INHIBITION OF BREAST AND PROSTATE CANCER CELL GROWTH BY 3,3'-DIINDOLYLMETHANE AND RELATED COMPOUNDS

A Dissertation

by

LEELA KOTHA

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

December 2006

Major Subject : Genetics

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Approved by:

Chair of Committee,	Stephen Safe
Committee Members,	Bhanu Chowdhary
	Robert Burghardt
	Timothy Phillips
Chair of Genetics Faculty,	James Wild

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ABSTRACT

Inhibition of Breast and Prostate Cancer Cell Growth by 3,3'-Diindolylmethane and Related Compounds. (December 2006)

Leela Kotha, B.S., St. Xavier's College;

M.S., Bombay University

Chair of Advisory Committee: Dr. Stephen Safe

Selective receptor modulators have been developed for steroid hormone receptors as a new class of mechanism-based drugs for treatment of hormone related diseases. We investigated an alternative mechanism-based strategy for treating various cancers with selective aryl hydrocarbon receptor modulators (SAhRMs), such as diindolylmetane/(DIM), 2,3,7,8-tetrachlorodibenzo-p-dioxin/(TCDD), and 6-6-methyl-1,3,8-trichlorodibenzofuran/(MCDF) that exhibit antiproliferative activity in several cancer cell lines. MDA-MB-453 and BT-474 are estrogen receptor/(ER) negative breast cancer cell lines that express a functional aryl hydrocarbon receptor/(AhR) and treatment with SAhRMs significantly inhibited MDA-MB-453/BT-474 cell proliferation but did not significantly affect the percent distribution of cells in G0/G1/S/G2/M phases of cell cycle. TCDD and the SAhRMs had minimal effects on the expression of various cellular kinases. These data coupled with results obtained for other activated kinase pathways demonstrate that TCDD and SAhRMs uniquely inhibit growth of ER-negative MDA-MB 453/BT-474 breast cancer cells through kinase-

independent pathways. However, the SAhRMs induced HES-1, an antiproliferative transcription factor, in both cell lines and this might represent a possible mechanism for the growth inhibitory effects observed with these compounds.

We proved that ring substituted DIMs exhibit androgenic/antiandrogenic activities in androgen receptor/(AR)-positive LNCaP/22RV1 prostate cancer cell lines resulting in antiproliferative activities. These antiproliferative activities were accompanied by antiandrogenic activity and structure-dependent down regulation of AR. The ring-substituted DIMs also induced both non-steroidal anti-inflamatory drug-induced gene-1/(NAG-1) and activating transcription factor 3/(ATF-3), two anti-proliferative/apoptotic genes which are responsible in part for the inhibitory effects of these compounds on the proliferation of prostate cancer cells.

DEDICATION

I would like to dedicate this work, in loving memory, to my grandfather Kollipara Radhakrishnamurty, who I love and miss dearly.

I would also like to dedicate to my mother, Nagachandravathi Kotha, and father, Sambasivarao Kotha without whom I would not be where I am today. They are the WORLD to me.

ACKNOWLEDGEMENTS

I would like to thank Dr. Stephen Safe for his understanding, guidance immense patience and support for the duration of my graduate career, without whom none of this is possible. I am forever indebted to him. I would also like to thank my committee members Dr. Robert Burghardt, Dr. Bhanu Chowdhary and Dr. Timothy Phillips for their guidance, support and valuable advice during my study. I would like to thank Lorna Safe for all her kindness, support and helping nature. I would like to thank Kim Daniel, Kathy Mooney and Julia Williams for always being willing to help and assist.

I would like to thank all the current and past Safe lab members, especially Dr. Andrew McDougal, Dr. Mark Wormke, Dr. Matt Stoner for scientific discussions and help in the lab.

Thanks to my entire family, especially to my sister Pallavi, for making me laugh when times were hard and I felt lost. I will never forget our “CODE” sis. I would like to especially thank my Dad for being just a phone call away to keep me in high spirits with his humor many a night when I lay awake in bed feeling home sick. And last but not least, my MOM who I can’t thank enough. She is the epitomy of the word MOM. I aim to reach your standards in understanding, love, friendship and patience. I LOVE YOU MA.

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INTRODUCTION

1.1 Cancer

1.1.1 Statistical data

Cancer is the second leading cause of premature death in the world and cancer mortality rates are the second only to heart disease. According to the global cancer statistics it is estimated that 10.9 million new cases, 6.7 million deaths and 24.6 million people are living with cancer within 5 years of diagnosis in the year 2002 (1). Lung cancer is the main cancer in the world today, both in the number of cases (1.35 million) and deaths (1.18 million) and it has the highest fatality rate at 0.87 (1). Breast cancer, the second most common cancer overall with 1.15 million new cases annually ranks 5th in mortality since it has relatively good prognosis after treatment. Breast cancer is the most prevalent disease of women in the world with an estimated 4.4 million survivors living up to 5 years after diagnosis and this disease was responsible for 411,000 deaths in year 2002 (1). There are 934,000 cases and 700,000 deaths from stomach cancer and 626,000 cases and 598,000 deaths from liver cancer worldwide. In men although lung cancer is the most common cancer worldwide, prostate cancer is ranked second with 679,000 cases and 221,000 deaths in 2002 (Tables 1, 2) (1).

This dissertation follows the style and format of Cancer Research.

Table 1. Worldwide incidence of cancer by sex and tissue/organ site (1).

INCIDENCE				
Rank	CANCER SITE	Males	Females	Total
1	Lung	965,241	386,891	1,352,132
2	Colo/rectal	550,465	472,687	1,023,152
3	Stomach	603,419	330,518	933,937
4	Liver	442,119	184,043	626,162
5	Esophagus	315,394	146,723	462,117
6	Bladder	273,858	82,699	356,557
7	Non-Hodgkin lymphoma	175,123	125,448	300,571
8	Leukemia	171,037	129,485	300,522
9	Oral Cancer	175,916	98,373	274,289
10	Pancreas	124,841	107,465	232,306
11	Kidney	129,223	79,257	208,480
12	Brain	108,221	81,264	189,485
13	Melanoma of skin	79,043	81,134	160,177
14	Larynx	139,230	20,011	159,241
15	Thyroid	37,424	103,589	141,013
16	Multiple myeloma	46,512	39,192	85,704
17	Nasopharynx	55,796	24,247	80,043
18	Hodkins lymphoma	38,128	24,111	62,239
	Female cancers			
	Breast		1,151,298	1,151,298
	Cervix uteri		493,243	493,243
	Corpus Uteri		198,783	198,783
	Ovary		204,499	204,499
	Male Cancers			
	Prostate	679,023		679,023
	Testis	48,613		48,613

Table 2. Worldwide mortality of cancer by sex and tissue/organ site (1).

MORTALITY				
Rank	CANCER SITE	Males	Females	Total
1	Lung	848,132	330,786	1,178,918
2	Stomach	446,052	254,297	700,349
3	Liver	416,882	181,439	598,321
4	Colo/rectal	278,446	250,523	528,969
5	Esophagus	261,162	124,730	385,892
6	Pancreas	119,554	107,749	227,303
7	Leukemia	125,142	97,369	222,511
8	Non-Hodgkin lymphoma	98,865	72,955	171,820
9	Bladder	108,310	36,699	145,009
10	Brain	80,034	61,616	141,650
11	Oral Cancer	80,736	46,723	127,459
12	Kidney	62,696	39,199	101,895
13	Larynx	78,629	11,327	89,956
14	Multiple myeloma	32,696	29,839	62,535
15	Nasopharynx	34,913	15,419	50,332
16	Melanoma of skin	21,952	18,829	40,781
17	Thyroid	11,297	24,078	35,375
18	Hodkins lymphoma	14,460	8,352	22,812
	Female cancers			
	Breast		410,712	410,712
	Cervix uteri		273,505	273,505
	Ovary		124,860	124,860
	Corpus Uteri		50,327	50,327
	Male Cancers			
	Prostate	221,002		221,002
	Testis	8,878		8,878

1.1.2 Stages of carcinogenesis

All cells undergo complex changes in response to hormones, growth factors, cellular regulatory molecules and environmental insults. Cells divide, differentiate, some secrete milk proteins while others secrete hormones essential for the maintenance of the homeostasis and eventually all cells are destined to the same common event leading to death or apoptosis. The etiology of any cancer involves a complex interplay of genetic, hormonal and dietary factors. Initiation of a tumor cell is often attributed to irreversible damage or mutations caused to the DNA and this DNA damage can be induced by carcinogens such as tobacco smoke, synthetic chemicals, endogenous biochemicals, ionizing radiation, electromagnetic fields and viruses.

There are more than 100 distinct types of cancer and they all have acquired unregulated proliferative capabilities and the ability to disperse and metastasize from the original tumor to distal sites. Carcinogenesis is a multistage, multimechanism, process consisting of a single cell that has been irreversibly blocked from terminal differentiation (Figure 1). The initiation step which may be irreversible, can be triggered by a single exposure to a certain physical or chemical agent. Initiated cells may not produce a cancer during the lifetime of the organism, but sustained exposure to a non-carcinogenic agent or

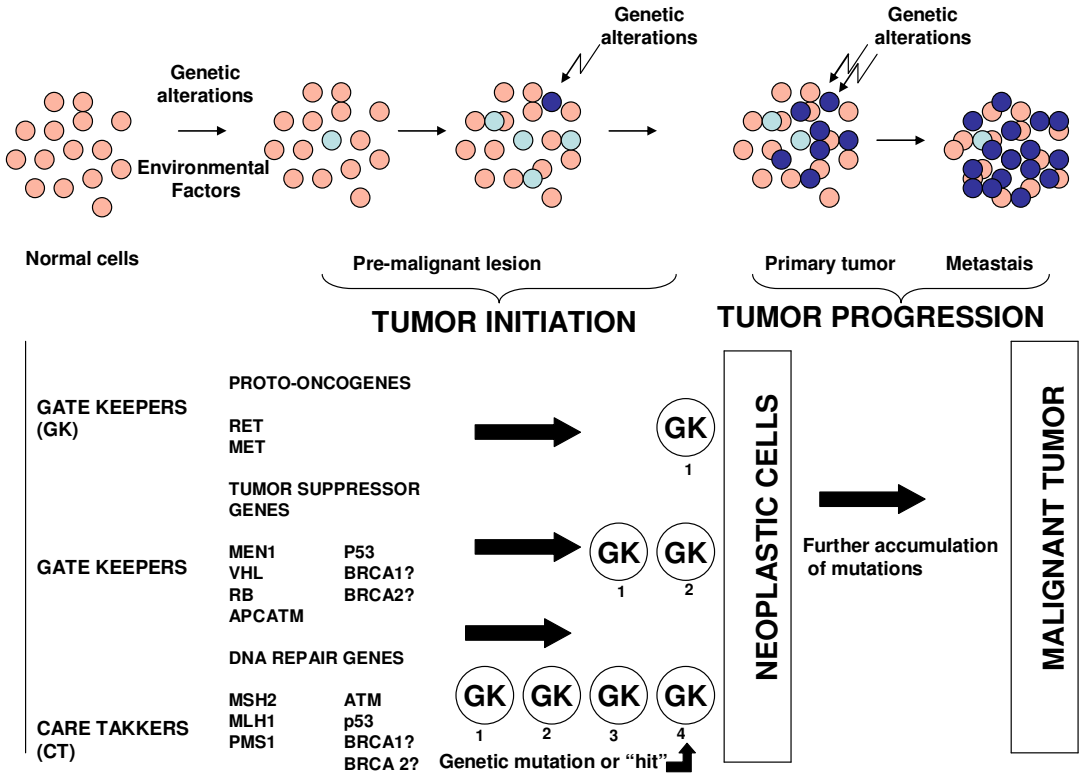


Figure 1. Stepwise malignant progression of human cancer (2, 3).

promoters can give rise to a tumor. Tumor promotion is potentially reversible as DNA damage could be repaired or it could result in clonal expansion of the initiated cell by a combination of growth stimulation and inhibition of apoptosis. Tumor promotion depends on sustained exposure to noncarcinogenic, and nonmutagenic agents or promoters such as phenobarbital, DDT, phorbol ester, and polybrominated biphenyls (4-6). In the promotion stage a single initiated cell undergoes clonal expansion and selective proliferation while simultaneously the

cell is prevented from undergoing apoptosis. The expanded initiated cell acquires sufficient mutations and epigenetic alterations to become growth stimulus independent and resistant to growth inhibitors and apoptosis which marks the progression stage of cancer (4, 7, 8).

1.1.3 Physiological alteration of cancer cells

Essentially all cancer cell genotypes are due to the manifestation of six critical alterations in cell physiology that collectively dictate uncontrolled malignant growth (8, 9) (Figure 2). These alterations include self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death/apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (8, 9).

1.1.3.1 Self sufficiency in growth signals

Normal and neoplastic cells differ in their dependence on mitogenic stimulation. The proliferation of normal cells requires the presence of extracellular growth factors whereas cancer cells have reduced dependence on external mitogenic stimulation (8-10). Cancer cells constitutively over-express growth factors and oncogenes that generate mitogenic signals; for example, growth factors like platelet-derived growth factor (PGDF) and transforming growth factor (TGFα) are highly expressed in glioblastomas and sarcomas (8, 9). In addition, membrane receptors such as epidermal growth factor receptors

(EGF-R/erbB2) and HER2/neu are also over-expressed and this enhances sensitivity to ambient levels of growth factors that normally would not trigger cell proliferation (2, 8)**.** Tumors of the stomach, brain and breast over express the EGF-R/erbB2 while stomach and mammary carcinomas over express HER2/neu receptors(8). In addition ligand independent activation of growth factor receptors is frequently observed due to alterations in the receptors such as the truncated version of the EGF-R which lacks the cytoplasmic domain and is constitutively active (8, 9).

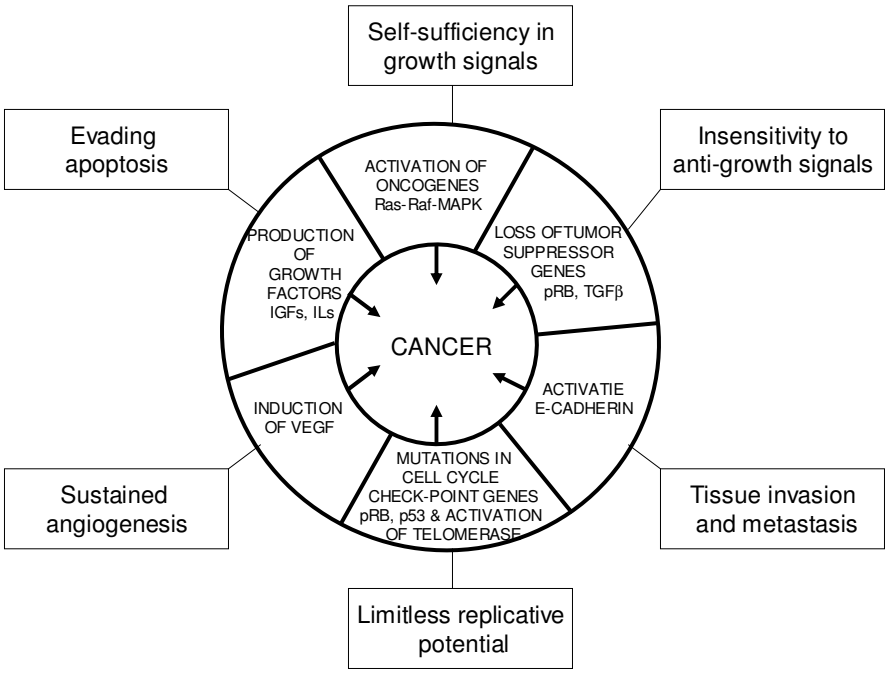


Figure 2. Acquired capacities of cancer cells (8).

Activation of oncogenes also enhances constitutive mitogenic signals. K-Ras mutations are highly prevalent in lung pancreatic and colon cancers and mutations in B-Raf protein are found in up to 66% of melanomas (11, 12). Ras activates a cascade of serine-threonine kinases like MEK, culminating in the activation of mitogen-activated protein kinase (MAPK) which in turn moves to the nucleus, and phosphorylates transcription factors responsible for genes involved in cell growth and survival (8, 9).

1.1.3.2 Insensitivity to antigrowth signals

Antigrowth signals can block proliferation by two distinct mechanisms. Cells may be forced out of the active proliferative cycle into the quiescent G0 state from which they may re-emerge at a later time, or they may enter into a postmitotic differentiation state. Many of the antiproliferative signals are regulated by the retinoblastoma (pRB) protein and its two related proteins p107 and p130. Hyperphosphorylated pRB blocks proliferation by sequestering E2F transcription factors that control expression of genes essential for progression from G1 to S phase (13). In many cancers such as retinoblastomas, osteosarcomas, cervical carcinomas and breast cancer, the pRB protein is not expressed because of mutations that disable the RB gene. Analysis of inactivating mutations in the RB gene indicated that most are the result of C-T transitions at CpG dinucleotides (CpG islands). These recurrent mutations may be a result of the deamination of 5-methylcystine within these CpG islands.

Many of these mutations result in truncated proteins as a result of premature termination of protein synthesis either through introduction of chain termination sequences or altered splice sites resulting in changes in the mRNA processing (14, 15). In cervical carcinomas, the pRB protein is sequestered and tagged for degradation by the E7 oncoprotein of type 16 and 18 human papillomaviruses (9, 16).

1.1.3.3 Evading apoptosis

Resistance to apoptosis can be acquired by cancer cells through a variety of changes. The functions of the p53 tumor suppressor gene which monitors stress and directs the cell toward an appropriate repair response are mutated in many tumors. p53 is responsive to many signals including anoxia, insufficiency of nucleotides for DNA synthesis, inappropriate activation of oncogenes, and DNA lesions such as single stranded breaks and covalent adducts (17). Loss of p53 function is observed in more than 70% of all human carcinomas, and results in the loss of a key DNA damage sensor that induces the apoptotic effector caspases (18). Analysis of p53 mutations has revealed hot spots which are localized to exons III, IV, V which encode for regions of the protein involved in DNA binding (17, 19). Within these exons are hotspot codons 157, 245, 248 and 273 which harbor CpG sites that are highly susceptible to DNA adduct formation (20-22).

1.1.3.4 Limitless replication potential

The ends of chromosomes, telomeres, act as counting devices for determining the number of cell generations. In 1978, Elizabeth Blackburn, working with the ciliated protozoan, Tetrahymena, found that telomeres consist of simple hexameric repeats of nucleotides TTGGGG (23). These telomeres are composed of several thousand repeats of these short 6 base-pair sequences and 50-100 repeats are lost every generation resulting in eventual exposure of chromosomal DNA due to lack of protection by telomeres. The unprotected chromosomal ends give rise to end-to-end chromosomal fusions, yielding the karyotypic disarray associated with cellular crises which trigger apoptosis or removal of the damaged cell (8, 24). To overcome this flaw, most human malignancies have telomerase activity in order to maintain telomerase length. Telomerase is an enzyme which adds hexanucleotide repeats onto the ends of chromosomes and thus permits unlimited multiplication of cancer cells. The extent of activation seems to be positively correlated with the aggressiveness of the disease; 98% of small cell lung carcinomas, 100% of invasive breast carcinomas, 70% of ductal carcinomas over-express telomerase activity (17). Moreover, ectopic expression of telomerase in presenescent cells renders them immortal (25, 26). Homozygous knockout mice of the cell cycle inhibitor p16 are tumor prone, particularly when exposed to carcinogens, and the tumors that arise have increased telomerase activity. However when carcinogens were

applied to mice that lacked telomerase activity, tumor incidence was reduced (27).

1.1.3.5 Sustained angiogenesis

Tumor growth is blocked when it reaches a 2 mm diameter unless adequate blood supply is provided. To overcome this deficit, tumors acquire elevated angiogenic activity to achieve greater access to the circulatory system. Tumors release many angiogenic signals, which attract and stimulate endothelial cells (9). Examples of angiogenesis initiating signals are vascular endothelial growth factor (VEGF) and fibroblast growth factors 1 and 2 (FGF1/2) which bind to transmembrane tyrosine kinase receptors on the surface of the newly recruited endothelial cells. (8). The VEGF gene is also under transcriptional control of the Ras oncogene, hence activation of the Ras oncogene results in up-regulation of the VEGF gene. Cancer cells express higher levels of angiogenic factors such as VEGFs and FGF in combination with down regulation of expression of inhibitors of angiogenesis such as thrombospondin-1 than compared to normal cells (8, 28, 29).

1.1.3.6 Ability to invade and metastasize

Primary human tumors acquire the ability to invade to other tissues and metastasize to form secondary tumors (2, 8). Several proteins involved in the tethering of cells to their surrounding tissue are altered in cells possessing

invasive capabilities. These affected proteins include cell-cell adhesion molecules (CAMs) notably members of the immunoglobulin and calcium dependent cadherin families, both of which mediate cell-to-cell interactions which link cells to extracellular matrix substances. The most widely observed alteration in cell-to-environment interactions in cancer involves E-cadherin, a homotypic cell-to-cell interaction molecule ubiquitously expressed on epithelial cells. Coupling of adjacent cells by E-cadherin conveys anti-growth and other signals via cytoplasmic contact with β-catenin to intracellular signaling circuits that include the Lef/Tcf transcription factors (8, 30, 31). Examination of cancer cells revels that E-cadherin function is apparently lost in a majority of the epithelial cells through mutations of the E-cadherin or β-catenin genes, transcriptional repression, or proteolysis of the extracellular cadherin domain. Forced expression of these genes in transgenic mouse model of carcinogenesis impairs the invasive and metastatic phenotype, whereas interference with these genes enhances both capabilities. (8, 30, 31).

