenesis include fibroblast growth factor-1 (FGF-1), FGF-2, vascular endothelial growth factor (VEGF), TNF-α, angiogenin, transforming growth factor-α (TGF-α), TGF-β, platelet-derived endothelial cell growth factor (PD-ECGF), pleiotropin and interleukin-8 [270]. Negative regulators of angiogenesis include thrombospondin, cartilage-derived inhibitor, platelet-factor 4, angiostatin, interferon-α and interferon-β.

1.3.2.5.1. Overview of VEGF family

The best characterized angiogenic factor is VEGF which functions as both an EC mitogen and can also increase vascular permeability [268]. VEGF is unique among growth factors due to its specificity for vascular ECs [271]. VEGF can bind 3 EC receptors: VEGFR-1/Flt1, VEGFR-2/Flk1 and VEGFR-3/Flt4, with both VEGFR1 and VEGFR2 being part of the receptor tyrosine kinase family. There are several ligands

involved in the VEGF/VEGF network including VEGF-A, -B, -C, -D, -E and placental growth factor.

A multitude of growth factors and cytokines can stimulate VEGF production such as platelet-derived growth factor, epithelial growth factor, TNF-α, TGF-β1, interleukin 1-β. Hypoxia is also a key regulator of VEGF expression in both normal and malignant cells. mRNA levels are increased in tumor cells near necrotic areas and in cell culture studies there is a substantial increase in VEGF mRNA seen in cells grown under hypoxic conditions compared to normoxic conditions elicited in cultured cells [272,273].

Hypoxic conditions lead to upregulation of growth factor receptors which induce a cascade of events including vascular permeability and matrix metalloproteinases, the latter being of fundamental importance in terms of tissue invasion. These events lead to the formation and movement of ECs resulting in vessel extension and formation of a vascular network [268]. VEGF also increases expression of a variety of apoptosis inhibitors including Bcl-2 [274].

1.3.2.5.2. Angiogenesis in breast cancer

Recent reports have shown that breast cancer cells may express cell surface VEGFRs and this ligand/receptor combination may function as an autocrine loop in addition to the paracrine loop between breast cancer cells and endothelial cells [275]. VEGF is also an indicator of breast cancer progression. VEGF-overexpressing breast cancer cells have immense growth and metastatic potential based on data from xenograft studies [276]. HER2-overexpressing cancers are typically a more invasive form of

breast cancer and recently HER2 overexpression has been positively correlated with VEGF overexpression and this may be essential for the poor prognosis of patients overexpressing high levels of HER2 [277,278]. VEGF is critical for tumor progression since tumor growth is angiogenesis-dependent and angiogenesis is VEGF-dependent [270].

1.3.2.6. Tissue invasion and metastasis

Metastasis refers to the ability of cancer cells to invade other tissues and systems including the vasculature and lymphatic system and is a critical step in cancer progression [279]. Metastatic tumors are the primary cause of death in cancer patients and tumor invasion and metastasis are critical activities of aggressive tumors.

Normal cells require adhesion to grow and anchorage-independent growth is a fundamental feature of invasive and metastatic tumors [280]. The extracellular matrix is normally impermeable to cell migration and therefore major changes occur in the relationship between cancer cells and the extracellular matrix during metastasis and include: (1) disruption of cell-cell contacts; (2) loss of epithelial cell polarity and acquisition of a motile phenotype; (3) disruption of cell-extracellular matrix interactions.

1.3.2.6.1. Cell-cell adhesions

Cell-cell contacts allow for adhesion and intercellular communication. These adhesive contacts are limiting factors for tumor metastasis and must be broken down for tumor cells to invade other tissue. Breast epithelial cells are normally attached via specialized cell-cell contacts to the basement membrane, which are thin “sheets” of connective tissue containing collagen type IV, laminins, entactins (nidogen) and

proteoglycans [281,282] and breaching this membrane is a key event in cancer metastasis.

Laminins are a family of 12 distinct heterotrimeric glycoproteins that are major basement membrane components [279]. Several surface proteins act as receptors for laminins and these include members of the integrin family, dystroglycan and heparan sulfate [282,283]. The predominant receptors on mammary epithelial and breast carcinoma cells are integrins [279] and integrins are receptors for extracellular matrix components and metastasis alters their expression.

Integrins form heterodimeric receptors for extracellular matrix molecules including laminins. There are 18 α-subunits and 8 β-subunits and each integrins consists of a noncovalently linked α- and β-subunit [154]. There are at least 25 distinct pairings that are specific for a unique set of ligands [284]. Integrins are key regulators of cell adhesion but also play a role in influencing cell shape, survival, proliferation, gene transcription and migration [285].

Ligand binding to integrin receptors triggers a cluster formation of integrins and cytoskeletal components including actin-associated proteins such as α-actinin, vinculin, tensin and paxillin [286]. Kinases that phosphorylate cytoskeletal proteins are also activated by integrins that regulate cell shape and migration [154]. The cytoplasmic tail of integrins can recruit regulatory proteins for integrin adhesiveness to the extracellular matrix [154]. The α6β4 integrin is a key component in many epithelia for adhering epithelial cells to the basement membrane via hemidesmosomes [178].

Hemidesmosomes link the intermediate filament cytoskeleton with laminins in the basement membrane.

1.3.2.6.2. Epithelial-mesenchymal transition and acquisition of motile phenotype

The epithelial-mesenchymal transition (EMT) refers to a process where epithelial cells lose their polarity and cell-cell contacts, undergo cytoskeletal remodeling and acquire mesenchymal and migratory characteristics [144,287]. EMT most likely involves disassembly of cell-cell connections including tight junctions, adherens junctions and desmosomes. Growth factors can activate EMT by means of receptor tyrosine kinase activation and these factors include scatter factor/hepatocyte growth factor, FGF, EGF, IGF-I and IGF-II (Figure 1.15) [144]. The majority of growth factors turn on EMT through MAPK activation, but Src, PI3K or Rac may also be involved [288]. Normal breast cell lines respond to TGF-β, which cooperatively acts with Ras to induce EMT [289].

Cadherins are proteins critical for epithelial cell-cell adhesion. The ectodomain of cadherin functions in homophilic adhesion and the cytoplasmic domain is essential for interaction with the actin cytoskeleton via the β-catenin-α-catenin complex [290]. E-cadherin expression is frequently lost during cancer progression and is a hallmark of EMT [287].

Slug and Snail are transcriptional repressors that can downregulate E-cadherin expression [173,288]. Snail is a zinc finger protein that recognizes E-box motifs in the E-cadherin promoter [291]. Slug also binds with lower affinity to the same region of the E-cadherin promoter [288]. SIP1 is downstream of TGF-β and is overexpressed in E-cadherin deficient cell lines and also binds to E-boxes in the E-cadherin promoter and like Snail, exhibits similar repressor effects [292]. Overexpression of Snail and SIP1 inhibit E-cadherin adhesion and promote invasion [292]. Recently, Twist has been added to the list of repressors of E-cadherin and subsequent induction of EMT [293].

Migration is a key step in invasion and metastasis and involves lamellipodia that attach to the extracellular matrix and propels the cell forward [294]. In the metastatic stages of cancer, tumor cells express vimentin, which is typical of migratory motile mesenchymal cells instead of cytokeratin, which is characteristic of epithelial cells [158]. Cell migration is initiated by polarizing the cell to have distinctive front and rear advancing and tracking regions. Lamellipodia are formed by actin polymerization and decreased cell membrane tension. The leading edge also is flooded with receptors and signaling molecules to allow for new adhesive cell-cell contacts to be formed. At the rear end of the cell, cell-cell integrins contacts are released to allow for migration [154].

1.3.2.6.3. Extracellular matrix remodeling

Penetrating the basement membrane is a key event in cancer metastasis [295]. A three-step model of invasion has been proposed suggesting that tumor cells attach themselves to the basement membrane secrete proteases to dissolve the basement membrane to facilitate access to the basement membrane. Extracellular matrix remodeling also occurs during metastasis and involves extracellular matrix degradation via proteases and the synthesis of new extracellular matrix proteins [154]. These proteases break down the basement membrane and allow access of epithelial cells to stroma and other tissues. Breaching the basement membrane necessitates secretion of specific proteases for the extracellular matrix in close proximity to the cancer cells and proteases to stop excess matrix degradation.

Matrix metalloproteases (MMPs) are critical enzymes involved in tissue remodeling and destruction of the microenvironment [158]. In the context of extracellular matrix remodeling, MMP-2 and MMP-9 can degrade a major component of the basement membrane, type IV collagen [296]. MMPs are secreted as inactivated zymogens and can be activated by other proteases that regulate activation and localization of MMPs and tissue inhibitors of MMPs (TIMPs) are critical in this process [154]. Overexpression of MMPs is a critical step in tumor invasion and metastasis since this destroys barriers and allows access of cancer cells to other tissues [297]. MMPs are also critical in the loss of epithelial cell identity since MMPs can destroy factors critical for epithelial differentiation [158].

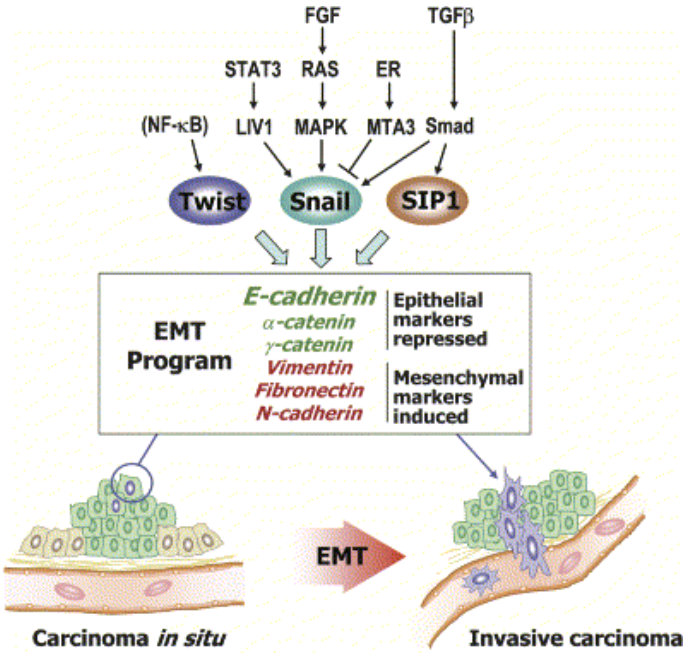


Figure 1.15: Regulators of the Epithelial-Mesenchymal Transition (EMT). Twist, Snail, SIP1 repress E-cadherin transcription via E boxes and activate expression of genes that triggers EMT. Twist, Snail and SIP1 are regulated by various pathways in tumor cells and those shown in parentheses are involved during development. [287]

1.3.2.6.4. Integrins, E-cadherins and breast cancer

Integrins are receptors involved in facilitating contact between the extracellular matrix and cellular cytoskeleton. Integrins expressed on epithelial and myoepithelial cells of the breast include α2β1, α3β1, α5β1, α6β1, α6β4, αvβ6 [298]. The α6 integrins (α6β1, α6β4) have been linked to aggressive forms of breast cancer and have been the main focus of investigators for this reason. Reduced survival times in women were significantly correlated with high expression of α6 subunits [299]. Coexpression of α6β4 and laminin in breast tumors has been associated with a poor prognosis [300]. Although migration principally involves disassembly of cell-cell contacts such as hemidesmosomes, α6β4 integrin has recently been shown to promote migration through its function in the formation and stabilization of filopodia and lamellae [181]. In addition, decreased expression of α1, α6, β1, β4 integrin subunits is associated with neoplastic formation in breast tissue [301].

E-cadherin is another protein linked to breast cancer due to its critical role in EMT initiation. In vitro and in vivo studies have shown that E-cadherin loss increases tumor cell invasiveness and adenomas convert to carcinomas, respectively [144]. E-cadherin expression is inversely correlated with tumor grade and stage and hence is considered a tumor suppressor gene [287]. Approximately half of breast lobular carcinomas have E-cadherin mutations [144]. An inverse relationship exists between E-cadherin and Twist expression in invasive lobular carcinomas [293].

1.4. Current Drug Strategies

Despite scientific advances, metastatic breast cancer remains an incurable disease and treatment is used to delay disease progression, improve the quality of life and prolong life [302,303]. Adjuvant therapy is a critical component of treatment regimens for women with primary breast cancer and the chosen therapy is based on characteristics that can be divided into 3 categories: patient characteristics, disease characteristics and biologic features [304].

1.4.1. Endocrine therapy

Endocrine therapy is universally employed for hormone receptor positive breast tumors and the agents used are based on menopausal status. In premenopausal women, the therapeutic options include tamoxifen or ovarian ablation/suppression. Ovarian ablation significantly increases survival in women less than 50 years of age [305]. Tamoxifen has exhibited favorable therapeutic effects in both premenopausal and postmenopausal women, reducing recurrence and death from breast cancer [306].

In postmenopausal women, tamoxifen and aromatase inhibitors are both used, the latter of which is only effective in the absence of ovarian function [307]. A comparison of the aromatase inhibitor anastrozole and tamoxifen showed anastrozole to be comparable to tamoxifen with respect to objective response and clinical benefit and significantly better than tamoxifen for delayed disease progression [308]. Letrozole, an alternative aromatase inhibitor, showed better objective response, clinical benefit and time to progression compared to tamoxifen [309]. In addition, aromatase inhibitors are

beneficial since they do not have an enhanced risk of endometrial cancer like tamoxifen but are associated with accelerated bone loss [304].

1.4.2. Cytotoxic drugs

Cytotoxic drugs inhibit cell proliferation by an array of mechanisms and have been studied for the past 40 years in treating cancer patients. An extensive range of single agent and combination therapies have been investigated. Early chemotherapeutic drugs functioned as DNA alkylating agents including cyclophosphamide or antimetabolite agents including methotrexate and fluorouracil and were frequently utilized in the 1970s [310]. In the next decade, cyclophosphamide was used in combination with the anthracycline doxorubicin [311]. Currently, the most common cytotoxic drugs used in breast cancer treatment are the anthracyclines, doxorubicin and epirubicin and the taxanes, paclitaxel and docetaxel [302].

1.4.2.1. Anthracyclines

Anthracyclines function by interacting with DNA, inhibiting tumor cell proliferation and gene expression and production of free radicals that will ultimately result in tumor cell death [312]. Cardiotoxicity is one of the major toxic effects associated with anthracyclines and has prompted more recent formulations to include liposomal anthracyclines, which have a more favorable cardiac safety profile [313].

Single agent use of doxorubicin produced response rates of 40-50% in untreated patients and 32-36% in previously treated patients [307]. Comparative response rates of other chemotherapeutic agents are summarized in Table 1.11. Anthracyclines used in combination with other chemotherapeutic agents has produced enhanced response rates

in the range of 60-85%, however, with no statistically significant change in survival [314].

Table 1.11: Response rates with single-agent chemotherapy administered to patients with metastatic breast cancer. (reviewed in [307])

CHEMOTHERAPEUTIC AGENT	Response Rates (%)	
	First-line	Second-line
Doxorubicin (including liposomal formulations)	40-50	32-36
Epirubicin	52-68	25-35
Paclitaxel	29-63	19-57
Docetaxel	47-65	39-58
Capecitabine	20-30	20-27
Gemcitabine	23-37	13-41
Vinorelbine	40-44	17-36

1.4.2.2. Taxanes

Taxanes are commonly employed as a first line of treatment of metastatic breast cancer. Both paclitaxel and docetaxel interfere with mitosis by binding to the β-subunit of tubulin, which leads to the formation of stable nonfunctional microtubule bundles [315]. The effects of paclitaxel and docetaxel are active in G_2/M and S phase respectively of the cell cycle [315]. The pharmacokinetics of the two taxanes are slightly different; docetaxel has linear pharmacokinetics and longer plasma half-life and intracellular retention time.

In early single agent studies with either paclitaxel or docetaxel as the first agent to treat metastatic breast cancer, response rates were as high as 60% [316,317]. A comparison of docetaxel given 3 weeks at 100 mg/m^2 and doxorubicin 75 mg/m^2 showed docetaxel being more active therapeutically and less toxic than doxorubicin [318]. A comparison of the two taxanes showed that although docetaxel had a higher overall survival than paclitaxel, there was a higher toxicity in the form of neutropenia and treatment-related death [319]. Taxanes has also been effective in patients that have developed resistance to anthracyclines [320].

Taxanes plus doxorubicin as a treatment regimen elicited response rates from 28-58% [311]. Docetaxel/doxorubicin versus doxorubicin/cyclophosphamide combination treatments showed the taxane-containing combination had a significantly improved response rate and time to cancer progression [321]. However, other taxane treatment combinations produced synergistic toxic effects, such as between the HER2 humanized antibody, trastuzumab, and taxanes [322].

1.4.3. Kinase inhibitors

ErbB receptors respond to various growth factor ligands and their overactivation can lead to uncontrolled cell growth. ErbB2/HER2/neu is frequently overexpressed in 25-30% of breast tumors and is associated with a more invasive form of breast cancer and poor prognosis [323]. Overexpression of EGFR/HER1 occurs in 16-48% of human breast cancer cases and has also been linked with an invasive metastatic breast cancer phenotype [324,325]. ErbB positive tumors are customarily resistant to antiestrogen therapy [326] and cytotoxic agents have been traditionally used. Alternative drug targets

for HER positive tumors have been extensively studied that have fewer side effects than their cytotoxic counterparts; both HER1- and HER2-targeted treatments have been investigated clinically for use in treating metastatic breast cancer with promising antitumorigenic effects.

1.4.3.1. HER2 inhibitors

Trastuzumab is a monoclonal antibody that recognizes the extracellular domain of HER2 and is effective when used alone or in combination with other chemotherapeutic agents when treating HER2 positive tumors [327,328]. The mechanisms of trastuzumab action include: (1) reduced receptor signaling; (2) G1 arrest via p27 modulation; (3) induction of apoptosis; (4) inhibition of angiogenesis; (5) immune-related mechanisms of cytotoxicity; (6) inhibition of HER2 extracellular domain cleavage; (7) inhibition of DNA repair [329]. Another HER2-targeted agent is pertuzumab, which inhibits HER2 dimerization and is currently in clinical trials [330]. In addition, small-interfering RNAs (siRNAs) against HER2 expression slowed cell proliferation, increased apoptosis and reduced tumor growth in ovarian and breast cancer cells [331].

1.4.3.2. HER1 inhibitors

Gefitinib is a small molecule receptor tyrosine kinase inhibitor with specificity towards EGFR by being a competitive inhibitor of adenosine triphosphate (ATP) receptor binding [330]. Gefitinib has proven effective in vitro not only in EGFR overexpressing cells but also HER2-expressing cells presumably by reducing EGFR/HER2 heterodimer phosphorylation [332]. However, these results did not

necessarily translate into successful clinical trials. Gefitinib had low efficacy in a pre-treated population are metastatic breast cancer. The exception is in tamoxifen-resistant ER positive tumors, where a therapeutic effect was achieved with gefitinib treatment [330].

1.4.4. Antiangiogenic therapy

Angiogenesis is an absolute requirement of primary tumors due to the role of new blood vessels in tumor growth and invasion [333]. In solid tumors, angiogenesis is critical in order for the tumor to grow beyond 2-3 mm [334]. There have been 4 classes of antiangiogenic agents studied: (1) Endothelial toxins; (2) Growth factor/receptor antagonists; (3) Protease inhibitors; and (4) Natural inhibitors [335].

1.4.4.1. Endothelial toxins

Endothelial chemotaxis and migration are important steps in the angiogenic process and perturbations can impede angiogenesis. Integrins are key targets in this process since they link endothelial cells to the extracellular matrix components, particularly integrin $\alpha_V\beta_3$ [336]. Vitaxin™ is a monoclonal antibody against $\alpha_V\beta_3$ and has shown growth inhibitory and antitumorigenic effects in vitro and in vivo [337].

1.4.4.2. Growth factor antagonists

There are a variety of factors that regulate angiogenesis including growth factors and external stimuli. VEGF is considered the predominant factor that initiates angiogenesis. It is expressed in a variety of tumor types and a higher expression of VEGF mRNA is seen in invasive ductal carcinoma, metastatic ductal carcinoma compared to lobular carcinoma [338]. In invasive HER2 positive tumors, VEGF is

upregulated and may be involved in the switch to an aggressive phenotype [339]. VEGF stimulates endothelial cell proliferation and increases microvascular permeability, which leads to the release of plasma protein that can change extracellular matrix components and promote new blood vessel formation [340]. Since VEGF plays such a critical role in angiogenesis, it has become a common chemotherapeutic target by pharmaceutical companies.

Bevacizumab is an antibody directed against VEGF-A and has shown promise in animal and clinical studies [341,342]. Combination therapy of bevacizumab and capcitabine showed improved therapeutic benefit when compared to single treatments of either drug [343].

1.4.4.3. Protease inhibitors

Another key step in the angiogenic process is breakdown of the basement membrane and stroma by a specific class of proteases, the MMPs. MMP inhibitors have shown promise in xenograft studies with breast cancer cells but not when tumors are large and breast cancer is in an advanced stage [344]. Clinical trials involving MMPs inhibitors for treatment at the earlier micrometastatic stage of breast cancer showed antitumorigenic effects after treatment but also lead to high musculoskeletal toxicity [345].

1.4.4.4. Endogenous antiangiogenics

Certain naturally-occurring chemicals have exhibited antiangiogenic effects. 2-Methoxyestradiol (2ME2) is metabolic product of estradiol and inhibits both proliferation and angiogenesis [346]. Clinical trials of 2ME2 have prompted the

development of new formulations since there was a significant conversion to an inactive metabolite in patients administered the drugs [347].

1.4.5. Proteasome inhibitors

Ubiquitination refers to a process of regulating proteins whereby the highly conserved 76 amino acid protein ubiquitin can be covalently conjugated to lysine residues [348]. This process is carried out by 4 critical enzymes: ubiquitin-activing enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3) and the 26S proteasome [349]. E1 binds to and activates ubiquitin, which is an ATP-dependent process, then passes ubiquitin to E2 and is then transferred to the lysine residue by way of E3 that acts as a scaffolding protein. The polyubiquitinated form is then targeted for degradation via the 26S proteasome. E3 confers substrate specificity [350].

The ubiquitin-proteasome pathway critically regulates a number of key signaling pathways. Nuclear factor kappa-B (NF-κB) is a transcription factor and integral player in immune and inflammatory responses, apoptosis and cell proliferation [351,352]. Temporal expression of cyclins and CKIs in the cell cycle is essential for successful progression and is regulated by ubiquitin-proteasome dependent degradation [350]. The ubiquitin-proteasome machinery is critical in terms of generating antigenic peptides from cytosolic proteins [352].

Growth factors receptors can be degraded through both ligand-dependent and ligand-independent degradation. The ligand-dependent mechanism involves a rapid reduction in cell surface expression of EGFR upon ligand binding. This downregulation involves two steps: internalization of membrane receptor and receptor degradation [353].

Internalization of EGFR is accomplished by recruitment of Cbl proteins upon ligand binding that have E3 and adaptor protein capabilities and therefore the ubiquitin-proteasome pathway is crucial in terms of regulation of growth factor expression.

1.4.5.1. Indirect effects on ubiquitin-proteasome function

Several drugs indirectly influence the ubiquitin proteasome pathway by either increasing or inhibiting the function of this pathway. Examples of drugs and their effects on the proteasome pathway are summarized in Table 1.12. Camptothecin and fulvestrant are chemotherapeutic agents that act by stimulating the proteasome pathway. Camptothecin inhibits the religation step of topoisomerase 1 by stimulating the ubiquitination and subsequent degradation of topoisomerase 1 [354]. Fulvestrant partially functions as a pure estrogen receptor antagonist by activating proteasome-dependent degradation of ERα [355]. Arsenic is an example of a drug that inhibits proteasome function by inhibiting the action of IκB kinase and thus preventing the necessary phosphorylation of IκB that leads to proteasome-mediated degradation of IκB [356]. Trastuzumab is an antibody that targets ErbB2 and recent work has shown that it recruits Cbl proteins and stimulates the subsequent ubiquitination and degradation of ErbB2 via the proteasome pathway [357].

Table 1.12: Drugs that influence ubiquitin-proteasome activity. [350,358,359]

Drug class	Action and mechanism
Chemotherapeutic agents	
Aclarubicin	Inhibits the chymotrypsin-like proteolytic activity of the proteasome
All-*trans* retinoic acid	May accelerate PML fusion protein degradation through the proteasome
Arsenic trioxide	Inhibits ubiquitination and degradation of IKB through effects on the IKB kinase
Camptothecin	Stimulate ubiquitination and degradation of topoisomerase 1
Geldenamycin	Inhibits HSP90 ATPase, stimulating proteasomal degradation of client proteins
PS-341	Inhibits the chymotrypsin-like activity of the proteasome
Trastuzumab	Stimulates ubiquitination and subsequent degradation of ErbB2
Vinblastine, Vincristine	Inhibits the chymotrypsin-like, trypsin-like- and peptidyl-glutamyl peptide hydrolyzing proteasome activities
Immunosupressive agents	
Cyclosporin A	Uncompetitive inhibitor of the proteasomal chymotrypsin-like activity
Rapamycin	Inhibits proteasome function by inhibiting the proteasome activator PA28
Miscellaneous agents	
Fulvestrant	Stimulates proteasome-dependent proteolysis of ERα
Tannic acid	Inhibits the chymotrypsin-like activity of the proteasome
Lovastatin	Mechanism unknown, but appears structurally similar to proteasome inhibitor lactacystin
Anti-retroviral drugs	Inhibits the chymotrypsin-like and trypsin-like proteasome activities
Bortezomib	Inhibits 26S proteasome activity

1.4.5.2. Direct effects on ubiquitin-proteasome

Several agents directly target the ubiquitin-proteasome system. The immunosuppressive agent rapamycin inhibits the expression of proteasome activator PA28 and therefore inhibits proteasome function [360]. Other chemotherapeutic drugs

inhibits the proteasome including aclarubicin, vinblastine and vincristine [361,362]. Peptide aldehydes such as MG132 and MG115 are inhibitors of the 20S proteasome and have been used extensively to study the role of proteasomes in various cellular functions [363]. Bortezomib is an inhibitor or the 26S proteasome however, clinical trials of bortezomib used as a single agent against metastatic breast cancer did not yield any therapeutic benefit [359].

1.4.6. Gene replacement therapy

Gene therapy is a promising new field of cancer treatments and is a method for transferring genetic material that will either promote cell death or inhibit cell growth by gene transfer. Gene therapy can be classified into 4 strategies: (1) suppression of oncogenes or transfer of tumor suppressor genes; (2) enhancement of immunological response; (3) transfer of suicide genes; (4) protection of bone marrow using drug resistance genes [364]. There are 3 primary methods for gene transfer: (1) transient transduction of naked DNA via lipofection; (2) transduction of adenoviral vector or vaccinia virus vector; (3) transduction of retroviral vector [365]. Only transduction of a retroviral vector leads to stable expression; the two former procedures for gene transfer lead to transient expression of the desired genes.

1.4.6.1. Oncogene suppression or tumor suppressor gene transfer

Tumor suppressor genes are critically involved in apoptosis induction or suppression of tumor growth and their loss due to functional defects or absence of one or more tumor suppressor genes leads to cancer. Gene replacement therapy is based on the

principle that the addition of functional copies of tumor suppressor genes will lead to a reduction of tumor cell growth and cancer progression [366].

The most commonly altered tumor suppressor gene in cancer is p53 and p53 mutations will lead to cell cycle arrest or apoptosis [365]. Viral introduction of p53 into human breast cancer cells can restore cellular homeostasis in terms of balanced cell growth and death [366]. An added benefit of this technique was that in addition to cell death observed in p53-transduced cells, cells surrounding the transduced cells are also killed and this is dubbed the bystander effect [367]. The bystander effect is advantageous because it reduces the need for high transduction efficiency in order to achieve desired efficacy [368].

Other gene abnormalities common in breast cancer patients include overexpression of ErbB2, c-myc and cyclin D1 [369]. ErbB2 overexpression is common in invasive forms of breast cancer and associated with a poor prognosis and hormone therapy resistance [369]. ErbB2 gene therapy in patients overexpressing ErbB2 decreases the number of tumors cells and reduces ErbB2 expression [370]. Transfer of c-myc and c-fos genes under control of the MMTV promoter inhibit tumor formation in murine models [371].

1.4.6.2. Suicide gene therapy

Gene transfer of drug-activating enzymes leads to increased cellular concentrations of activated prodrugs and this results in increased death of tumor cells. The bystander effect is also relevant for suicide gene therapy such that cells adjacent to transduced tumor cells are also affected [365]. Cytosine deaminase gene transfer and

subsequent treatment of breast cancer patients with fluorocytosine (prodrug form) yielded positive tumor reduction results [372]. CYP2B6 gene therapy and cyclophosphamide co-treatment was antitumorigenic in Phase I clinical trials for patients with metastatic breast cancer [373].

1.4.6.3. Bone marrow protection by drug resistance

High dose chemotherapy cannot completely kill residual disease and this stage of the disease is also associated with insufficient bone marrow function. One approach is to transplant drug-resistant gene-transduced hematopoietic stem cells, resulting in normal bone marrow cells that are resistant to the effects of chemotherapeutic drugs. The multi-drug resistant 1 (MDR1) gene excretes various drugs from the cytoplasm and MDR1 gene transfer to bone marrow cells is a new therapy for treating cancer patients. Clinical trials involving advanced breast cancer patients that received MDR1 gene transfer in combination with docetaxel exhibited therapeutic benefits compared to conventional therapy. [365]

1.4.7. Immunotherapies

The immune system has originally been thought to induce a response based on recognizing self versus non-self [374]. However, more recent evidence supports the danger theory which suggests that cells experiencing stress release “alarm” signals [375] and therefore the microenvironment surrounding the cell in terms of immunostimulatory molecules versus immunosuppressive molecules are critical in eliciting a T-cell mediated response [376]. The immune cells involved in responding to these signals are summarized in Table 1.13.

Table 1.13: Function of cells in the immune response. Abbreviations used: IL=interleukin; IFN-γ=interferon gamma [377]

Cell Type	Function
CD8+ cytotoxic T cells (CTL)	Kills cells expressing antigens
CD4+ T helper cells (CTh) • Th1 • Th2	Augment antigen-specific immune response via cytokine secretion Secrete cytokines (e.g. IL-2, IFN-γ) that stimulate proliferation and activity of cytotoxic T cells Secrete cytokines (e.g. IL-4, 5, 6, 10) that results in more effective antibody production
B cells	Signals from CD4+ cells trigger antibody-producing cells that secret antibodies
Natural Killer (NK) cells	Constitutively kill small set of hematopoietic cells IL-2 or IL-15 stimulation permits recognition of broader range of altered "self"

1.4.7.1. Cytokine treatment

Cytokines are critical molecules involved in the regulation of inflammation, cell growth and differentiation [377] and delivery of these molecules via gene therapy can enhance the immune response. IL-2, Il-12, GM-CSF, TNF, IFN-γ are all cytokine genes that have been genetically transferred with the intention of boosting the immune system [365].

1.4.7.1.1. Interferon-γ(IFN-γ)

Interferons (IFNs) are natural glycoproteins that enhance the immune response by increasing the expression of cell surface proteins including major histocompatiblity complexes (MHC) [378] and IFNs play a role in the activity of other cytokines. IFNs can be divided into 2 classes: Type I and Type II IFNs [379]. Type I IFNs typically respond to viral infection and include IFN-α and IFN-β [380]. In contrast, Type II IFNs

such as IFN-γ are induced by immune and inflammatory stimuli and are produced by immune cells including T lymphocytes and NK cells [381,382]. IFN-γ acts as a homodimer with its receptor that is ubiquitously expressed on the surface of normal cells. Upon receptor activation, JAKs are activated, leading to activation of the JAK/STAT pathways that binds to gamma-interferon activator sets (GAS) elements and interferon-stimulated response elements (ISREs) in promoter regions of target genes [383,384].

IFN-γ is downregulated via IL-10 [385] whereas IL-2 acts synergistically with IFN-γ to reduce tumor size [386]. Although IL-10 is present in normal tissue, it is absence from breast tumors and may be explain why IFNγ, IL-2, IL-4 are also not detected in tumors [387]. Clinical studies with IFN-γ and hormone therapy as a combination therapy produced a favorable response in breast cancer patients [388].

1.4.7.1.2. IL-2

IL-2 induces proliferation of key immune cells including NK and T cells and its therapeutic benefit through gene transfer has been documented in melanoma and renal cell carcinoma patients [377]. Antitumorigenic effects are also observed in murine breast cancer models after administration of IL-2 gene therapy [389].

1.4.7.2. Vaccines targeting breast cancer

Cancer vaccine immunotherapy uses T cells and exploits their anti-tumor cytotoxic T lymphocyte function to specifically target and kill tumor cells [390]. Breast cancers exhibit low antigenicity and are non-responsive to immune therapy, however, more recent studies have uncovered breast cancer-associated antigens [365]. Tumor-specific antigens that are integrally required for this therapy fall into 3 categories:

tissue-specific differentiation antigens, tumor-specific shared antigens, tumor-specific unique antigens [390]. Breast cancer-specific antigens include HER-2/neu [391], autoimmunogenic cancer/testis antigen (NY-ESO-1) [392], carcinoembryonic antigen (CEA) [393], melanoma-associated antigen 1 (MAGE-1) [394] and Mucin 1 (MUC-1) [395]. Cloned tumor antigens are advantageous because the delivery of antigens can be controlled and they are safer since the likelihood of inducing an immune response for tumor-specific antigens is decreased [376].