Alterations in the expression of CAMs in the immunoglobulin superfamily also appears to play critical roles in invasion and metastasis. CAMs can undergo a switch in expression from a highly adhesive to poorly adhesive forms in Wilms' tumors, neuroblastoma, and small cell lung cancer and reduction in their overall expression is observed in invasive pancreatic and colorectal cancers. (32).

1.1.4 Environmental and life style contributions to cancer incidence

The health of an individual and the population is a result of the interaction between their genetics and environmental factors. Nutrition is an important environmental factor in cancer incidence (33) and it has been estimated that nutritional factors contribute 20-60% in development of all cancers worldwide and approximately one third of cancer deaths in Western countries (34, 35) (Table 3). Many studies have been conducted in an attempt to support and clarify which specific nutritional factors contributed to cancer incidence. However, with the exception of the contributions from obesity, physical inactivity, alcohol and lack of dietary folate and calcium, the remaining nutritional factors that increase or decrease the risks for human cancers are still largely unknown (34). During the past 50-70 years, there have been major lifestyle changes which have lead to increased average body weight and overall obesity. The MONICA project, sponsored by the World Health Organization (WHO) showed that in the 1990s 20% of all individuals in parts of Western Europe and the USA and almost 35% of the population in the Eastern European countries were obese (36). In developing countries, the prevalence of obesity is generally much lower, but it has reached 10-15% in some urban areas of Namibia, Zimbabwe, Turkey, Guatemala, Honduras, Mexico, and Peru (36).

The high prevalence of obesity in developed countries can be explained by a lifestyle characterized by over-consumption of energy combined with low physical activity. Obesity is a well known risk factor for cardiovascular disease.

Table 3. Influences of diet and nutrition on various types of cancer (34).

	Breast	Prostate	Lung	Colon	Stomach	Esophagus	Endometrial
Macronutrients/ energy balance							
Obesity	++	+		++	+	+	++
Glycemic index/ Load	+	+		++			+
Animal fat							+
Foods							
Red/processed Meat		+		+			
Fruits	-	-	-	-	-	-	-
Vegetables		-		-	-	-	-
Nutrients	-						
Folic acid	++		-	--			
Alcohol				++		++	
Calcium		+		-			
Vitamin D		-		-			
B-Carotene supplements			++		-		
Lycopene-containing foods		-	-		-		

++ indicates strong correlation with increase incidence of cancer
+ indicates slight correlation with increased incidence of cancer
-- indicates strong correlation with decreased incidence of cancer
- indicates slight correlation with decreased incidence of cancer

and diabetes, and epidemiological studies are providing increasing evidence for a link between body weight and cancer risk. A 16 year study was conducted by the American Cancer Society consisting of more than 900,000 US adults (404,576 men and 495,477 women) who were free of cancer at enrollment in 1982, and they reported 57,145 deaths from cancer during the 16 years of follow-up. Death rates were approximately 52% and 62% higher for men and women respectively who were obese according to their body-mass index (BMI- the weight in kilograms divided by the square of the height in meters) compared to individuals with normal weight. In both men and women body mass index was also significantly associated with higher rates of death from cancers of the esophagus, colon and rectum, liver, gallbladder, pancreas and kidney, and similar results were observed for non-Hodgkin's lymphoma and multiple myeloma. Significantly increased incidence of death was observed for cancers of the stomach and prostate in men and breast, uterus, cervix, and ovary in women. Based on these observations, the American Cancer Society estimated that current patterns of overweight and obesity in the US could account for 14% of all deaths from cancer in men and 20% of cancer deaths in women (37, 38).

Body weight modulates metabolic factors that in turn affect cancer risk, particularly on circulating levels of growth factors and steroid hormones and their binding factors. These effects vary with gender and the menopausal status in women. A linear increase in circulating levels of insulin occurs with increasing

BMI in both men and women. Insulin controls the uptake and use of glucose in peripheral tissues. With increasing caloric consumption and weight gain, tissues become insensitive to insulin and this is compensated by production of more insulin resulting in hyperinsulinemia. Insulin-like growth factors (IGFs) are mitogens that regulate energy-dependent growth processes (39). IGF-1 stimulates cell proliferation and inhibits apoptosis and has strong mitogenic effects in a wide variety of cancer cell lines. The synthesis of IGF-1 and its main binding protein, IGF-1 binding protein-3 (IGFBP-3) are regulated by pituitary growth hormone (GH). More than 90% of IGF-1 is bound to circulating IGFBP-3. Obesity and other conditions related to the hyperinsulinemia result in elevated blood glucose levels, decreased levels of IGF-binding proteins 1 and 2 and higher levels of free plasma IGF-1. Insulin and free IGF-1 interact with and regulate the synthesis and bioactivity of sex steroids that affect the development and progression of certain cancers (Figure 3) (40). Chronic hyperinsulinemia inhibits hepatic synthesis of sex-hormone binding protein/globulin (SHBG), resulting in increased levels of bioavailable androgens and estrogens that are not bound to SHBG and these free steroid hormones determine the available estrogen and androgen levels essential for growth and proliferation of cancer cells (37, 38).

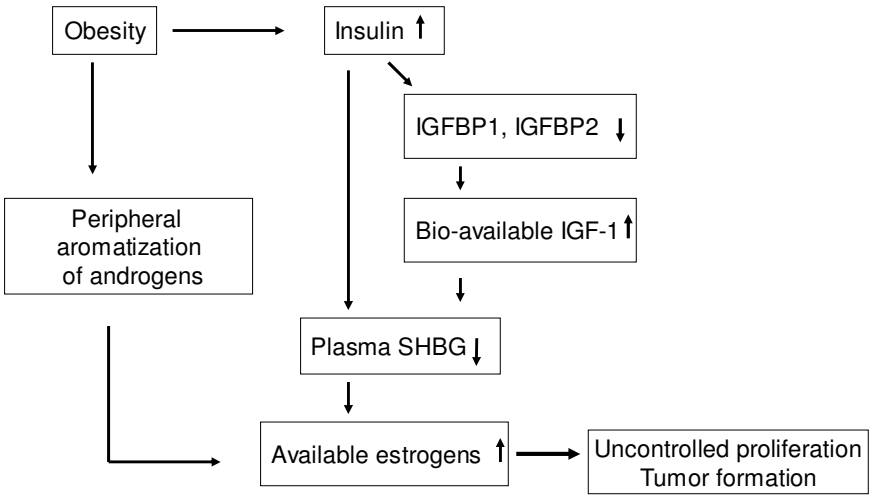


Figure 3. Mechanism underlying obesity and cancer (38).

1.1.5 Epigenetic contributions to cancer incidence

Aberrant DNA methylation of specific gene promoter regions is a key mechanism for inactivation of genes that suppress tumorigenesis (Tables 4,5). In normal cells, the pattern of DNA methylation is conserved after DNA replication and cell division by the methylation of cysteine by a maintenance DNA methyltransferase (DNMT1). DNA methylation of genes occurs primarily in the promoter regions that contain CpG islands, which are defined as a 1 kb stretch of DNA that contain guanine rich sequences at a higher frequency than the rest of the genome. Genes that are involved in every step of tumor formation can be silenced by methylation (41). Genomic screening of 98 different primary tumors has revealed that on an average there exists approximately 600 aberrantly methylated CpG islands in each tumor (41, 42).

Table 4. Different types of genes silenced by aberrant DNA methylation (41).

Acquired Capability	Gene silenced by DNA methylation	Gene function
Insensitivity to antigrowth signals	P16CDKN2A	Cyclin-kinase inhibitor induce differentiation cell cycle arrest
Self Sufficiency in growth signals	RASSF1A Caspase 8 TMS1 DAP kinase p14 ARF	Regulation of RAS pathway Initiate apoptosis Proapoptotic Proapoptotic Proapoptotic
Limitless replicative potential	Rb	Tumor suppressor gene
Sustained angiogenesis	Thrombospondin-1	Angiogenesis inhibitor
Increased invasion and metastasis	E-cadherin TIMP3 hMLH1	Suppress metastasis Inhibit metastasis DNA mismatch repair
Genome instability	MGMT BRCA1	Repair alkylated guanine Repair DNA damage

Table 5. Epigenetic genes disrupted in human cancer (43).

Function	Gene	Alteration	Tumor Profile
DNA methyltransferases	DNMT1	Overexpression	Multiple types
	DNMT3b	Overexpression	Multiple types
Methyl-CpG binding proteins	MeCP2	Overexpression, mutations	Multiple types
	MBD1	Overexpression, mutations	Multiple types
	MBD2	Overexpression, mutations	Multiple types
	MBD3	Overexpression, mutations	Multiple types
	MBD4	Inactivating mutations	Colon, stomach, endometrium
Histone acetyltransferases	p300	Mutations	Colon, stomach, endometrium
	CBP	Mutations, homozygous deletions	Colon, stomach, endometrium, lung
	pCAF	Rare mutations	Colon
	MOZ	Translocations	Haematological malignancies
	MORF	Translocations	Haematological malignancies
Histone deacetylases	HDAC1	Imbalanced expression	Multiple types
	HDAC2	Imbalanced expression	Multiple types
Histone methyltransferases	RIZ1	CpG island hypermethylation, mutation	Multiple types
	MLL1	Translocations	Haematological malignancies
Chromatin remodeling factors	EMSY	Gene amplification and over expression	Breast
	BRMS1	Loss of expression	Breast
	MTA1	Overexpression	Breast, Haematological malignaincies
	MTA3	Overexpression	Breast, Haematological malignaincies
SWI/SNF family proteins	PASG	Mutations	Haematological malignancies
	BRG1	Homozygous deletion, mutation	Lung
	HLTF	CpG island hyper methylation	Multiple types

Although it is unclear which specific molecular events lead to aberrant methylation, there are several hypotheses that try to explain this process. One hypothesis is that it could be due to infidelity of DNMT1. The substrate for this DNA methyltransferase is hemimethylated DNA. Methylation occurs immediately after DNA replication with the primary function to ensure that the identical methylation patterns of the parental cell are passed on to the next generation daughter cell. The DNA methyltransferase methylates genomic DNA at very high fidelity, but commits errors at a very low frequency (41, 44). Other DNA methylating enzymes that could be involved in aberrant DNA methylation are de novo methyltransferases which include DNMT3a and DNMT3b which use unmethylated DNA as their template and play an important role in embryonic development (41, 45, 46). Another possible mechanism responsible for gene hypermethylation could be due to a faulty repair mechanism of aberrantly methylated DNA. DNA demethylase is a relatively new enzyme that has the potential to function as a repair enzyme, and correct aberrantly methylated CpG sequences. The factors that control the template specificity of DNA demethylase have not been identified and further research is required to clarify its role in regulating cell growth and differentiation (41, 47).

Chromatin remodeling also plays an important role in the regulation and expression of certain genes. Modification of the N-terminal group of lysine in histones by acetylation and deacetylation changes the configuration of nucleosomes and chromatin structure. The acetylation of lysine by histone

acetyltransferase removes a positive charge on DNA and the compact structure of the DNA is lost resulting in opening of the chromatin structure, which facilitates gene transcription. Histone deacetylase (HDAC) removes acetyl groups from lysines and reverses this process and restores the positive charges on lysines resulting in a compact chromatin structure which represses transcription and leads to silencing of genes (41). Aberrant deacetylation of histones in nucleosomes is due to dysregulation of specific HDACs and may be associated with neoplastic transformation.

In summary, it is apparent that many factors merge together to result in a malignant phenotype. There is a fine balance between gene expression and environmental and behavioral factors and their interactions which results in cellular events that lead to cancer initiation, promotion and progression.

1.2 Breast cancer

1.2.1 Mammary gland development

An understanding of breast development and its morphology is important for determining the underlying causes of breast cancer. The mammary gland is a structurally dynamic organ which undergoes changes with age, menstrual cycle and reproductive status. Mammary gland development is initiated in the embryo, and major changes occur during the pre-pubertal phase under the main stimuli of pituitary and ovarian hormones (48, 49). The mammary gland first appears around day 10 of embryonic development as five pairs of placodes, and

by day 15 the placodes have formed epithelial buds surrounded by mesenchymal layers. These structures develop into branching networks of ducts terminating in end buds through the signaling between the epithelium, the mammary mesenchyme and the stromal fat pad (48, 49). Mammary gland development during the childhood primarily keeps pace with the general growth of the body until the approach of puberty or adolescence. The adolescent period begins with the first signs of sexual change at puberty and terminates with sexual maturity (48, 49). Changes in the hormonal environment during puberty are the major factors controlling mammary gland development. In the male, further development is inhibited by production of testosterone. With the approach of puberty, the rudimentary mammae begin to show growth activity both in the glandular tissue and the surrounding stroma. Glandular growth is due to proliferation and division of small bundles of primary and secondary ducts which grow and divide by repeated bifurcations form the main axis and successive secondary axes. The ducts grow and divide to form club-shaped terminal end buds which give rise to alveolar buds (49). Alveolar buds cluster around a terminal forming the lobule type 1 or virginal lobule and each cluster is composed of approximately 11 alveolar buds. Alveolar buds and terminal ducts are lined by a two-layer epithelium. Lobule formation in the female breast occurs with in 1-2 years after onset of the first menstrual period. Full differentiation of the mammary gland is a gradual process taking many years, and in some cases, if pregnancy does not occur, is never fully attained (48-50).

The breast of an adult woman contains three types of lobules, type 1, 2 and 3. The breast of nulliparous women is mostly comprised of undifferentiated structures, such as the terminal end buds and type 1 lobules, which are potential sites for development of preneoplastic lesions which eventually progress into invasive carcinomas. Type 2 lobules are present in moderate numbers during the early years but decrease drastically after age 23. In parous women, type 3 lobules are the predominant structures during pregnancy until the fourth decade of life after which the number of type 3 lobules decrease. At this stage breasts of both nulliparous and parous women are similar and they both have mostly type 1 lobules, but the type 1 lobules in parous women are different because they are fully differentiated and are therefore less susceptible for development into malignant tumors (48-50).

The normal mammary gland is composed of two cellular components, the mesenchymal component of fatty stroma containing the blood vessels and nerves and the epithelial compartment of ducts and lobules. The epithelial component is made up of three distinct cell types, the epithelial cells which line the ducts, the alveolar cells which line the alveoli and the myoepithelial cells that are between the epithelial cells and the alveolar cells and the basement membrane. Tumors and malignancies in humans and rodent models usually occur in the epithelial cells, specifically the undifferentiated terminal end buds, whereas benign lesions usually occur in both epithelial and myoepithelial cells (48, 49)

Mammary gland development and differentiation is maximal during pregnancy when the duct system of the gland branches and enlarges. There is active cell division and increase in size in the epithelial cells of the newly formed lobules. This phase is driven by the hormones 17β-estrodial (E2), progesterone and prolactin. Estrogen mainly acts on the ductal system; progesterone promotes alveolar development in the second and third trimester. At parturition, there is a drop in estrogen and progesterone blood levels. There is an increase in prolactin release from the anterior pituitary in response to the suckling stimulus from the infant. As suckling continues, there is large volumes of milk produced, and at the end of this period, the epithelial cells are lost due to apoptosis and mammary glands reduce in size and return to a resting phase (48, 49).

1.2.2 Life style and behavioral risk factors for breast cancer

The major breast cancer risk factors include obesity, alcohol consumption, physical activity, exogenous hormones such as oral contraceptives or hormone replacement therapy. Diet and nutrition are also important however the precise contributions of various components of the diet are unclear. Reproductive factors such as early menarche (before age 12) greater frequency of ovulatory cycles, late age of first birth or multiparity, lack of lactation, late menopause, greater number of lifetime ovulatory cycles and greater time between menarche and menopause also increase the risk of breast

cancer (51-53). The reproductive risk factors suggest that a woman's overall lifetime exposure to estrogens enhances breast cancer incidence. The Nurses' Health Study reported that women who were engaged in an average of 7 or more hours per week of physical activity had an 18% lower chance of developing breast cancer than women who engaged in less than 1 hour per week of such activities (54). Increased breast cancer risk is associated with alcohol use, obesity, increased meat (red) intake, diets rich in fat and low in fiber. Epidemiological data suggest that consumption of soy products is associated with a lower risk of breast cancer (51, 55, 56). However, recent evidence suggests that one component of soy, genistein, may promote the growth of some estrogen-sensitive tumors and reduce the efficiency of antiestrogen tamoxifen (51, 57, 58). Phytoestrogens can act as weak estrogens and as antagonists depending on the hormonal status of the host. High phytoestrogen intake can compete with endogenous estrogens in premenopausal women and reduce overall estrogen exposure of the target tissue; phytoestrogens can also increase estrogenic response in women with low endogenous levels of estrogens in post-menopausal women (51, 52). Specific micronutrients have been associated with reduced risk of breast cancer and these include carotenoids, folate, calcium, vitamin D, lycopene and vitamin C (51, 54). Many of these studies on micronutrients give variable results and may not be conclusive.

1.2.3 Genetic risk factors

Approximately 10-15% of all breast cancers are thought to be familial, with about one third of these cases attributed to inherited cancer-susceptibility genes, designated BRCA1 and BRCA2 (59). BRCA1 gene was identified by linkage analysis in 1990 and linkage was established to the long arm of chromosome 17 at region q21 with the help of polymorphic markers. Familial breast cancer, characterized by frequent occurrence of male breast cancer is linked to the BRCA2 locus located on 13q. Along with BRCA1 and BRCA2 a third gene, p53 located on 17p13, when inherited in a mutated from can confer high lifetime risks for developing breast cancer (59-61).

Germ-line mutations of BRCA1 and BRCA2 genes account for approximately 40% of inherited breast cancer cases. It is estimated that in families that have mutations in BRCA1 or BRCA2 , the cumulative risk of developing breast cancer is 71% up to the age of 70, which corresponds to 10-20 fold increased risk compared to women who do not have the familial gene mutations (59, 60). Mutations in BRCA1 and BRCA2 are associated with very early-onset of breast cancer. As many as 33% of women under the age of 29 with breast cancer carry a mutation in BRCA1 or BRCA2. These individuals also have a 44% greater chance for developing ovarian cancer by age 70 (59).

BRCA1 protein is expressed in a wide range of tissues, including the breast. It is highly expressed in tissues containing rapidly proliferating cells that are involved in differentiation. The BRCA1 gene consists of 24 exons and the

size of the mRNA is 7.8 kb and expression is increased during G1 to S phase. The gene product of BRCA1 is a phosphorylated protein that consists of 1863 amino acids and has a molecular weight of 220 kDa. A ring finger structure is present at the N-terminal region and a binding site for RAD 51 has been identified in the central part of the primary structure. Two BRCT domains are present at the C-terminal region and take part in DNA repair and may be involved in transcriptional activation and chromatin remodeling. BRCA1 has many functions, including repair of oxidative DNA damage and BRCA1 is also involved with homologous recombination and repair in combination with RAD51 (62). BRCA1 regulates gene expression as a co-activator in a p53 dependent manner and as a co-repressor of estrogen induced transcription. BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor (ER) and acts as a ligand reversible barrier to transcriptional activation by unliganded ER. BRCA1 deficient cells exhibit decreased cell growth and hypersensitivity to ionizing radiation, giving rise to DNA damage associated with chromosomal abnormalities including breaks in DNA and aneuploidy in the successive daughter cells. Therefore it is believed that BRCA1 contributes to the maintenance of chromosomal stability (62-64).

BRCA2 gene has a total length of 70 kb and consists of 27 exons and has a transcriptionally active product of approximately 11.5kb. The BRCA2 gene is highly expressed in the thymus, testis, mammary tissue and the ovaries (62, 65) and the expression of BRCA2 is cell-cycle dependent. The protein is 378 kDa

and has 3418 amino acids with no homology to BRCA1 protein although it shares similar structural features and expression patterns. BRCA2 also has a RAD51 binding site and contributes to the maintenance of chromosomal stability and homologous recombination (62, 66).

Since BRCA1 and BRCA2 are expressed in the same tissue and cell types they interact with each other and RAD51 along with other proteins involved in DNA repair and recombination. BRCA1 is phosphorylated by a protein kinase called ATM (ataxia-telangiectasia) as a result of DNA damage and the hyperphosphorylated BRCA1 co-localizes with BRCA2 and RAD 51 protein in the nuclear foci at S-phase of the cell cycle(59, 66-68).

Breast cancer is also found in rare genetic syndromes, such as Li-Fraumeni syndrome (LFS). The spectrum of cancers in LFS families include soft tissue sarcomas, brain tumors, osteosarcomas, leukemia, and adenocorticol carcinomas and characteristically develop at very early age with presence of multiple tumors (59, 62, 66). Genetic analysis shows that families at risk have a 50% probability of developing invasive cancers by age 30 and more than a 90% chance by age 70. The tumor suppressor gene p53 is often reported to be mutated in the LFS families. Germline mutations in the p53 gene account for up to 1% of breast cancer cases diagnosed under the age of 35 (59).

Carriers of homozygous or compound heterozygous mutations in the ATM gene suffer from the rare recessive disorder ataxia-telangiectasia (AT). AT is a degenerative disorder that is characterized by progressive cerebral ataxia, a

weakened immune system, high sensitivity to ionizing radiation, distinctive dilated blood vessels in the eyes and skin and increased susceptibility to cancer. ATM is a protein kinase that phosphorylates BRCA1, activating its DNA repair response upon DNA damage. Heterozygous carriers of ATM mutations exhibit a five-fold increased risk for breast cancer (66-68).

1.2.4 Environmental risk factor

There are well known geographical variations in breast cancer rates suggesting a role for the environment in the etiology of breast cancer. Breast cancer incidence in the US are among the highest in the world and rates in Western industrialized countries are as much as five-to-eight fold higher than rates in Asia and Africa (69). Studies indicate that as populations migrate from the low to high-risk geographical areas the incidence of breast cancer in these individuals approaches that of the host country within one or two generations suggesting that environmental factors play a major role in determining incidence of breast cancer (69-71) . Female age, reproductive cycles, inheritance patter of breast cancer susceptibility genes (BRCA1 and BRCA2), and other known factors explain only about 40% of breast cancer cases in the US and it is estimated that up to 60% of breast cancer cases have an environmental etiology (69).

Most scientists believe that breast cancer is the result of complex interactions of internally and externally introduced factors that occur over long

periods of time. A number of environmental chemicals induce mammary cancer in rodents and similar effects in humans, and these genotoxic agents are broadly classified as tumor initiators or tumor promoters. These compounds act at many junctions during the development of breast cancer and some of them act by modulating endocrine pathways. These chemicals and other endocrine disruptors have industrial origins and enter the environment as industrial by-products/wastes or through their use in production of pesticides and other industrial chemicals (Tables 6,7) (69).

Some chemicals are present both in the environment and workplace. These chemicals may be used in manufacture of glues, paints, varnishes, solvents and many other products. Band and coworkers (2000) have noted increased incidence of breast cancer linked to occupations involving exposure to solvents used in the printing and publishing, laundry and dry cleaning industries, mechanics, aircraft workers, automotive repair workers, gasoline service station workers are all exposed to solvents and exhibit increased rates of breast cancer (72). Only one case control study assessing breast cancer risk found an increased risk for post-menopausal breast cancer among those working in the laundry and dry cleaning industries (72). A cohort study of women employed in coiling and wire drawing in the manufacture of light bulbs where methylene chloride and trichloroethylene were used had an excess of breast cancer incidence among those who worked for more than 5 years (73). In another study increased rates of breast cancer were observed in pre and

postmenopausal women working in the publishing and printing industry (72). It should be noted that all these studies suffer some shortcomings since the occupational studies rely on historical assessment of exposure to organic solvents which are short-lived in the body (69).

Exposure to metals may also be associated with breast cancer risk. Martin and coworkers (2003) demonstrated that in MCF-7 breast cancer cells, divalent cadmium, copper, cobalt, nickel, lead, mercury, tin, chromium, arsenic, selenite and vanadate activated responses mediated by ERα. In addition, the metalloestrogen-induced activation of ERα was more potent than phytoestrogens, and most other environmental estrogenic compounds (74).

Cadmium in particular was shown to induce estrogenic responses along with increased uterine weights, hyperplasia, hypertrophy of the endometrial lining and increased mammary epithelial density in female rats (74). However, the effects of heavy metals on breast cancer rates in humans has not been fully determined.

Viral exposure may be involved in the genesis of breast cancer. Two possible viral candidates are the Epstein-Barr virus (EBV) and mouse mammary tumor virus (MMTV) (69). The EBV is a ubiquitous human γ herpes virus that infects and establishes a mostly asymptomatic life-long infection in B lymphocytes. The EBV genome has been detected in subset of breast tumor specimens and in a study by Yasui and colleagues (2001) it was noted that breast cancer rates were higher in individuals who had a history to

Table 6. Chemicals associated with increased incidence of mammary gland tumors in rats and mice tested by US National Toxicology program (75).

Chemical	**Use**
Benzene	Gasoline solvent
2,2'-bis(Bromomethyl)-1,3-propanediol	Flame retardant
1,3-Butadiene	Auto exhaust, rubber manufacturing, gasoline
C.I. acid red 114	Dye for jute, silk , leather
C.I.basic red 9 monohydrochloride	Dye for paper, textiles, leather
2-chloroacetophenone	Flame retardant
Chloroprene	Neoprene manufacture
Clonitralid	Molluskicide
Cytembena	Pharmaceuticals
2,4-diaminotoluene	Intermediate in dye industry
1,2-Dibromo-3-chloropropane	Soil fungicide and pesticide
1,2-Dibromoethane	Soil fungicide, lead scavenger in gasoline
2,3-Dibromo-1-propanol	Flame retardant
1,1-Dichloroethane	Solvent
1,2-Dichloroethane	Solvent and intermediate in insecticide manufacture
Propylene dichloride	Solvent in dry cleaning fluids, fumigant
1,2-Dimethoxybenzidine dihydrochloride	Dye intermediate
3,3-Dimethoxybenzidine dihydrochloride	Dye intermediate
2,4-Dinitrotoluene	Dye intermediate, explosives, propellants
Ethylene oxide	Sterilizing gas for medical equipment
Furosemide	Pharmaceuticals
Glycidol	Intermediates in pesticides and fragrances, plastic stabilizers
Hydrazobenzene	Dye intermediates, tobacco pesticides, motor oil
Isophosphamine	Pharmaceuticals
Isoprene	By-product of ethylene production
Methylene chloride	Solvent, furniture stripper, adhesives
Nitromethane	Rocket and engine fuel. Solvent, mining explosive
1,2,3-trichloropropane	Chemical intermediate, solvent for paint remover
Sulfallate	Herbicide

Table 7. Endocrine disruptors (75).

Compound	Exposure / Uses
Pesticides	
Atrazine	Selective herbicide
Chlordane	Insecticide, acaricide, veterinary pharmaceutical
Chlorpyrifos	Insecticide, acaricide
Cypermethrin	Insecticide
Lindane	Insecticide
Malathion	Insecticide
Methoxychlor	Insecticide Veterinary pharmaceutical
Pentachlorophenol	Insecticide and wood preservative
Permethrin	Insecticide
Toxaphene	Insecticide
Tributyl tin	Biocide, rodent repellant
Vinclozolin	Agriculture fungicide
Organochlorines and PAHs	
PAHs	Industrial air pollutants, smoke from coal/coke-burners, tobacco tar
Polybrominated biphenyls	Flame retardants
polybrominated diphenul ethers	Flame retardants
PCBs (Aroclor 1254)	Transformers and electrical equipment (NO LONGER IN USE)
Dioxins and furans	Products of incineration, paper manufacturing and herbicides
Phenols and akkylphenols	
Biphenol A	Polycarbonate and polyester-styrene resins
4-tert-butylphenol	Intermediates in manufacture of varnish and lacquer resins, soap, antioxidants
Nonylphenol polyethoxylate	Surfactants, detergent, defoaming agents, pesticide formulation, rubber industry
Phthalates	
Butyl benzyl phthalate	Commercial plasticizer for polyvinyl chloride polymers
Di/n-butyl/ethyl phthalate	Personal care products such as nail polish, perfume, hair spray, inks, adhesives
Parabens	
Butyl, ethyl, methyl, propyl paraben	Pharmaceutical aid (antifungal), preservative in foods, creams, lotions, ointments
Other organics	
Amsonic acid	Dyes, bleaching agents, optical brighteners or fluorescent whitening agents
Styrene	Manufacturing of plastics, synthetic rubber, resins, insulator
Vinyl acetate	Production of polymers, adhesives, paints, food packing
Metals	
Cadmium, lead	Batteries, plastic stabilizers, pigments
Mercury	Thermometers, dentistry, pharmaceuticals, agricultural chemicals
Phytoestrogens	
Genistein, Coumestrol, Zearalenone	Soy, grains, grain molds

mononucleosis, (caused by EBV) (76). In one study, MMTV-like envelope protein gene sequences were detected in 37% of human breast tumors, but not in normal breast tissue Future work may involve the use of vaccines to prevent or modify the primary infection to reduce breast cancer risk (77).

Organochlorines (OC) are a group of synthetic chemicals that are abundant in the environment as pesticides or industrial by-products. The most abundant of the OCs are dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCB), which were introduced in the US in 1945 and then were banned in the 1970s (69, 78). DDT was widely used in the US as an insecticide in forestry and agriculture from 1945 until the early to mid 1960s. PCBs were used in dielectric fluids in transformers and capacitors, as plasticizers, lubricants and heat transfer fluids and in manufacture of paper and paint until they were banned in 1977 (69). OCs degrade slowly as they accumulated in the food chain due to their chemical stability and they are found in human adipose tissue, blood and breast milk. The most prevalent OC residue found in human tissues is dichlorodiphenyltrichloroethylene (DDE) which is the major metabolite of DDT (69). A case control study conducted in 1993 by Wolff and colleagues in New York showed that there was a two to four-fold increase in the occurrence of breast cancer among women with the highest serum levels of DDE and PCBs compared to women with the lowest levels (79). However, many studies conducted since then did not find an increased risk of breast cancer with exposure to OCs (80-84).

1.2.7 Role of ER in breast cancer

Estradiol (E2) is a steroid hormone that exhibits important biological functions in target tissues such as the female and male reproductive tract. The growth of the mammary gland and uterine endometrium during pregnancy and menstrual cycle is dependent on estrogen. E2 is also important in the functioning of other tissues, such as bone, liver, brain and the cardiovascular system. In addition to its role as a proliferative agent in normal cells, E2 has also been linked to the initiation, promotion and progression of breast and endometrial cancers.(85). . One of the earliest studies showing a relationship between breast cancer and ovarian hormones was the observed regression of breast cancer after removal of ovaries, the major site of estrogen production in premenopausal women (86). The classical scheme of ER action involves ligand (E2) binding to the ER, dissociation of heat shock proteins from the ER and receptor dimerization. The dimer then binds to DNA sequences called estrogen response elements (ERE) that are located in the regulatory regions of responsive target genes which recruit coactivators and other transcription factors to activate gene expression (87)

1.2.8. Expression of the Estrogen Receptor (ERα) in breast cancer

Breast cancers can be divided into two subtypes based on their expression of ERα. The ER status of breast tumors is important because circulating estrogens can bind ER and stimulate cell division and tumor growth where as ER-negative breast tumors are resistant to the effects of estrogens (88).

Most invasive breast cancers (IBCs) are derived from premalignant breast lesions, which in turn arise from normal epithelial cells lining the terminal duct lobular units (TDLU) (88, 89). All epithelial cells or their ancestors must express ER at some time during their lifecycle. Immunochemical examination of a normal adult woman would show that nearly all TDLUs contain ER positive cells, but only 30% on average are ER positive at any given time. These ER positive cells are mostly non-dividing. In the 30%, the fraction of cells that are dividing only account for less than 5% of the overall population (88). A normal breast consists of largely ER negative non-dividing cells and they may arise from ER positive or other wise estrogen-responsive stem cells that have differentiated in diverse directions (88)

ER expression and growth of breast cancer are closely correlated. IBCs contain on average 75% ER positive cells, but this can vary from less than 1% to 100%. In ER positive IBCs, the proportion of dividing cells that are ER positive also varies from less than 1% to nearly 100%. The remaining 25% of the cells which are ER negative exhibit estrogen independent proliferation (88, 90).

ER-negative IBCs are histologically high grade, rapidly proliferating tumors derived from ER-negative ductal carcinomas in situ (DCIS) and the two types of cells share many similarities. Most DCIS have evolved from atypical ductal hyperplasias (ADH) in which nearly all cells are ER-positive, however ER-negative cells are present in all premalignant lesions including ADH and they may be involved as progenitor cells in the development of ER-negative DCIS. Human mammary epithelial cells show gene expression patterns similar to basal IBCs, which are also ER-negative, suggesting that they may represent a stem cell population for ER-negative breast cancer (91, 92)

1.2.9 Treatment and prevention of breast cancer

Surgical removal of cancerous tissue is a very highly effective method of treatment for node negative breast cancer, however this does not affect distal metastasis where the cancer has spread to surrounding tissues such as the lymph nodes. Treatment of gross micrometastasis and multifocal isolateral and contralateral breast cancer requires a mastectomy, adjuvant and radiation therapy. Depending on the extent of cancer, two types of surgical procedures are available. Breast conserving surgery involves complete removal of neoplastic tissue and a small amount of normal tissue and the cosmetic effects can be addressed with post reconstructive surgery. This initial option is limited to patients with early stage breast cancer. The second procedure is a total mastectomy, which involves the complete removal of the mammary gland,

axillary lymph nodes and fascia of the underlying pectoral muscle and this is the primary option for patients with late stage breast cancer as well as for women with a high genetic/familial risk of breast cancer (93, 94).

Endocrine therapy is another method for managing breast cancer. Normal breast tissue is hormonally regulated and mammary neoplasia progresses from a state of hormone-responsiveness (dependent) to hormone-nonresponsiveness (independent). Neoplastic cells initially take advantage of the growth hormones of the endocrine system to rapidly proliferate in a hormone-dependent manner and endocrine therapy eliminates the production of or antagonizes the effects of these growth/steroid hormones (95).

The most primitive form of hormone ablation is removal of endocrine glands namely the ovaries, adrenals and pituitary (96). More than 100 years ago, George Beatson showed that metastatic breast cancer could be forced into remission by bilateral removal of the ovaries, as it is the primary site of estrogen production and this surgery increases the lifespan of premenopausal breast cancer patients. The decrease in E2 production can be as much as 20-fold (200pg/ml to 10 pg/ml) (97, 98). Since androgens are precursors of E2 that are primarily produced in the adrenal cortex, adrenalectomy prevents androgen production and their conversion into E2. The pituitary gland secretes LH and FSH to induce E2 and progesterone production in the adipose tissue. Hence a hypophysectomy stops production of LH and FSH so they are no longer able to stimulate production of E2 in the adipose tissue. A hypophysectomy is usually

recommended for postmenopausal women as the adipose tissue is still a source of E2 production (96). Although surgical techniques are effective against hormone-responsive tumors, these procedures are both invasive and irreversible; hence they have been replaced by specific antiestrogenic drugs.

Chemotherapeutic treatment of cancer has made significant advances in the past three decades. This was made possible due to the increased knowledge and understanding of the roles of membrane receptors, signal transduction pathways and transcription factors involved in the tumorigenic process.

1.2.10 Selective estrogen receptor modulators (SERMs)

The ER is essential for normal breast development and for mammary carcinogenesis. This receptor has been an important target for drug development. SERMS play a key role in breast cancer chemotherapy. These agents antagonize the effects of estrogens in some tissues and mimic their action in others (99). The introduction of the antiestrogen tamoxifen in the early 1970s represented a landmark in the treatment of breast cancer. Over the past three decades tamoxifen has been proven to be effective for treatment of early and advanced breast cancer and has been successfully used for chemoprevention of breast cancer in high risk pre and postmenopausal women (100-102).

Before the breakthrough discovery of tamoxifen, in the early 1950s two antiestrogenic drugs namely Mer-25 and clomiphene were introduced. This was followed by the development of a series of triarylethylene derivatives with alkyl substitutes for chlorine in the clomiphene molecule for treating hormone dependent tumors. However animal studies revealed that clomiphene and related compounds lead to formation of cataracts due to accumulation of desmosterol, a cholesterol precursor (102-104).

Tamoxifen was first used in a clinical trial in 1969 consisting of 46 premenopausal patients with advanced breast cancer. When patients were administered 10-20 mg tamoxifen daily for 3 months, a remission rate of 22% was achieved and this was comparable to that seen with stilboestrol (the drug used at that time), but with reduced toxicity. Thirty-five randomized clinical studies involving 5160 patients comparing tamoxifen with other endocrine therapies, showed that tamoxifen had clear therapeutic advantages (102, 105).

The antitumorigenic effects of tamoxifen are mediated by its anti-estrogenic activity. E2 binds ER and initiates a sequence of events that lead to activation of gene transcription and enhanced cell proliferation. Tamoxifen competitively inhibits binding of estrogen to the ER and the tamoxifen-ER complex homodimerizes leading to attenuation of estrogen responsive gene expression. This results in decrease of cell proliferation by arresting cells in G0/G1 phase of cell cycle (102, 106). Tamoxifen can affect malignant breast epithelial cells indirectly by modulating level of cytokines in a local and systemic

manner. It stimulates production of transforming growth factor β (TGFβ) which is an inhibitory growth factor which acts on breast carcinoma cells in a negative paracrine manner. Tamoxifen also decreases the production of IGF-1 a potent mitogen for breast cancer and tamoxifen also induces apoptosis in both ER-positive and ER-negative breast cancer cells (101, 102, 107, 108). Tamoxifen, although the leading drug of choice for breast cancer treatment, has adverse side effects which include hot flashes, endometrial cancer, abnormal bleeding and pain, rare reports of thromboembolic events, stroke, deep vein thrombosis, and pulmonary embolisms. However, it has been concluded that its benefits in saving lives by far exceeds the incidence and severity of the in side effects (102). Raloxifene is another SERM with estrogen antagonist action in both breast and uterus and agonist action in bone. It is potentially less hazardous than tamoxifen, as it has not been associated with increased risk of endometrial cancer (99).

Unfortunately, most patients who respond well to treatment with SERMs such as tamoxifen will eventually have a reoccurrence of resistant tumors. These tumors may express a functional ER and their resistance may be due to numerous mechanisms including increased local metabolism of tamoxifen to unstable or less potent metabolites, mutations in ER, variations in co-regulator expression and recruitment or interaction with other signaling pathways. The precise mechanism of tamoxifen resistance is still unknown and requires further research (99, 109).

1.2.11 Aromatase inhibitors in breast cancer

Estrogen depravation is an effective treatment for breast cancer and introduction of tamoxifen, the antiestrogen, as first-line treatment has markedly improved breast cancer survival. However tamoxifen also exhibits partial ER agonist activity, and may also be involved in the later development of tamoxifen resistance. Hence there is a pressing need to find alternatives to tamoxifen for treatment of this disease. A promising alternative for decreasing E2 has been the development of inhibitors of aromatase, the enzyme that converts androstenedione and testosterone to estrone and E2. This enzyme is a member of the cytochrome P450 class and is highly expressed in the placenta and in granulosa cells of the ovarian follicles, but is also present in smaller amounts in peripheral tissues such as adipose tissue, liver, muscle, brain and breast tissue (96, 101, 110).

Aromatase inhibitors can be classified into two subtypes, steroidal and nonsteroidal or type 1 and type 2 agents respectively. Steroidal inhibitors include formestane and exemestane which are analogues of androstenedione (substrate) and irreversibly bind aromatase at the substrate binding side, and are known as enzyme inactivators. Nonsteroidal inhibitors such as fadroxole, anastrazolem and letroxole reversibly bind the heme group of the enzyme. Thus different types of aromatase inhibitors can be used sequentially in order to prolong their clinical response. However the clinical significance of these different mechanisms of action on aromatase inhibitors is not yet completely

understood. Several clinical trails have shown that aromatase inhibitors as second-line treatment are effective for the treatment of breast cancer in postmenopausal women who have experienced disease progression while on tamoxifen. (96, 101, 110)

The treatment of breast cancer has come a long way since its beginning. Endocrine therapy and antiestrogen treatment regimens provide the latest therapeutic methods for treating ER-positive breast cancer. However there are, as yet no mechanism based drugs for treatment of ER-negative breast cancers which are primarily treated with cytotoxic drugs and the overall prognosis is very poor. Hence research leading to the discovery of mechanism-based drugs for treatment of these cancers is essential and some of these aspects will be discussed later in this dissertation.

1.2.12 The aryl hydrocarbon receptor (AhR)

The AhR is a ligand activated nuclear transcription factor that was first identified by Poland and coworkers (1976) in the hepatic cytosol of mice using ^{3}H labeled 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as a radioligand (111). The AhR belongs to the periodicity/AhR nuclear translocator (ARNT)/single-minded (PAS) and basic helix-loop-helix (bHLH) protein family (112-115). Members of the PAS family include the hypoxia responsive genes such as hypoxia inducible factors (HIF1-α , HIF2-α/HLF) which are involved in oxygen sensing and angiogenesis, proteins involved in neurogenesis such as

single minded protein(Sim), transcription factors such as steroid receptor coactivator 1 and transcriptional intermediate factor 2 (SRC-1, TIF2) and circadian rhythm proteins such as CLOCK, BMAL1 and ARNT (113, 114, 116, 117).

1.2.13 Molecular structure of AhR

The AhR and ARNT proteins are transcription factors that contain several domains that are responsible for DNA binding, ligand binding, and interactions with other proteins that are required for ligand-induced transactivation (Figure 4). The N-terminal region of these proteins contains a bHLH motif which is also shared by the family of transcription factors which include the protooncogenes Myc and MycD. Adjacent to the C-terminal region of the bHLH domain is a sequence of 250 amino acids termed PAS. This is a consensus region which was originally identified as a conserved region between Drosophila period (per), human ARNT, and Drosophila Sim. Within this 250 amino acid sequence are two imperfect repeats of 50 amino acids PAS-A and PAS-B which functions as interactive surfaces for hetero or homo dimer formation with the Hsp 90 complex (unliganded form) and binding of receptor ligands. The HLH region is mainly responsible for dimerization with the ARNT and DNA binding (113, 114, 118-120).

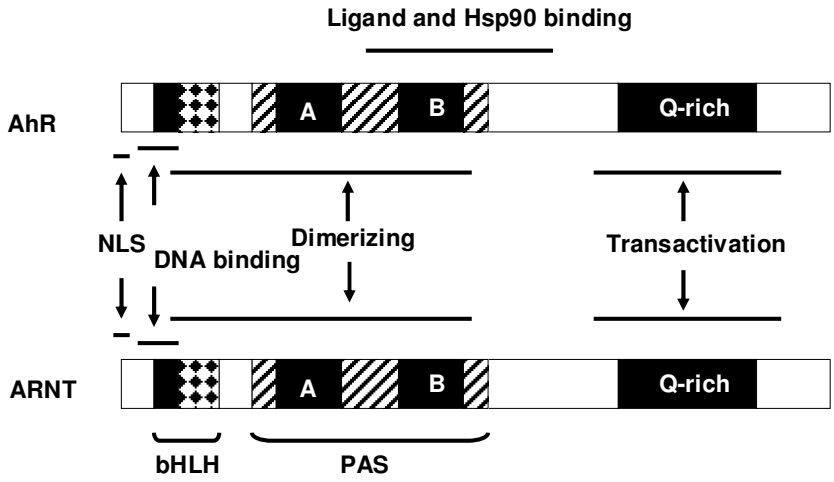


Figure 4. Structure domains of AhR and ARNT (121).