1.4.7.3. T-cell therapy

Clonal expansion of tumor-specific T cells ex vivo is a therapeutic strategy to kill tumor cells. A section of the primary tumor is surgically removed and anti-tumor T lymphocytes are generated. These T-lymphocytes are transferred into patients, migrate to tumor sites and ultimately kill tumor cells [390]. Initial trials in breast cancer patients showed an increase in survival rate [396].

1.4.8. Apoptosis inducers

Apoptosis is a major death pathway that is evaded by tumor cells through various molecules that create barriers to cell death [397]. The Bcl-2 family of proteins, IAPs and death receptors function as the major regulatory molecules of this pathway and have all been studied as potential therapeutic targets to encourage apoptosis-induced death of cancer cells [398].

1.4.8.1. Bcl-2 proteins

Overexpression of Bcl-2 and other family members has been linked with resistance to cytotoxic agents due to the ability of these compounds to block cell death

[397]. Numerous agents reduce expression of anti-apoptotic members of the Bcl-2 family and these include antiestrogens, retinoids, Vitamin D and HDAC inhibitors [399-402].

Chemicals have also been designed to specifically target Bcl-2 that bind to the regulatory sites of Bcl-2 proteins or bind to the BH3 binding site on Bcl-2. Natural products including gossypol, epigallecatechin gallate, theaflavin, chelerythrine and antimycin have shown to be BH3-mimicking chemical antagonists [403-406].

DNA-based drugs for Bcl-2 proteins are based on the principle that antisense nucleotides can selectively bind to mRNA targets and induce ribonuclease-mediated mRNA degradation [397]. Oblimersen sodium is a Bcl-2 antisense inhibitor that has been successful in Phase III clinical trials for treatment of chronic lymphocytic leukemia but not for treatment of refractory multiple myeloma [407].

1.4.8.2. IAP proteins

IAP-family proteins are critical negative regulators of apoptosis that directly bind to and inactivate caspases. Small molecules have been developed that inhibit IAPs by displacing caspases and hence promoting apoptosis [397]. Survivin and XIAP antisense are both undergoing Phase I clinical trials [408]. Many other inhibitors of XIAP are currently being developed both in academia and industry [397].

1.4.8.3. Death receptor pathway

TRAIL is a member of the TNF family and is a death receptor that induces apoptosis in response to extracellular stimuli. However, breast cancer cells are frequently resistant to TRAIL-induced apoptosis [409]. A new breast cancer therapy strategy involves the use of interferon-γ to sensitize tumor cells to TRAIL-induced apoptosis [410].

IFN-γ inhibits mammary tumor growth in murine models [411,412] and an increase in IFN-γ levels in tumors is positively correlated with a good prognosis [388]. IFN-γ can enhance sensitivity of tumor cells to apoptosis [410] and the mechanism of IFN-γ in producing these effects has also been extensively studied. Although IFN-γ upregulates CD95-induced apoptosis [248], this is not a viable therapeutic approach since normal cells also undergo enhanced CD95-mediated apoptosis [413]. IFN-γ also increases the expression of TRAIL and decreases expression of TRAIL decoy receptors, ultimately increasing apoptosis [414]. In vitro studies showed that caspase pathways and Bcl-2 protein levels were modulated in MCF-7 and MDA-MB-231 breast cancer cells after treatment with IFN-γ.

1.5. DIM as a Model for Development of Mechanism-Based Drugs

Recent advances in cancer chemotherapy have involved the development of drugs that specifically target cancer cells rather than normal cells. The chemopreventive properties of DIM have previously been discovered and studies on the mechanism of action of DIM have revealed cancer-specific and cell-context dependent targets of growth inhibition that could be exploited for therapeutic purposes.

1.5.1. DIM

Cruciferous vegetables (broccoli, brussel sprouts, cabbage, cauliflower, bok choy) contain dietary indoles that have a protective effect against a number of cancers including breast cancer [123-127,415]. Indole-3-carbinol (I3C) is a phytochemical that exhibits antiproliferative and antitumorigenic effects in vitro and in vivo [130,131,133-135]. DIM is an acid condensation product of I3C (see Figure 1.16) [128,129] and extensive research has revealed the chemotherapeutic potential of this compound. Multiple molecular mechanisms of cellular action have been suggested to play a role in DIM-induced growth inhibition and are described below.

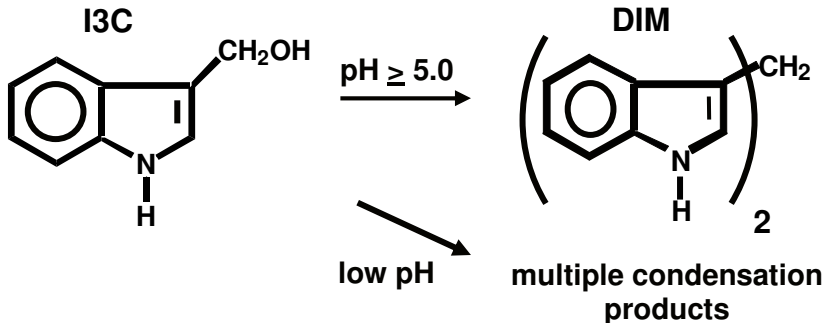


Figure 1.16: Conversion of I3C into condensation products under acidic conditions.

1.5.1.1. Modulation of cell cycle

A number of papers suggest that the growth inhibitory effects of DIM are due to modulation of cell cycle proteins and cell cycle arrest. In MCF-7 breast cancer cells, I3C and DIM induce G_1 cell cycle arrest, increase p21 gene expression through Sp1 promoter elements and stimulate cdk6 through Sp1 and Ets6 promoter elements [134,416,417]. I3C induces G1 cell cycle arrest in prostate cancer cells [132] whereas in HepG2 cells treated with DIM, S-phase retardation was observed [418].

1.5.1.2. Induction of apoptosis

Apoptosis is a regulated program of cell death and has been induced by DIM through a variety of mechanisms in cancer cell lines. DIM induced caspase-8-dependent apoptosis in colon cancer cells [419]. Prostate cancer cells treated with DIM exhibited signs of apoptosis which could be partially reversed through downregulation of the androgen receptor (AR) [420-422]. Induction of endoplasmic reticulum stress and subsequent induction of apoptosis was also induced after treatment of pancreatic cells with DIM [423]. DIM also sensitizes TRAIL-resistant liver and colon cancer cells to TRAIL-mediated apoptosis by downregulation of c-FLIP [424]. In breast cancer cells, DIM induced apoptosis [425,426] and this induction involved the inhibition of the nuclear translocation of NF-κB in MCF-7 breast cancer cells [427]. In addition, the pro-apoptotic protein, non-steroidal anti-inflammatory drug-activated gene 1 (NAG-1) was upregulated by DIM in colon cancer cells [428].

1.5.1.3. Other cellular effects

DIM causes a number of biological effects in addition to apoptosis induction and cell cycle arrest. In MCF-7 breast cancer cells, DIM inhibited mitochondrial H^+-ATP synthase [429] and activated IFN-γ through upregulation of JNK and p38 [430,431]. DIM is also an aryl hydrocarbon receptor (AhR) agonist and inhibited breast cancer cell growth through negative crosstalk with ER [131]. DIM inhibited angiogenesis in *in vivo* breast cancer xenograft models [432]. DIM increased TGF-α in human endometrial cancer cells [433].

1.5.2. Ring-DIMs

Ring-DIMs constitute a new class of anticancer agents and their chemical structure is illustrated in Figure 1.17. The chemotherapeutic effects of DIM and ring-substituted DIMs on mammary tumor growth in DMBA-induced female Sprague-Dawley rats and their antigrowth effects in ER-positive MCF-7 and T47D breast cancer cells have been investigated. At a dose of 5/mg/kg/48h (X10), DIM inhibited tumor

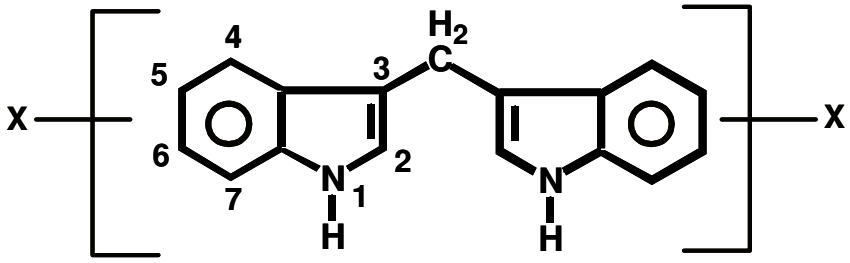


Figure 1.17: Chemical structure of Ring-DIMs. X=Br, Cl, I, OCH3

growth, and several ring-substituted DIMs were antitumorigenic at doses ≤1mg/kg/48h (X10) [131,434,435]. The *in vitro* antiestrogenic effects of DIM and ring-substituted

DIMs were also observed in ER-positive MCF-7 and T47D breast cancer cells. 1,1'-, 2,2'-, 5,5'-dimethylDIM and 1,1',2,2'-tetramethylDIM were the four most potent methyl-substituted ring-DIMs and 5,5'-dibromoDIM was the most potent compound in the dihalo ring DIM series [131,434,435]. The DIM compounds bind the aryl hydrocarbon receptor (AhR) [131,434,435] and the results suggest that the observed antiestrogenic responses may be due, in part, to inhibitory AhR-ERα crosstalk.

1.5.3. C-DIMs

Chemical modifications of DIM yield a novel class of synthetic compounds termed the 1,1-bis(3-indolyl)-1-(p-substitutedphenyl)methanes as depicted in Figure 1.18.

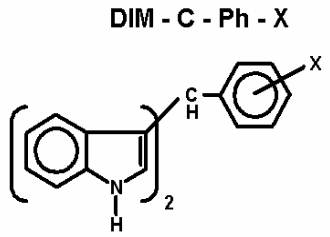


Figure 1.18: Chemical structure of 1,1'-bis(3-indolyl)-1-(p-substitutedphenyl)methanes.

Research on the growth inhibitory effects of a series of these compounds with various substituents (X) showed that many of the C-substituted DIMs induced nuclear-receptor dependent and independent effects including induction of caveolin proteins, induction of ER stress, G_0/G_1 cell cycle arrest and induction of the pro-apoptotic molecule NAG-1 in a cell-context dependent manner (Figure 1.19).

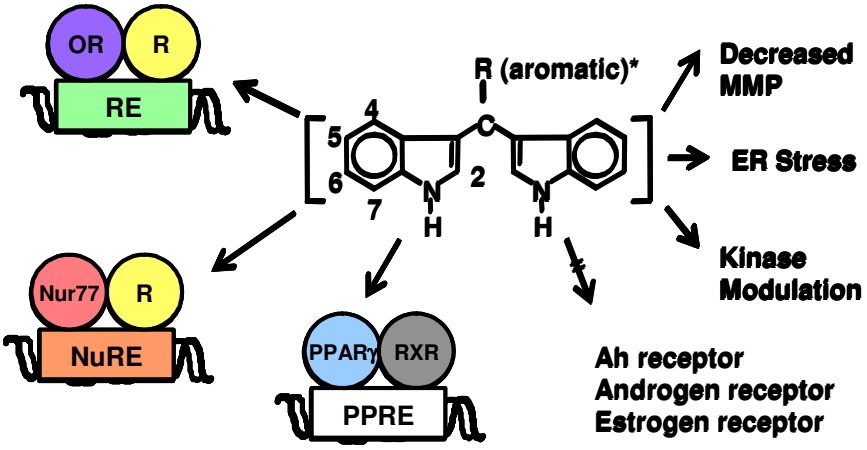


Figure 1.19: Mechanisms of growth inhibition induced by C-DIMs. C-DIMs activate both receptor-dependent and independent mechanisms of growth inhibition that is highly structure and cell context-dependent. C-DIMs activate several nuclear receptors including Nur77, PPARγ and other orphan nuclear receptors (OR) and also exert inhibitory effects on other nuclear receptors including the estrogen receptor and androgen receptor. Receptor-independent mechanisms of growth inhibition include decreased MMP, induction of ER stress and kinase modulation.

1.5.3.1. Nuclear receptors

Nuclear receptors are important targets for cancer chemotherapy because they transcriptionally regulate key genes and are differentially expressed in tissues, which allows for the potential of targeting tumor cells overexpressing these receptors. The nuclear receptor superfamily is comprised of steroid receptors, nonsteroidal receptors and orphan receptors. The steroid hormone receptors encompass AR, ER, PR, mineralocorticoid receptor (MR) glucocorticoid receptor (GR). Thyroid hormone receptor (TR), vitamin D receptor (VDR), retinoic-acid receptor (RAR), 9-cis-retinoic acid receptor (RXR) and ecdysone receptor (EcR) are included in the group of nuclear receptors that form transcriptionally active receptor-RXR heterodimers. In addition, several orphan receptors also form heterodimers with RXR and this include the

peroxisome proliferator activated receptor (PPAR), steroidogenic factor 1 (SF-1), nerve growth factor-induced receptor (NGFI) and X-linked orphan receptor DAX-1. [436,437]

Nuclear receptors contain a linker region (D) and three structural domains: an N-terminal (A/B) domain, DNA binding (C) domain and ligand binding domain (E) (see Figure 1.20). The A/B region contains the constitutive activation function 1 (AF-1) domain and is the least conserved domain whereas the DNA binding domain (DBD) is the most highly conserved region of nuclear receptors [438]. The DBD is comprised of two zinc finger motifs that allow specific recognition of inverted DNA repeats in the case of steroid receptor or direct repeats in the the case of RXR-heterodimeric receptors

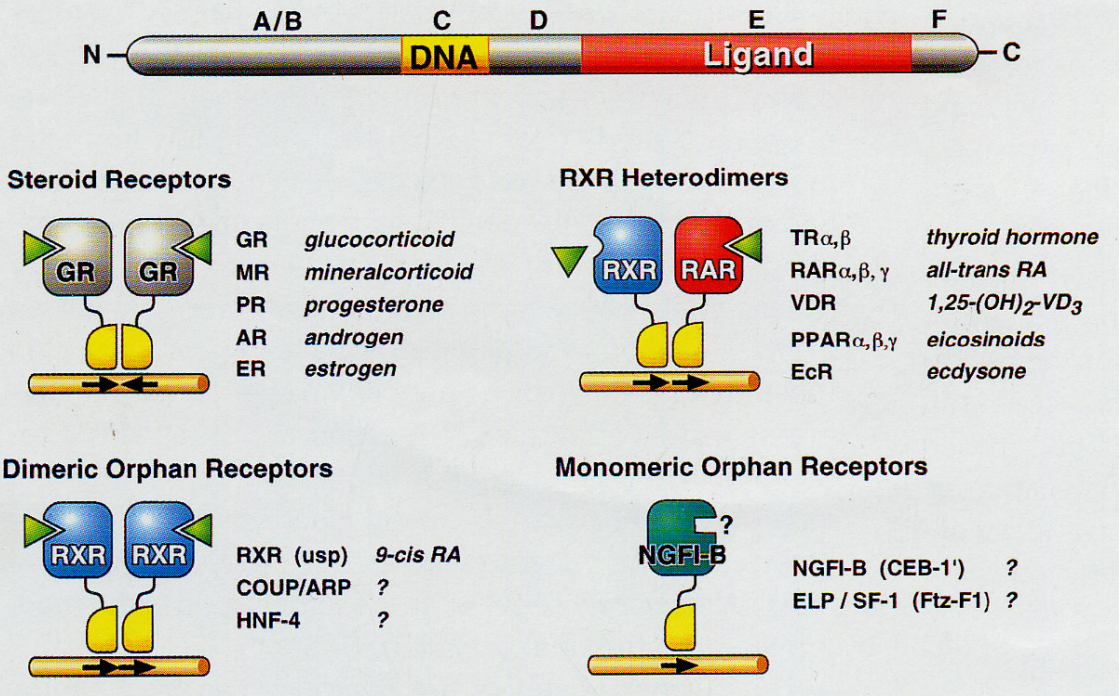


Figure 1.20: Nuclear receptor structure domains. A typical nuclear receptor contains a variable N-terminal region (A/B), conserved DNA binding domain (C), variable hinged region (D), conserved ligand binding domain (E) and a variable C-terminal region (F). Nuclear receptors can be divided into 4 categories based on their ligand binding. [440]

[437]. The AF-2 domain is located in the ligand binding domain and is responsible for ligand-dependent activation of the receptor [439].

Steroid hormone receptors typically exist in complexes with chaperone proteins either in the nucleus or cytoplasm; upon ligand binding, the receptors dissociate from chaperone proteins and bind as a homodimer to hormone response elements on the DNA promoter of target genes [440]. RXR-heterodimeric receptors are complexed with corepressor proteins typically bound with RXR to cognate DNA binding sites and upon ligand binding undergo a conformational change which results in the loss of corepressors and recruitment of coactivators [437]. In addition, some nuclear receptors including PPARγ can be activated through ligand-independent activation in the N-terminal region [441].

Nuclear receptor ligands have chemical similarities. They are small, rigid, relatively hydrophobic molecules with polar groups capable of forming hydrogen bond or electrostatic bonds at the end of the molecule. The ligand-binding cavity of PPARγ is significantly larger than other nuclear receptors and optimized to bind to a wide variety of endogenous and exogenous ligands. [437]

1.5.3.1.1. PPARγ

PPARγ is a member of the nuclear receptor superfamily and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 may be an endogenous ligand for this receptor [442]. The PPAR subfamily includes α, β(δ) and γ sub-types which are differentially expressed in various tissues and exhibit some overlapping or interconnected activities such as in cholesterol transport, metabolism and function [443]. PPARα regulates genes involved in fatty acid

utilization during fasting and PPARα agonists are drugs used for treating patients with hyperlipidemia. PPARγ is primarily expressed in adipocytes and plays a role in adipocyte differentiation. Thiazolidinediones (TZDs) such as rosiglitazone and pioglitazone are PPARγ agonists which are widely prescribed as insulin-sensitizing agents for treatment of type II diabetes [443-446].

PPARγ is overexpressed in human primary and metastatic breast adenocarcinomas [447] and therefore is a therapeutic target for metastatic breast cancer. PPARγ agonists exhibit antitumorigenic activity against a wide variety of cancers including breast cancer [448-450]. In colon cancer models, there is conflicting evidence since the PPARγ agonists troglitazone and pioglitazone promotes or inhibits tumorigenesis in colon cancer mouse models, respectively [451,452]. The antineoplastic effects of PPARγ ligands have been characterized and include induction of apoptosis, cell cycle arrest and terminal differentiation [447,448,453,454].

C-DIMs containing substituents p-CF_3, p-tBu, p-C_6H_5 are PPARγ-active compounds in a number of cancer types including breast, colon, ovarian and pancreatic cell lines [455-458]. The IC50 values of DIM-C-p$PhCF_3$, DIM-C-pPhtBu and DIM-C-pPhC_6H_5 for growth inhibition were 1-5 μM, which was similar to the dose required for PPARγ-dependent transactivation [459]. PPARγ-dependent upregulation of p21 was reported in both pancreatic and ovarian cancer cell lines treated with C-DIMs [455,458]. PPARγ-dependent activation of phospho-Akt was also reported in colon cancer cells treated with C-DIMs [460].

1.5.3.1.2. Nur77

Nur77 was originally discovered as a nerve growth factor inducible gene in PC-12 cells [461] and like PPARγ, Nur77 is overexpressed in cancers including prostate and breast cancer [462,463]. Nur77 exhibits both survival and death characteristics. Nur77 stimulates cell cycle progression and proliferation in lung cancer cells [464] whereas a variety of apoptosis-inducing agents also stimulate Nur77 and lead to the induction of apoptosis [463,465,466]. In several cancer cell lines, DIM-C-pPh, DIM-C-pPhCF_3 and DIM-C-pPhOCH_3 activated Nur77 and these compounds also induced cell death in pancreatic cancer cells and kidney cancer cells by means of Nur77-dependent TRAIL-mediated apoptosis and caspase-dependent PARP cleavage [467,468].

1.5.3.2. Caveolins

Caveolins-1, -2 and -3 are membrane proteins that are constituents of sphingolipid and cholesterol-rich invaginations in cell membranes termed caveolae [469]. Caveolins are considered differentiation markers and are most commonly expressed in terminally differentiated cells [470,471] whereas tumor cells have reduced expression of caveolin proteins [472,473]. PPARγ ligands induced terminal differentiation in breast cancer cells [447] and also induce caveolin expression in both colon and breast cancer cells [474]. Since C-DIMs activate PPARγ, caveolin expression was assayed in colon cancer cells and caveolin-1 and caveolin-2 were upregulated after treatment with C-DIMs [457]. This upregulation was mediated by PPARγ and represents a receptor-dependent pathway of terminal differentiation induced by C-DIMs.

1.5.3.3. NAG-1

NAG-1/MIC-1/GDF-15/placental transforming growth factor-β (PTGF-β)/PLAB is a member of the TGF-β superfamily and has been shown to induce apoptosis and inhibit tumorigenesis in vitro and in vivo [475,476]. Although highly expressed in mature epithelial cells lining the intestine, NAG-1 expression is reduced in human colon cancer samples [477]. PTGF-β caused cell cycle arrest and induced apoptosis by inhibiting cyclin D1 expression in breast cancer cells [478]. Overexpression of NAG-1 in MDA-MB-468 and MCF-7 breast cancer cells inhibited cell growth [479]. The PI3-K/Akt/GSK-3β pathway [480], protein kinase C pathway [481], early growth response gene-1 (Egr-1) [482,483], activating transcription factor 3 (ATF3) [484] have been identified as upstream regulators of NAG-1 in different cell lines.

Many different compounds induce NAG-1 including PPARγ agonists and DIM [428,482-488], and therefore induction of NAG-1 expression by PPARγ-active C-DIMs was also investigated. C-DIMs induced NAG-1 expression in HCT-116 colon cancer cells and this was dependent on the PI3-K-dependent activation of Egr-1 and NAG-1 induction and NAG-1 induction could be reversed by cotreatment with a PI3-K inhibitor [489]. This data represented a receptor-independent pathway for C-DIMs and involved activation of PI3-K and Egr-1 that results in induction of NAG-1 expression and apoptosis.

1.5.3.4. ER stress

The endoplasmic reticulum is the primary site of protein synthesis and folding and requires ATP and calcium to effectively carry out these processes [490]. Under stress conditions that perturb calcium or ATP levels, there is an accumulation of unfolded proteins and this cellular state is termed ER stress [491]. Unfolded protein response (UPR) is a protective mechanism for the cell to combat ER stress and involves three transmembrane ER receptors: pancreatic ER kinase-like (PERK), activating transcription factor 6 (ATF6) and inositol-requring enzyme 1 (IRE1) (see Figure 1.21). GRP78 functions as a chaperone protein for these receptors to maintain their inactive state and once unfolded proteins accumulate, GRP78 dissociates and this activates the receptors. UPR is considered a survival response but under prolonged conditions of stress, UPR can trigger apoptosis through activation of CHOP, JNK and Bcl-2 family of proteins. [491]

Induction of ER stress was seen in several ovarian cancer cell lines treated with the C-DIM, DIM-C-pPhtBu [458]. DIM-C-pPhtBu induced GRP78 expression and the JNK pathway. In addition, CHOP was activated in response to treatment of ovarian cancer cells with DIM-C-pPhtBu and CHOP bound to response elements in the DR5 promoter resulting in induction of DR5. DR5 is associated with the extrinsic pathway of apoptosis which was induced by DIM-C-pPhtBu treatment. This represents a novel mechanism of cell death induced by C-DIMs.

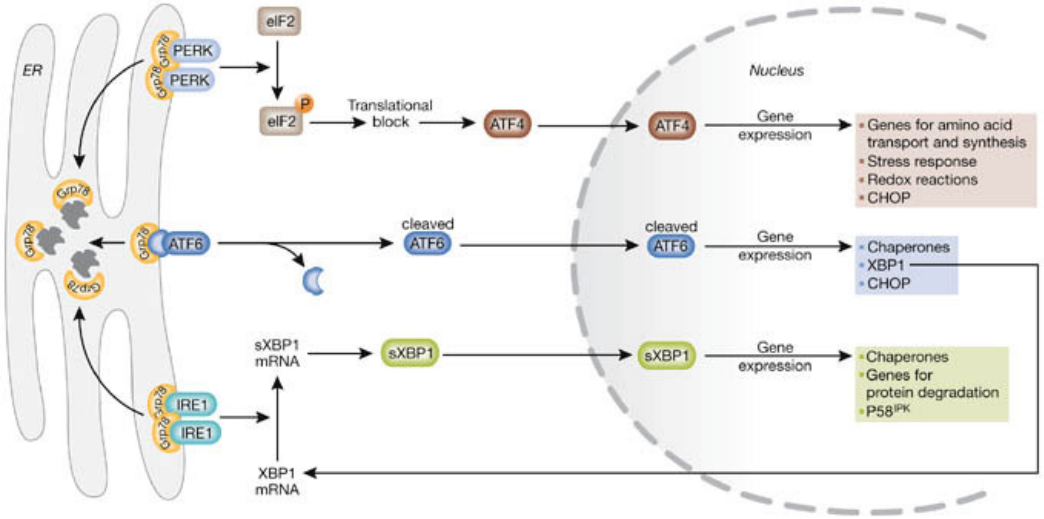


Figure 1.21: Mediators of the unfolded protein response (UPR). UPR involves three ER transmembrane receptors: PERK, ATF6 and IRE1 that are present in the inactive state due to GRP78 functioning as a chaperone protein. Accumulation of unfolded proteins leads to the dissociation of GRP78 and activation of the receptors, which induce expression of genes involved with survival, protein degradation and apoptosis such as CHOP. [491]

1.5.3.5. Cell cycle

Cancer cells treated with C-DIMs exhibit changes in cell cycle proteins and cell cycle progression. In both ovarian and pancreatic cancer cells, C-DIMs induced p21 and cells were arrested in the G_1 phase of the cell cycle [455,458]. In addition to G_1 cell cycle arrest, C-DIMs induced proteasome-dependent degradation of cyclin D1 in MCF-7 breast cancer cells and this response was also PPARγ-independent [459].

CHAPTER II

1,1-BIS(3'-INDOLYL)-1-(P-SUBSTITUTEDPHENYL)METHANES INHIBIT PROLIFERATION OF ESTROGEN RECEPTOR-NEGATIVE BREAST CANCER CELLS THROUGH ACTIVATION OF MULTIPLE PATHWAYS

2.1. Synopsis

1,1-Bis(3'-indolyl)-1-(p-substitutedphenyl)methanes containing para trifluoro-methyl (DIM-C-pPhCF_3), t-butyl (DIM-C-pPhtBu) and phenyl (DIM-C-pPhC_6H_5) groups activate peroxisome proliferator-activated receptor γ (PPARγ) in estrogen receptor-negative MDA-MB-231 and MDA-MB-453 breast cancer cells. These PPARγ-active compounds inhibit breast cancer cell proliferation, however, inhibition of G_0/G_1 to S phase progression and cyclin D1 downregulation was observed in MDA-MB-231 but not MDA-MB-453 cells. Nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1), a transforming growth factor β-like peptide, was also induced by these compounds and response was dependent on cell-context dependent activation of kinase pathways. Despite the induction of NAG-1 and downregulation of the antiapoptotic protein survivin in both MDA-MB-231 and MDA-MB-453 cells treated with these methylene-substituted diindolylmethanes (C-DIMs), this was not accompanied by apoptosis. Nevertheless the cytotoxicity observed for the C-DIMs in vitro was complemented by inhibition of tumor growth in athymic nude mice bearing MDA-MB-231 cells as xenografts and treated with DIM-C-pPhC_6H_5 (40 mg/kg/d). The inhibition of tumors derived from highly aggressive MDA-MB-231 cells suggests a potential role for the C-DIM compounds in the clinical treatment of ER-negative breast cancer.

2.2. Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of transcription factors [444,492,493]. The PPAR subfamily includes α, β(δ) and γ sub-types that bind fatty acids and lipids and the receptors play a role in lipid metabolism and metabolic diseases that include insulin-resistance, coronary artery disease and hyperlipidemia [443-446]. PPARs are differentially expressed in various tissues and exhibit some overlapping or interconnected activities such as in cholesterol transport, metabolism and function [443]. PPARα regulates genes involved in fatty acid utilization during fasting and PPARα agonists are drugs used for treating patients with hyperlipidemia. Thiazolidinediones (TZDs) such as rosiglitazone and pioglitazone are PPARγ agonists which are widely prescribed as insulin-sensitizing agents for treatment of type II diabetes [443-446].

PPARγ is overexpressed in multiple tumor types and their derived cancer cell lines [494,495] and is considered a potential target for development of anticancer drugs. Not surprisingly several different structural classes of PPARγ agonists have been identified and their anticancer activities have been investigated and these include TZDs, 15-deoxy-$\Delta^{12\text{-}14}$-prostaglandin J2 (PGJ2), 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and related compounds, 1,1-bis(3'-indolyl)-1-(p-substituted-phenyl)methanes [methylene-substituted diindolylmethanes (C-DIMs)] and several other chemical classes [444,456,492,493,496-502]. All PPARγ agonists typically induce fat cell differentiation and using RNA interference and PPARγ antagonists several PPARγ-dependent responses have been characterized in various cancer cell lines. For example

CDDO induces the tumor suppressor gene caveolin 1 in colon cancer cells and this response was inhibited by the PPARγ antagonist GW9662, however, many other CDDO-induced responses were receptor-independent or the role of PPARγ was not investigated [503-509].

Research in this laboratory has identified 1,1-bis(3'-indolyl)-1-(p-trifluoromethylphenyl)methane (DIM-C-pPhCF_3), 1,1-bis(3'-indolyl)-1-(p-tbutylphenyl) methane (DIM-C-pPhtBu), 1,1-bis(3'-indolyl)-1-(p-biphenyl)methane (DIM-C-pPhC_6H_5) as PPARγ agonists in several different cancer cell lines [423,455-458,460,468,489,510,511]. The PPARγ-active C-DIMs induce PPARγ-dependent transactivation in several different cancer cells and their induction of caveolin-1 and p21 was also PPARγ-dependent but varied with cell context. C-DIM compounds also induce receptor-independent responses including endoplasmic reticulum (ER) stress, activation of multiple kinases, induction of non-steroidal anti-inflammatory drug activated gene-1 (NAG-1) and proteasome-dependent downregulation of cyclin D1. A previous study showed that PPARγ-active C-DIMs inhibited growth of ER-positive MCF-7 breast cancer cells, induced apoptosis and downregulated expression of estrogen receptor α and cyclin D1 proteins [456]. In this study, we show that DIM-C-pPhCF_3, DIM-C-pPhtBu, DIM-C-pPhC_6H_5 induce PPARγ-dependent transactivation in ER-negative MDA-MB-231 and MDA-MB-453 breast cancer cells and inhibit proliferation with IC_{50} values ≤ 5 μM for most compounds in both cell lines. There were both differences and similarities in the effects of C-DIMs in the two cell lines including an increase in the distribution of cells in G_0/G_1 and decreased S phase in MDA-MB-231 but not in MDA-MB-453 cells.

PPARγ-active C-DIMs did not affect expression of the cell cycle genes p21, p27 or cyclin D1 in MDA-MB-453 cells but induced proteasome-dependent degradation of cyclin D1 in MDA-MB-231 cells. These compounds did not induce caveolin-1 or activate ER stress in either cell line but induced NAG-1 and ATF3 in both ER-negative cells. In addition the C-DIM compounds downregulated the antiapoptotic protein survivin but did not induce apoptosis in these cells. In athymic nude mice bearing MDA-MB-231 cells as xenografts, treatment with 40 mg/kg/day of DIM-C-pPhC_6H_5 significantly inhibited tumor growth and confirmed the anticarcinogenic activity of C-DIMs both in vivo and in vitro.

2.3. Materials and Methods

2.3.1. Cells, chemicals and other materials

MG132 was obtained from Sigma Chemical Co. (St. Louis, MO). The human breast cancer cell lines MDA-MB-231 and MDA-MB-453 were obtained from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231 cells were maintained in DMEM:F-12 supplemented with 0.22% sodium bicarbonate, 10% fetal bovine serum (FBS), and 2 ml/L antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO). MDA-MB-453 cells were maintained in RPMI supplemented with 0.15% sodium bicarbonate, 0.12% HEPES, 10% fetal bovine serum (FBS), and 2 ml/L antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO). Cells were grown in 150 cm^2 culture plates in an air/CO_2 (95:5) atmosphere at 37°C and passaged approximately every 5 days. p21 (C-19), p27 (C-19), cyclin D1 (M-20), cyclin D3 (C-16), caveolin-1 (N-20), caveolin-2 (N-20), p-Erk (K-23), Erk (E-4), p-Akt (Ser473), Akt

(H-136), p-c-Jun (KM-1), c-Jun (D), Grp78 (H-129), ATF-3 (C-19), survivin (FL-142) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The NAG-1 antibody was purchased from Upstate (Lake Placid, NY). Horseradish peroxidase substrate for Western blot analysis was purchased from NEN Life Science Products (Boston, MA).