1.2.14 Mechanism of AhR mediated transactivation

The AhR is the only member of PAS family activated by a ligand (Figure 5). The unliganded AhR is a cytosolic protein complexed with heat-shock protein (Hsp) 90, the co-chaperone protein p23 and the immunophilin-like AIP/ARA9/XAP2 protein. Binding of Hsp90 to the AhR at the PAS-B domain masks the nuclear localization signal resulting in the cytoplasmic localization of the AhR in many cells. Ligand binding results in conformational changes and exposure of the nuclear translocator sequence; the liganded AhR translocates into the nucleus where it switches partners from Hsp90 to ARNT. In its transcriptionally active form, the AhR is a hetero dimer of the AhR and the ARNT proteins, which interacts with the genomic cis-acting dioxin-response elements (DREs) in the promoter regions of the Ah-responsive genes such as CYP1A1.

The AhR is a transcription factor that binds a diverse group of natural and man-made compounds; however the endogenous ligand has not been identified. (121-124)

The physiological role of the AhR-ARNT hetero dimer has been investigated in AhR transgenic knockout mice. These mice typically have problems in liver development, they exhibit poor fecundity and weight loss, suggesting that the AhR-ARNT complex regulates constitutive functions in the absence of exogenous ligand, and this is consistent with the biochemical studies showing that AhR-ARNT alone may act as a transcription factor (125, 126). In addition, the AhR influences the cell cycle since mouse embryonic fibroblast from AhR deficient mice exhibit a prolonged doubling time compared to wild type cells. Analysis of these fibroblast cells by flow cytometry showed that the slow growth rate of these AhR deficient cells was due to prolonged G1 phase of the cell cycle (127-129). There is also a significant increase in TGF-β expression and down-regulation of mitotic kinases such as Cdc2 and other cell cycle kinases responsible for proper timing of cell division (112, 127, 130) .

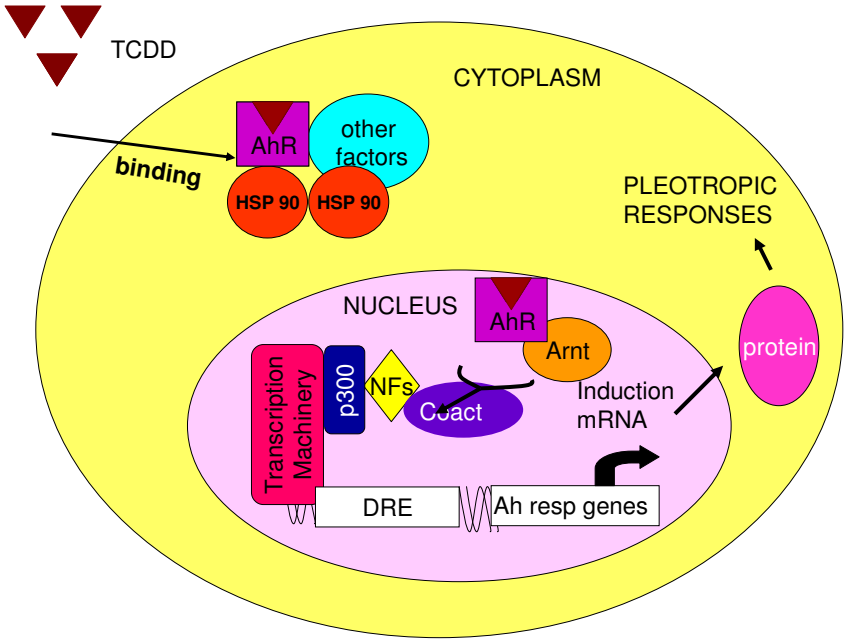


Figure 5. Mechanism of AhR action (121).

1.2.15 Toxic responses mediated by AhR

The AhR was initially identified in the mouse liver cytosolic extracts as the intracellular receptor that bound the environmental toxicant TCDD with high affinity and subsequently activated the CYP1A1 gene which is one of the most widely characterized AhR-dependent biochemical responses which subsequently activates a battery of genes which catalyze the oxidative metabolism of diverse substances (131-133). The AhR also binds structurally related polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs)

which contain 3 or 4 lateral chlorine substituents (2, 3, 7, and 8) and these compounds are also environmental contaminants formed as byproducts of combustion and sewage treatment during production of pesticides and various industrial chemicals and during the chlorine-dependent bleaching process in paper production (134-136). TCDD and other AhR agonists are toxic in laboratory animals and in mammalian cell culture and their toxic effects include carcinogenicity and tumor promotion, teratogenicity, immunosuppression, neurotoxicity, hepatotoxicity, a wasting syndrome, chloracne and associated dermal effects as well as disruption of the endocrine and reproductive pathways (115, 131, 137). In cells, TCDD induces cytokines, hormones and growth factors and their receptors as well as phase I and phase II drug-metabolizing enzymes such as CYP1A1, CYP1A2, CYP1B1, glucuronosyltransferase and glutathione-S-transferases (137, 138). The AhR also binds with moderate affinity to chemoprotective phytochemicals such as indole-3-carbinol (I3C), flavonoids, and carotenoids, which exhibit agonist/antagonist activities in rodent mammary tumors (112, 139, 140).

1.2.16 Inhibitory AhR-ER crosstalk

The ligand-activated AhR complex inhibits estrogen-induced gene expression and their derived proteins or dependent activities and also inhibits growth of estrogen-dependent mammary tumors in rodent models. TCDD and related compounds have been used to study the AhR-mediated responses in

ER-positive and ER-negative human breast cancer cells. ER is a ligand activated transcription factor that is a member of the nuclear receptor family. As mentioned earlier, the ER plays an important role in normal female physiological development and has also been associated with the promotion of breast and endometrial cancer. Drugs used for treatment of early stage ER-positive breast cancer target the ER and these include antiestrogens such as tamoxifen, that block estrogen action in mammary tumors. Several studies also show that ligands for the AhR inhibit multiple estrogen-induced responses including the inhibition of mammary tumor growth in cells expressing AhR. AhR-dependent inhibition of E2 induced responses has been observed in the rodent uterus and mammary tumors as well as in breast cancer cell lines (119, 130, 141).

Kociba and co-workers (1978) reported the effects of long term dietary exposure to TCDD on tumor development in both male and female Sprague-Dawley rats. There was an increase in hepatocellular carcinomas and keratinizing squamous cell carcinomas in female but not male rats. In contrast, several age-dependent spontaneous tumors in the endocrine organs and the female reproductive tract were decreased in rats exposed to TCDD in the diet for up to two years. The incidence of both mammary and uterine tumors were decreased in female rats exposed to TCDD suggesting that ligand-dependant activation of the AhR inhibits formation/ and or growth of E2 dependent tumors. These initial observations gave rise to the idea of inhibitory AhR- ER crosstalk (121, 142).

Gierthy et al (1993) demonstrated that TCDD at 5 μg/kg/week inhibited tumor growth in B6D2F1 immunosuppressed mice bearing MCF-7 cells as a xenograft and an implanted E2 pellet (143). In MDA-MB-231 cells which are ER-negative and have a nonfunctional AhR, crosstalk is restored between the two receptors upon transient transfection of human ER. This also demonstrates that cross-talk occurs between the two receptors. (144). Moreover, in breast cancer cells expressing a functional AhR and ER, TCDD significantly inhibits E2-induced cell proliferation, cathepsin D secretion and mRNA levels, pS2, prolactin receptor and mRNA, secretion of tissue plasminogen activator, and progesterone receptor (PR) and mRNA. (143, 145-153). Inhibitory AhR-ER crosstalk activated by TCDD inhibits E2 induced MCF-7 cell proliferation and multiple hormone induced cell cycle enzymes and these include cyclin D1 and E2F1 protein and mRNA, cyclin dependent kinase-2 (cdk-2), cdk-4 and cdk-7 activities, cdc25 phosphatase protein and RB protein phosphorylation (154-156).

Studies have shown that TCDD and related compounds are antiestrogenic in rat and mouse uterus. Treatment of ovariectomized or immature rats and mice with E2 increases uterine wet weight and DNA synthesis. Cotreatment with TCDD inhibits E2-induced increase in uterine wet weight, DNA synthesis, EGFR binding and mRNA, progesterone receptor (PR) binding, peroxidase activity, and c-fos mRNA levels (154, 155, 157-161). Studies have also shown that incidence of spontaneous mammary and uterine

tumors in female Sprague-Dawley rats administered TCDD in the diet for up to 2 years showed that TCDD inhibited tumor formation and tumor growth. Subsequent studies have demonstrated that treatment with TCDD inhibits E2 dependent mammary tumor formation and growth in carcinogen-induced mice and athymic nude mice bearing human breast cancer cell xenografts (143, 162). This confirms the finding in Seveso, Italy where women accidentally exposed to high levels of TCDD exhibited a decreased incidence of mammary cancer.(163)

1.2.17 Mechanism of inhibitory AhR-ER cross-talk

There are several possible mechanisms proposed for inhibitory AhR-ER crosstalk and one of these involves enhanced metabolism of E2. Several studies have demonstrated that TCDD and other AhR agonists induce CYP1A1 and CYP1B1 in breast cancer cells resulting in enhanced metabolism of E2 and thereby depleting intracellular hormone levels resulting in decreased hormone-responsiveness (164-166). However, there is evidence to support that alterations in E2 metabolism are insufficient to mediate AhR mediated antiestrogenic response. For example, TCDD inhibits cathepsin D mRNA levels in MCF-7 cells with in 30-60 min and this precedes induction of CYP1A1 protein and E2 metabolism (148, 150, 167). Furthermore, rodents treated with TCDD do not show altered E2 levels, and several selective AhR agonists do not induce CYP1A1 protein in breast cancer cells or rodent models but these compounds inhibit tumor growth (150, 168).

Another mechanism of inhibitory AhR-ER crosstalk involves direct interaction of the AhR with inhibitory DREs (iDREs). In most cases where genes such as CYP1A1 are activated by the AhR, the AhR/ARNT complex interacts with a DRE located in the gene promoter. Binding of the AhR/ARNT hetero dimer complex to DREs induces remodeling of chromatin structure enabling the association of transcription factors in the gene promoter and enhancer region resulting in gene transcription. Studies in this laboratory have identified iDREs that would account for the inhibitory effects of AhR-ER cross-talk on some genes (121). Cathepsin D is one such gene with an iDRE in the promoter region. The Cathepsin D gene promoter contains an estrogen responsive ERE1/2(N23) Sp1 motif that is estrogen responsive and this response is inhibited by cotreatment with TCDD. Closer examination of the sequence between the ERE half-site and the GC rich Sp1 binding sites revealed the presence of an overlapping core inhibitory DRE motif. Mutation of the DRE motif did not effect reporter gene activity induced by E2, however cotreatment with TCDD did not affect E2-induced responses (169-171). Subsequent studies have identified iDREs in the 5' flanking region of the c-fos, pS2, and Hsp 27 genes (172).

A third mechanism by which AhR agonists such as TCDD may inhibit estrogen-responsive gene expression is by ligand-activated degradation of ER resulting in limiting levels of ERα expression. Initial reports by Harris and coworkers (1990) demonstrated that for a series of AhR agonists, their potency to induce degradation of ER in MCF-7 cells correlated with their binding affinity

for the AhR (172). Wormke et al. (2003) showed that both E2 and TCDD activated proteasome-dependent degradation of ERα and AhR proteins in MCF-7 and T47D cells respectively (130). In addition TCDD induced degradation of ERα protein, where as E2 did not activate proteasome dependent degradation of the AhR. Thus, TCDD not only activates protease dependent down-regulation of its own receptor, but also induces degradation of ERα protein via proteasome dependent pathways and the combined effects of E2 plus TCDD may result in limiting levels of ER α for activation of some genes (130). It is also possible that the ligand bound AhR may compete with ER for cofactors leading to decreased E2-induced transactivation (173-175). Studies in this laboratory and others have shown that the AhR interacts with ERα coactivators, namely SRC-1, RIP 140 and ERAP140 and corepressor SMRT. These interaction may also contribute to inhibition of ER-mediated responses by AhR agonists (131, 137, 173-175).

1.2.18 Selective AhR modulators (SAhRMs) for treatment of breast cancer

As discussed above, the AhR is a potential target for breast cancer therapeutics. Alkylated PCDFs and substituted diindolylmethanes (DIMs) represent two classes of SAhRMs (Figure 6) which exhibits relatively low toxicity but induces antiestrogenic responses. The toxicity associated with TCDD and related compounds is due to their coplanar structure and substitution with

chlorine groups at the lateral (2,3,7,8) positions resulting in high AhR binding affinities and long term occupation of the AhR (131, 137). PCDDs or PCDFs with chlorine substitutions at only 2 lateral positions exhibit decreased AhR binding affinity and toxicity (176). Moreover, substitution of chlorine with alkyl groups such as a methyl substituents slightly decrease AhR binding however these alkyl PCDFs exhibit decreased toxicity and decreased activity as inducers of hepatic CYP1A1 in rats (177-181).

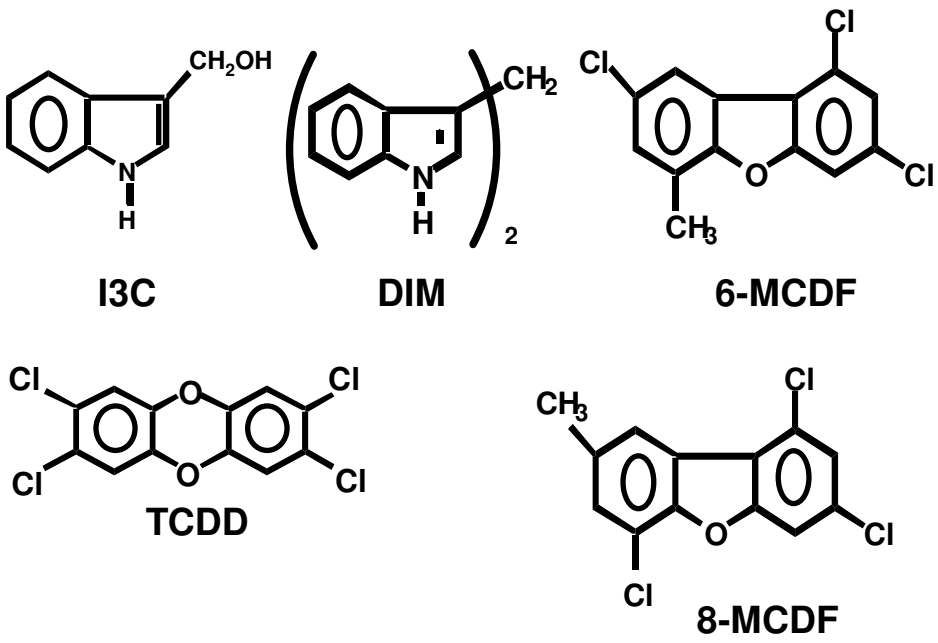


Figure 6. Selective AhR modulators

1.2.19 Alkyl-substituted polychlorinated dibenzofurans

6-Methyl-1, 3, 8 trichlorodibenzofuran (6-MCDF) and other 6-alkyl substituted analogs were developed as AhR antagonists and in a series of studies this compound was shown to be relatively non-toxic. The 2,4,6,8- or 1,3,6,8- substituted PCDFs with at least one alkyl group bound the AhR with moderate affinity, exhibited minimal toxicity and partial AhR antagonist activity. Cotreatment studies with TCDD and 6-MCDF showed that 6-MCDF inhibited TCDD-induced CYP1A1 gene expression in various cell lines and in rat liver (177-179). 6-MCDF also inhibited immunotoxicity, cleft plate and hepatic porphyria induced by TCDD in mice. Thus 6-MCDF was shown to be an AhR antagonist. (177-179, 182). 6-MCDF competitively binds to the AhR and in gel mobility shift assays this complex appears to be distinct from that of the TCDD-AhR complex, indicating that 6-MCDF may induce a different conformation of the AhR compared to the AhR bound to TCDD. This may explain the reason why 6-MCDF does not readily induce the gene cascades associated with AhR-mediated toxicity (179, 182-184).

Results in the Safe laboratory have shown that AhR agonists such as 6-MCDF and 8-MCDF inhibit carcinogen induced (7,12-dimethylbenz[a]anthracene-DMBA) mammary tumor growth in female Sprague Dawley rats by 6-MCDF and 8-MCDF (168). Both compounds cause a dose dependent decrease in mammary tumor growth at does of 5, 10 and 25 mg/kg/week and there was no induction of hepatic CYP1A1 in the treated

animals. The effects of 6-MCDF, TAM and their combination were also extensively studies in the rat mamnmary tumor model. 6-MCDF and TAM alone inhibited mammary tumor growth at doses of 100 and 50 μg/kg/day respectively, where as cotreatment with 100, 50, 25 μg/kg/day of each compound resulted in complete inhibition of mammary tumor growth in all cotreatment groups and in some groups the final tumor volumes were less than the initial volumes indicating growth stasis. Data analysis showed that 100, 50 μg/kg doses are additive and the interaction at 25 μg/kg is synergistic. Body and organ weights were unchanged in all treatment groups, and there was no induction of CYP1A1-dependent EROD activity indicating absence of any AhR mediated effects on the liver (168).

The effects of TAM, 6-MCDF and their combination were determined in ovariectomized female Sprague Dawley rats using TAM (400 μg/kg/day) for 20 days in combination with 6-MCDF at 800 μg/kg/day. Results showed that TAM significantly induced uterine wet weight, peroxidase activity, and levels of PR binding. However, 6-MCDF at the given dose significantly inhibited uterine peroxidase activity and did not induce wet weight or PR binding. In the cotreatment studies, there were minimal changes in uterine wet weight compared to treatment with TAM alone. In addition cotreatment studies showed significant inhibition of TAM –induced peroxidase activity and PR binding.(185-188). These results suggest that 6-MCDF in combination with TAM will act synergistically to inhibit mammary tumor growth and block the potential

endometrial effects of TAM in the endometrium where the prolonged used of TAM is a risk factor for endometrial cancer (168).

1.2.20 I3C and DIM

Diets enriched in cruciferous vegetables have been associated with protection against multiple cancers, including breast cancer in women (34, 185, 189, 190) and there has been research on the chemoprotective mechanisms associated with phytochemicals produced by these plants. I3C is found in cruciferous vegetables as a cyanogenic glucosinolate which can be hydrolyzed by myrosinase to give the free indole derivative(191, 192). I3C is known to inhibit carcinogen-induced initiation and spontaneous tumor formation in various cancers such as breast, lung and endometrium (193). I3C is an unstable compound and rapidly undergoes acid-catalyzed condensation reactions in the human gut to give a complex mixture of products including DIM, 5,6,11,12,17,18-hexahydrocyclononal [1,2-β:4,5-β':7,8-β ''] triindole, [2-(indole-3-ylmethyl)-indole-3-yl] indol-3-ylmethane, 3,3'-bis(indole-3-ylmethyl) indolenine, cyclic and linear tetramers of I3C and indole (3,2-β) carbazole (ICZ) (140, 191, 192, 194). I3C binds weekly to the AhR and the higher molecular weight condensation products exhibit increased binding affinity for the AhR (140, 195).

The effects of consumption of I3C have been extensively studied. Initial reports showed that high doses of I3C fed in the diet or administered by oral intubation to rodents greatly decreased the incidence of spontaneous and

carcinogen induced tumors of mammary gland, endometrium, lung, colon, skin, liver and cervix (196-199). Studies have shown that treatment with I3C prevents the formation of DMBA-induced mammary tumors in rats, and dietary supplementation with cabbage or broccoli both of which are good sources of I3C, also resulted in decreased mammary tumor formation in DMBA-treated rats (199). Furthermore, dietary administration of DIM at 5 mmol 20 h prior to treatment with DMBA inhibits tumor formation in the carcinogen induced rat mammary tumor models (193).

The antiproliferative effects of DIM were examined in human breast cancer cell lines by examination of incorporated ^{3}H and flow cytometry. Cell proliferation was inhibited up 90% and also resulted in a G1 arrest (193). . The G1 cell cycle arrest of human breast cancer cells by DIM was a result of interactions with specific proteins that subsequently control the transcription of critical cell-cycle genes. Cell cycle arrest in MCF-7 cells is accompanied by downregulation of Cdk6 and stimulation of p21 gene expression. Similar results are also observed in prostate cancer cells (LNCaPs) (193, 200). DIM also caused breast cancer cells to undergo programmed cell death as monitored by DNA fragmentation and chromatin condensation. Apoptosis was accompanied by decrease in the apoptosis inhibitory protein Bcl-2 and increase in the levels of the proapoptotic protein Bax (201, 202). In summary, the transcriptional control of cell cycle gene expression is mediated by a combination of the specific effects of I3C and DIM.

1.3. Prostate cancer

1.3.1 Statistics

Prostate cancer is the fifth most common cancer worldwide and second most common in men. There were 679,000 new cases of prostate cancer diagnosed worldwide in 2002. The prognosis is relatively good, hence has a less prominent cause of mortality with 221,000 deaths worldwide in 2002. Approximately 75% of all cases are in men aged 65 or more and mortality rates are relatively high in the Carribean, Southern and Central Africa, Northern and Western Europe, Australia/New Zealand and North and South America and low in Asian populations and in North Africa. Variations in mortality rates and incidence between China and the US are 16-fold and approximately 80-fold respectively (1).

1.3.2 Prostate structure and development

The development of the male reproductive system is dependent upon many factors. The development starts at the embryonic stage where there interaction of the mesenchymal-epithelium and the fetal androgens first begins (203, 204). In the fetus, testosterone stimulates budding of the prostate epithelium from the urogenital sinus which then produces growth factors such as sonic hedgehog to activate the underlying mesenchyme (204). This is followed by branching and morphogenesis of the prostate and the urogenital mesenchyme which express very high levels of the androgen receptor (AR).

The AR is however undetectable in the epithelium at this time during development. The mesenchyme produces paracrine growth factors that aid in glandular morphogenesis and epithelial cell growth in the developing prostate. During the maturation process, the AR can be detected in the secretory cells layer and in the adult prostate in the stromal and secretory epithelial cells (205).

A normal adult prostate is composed of a glandular epithelial and a fibromuscular stromal component and a balance of proliferation and cell death is maintained in these two compartments. The concept of zonal anatomy of the prostate suggested by McNeal in the 1980s forms the current basis for describing the location of the neoplastic cells in the prostate. According to this concept, the glandular part of the prostate is composed of a small central zone surrounded by a large peripheral zone which make up 95% of the gland. The remainder is composed of the transition zone and the peri-urethral glands. About 70% of prostate cancers occur in the peripheral zone and 20% in the transition zone and the remaining fraction of the cancers originate in the central zone. Benign prostatic hyperplasia (BPH) develops mainly from the peri-urethral stroma and glands of the transition zone (205).

Most prostate cancers originate from the glands of the peripheral zone and hence are classified as adenocarcinomas. Carcinogenesis occurs in a multistage process involving progression from a low-grade small, latent carcinoma to high grade metastatic disease (Figure 7). Normal prostate epithelial cells undergo multiple cellular, biochemical, and genetic alterations,

elevated levels of androgens such as testosterone and dihydrotestosterone (DHT) leading to neoplastic changes characteristic of prostate cancer (206).

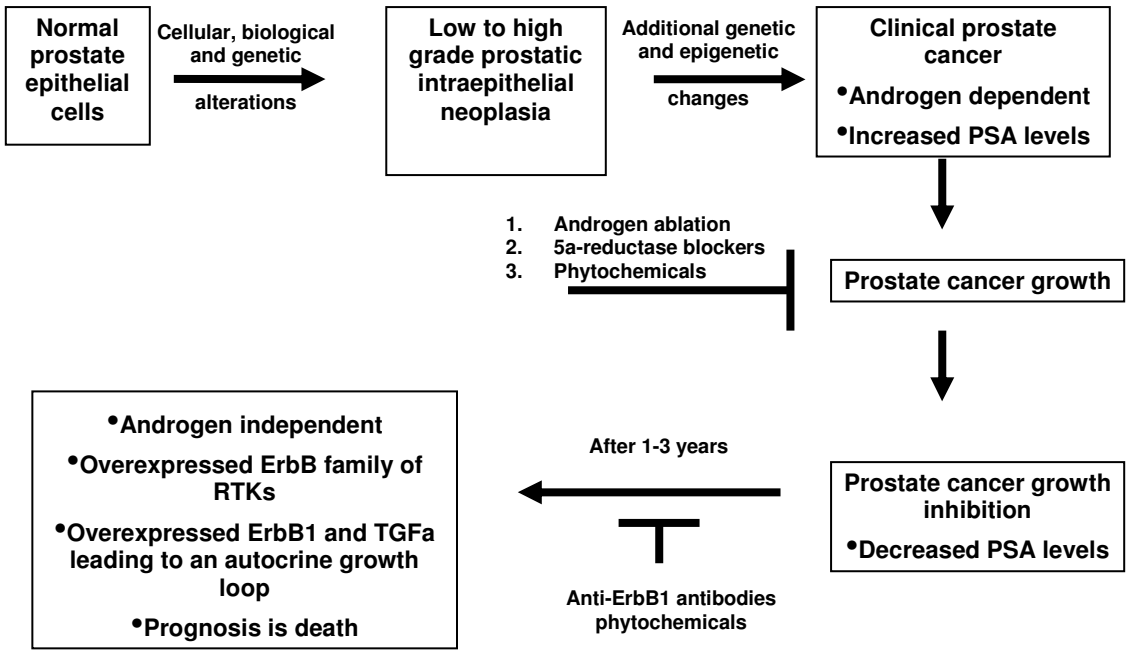


Figure 7. Steps in progression of prostate cancer (207).

1.3.3 Environmental risk factors associated with prostate cancer

Prostate cancer occurrence differs markedly across different populations varying from approximately 1 per every 100,000 reported for China, to 45-65 per 100,000 Caucasians in the US; rates for people of African descent are as high as 100 per 100,000. Prostate cancer incidence in Western Europe is 20-30 per 100,000 with some variations among the countries. The rates of clinical prostate cancer vary geographically whereas latent prostate cancer is still equally

distributed in areas with high and low prostate cancer. This strongly suggests that environmental factors may be important for the progression from latent to clinical prostate carcinoma (208, 209). It has been reported that among migrants moving from countries with a low prostate cancer incidence, such as Japan, to North America, their incidence rate approaches incidence rates observed among Caucasian Americans within a generation. Men from the same ethnic group but raised in different environments have a risk of prostate cancer comparable to the incidence of their residing country rather than their country of origin (189).

It is estimated that 40% of prostate cancers can be explained by genetic factors, and there is strong evidence from migration studies that lifestyle and environmental factors also have a major influence on development of this neoplasia (210). Among the environmental factors, the diet plays a major role in the initiation, promotion and progression of prostate cancer. Several studies have identified dietary substances with antioxidant properties that have an inhibitory effect on development and progression of prostate cancer. Many of these substances such as carotenoids and lycopenes, retinoids and vitamin A, vitamin E, vitamin C, selenium (Se), and phenol containing substances have effects that extend beyond just their antioxidant properties (211-213).

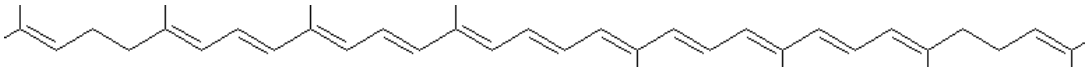
1.3.3.1 Carotenoids and lycopenes.

Approximately 600 carotenoids are found in nature and some of these are pure hydrocarbons (carotenes) while others may contain oxygenated functional groups (xanthophylls). The major carotenoids include α/β-carotene, β crypotoxanthin, lutein, and lycopene (Figure 8). Carrots, winter squash, and pumpkin are a major source of α-carotene while fruits such as mangoes, apricots, cantaloupe and vegetables such as carrots, red peppers, pumpkin, sweet potatoes, broccoli and leafy green vegetables are a rich source of β -carotene. β -Cryptoxanthine is found primarily in mangoes, apricots, sweet potatoes, broccoli and leafy green vegetables and lutein is found predominantly in green vegetables such as brussels sprouts, green beans, peas, zucchini and corn. Main sources of lycopene are tomatoes, water-melon, and pink grapefruit (214-216).

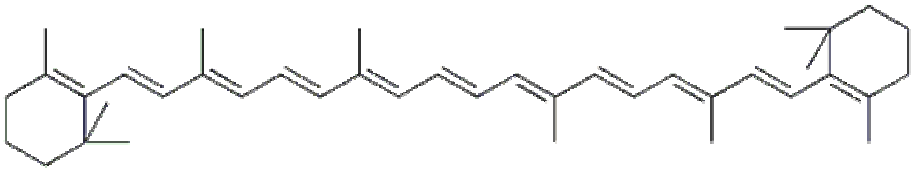
The chemical structure of carotenoids enables them to absorb UV light and act as antioxidants. In addition, β-carotene inhibits cancer cell growth in a cell cycle-independent manner and also induces apoptosis (214, 215). In addition to acting as quenchers for reactive oxygen species, carotenoids also interfere with free radical-initiated reactions such as lipid peroxidation. (217).

The link between lycopene intake and development or progression of prostate cancer has been extensively investigated. Several studies have reported a significant inverse association between the dietary intake of tomatoes and blood lycopene levels, and the risk of prostate cancer (218). A population

based case-control study conducted in New Zealand showed a link between lycopene consumption and prostate cancer revealed that increased intake resulted in decreased prostate cancer, however no correlation was observed for β-carotenoids (219). Another randomized, placebo-controlled study by Gann and colleagues (1999) compared plasma lycopene levels in 578 men with prostate cancer with 1294 age and smoking status matched controls and observed an inverse correlation between plasma lycopene levels and prostate cancer risk. (220). Similar results were observed in a Chinese population by Binns and colleagues who noted an odds ratio (OR) of 0.18 (95% CI0.08-0.41) for lycopene in a questionnaire-based case-control study (221). Some studies have demonstrated an inverse correlation between dietary lycopene intake and both serum IGF-1 levels and risk of prostate cancer. Lycopenes may exert protection against prostate cancer, particularly early in the disease process by decreasing plasma IGF-1 levels by an unknown mechanism (222-224). In vitro studies using prostate cancer cell lines namely PC-3, DU145, LNCaP by Kotake and co-workers (2001) demonstrated the antiproliferative effects of carotenoids found in tomatoes. They discovered that both monoethoxy and acyclic carotenoids significantly reduced prostate cancer cell viability and in addition they demonstrated that the reduced viability was mediated by apoptosis.(225).



Lycopene



β-carotene

Figure 8. Lycopene and β-carotene

1.3.3.2 Retinoids and vitamin A

Retinoids include retinal (vitamin A) retinoic acid, and retinol (Figure 9). Vitamin A or retinal is a conversion product of β-carotene in the gut but its main sources are derived from foods of animal origins such as liver and fish oil, whereas β-carotene found in carrots, and green vegetables is chemically converted into retinol in the gut (226). Vitamin A exhibits anti-oxidant activity; however the compound alone is unable to quench singlet oxygen species but rather exerts its effects by affecting tissue levels of other antioxidants such as a-tocopherol and Se. Retinoids are classified as antiproliferative biomolecules which may also induce apoptosis (227, 228) These compounds also act as transcriptional regulators by inhibiting DNA polymerase activity in G1 phase of the cell cycle in some cell types. (229, 230).

Studies by Sharp and co-workers (2001) using the synthetic retinoid N-(4-hydroxyphenyl)-retinamide (4-HPR) showed that in vitro cell growth was inhibited in 4 different prostate cell lines (DU145, RWPE-1, WPE1-7, WPE1-10) and histological staining revealed that this was achieved by a mechanism in which both tumor suppressor protein p53 and pRB were downregulated. This suggests that inhibition of prostate cancer cell proliferation is achieved at several stages of disease progression(231). Epidemiological studies in men older than 70 years demonstrated an inverse relationship between dietary vitamin A intake and prostate cancer (212, 232). Men with BPH had plasma vitamin A levels five to eight fold higher than those men with prostate cancer. A study by Rohan et al.(1995) comparing the diets of 207 men with prostate cancer and 207 randomly selected men with BPH revealed that higher dietary retinol intake was associated with a reduced risk of prostate cancer. Thus, increased dietary intake of vitamin A may be beneficial in reducing the incidence of prostate cancer in older men who already have a higher age-related risk of developing prostate cancer (233). In addition, treatment with retinoids appears to increase the expression of prostate-specific membrane antigen, a marker that is associated with more differentiated histology in prostate cancer tissues (234). Also, Slawin et al. (1993) demonstrated that mice fed a diet enriched with synthetic retinoid, fenertinide, had a 49% reduction in prostate tumors and 52% reduction in tumor mass as compared to mice whose diet lacked fenretinide (235).

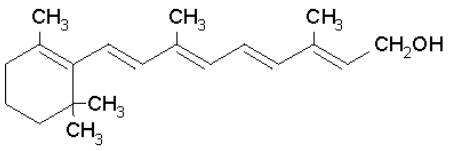


Figure 9. Vitamin A

1.3.3.3 Vitamin E

Vitamin E refers to eight structurally related compounds found in vegetable oils, nut oils, egg yolk, various cheeses, whole grain products and vegetables like carrots, sweet potatoes and tomatoes. These compounds are fat soluble and are absorbed efficiently from the gut. The antioxidant properties of vitamin E are due to the chemical structure and the reactivity of the phenolic hydrogen ion and C6 hydroxyl group which provides the unpaired electron that stabilize various free radical species (236). The major forms of vitamin E are α, β, δ and γ-tocopherol (Figure 10). α-Tocopherol has the greatest antioxidant activity followed by β-tocopherol and γ-tocopherol (both are roughly equal) followed by δ-tocopherol which has the least activity (236). Vitamin E scavenges free radicals, inhibits peroxidation of lipid membranes and reduces carcinogen-induced DNA damage. In addition α-tocopherol binds highly reactive genotoxic electrophiles such as hydroxyl, superoxide, lipid peroxy, hydroperoxyl and nitrogen oxide derived free radicals and these interactions protect against DNA and biological membrane damage by free radicals (237). In a large study of

smokers, both incidence and mortality from prostate cancer were significantly lower in men who were given 50 mg of vitamin E (a-tocopherol) for 5-8 years(238). The United States Health Professional Study (USHPS) conducted on 47,780 healthy male health professional, including smokers and non smokers who were administered a dietary lifestyle questionnaire followed by 11 years of monitoring for prostate cancer showed no association between dietary vitamin E supplementation and decreased prostate cancer. However there was an inverse association between dietary vitamin E supplementation and a decrease of metastatic and fatal cases of prostate cancer among the smokers in this group of professionals(239).

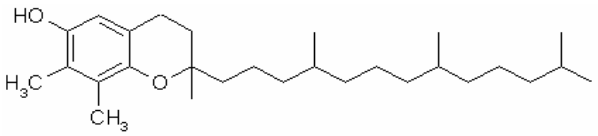


Figure 10. γ-tocopherol

1.3.3.4 Vitamin C

Vitamin C also known as ascorbic acid is present in foods of both plant and animal origin (Figure 11). Some sources include potatoes, fruits, vegetables and organ meats such as kidney and liver. Vitamin C is thought to protect cytoplasmic and membrane components of cells from oxidative damage by scavenging free radicals generated during cellular metabolism as well as from free radical induced damage. It does so by donating one or two of its electrons

during a redox reaction resulting in production of an ascorbate free radical (AFR) (240, 241). In addition, vitamin C inhibits cellular DNA synthesis, growth, and viability by inhibition of G1 phase of cell cycle and by triggering DNA degradation (242). In vitro studies using prostate cancer cell lines namely LNCaP and DU145 cells have shown that vitamin C causes a dose and time dependent decrease in cell number, viability and DNA synthesis (242). Epidemiological results however have shown mixed results about the beneficial role of dietary vitamin C in prostate cancer. Most studies found no association between increased serum and dietary vitamin C content and reduced risk of prostate cancer. The study by Deneo-Pellegrini et al. (1999) however, has demonstrated that the beneficial effects of vitamin C in reducing risk of prostate cancer are dose dependent, hence raising the possibility that studies showing no association may have not included individuals with adequate vitamin C levels to demonstrate such an association (213, 243, 244). The European Prospective Investigation into Cancer and Nutrition (EPICN) which evaluated plasma vitamin C levels and cancer mortality in both males and females whose diet was augmented with low and high doses of vitamin C showed an inverse relationship to cancer mortality in both the sexes (245).

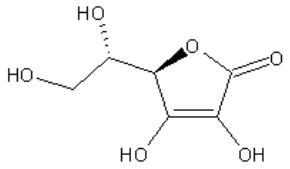


Figure 11. Vitamin C

1.3.3.5 Selenium (Se)

Plants are major source of Se and its concentration varies according to geographic region and soil content. The major dietary sources of Se are bread products and meats (246). Se is a constituent of the enzyme glutathione peroxidase (GPO) and in concert with glutathione (GSH) GPO plays a crucial role in preventing harmful effects of free-radical induced lipid membrane damage. The multi-complexed enzyme system along with GSH prevents free radical formation by converting hydrogen peroxide to harmless water. Se also induces apoptosis and inhibits cell growth by arresting or blocking various steps in the cell cycle (247). In prostate cancer DU145 cells, Se induces apoptosis by activation of multiple caspases such as caspase-3, -7,-8, and -9, along with the release of mitochondrial cytochrome c accompanied by DNA laddering (248). In vivo studies by Hardell et al. (1995) have shown that average plasmas levels of Se were significantly higher in patients with BPH than those with prostate cancer. In a similar study by Criqui et al. (1991) plasma Se levels were higher in patients of the control group than those who died of prostate cancer whose plasma was stored under similar conditions of time and temperature. (249, 250).

However in one prospective study, it was found that increased Se levels in men who later developed prostate cancer correlated with reduced risks of advanced disease (251) although it was not observed in another study (252). In addition, a placebo controlled study by Clark et al (1998) reported that geographical areas of the US with low Se soil content, exhibited a significantly lower incidence of prostate cancer among men receiving dietary supplements enriched with Se (253, 254).

1.3.4 Lifestyle risk factors associated with prostate cancer

Among the lifestyle factors that play a substantial role in development of prostate cancer are obesity and smoking. Obesity is often associated with a more sedentary life style and decreased frequency of physical activity. Obesity caused an increase in circulating estrogen levels and decreasing androgen levels since they are aromatized to form estrogens in adipose tissue. This results in an increase of prostate cell sensitivity to androgens and hence increases the risk of prostate cancer (255).

Andersson and colleagues (1997) discovered that BMI was positively associated with increased mortality from prostate cancer, and similar results were observed by Rodriguez and coworkers (2001) upon examination of 1590 deaths from prostate cancer in two cancer prevention studies. Another study (Hsing et al. 2000) among a Chinese male population cohort showed that the highest quartile of waist-to-hip ratios among the subjects conferred a 2.71 times

greater risk of prostate cancer compared to lowest quartile. In contrast, other studies (Health Professionals Follow-up Study, Baltimore Longitudinal study of Aging and Swedish Twin Registry) showed that there was not a statistically significant excess risk of prostate cancer associated with BMI or other measures of obesity. Thus it appears to be that obesity confers an excess risk of prostate cancer mortality rather than incidence (256-258).

The role of smoking in prostate cancer is not clear, however most of the studies that used incident prostate cancer cases in their analyses observed no strong association between smoking and risk of prostate cancer development. However there are some studies for example one among the Swedish construction workers which consisted of 135,000 subjects of which 2300 subjects developed prostate cancer during 20 years of follow-up which indicate a significantly increased risk for prostate cancer. In two other studies conducted in Iowa and California there was a significant positive association between smoking and risk for prostate cancer. In addition there was a dose-response relationship observed in the Swedish and the Iowa cohort studies (259-262). Also the Health Professional Follow-Up Study found that men who had smoked 15 or more pack-years of cigarettes within the preceding 10 years were at a higher risk of metastatic prostate cancer and fatal prostate cancer relative to non-smokers, and they also noted a dose-response relation between smoking and prostate cancer. (263).

1.3.5 Genetic risk factors for prostate cancer

1.3.5.1. Hereditary risk factors in prostate cancer

Prostate cancer can be divided epidemiologically into hereditary and sporadic forms. Some of the important hereditary risk factors for prostate cancer can be attributed to the following genes: ELAC2, CHEK2, RNASEL, BRCA1 and BRCA2. The first hereditary prostate cancer gene to be discovered was the ECLA2 gene located on chromosome 17p. Although the entire function of this gene is not clearly known it has been proposed as a metal dependant hydrolyase, and its overall role in prostate cancer seems to be minimal (264, 265). This gene consists of 24 exons coding for a protein of 826 amino acids, which displays 98.9% homology with the chimpanzee gene. Mutational analysis of this gene revealed a frame shift mutation that segregates with prostate cancer in a high risk pedigree. Analysis of another large pedigree revealed another missense mutation, Arg781His, which occurs in a very highly charged stretch of amino acid residues near the C terminus of the protein which also segregates with the disease (265). Upon extensive screening two more missense variants were identified namely Ser217Leu and Ala514Thr which lie at the border of a histidine motif and thus may affect the protein function (265) An extensive study by Rebbeck et al (2000) consisting of 359 prostate cancer cases and 266 male controls matched for age and race revealed that the Leu217 and Thr541 had a mutation frequency of 31.6% and 2.9% respectively and the relative risk of prostate cancer was increased in men carrying the Leu217/Thr541 allele (266).

The CHEK2 gene is an upstream regulator of p53 in the DNA damage signaling pathway. CHEK2 mutations have been identified in both sporadic and familial cases of prostate cancer (264, 265). This gene is located on chromosome 22q and encodes for cell-cycle checkpoint kinase2 (chk2) a mediator of cells' response to DNA damage. Upon DNA damage chk2 phosphorylates several substances such as p53, BRCA1, Cdc25A and Cdc25C which are involved in DNA repair and apoptosis. It has been suggested that germ-line CHEK2* 1100delC resulting in truncated protein along with other CHEK2 mutations confer susceptibility to prostate cancer (267). In a Swedish study by Wagenius and co-workers (2005) CHEK2* 1100delC mutation was found at a frequency of 1.6% in sporadic cases diagnosed before the age of 55 and similar results were observed for hereditary prostate cancer. The CHEK2* 1100delC mutation was found at a greater frequency in a study conducted among Finnish patients with hereditary prostate cancer (268).

Many important features are shared between breast and prostate cancer. They both share hormone dependency and a tendency for bone metastasis and epidemiological studies have shown evidence to link these two cancers in inheritance. The tumor suppressor gene BRCA1 has been linked to a subset of hereditary breast cancers. In some studies, a slight increase in risk of prostate cancer was detected in individuals carrying known BRCA1 truncating mutations, especially those who were younger in their age at diagnosis (68). However

several other studies have found no association between BRCA1 mutations and prostate cancer risk (269).