2.3.2. Cell proliferation assays

Cells were plated at a density of 3-5 x 10^4/well in 12-well plates and media was replaced the next day with DMEM:F-12 media containing 2.5% charcoal-stripped FBS and either vehicle (DMSO) or the indicated ligand and concentration. Fresh media and compounds were added every 48 h. Cells were counted at the indicated times using a Coulter Z1 cell counter. Each experiment was completed in triplicate and results are expressed as means ± SE for each determination.

2.3.3. Transient transfection assays

Breast cancer cells were plated in 12-well plates at 1.5 x10^5 cells/well in DMEM:F12 media supplemented with 2.5% charcoal-stripped FBS. PPARγ-Gal4 (0.2 μg/well), Gal4-LUC (0.5 μg/well) and pCDNA3.1-His-LacZ (0.04 μg/well, Invitrogen, Carlsbad, CA) expression plasmid (for normalization of transfection efficiency) were transiently cotransfected into MDA-MB-231 and MDA-MB-453 cells using Lipofectamine according to the manufacturer's protocol. After 5-6 h, cells were treated for 24 h with fresh 2.5% stripped bovine medium containing 1, 5, or 10 μM DIM-C-pPhCF_3, DIM-C-pPhtBu, DIM-C-pPhC_6H_5 dissolved in DMSO, or DMSO alone as a solvent control. After 24 h of treatment, cells were then lysed with 150 μL of 1 X

reporter lysis buffer from Promega (Madison, WI) and 30 μL of cell extract were used for luciferase and β-gal assays. Significance was determined by SuperANOVA ($p < 0.05$).

2.3.4. Fluorescence-Activated Cell-Sorting assays (FACS)

MDA-MB-231 cells and MDA-MB-453 cells were treated with either the vehicle (DMSO) or the indicated compounds for 48 h. Cell were trypsinized, centrifuged and resuspended in staining solution containing 50 mg/mL propidium iodide, 4 mM sodium citrate, 30 units/mL RNase, and 0.1% Triton X-100. After incubation at 37^{O}C for 10 min, sodium chloride was added to give a final concentration of 0.15 M. Cells were analyzed on a FACS Calibur flow cytometer using CellQuest acquisition software. PI fluorescence was collected though a 585/42nm bandpass filter, and list mode data were acquired on a minimum of 20,000 single cells defined by a dot plot of PI width versus PI area. Data analysis was performed in ModFit LT using PI width versus PI to exclude cell aggregates.

2.3.5. Western blots

MDA-MB-231 and MDA-MB-453 cells were seeded in DMEM:F-12 media containing 2.5% charcoal-stripped FBS for 24 h and then treated with either the vehicle (DMSO) or the indicated compounds. Whole cell lysates were obtained using high salt buffer [50 mM HEPES, 500 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol and 1% Triton X-100 pH 7.5 and 5 μL/mL of Protease Inhibitor Cocktail]. Protein samples were incubated at 100^{O}C for 2 minutes, separated on 10% SDS-PAGE at 120 V for 3-4 h in 1 X running buffer [25 mM Tris-base, 192 mM glycine, and 0.1% SDS (pH 8.3)] and

transferred to polyvinylidene difluoride membrane at 0.2 V for 16 h at $4^{O}C$ in 1 X transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 0.025% SDS). The PVDF membrane was blocked in 5% TBST-Blotto [10 mM Tris-HCl, 150 mM NaCl (pH 8.0), and 0.05% Triton X-100 and 5% non-fat dry milk] with gentle shaking for 30 min and incubated in fresh 5% TBST-Blotto at 1:200-1:1000 with primary antibody overnight at $4^{O}C$ with gentle shaking. After washing with TBST for 10 min, the PVDF membrane was incubated with secondary antibody (1:5000) in 5%TBST-Blotto for 2 h. The membrane was washed with TBST for 10 min and incubated with 10 mL of chemiluminescence substrate for 1.0 min and exposed to Kodak X-OMAT AR autoradiography film.

2.3.6. Anti-tumor efficacy studies

Athymic nude mice ($Nu^{-/-}$) were injected i.p. with 75 μg of a rat anti-mouse asialo GM1 antibody (Wako Chemical Company, Richmond, VA) to reduce natural killer cells. Injections were done on days -4 and -2 prior to the injection of MDA-MB-231 cells. At day 0, mice were injected subcutaneously in the left flank with 10^7 MDA-MB-231 cells in 100-200 μL serum-free medium. Three groups of mice (12 mice/group) were then treated i.p. with 40 mg/kg DIM-C-pPhC_6H_5 in 50 μL placebo, 50 μL placebo or 50 μL PBS, every day for 35 total injections starting at day 4 post-tumor inoculations. Tumor size was measured with calipers, based on the formula L X W^2 where L is the length and W is the width of the tumor. Moribund mice and mice whose tumor burdens exceeded 20% of their body weight were euthanized following institutional regulations.

2.4. Results

Previous studies showed that DIM-C-pPhCF$_3$, DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$ activated PPARγ and inhibited growth of breast, bladder, colon, ovarian and pancreatic cancer cells [423,455-458,460,468,489,510,511]. Results in Figures 2.1A and 2.1B show that these PPARγ-active C-DIMs significantly induced transactivation in ER-negative MDA-MB-231 and MDA-MB-453 cells transfected with PPARγ-GAL4/pGAL4. Rosiglitazone, a thiazolidinedione PPARγ agonist also induced transactivation in both cell lines. GW9662 is a PPARγ antagonist and the induction of transactivation by DIM-C-pPhCF$_3$, DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$ in MDA-MB-231 (Figure 2.1C) and MDA-MB-453 cells (Figure 2.1D) transfected with PPARγ-GAL4/pGAL4 was inhibited after cotreatment with GW9662. These results demonstrate that the PPARγ-active C-DIMs activate PPARγ in ER-negative breast cancer cell lines and these results were similar to those previously reported for the same compounds in MCF-7 cells [456].

PPARγ-active C-DIMs inhibit growth of pancreatic, colon, bladder and ovarian cancer cells and these compounds also inhibited proliferation of ER-positive MCF-7 cells [423,455-458,460,468,489,510,511]. Results illustrated in Figure 2.2 show that DIM-C-pPhCF$_3$, DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$ inhibit proliferation of ER-negative MDA-MB-231 and MDA-MB-453 breast cancer cells and IC$_{50}$ values were generally between 1-5 μM. The only exception was the decreased potency of DIM-C-

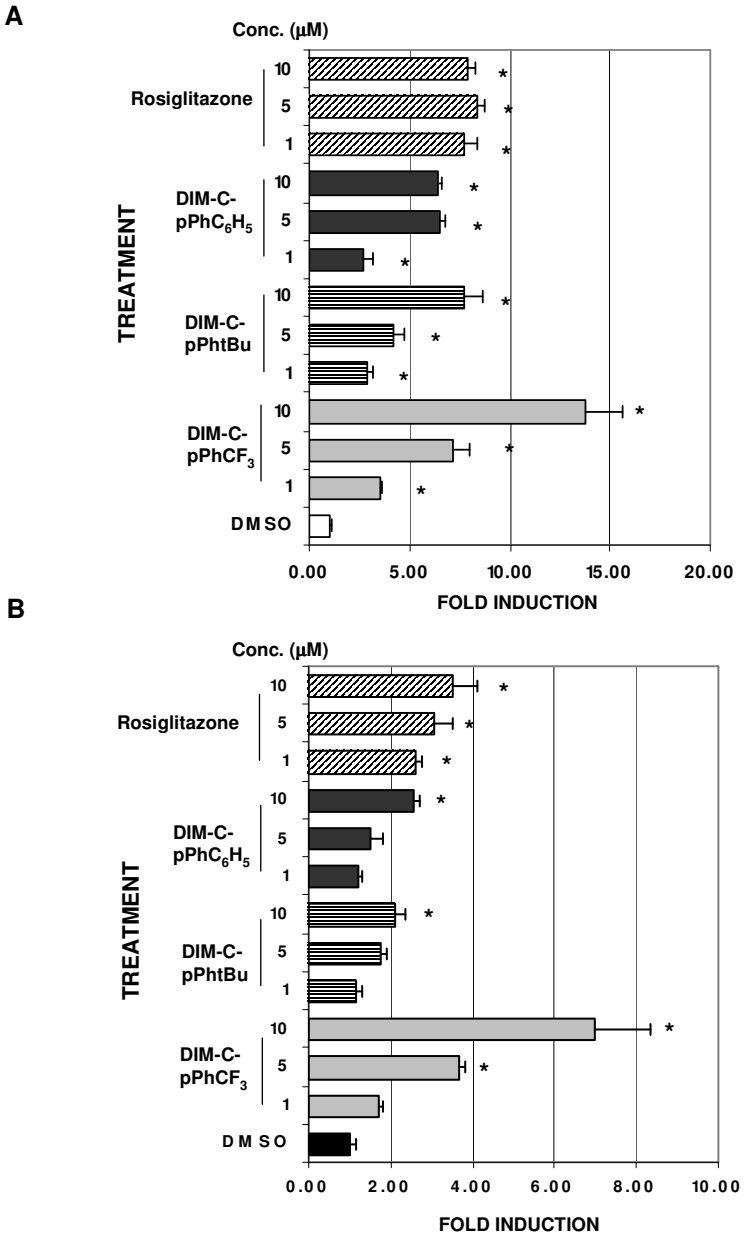
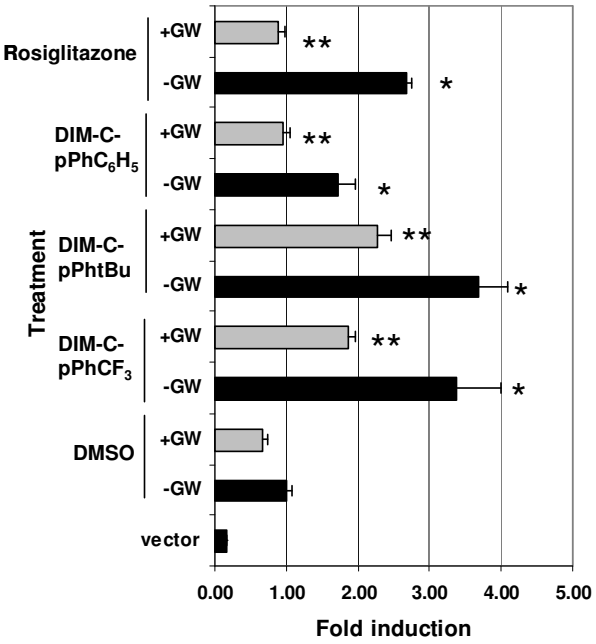


Figure 2.1: Ligand-induced activation of PPARγ and effects of PPARγ antagonists. Ligand-dependent activation of PPARγ-GAL4/pGAL4 in MDA-MB-231 [A] and MDA-MB-453 [B] cells. Cells were transfected with PPARγ-GAL4/pGAL4, treated with 1, 5, or 10 μM of DIM-C-pPhCF$_3$, DIM-C-pPhtBu, DIM-C-pPhC$_6$H$_5$ and rosiglitazone (positive control), and luciferase activity was determined as described in Materials and Methods. Results of all transactivation studies in this figure are presented as means ± SE for at least three separate determinations for each treatment group, and significant ($p < 0.05$) induction compared with solvent (DMSO) control is indicated by an asterisk. Inhibition of transactivation in MDA-MB-231 [C] and MDA-MB-453 [D] cells by PPARγ antagonist GW9662. Cells were transfected with PPARγ-GAL4/pGAL4, treated with 10 μM rosiglitazone or C-substituted DIMs alone or in combination with 7.5 μM GW9662, and luciferase activities were determined as described in [A]. Significant ($p < 0.05$) inhibition of induced transactivation by GW9662 is indicated (**).

C



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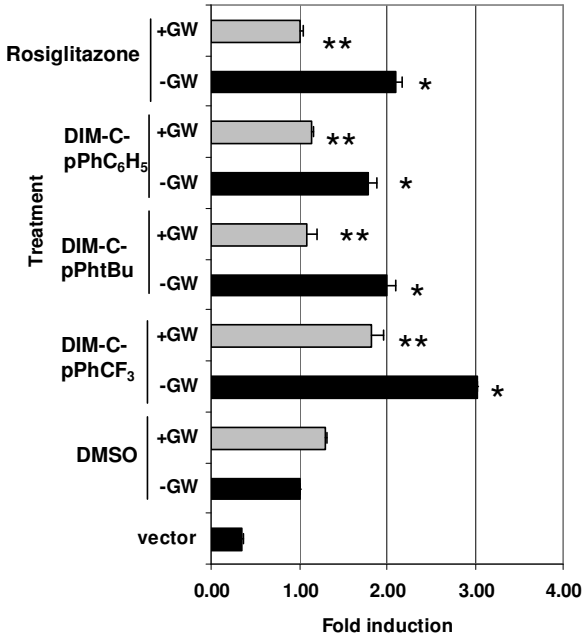


Figure 2.1 (Continued)

pPhtBu in MDA-MB-453 cells where the IC_{50} was slightly greater than 5 μM. PPARγ-active C-DIMs also affected the % distribution of MDA-MB-231 cells in G_0/G_1, S and G_2/M phase of the cell cycle (Figure 2.2C). Even at concentrations as low as 1 or 5 μM the C-DIM compounds consistently increased the % of cells in G_0/G_1 and decreased the % of cells in S phase of the cell cycle. In contrast, these same concentrations had minimal effects on the % distribution of MDA-MB-453 cells in G_0/G_1, S or G_2/M phase of the cell cycle (Figure 2.2D) indicating some cell context-dependent differences in the effects of C-DIMs on cell cycle progression.

PPARγ agonists including C-DIMs affect expression of cell cycle proteins in some cell lines and results in Figure 2.3A show that after treating MDA-MB-231 cells for 12 h with 10 μM C-DIMs there was decreased expression of cyclin D1 and slightly increased levels of p21 or p27 proteins and similar results were observed after treatment for 24 h (data not shown). In contrast, the PPARγ-active C-DIMs did not affect cyclin D1, p21 or p27 levels in MDA-MB-453 cells after treatment for 12 h (Figure 2.3B) or 24 h (data not shown). The C-DIM-induced downregulation of cyclin D1 in MDA-MB-231 cells was inhibited in cells cotreated with the proteasome inhibitor MG132 (Figure 2.3C) and this PPARγ-independent response was previously observed with these compounds in MCF-7 cells (17). Thus the major effects of the PPARγ-active C-DIMs was induction of proteasome-dependent degradation of cyclin D1 (Figure 2.3C) which correlates with the inhibition of G_0/G_1 to S phase progression in MDA-MB-231 cells (Figure 2.2D).

PPARγ-active C-DIMs induce several growth inhibitory factors or pathways in cancer cell lines including receptor-dependent induction of caveolin-1 and receptor-

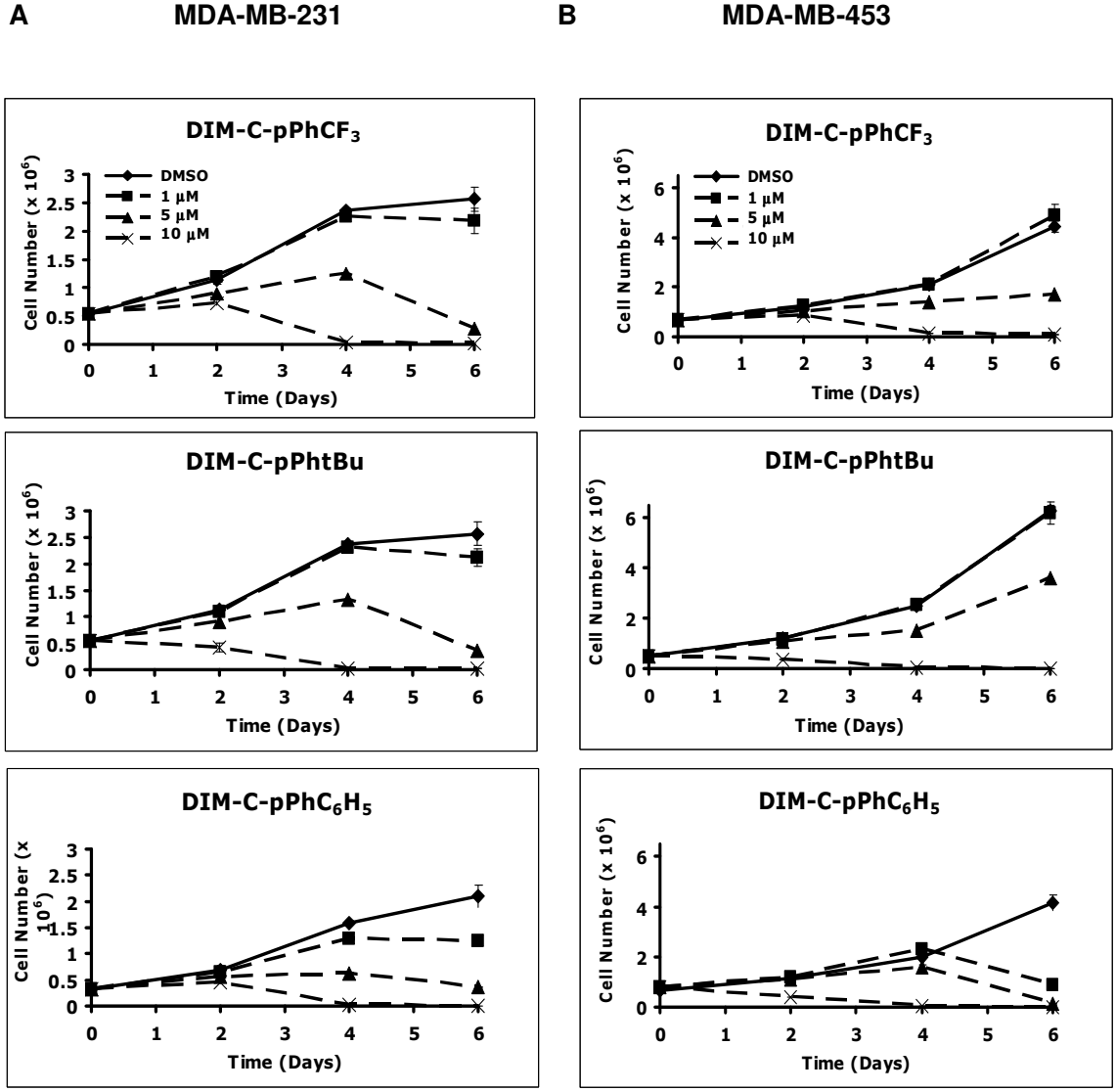
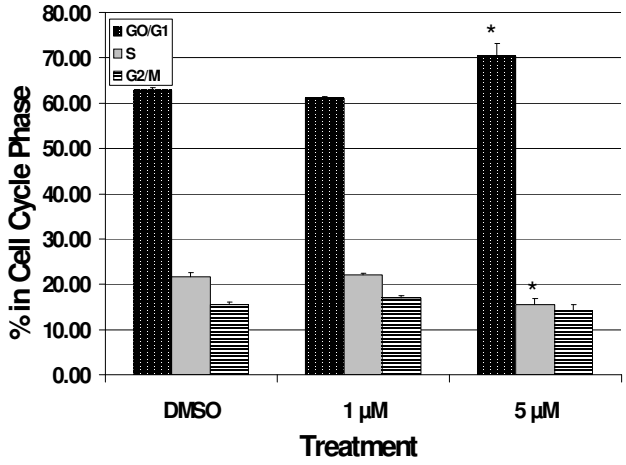


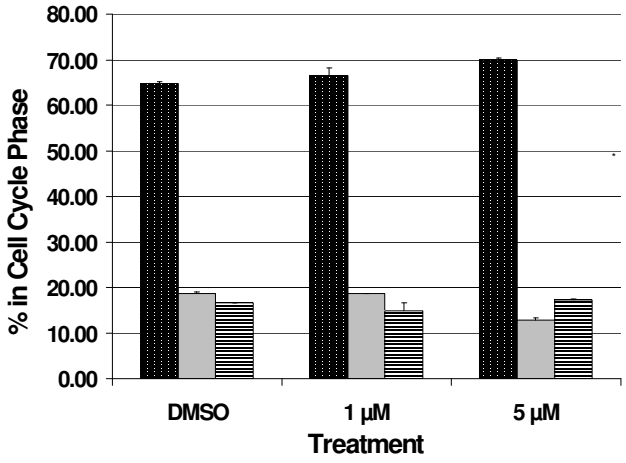
Figure 2.2: Growth inhibition studies and Fluorescence-Activated Cell Sorting (FACS) analysis. MDA-MB-231 [A] and MDA-MB-453 [B] breast cancer cells were treated with 1 to 10 µM DIM-C-pPhCF$_3$, DIM-C-pPhtBu, and DIM-C-pPhC$_6$H$_5$ for 6 days, and cell numbers were determined using a Coulter Counter as described in the Materials and Methods. Results are expressed as means ± SE for three separate determinations at each time point. MDA-MB-231 [C] and MDA-MB-453 [D] cells were treated for 48 h with 1-5 µM DIM-C-pPhCF$_3$, DIM-C-pPhtBu, and DIM-C-pPhC$_6$H$_5$ and analyzed by FACS analysis as described in the Materials and Methods. Experiments were carried out in 2.5% charcoal-stripped serum and DMSO served as the solvent control. Results are expressed as means ± SE for three separate determinations for each treatment group and significance ($p<0.05$) is indicated by an asterisk.

C

DIM-C-pPhCF$_3$



DIM-C-pPhtBu



DIM-C-pPhC$_6$H$_5$

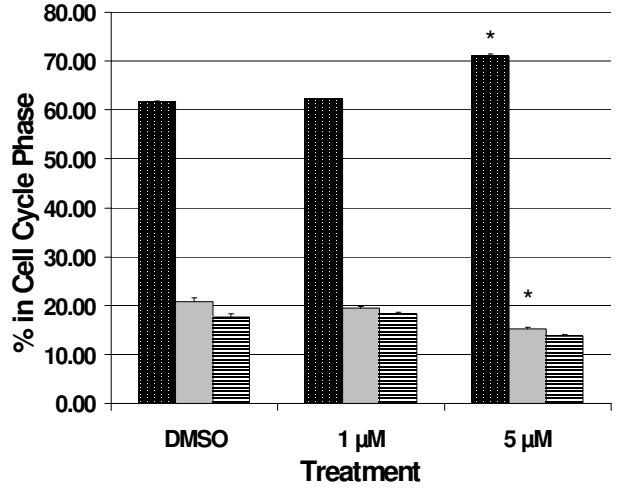


Figure 2.2 (Continued)

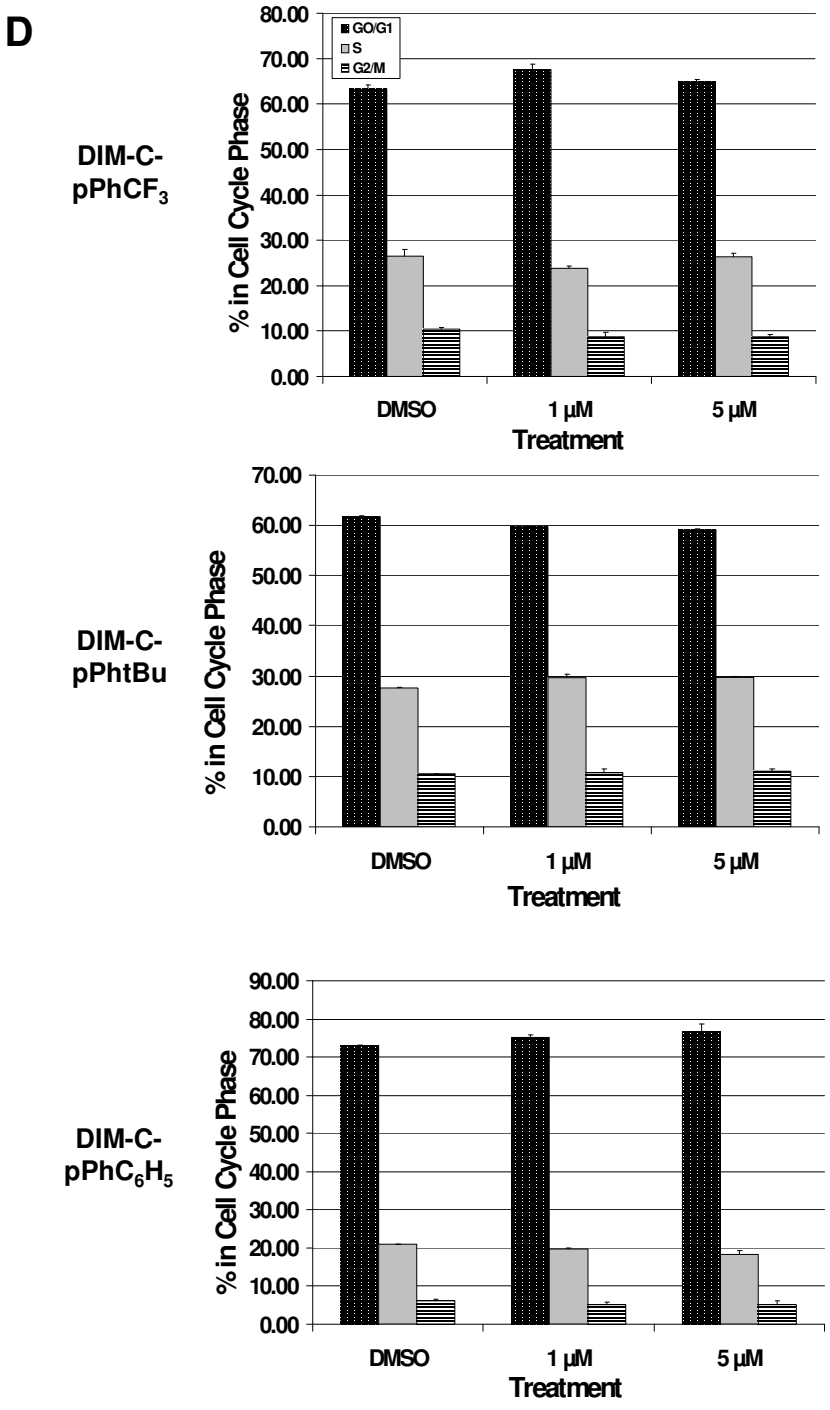


Figure 2.2 (Continued)

independent induction of ER stress [423] and NAG-1 [489]. Results illustrated in Figure 2.3D show that 5-10 μM of the C-DIM compounds did not affect expression of the stress protein GRP78 whereas 0.3 μM thapsigargin (positive control) induced GRP78 in MDA-MB-231 and MDA-MB-453 cells respectively. Figure 2.3E shows that caveolin 1 expression is not induced in MDA-MB-453 cells by the C-DIM compounds whereas in MDA-MB-231 cell concentration-independent effects on caveolin-1 expression were observed. In contrast, the same compounds induced caveolin 1 in colon cancer cells and cotreatment with PPARγ antagonists blocked induction [489].

NAG-1 is a proapoptotic and growth inhibitory protein induced in cancer cell lines by diverse agents including C-DIM compounds ([475,481-483,483,489]and results in Figures 2.4A and 2.4B show that NAG-1 is induced in MDA-MB-453 and MDA-MB-231 cells after treatment with 10 μM DIM-C-pPhCF_3, DIM-C-pPhtBu and DIM-C-pPhC_6H_5. ATF3, which is ofen co-induced with NAG-1 was also elevated in the ER-negative breast cancer cells treated with PPARγ-active C-DIMs. Using DIM-C-pPhC_6H_5 as a model, we showed that NAG-1 induction was not inhibited by the PPARγ antagonist GW9662 (Figures 2.4A and 2.4B). Similar results were observed for PPARγ-active C-DIMs in colon cancer cells where induction of NAG-1 was also PI3-K dependent [489]. NAG-1 is induced through multiple pathways including activation of kinases in colon cancer cells where PPARγ-active C-DIMs induced NAG-1 through PI3-K dependent activation of Egr-1 which binds the proximal region of the NAG-1 promoter [489]. The induction of NAG-1 by DIM-C-pPhC_6H_5 in the absence or

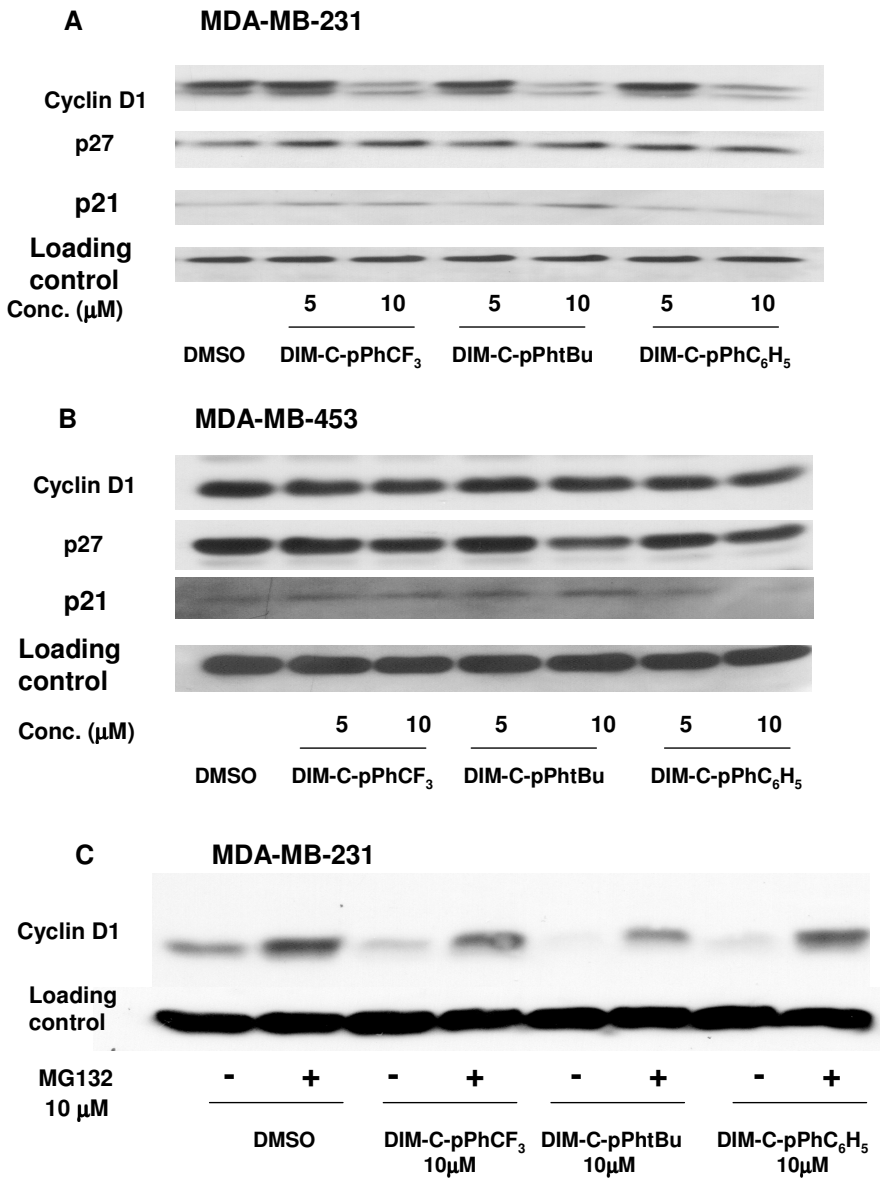
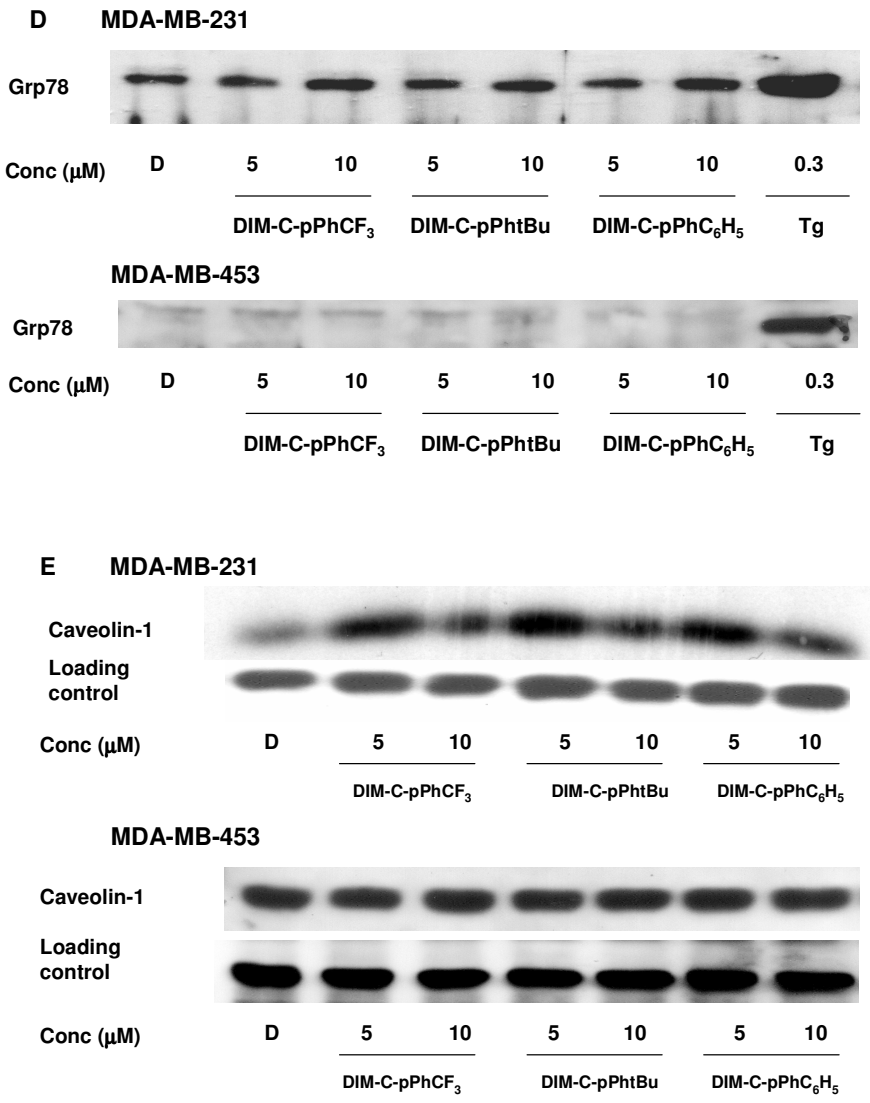


Figure 2.3: Modulation of cell cycle, ER stress and caveolin proteins. MDA-MB-231 [A] or MDA-MB-453 [B] cells were treated with 5 or 10 μM DIM-C-pPhCF$_3$, or DIM-C-pPhC$_6$H$_5$ for 12 h and whole cell lysates were analyzed by Western blot analysis as described in Materials and Methods. MDA-MB-231 [C] cells were pre-treated for 30 minutes with 10 μM proteasome inhibitor MG132 or vehicle control and subsequently treated for 12 h with DMSO, 10 μM DIM-C-pPhCF$_3$, or DIM-C-pPhC$_6$H$_5$ for 12 h and whole cell lysates were analyzed by Western blot analysis as described in Materials and Methods. Experiments were done in triplicate and results shown are typical of Cyclin D1, p21, and p27 protein levels for treatment replicates. MDA-MB-231 and MDA-MB-453 [D,E] cells were treated with DIM-C-pPhCF$_3$, DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$ or 0.3 μM thapsigargin (Tg) (positive control for ER stress) for 24 h [D] or 72 h [E] and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. Experiments were done in triplicate and results shown are typical of treatment replicates.