The BRCA2 gene, however, has been consistently shown to play a significant role in prostate cancer. Germ-line mutations in the BRCA2 gene account for 30-35% of familial breast cancers and an association between BRCA2 mutations and prostate cancer has been noted in breast-ovarian cancer families with BRCA2 mutations. A common founder mutation (6174delT) which has been identified in an Ashkenazi Jewish population has been significantly associated with increased risk of prostate cancer. Another founder mutation, a 5-bp deletion among Icelandic population has been associated with increased risk of prostate cancer. An estimated 5% of prostate cancer in familial clusters have been linked to germ-line mutations in BRCA2 and hence these mutations are significant in prostate cancers diagnosed at a younger age (270).

RNASEL is a ribonuclease that degrades viral and cellular RNA resulting in cellular apoptosis upon viral infection (264). The RNASEL gene is located on chromosome 1q25 and with the help of positional cloning and candidate gene methods a non-sense mutation and a mutation in an initiation code have been shown to segregate independently in hereditary prostate cancer. A founder frame shift mutation 471delAAAG has been identified in Ashkenazi Jews, at a relatively high frequency (4%) and in the model human prostate cancer cell line, LNCaP. The frequency of this mutation was higher among prostate cancer patients (6.9%) as compared to (2.4%) in unaffected elderly men in this group of

population (270, 271). The role of RNASEL in prostate cancer was strongly supported by a Finnish study consisting of 116 families with hereditary prostate cancer. A truncating mutation, E265X was found at a significantly higher frequency 4.3%, in hereditary prostate cancer than in the control cases (1.8%). In addition the frequency of the E265X mutation increased with the number of affected members per family such that families with four or more affected individuals have a frequency of E265X of 9.5%. There was no significant increase in the frequency of E265X in unselected prostate cancer patients compared to controls(271).

1.3.5.2 Sporadic prostate cancer

The genomic alterations in prostate cancer that are responsible for sporadic cancer are mostly somatic changes. A role for a number of genes has been identified for their role in sporadic prostate cancer. For hereditary cancer, the relevant mutations are mainly at the sequence level, but for sporadic cancer, the relevant mutations include alterations in both sequence and copy number (270).

Early stage prostate cancer often remains euploid and numerical and structural chromosomal alterations accumulate at advanced stages. Deletion of chromosomal segments predominate, while gains of chromosomal segments and amplifications become more frequent in advanced prostate cancer (205).

Mutations of a substantial number of tumor suppressor genes have been identified in all human cancer cells. Among the established tumor suppressor genes, p53 and PTEN are clearly involved in the progression of prostate carcinoma. Losses of chromosome 17p and 10q corresponding to p53 and PTEN respectively, occur with moderate frequency in advanced cancers. Loss of one allele is accompanied by point mutation in the remaining copy of p53 leading to its functional inactivation(272). In the case of PTEN, point mutations have been identified in one allele and the other copy is transcriptionally silenced. PTEN is one of the few genes that is implicated in prostate cancer by frequent somatic mutations in aggressive prostate cancer. PTEN is more frequently mutated in prostate cancer metastases indicating its role in progression of prostate cancer (270).

KLF-6 is another tumor suppressor gene and its gene product acts as a zinc finger transcription factor and has a role in cell proliferation and differentiation. Somatic mutations of KLF-6 in prostate cancers have been confirmed in many studies, and in addition loss of KLF-6 expression by regulatory mechanisms also occurs in prostate cancer. In addition a germline SNP in KLF-6 has been confirmed in a tri-institutional study of 3411 men there was a significant association of this mutation with an increased relative risk of prostate cancer (273).

The altered expression of several prominent oncogenes also contributes to prostate cancer development and progression. A very consistent finding in

metastatic cancers is over-expression of MYC which is due to increased copy number by chromosomal gains or amplification. Comparative genome hybridizing studies have shown that gain of 8q, including 8q24 involving MYC and 8q21 is one of the most frequent alterations in prostate cancer (274). In vivo studies using transgenic mouse models over-expressing MYC in the prostate leads to prostatic intraepithelial neoplasia and induction of prostate carcinomas. Moreover, over-expression of MYC, can also immortalize human prostatic epithelial cells (275).

In addition to altered oncogenes and tumor suppressor genes, prostate cancer has been shown to harbor deregulated expression of several growth factors including tyrosine kinase receptors EGFR, FGFR2c and ErbB2. Chromosomal gain may contribute to over-expression of many of these growth factors/receptors (276). Although examination in finer detail will reveal a much larger number of genes that are responsible for the initiation, progression and promotion of prostate cancer, the genes mentioned above are some of the critical genes involved in the disease.

1.3.6 Treatment of prostate cancer

Treatment of prostate cancer is highly dependent on the stage and advancement of the cancer (Table 8). There are multiple treatment options based on individual diagnosis and progression of the disease. Management options for clinically localized prostate cancer include radical prostatectomy,

Table 8. Staging of the primary tumor and regional lymph nodes (277).

T- Primary tumor		ABCD
TX	Primary tumor cannot be assessed	
T0	No evidence of primary tumor	
T1	Clinically unapparent tumor not palpable or visible by imaging	
T1a	Tumor incidental histological finding in 5% or less of tissue resected	A1
T1b	Tumor incidental histological finding in more than 5% or less of tissue resected	A2
T1c	Tumor identified by needle biopsy	B0
T2	Tumor confined within the prostate	
T2a	Tumor involves one lobe	B1
T2b	Tumor involves both lobes	B1
T3	Tumor exceeds through the prostatic capsule	
T3a	Extracapsular extension (unilateral or bilateral)	C1
T3b	Bilateral extracapsular extension	C1
T3c	Tumor involves seminal vesicles	C2
T4	Tumor invades bladder neck, external spincter, rectum, levator muscles and/or pelvic wall	C2
N	Regional lymph nodes	
NX	Regional lymph nodes cannot be assessed	
N0	No regional lymph node metastasis	D
N1	Regional lymph node metastasis	

radiotherapy and cancer surveillance. Adjuvant therapy in the form of androgen deprivation, radiotherapy and hormone therapy is available for patients with more advanced cancers. Current treatment options for early prostate cancer include radical prostatectomy, radiotherapy and hormone therapy consisting of androgen ablation(278).

Radical prostatectomy is a curative procedure intended for treatment of localized prostate cancer, which consists of surgical removal of the prostate and the seminal vesicles. It was first discovered by H. H. Young in 1905. Since then many advancements have been made in this technique which enables the patients to maintain their quality of life, in terms of urethral control and erectile function. Radical retropubic prostatectomy (RRP) is offered to men with clinically localized prostate cancer (T1-3a, Nx/0, M0) and a life expectancy of 10 years or longer (279). In general PSA levels serve as a good method for monitoring the tumor free status following RRP. A few long term (15 years) follow-up studies have shown that PSA progression-free survival has been reported to be 40-60% after 15 years (279).

Radical radiation therapy is currently used to treat more than 60,000 men each year in achieving total tumor eradication in these men who are presented with early prostatic carcinoma (280).. There are no randomized studies that compare the outcome of surgery (prostatectomy) with either radiotherapy or brachytherapy for patients with clinically localized low-risk prostate cancer. However there is substantial documentation from large single-institutional and multi-institutuional studies of patients with this disease category (PSA < 10, gleason score ≤ 6, ≤ T2b) showing that the outcome of external beam radiotherapy is similar to those of surgery (277). A successful outcome requires a patient to have no PSA-based evidence of relapse with long-term follow-up of 10 years or more(280).

1.3.6.1 Antiandrogen hormone therapies

Androgen deprivation by hormone therapy has been the mainstay of treatment for managing advanced prostate cancer. Castration-based therapy is the predominant form of androgen deprivation therapy; however it is associated with adverse side effects such as decreased muscle mass, loss of bone mass and osteoporosis along with quality of life issues such as maintenance of sexual activity and physical ability. Hence alternative hormone therapies that are both effective and better tolerated are essential (281, 282).

Androgen blockade for the treatment of prostate cancer has significantly improved since its inception in the 1930 where the methods used were irradiation of the testis or radiotherapy. Androgen blockade can now be achieved by pharmacological means with the introduction of luteinizing hormone releasing hormone (LHRH) analogs. LHRH analogs are equally effective as castration and have the advantage of being reversible. Leuprolide (Lupron®, Viadur ®) and Goserelin (Zoladex ®) are the two most commonly used for treatment of prostate cancer in the US (283, 284). However, LHRH analogs are associated with many disadvantages such as initial transient “flare” of symptoms during therapy, loss of sexual potency, hot flashes fatigue, anemia and osteoporosis (283).

Antiandrogens are a class of drugs with a different mechanism of action to castration therapies. Antiandrogens compete with circulating androgens for binding sites on their receptors with in the prostate cell, thus promoting

apoptosis and inhibiting prostate cancer growth (Figure 12) (283). Antiandrogens are classified into two structurally distinct classes namely steroidal antiandrogens which include cyproterone acetate (CAP) and non steroidal antiandrogens which include bicalutamide [Casodex], flutamide, nilutamide. Both classes of chemicals act as competitors of androgens at the receptor level. While this is the main mechanism of action for non-steroidal antiandrogens, steroidal antiandrogens also have progestational activity that decreases serum testosterone levels, leading to loss of libido and sexual function (282, 285). In contrast, the non-steroidal antiandrogens do not suppress testosterone and estrogen levels, allowing sexual potency to be retained (282).

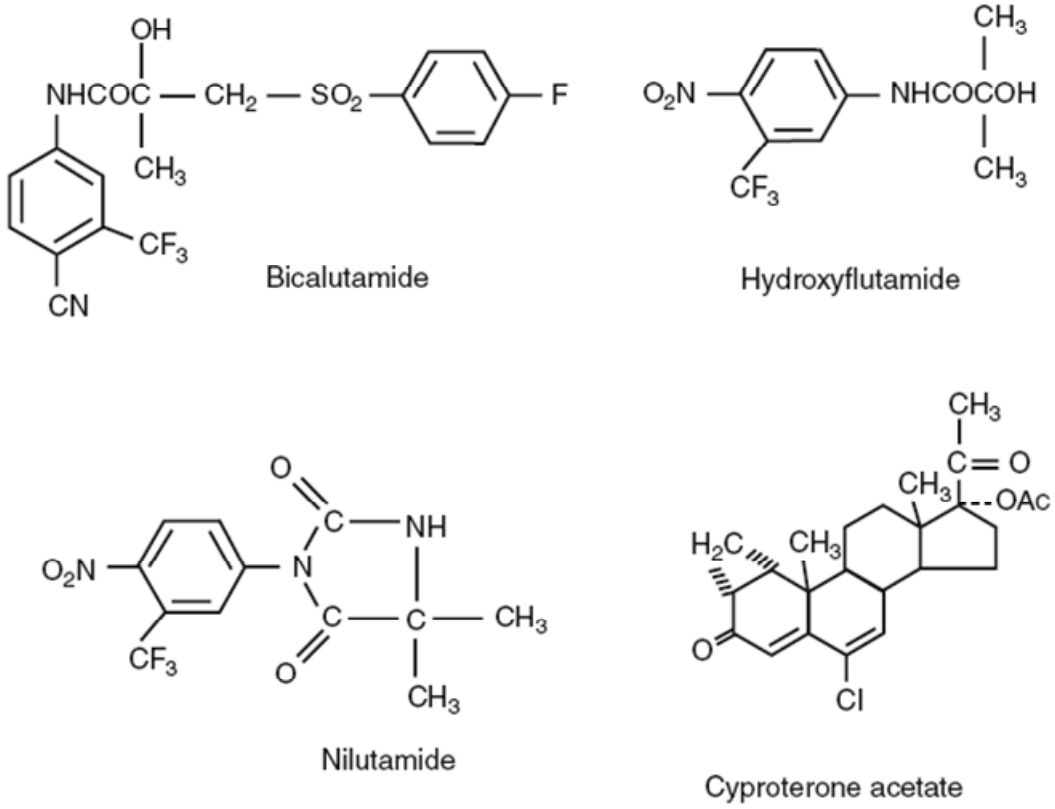


Figure 12. Structures of available antiandrogens (282).

Comparative studies of non-steroidal antiandrogen monotherapy versus castration alone in patients with locally advanced disease have been carried out with bicalutamide only. A study pooling two open-labeled, multicenter studies of identical design has evaluated efficiency of bicalutamide monotherapy to castration. The study consisting of 480 patients with T3-4 upto N0 disease stages received either 150 mg of bicalutamide or castration. After a follow-up of 6.3 years, mortality was 56% with no significant difference between the two

groups in terms of overall survival or time of progression for bicalutamide relative to castration (286). The early prostate cancer program (EPC) conducted 3 randomized double-blind, placebo controlled studies to compare bicalutamide as an adjuvant to therapy of curative intent in men with locally advanced disease. The study consisted of 8,113 men with localized or locally advanced prostate cancer who were randomized to bicalutamide 150 mg or placebo, in addition to standard care of radiotherapy, radical prostatectomy, or watchful waiting. The follow-up analysis (7.5 years) revealed that 150 mg of bicalutamide significantly improved progression-free survival compared with radiotherapy or radical prostatectomy alone. In addition, bicalutamide 150 mg adjuvant to radiotherapy was associated with a significant improvement in overall survival, largely because of a reduced risk of prostate cancer-related deaths (16% in bicalutamide group vs. 24.3% with standard care alone) (287). In a randomized trial evaluating flutamide at 750 mg adjuvant to radical prostatectomy compared with radical prostatectomy alone in patients with lymph-node negative prostate cancer there was a significantly increased progression free survival.(288).

One of the most important advantages of non-steroidal antiandrogens is that they allow patients to maintain serum testosterone and estrogen levels, thus offering potential benefits on quality of life when compared with castration based therapies (282). In an Italian study comparing bicalutamide 150 mg monotherapy with combined androgen blockade (CAB) using flutamide 750 mg plus goserelin 3.6 mg (LHRH analog), significantly fewer patients in the bicalutamide group

reported erectile dysfunction (69.2% vs. 93.3% respectively) or loss of libido (59.6% vs. 85.5 respectively) compared with CAB alone.(289). Hence it is clear that non-steroidal antiandrogens are an effective treatment option with locally advanced prostate cancer and are advantageous over castration based regimens.

1.3.7 The androgen receptor (AR)

The AR gene is a member of the steroid hormone receptor gene family which includes the glucocorticoid receptor, progesterone receptor, mineralocorticoid receptor and s(290). The AR is located on chromosome Xq11-12 and its genomic organization is conserved throughout mammalian evolution from rodents to man. The AR gene spans approximately 90 kb of DNA containing eight exons that code for 2757 base pair open reading frame with a 10.6-kb mRNA (Figure 13) (291, 292). The first exon codes for the N-terminal domain that is the transcriptional regulatory region of the protein, also known as the activation function 1 (AF1) domain. Exon 2 and 3 code for the central DNA-binding domain (DBD) and exons 4-8 code for the C-terminal steroid hormone/ligand binding domain (LBD) (291, 292).

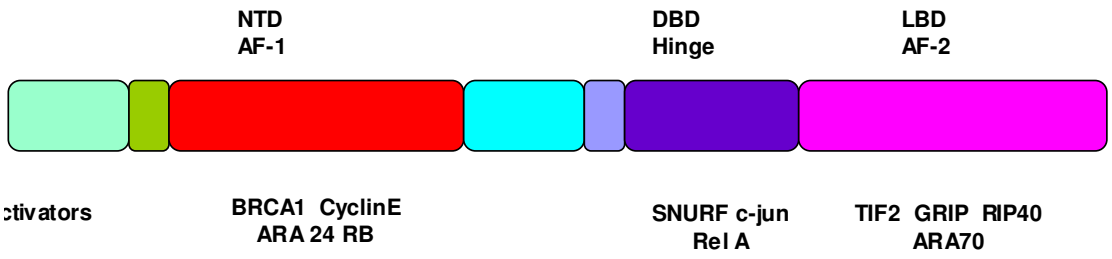


Figure 13. AR structural organization (293).

The AR is a phosphoprotein that mediates the action of testosterone and dihydrotestosterone (DHT) by acting as a transcription factor. The AR is found in many tissues of both sexes but is most abundant in male sex tissues. The AR has a pivotal role in growth and differentiation of the male urogenital structures and is also essential for the initiation and maintenance of spermatogenesis (294).

1.3.7.1 Molecular mechanism of AR action

The AR is a large multi-protein complex comprised of heat shock proteins bound to the ligand binding domain in its transcriptionally inactive form. Many unliganded steroid hormone receptors reside in the cytoplasm where they are sequestered by such chaperone molecules. Ligand binding induces conformational changes in receptors, which leads to shedding of the chaperones and translocation into the nucleus (295-299). However in the case of the AR

there is evidence showing that the major portion of the receptor is located in the nucleus (292) and localization of the AR may also be cell type specific (300, 301). In the absence of ligand, the LBD represses AF-1-mediated activation and agonist binding phosphorylates the AR at three or more sites and one of these is required for transactivation (292).

Like other nuclear receptors, AR activity is also modulated by coactivator and corepressor complexes. Agonist binding results in recruitment of coactivators to the AF1 regions resulting in formation of a protein complex which then interacts with other transcriptional mediators and cofactors as well as the basal transcriptional machinery to modulate target gene transcription(302, 303). There are mainly two types of coactivators; type 1 coactivators function by influencing chromatin remodeling, or recruitment of general transcription factors associated with the RNA polymerase II holocomplex. The p160 family of coactivators, which include steroid receptor coactivator 1 (SRC-1), SRC-2, also known as glucocorticoid receptor interacting protein (GRIP/TIF2), SRC-3, cyclic AMP response element binding protein/p200 (CBP/p300) act as transcriptional coactivators for AR and are included in type 1 coactivators (304, 305). Type II coactivators act by influencing appropriate folding of the AR or promoting between the N- and C-terminal domain interactions. A number of AR associated proteins including ARA70, ARA 55, ARA54 which stabilize ligand-bound AR are included in this category.(306). The cell cycle regulatory proteins such as cyclin E and the RB also function as coactivators of AR in its transactivation regulatory

activity, and this may be a factor linking the growth promoting activity of AR signaling with regulators of cell cycle progression (293, 306, 307).

1.3.7.2 AR and prostate cancer

The AR and prostate cancer are closely linked since the prostate gland depends on androgens for its development and maintenance. Congenital dysfunction or deficiency mutations of the 5-a-reductase in genetic males causes minimal development or absence of the prostate gland (308). Mice that have been castrated for less than a week will experience involution of the prostate due to epithelial cell apoptosis (309). The role of AR in promotion of prostate cancer is evident in transgenic mice engineered for elevated AR expression in the prostate since they show very high turnover of prostatic epithelial cells and develop prostatic intraepithelial neoplasia later in life (310). Although it is evident that androgens are important in the development of prostate cancer, it is difficult to correlate relative levels of serum androgens with prostate cancer risk since many other factors may play an equally important role in prostate cancer promotion (292).

The AR gene is normal and expressed in primary prostate carcinomas, but following androgen deprivation therapy, a number of AR gene alterations occur. These alterations cause the receptor to be more sensitive to low levels of circulating androgens and also affect the receptor's ability to recognize a wider spectrum of ligands that activate the AR (292, 294). Prostate cancer cells adapt

to hormone deprivation therapy by amplifying AR gene copy number. Approximately 25-30% of patients who experience disease reoccurrence exhibit this phenomenon. Experiments have shown that gene amplifications are not found in pretreated tissue from patients in whom AR gene amplification occurred following hormone ablation therapy.(311-313). AR amplification was associated with increased mRNA expression and patients harboring AR amplifications had longer response duration to initial hormone therapy and a longer median survival after recurrence (313). Also, patients with AR amplifications had an increased likelihood of responding to second-line hormone therapy than patients lacking amplification (313).

Point mutations in the AR lead to altered responses to antiandrogens and binding affinities to a larger number of agonists. The first AR mutation in prostate cancer was discovered in the well-characterized LNCaP cell line which harbors a point mutation at codon 877 of the hormone-binding domain. The mutant cell lines recognize the antiandrogens cyproterone acetate and hydroxyflutamide, the active metabolite of flutamide as agonists (314, 315). Since then, point mutations have been reported in hormone sensitive prostate cancers and more frequently from androgen-independent tumors (316, 317). Marcelli et al. (2000) reported that AR point mutations occurred in 8 of 38 (21%) regional lymph nodes from hormone-sensitive patients (318). Tilley et al. (1996) reported AR mutations in 11 of 25 (44%) of prostate gland tumor samples from hormone-sensitive patients (319).

AR mutations in prostate cancer cluster in three regions of the gene. In the LBD, mutations cluster in the loop between helices 3 and 4 which is common to many steroid hormone receptors. Mutations in this regions enables the AR to bind to a greater spectrum of steroid hormones and pharmacological antiandrogens(317, 320, 321). A second cluster is located in the region 874 to 910 that flanks AF-2, and affects binding of p160 coactivator molecules and the AR NTD (322). Mutations are also found in the hinge region that borders the DBD and the LBD. Mutations in this region affects AR interactions with corepressors resulting in diminished efficacy of antiandrogens and this may explain the sensitization of the AR to ligand interactions in late-stage prostate cancer (323).