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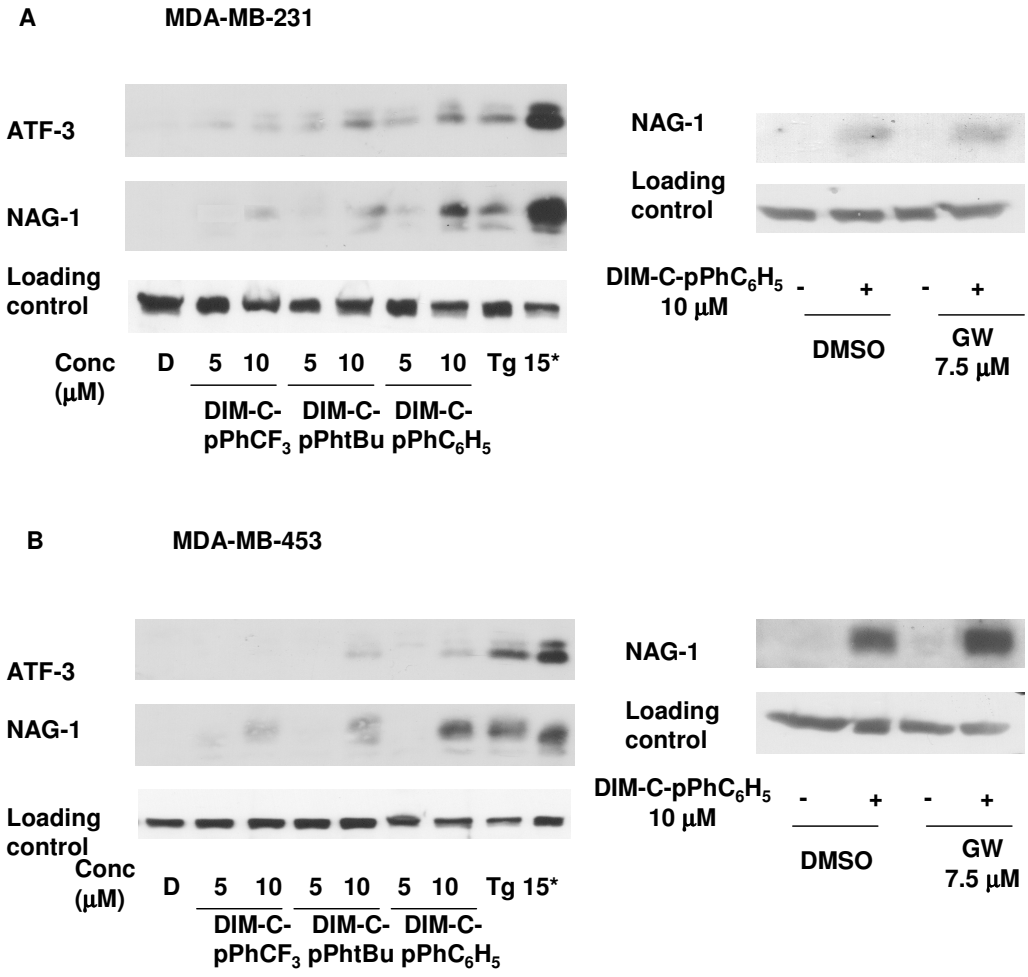
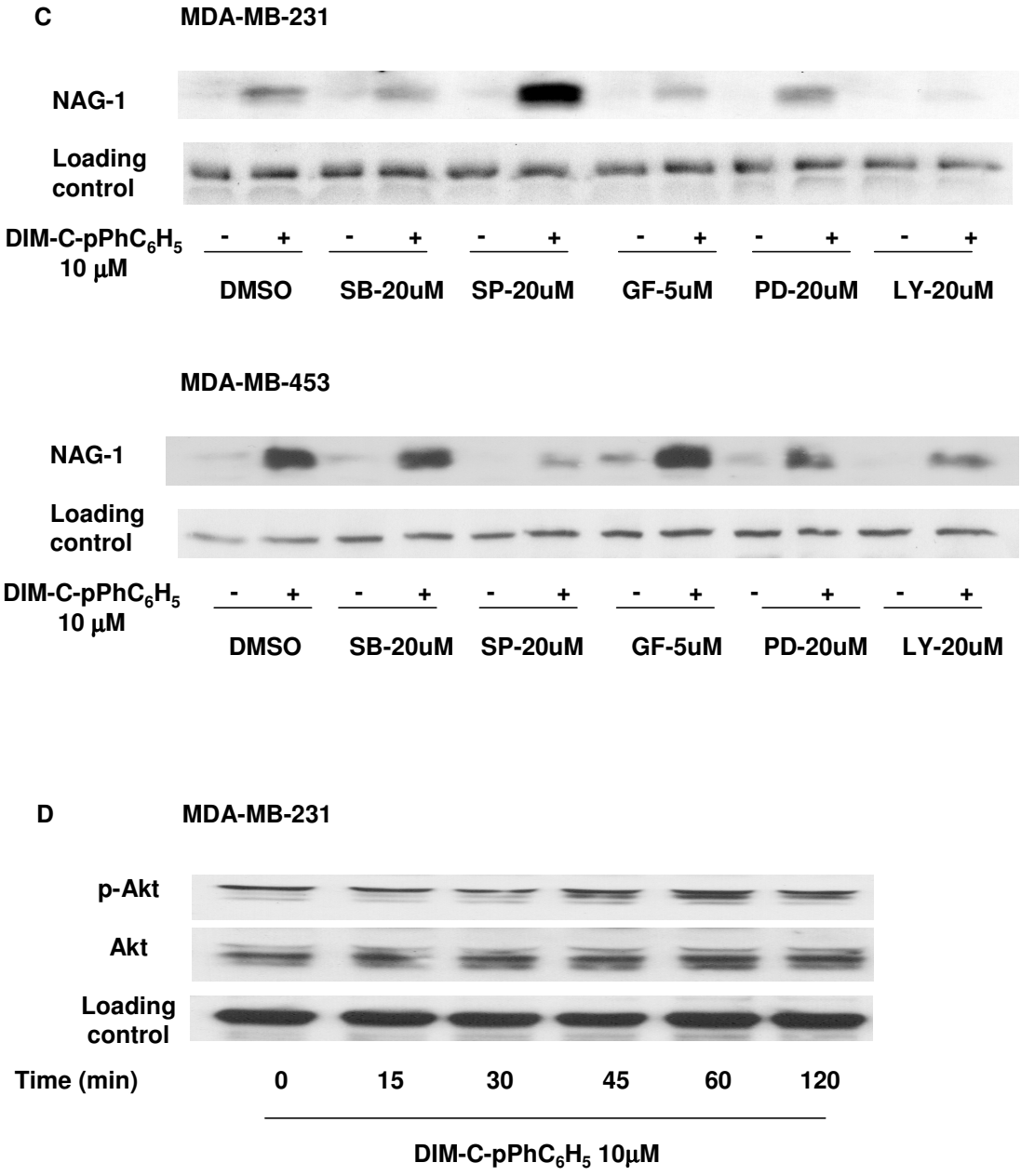


Figure 2.4: Induction of NAG-1 proteins. MDA-MB-231 [A, left] and MDA-MB-453 [B, left] cells were treated with DMSO or 5-10 μM DIM-C-pPhCF$_3$, DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$ or 0.3 μM thapsigargin (Tg) for 24 h and whole cell lysates were analyzed by Western blot analysis for NAG-1 and ATF3. 15* represents whole cell lysate sample isolated from SW480 cells and treated with 15 μM DIM-C-pPhtBu and based on previous published data, served as a positive control for NAG-1 and ATF3 induction. PPARγ-independent activation of NAG-1. MDA-MB-231 [A, right] or MDA-MB-453 cells [B, right] were treated for 24 h with DMSO or 10 μM DIM-C-pPhC$_6$H$_5$ and with or without co-treatment with 10 μM PPARγ inhibitor GW9662 and whole cell lysates were analyzed by Western blot analysis and probed for NAG-1. Role of kinases in NAG-1 induction [C]. MDA-MB-231 and MDA-MB-453 cells were treated with DMSO or 10 μM DIM-C-pPhC$_6$H$_5$ and with or without co-treatment of the following kinase inhibitors: 20 μM p38 MAPK (SB203580), 20 μM JNK (SP600125), 5 μM PKC (GF109203X), 20 μM p44/42 MAPK (PD98059) and 20 μM PI3-K (LY294002) for 24 h and whole cell lysates were probed for NAG-1. Activation of kinases in 0-120 min treatment with 10 μM DIM-C-pPhC$_6$H$_5$ [D,E]. Whole cell lysates isolated from MDA-MB-231 [D] cells were analyzed by Western blot analysis and probed for p-Akt and Akt. MDA-MB-453 [E] cells were analyzed by Western blot analysis for p-Akt, Akt, p-Erk, Erk, p-c-Jun, c-Jun. Experiments were done in triplicate and results shown are typical of treatment replicates.



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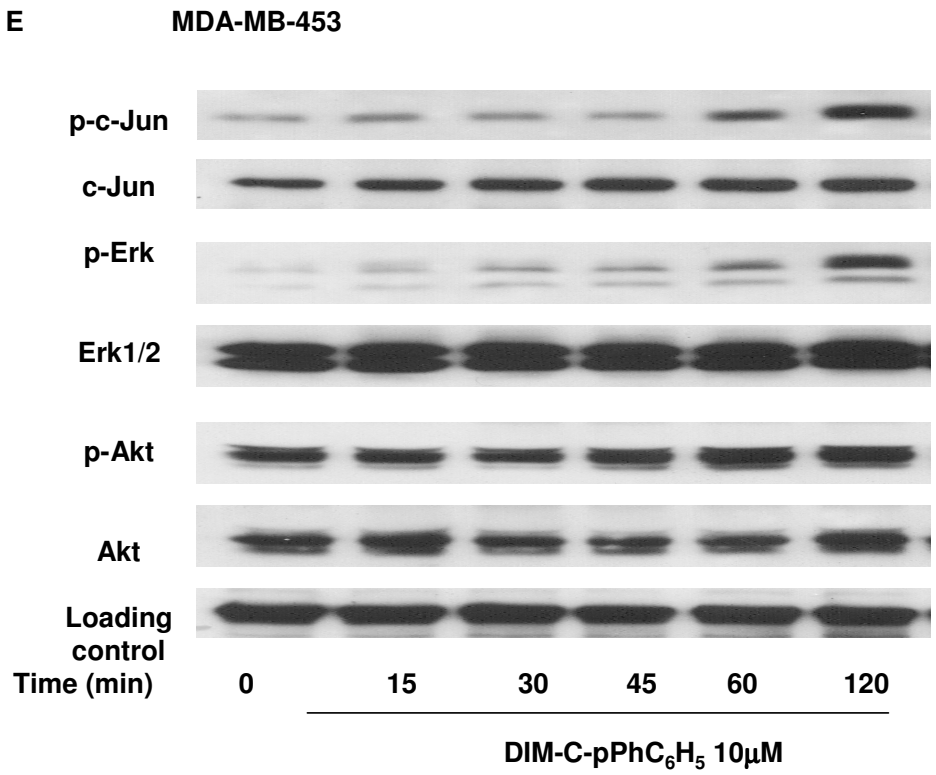

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presence of inhibitors of p38 MAPK (SB203580), JNK (SP600125) PKC (GF109203X), p44/42 MAPK (PD98059) and PI3-K (LY294002) is summarized in Figure 2.4C. In MDA-MB-453 cells inhibition of NAG-1 induction by DIM-C-pPhC_6H_5 was observed in cells cotreated with p44/42 MAPK, PI3-K and JNK inhibitors with the latter inhibitor being the most effective. In contrast only LY294002 (PI3-K inhibitor) blocked induction of NAG-1 by DIM-C-pPhC_6H_5 in MDA-MB-231 cells and the JNK inhibitor actually enhanced induction of NAG-1 by the C-DIM compound (Figure 2.4C). Results in Figure 2.4D show that DIM-C-pPhC_6H_5 induces PI3-K-dependent phosphorylation of Akt in MDA-MB-231 cells and phosphorylation of MAPK, Akt and c-Jun in MDA-MB-453 cells (Figure 2.4E) and these induced kinases play a role in the induction of NAG-1 in the same cell lines.

Previous studies showed that DIM, ring-substituted DIMs and C-DIMs induced apoptosis in ER-positive MCF-7 cells and the former two compounds did not induce apoptosis in MDA-MB-231 cells [425,456]. The effects of the PPARγ-active C-DIM compounds on induction of apoptosis were investigated in both MDA-MB-231 and MDA-MB-453 cells by determining caspase-dependent PARP cleavage, bax/bcl-2 expression (Figures 2.5A and 2.5B) and effects on the antiapoptotic protein survivin (Figures 2.5C and 2.5D). There was an increase in bax and decrease in bcl-2 expression observed after treatment of MDA-MB-231 and MDA-MB-453 cells with PPARγ-active C-DIMs, however, activation of the intrinsic pathway for apoptosis as evidenced by caspase-dependent PARP cleavage was not observed. The antiapoptotic protein, survivin, was expressed in MDA-MB-231 and MDA-MB-453 cells and treatment with

PPARγ-active C-DIM compounds decreased survivin protein expression (Figures 2.5C and 2.5D), however, this response was insufficient for activation of caspase-dependent apoptotic pathways.

Results in Figure 2.6 show that treatment with DIM-C-pPhC$_6$H$_5$ (40 mg/kg/d) decreased tumor growth in athymic nude mice bearing MDA-MB-231 cells as xenografts. These results, coupled with the cancer cell growth inhibition studies (Figure 2.2) clearly show that PPARγ-active C-DIMs effectively inhibit growth of ER-negative breast cancer cells and this was comparable to inhibition of colon and bladder tumor growth in mouse xenograft models [460,511].

2.5. Discussion

Several different structural classes of PPARγ agonists have been identified and many of these including PGJ2, thiazolidinediones and CDDO inhibit proliferation of breast and other cancer cell lines through multiple receptor-dependent and independent pathways [454,459,503-509,512-517]. For example in one study 10 μM PGJ2 induced rapid morphological changes associated with apoptosis in MDA-MB-231 cells whereas 100 μM troglitazone induced minimal apoptosis after prolonged (50 h) treatment [454]. Other studies also show similar differences between induction of apoptosis by PGJ2 and troglitazone in MDA-MB-231 cells. CDDO is a triterpenoid acid and a PPARγ agonist that induces both receptor-dependent and independent responses in cell lines and tumors derived from multiple tissues including breast cancer cells. CDDO alone did not induce apoptosis in ER-positive T47D or ER-negative MDA-MB-453 cells [516], however, CDDO enhanced TRAIL-induced apoptosis in both cell lines. PPARγ-active C-DIM

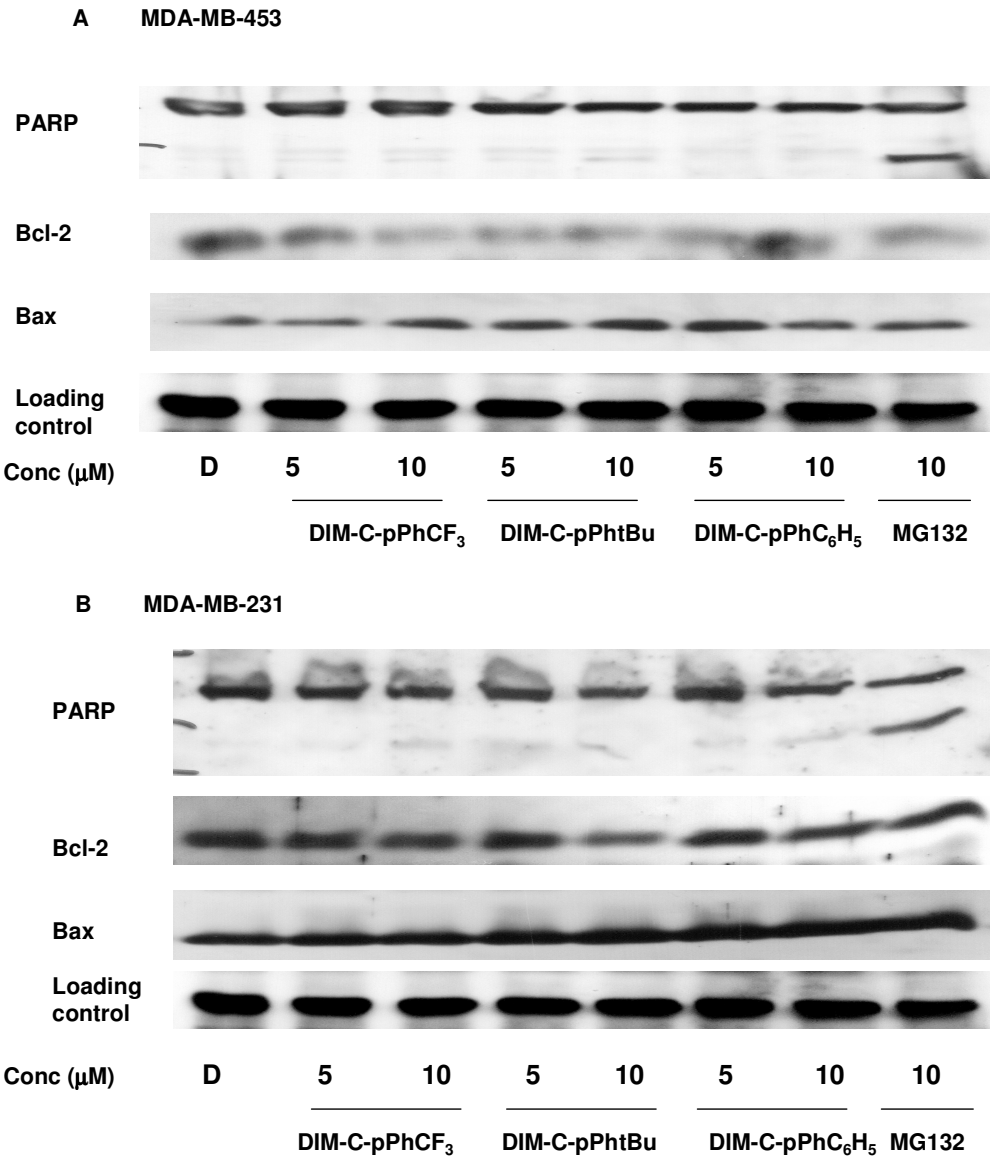
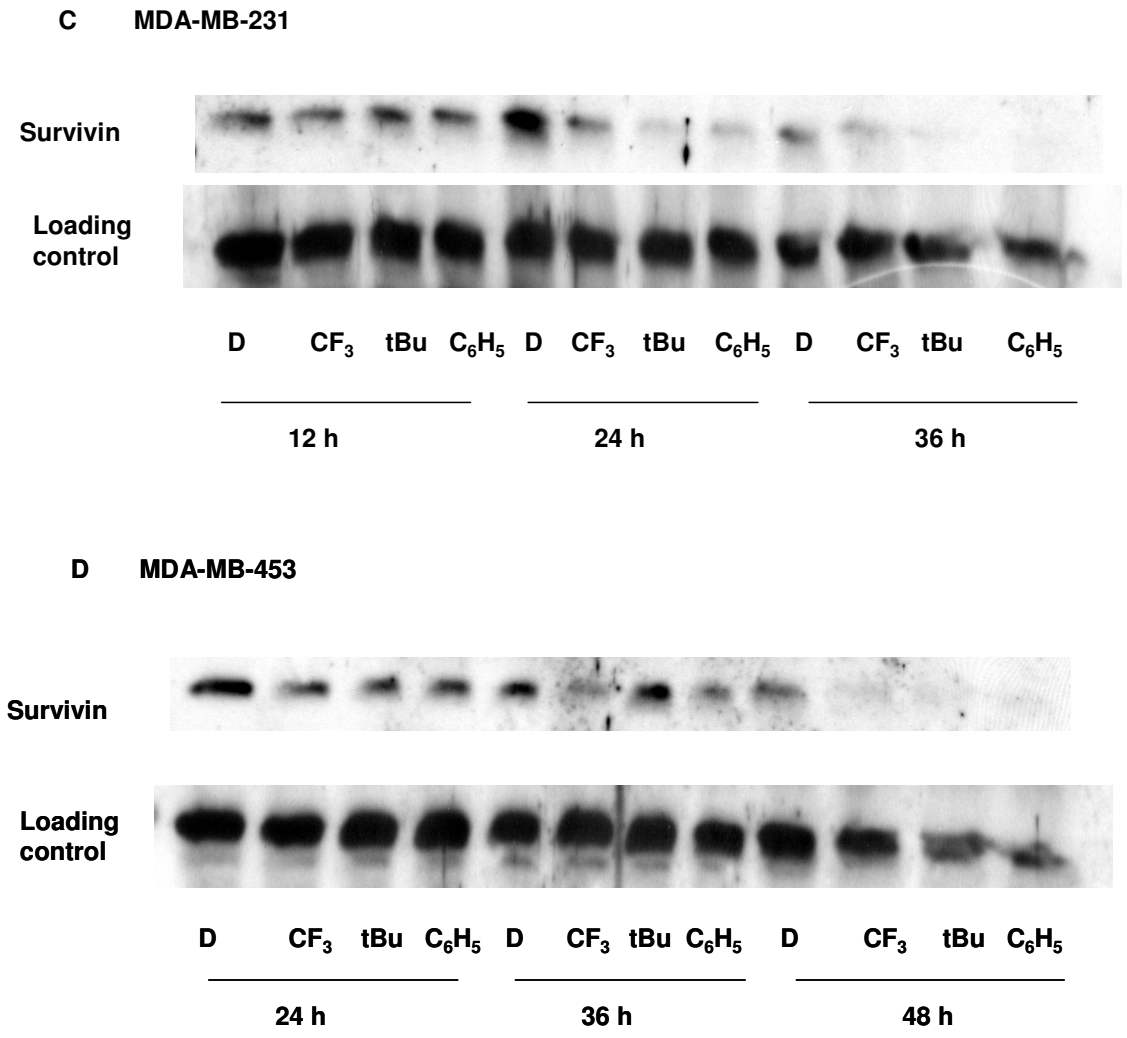


Figure 2.5: Effects of PPARγ agonists on apoptosis. MDA-MB-231 [A] and MDA-MB-453 cells [B] were treated with DMSO or 5-10 μM DIM-C-pPhCF$_3$, DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$ or 10 μM MG132 for 24 h and whole cell lysates were analyzed by Western blot analysis for PARP112/85, bcl-2 and bax. Time-dependent activation of survivin. MDA-MB-231 [C] and MDA-MB-453 cells [D] were treated for 12-36 h or 24-48 h with DMSO or 10 μM DIM-C-pPhCF$_3$, DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$. Whole cell lysates were analyzed by Western blot analysis and probed for survivin. Experiments were done in triplicate and results shown are typical of treatment replicates.



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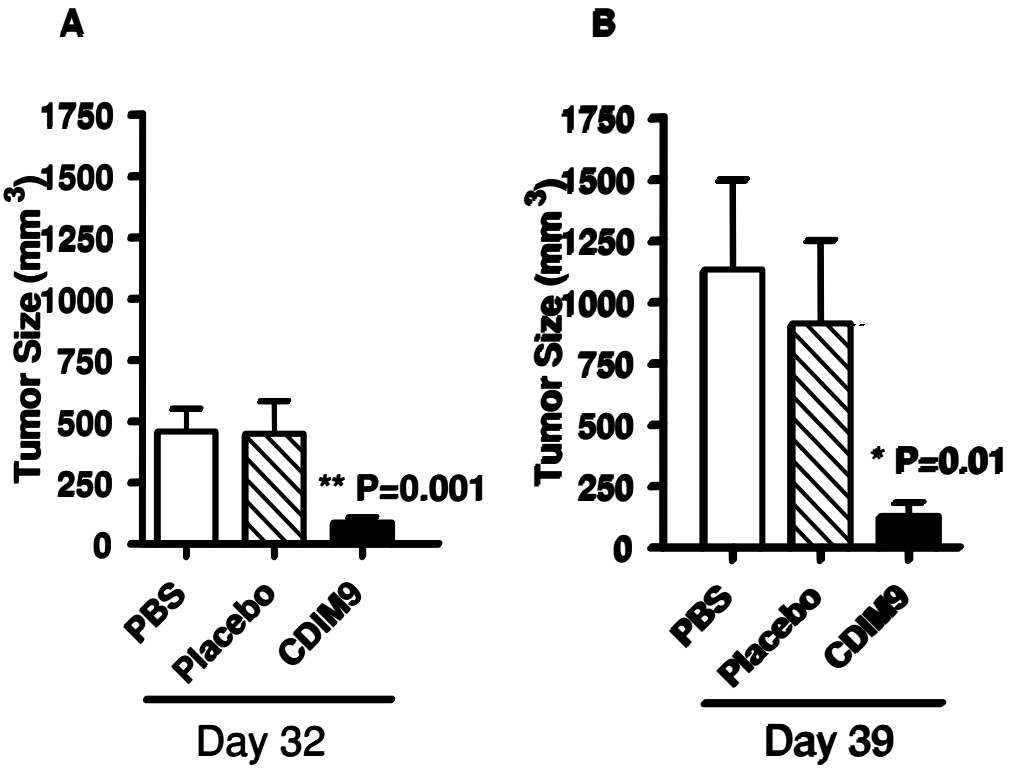


Figure 2.6: In vivo antitumorigenic activity of DIM-C-pPhC$_6$H$_5$. MDA-MB-231 s.c. tumor size (mean ± SE) in athymic mice treated i.p. daily starting at day 4 post-tumor inoculation for 35 total doses with PBS (50 µL), placebo (50 µL) and DIM-C-pPhC$_6$H$_5$ (40 mg/kg in 50 µL placebo). The tumor sizes were measured on day 35 and day 39. On day 32, the tumor sizes were 446.1±138.3 mm^3 (placebo treated), 454.3±97.5 mm^3 (PBS treated), 83.3±25.1 mm^3 (DIM-C-pPhC$_6$H$_5$ treated) (t-test, P=0.001 versus PBS treated group); On day 39, the tumor sizes were 909.9±342.6 mm^3 (placebo treated), 1129±371.9 mm^3 (PBS treated), 124.7±54.8 mm^3 (DIM-C-pPhC$_6$H$_5$ treated) (t-test, P=0.01 versus PBS treated group).

compounds have been identified in this laboratory as a new class of PPARγ agonists and several receptor-dependent and independent responses have been characterized. Results of transactivation studies (Figure 2.1) clearly demonstrate that the three PPARγ-active C-DIMs induced PPARγ-dependent activity in MDA-MB-231 and MDA-MB-453 cells as previously reported in other cancer cell lines [455-458,460,468,489,510,511].

PPARγ agonists typically inhibit cancer cell growth by arresting cells in G_0/G_1 and this is often associated with modulation of cyclin D1 (decreased) or induction of the cyclin-dependent kinase inhibitor p21 [455-457,512]. PPARγ-active C-DIMs inhibit growth of ER-negative MDA-MB-231 and MDA-MB-453 cells (Figure 2.2) but induced only a modest increase in cells in G_0/G_1 and decrease in cells in S-phase in MDA-MB-231 but not in MDA-MB-453 cells (Figures 2.2C and 2.2D). These results were observed over several time points (12, 24, 48 and 96 h) using 1 or 5 μM concentrations of the C-DIM compounds which are sufficient to inhibit cell proliferation (Figure 2.2). The growth inhibitory effects of the PPARγ-active C-DIMs on cell cycle proteins were also different in the two ER-negative cell lines where 5-10 μM C-DIMs induced cyclin D1 downregulation in MDA-MB-231 but not MDA-MB-453 cells (Figures 2.3A-2.3C). This was most pronounced after treatment for 12 h and was reversed by the proteasome inhibitor MG132 (Figure 2.3C). The results obtained in MDA-MB-231 cells were comparable to the effects of the same C-DIM compounds and PGJ2 in ER-positive breast cancer cells where proteasome-dependent degradation of cyclin D1 was also reported [456,459]. p21 and p27 proteins were only slightly induced in MDA-MB-231 cells in a concentration-independent manner and studies with p21 promoter constructs

showed that the PPARγ-active C-DIMs did not induce transactivation in cells transfected with these constructs (data not shown). The results suggest that the inhibition of G_0/G_1 to S phase progression in MDA-MB-231 cells treated with C-DIMs is primarily due to decreased cyclin D1 expression whereas other factors must be involved for inhibition of MDA-MB-453 cell proliferation.

PPARγ-active C-DIMs induce receptor-dependent caveolin 1 and receptor-independent ER stress in pancreatic, colon, bladder and ovarian cancer cells [423,455,457,458,460,468,475,482,489,511], however, neither caveolin (Figure 2.3E) or GRP78 (an ER stress marker) (Figure 2.3D) were induced in MDA-MB-231 or MDA-MB-453 cells. In contrast, the TGFβ-like peptide NAG-1 was induced by 10 μM C-DIMs in both cell lines and was accompanied by a slight induction of ATF3 (Figs. 4A and 4B) which is often coinduced with NAG-1 [428,489]. NAG-1 is induced by several proapoptotic and growth inhibitory agents including C-DIMs, troglitazone and PGJ2, however, the mechanisms of induction are variable [428,460,475,481-483,489]. Previous studies showed that activation of NAG-1 in colon cancer cells was associated with PI3K-dependent activation of early growth response-1 (Egr-1) gene which activates NAG-1 through specific proximal promoter sequences [489]. In contrast activation of NAG-1 by the phorbol ester TPA was PKC-dependent [481]. In ER-negative MDA-MB-453 cells, activation of NAG-1 by DIM-C-pPhC_6H_5 was decreased in cells cotreated with the JNK inhibitor SP600125, the MAPK and PI3K inhibitors PD98059 and LY294002 respectively (Figure 2.4C). These results correlated with DIM-C-pPhC_6H_5-dependent enhancement of all three kinase pathways in the same cell line (Figure 2.4E).

However, in MDA-MB-231 cells, only the PI3K inhibitor LY294002 blocked induction of NAG-1 by DIM-C-pPhC_6H_5 in MDA-MB-231 cells (Figure 2.4C) and only this kinase was induced by the C-DIM compound in this cell line (Figure 2.4D). Thus induction of NAG-1 by DIM-C-pPhC_6H_5 in ER-negative breast cancer cells was due to cell context-dependent activation of kinases; and current studies in this laboratory are investigating upstream targets that are activated by C-DIMs and mediate selective activation of kinase pathways in different cell lines.

Although NAG-1 is associated with apoptotic activity, we did not observe caspase-dependent PARP cleavage after treatment of MDA-MB-453 and MDA-MB-231 cells with PPARγ-active C-DIM compounds whereas the proteasome inhibitor MG132 (positive control) induced PARP cleavage (Figs. 5A and 5B). Bax/bcl-2 ratios were increased in MDA-MB-453 cells treated with 5 or 10 μM PPARγ-active C-DIMs. However, increased bax/bcl-2 ratios combined with C-DIM-dependent downregulation of the antiapoptotic protein survivin in both cell lines (Figs. 5C and 5D) was not accompanied by activation of caspase-mediated apoptosis. The failure to induce apoptosis was not unprecedented since other studies also show that ER-negative breast cancer cells are somewhat resistant to PPARγ agonist-induced apoptosis [425,454,516]. Treatment of MDA-MB-231 cells with high concentrations of troglitazone (100 μM) for 50 h induced apoptosis in only 30% of the cells [454] and CDDO did not cause caspase-dependent PARP cleavage in ER-negative MDA-MB-468 cells [459]. High concentrations of troglitazone (50 μM) were cytotoxic to ER-negative MDA-MB-435 cells and decreased survivin expression [518]; and similar results were observed for the

C-DIM compounds (Figs. 2.5C and 2.5D). Moreover, like the C-DIM compounds, troglitazone also did not induce apoptosis in MDA-MB-435 cells. However, the lack of induction of apoptosis by PPARγ-active C-DIMs did not affect their in vitro cytotoxicity or their inhibition of tumor growth in athymic nude mice bearing MDA-MB-231 cells as xenografts (Figure 2.6).