There have also been several studies showing that the AR in prostate cancer cell lines can be activated in the absence of androgens or by decreased levels of androgens. Initial studies in LNCaP cells showed the growth factor interactions with IL-6 and the IGF-1, keratinocyte growth factor(KGF), and EGF stimulated AR transactivation function in the absence of androgen (293, 324). These results correlate with the observation that IL-6 levels are elevated both in the serum as well as tissue extracts of patients with prostate cancer. Also KGF, IGF-1, and EGF are produced and secreted by fibroblasts of prostatic stoma tissue and these growth factors are known to affect paracrine regulation of prostate epithelial cells via their cognate cell surface receptors expressed in epithelial cells. The ligand dependent interaction causes activation of receptor

tyrosine kinase activity of the cell surface receptors in epithelial tissue which leads to activation of a cascade of kinase-driven intracellular signaling pathways. With the help of pharmacological inhibitors , it was demonstrated that the Ras-Raf-MEK-ERK pathway of MAP kinase signaling is a major pathway in the crosstalk of AR with IL-6/IGF/EGF/KGF (293, 324). High grade prostate carcinomas are frequently exhibit activated PI3K/Akt signaling (325). Activation of Akt triggers antiapoptotic signals by phosphorylation and induces inactivation of the proapoptotic proteins Bad and Caspase-9 (326, 327).

Like androgens, estrogens can also promote growth of the hypertrophic prostate tissue and prostate adinocarcinomas. The b form of ER (ERβ) is the primary mediator of estrogen action in the prostate (328). The normal human prostate epithelium shows significant expression of the ERβ and E2 stimulates proliferation of LNCaP prostate cancer cells. Exposure of LNCaP cells to estrogen or androgen triggers association of AR and ERβ to the cytoplasmic tyrosine kinase Src and the AR-Src-ERβ ternary complex activates the Src/Raf/-1/ERK-2 pathways, causing S-phase entry and prostate cancer cell proliferation (329).

With all the molecular knowledge available at hand about the many pathways active in prostate cancer progression and metastasis, combined with the ongoing research commitments in this field, new methodologies and new drugs will become available to the growing demands of the disease.

1.4 Research objectives

The AhR is a ligand activated nuclear transcription factor that mediates responses to toxic halogenated aromatic compounds such as TCDD, polynuclear aromatic hydrocarbons, combustion products and phytochemicals such as flavonoids and I3C. The AhR complex is a heterodimer containing AhR and ARNT which bind genomic DREs in regulatory regions of Ah-responsive genes (122, 132). SAhRMs are relatively non-toxic AhR agonists that inhibit E2-induced signaling pathways and growth of E2-dependent mammary tumors in rodent models.

1.4.1 Objective 1 (breast cancer study)

The first objective is to investigate the potential growth inhibitory actions of SAhRMs in ER-negative breast cancer cells that express a functional AhR. This study has identified two new Ah-responsive ER-negative breast cancer cell lines namely BT-474 and MDA-MB-453 cells. Preliminary results from these cell lines show that the SAhRMs-6-MCDF and DIM inhibit growth of these cells and TCDD induces CYP1A1 and DRE-dependent activities (330). This study describes the growth inhibitory effects of SAhRMs in ER-negative breast cancers and investigates their mechanism of cell growth inhibition. A major objective in this laboratory is to develop new mechanism-based treatments for later stage ER-negative breast cancers, since these tumors are now treated with

cytotoxic drugs which exhibit adverse toxic side effects in multiple tissues and organs.

1.4.2 Objective 2 (prostate cancer study)

Epidemiological studies show that high consumption of cruciferous vegetables such as cauliflower, broccoli and brussels sprouts are associated with decreased risks for several cancers and I3C is highly expressed in these vegetable and the this compound induces several anticarcinogenic activities (185, 188, 190, 331-333). At low pH in the gut, I3C is converted into structurally diverse condensation products including DIM and both compounds induce many of the same responses in vitro (140, 194, 334). Research in this laboratory has focused on using DIM as a building block for synthesis of both ring- and methylene-substituted DIMs (C-DIMs) as a novel class of anticancer drugs.

The major objective of this study will be to determine the structure-dependent antiandrogenic activities of a series of ring-substituted DIMs and identify potentially new antiandrogens for treatment of prostate cancer. A series of symmetrical, dichloro and dibromo DIM isomers will be investigated as antiandrogens and androgens in AR-responsive LNCaP and 22Rv1 prostate cancer cells transfected with the androgen-responsive pPB construct, which contains the probasin gene promoter linked to the luciferase gene. We expect to

see structure dependent activation with the series of DIM compounds under investigation.

The antiandrogenic/androgenic responses of few model ring substituted DIM compounds will be further investigated by determining their effects on AR levels and expression of androgen-responsive proteins such as PSA and FKBP51 in LNCaP cells since these proteins play a major role in the prognosis of prostate cancer treatment (335). We expect some of these compounds will act by decreasing AR levels and mechanism of this response will be investigated.

1.4.3 Objective 3 (prostate cancer study)

The second objective of the prostate cancer study will be to determine the mechanism of action of the ring-substituted DIMs. Preliminary results show that ring-substituted DIMs inhibit growth of prostate cancer cells lines (LNCaP and 22Rv1 cells). It has previously been shown that overexpression of non-steroidal anti-inflammatory drug-induced gene-1 (NAG-1) in breast cancer cells results in growth arrest and apoptosis. And similar results were observed in colon cancer cells (336-338). In addition to NSAIDs, other agents that induce NAG-1 include phorbol esters, cyclooxygenase inhibitors, genistein, plant polyphenolics, diallyl disulfide, retinoids, I3C, DIM, and peroxisome proliferator activated receptor γ(PPARγ) agonists (336, 337, 339-341). Several mechanisms of NAG-1 induction have been described and these are dependent not only on the

structure or class of inducing agent but also on cell context. We propose to examine the effects of ring substituted DIMs on induction of NAG-1 induction in prostate cancer cells.

CHAPTER II

INHIBITION OF ESTROGEN RECEPTOR NEGATIVE BREAST CANCER CELL GROWTH BY ARYL-HYDROCARBON RECEPTOR AGONISTS

2.1 Introduction

The aryl hydrocarbon receptor (AhR) was initially identified as a receptor that bound the environmental toxicant TCDD with high affinity and studies with AhR knockout mice have confirmed a role for this protein in mediating TCDD-induced toxicity (111, 115, 124-126, 131, 133, 137). The mechanism of AhR action is similar to that described for other ligand-activated receptors and was derived from early studies on AhR mediated induction of CYP1A1 gene expression (reviewed in (115, 124, 133, 342)). The unbound AhR is associated with heat shock protein 90 (Hsp90) and, in the presence of a ligand, the bound receptor forms a hetero dimeric nuclear AhR complex containing the AhR and AhR nuclear translocator (ARNT) proteins. The complex binds DREs in target gene promoters to induce transcriptional activation.

TCDD modulates an increasing number of biochemical, toxic and endocrine responses suggesting that this signal transduction pathway should be useful for developing SAhRMs for treating various diseases. The linkage of the AhR with toxic halogenated aromatics hindered development of SAhRMs;

however, an increasing number of studies have shown that the AhR binds structurally-diverse naturally-occurring compounds including phytochemicals that exhibit multiple chemoprotective and anticarcinogenic activities (139, 140, 195, 343-348). Some of these phytochemical compounds that bind the AhR include I3C and related condensation products, various carotenoids, flavonoids, and the antioxidant resveratrol (found in wine and grape juice).

Research in this laboratory has focused on one of the most intriguing AhR mediated responses, namely the tissue specific inhibition of estrogen-induced responses (183, 349-351). Kociba and coworkers (352) initially reported that dietary administration of TCDD to female Sprague Dawley rats inhibited age dependent spontaneous mammary and uterine tumor formation. Subsequent research in several laboratories has demonstrated that TCDD inhibits 17β-estradiol (E2)-induced uterine wet weight and uterine peroxidase activity, progesterone receptor (PR) binding, (ER) binding, epithelial growth factor receptor (EGFR) binding, c-fos and EGFR mRNA in female rodents (154, 155, 157-161) . Moreover, TCDD also inhibited E2-regulated endometriosis (353) and mammary tumor formation or growth in rodent (mice or rats) models (143, 162, 354) . TCDD has been used to investigate AhR-responsiveness and inhibitory AhR-ERα crosstalk in ERα-positive breast cancer cell lines (145, 146, 148, 150-152, 167, 169, 170, 172, 355-367).TCDD inhibited E2 induced breast cancer cell growth, cell cycle progression as well as a number of E2 responsive genes/proteins including PR mRNA/protein, pS2 mRNA/protein, cathepsin D

mRNA/protein, prolactin receptor mRNA, vitellogenin A2, cyclin D1, creatine kinase B gene, and phosphorylation of RB protein, cyclin-dependent kinase (cdk) 2 and cdk4. Recent studies also show similar inhibitory AhR-ERα interactions in Ishikawa and ECC1 human endometrial cancer cell lines (141, 368, 369). Mechanistic studies show that AhR-mediated inhibition studies of ER-mediated gene expression is complex and may involve multiple pathways (183, 349-351). Studies in this laboratory and others have identified inhibitory dioxin response elements (iDREs) GCGTG, as genomic targets for direct interaction with the nuclear AhR complex, and functional iDREs have been characterized in promoter regions of cathepsin D, c-fos, pS2 and Hsp 27 genes (150, 169, 170). Mutation of these sequences results in loss of inhibitory AhR-ER crosstalk for these genes, and this represents a unique model for interactions between two ligand-activated signaling pathways. In addition, AhR ligands induce proteasome-dependent degradation of ERα (370) and the AhR complex may also directly sequester DNA-bound ER (371).

Most research on the effects of TCDD and other AhR agonists in breast cancer cells has focused on inhibitory AhR-ERα crosstalk in E2-responsive breast cancer cell lines (167, 358-360). It was initially reported that ER-negative breast cancer cells are Ah-nonresponsive (365) and this is typified by MDA-MB-231 cells that express both AhR and ARNT, however, TCDD does not induce CYP1A1 in this cell line. This was later shown to be due, in part, to over expression of Hsp90 which prevented TCDD induced transformation and

activation of the cytosolic AhR receptor complex (372). One study reported the TCDD inhibited growth of ER-negative MDA-468 breast cancer cells and this was the first example of ER-negative cell line that expressed the AhR and was Ah-responsive (369).

In this study we have investigated the Ah-responsiveness of two ER-negative breast cancer cell lines MDA-MB-453 and BT474 cells that overexpress the oncogene epidermal growth factor receptor 2 (EGFR2/ErbB2). The results show that the AhR is expressed in both cell lines and treatment with TCDD induces CYP1A1 protein and ethoxyresorufin-O-deethylase (EROD) activity. TCDD and related compounds inhibit MDA-MB-453 and BT474 cell growth and analysis of several proteins and kinases have identified hairy and enhancer of split homologue-1 (Hes-1) as a TCDD inducible protein associated with AhR dependent inhibition of cell proliferation in these cell lines.

2.2 Materials and methods

2.2.1 Cell lines and reagents

MB-453 and BT474 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). Fetal bovine serum was obtained from JRH Biosciences, Lenexa, KS. Cells were maintained in RPMI 1640 (Sigma Chemical St. Louis, MO) supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% FBS, and 10 mL/L of

100x antibiotic antimycotic solution (Sigma). Cells were maintained at 37°C in the presence of 5% CO_2. Antibodies for AhR, CYP1A1, Sp1, pERK, ERK, pAkt, CD1, p27, pErbB2 and ErbB2 were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). TCDD was prepared in this laboratory. DIM was prepared by dimerization of I3C and 6-MCDF was synthesized in this laboratory and was greater than 98% pure.

2.2.2 Western blot analysis

MDA-MB-453 and BT474 breast cancer cells were seeded in 100 mm plates in DMEM: Ham's F-12 media containing 2.5% charcoal-stripped FBS and allowed to attach overnight. The next day, cells were treated with DMSO, different concentrations of TCDD, DIM 6-MCDF or other chemicals for different time points as indicated. For experiments where MG132 or calpain were used, cells were pretreated for 30 min. Whole cell lysates cells were scraped in 500 µL of lysis buffer [50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% v/v glycerol, 1% Triton X, and 5 µL/ml of Protease Inhibitor Cocktail (Sigma)]. The lysates were incubated on ice for 1 - 1.5 hr with intermittent vortexing followed by centrifugation at 40,000 *g* for 10 min at 4°C. Protein levels were estimated using Bradford reagent; equal amounts of protein were diluted with loading buffer and boiled for 4 min, and loaded onto 10% SDS-polyacrylamide gel. After electrophoresis, gels were transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) using an

electroblotting apparatus overnight at 4°C in transfer buffer containing 48 mM Tris-Cl, 29 mM glycine, and 0.025% SDS. The membranes were blocked with TBS [10 mM Tris-HCl (pH 8) and 150 mM sodium chloride] plus 5% milk (blotto-buffer) for 1 hr, and then incubated in primary antibodies (AhR, Sp1, CYP1A1, pERK, ERK, p27, pAkt, CD1, pErbB2, PARP, Hes-1). at 1:1000 dilution in blotto buffer at 4°C overnight, followed by one min washes (2X) and incubation with secondary antibody for 3 - 5 hr at 4°C. Membranes were then rinsed with water and incubated in enhanced chemiluminescence (ECL) reagents (Perkin Elmer, Boston, MA) for 1 min, removing excess ECL with paper towelette. The membrane was sealed in plastic wrap and photographed for immunoreactive bands using ECL hyperfilm.

2.2.3 Cell proliferation assays

Breast cancer cells were maintained in RPMI media with phenol red and were seeded into 6-well plates at a density of 50,000 cells/well in DME-F12 plus 5% FBS stripped with dextran treated charcoal. After allowing 24 hr to attach, cell were treated with test compounds in DMSO (1% final volume) or DMSO alone every 48 hr in fresh media. Cells were harvested every 48 hr by trypsinization and counted using a Coulter Z1 cell counter (Coulter Electronics, Hialeah, FL).

2.2.4 Ethoxyresorufin-O-deethylase (EROD) analysis of breast cancer cells

Trypsinized cells were plated into 48-well tissue culture plates allowed to attain 70% confluency and treated with DMSO, TCDD, DIM or 6-MCDF for 24 hr. Cells were then washed with PBS, 200 µl PBS was added to each well and cells were incubated in a 37^0 C water bath for 2 min. The reaction was started by addition of 50 µl of ethoxyresorufin (1 mg/40 ml methanol) and incubated in 37^0 C water bath for 10 min. The reaction was stopped by addition of 100 µl fluorescamine. Protein activity was measured at 400/460 excitation/ emission and EROD activity was measured immediately afterward at 530/590 excitation emission using a cytoflor 2350 fluorescence measurement system.

2.2.5 Statistical analysis

Statistical differences between different groups were determined by ANOVA and Scheffe's test for significance. The data are presented as mean ± SE for at least three separate determinations for each treatment.

2.3 Results

MDA-MB 453-and BT474 breast cancer cell lines are highly aggressive cells that over-express ErbB2 and this study investigates the Ah-responsiveness of these cells and the effects of AhR ligands on their proliferation. Figure 14A summarizes the effects of 10 nM TCDD, 1, 2, and 5 µM 6-MCDF on proliferation

of MDA-MB-453 cells and growth inhibition was observed at all concentrations. In a parallel study the growth inhibitory effects of 10 nM TCDD, 1, 5 and 10 μM DIM were also determined (Figure 14B) and growth inhibition was observed at all concentrations of DIM demonstrating that 3 prototypical AhR agonists inhibited growth of an ER-negative breast cancer cell line. MDA-MB-453 cells exhibit constitutively activated kinase activities which are responsible in part, for their enhanced proliferation (373-375). The results in Figure 14C show that the MAPK and PI3K inhibitors UO126 and LY 274002 respectively also inhibit growth of MDA-MB-453 cells and both compounds are more potent than TCDD. Results in Figure 15A show 10 nM TCDD, 10 and 20 μM DIM inhibited growth of BT474 and similar results were observed for 2 and 5 μM 6-MCDF, 5 μM UO126 and 10 μM LY294002 (Figure 15B).

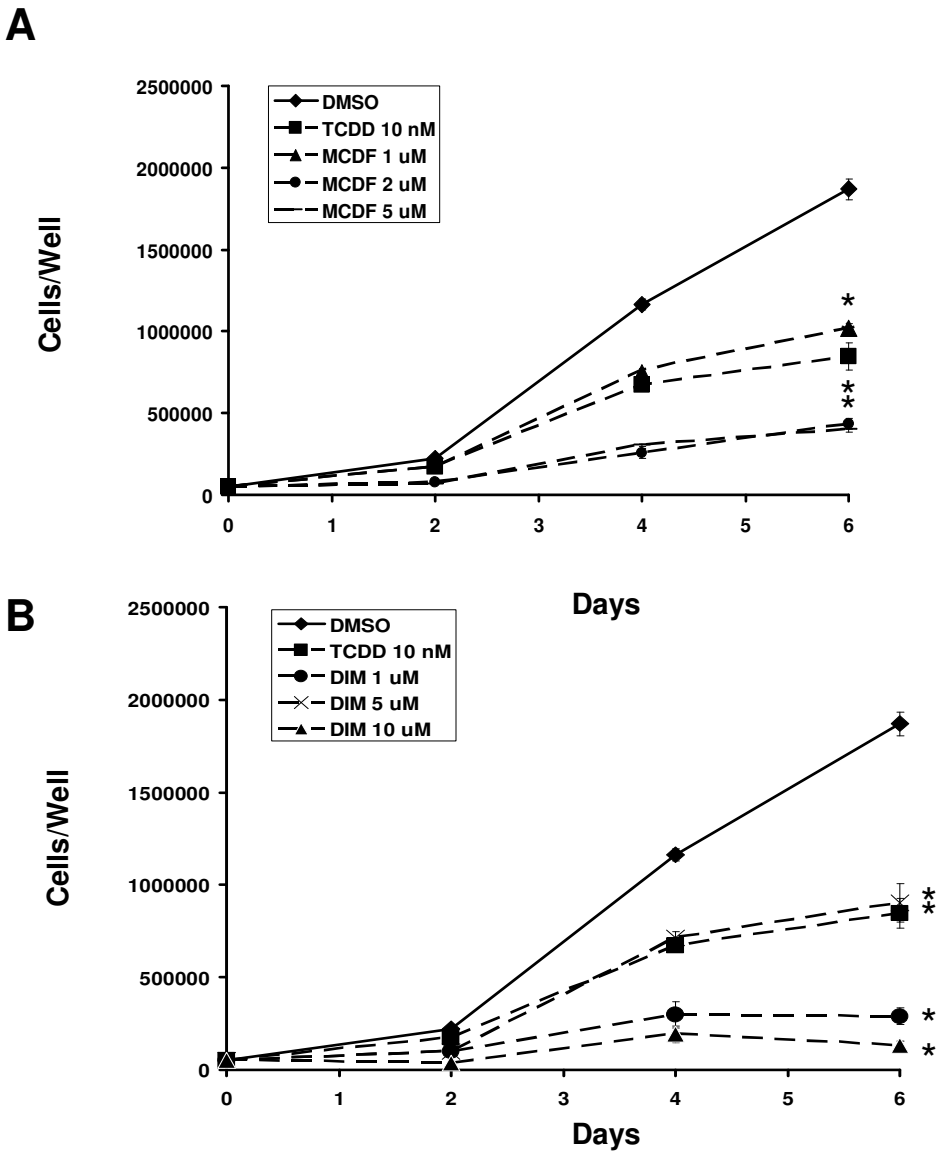


Figure 14. Cell proliferation assays with SAhRMs in MDA-MB-453 cells. MDA-MB-453 cells were treated with (A) TCDD and 6-MCDF (B) TCDD and DIM.

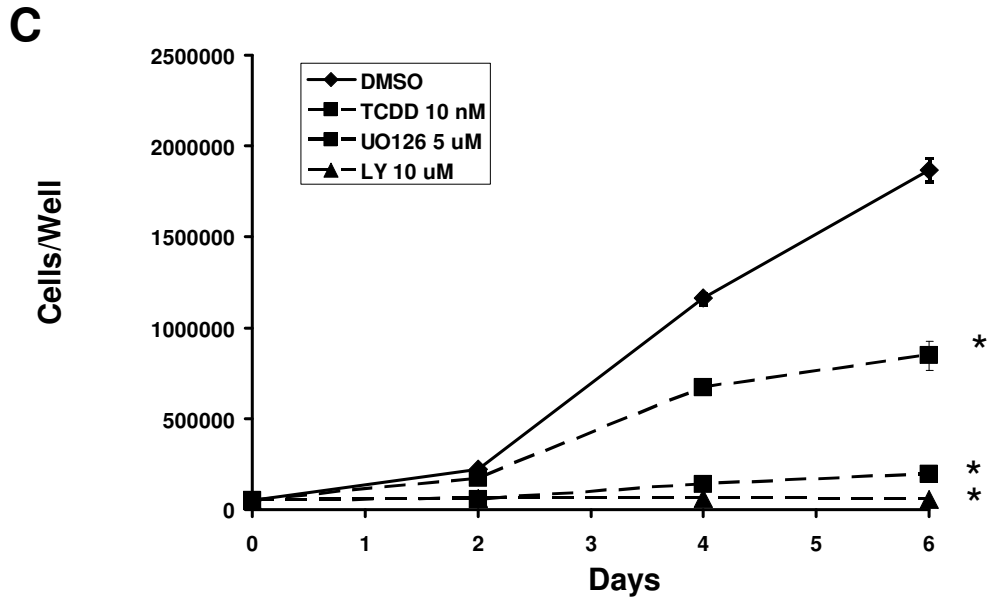


Figure 14. (continued) (C) MDA-MB-453 cells were treated TCDD, UO126 and LY and cell numbers were determined after 2,4 and 6 days as described in Materials and methods. Data presented as means of three replicate determinations for each treatment group. Significant inhibition ($p<0.05$) of cell proliferation ± SE (to DMSO) is indicated as "*" in figures (6 day point).