In summary, PPARγ-active C-DIM compounds induced PPARγ-dependent transactivation in MDA-MB-231 and MDA-MB-453 cells and IC50 values for growth inhibition were ≤ 5 μM in both cell lines. However, it was apparent that the effects of the PPARγ-active C-DIMs on distribution of cells in G_0/G_1 and S-phase, expression of cyclin D1 and induction of NAG-1 were dependent on cell context whereas NAG-1 was induced in both cell lines. Despite the potent antiproliferative effects of these compounds in vitro and in vivo and their downregulation of the antiapoptotic survivin protein and induction of the proapoptotic NAG-1 protein in both cell lines, we did not observe caspase-dependent PARP cleavage (Figure 2.5) or other indicators of apoptosis such as positive Annexin V staining (data not shown). In ongoing studies, we are investigating the potential role of other cell death pathways in breast cancer cells and tumors in order to delineate critical markers associated with the potent anticarcinogenic activity of C-DIMs. Results of this study are similar to those reported for TZDs and CDDO in ER-negative cancer cell lines [516,518] and similarities and differences in the effects of these compounds and C-DIMs are also being investigated.

CHAPTER III

INDUCTION OF AUTOPHAGY BY A NEW SERIES OF BREAST CANCER CHEMOTHERAPEUTIC AGENTS

3.1. Synopsis

Indole-3-carbinol is a phytochemical found in cruciferous vegetables and one of its major metabolites, 3,3'-diindolylmethane (DIM), exhibits a broad range of anticancer and antitumorigenic activities. A novel series of methylene--substituted DIMs (C-DIMs), namely 1,1-bis(3'-indolyl)-1-(p-substitutedphenyl)methanes containing t-butyl (DIM-C-pPhtBu) and phenyl (DIM-C-pPhC_6H_5) groups inhibit proliferation of invasive estrogen receptor-negative MDA-MB-231 and MDA-MB-453 human breast cancer cell lines with IC50 values between 1-5 μM. C-DIM-induced cell death could not be reversed by zVAD-fmk, a pan-caspase inhibitor in either cell line. MDA-MB-231 and MDA-MB-453 cells treated with C-DIMs did not exhibit Annexin V-PI staining patterns consistent with apoptosis, suggesting that C-DIMs did not induce Type I (apoptotic) programmed cell death. Cell necrosis, as indicated by propidium iodide (PI) staining and lactate dehydrogenase (LDH) release, was also negative after C-DIM treatment of MDA-MB-453 cells. Autophagic cell death is an important cell death pathway in breast cancer cells after treatment with several chemotherapeutic agents including tamoxifen, EB1089 and sulforaphane and therefore we investigated the possibility that C-DIMs may also induce autophagy in breast cancer cells. Treatment of MDA-MB-231 and MDA-MB-453 cells with C-DIMs resulted in an accumulation of light chain associated-protein 3 (LC3)-II compared to LC3-I protein and this is a characteristic marker of autophagy

which is also observed in cells treated with a vitamin D analog and paclitaxel analogs in breast cancer cells. Transient transfection of green fluorescent protein-LC3 also revealed that treatment with C-DIMs induced a redistribution of LC3 to autophagosomes after C-DIM treatment. In addition, the autofluorescent drug monodansylcadaverine (MDC), a specific autophagolysosome marker, accumulated in vacuoles after C-DIM treatment. Western blot analysis of lysates from cells treated with C-DIMs showed the Beclin 1/Bcl-2 protein ratio increased suggesting that C-DIM compounds may represent a new mechanism-based agent for treating drug-resistant ER-negative breast tumors through induction of autophagic cell death.

3.2. Introduction

Autophagy is an evolutionarily conserved mechanism of survival triggered by stress-associated damage or nutrient deprivation and is characterized by the formation of double-membrane bound cytoplasmic vesicles called autophagosomes [519]. Autophagosomes subsequently fuse with lysosomes to form autophagolysosomes and the vacuolar content is then degraded by lysosomal enzymes. Under nutrient-deprived conditions, autophagy is a form of cell survival and a means to generate ATP and amino acids from degraded proteins and organelles. Autophagy, however, can only be considered a short-term mechanism of survival since degradation of most organelles will eventually lead to cell death [147]. Type II autophagic cell death is a non-apoptotic form of programmed cell death and is morphologically characterized by the accumulation of autophagic vacuoles in cells lacking hallmarks of apoptosis such as nuclear condensation and cell fragmentation [161].

Both autophagy and autophagic cell death are regulated by the autophagy-related gene (Atg) family. Atg6/Beclin 1 is involved in the class III PI3K complex, which is essential for autophagosome formation and Beclin 1 overexpression-induced autophagy and inhibited tumorigenicity in breast cancer cells [165,520]. The anti-apoptotic protein Bcl-2 has an inhibitory action on Beclin 1 and the subsequent formation of autophagosomes [521]. Atg8/Light-chain associated-protein 3 (LC3) is also critical for autophagosome formation and becomes lipidated during activation of autophagy [522]. LC3 can be present as either an 18 kDa cytosolic protein, LC3-I, or cleaved to a 16 kDa LC3-II derivative that localizes to autophagosomal membranes, the latter form serving as a marker protein for autophagic vacuoles [166]. Atg5 and Atg12 are ubiquitinated by E1-like enzymes Atg7 and Atg10 and are involved in the recruitment of proteins to autophagosomal membranes [523,524]. Although the same autophagy-related genes play a role in autophagy and autophagic cell death, both Atg5 and Atg6/Beclin 1 are upregulated in autophagic cell death and remain low in autophagy [172].

Autophagic (Type II) cell death is considered a controversial death pathway. Although autophagy may be induced in cells, it may be a mechanism of promoting cell survival, not cell death. Initial evidence supporting the concept of Type II non-apoptotic cell death was illustrated when cell death could be induced in several cell lines treated with caspase inhibitors [171]. In addition, autophagic cell death is a pathophysiologically relevant process since it is associated with retinal degeneration and degeneration of neurons in certain pathologies [525,526]. Although apoptosis has long been considered the central form of cell death evaded by tumor cells, Type II cell death

may also be relevant since reduction of autophagy is observed in tumor cells and is associated with tumor promotion since Beclin 1 is a haploinsufficient tumor suppressor gene [165,527-530]. Moreover, autophagy may be causally linked to the ability of tumor cells to evade immune surveillance since autophagy has been linked to antigen processing [531].

Autophagic cell death has been reported to be induced by a number of chemotherapeutic agents in various types of cancers [532-537]. Sulforaphane is a phytochemical present in cruciferous vegetables and induced autophagic cell death in human prostate cancer cells [532]. Autophagic cell death was the predominant form of cell death induced by taxol, taxol derivatives and tamoxifen in breast cancer cells [533,534]. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 induced autophagic cell death in pancreatic cancer cells [535]. Autophagic cell death was also induced in response to radiation treatment in breast, colon and prostate cancer cell lines [536]. Temozolide and arsenic trioxide induced autophagic cell death in glioma cells [537].

Studies in this laboratory have investigated the mechanisms of cell death induced by a new series of anticancer agents that are derivatives of phytochemicals found in crucifers. Epidemiology studies have positively linked consumption of cruciferous vegetables to decreased risk of certain types of cancer [123-127]. Mechanisms of growth inhibition induced by DIM, an oligomerization product of the cruciferous phytochemical indole-3-carbinol, have shown anticancer and antitumorigenic effects in vitro and in vivo [130-135]. The mechanisms of growth inhibition induced by DIM have been well-studied and include G0/G1 cell cycle arrest, induction of ER stress, induction

of apoptosis, aryl hydrocarbon receptor (AhR)-dependent antiestrogenicity and downregulation of the androgen receptor (AR) [131,134,416,419,420,423,425,426,538]. C-substituted DIMs (C-DIMs) represent a new class of anticancer agents that activate receptor-dependent and receptor-independent pathways in a cell-context dependent manner. In colon, pancreatic, ovarian, prostate, bladder and breast cancer cells, C-DIMs activate nuclear receptors PPARγ and Nur77 [455-458,460,468,511]. In ER-negative breast cancer cells, the effect of PPARγ-active C-DIMs on the cell cycle, induction of the pro-apoptotic protein NAG-1 and activation of kinases is primarily receptor-independent. Although C-DIMs modulated Bax and Bcl-2 protein expression, PARP was not cleaved, suggesting a caspase-independent form of cell death. The mechanism of cell death induced by C-DIMs in breast cancer cells therefore required further examination.

In the present study, mechanisms of cell death induced by PPARγ-active C-DIMs in breast cancer cells were investigated. Treatment of ER-negative MDA-MB-231 and MDA-MB-453 cells with C-DIMs did not activate caspases or increase Annexin V staining indicating that apoptotic cell death was not activated. Measurement of LDH release and propidium iodide (PI) staining suggested that necrosis was not the predominant form of cell death induced in breast cancer cells treated with C-DIMs. In contrast, autophagolysosomes were positively stained with monodansylcadaverine (MDC) after treatment with C-DIMs and there was a significant increase in LC3-II/LC3-I and Beclin 1/Bcl-2 protein ratios. In addition, after treatment with C-DIMs transfected GFP-LC3 localized to autophagosomal membranes of cells. These data support

autophagic cell death as a novel mechanism induced by C-DIMs in ER-negative breast cancer cells.

3.3. Materials and Methods

3.3.1. Cells, chemicals and other materials

NADH, zVAD-fmk and propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO). Monodansylcadaverine (MDC) was purchased from Fluka (Buchs, Switzerland). The human breast cancer cell lines MDA-MB-231 and MDA-MB-453 were obtained from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231 cells were maintained in DMEM:F-12 supplemented with 0.22% sodium bicarbonate, 10% fetal bovine serum (FBS), and 2 ml/L antibiotic solution (Sigma Chemical Co., St. Louis, MO). MDA-MB-453 cells were maintained in RPMI supplemented with 0.22% sodium bicarbonate, 10% fetal bovine serum (FBS), and 2 ml/L antibiotic solution (Sigma Chemical Co., St. Louis, MO). Cells were grown in 150 cm^2 culture plates in an air/CO_2 (95:5) atmosphere at 37°C and passaged every 5 days. Beclin 1 (H-300) and Bcl-2 (N-19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The LC3 antibody was purchased from MBL International (Wobrum, MA). Horseradish peroxidase substrate for Western blot analysis was purchased from NEN Life Science Products (Boston, MA).

3.3.2. Cell proliferation assay

MDA-MB-231 and MDA-MB-453 cells were seeded at a density of 3-5 x 10^4/well in 12-well plates and media was replaced the next day with DMEM:F-12 media containing 2.5% charcoal-stripped FBS and pre-treated with 20 μM zVAD-fmk or

vehicle control for 30 minutes. Cells were then co-treated with zVAD-fmk or vehicle control and DMSO, 10 μM DIM-C-pPhCF_3, DIM-C-pPhtBu or DIM-C-pPhC_6H_5 for 48 h. Cells were then counted using a Coulter Z1 cell counter. Each experiment was completed in triplicate and results are expressed as means ± SE for each determination.

3.3.3. Annexin V staining

MDA-MB-231 and MDA-MB-453 cells were seeded at a density of 2.5-5 x 10^5/well in 6-well plates and media was replaced the next day with DMEM:F-12 media containing 2.5% charcoal-stripped FBS and DMSO, 10 μM DIM-C-pPhCF_3, DIM-C-pPhtBu, DIM-C-pPhC_6H_5 for 48 h. Cells were also treated for 24 h with 10 μM MG132. Cells were then harvested according to the Annexin-V-FITC protocol provided by BD Biosciences. Briefly, floating cells were transferred to an Eppendorf tube and centrifuged for 2 minutes at 3000 g. The adherent cells were trypsinized and transferred to the same Eppendorf which was spun and the supernatant was removed. Cells were resuspended in 85 μL of 1X Annexin V binding buffer and 10 μL of PI solution (5 ug/mL). Five μL of Annexin V-FITC conjugate was added to each sample and then incubated in the dark for 15 minutes before analysis by flow cytometry. Cells early in apoptosis positively stained for Annexin V whereas cells in the later stages of apoptosis stained positively for Annexin V and propidium iodide due to late apoptotic cells having leaky plasma membranes.

3.3.4. PI and Hoechst staining

Vybrant Apoptosis Assay Kit #7 was used from Molecular Probes (Eugene, OR) according to the manufacturer's directions. Briefly, monolayers of cells were cultured

for 48 h in 2-well Coverglass Chamber slides. Slides were washed with culture medium without serum or phenol red, and labeled with Hoechst 33343 and propidium iodide at a final concentration of 5 μg/mL and 1.0 μg/mL, respectively. The slides were incubated for 30 min on ice and visualized using a BioRad Radiance 2000 Multiphoton microscope. At least 3 areas per well were analyzed. Two wells were analyzed per treatment and per time point.

3.3.5. LDH assay

MDA-MB-231 and MDA-MB-453 cells were treated for 48 h with DMSO or C-DIMs as indicated. An extra 3 wells were treated for 1 h or until all cells had lysed with 0.3% Triton X in PBS. For each sample, 200 μL of 1.22 mM pyruvate in 50 mM phosphate buffer and 4 μL of 12.4 mg/mL NADH dissolved in 50 mM phosphate buffer were each added to a 96-well plate. 20 μL of supernatant from treated cells was then added and the plate was incubated at 37 degrees for 30 min. The LDH concentration was measured at 390 nm and the treated groups were compared to Triton X, which was considered 100 % LDH release.

3.3.6. MDC staining

Monolayers of cells were cultured for 48 h in 2-well Coverglass Chamber slides and treated as indicated. Slides were washed with culture medium without serum or phenol red. Cytoplasmic vacuoles were stained with MDC according to the method described elsewhere [539]. Briefly, cells were exposed to 50 μM of MDC for 10 minutes at 37 degrees and visualized using a BioRad Radiance 2000 Multiphoton

microscope. At least 3 areas per well were analyzed. Two wells were analyzed per treatment and per time point.

3.3.7. Western blots

MDA-MB-231 and MDA-MB-453 cells were seeded in DMEM:F-12 media containing 2.5% charcoal-stripped FBS for 24 h and then treated with either the vehicle (DMSO) or the indicated compounds. In experiments where indicated, cells were pre-treated for 30 min with 10μM of the proteasome inhibitor MG132. Whole cell lysates were obtained using high salt buffer [50 mM HEPES, 500 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol and 1% Triton X-100 pH 7.5 and 5 μL/mL of Protease Inhibitor Cocktail]. Protein samples were incubated at 100^0C for 2 minutes, separated on 10-15% SDS-PAGE at 120 V for 3-4 h in 1 X running buffer [25 mM Tris-base, 192 mM glycine, and 0.1% SDS (pH 8.3)] and transferred to a polyvinylidene difluoride (PVDF) membrane at 0.9 A for 90 minutes at 4^OC in 1 X transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 0.025% SDS). The PVDF membrane was blocked in 5% TBST-Blotto [10 mM Tris-HCl, 150 mM NaCl (pH 8.0), and 0.025% Triton X-100 and 5% non-fat dry milk] with gentle shaking for 30 min and incubated in fresh 5% TBST-Blotto with 1:200-1:1000 primary antibody overnight at 4^0C with gentle shaking. After washing with TBST for 10 min, the PVDF membrane was incubated with secondary antibody (1:5000) in 5%TBST-Blotto for 2 h. The membrane was washed with TBST for 10 min and incubated with 10 mL of chemiluminescence substrate for 1.0 min and exposed to Kodak X-OMAT AR autoradiography film. Band intensities were evaluated

by scanning laser densitometry (Sharp Electronic Corporation, Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corporation, Billerica, MA).

3.3.8. GFP-LC3 localization

Monolayers of cells were cultured for 48 h in 2-well Coverglass Chamber slides and treated as indicated. The GFP-LC3 plasmid was kindly provided by Dr. Tamotsu Yoshimori (Osaka University, Osaka, Japan). MDA-MB-231 cells were transfected with 500-600 ng/well of GFP-LC3 plasmid using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. MDA-MB-453 cells were transfected with 600 ng/well of GFP-LC3 plasmid using GeneJuice transfection reagent (EMD Biosciences, Madison, WI) according to the manufacturer's protocol. Cells with GFP-LC3 expression were counterstained with Hoechst DNA dye from Molecular Probes (Eugene, OR). Slides were examined by fluorescence microscopy using a Zeiss Stallion Dual Detector Imaging System. A 3 dimensional image of cells was captured in z Z-stack. Optical slices of cells were imaged containing GFP fluorescence, the Hoechst-stained DNA and DIC image. The entire z-stack was compiled and subjected to deconvolution. Cells were examined in more than five fields per slide on multiple slides. Data represents the average of all the fields.

3.3.9. Statistical analysis

Statistical differences between different groups were determined by ANOVA and Fisher's test for significance using SuperAnova software. The data are presented as mean ± standard error for at least 3 separate determinations for each treatment.

3.4. Results

PPARγ-active C-DIMs activate caspase-dependent apoptosis in colon cancer cells [468], however, previous studies reported that C-DIMs did not induce caspase-dependent PARP cleavage in ER-negative MDA-MB-231 and MDA-MB-453 cells. To verify that the cell death pathway induced by C-DIMs was independent of caspase activation, MDA-MB-231 and MDA-MB-453 cells were co-treated with a pan-caspase inhibitor, zVAD-fmk. There was no statistically significant reversal of C-DIM-induced cell death after with treatment with zVAD-fmk (Figure 3.1), suggesting that C-DIM compounds did not induce caspase-dependent cell death in ER-negative breast cancer cells.

MDA-MB-231 and MDA-MB-453 cells were treated with C-DIMs for 48 h, co-stained with Annexin V-FITC conjugate which is a marker of apoptosis and propidium iodide (PI) and analyzed by flow cytometry (Figure 3.2). PI is a DNA dye that is impermeable to cells with intact membranes and is a marker of late apoptotic or necrotic cells that have lost membrane integrity. Early apoptotic cells stain positive for Annexin V whereas cells in the later stages of apoptosis stain positively for both Annexin V and PI. The majority of cells treated with solvent control DMSO clustered in the quadrant negative for both Annexin V and PI. After treatment with the apoptosis-inducing

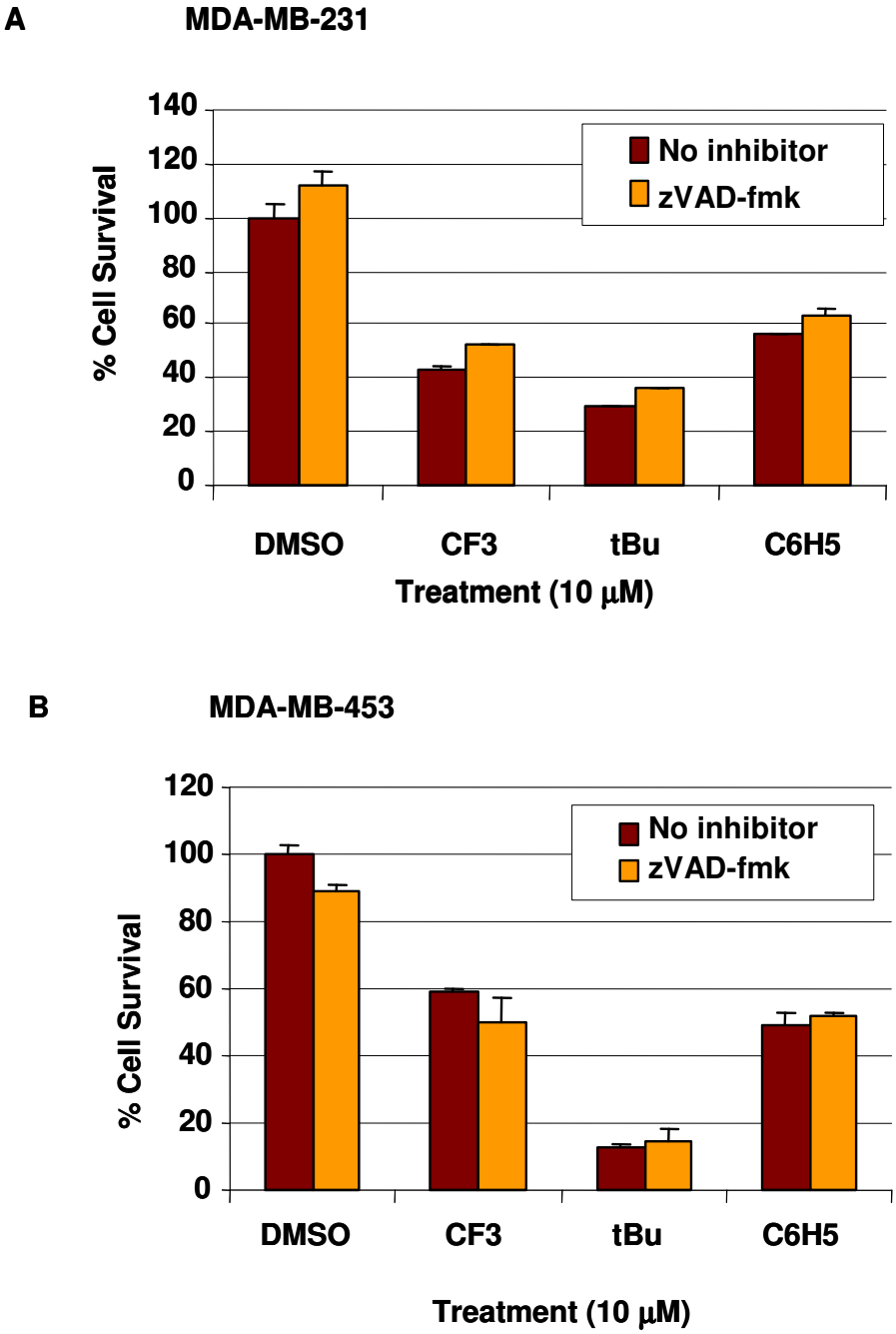


Figure 3.1: Influence of pan-caspase inhibitor zVAD-fmk on growth inhibition induced by C-DIMs. MDA-MB-231 [A] and MDA-MB-453 [B] cells were treated for 48 h with 10 µM DIM-C-pPhCF$_3$, DIM-C-pPhtBu or DIM-C-pPhC$_6$H$_5$ and with or without 20 µM zVAD-fmk. Cells were counted as described in Materials & Methods. Results of all proliferation studies are presented as means ± SE for at least three separate determinations for each treatment group. Significantly ($p<0.05$) decreased growth (compared to DMSO) in cells treated with C-DIMs alone is indicated (*).

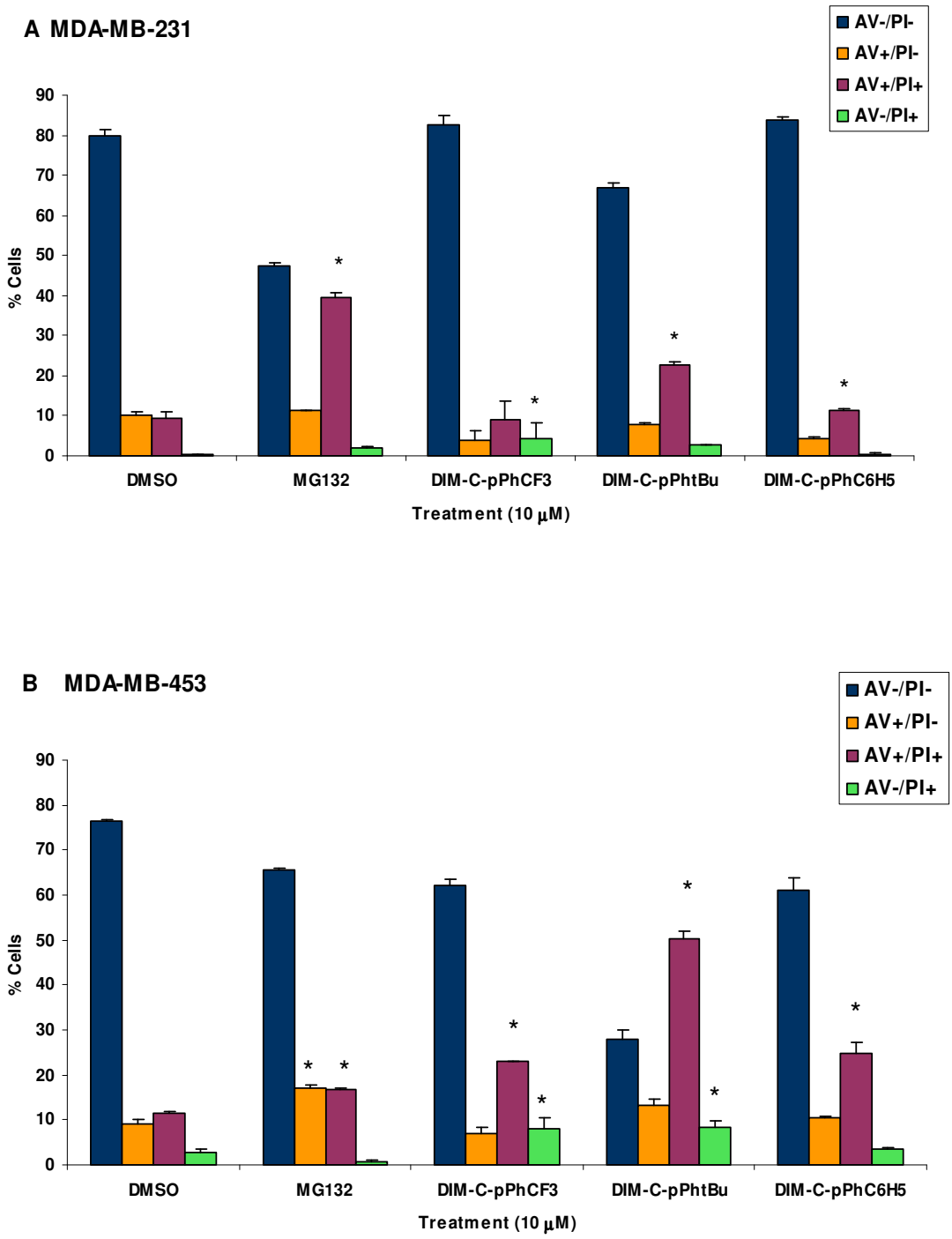


Figure 3.2: FACS analysis of cells co-stained with Annexin V and PI. MDA-MB-231 [A] and MDA-MB-453 [B] cells were treated for 48 h or 24 h with 10 µM MG132. Floating and adherent cells were incubated with Annexin V and PI and analyzed by flow cytometry as described in Materials & Methods. Results of all proliferation studies are presented as means ± SE for at least three separate determinations for each treatment group. Significance ($p<0.05$) compared to DMSO is indicated (*).

compound MG132, there was a slight increase in MDA-MB-231 cells staining positively for Annexin V alone and a statistically significant increase in cells staining positive for Annexin V and PI, suggesting that cells treated with MG132 were in the late stages of apoptosis. In contrast, after treatment of MDA-MB-231 with C-DIMs, there was a decrease in the number of cells staining positively for Annexin V alone and an increase in cells staining positive for Annexin V and PI. There was also an increase in both MDA-MB-231 and MDA-MB-453 cells staining for PI alone after treatment with DIM-C-pPhCF3. There was a statistically significant increase in MDA-MB-453 cells staining positively for Annexin V alone or both Annexin V and PI, signifying that these cells were exhibiting the prototypical characteristics of early and late stages of apoptosis. In contrast, MDA-MB-453 cells treated with C-DIMs exhibited an increase in cells staining for Annexin V and PI (Figure 3.2B). The failure of C-DIMs to induce Annexin V alone indicates that the ER-negative breast cancer cells do not undergo the early stages of apoptosis. The increased number of cells staining positive for both Annexin V and PI are indicative of C-DIM-induced loss of cell membrane integrity but do not allow us to identify which specific cell death pathway is activated.

Only a few cells were stained with PI after treatment of MDA-MB-231 cells with C-DIMs and no PI staining was observed in MDA-MB-453 cells treated with C-DIMs (Figures 3.3A and 3.3B). These results contrast with previous results in MDA-MB-231 cells treated with 5,5'-dibromoDIM where positive PI staining was observed [425]. LDH release from cells was significantly elevated after treatment with the lytic agent, Triton X in both cell lines however, among the three PPARγ-active C-DIMs only

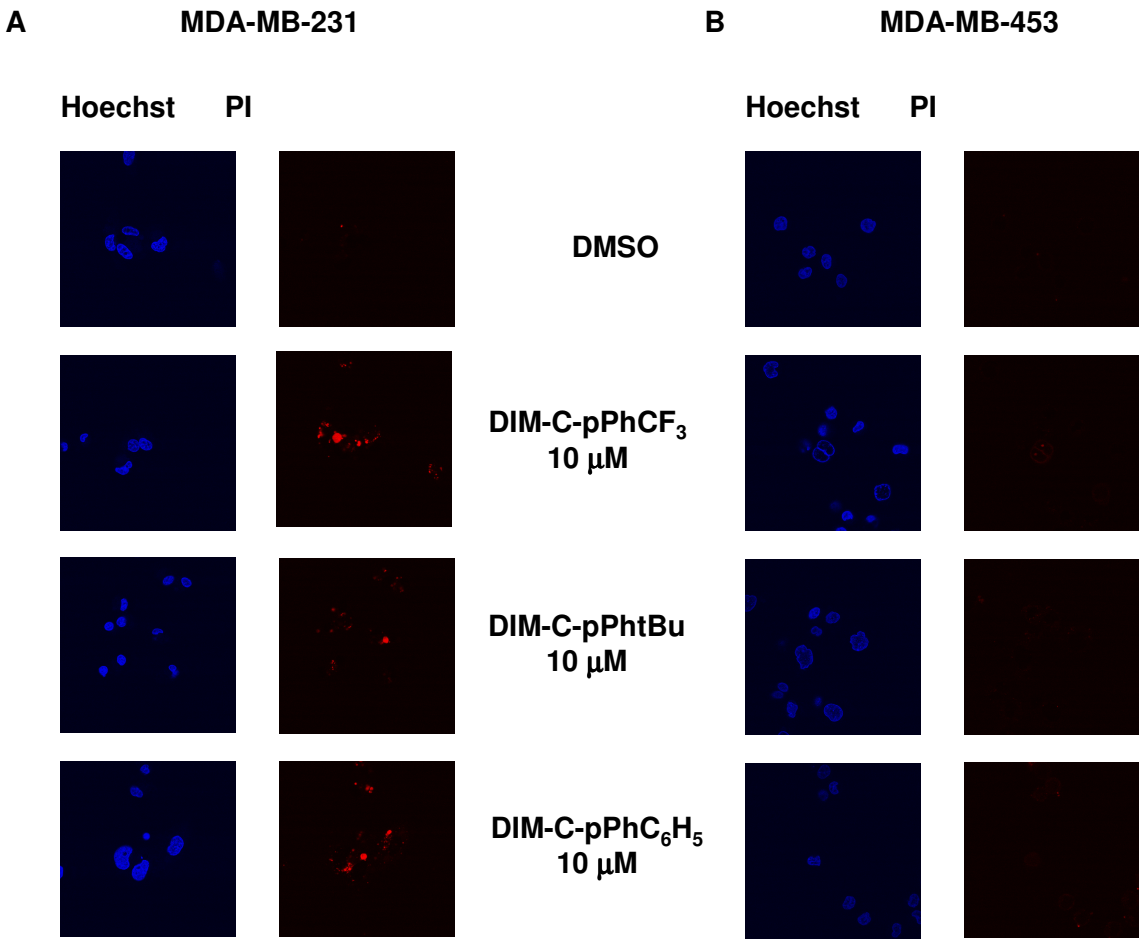


Figure 3.3: Necrosis detection by PI staining and LDH release. MDA-MB-231 [A] and MCF-7 [B] cells were treated for 24 h with DMSO or 10 μM DIM-C-pPhCF$_3$, DIM-C-pPhtBu or DIM-C-pPhC$_6$H$_5$. Two fluorescent DNA dyes Hoechst and propidium iodide, were loaded into cells and incubated on ice for 30 min and visualized using confocal microscopy as described in the Materials and Methods. The same microscopic field of MDA-MB-231 [A] or MDA-MB-453 [B] cells stained with Hoechst and propidium iodide are shown and are representative of other cell areas with the same treatment. At least 3 areas were scanned for each well and two wells were analyzed per treatment and per time point. LDH release after treatment with C-DIMs in MDA-MB-231 [C] and MDA-MB-453 cells [D]. Cells were treated for 48 h or 1 h with 0.1% Triton X and the supernatants were analyzed for LDH as described in the Materials and Methods. Triton X served as a positive for 100% cell lysis and LDH release. Results are presented as the means ± SE for at least three separate determinations for each treatment group. Statistical significance of treatments compared to DMSO ($p<0.05$) are represented by an asterisk.

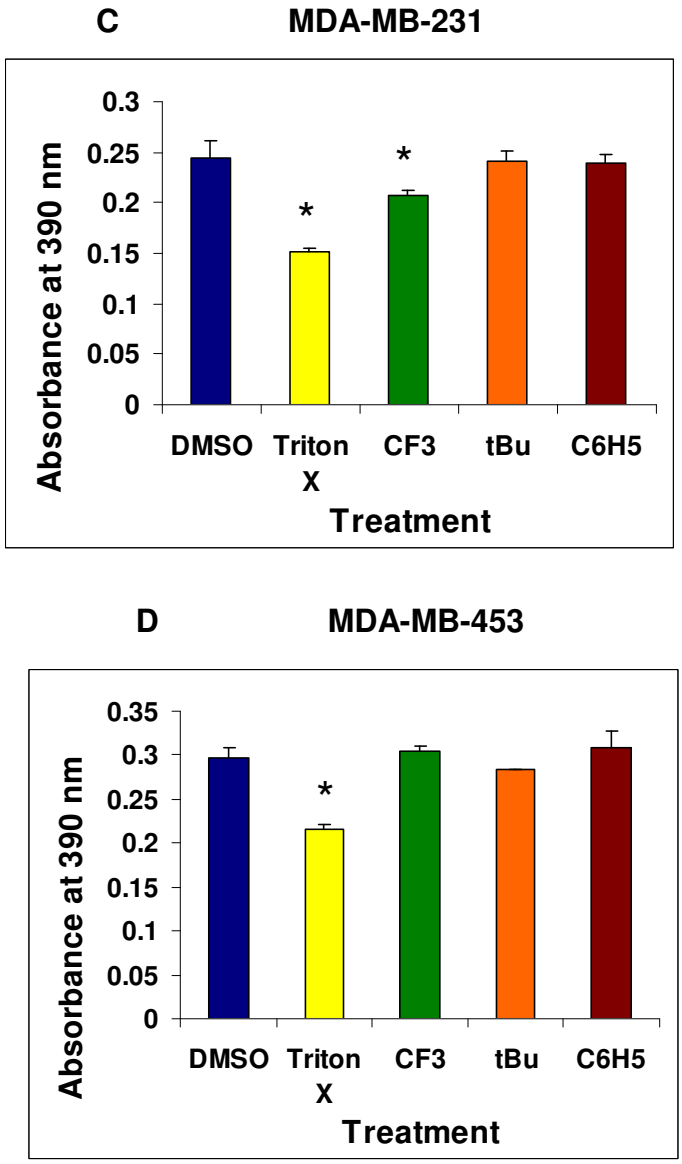


Figure 3.3 (Continued)

DIM-C-pPhCF$_3$ significantly increased levels of LDH release in MDA-MB-453 cells and the C-DIMs did not induce LDH release in MDA-MB-231 cells. Thus only DIM-C-pPhCF$_3$ significantlly induced necrosis in one of the two cell lines suggesting that necrosis was not the predominant death pathway induced by PPARγ-active C-DIMs in ER-negative breast cancer cells.

Several drugs that are cytotoxic to cancer cell lines induce autophagic cell death [532-537,540-544] and therefore the effects of C-DIMs on MDC localization, a positive marker for autophagolysosomes, was investigated. The C-DIMs clearly induce MDC in vacuoles in MDA-MB-231 cells whereas in cells treated with DMSO a smaller number of vacuoles was observed indicating low basal levels of autophagy in these cells (Figure 3.4A). Thus, treatment with C-DIMs increased the number and/or size of vacuoles in this cell line. MDC was also localized in vacuoles in MDA-MB-453 cells treated with DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$ (Figure 3.4B) and after treatment with DIM-C-pPhtBu, large MDC-stained vacuoles were observed in both cell lines. These results suggest that C-DIMs induced autophagy in MDA-MB-231 and MDA-MB-453 cells.

LC3 is critical for autophagosome formation and after induction of autophagy LC3-I is lipidated to LC3-II which localizes to autophagosomal membranes and the ratio of LC3-II to LC3-I protein expression is an indicator of autophagosome formation [166]. After treatment of MDA-MB-231 cells with C-DIMs the cleaved form of LC3 was increased and this was particularly evident in MDA-MB-231 cells treated with DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$ (Figure 3.5A). In MDA-MB-453 cells, the cleaved LC3-II protein accumulated only after treatment with DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$

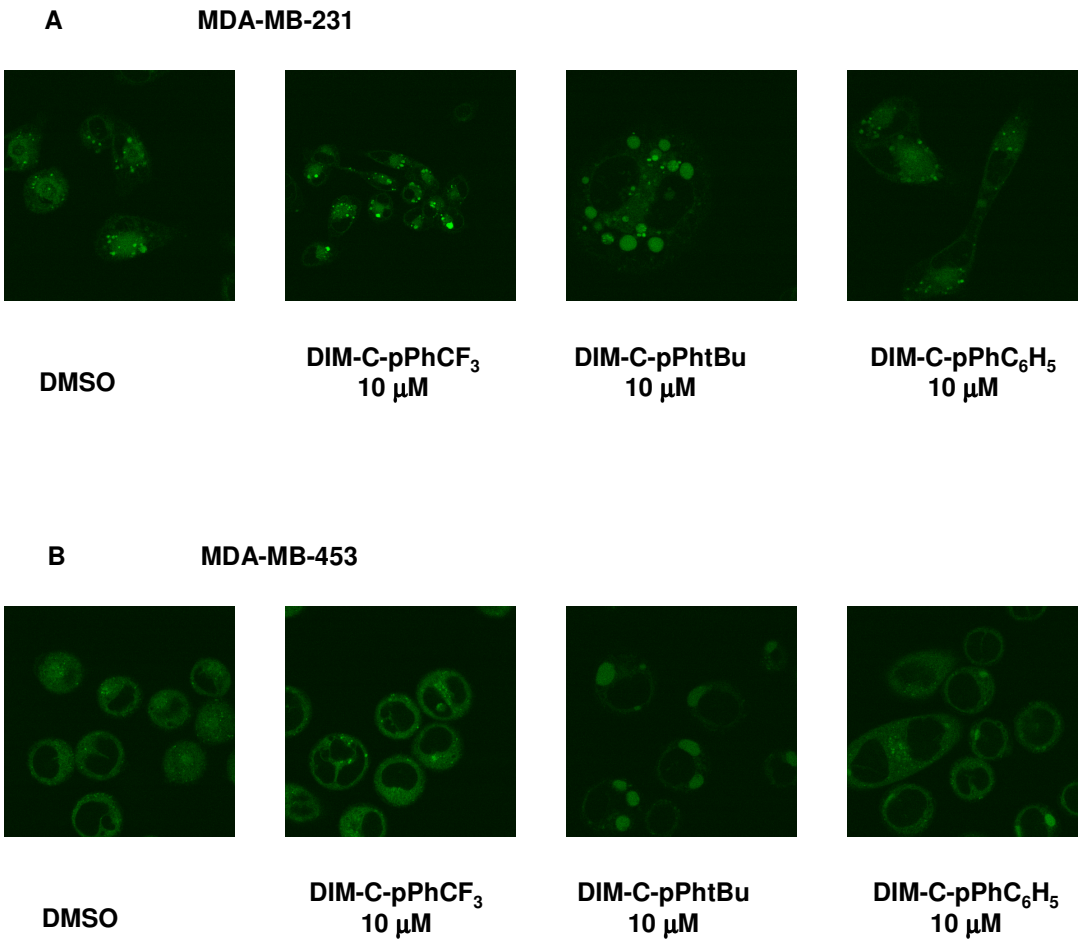


Figure 3.4: Detection of lysosomal activity with monodansylcadaverine (MDC) staining. MDA-MB-231 [A] and MDA-MB-453 cells [B] were treated with DMSO or 10 μM DIM-C-pPhCF$_3$, DIM-C-pPhtBu or DIM-C-pPhC$_6$H$_5$ for 24 h. MDC was loaded into cells and incubated at 37 degrees for 10 minutes and visualized using confocal microscopy as described in the Materials and Methods. At least 5 areas were scanned for each well and images represented are characteristic of treatment replicates.

(Figure 3.5C). Densitometric analyses of LC3-II to LC3-I protein expression in MDA-MB-231 (Figure 3.5A) and MDA-MB-453 (Figure 3.5C) cells revealed a statistically significant increase in the LC3-II/LC3-I ratio and this is consistent with increased autophagosome formation. Confocal microscopy images of a GFP-LC3 construct transiently transfected into the two cell lines showed that in untreated or DMSO treated cells there was a diffuse GFP staining pattern whereas after treatment with C-DIMs, GFP-LC3 was primarily localized in vacuoles (Figures 3.5B and 3.5D). In MDA-MB-231 cells there was an increase in the number of vacuoles positively stained for GFP-LC3 after treatment with DIM-C-pPhtBu and DIM-C-pPhC_6H_5 compared to DMSO (Figure 3.5B). The effect of DIM-C-pPhCF_3 was less pronounced than with the other two compounds in MDA-MB-231 cells and this was consistent with the higher necrotic activity of this compound (Figure 3.2). In MDA-MB-453 cells, the size of the vacuoles was substantially increased after treatment with DIM-C-pPhtBu whereas only the number of vacuoles increased in cells after treatment with DIM-C-pPhC_6H_5 (Figure 3.5D). These data suggest that C-DIM compounds activated the autophagic cell death pathways in MDA-MB-231 and MDA-MB-453 cells.

Although the same autophagy-related genes (Atg5, Atg6, Atg7) play a role in autophagy and autophagic cell death, both Atg5 and Atg6/Beclin 1 are upregulated in autophagic cell death and remain low in autophagy [172]. The anti-apoptotic protein Bcl-2 has an inhibitory action on Beclin 1 and the subsequent induction of autophagy [521] and therefore the effects of C-DIMs on expression of these proteins were further investigated. Treatment of MDA-MB-231 cells with C-DIMs increased Beclin 1 and

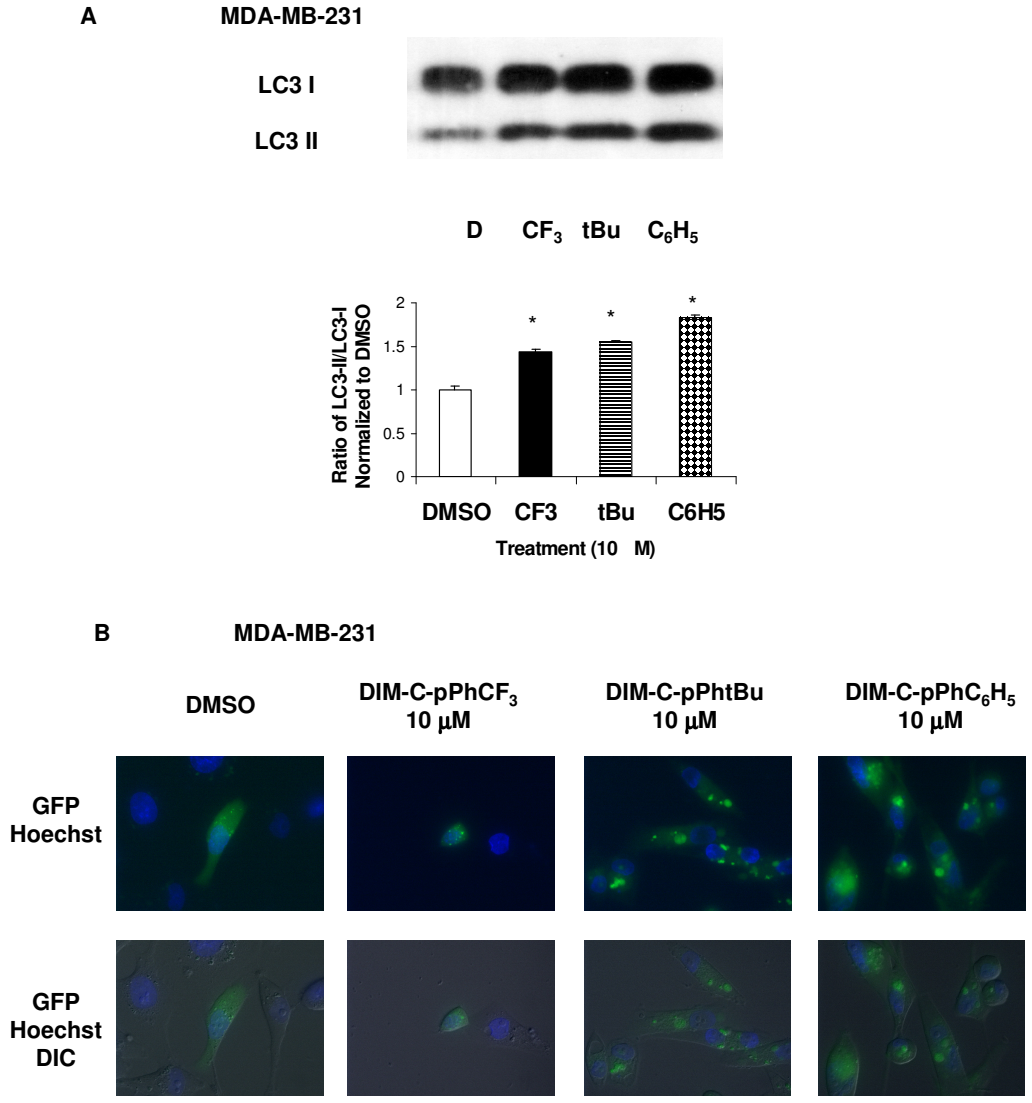
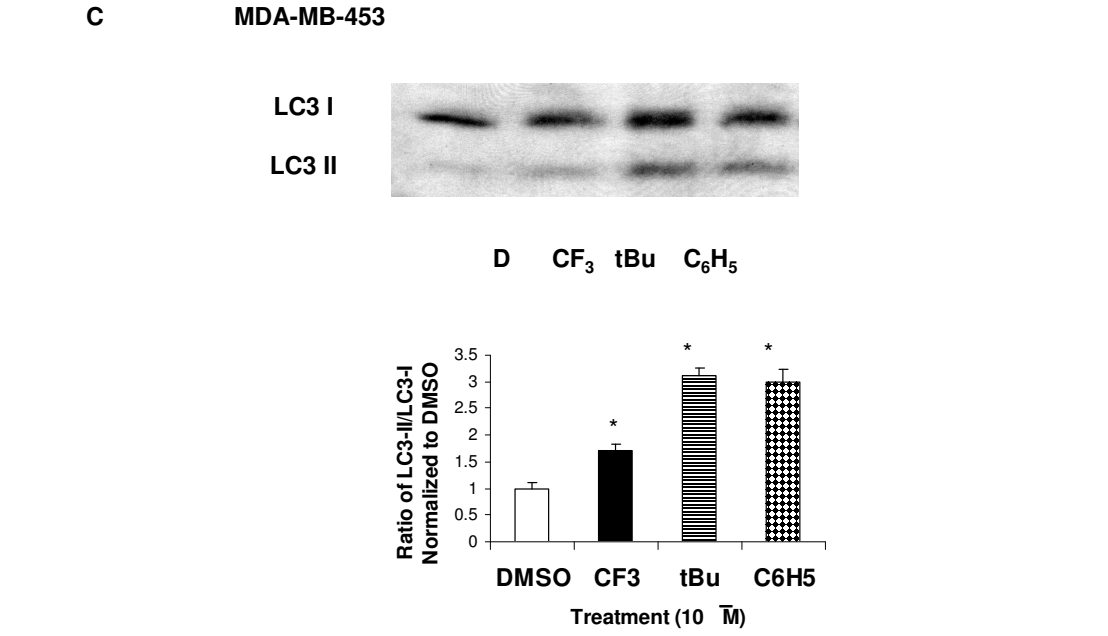


Figure 3.5: LC3 cleavage and translocation after treatment with C-DIMs. MDA-MB-231 [A] and MDA-MB-453 cells [B] were treated for 24 h with DMSO or 10 DIM-C-pPhCF$_3$, DIM-C-pPhtBu or DIM-C-pPhC$_6$H$_5$ and whole cell lysates were analyzed as described in Materials & Methods. Experiments were carried out in triplicate and results shown are typical of LC3 protein levels for treatment replicates. Densitometric analyses of LC3-II (cleaved form) and LC3-I protein expression in MDA-MB-231 [A] and MDA-MB-453 [B] cells was carried as described in the Materials and Methods and results are expressed as means ± SE for 3 replicate determinations for each treatment group. *Significantly different from DMSO ($p<0.05$). Translocation of transiently transfected GFP-LC3 plasmid. GFP-LC3 was transiently transfected into MDA-MB-231 [C] and MDA-MB-453 [D] and cells were treated with DMSO or 10 µM C-DIMs as outlined in the Materials and Methods. Confocal microscopy images of live cells were captured after treatment with C-DIMs for 24 h and differential interference contrast (DIC) and DIC/GFP overlay images of the same microscopic field are illustrated. At least 3 areas were scanned per well and the images depicted are representative of treatment replicates.



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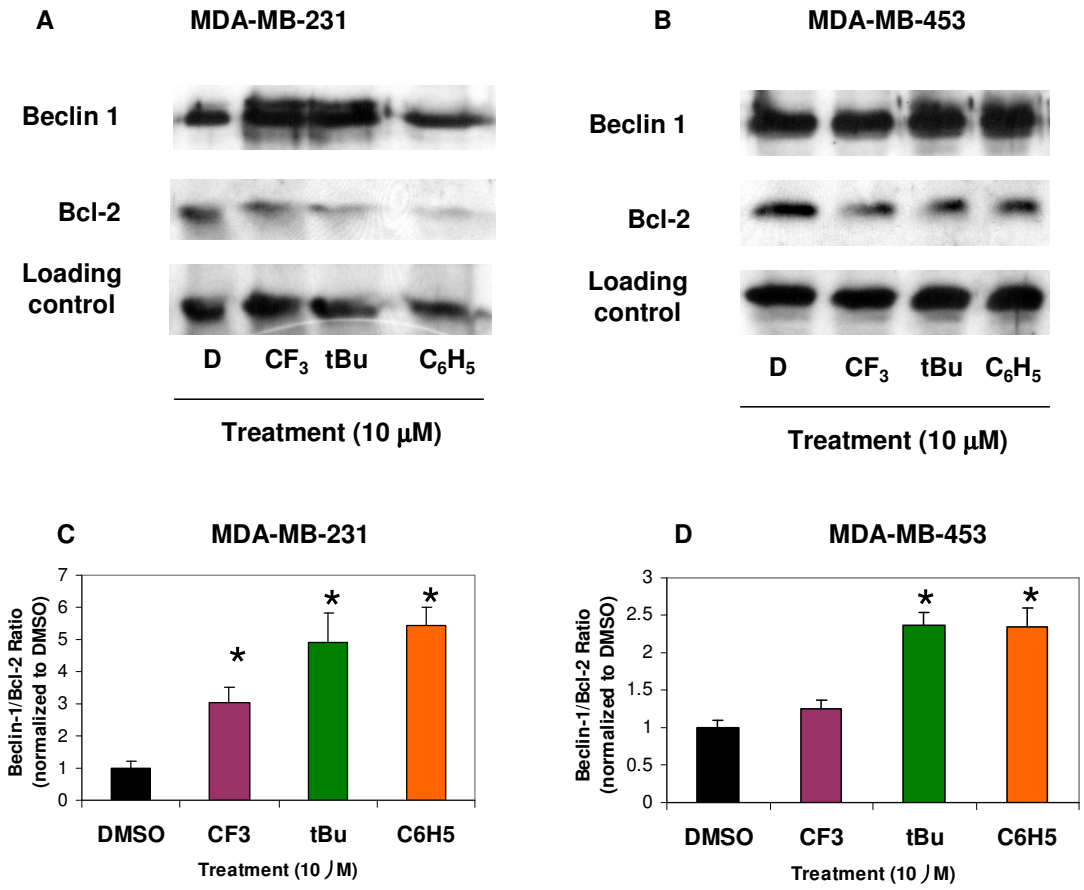

Figure 3.6: Modulation of autophagy-related gene Beclin 1 and Bcl-2 protein expression. MDA-MB-231 [A] and MDA-MB-453 [B] cells were treated for 24 h with DMSO or C-DIMs and whole cell lysates were isolated and analyzed by Western blot analysis according to procedures outlined in the Materials and Methods. Densitometric analyses of Beclin 1 and Bcl-2 protein expression in MDA-MB-231 [C] and MDA-MB-453 [D] cells was carried as described in the Materials and Methods and results are expressed as means ± SE for 3 replicate determinations for each treatment group. *Significantly different from DMSO ($p<0.05$).

decreased Bcl-2 protein levels (Figure 3.6A) and densitometric analyses of these proteins showed that C-DIMs increased Beclin 1/Bcl-2 ratios compared to that observed for DMSO (Figure 3.6C). Beclin 1 protein levels increased only after treatment with DIM-C-pPhtBu and DIM-C-pPhC_6H_5 and Bcl-2 protein was decreased in MDA-MB-453 cells treated with all 3 C-DIM compounds (Figure 3.6B). However, a statistically significant increase in Beclin 1/Bcl-2 protein ratios was only observed in cells treated with DIM-C-pPhtBu and DIM-C-pPhC_6H_5 (Figure 3.6D). These results suggest that PPARγ-active C-DIMs induce cell death (Figure 3.1) in ER-negative breast cancer cells through activation of the autophagic death pathway and this demonstrates the versatility of this novel class of anticancer drugs which activate receptor-independent apoptotic and autophagic cell death in different cancer cell lines.

3.5. Discussion

C-DIMs inhibit ER-negative MDA-MB-231 and MDA-MB-453 breast cancer cell growth and tumor growth in athymic nude mice bearing MDA-MB-231 cells as xenografts. C-DIMs represent a novel series of compounds that exert their growth inhibitory and antitumorigenic effects in a cell context-dependent manner. C-DIMs transactivate PPARγ in a number of cancer cell types including ovarian, bladder, colon, pancreatic and ER-negative and ER-positive breast cancer cells [455-458,460,511]. PPARγ agonists inhibit cancer growth by molecular mechanisms that include G_1 cell cycle arrest, induction of apoptosis and terminal differentiation [447,448,453,454]. PPARγ-active C-DIMs and other PPARγ agonists also activate PPARγ-independent growth inhibitory pathways including induction of NAG-1, ER stress and apoptosis

[458,489,503,545-548]. C-DIMs induce NAG-1 which is proapoptotic in some cancer cell lines, however, in MDA-MB-231 and MDA-MB-453 cells these compounds induced NAG-1 but not apoptosis. In addition, induction of other receptor-dependent and independent proapoptotic and differentiation pathways such as ER stress and caveolin 1 was not observed in ER-negative breast cancer cells. The precise mechanism of cell death induced by C-DIMs in ER-negative breast cancer cells treated with C-DIMs still remains elusive.

EB1089 is a vitamin D analog that induced caspase-independent cell death in ER-positive MCF-7 and ER-negative MDA-MB-231 and MDA-MB-453 breast cancer cells and similar results were observed for C-DIMs which also induced caspase-independent cell death since growth inhibition could not be reversed after co-treatment with the pan-caspase inhibitor zVAD-fmk (Figure 3.1). We also confirmed that C-DIMs did not induce Annexin V staining indicative of early apoptotic cells in ER-negative breast cancer cells, whereas the proapoptotic agent MG132 did induce cells staining with Annexin V alone (Figure 3.2). These results coupled with previous studies showing that C-DIMs also did not induce caspase-dependent PARP cleavage in these cell lines confirm that these compounds do not induce cell death through activation of apoptotic pathways. Other chemotherapeutic agents also induce non-apoptotic cell death in breast cancer cells. For example, the cytotoxic drug paclitaxel and related analogs induced cell death in breast cancer cells, however, only a small percentage of cells were undergoing apoptosis [534]. Thus, the current studies suggest that an alternative form of cell death is induced by C-DIMs in ER-negative breast cancer cells and this contrasts with the

induction of caspase-dependent apoptosis in ER-positive MCF-7 cells treated with C-DIMs [456].

Necrosis is an alternative form of cell death and although initially considered an uncontrolled form of cell demise, there is increasing evidence supporting the concept of programmed necrosis [549]. For instance, DNA damaging agents induced programmed cell death in $Bax^{-/-}Bak^{-/-}$ cells but only in those cells actively proliferating and these observations suggested an intrinsic cellular control point that decides cellular fate [550]. This form of cell death is particularly appealing for chemotherapeutic agents since many tumor cells have dysfunctional apoptotic pathways and therefore apoptosis-inducing agents are not always effective for treating cancer [549]. In MDA-MB-231 cells, minimal staining of PI and a statistically significant release of LDH was observed only after treatment with DIM-C-pPhCF_3; however, no signs of necrosis were seen after treatment of these cells with DIM-C-pPhtBu or DIM-C-pPhC_6H_5 (Figures 3.3A and 3C). The lack of LDH release and PI staining in MDA-MB-453 cells treated with C-DIMs (Figures 3.3B and 3.3D) suggested that necrosis was not induced by all 3 compounds. These results also indicate significant differences between C-DIMs and the structurally-related ring-substituted DIMs where 5,5'-dibromoDIM induced necrotic cell death in ER-negative breast cancer cells whereas DIM-C-pPhtBu and DIM-C-pPhC_6H_5 did not activate this cell death pathway in the same cell line [425].

Autophagic cell death is an alternative form of programmed cell death where cells lack the hallmarks of apoptosis but there is an accumulation of autophagic vacuoles in the cytoplasm [161]. Structurally diverse chemotherapeutic agents have been reported

to induce autophagic cell death in various cancer cell lines [532-537]. For example, the PPARγ agonist PGJ2 induced autophagic cell death in prostate cancer cells [535] and the cruciferous phytochemical sulforaphane induced autophagic cell death in prostate cancer cells [532]. Therefore we also examined the effects of C-DIMs on activation of autophagy in MDA-MB-231 and MDA-MB-453 breast cancer cells. There are several biochemical methods for detecting autophagic activity including acidic dyes that label vacuoles which exhibit lysosomal activity [539]. Autophagic cell death in prostate cancer cells treated with sulforaphane exhibited lysosomotropic staining of cytoplasmic vacuoles with acridine orange [532]. Other chemotherapeutic candidate drugs induce signs of autophagy in breast cancer cells [533,534,543,551]. Prenylated flavones inhibited cell growth and induced autophagy in both ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells and this response was typified by the formation of cytoplasmic vacuoles that stained with the autophagic marker MDC [551]. The vitamin D analog EB1089 also inhibited cell growth in MCF-7 breast cancer cells and increased uptake of MDC into cytoplasmic vacuoles [543]. These observations were similar to effects of C-DIMs on ER-negative breast cancer cells which also exhibited increased uptake of MDC into vacuoles (Figure 3.4) suggesting that these compounds induce autophagy. Therapeutic agents such as EB1089 and prenylated flavones typically induce punctate lysosomotropic staining whereas treatment with DIM-C-pPhtBu in particular induced formation of very large MDC-stained vacuoles (Figure 3.4). These differences in staining may be due in part to differences in the size of autophagosomes in the size of autophagosomes in various cancer cell lines [552].

LC3, a critical protein involved in the early stage of autophagosome formation, becomes lipidated upon induction of autophagy [522]. Induction of LC3-II protein expression, an increase in the ratio of LC3-II/LC3-I expression and translocation of LC3 to autophagosomal membranes are diagnostic molecular markers indicative of autophagy [166]. For example, breast cancer cells treated with camptothecin and paclitaxel analogs both increased the ratio of LC3-II/LC3-I protein expression [534,553]. Treatment of MDA-MB-231 and MDA-MB-453 cells with C-DIMs also induced a statistically significant increase in the LC3-II/LC3-I protein expression ratio (Figures 3.5A and 3.5B), which is consistent with the induction of autophagy by these compounds. With the exception of DIM-C-p-PhCF_3 in MDA-MB-453 cells, treatment with C-DIMs also induced translocation of GFP-LC3 from the cytoplasm to autophagosomal membranes (Figures 3.5C and 3.5D). Chemotherapeutic agents EB1089 or radiation that induce autophagic cell death typically induce GFP-LC3 staining patterns in breast cancer cells similar to those observed in this study with C-DIMs [536,543].

An important difference between autophagy as a survival mechanism versus autophagic cell death involves the Atg5 and Atg6/Beclin 1 genes, which are upregulated in autophagic cell death and remain low in autophagy [172]. The anti-apoptotic protein Bcl-2 is also critically involved as a negative regulator of the induction of autophagy by Beclin 1 [521]. Ceramide [554], camptothecin [555] and EB1089 [543] induce autophagic cell death in breast cancer cells and all of these agents increase Beclin 1 protein expression. These observations are similar to the significant increase of Beclin 1 protein levels and Beclin 1/Bcl-2 ratios in MDA-MB-231 and MDA-MB-453 cells after

treatment with C-DIMs (Figure 3.6) and suggest that these ER-negative breast cancer cells are undergoing autophagic cell death in response to C-DIMs.

In summary, results of this study demonstrate the C-DIM compounds clearly decrease ER-negative breast cancer cell survival and like some other cancer chemotherapeutic drugs induce autophagic cell death. These results are in contrast to the mechanisms of action of C-DIMs in colon, pancreatic, ovarian, prostate and bladder cancer cell lines where these same compounds induce both PPARγ-dependent and independent growth inhibitory and proapoptotic responses [456-458,460,468,489,511]. The mechanisms associated with the cell context-dependent differences in the anticancer activities of C-DIMs and their mechanisms of induction of autophagic cell death in ER-negative breast cancer cells are currently being further investigated. The unusual activity of C-DIMs is also being exploited for the clinical treatment of ER-negative/highly invasive breast tumors that normally only respond to highly cytotoxic drugs which induce serious toxic side effects.

CHAPTER IV

INHIBITION OF BREAST CANCER CELL GROWTH AND INDUCTION OF CELL DEATH BY 1,1-BIS(3'-INDOLYL)METHANE (DIM) AND 5,5'-DIBROMODIM*

4.1. Synopsis

1,1-Bis(3'-indolyl)methane (DIM) and the 5,5'-dibromo ring substituted DIM (5,5'-diBrDIM) inhibited growth of MCF-7 and MDA-MB-231 breast cancer cells, and IC_{50} values were 10 - 20 and 1 - 5 μM, respectively, in both cell lines. DIM and 5,5'-diBrDIM did not induce p21 or p27 protein levels or alter expression of Sp1 or Sp3 proteins in either cell line. In contrast, 10 μM 5,5'-diBrDIM downregulated cyclin D1 protein in MCF-7 and MDA-MB-231 cells 12 and 24 hr after treatment. DIM (20 μM) also decreased cyclin D1 in MCF-7 (24 hr) and MDA-MB-231 (12 hr), and the DIM/5,5'-diBrDIM-induced degradation of cyclin D1 was blocked by the proteasome inhibitor MG132. Both DIM and 5,5'-diBrDIM induced apoptosis in MCF-7 cells and this was accompanied by decreased Bcl-2, release of mitochondrial cytochrome c, and decreased mitochondrial membrane potential as determined by the red/green fluorescence of JC-1. DIM and 5,5'-diBrDIM induced extensive necrosis in MDA-MB-231 cells; however, this was accompanied by decreased mitochondrial membrane potential primarily in cells treated with 5,5'-diBrDIM but not DIM. Thus, DIM and 5,5'-

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diBrDIM induce cell death in MCF-7 and MDA-MB-231 cells by overlapping and different pathways, and the ring-substituted DIM represents a novel class of uncharged mitochondrial poisons that inhibit breast cancer cell and tumor growth.

4.2. Introduction

Epidemiology studies indicate that increased consumption of cruciferous vegetables is associated with decreased rates of multiple cancers [126] [123-125,127]. Phytochemicals identified in these vegetables exhibit anticarcinogenic activity in laboratory animal and cell culture studies, and one of these compounds is indole-3-carbinol (I3C) which occurs as a glycoside conjugate, glucobrassican [reviewed in [417,556,557]]. I3C conjugates are hydrolyzed in the acid environment of the gut and form several condensation and rearrangement products including 1,1-bis(3'-indolyl)methane (DIM) [128,129]. Several studies show that cruciferous vegetables (or their extracts), I3C and DIM exhibit antitumorigenic activity in *in vivo* rodent models and inhibit growth of cancer cells lines *in vitro* [130-135,416,426,558-565]. For example, I3C inhibited growth and/or formation of 7,12-dimethylbenz[a]anthracene (DMBA)-induced and spontaneous age-dependent mammary tumors and decreased the incidence of spontaneous endometrial tumors in rodent models [130,133,561,562]. Studies in this laboratory have investigated the chemotherapeutic effects of DIM and ring-substituted DIMs on mammary tumor growth in DMBA-induced female Sprague-Dawley rats. At doses of 5 mg/kg/every second day, DIM inhibited tumor growth, and several ring-substituted DIMs, including the 5,5'-dibromo analog (5,5'-diBrDIM), were antitumorigenic at doses ≤ 1 mg/kg/every second day [131,434,435].

The *in vivo* antiestrogenic effects of DIM and ring-substituted DIMs were also observed in estrogen receptor (ER)-positive MCF-7 and T47D breast cancer cells. The DIM compounds bind the aryl hydrocarbon receptor (AhR) in the μM range [131,434,435], and the results suggest that the observed antiestrogenic responses may be due, in part, to inhibitory AhR-ERα crosstalk. However, there is also evidence that DIM inhibits growth of ER-negative breast cancer cells and other ER-independent cancer cell lines. These responses are accompanied by cell context-dependent induction of apoptosis, p21 expression, and/or inhibition of $G_0/G_1 \rightarrow S$ phase progression [132,134,416,417,426,563,565]. Many of these studies used a relatively high concentration (50 μM) of DIM, and the data suggest that DIM acts primarily through AhR-independent pathways. The major objectives of this study were (a) to investigate growth inhibition and apoptosis induced by DIM and 5,5'-diBrDIM in Ah-responsive (MCF-7) and non-responsive (MDA-MB-231) breast cancer cell lines, (b) to determine the effect of lower doses (< 20 μM) of these compounds on critical cell cycle genes and apoptosis, and (c) to determine potential differences in the effects of DIM and 5,5'-diBrDIM in MCF-7 and MDA-MB-231 cells. The results of this study show that 1 - 20 μM DIM and 5,5'-diBrDIM significantly inhibited growth of both MCF-7 and MDA-MB-231 cells; this was accompanied by minimal effects on expression of p21 or p27 proteins but both compounds induced proteasome-dependent degradation of cyclin D1. DIM and 5,5'-diBrDIM also induced the release of cytochrome c and decreased Bcl-2 protein in MCF-7 and MDA-MB-231 cells; however, induction of PARP cleavage by these compounds was observed only in MCF-7 cells. The major difference between

DIM and 5,5'-diBrDIM in both cell lines was the rapid and persistent decrease in mitochondrial membrane potential (MMP) by 5,5'-diBrDIM, suggesting that the ring-substituted DIMs may be a new class of mitochondrial poisons for treatment of breast cancer.

4.3. Materials and Methods

4.3.1. Cells, chemicals and other materials

MG132 and JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide) and β-actin antibody (A1978) were obtained from Sigma Chemical Co. (St. Louis, MO). The human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231 cells were maintained in DME:F-12 supplemented with 0.22% sodium bicarbonate, 10% fetal bovine serum (FBS), and 2 ml/L antibiotic solution (Sigma Chemical Co., St. Louis, MO). MCF-7 cells were maintained in DME:F-12 supplemented with 0.22% sodium bicarbonate, 5% fetal bovine serum (FBS), and 2 ml/L antibiotic solution (Sigma Chemical Co., St. Louis, MO). Cells were grown in 150 cm^2 culture plates in an air/CO_2 (95:5) atmosphere at 37°C and passaged every 5 days. The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): p21 (C-19), p27 (C-19), cyclin D1 (M-20), Sp1 (PEP-2), Sp3 (D-20), Ah receptor (N-19), Bcl-2 (C-2), PARP (F-2), and cytochrome c (A-8). Horseradish peroxidase substrate for Western blot analysis was purchased from NEN Life Science Products (Boston, MA).

4.3.2. Cell proliferation assays

Cells were plated at a density of 5 x 10^4/well in 12-well plates, and culture media was replaced the next day with DME:F-12 media containing 2.5% charcoal-stripped FBS and either vehicle (DMSO) or the indicated ligand and concentration. Fresh media and compounds were added every 48 hr. Cells were counted at the indicated times using a Coulter Z1 cell counter. Each experiment was completed in triplicate and results are expressed as means ± SE for each determination.

4.3.3. Western blots

MDA-MB-231 and MCF-7 cells were seeded in DME:F-12 media containing 2.5% charcoal-stripped FBS for 24 hr and then treated with either the vehicle (DMSO) or the indicated compounds. In experiments where indicated, cells were pre-treated for 30 min with 10 μM of the proteasome inhibitor MG132. Whole cell lysates were obtained using high salt buffer [50 mM HEPES, 500 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol and 1% Triton X-100 pH 7.5 and 5 μL/mL of Protease Inhibitor Cocktail]. Protein samples were incubated at 100°C for 2 minutes, separated on 10% SDS-PAGE at 120 V for 3-4 hr in 1 X running buffer [25 mM Tris-base, 192 mM glycine, and 0.1% SDS (pH 8.3)] and transferred to a polyvinylidene difluoride (PVDF) membrane at 0.1 V for 16 hr at 4°C in 1 X transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 0.025% SDS). The PVDF membrane was blocked in 5% TBST-Blotto [10 mM Tris-HCl, 150 mM NaCl (pH 8.0), and 0.05% Triton X-100 and 5% non-fat dry milk] with gentle shaking for 30 min and incubated in fresh 5% TBST-Blotto with 1:1000 primary antibody for 5-6 hr with gentle shaking. After washing with TBST for

10 min, the PVDF membrane was incubated with secondary antibody (1:5000) in 5%TBST-Blotto for 2 hr. The membrane was washed with TBST for 10 min and incubated with 10 mL of chemiluminescence substrate for 1.0 min and exposed to Kodak X-OMAT AR autoradiography film. Band intensities were evaluated by scanning laser densitometry (Sharp Electronic Corporation, Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corporation, Billerica, MA). Quantitation of band intensities in DMSO and treatment groups was normalized to the loading control protein, β-actin. The fold-induction was determined by comparing band intensities in the treated vs. DMSO control groups.

4.3.4. Mitochondrial membrane potential

Monolayers of cells were cultured for 48 hr in 2-well Coverglass Chamber slides. Cells were treated with DMSO (vehicle control), 10 μM 5,5-diBrDIM, or 20 μM DIM for different time intervals (6, 12 or 24 hr). Slides were then washed with culture medium without serum or phenol red, and labeled with JC-1 [566-568] at a final concentration of 5 μg/mL for 10 min at 37°C. Following washing, cells were analyzed using a Meridian Ultima confocal microscope (Meridian Instruments, Okemos,MI). Excitation of JC-1 was done using a 488 nm wavelength and emission was collected using a dichroic 560nm SP in combination with 2 filters 530 nm/30 BP and 585 nm/42 BP. At least 8 areas per well were scanned. Two wells were analyzed per treatment and time point. Two experiments were conducted on different days.

4.3.5. Apoptosis assay

Vybrant Apoptosis Assay Kit #7 was used from Molecular Probes (Eugene, OR) according to the manufacturer's directions. Briefly, monolayers of cells were cultured for 48 hr in 2-well Coverglass Chamber slides. Slides were washed with culture medium without serum or phenol red, and labeled with Hoechst 33343, YO-PRO-1 and propidium iodide at a final concentration of 5 μg/mL, 100 nM and 1.0 μg/mL, respectively. The slides were incubated for 30 min on ice and visualized using a Meridian InSIGHT Point Confocal Microscope. At least 3 areas per well were analyzed. Two wells were analyzed per treatment and per time point. Short-term 50% EtOH treatment was initially utilized to ensure all 3 dyes could be visualized under conditions of necrosis. Hoechst 33343 is a vital blue fluorescing DNA stain that is membrane permeable and brightly stains the condensed chromatin of apoptotic cells, whereas nuclei of live cells exhibits lower fluorescence. The red propidium iodide is another DNA staining dye that only stains nuclei of cells with compromised plasma membrane and thus does not stain normal or apoptotic cells. The green fluorescent YO-PRO-1 dye can enter apoptotic cells in contrast to the propidium iodide and therefore apoptotic cells show green fluorescence only while dead cells show primarily red propidium iodide fluorescence and some green fluorescence.

4.3.6. Statistical analysis

Treatment-induced effects on the mitochondrial membrane potential as well as relative Western blot band intensities were statistically analyzed by ANOVA and Fisher's test for significance using SuperAnova software. The densitometric mean band

intensities of treatment groups are presented as the mean fold-induction normalized to the vehicle control (DMSO) ± SE for at least 3 separate determinations for each treatment. The MMP data are presented as the normalized ration (to vehicle control) of Jaggr/JC-1 ± SE for a minimum of 8 separate determinations for each treatment.

4.4. Results

MCF-7 and MDA-MB-231 breast cancer cells express the Ah [569]; although, MDA-MB-231 cells are Ah-nonresponsive and this may be due, in part, to overexpression of heat shock protein 90 which blocks ligand-dependent activation of the Ah [570]. One of the objectives of this study is to determine the role of the Ah receptor in mediating the growth inhibitory effects of DIM and 5,5'-diBrDIM in Ah-responsive MCF-7 and Ah-nonresponsive MDA-MB-231 cells. Preliminary cell proliferation studies in MCF-7 and MDA-MB-231 cells using bromo-, methyl- and chloro-substituents at 4,5,6,7 positions indicated that 5,5'-diBrDIM was the most active ring-substituted DIM, and this study investigated the growth inhibition and apoptosis induced by DIM and 5,5'-diBrDIM. The IC_{50} values for growth inhibition by 5,5'-diBrDIM were < 5 μM in both cell lines, whereas the corresponding IC_{50}'s for DIM were 10 - 20 μM (Figure 4.1). These *in vitro* differences between DIM and 5,5'-diBrDIM in breast cancer cell growth inhibition were comparable to their corresponding anticarcinogenic effects in DMBA-induced rat mammary tumor growth [19, 27, 28]. Based on the growth inhibitory effects of these compounds in both Ah responsive (MCF-7)

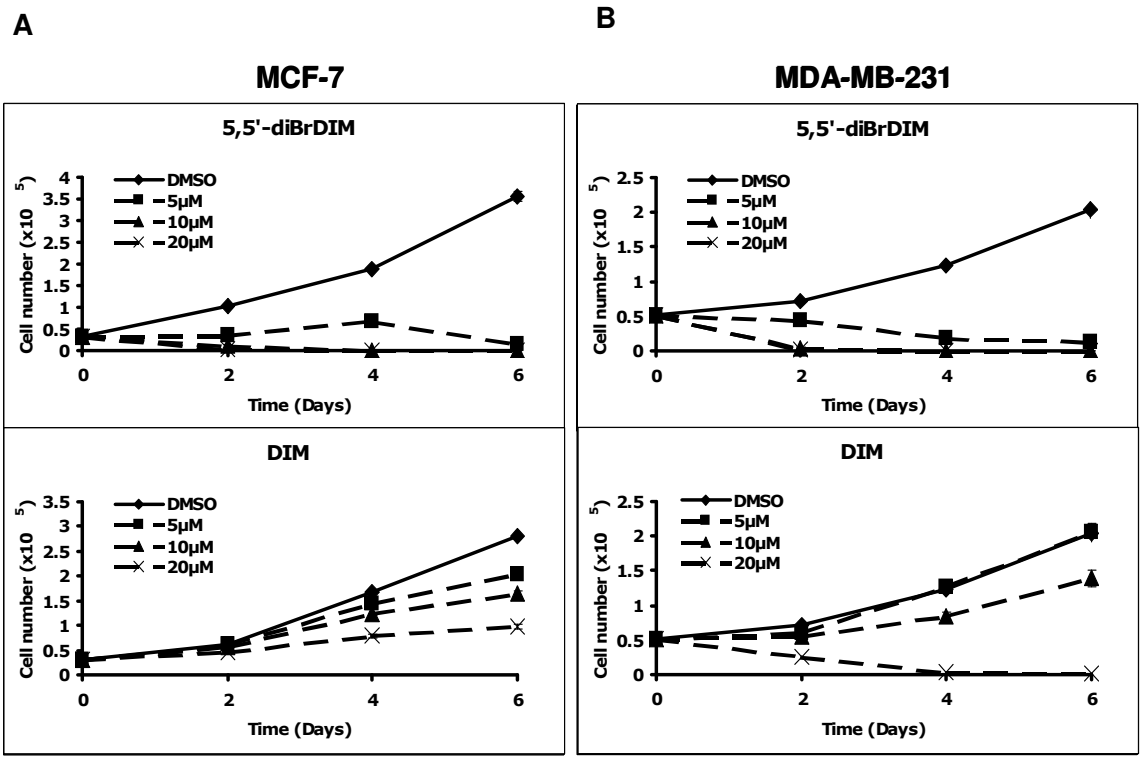

Figure 4.1: Inhibition of breast cancer cell growth by DIM and 5,5'-diBrDIM. MCF-7 [A] and MDA-MB-231 [B] cells. Cells were cultured for six days and treated every second day with DMSO (solvent control) or different concentrations of DIM or 5,5'-diBrDIM and the number of cells was determined as described in the Materials and Methods. Results are expressed as means ± SE for at least 3 separate determinations for each treatment group, significant ($p < 0.05$) growth inhibition was observed for ≥ 5 (MCF-7 cells) or ≥ 10 μM (MDA-MB-231 cells) for DIM or < 5 μM 5,5'-diBrDIM in both cell lines.

and non-responsive (MDA-MB-231) cells, it is evident that the antimitogenic activities of DIM and 5,5'-diBrDIM do not require a functional AhR.

Several studies report that I3C and DIM inhibit $G_0/G_1 \rightarrow S$ phase progression through modulation of key cell cycle regulatory proteins [134,416,417,563]. Results illustrated in Figures 4.2 and 4.3 summarize the effects of 1 - 10 μM 5,5'-diBrDIM and 5 - 20 μM DIM on p21, p27 and cyclin D1 levels in MDA-MB-231 and MCF-7 cells, respectively. Both MDA-MB-231 and MCF-7 cells express the Ah receptor, and levels were similar in DMSO (Figure 4.2B) and various treatment groups. The Ah receptor band staining was more intense using extracts from MDA-MB-231 cells. In MDA-MB-231 cells (Figure 4.2A), both compounds did not induce concentration-dependent changes on either p21 or p27 levels after treatment for 12 or 24 hr, whereas cyclin D1 protein levels were decreased by 5,5'-diBrDIM and DIM after treatment for 24 hr. A similar approach was used for MCF-7 cells (Figure 4.3); 10 μM 5,5'-diBrDIM decreased cyclin D1 levels (12 and 24 hr), and similar results were observed for 20 μM DIM after treatment for 24 hr. p27 levels were unaffected by 5,5'-diBrDIM or DIM, and p21 protein was increased by DIM (10 and 20 μM) and decreased by 5,5'-diBrDIM (5 and 10 μM) only at the 12 and 24 hr time points, respectively. In addition, other cell cycle proteins (cdk2, cdk4, cdk6) are unaffected by the treatments (data not shown).

Hong and coworkers [134] previously reported that 50 μM DIM induced p21 mRNA and protein levels for up to 72 hr in MCF-7 and MDA-MB-231 cells, and this was associated with enhanced Sp1 and Sp3 interactions with GC-rich motifs in the p21 promoter. Treatment of MCF-7 and MDA-MB-231 cells with 1 - 10 μM 5,5'-diBrDIM

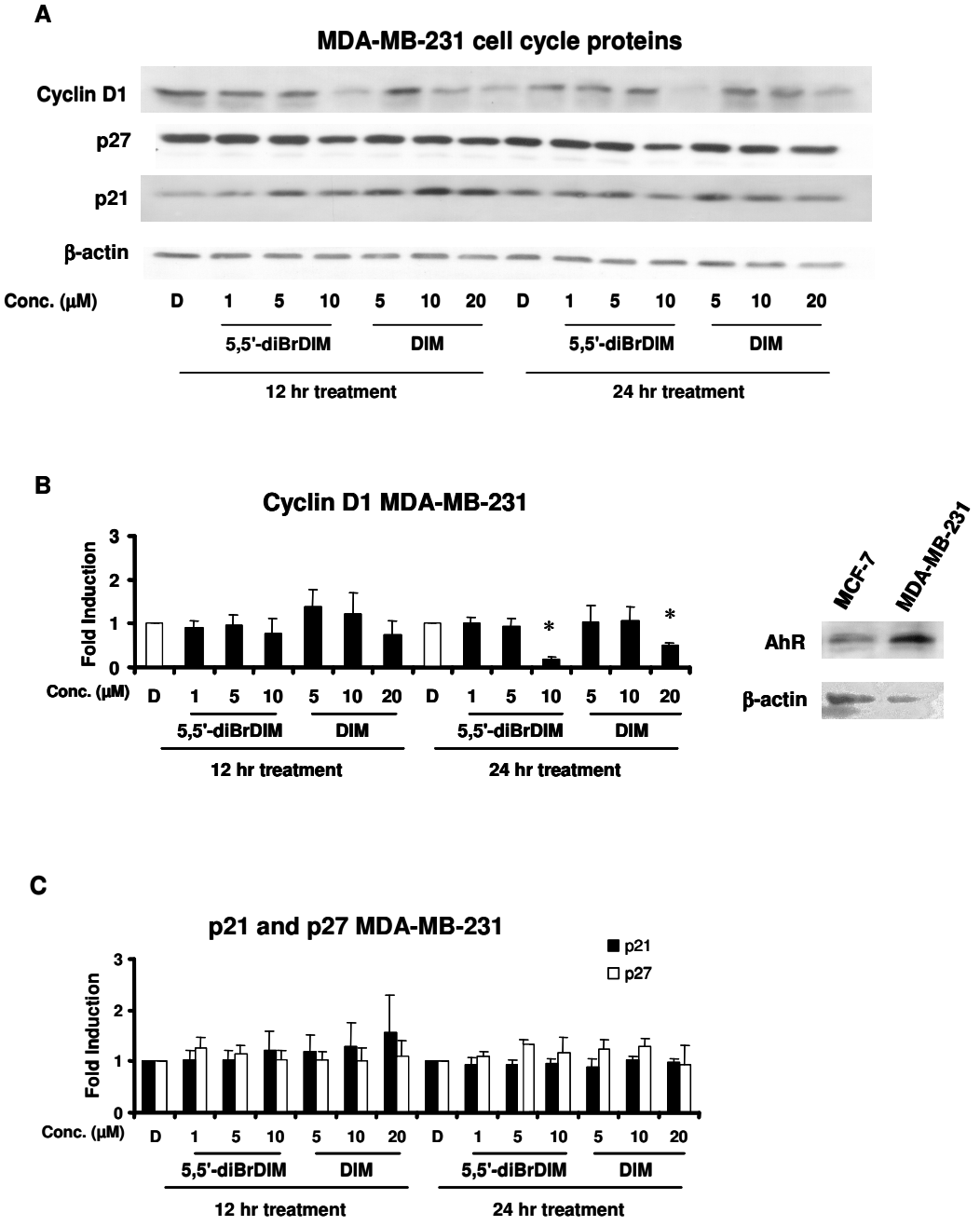

Figure 4.2: Effects of DIM and 5,5'-diBrDIM on cell cycle genes in MDA-MB-231 cells. [A] Western blot analysis. The effects of DMSO (D) (solvent control), DIM and 5,5'-diBrDIM on cyclin D1, p27 and p21 protein levels were determined by Western blot analysis of whole cell lysates from the different treatment groups as described in the Materials and Methods. Quantitation of cyclin D1 [B] and p27/p21 [C] protein levels. Based on results obtained for three separate experiments for each treatment, the levels of cyclin D1, p21 and p27 relative to DMSO (D) treatment were quantitated and results are expressed as means ± SE. Significant ($p < 0.05$) inhibition of cyclin D1 protein levels is indicated by an asterisk. The loading control was β-actin.

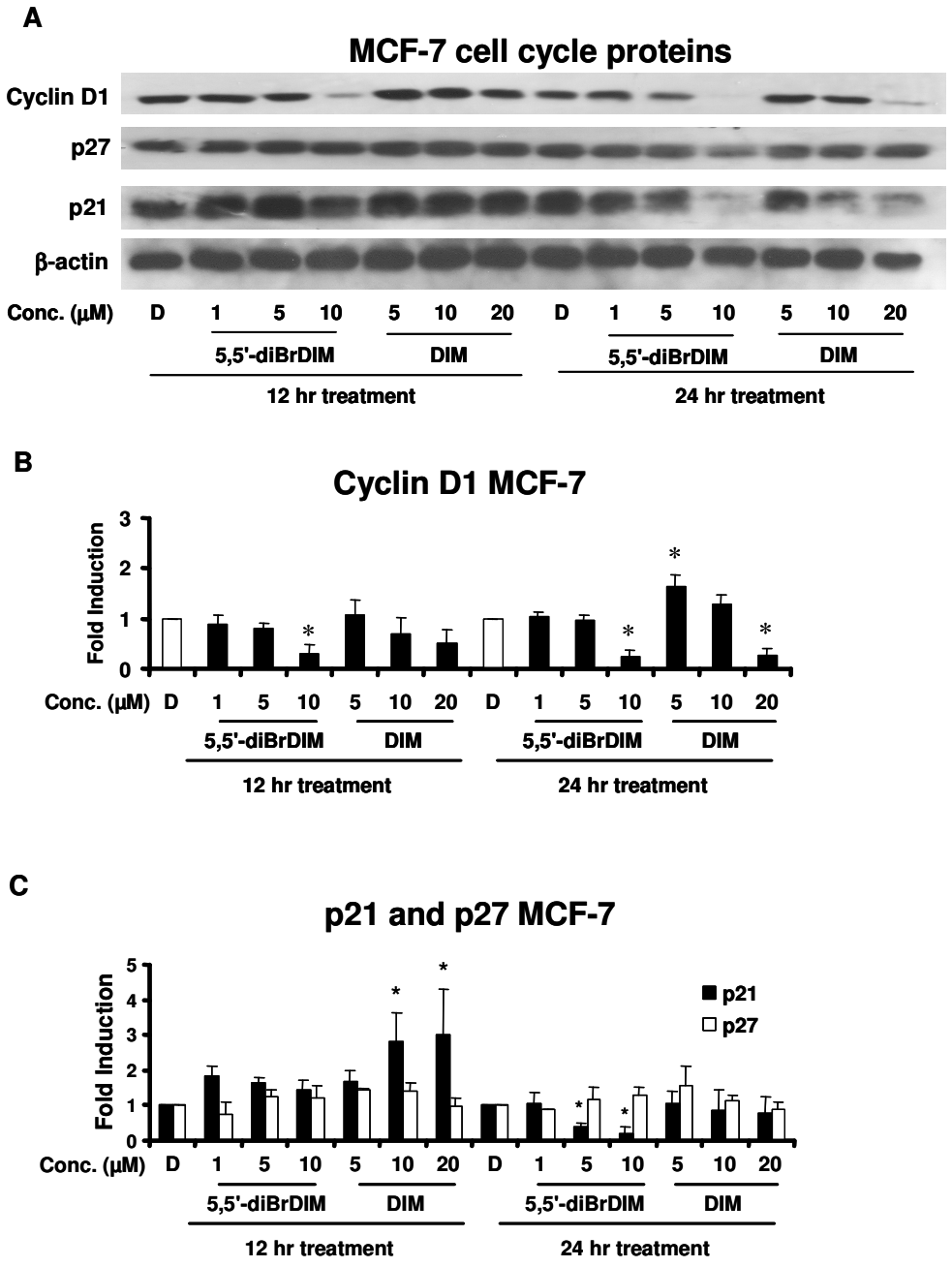

Figure 4.3: Effects of DIM and 5,5'-diBrDIM on cyclin D1, p21 and p27 protein expression in MCF-7 cells. [A] Western blot analysis. MCF-7 cells were treated with DMSO (D) or different concentrations of DIM or 5,5'-diBrDIM for 12 or 24 hr, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. Quantitation of cyclin D1 [B] and p27/p21 [C] protein levels. Levels of cyclin D1, p21 and p27 proteins in treatment groups were determined in 3 separate experiments as described in [A] and mean values ± SE relative to DMSO (D) are presented. Significant ($p < 0.05$) changes in levels relative to DMSO are indicated by an asterisk. The loading control was β-actin.

or 5 - 20 μM DIM (Figure 4.4) caused minimal changes in Sp1 or Sp3 levels in either cell line. Cellular responses to 50 μM DIM and higher concentrations of 5,5'-diBrDIM (≥ 20 μM) were not feasible in this study due to high cytotoxicity observed in the breast cancer cell lines within 24 hr after treatment (data not shown). Nevertheless, these data show that the growth-inhibitory activity of DIM or 5,5-diBrDIM at the doses and conditions used in this study was not related to modulation of Sp1/Sp3 ratios.

Carter and coworkers [571] have reported in a microarray experiment that DIM downregulates cyclin D1 gene expression in human keratinocytes and, in MCF-7 cells, a structurally-related series of methylene-substituted DIM compounds induce proteasome-dependent degradation of cyclin D1 [459]. Therefore, the effects of DIM and 5,5'-diBrDIM on activation of proteasome-dependent degradation of cyclin D1 was investigated in MCF-7 and MDA-MB-231 cells. The proteasome inhibitor MG132 significantly inhibited downregulation of cyclin D1 protein in MCF-7 cells treated with 10 μM 5,5'-diBrDIM (12 and 24 hr) or 20 μM DIM (24 hr) (Figure 4.5A). Cyclin D1 downregulation was also reversed by MG-132 in MDA-MB-231 cells treated with 10 μM 5,5'-diBrDIM (12 and 24 hr) or 20 μM DIM (12 hr). These results suggest that DIM and 5,5'-diBrDIM (ring-substituted DIM) induce proteasome-dependent downregulation of cyclin D1 in MCF-7 and MDA-MB-231 cells as previously reported for methylene-substituted DIMs in the former cell line [459].

Previous studies with I3C and/or DIM indicate that these compounds activate multiple pathways in breast and other cancer cell lines, and these include induction of apoptosis, modulation of Bcl-2/Bax levels, and alteration of MMP by I3C and/or DIM in

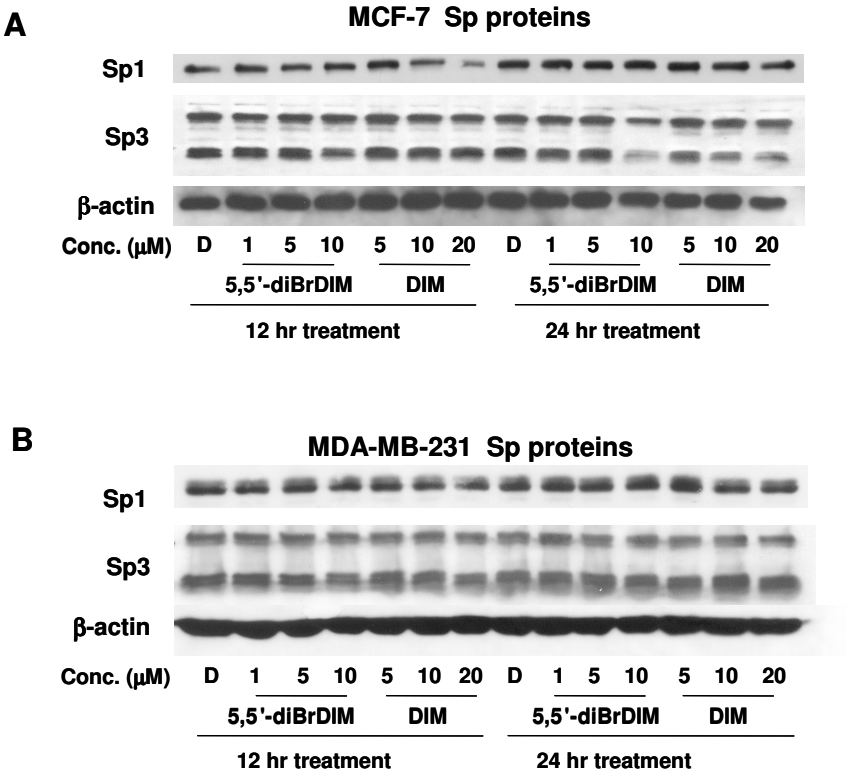

Figure 4.4: Sp1 and Sp3 protein levels in MCF-7 and MDA-MB-231 cells treated with DIM or 5,5'-diBrDIM. [A] MCF-7 cells and [B] MDA-MB-231 cells. The treatment protocols were identical to those described in Figures 2 and 3, and Sp1/Sp3 protein levels were determined by Western blot analysis and quantitated [relative to DMSO (D) treatment]. Results illustrated in the Figure are derived from the same whole cell lysates used for Western blot analytical data illustrated in Figures 2 and 3. Results of triplicate analysis (means ± SD) for Sp1 and Sp3 levels in both cell lines showed that Sp1 (5 μM DIM) and Sp3 (5 μM DIM) levels were increased (< 50%) in MCF-7 but not MDA-MB-231 cells (data not shown). The loading control was β-actin.

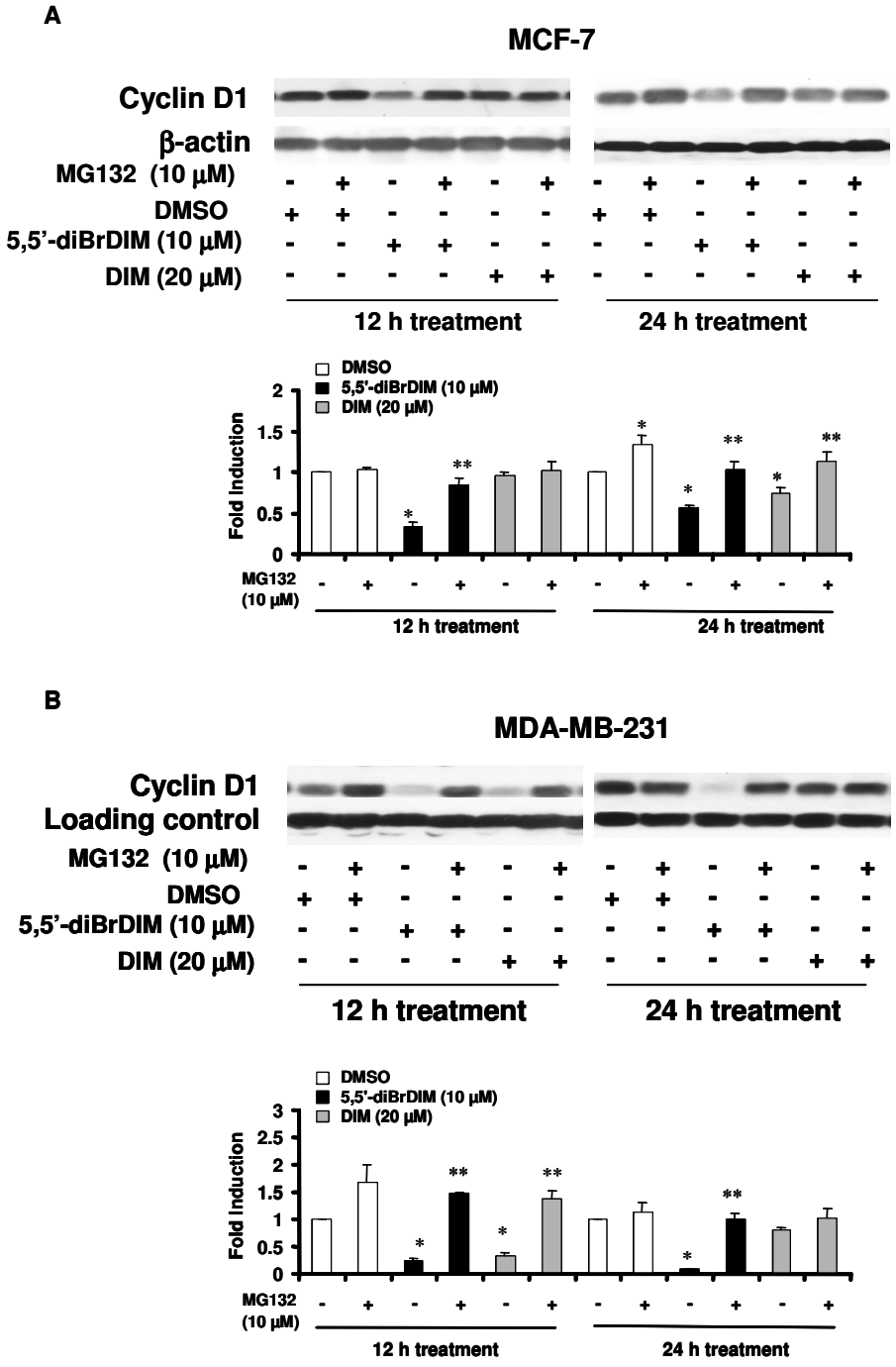

Figure 4.5: Effects of MG-132 on DIM-/5,5'-diBrDIM-induced downregulation of cyclin D1 protein in MCF-7 [A] and MDA-MB-231 [B] cells. Cells were treated with DMSO (D), 10 μM 5,5'-diBrDIM, 20 μM DIM, 10 μM MG-132, or their combinations (as indicated) for 12 or 24 hr, and whole cell lysates were examined by Western blot analysis as described in the Materials and Methods. Each treatment group was replicated (3X), and results are expressed as means ± SE relative to DMSO (control). Significantly ($p < 0.05$) decreased cyclin D1 (*) or inhibition of this decrease by MG-132 (**) are indicated. The loading control was β-actin.

MCF-7 and tumorigenic MCF10Ca1a cells [132,426,563,565,572]. We therefore further investigated the effects of 5,5'-diBrDIM and DIM on parameters associated with mitochondrial damage and cell death pathways. MDA-MB-231 cells were treated for 48 and 96 hr with 5 or 10 μM 5,5'-diBrDIM, 5, 10 or 20 μM DIM, and cell lysates were analyzed for PARP cleavage, Bcl-2 and cytochrome c protein levels (Figure 4.6A). PARP cleavage was not observed in any of the treatment groups; however, after 96 hr both DIM and 5,5'-diBrDIM caused a decrease in Bcl-2 and an increase in cytochrome c levels. After treatment of MDA-MBA-231 cells with 10 μM 5,5'-diBrDIM for 96 hr, there was extensive cell death and minimal protein was available for analysis (Figure 4.6A). As a control for this experiment, both cycloheximide (CHX) and the proteasome inhibitor MG-132 induced PARP cleavage in MDA-MB-231 cells (Figure 4.6B). A similar protocol was used for MCF-7 cells treated with DIM and 5,5'-diBrDIM for 48 and 72 hr (Figure 4.6C). 5,5'-diBrDIM (10 μM) induced PARP cleavage and increased cytochrome c levels after 48 hr and these responses as well as decreased Bcl-2 protein were observed after 72 hr. Comparable effects (but less pronounced) were also observed for DIM. The same mitochondrial responses were also investigated in MCF-7 and MDA-MB-231 cells treated for only 24 hr with higher concentrations of DIM (40 μM) and 5,5'-diBrDIM (20 μM) which were cytotoxic after > 30 hr treatment (Figure 4.6D). The results demonstrated that PARP cleavage, decreased Bcl-2 and increased cytochrome c protein levels could be observed within 24 hr in MCF-7 cells, whereas only Bcl-2 was affected in MDA-MB-231 cells at this time point. Despite the temporal

differences in the various indicators of cell death pathways in MCF-7 and MDA-MB-231 cells, nuclear staining of these cells

treated with DIM or 5,5'-diBrDIM showed specific treatment-related effects (Figure 4.7). MDA-MB-231 and MCF-7 cells were treated for 24 hr with 5 - 10 μM 5,5'-diBrDIM and 20 μM DIM, and normal, necrotic and apoptotic cells were distinguished by confocal microscopy using a fluorescent triple-dye assay. In MCF-7 cells (Figure 4.7A), treatment with 5,5'-diBrDIM (5 μM) and DIM (20 μM) for 24 hr increased the number of apoptotic cells, which were represented by an increase in the number of cells exhibiting both blue (Hoechst dye) and green (YO-PRO-1) fluorescence. Minimal necrosis (propidium iodide staining) was observed. Cells treated with 10 μM 5,5'-diBrDIM were primarily detached from this dish and could not be further evaluated. In contrast, the predominant cell death pathway in MDA-MB-231 cells treated with 5 - 10 μM 5,5'-diBrDIM was necrosis (Figure 4.7B) as evidenced by the intense propidium iodide nuclear staining. DIM (20 μM) induced blue and green staining with Hoechst and YO-PRO-1 dyes but no propidium iodide staining which indicated induction of apoptosis in MDA-MB-231 cells. Ethanol was used as a reference toxicant which induced blue, green and red staining with all three dyes.

We also examined decreased MMP in MCF-7 and MDA-MB-231 cells after treatment with DIM or 5,5'-diBrDIM using JC-1 which forms aggregates on the inner mitochondrial membrane of cells with higher MMP [566-568]. 5,5'-DiBrDIM induced a time-dependent decrease in MMP in MCF-7 cells; however, DIM induced an initial increase (6 and 12 hr) and only a slight decrease in MMP after 24 hr (Figures 4.8A and

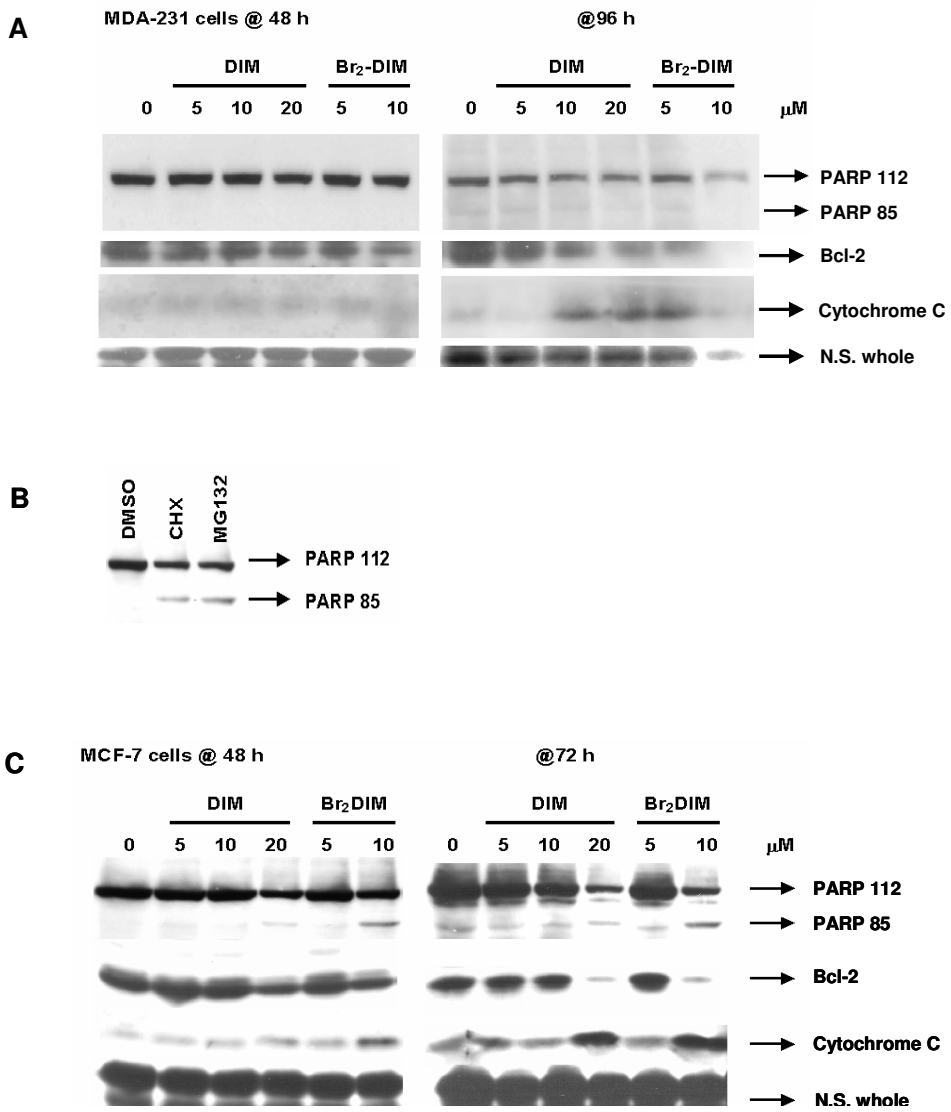

Figure 4.6: Effects of DIM and 5,5'-diBrDIM on MDA-MB-231 and MCF-7 cell mitochondrial proteins. [A] Mitochondrial proteins in MDA-MB-231 cells after treatment for 48 hr or 96 hr. Cells were treated with different concentrations of DIM or 5,5'-diBrDIM for 48 or 96 hr, and whole cell lysates or cytosolic fractions (for cytochrome c analysis) were isolated and examined by Western blot analysis as described in the Materials and Methods. Ten μM 5,5'-diBrDIM was cytotoxic to MDA-MB-231 cells after treatment for 96 hr, and decreased band intensities were due to the low yield of cells. [B] Treatment with cycloheximide (CHX) and MG-132. As a positive control for PARP cleavage, MDA-MB-231 cells were treated with 10 μM cycloheximide or MG-132, respectively, for 20 hr, and whole cell lysates were examined by Western blot analysis as described in the Materials and Methods. [C] Mitochondrial proteins in MCF-7 cells after treatment for 48 or 72 hr. Whole cell lysates or cytosolic fractions (for cytochrome c release) were isolated from the different treatment groups were examined by Western blot analysis as described in [A]. [D] Treatment of cells for 24 hr. MCF-7 and MDA-MB-231 cell mitochondrial proteins were determined after treatment with up to 40 and 20 μM DIM and 5,5'-diBrDIM, respectively, for 24 hr. Whole cell lysates or cytosolic fractions (for cytochrome c analysis) were examined by Western blot analysis as described in the Materials and Methods.

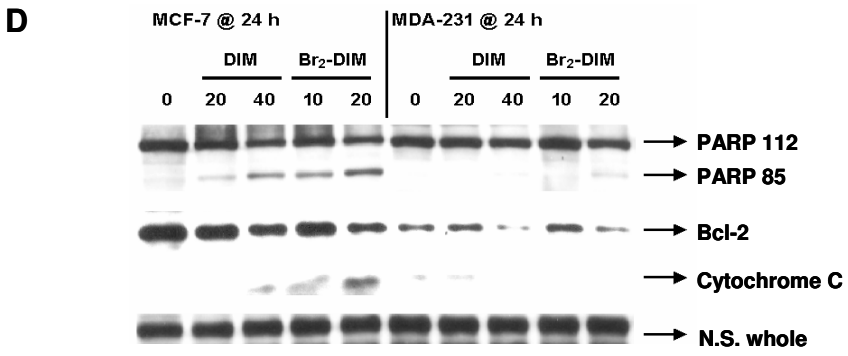

Figure 4.6 (Continued)

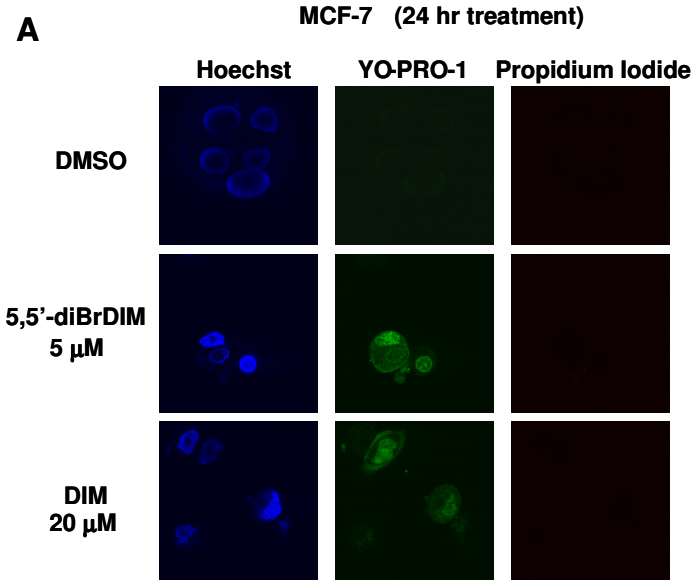

Figure 4.7: Characterization of 5,5'-diBrDIM and DIM-induced cell death in MCF-7 [A] and MDA-MB-231 [B] cells. MCF-7 and MDA-MB-231 cells were treated for 24 hr with DMSO, 5 or 10 μM 5,5'-diBrDIM, or 20 μM DIM. Three fluorescent DNA dyes Hoechst, YO-PRO-1 and propidium iodide, were loaded into cells and incubated on ice for 30 min and visualized using confocal microscopy as described in the Materials and Methods. Necrotic cells are identified by propidium iodide staining, whereas apoptotic cells exhibit green YO-PRO-1 and bright blue Hoechst fluorescence. The same microscopic field of MCF-7 [A] or MDA-MB-231 [B] cells stained with Hoechst, YO-PRO-1 and propidium iodide are shown and are representative of other cell areas with the same treatment. At least 3 areas were scanned for each well and two wells were analyzed per treatment and per time point. Five μM 5,5'-diBrDIM- and 20 μM DIM-treated MCF-7 cells exhibited minimal necrosis. The majority of cells treated with 10 μM 5,5'-diBrDIM detached from the dish and it was not possible to identify the cell death pathway using this assay. In MDA-MB-231 cells, 50% EtOH treatment was initially utilized as a positive control for necrotic cells and visualization of all three fluorescent dyes. MDA-MB-231 cells treated with 5 and 10 μM 5,5'-diBrDIM exhibited numerous propidium iodide stained cells, whereas cells treated with 20 μM DIM primarily showed YO-PRO-1 staining.

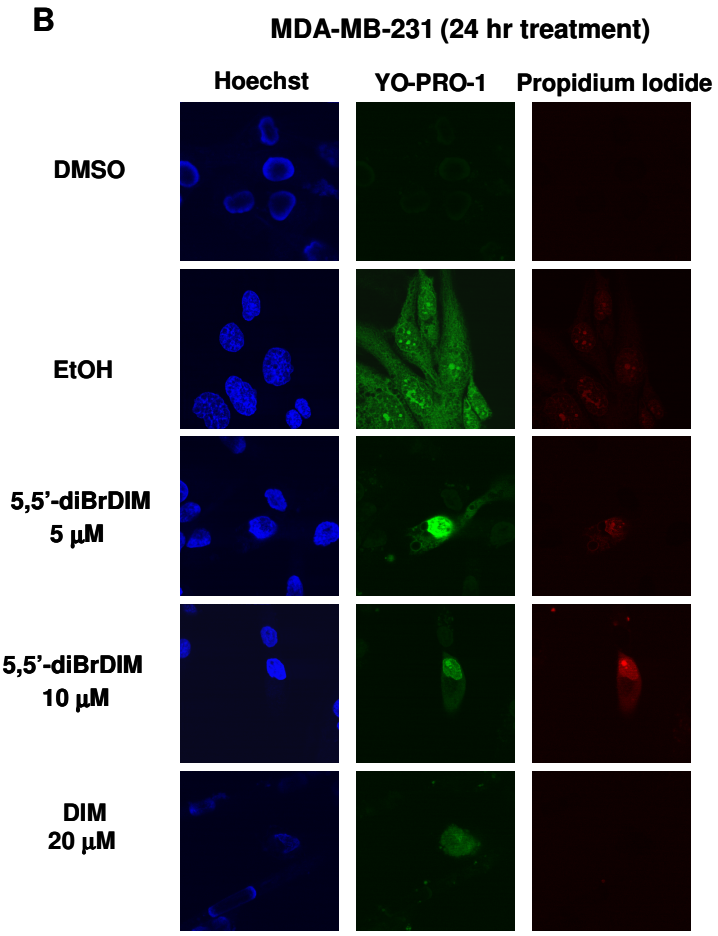

Figure 4.7 (Continued)

4.8B). 5,5'-DiBrDIM induced a highly significant decrease in the MMP in MDA-MB-231 cells 6, 12 and 24 hr after treatment, whereas DIM had only minimal effects on the MMP in MDA-MB-231 cells (Figures 4.8C and 4.8D). These results suggest that, although both DIM and 5,5'-diBrDIM induce downregulation of cyclin D1 protein, growth inhibition and necrosis in MDA-MB-231 cells, their effects on MMP are not the same, suggesting some differences in their mechanisms of action in which direct mitochondrial targeting is the predominant pathway for the ring-substituted 5,5'-diBrDIM.

4.5. Discussion

I3C and DIM exhibit anticarcinogenic activities in cancer cells and in *in vivo* models, and contribute to the chemoprotective effects associated with consumption of cruciferous vegetables [123-127,130-135,416,426,434,435,558-565]. DIM exhibits multiple activities and interacts directly with the trout ER, human androgen receptor and Ah [134,434,435]. Ligands that activate the Ah inhibit growth of breast, prostate, endometrial and pancreatic cancer cells [131,573-576], and both DIM and 5,5'-diBrDIM inhibited DMBA-induced rat mammary tumor growth at doses < 5 or < 1 mg/kg every second day, respectively [131,435]. Hong and coworkers [134] reported that DIM inhibited growth of Ah-responsive and non-responsive MCF-7 and MDA-MB-231 breast cancer cells and similar growth inhibitory responses were also observed for DIM and ring-substituted DIMs (Figure 4.1). MCF-7 cells were responsive to growth inhibition by these compounds and this may be due, in part, to activation of the AhR. However, the results observed in MDA-MB-231 cells treated with DIM and ring-substituted DIMs

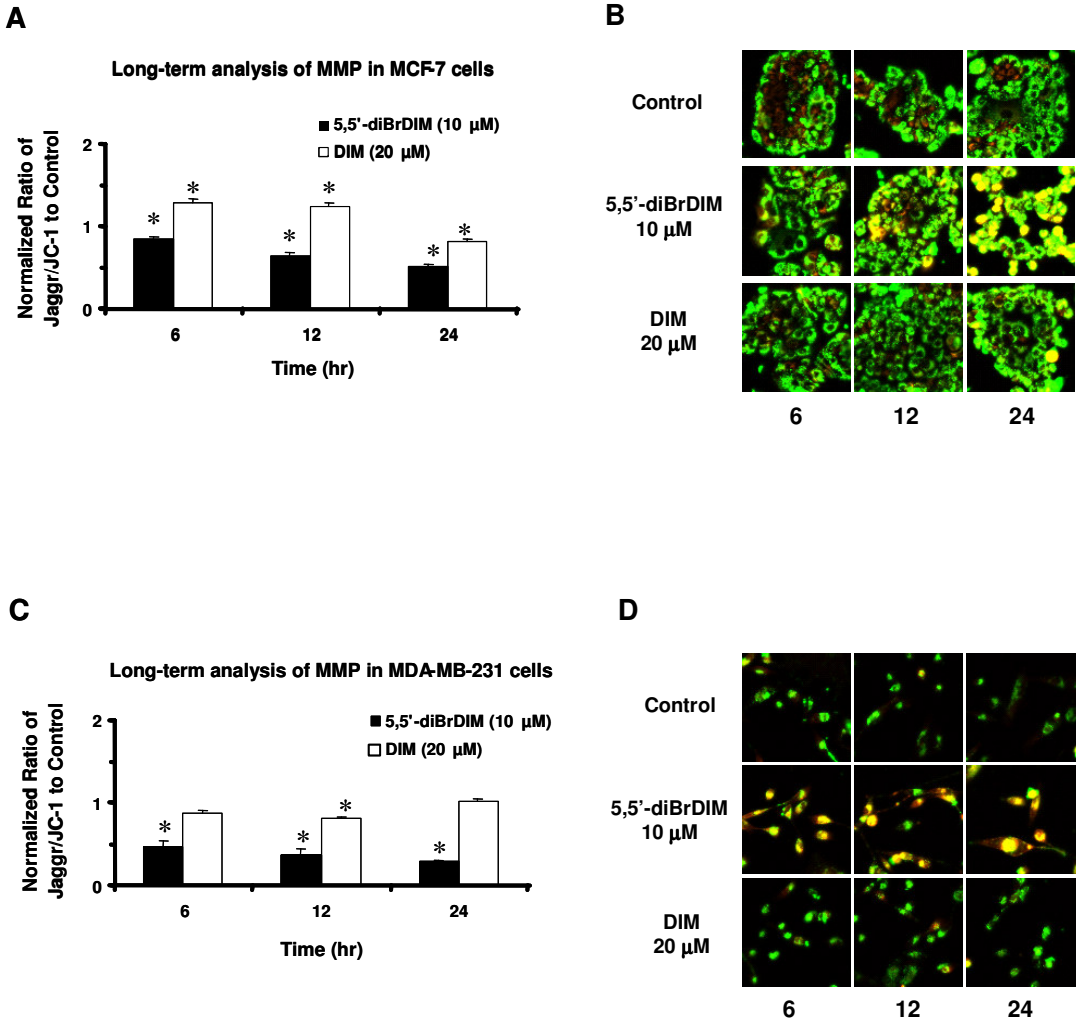

Figure 4.8: Time-dependent mitochondrial membrane depolarization after treatment of MCF-7 [A, B] or MDA-MB-231 [C, D] cells with 5,5'-diBrDIM and DIM. MCF-7 and MDA-MB-231 cells were treated for 6, 12 or 24 hr with DMSO, 10 µM 5,5'-diBrDIM and 20 µM DIM, and cells were loaded with the potential-sensitive JC-1 dye and visualized as described in the Materials and Methods. The ratio of Jaggr/JC-1 for each treatment was normalized to DMSO and these results are shown for MCF-7 [A] and MDA-MB-231[C] cells as the mean ± SE for at least 8 replicate determinations for each treatment group. Representative fluorescent images are also depicted in [B] and [D] for MCF-7 and MDA-MB-231 cells, respectively. Significance ($p<0.05$) is denoted by an asterisk (*).

(Figure 4.1) indicate that growth inhibition and cytotoxicity observed after treatment with DIM or ring-substituted DIMs is AhR-independent.

It was reported that concentrations of DIM ≥ 50 μM induced p21 mRNA and protein levels in MCF-7 and MDA-MB-231 cells, and this was associated with enhanced binding of Sp1 and Sp3 to GC-rich elements in the p21 gene promoter [134]. In this study, 20 μM DIM was cytotoxic in MDA-MB-231 and cytostatic in MCF-7 cells (Figure 4.1) and 10 μM concentrations of the ring-substituted DIMs were cytotoxic in both cell lines (Figure 4.1). Higher concentrations of DIM (40 μM) or 5,5'-diBrDIM (20 μM) could only be used in short term (24 hr) studies since these concentrations resulted in 100% cell death and detachment from the cell culture wells within 48 hr after treatment. DIM had minimal effects on p21 or p27 levels in MDA-MB-231 and MCF-7 cells (Figures 4.2 and 4.3), and a < 3-fold increase in p21 protein was observed only in MCF-7 cells treated with 10 or 20 μM DIM for 12 hr. We did not observe any changes in Sp1 or Sp3 levels in MCF-7 or MDA-MB-231 cells treated with 1 - 10 μM 5,5'-diBrDIM or 5 - 20 μM DIM (Figure 4.3) which were also growth inhibitory (Figure 4.1). These results suggest that the cytotoxic and cytostatic effects of DIM (< 20 μM) or 5,5'-diBrDIM (< 10 μM) in MCF-7 and MDA-MB-231 cells are independent of changes in Sp1, Sp3 and p21 expression as previously reported [134]; however, this does not exclude the reported effects of higher concentrations (e.g. 50 μM) of DIM on Sp1, Sp3 and p21 levels. The major effects of DIM and 5,5'-diBrDIM on cell cycle proteins associated with $G_1 \rightarrow S$ phase progression was decreased cyclin D1 protein expression

in both cell lines (Figures 4.2 and 4.3). Downregulation of cyclin D1 protein by DIM and 5,5'-diBrDIM in MCF-7 and MDA-MB-231 cells was blocked by the proteasome inhibitor MG-132 (Figure 4.5) as previously reported for methylene substituted DIMs in the former cell line [459]. These results indicate that both ring- and methylene-substituted DIM compounds which are di- or triarylmethane analogs, respectively, may exhibit common pathways for inducing proteasome-dependent degradation of cyclin D1, and we are currently investigating the mechanisms of this process.

Previous studies show that I3C and DIM also affect mitochondrial function [132,426,563,565,572] and treatment of MDA-MB-231 or MCF-7 cells with 50 μM DIM decreased Bcl-2 and increased Bax protein levels and the percentage of apoptotic cells [426]. In this study, we also observed decreased Bcl-2 protein and this was accompanied by increased levels of cytochrome c released into the cytosol in both cell lines treated with DIM or 5,5'-diBrDIM (Figure 4.6). In contrast, PARP cleavage was induced only in MCF-7 and not in MDA-MB-231 cells (Figure 4.6) and we previously also observed induction of PARP cleavage by methylene-substituted DIMs in MCF-7 cells [35]. Lipophilic cations [577-579] and other lipophilic chemicals such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 [535] target the mitochondria in some cell lines and induce mitochondrial membrane depolarization and increased mitochondrial membrane permeability. Cell death pathways induced by DIM and 5,5'-dibrDIM were investigated in MCF-7 and MDA-MB-231 cells using Hoechst 33343 dye, the green fluorescent YO-PRO-1 dye that can enter apoptotic cells, and propidium iodide which stains necrotic cell nuclei. DIM and 5,5'-diBrDIM induced YO-PRO-1 staining but no propidium iodide

staining in MCF-7 cells (Figure 4.7A) which is indicative of apoptosis. The results are consistent with the induction of PARP cleavage by the same compounds in MCF-7 cells (Figure 4.6). In contrast, only DIM induced apoptosis in MDA-MB-231 cells (Figure 4.7B) and treatment with 5,5'-dibrDIM resulted in propidium iodide-stained cells and necrosis. These results demonstrate that DIM and 5,5'-diBrDIM induce overlapping and different cell death pathways that are cell context-dependent.

Rahman and coworkers [563] also showed that I3C induced the loss of MMP in MCF-7 cells, and this may be due, part, to DIM which is formed in cell culture from spontaneous dimerization of I3C [580]. Results in Figure 4.8 illustrate the effects of DIM and 5,5'-diBrDIM on the fluorescence of JC-1, a dye that accumulates and oligomerizes in the inner mitochondrial membrane and exhibits a distinct red fluorescence. This response is associated with permeabilization of various mitochondrial pores/channels and release of pro-apoptotic/necrotic factors [577-579]. Using confocal microscopy and a potential-sensitive dye, it was evident that 5,5-diBrDIM decreased the ratio of red/green fluorescence indicative of decreased MMP, whereas the effects of DIM were minimal (Figure 4.8). DIM and 5,5-'diBrDIM induced apoptosis in MCF-7 cells; however, only the latter compound decreased MMP throughout the 24 hr treatment. 5,5'-diBrDIM primarily induced necrosis of MDA-MB-231 cells, and MMP was decreased after treatment with 5,5'-diBrDIM but not DIM, suggesting that introduction of the ring substituents significantly enhanced the mitochondriotoxic effects of this compound.

In summary, results of this study indicate that DIM and 5,5'-diBrDIM inhibit growth of MCF-7 and MDA-MB-231 breast cancer cells through multiple pathways including activation of proteasome-dependent degradation of cyclin D1, induction of cell necrosis, apoptosis and decreased MMP, and these effects were essentially Ah receptor-independent. For most responses, 5,5'-diBrDIM was significantly more potent than DIM, and 5,5'-diBrDIM significantly decreased MMP in both MDA-MB-231 and MCF-7 cells, whereas DIM had minimal effects on MMP. Preliminary results also indicate that other ring-substituted DIMs decrease MMP in breast cancer cells (data not shown). Current studies are focused on development of 5,5'-diBrDIM and related compounds as a new class of mitochondrial poisons and on determining their specific mechanisms of mitochondrial toxicity in different cancer cell lines.

CHAPTER V

SUMMARY

Cancer is the second-leading cause of death in the United States and 1 in 2 men and 1in 2 women are expected to develop cancer in their lifetime. Breast cancer accounts for one third of all new cancer cases in women in the US and over 41,000 are expected in 2006. Due to the high incidence of breast cancer, there is a great need to develop more effective treatments for this disease.

Cancer can be defined as abnormal cells that grow uncontrollably and have the ability to invade normal tissues and many cytotoxic chemotherapeutic agents kill cancer cells by targeting rapidly dividing cells. This therapeutic approach causes many adverse side effects to normal cells that are also rapidly proliferating. Current chemotherapeutic drug design strategies have focused on identifying targets specific to cancer cells, which include the 6 hallmarks of cancer: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis.

Breast cancer can be classified into 2 different categories: early stage ER-positive breast cancer and later stage ER-negative breast cancer. ER-positive breast cancer is defined by tumor cells that express ER and respond to the mitogenic effects of estrogen. This type of breast cancer can be treated effectively by drugs such as selective estrogen receptor modulators (SERMs) or aromatase inhibitors that exploit the estrogen-induced growth signaling pathway. SERMs inhibit E2 binding to ER and aromatase inhibitors interefere with the conversion of testosterone to estrogen. Although both are

effective, prolonged use of SERMs is associated with an increased risk of endometrial cancer and aromatase inhibitors can increase bone loss.

Epidemiology studies have linked increased consumption of cruciferous vegetables to decreased incidence of several cancers including breast cancer. DIM is the dimerization production of the cruciferous phytochemical indole-3-carbinol (I3C) and has both I3C and DIM exhibit growth inhibitory and antitumorigenic effects in vitro and in vivo cancer models. My research has investigated the mechanisms of growth inhibition in breast cancer cells for two series of substituted DIMs: methylene-substituted DIMs and ring-substituted DIMs. Both series of compound exhibit great potential for treating breast cancer and these C-DIM analogs are active against ER-positive and ER-negative breast cancer cells/tumors in both in vitro and in vivo models.

1,1-Bis(3'-indolyl)-1-(p-substitutedphenyl)methanes containing para trifluoromethyl (DIM-C-pPhCF_3), t-butyl (DIM-C-pPhtBu) and phenyl (DIM-C-pPhC_6H_5) groups activate peroxisome proliferator-activated receptor γ (PPARγ) in ER-negative MDA-MB-231 and MDA-MB-453 breast cancer cells. These PPARγ-active compounds inhibit breast cancer cell proliferation, however, inhibition of G_0/G_1 to S phase progression and cyclin D1 downregulation was observed in MDA-MB-231 but not MDA-MB-453 cells. Nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1), a transforming growth factor β-like peptide, was also induced by these compounds and response was dependent on cell-context dependent activation of kinase pathways. Despite the induction of NAG-1 and downregulation of the antiapoptotic protein survivin in both MDA-MB-231 and MDA-MB-453 cells treated with these methylene-

substituted diindolylmethanes (C-DIMs), this was not accompanied by apoptosis. Nevertheless the cytotoxicity observed for the C-DIMs in vitro was complemented by inhibition of tumor growth in athymic nude mice bearing MDA-MB-231 cells as xenografts and treated with DIM-C-pPhC_6H_5 (40 mg/kg/d).

Although C-DIMs inhibited breast cancer cell growth, the mechanism of inhibition required further investigation into apoptotic, necrotic and autophagic cell death. C-DIM-induced cell death could not be reversed by zVAD-fmk, a pan-caspase inhibitor in either cell line. Treatment of MDA-MB-231 and MDA-MB-453 cells with C-DIMs did not elevate annexin V-propidium iodide (PI) staining patterns consistent with apoptosis, suggesting that C-DIMs did not induce Type I (apoptotic) programmed cell death. Cell necrosis (Type III cell death), as indicated by propidium iodide (PI) staining and lactate dehydrogenase (LDH) release, was also negative after treatment of MDA-MB-453 cells with C-DIMs. Autophagic (Type II) cell death is an important cell death pathway in breast cancer cells after treatment with several chemotherapeutic agents including tamoxifen, EB1089 and sulforaphane, and therefore we investigated the possibility that C-DIMs may also induce autophagy in breast cancer cells. Treatment of MDA-MB-231 and MDA-MB-453 cells with C-DIMs resulted in an accumulation of light chain associated-protein 3 (LC3)-II compared to LC3-I protein and this is a characteristic marker of autophagy which is also observed in breast cancer cells treated with a vitamin D analog and paclitaxel analogs. Transient transfection of MDA-MB-231 and MDA-MB-453 cells with green fluorescent protein-LC3 also revealed that treatment with C-DIMs induced a redistribution of LC3 to autophagosomes after C-DIM treatment.

In addition, the autofluorescent drug monodansylcadaverine (MDC), a specific autophagolysosome marker, accumulated in vacuoles after C-DIM treatment. Western blot analysis of lysates from cells treated with C-DIMs showed the Beclin 1/Bcl-2 protein ratio increased, suggesting that C-DIM compounds may represent a new mechanism-based agent for treating drug-resistant ER-negative breast tumors through induction of autophagic cell death.

The growth inhibitory effects of ring-substituted DIMs were investigated in both ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. DIM and the 5,5'-dibromo ring substituted DIM (5,5'-diBrDIM) inhibited growth of MCF-7 and MDA-MB-231 breast cancer cells, and IC_{50} values were 10 - 20 and 1 - 5 μM, respectively, in both cell lines. DIM and 5,5'-diBrDIM did not induce p21 or p27 protein levels or alter Sp1 or Sp3 protein expression in either cell line. In contrast, 10 μM 5,5'-diBrDIM downregulated cyclin D1 protein in MCF-7 and MDA-MB-231 cells 12 and 24 h after treatment. DIM (20 μM) also decreased cyclin D1 in MCF-7 (24 hr) and MDA-MB-231 (12 hr), and the DIM/5,5'-diBrDIM-induced degradation of cyclin D1 was blocked by the proteasome inhibitor MG132. Both DIM and 5,5'-diBrDIM induced apoptosis in MCF-7 cells and this was accompanied by decreased Bcl-2, release of mitochondrial cytochrome c, and decreased mitochondrial membrane potential as determined by the red/green fluorescence of JC-1. DIM and 5,5'-diBrDIM induced extensive necrosis in MDA-MB-231 cells; however, this was accompanied by decreased mitochondrial membrane potential primarily in cells treated with 5,5'-diBrDIM but not DIM. Thus, DIM and 5,5'-diBrDIM induce cell death in MCF-7 and MDA-MB-231

cells by overlapping and different pathways, and the ring-substituted DIM represents a novel class of uncharged mitochondrial poisons that inhibit breast cancer cell and tumor growth.

In summary, ring-substituted DIMs are highly effective drugs for treatment of breast cancer and are more potent than DIM both in vitro and in vivo models. 5,5'-diBrDIM inhibited growth in both ER-negative and ER-positive breast cancer and acted as a mitochondrial poison. The PPARγ-active C-DIMs inhibit growth in ER-negative breast cancer cells and are antitumorigenic in mouse xenografts and therefore represent a new class of anticancer agents that exhibit immense therapeutic potential for treating invasive ER-negative breast cancer. These compounds exert their growth inhibitory effects through a variety of pathways including cell cycle arrest, induction of TGF-β-like peptides and autophagic cell death. In addition, the C-DIMs investigated in this study exhibit distinctive preferences in terms of cellular targets: DIM-C-pPhCF_3 is the most potent PPARγ agonist and inducer of p21; DIM-C-pPhtBu is the best mitochondrial poison; and DIM-C-pPhC_6H_5 induces the TGF-β-like peptide NAG-1 to the greatest extent. In summary, the substituted DIMs target multiple mechanisms in a cell-context dependent manner and this property contributes to the great therapeutic potential for application of these compounds in the treatment of invasive breast cancer.

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