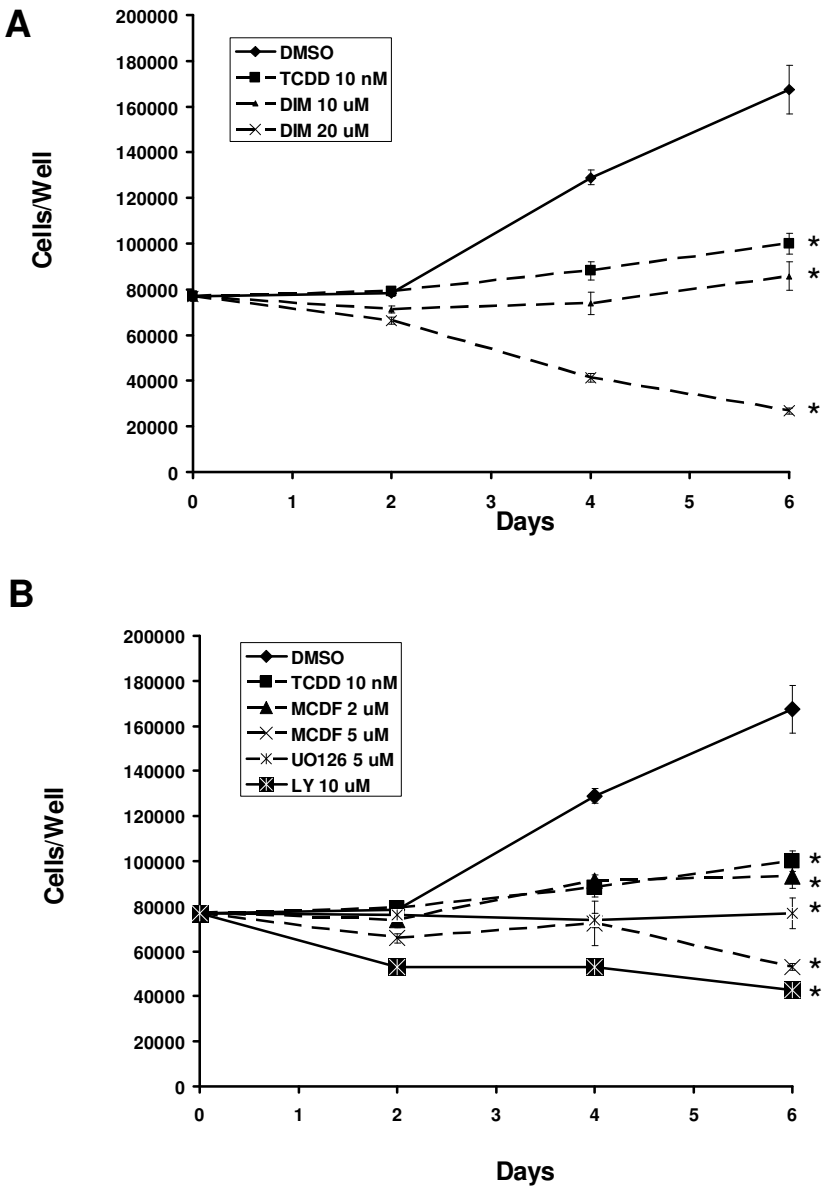


Figure 15. Cell proliferation assays with SAhRMs in BT474 cells. BT474 cells were treated with (A) TCDD and DIM or (B) TCDD, 6-MCDF, UO126 and LY and cell numbers were determined after 2,4 and 6 days as described in Materials and methods. Data presented as means of three replicate determinations for each treatment group. Significant inhibition ($p<0.05$) of cell proliferation ± SE (to DMSO) is indicated as "*" in figures (6 day point).

The Ah-responsiveness of TCDD, 6-MCDF and DIM was investigated in both cell lines by determining the induction of CYP1A1-dependent EROD activity which is a well characterized marker of AhR-active compounds (124, 131). TCDD (1, 10 and 100 nM) induced EROD activity in MDA-MB-453 (Figure 16A) and BT474 (Figure 16B) cells where as 6-MCDF (168, 184, 376, 377) and DIM (378) were inactive as inducers. These results may be due in part, to the low concentrations used in this enzyme induction study. Figures 16C and 16D show that 10 nM TCDD and to a lesser extent 6-MCDF decreased AhR expression levels in MDA-MB-453 and BT474 cells respectively and this is consistent with the previously reported, ligand activated proteasome dependent degradation of the AhR (370). In contrast, DIM did not affect AhR expression. This experiment was also repeated in both cell lines treated with the AhR agonists alone or in the presence of 5 μM MG132 (proteasome inhibitor) (Figures 16E and 16F) and TCDD induced degradation of the AhR was reversed by MG132. Only minimal changes in AhR expression were observed in cells treated with DIM and 6-MCDF and as a positive control the protease inhibitor calpain II did not effect TCDD- induced downregulation of the AhR (data not shown). Thus the effects of TCDD on AhR expression are similar to those observed in ER-positive breast cancer cells, and in other cancer cell lines derived from other tumors. Induction of CYP1A1 protein was also determined in BT474 and MDA-MB-453 (Figures 17A and 17B) and also showed that 10 nM TCDD induced expression of this

protein whereas lower but detectable induction was observed for higher concentrations

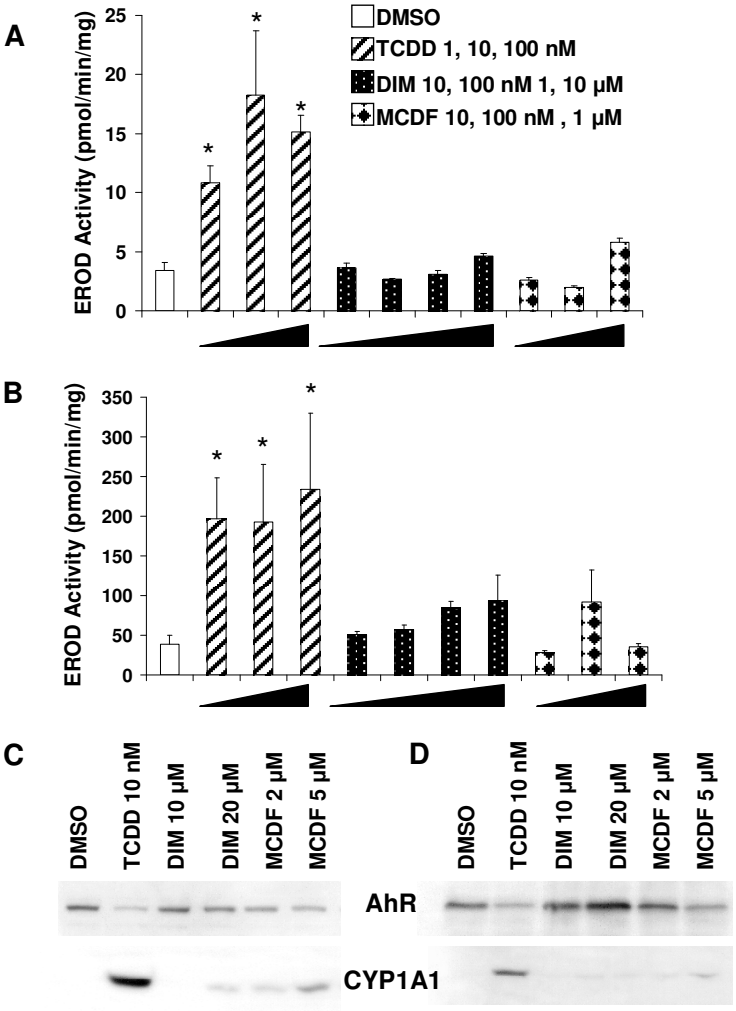


Figure 16. Ah-responsiveness of TCDD and SAhRMs DIM and 6-MCDF. Induction of CYP1A1 dependent EROD activity in (A) MDA-MB-453 cells and (B) BT474 cells. Cells were treated with TCDD and SAhRMs for 24 hr and EROD activity was determined as described in Materials and methods. Effects of TCDD and SAhRMs on AhR expression levels in (C) MDA-MB-453 cells and (D) BT474 cells. Cells were treated for 24 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. Effects of DIM and 6-MCDF on AhR expression and the proteasome pathway.

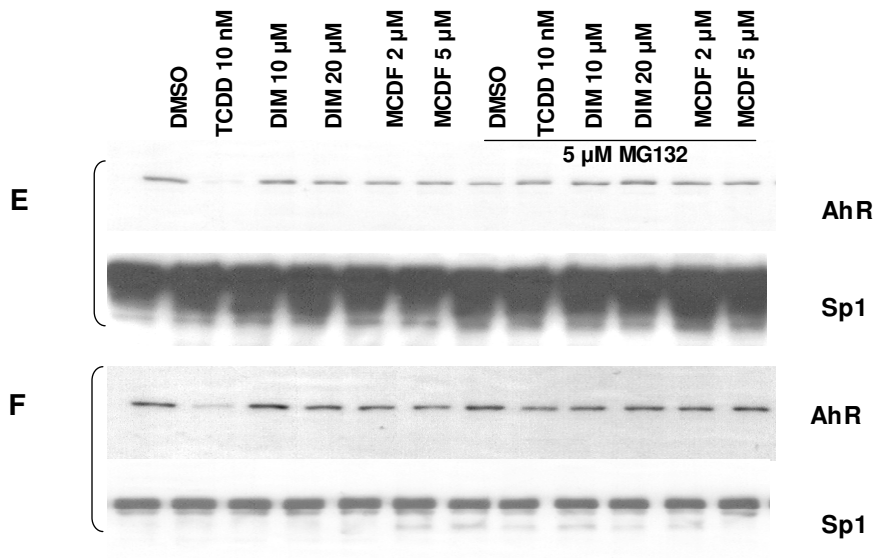


Figure 16. (Continued). (E) and (F) Analysis of the proteasome pathway in MDA-MB453 and BT 474 cells respectively. Cells were treated with different concentrations of compounds for 36 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. In case of MG132 cells were pretreated for 30 min. Sp1 was used as loading control for these experiments.

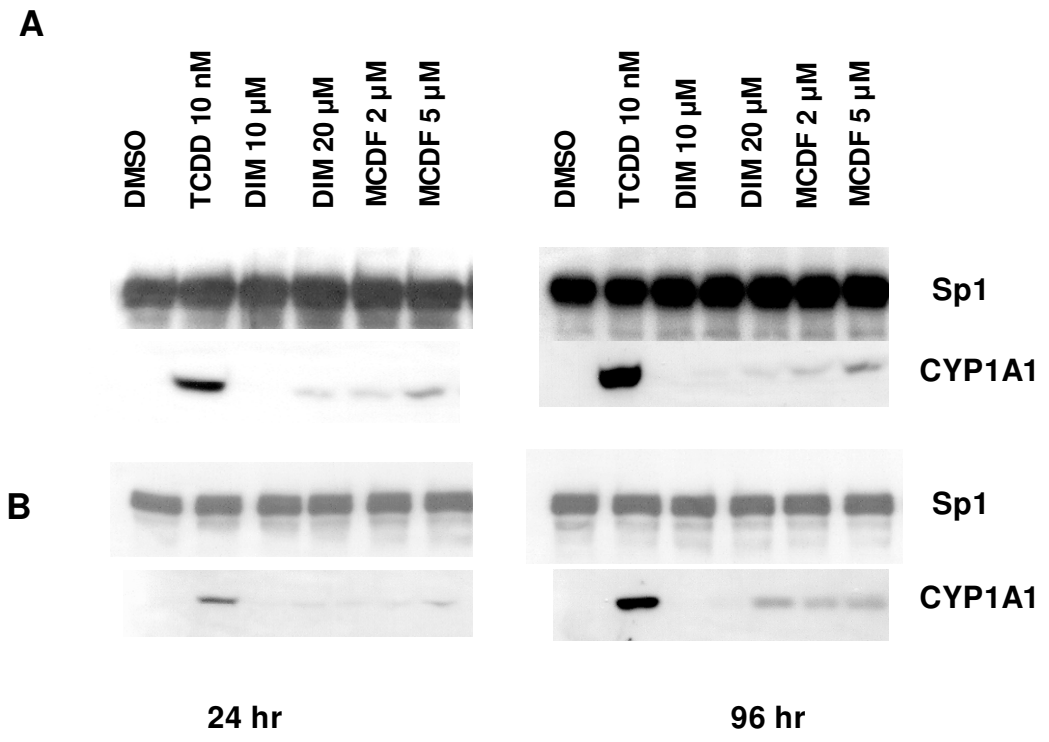


Figure 17. Induction of CYP1A1 by SAhRMs.
(A) MDA-MB-453 cells were treated with TCDD and different concentrations of SAhRMs for 24 hr whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. (B) The experiment was repeated in BT474 cells. Sp1 was used as a loading control for these experiments.

of both 6-MCDF and DIM after treatment for 24 hr. Induction responses were also observed after 96 hr, however, at this later time point the effects of DIM were decreased, and this may have been due to metabolism of this compound. In cells over-expressing ErbB2 several kinases such as MAPK and PI3K are constitutively expressed and contribute to proliferation of these cells(373-375). Results in Figure 18A compare the expression of phospho-ERK (pERK) in

BT474 cells treated for 12 or 24 hr with TCDD, 6-MCDF and DIM and the AhR agonists did not affect pERK or ERK proteins. The effects of these compounds on AhR protein served as a positive control and Sp1 protein was the loading control for this experiment. This study was repeated in BT474 cells treated with the same compounds for 12 and 96 hr (Figure 18B) and the results were similar at both the time points.

A similar approach was used in MDA-MB-453 cells treated with AhR agonists for 12 and 24 hr (Figure 19A) or 12 and 96 hr (Figure 19B). Similar results were observed in both cell lines treated with the same AhR agonists for 12, 24 or 96 hr clearly demonstrating that the MAPK pathway was unaffected by these compounds. As an additional positive control for experiments in Figures 18 and 19 we showed that pERK was decreased in both cell lines treated with the MAPK inhibitor UO126 but not by the PI3K inhibitor LY294002.

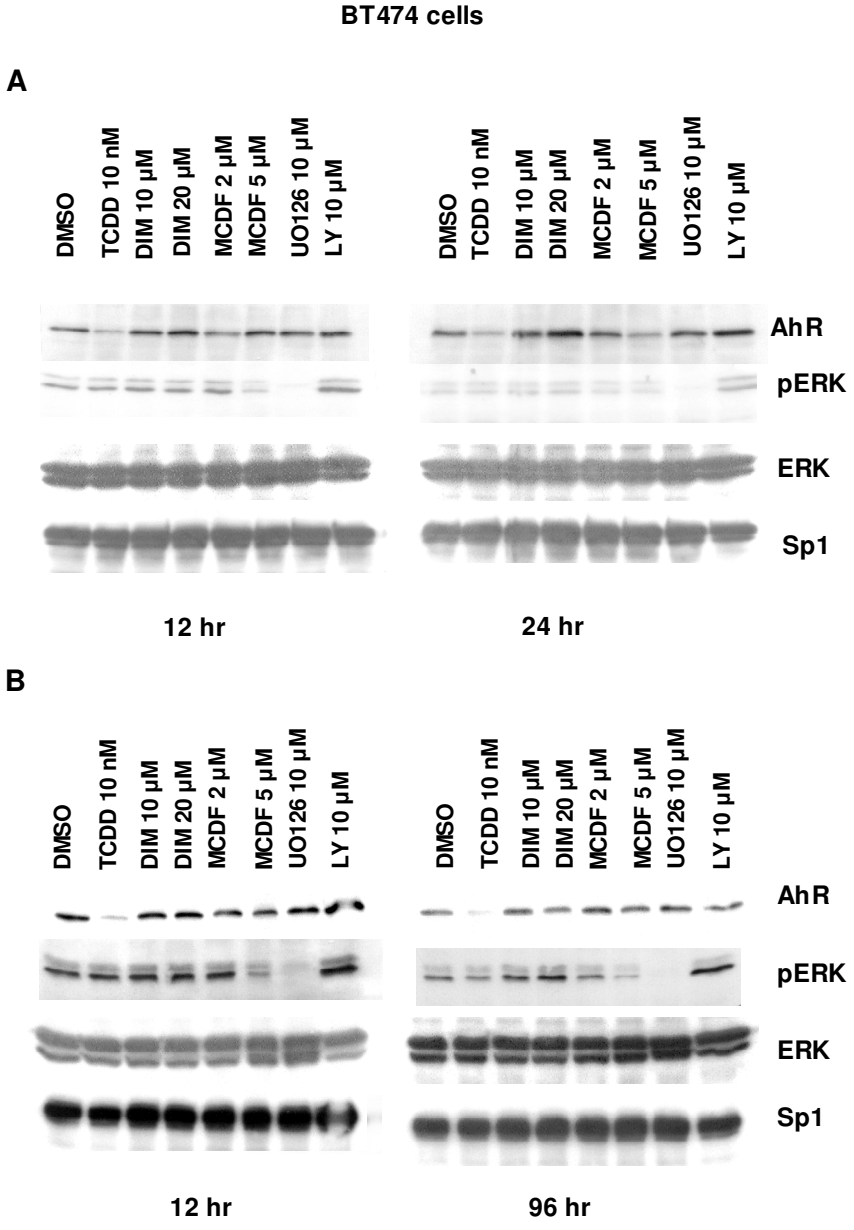


Figure 18.Cellular kinase expression and phosphorylation (BT474). (A) Phosphorylation of ERK in BT474 cells. Cells were treated with different concentration of compounds for 12 and 24 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. The experiment was repeated in BT474 cells for 12 and 96 hr (B). Sp1 was used as loading control for these experiments.

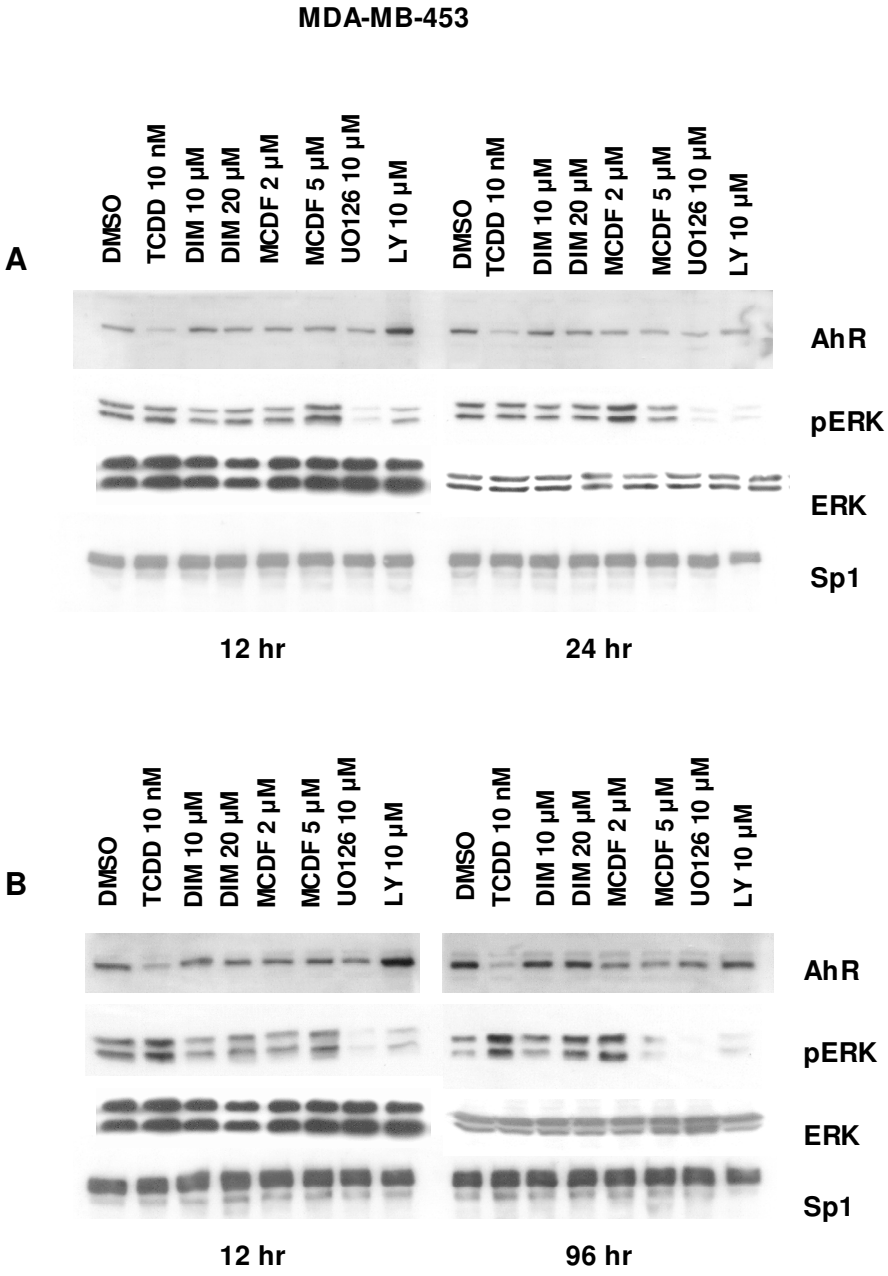


Figure 19. Cellular kinase expression and phosphorylation MDA-MB-453). (A) Phosphorylation of ERK in MDA-MB-453 cells. Cells were treated with different concentration of compounds for 12 and 24 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. The experiment was repeated in MDA-MB-453 cells for 12 and 96 hr (B). Sp1 was used as loading control for these experiments.

The effects of AhR agonists on the PI3K pathway were also investigated (Figure 20A) and the results showed that in BT474 cells the pAkt levels were unchanged by these compounds. Surprisingly, the PI3K inhibitor LY294002 was also inactive in this assay which was repeated several times. Moreover similar results were obtained in MDA-MB-453 cells indicating that AhR agonists did not affect pAkt expression. Moreover levels of cyclin D1 and p27 were also not altered by these compounds and FACS analysis indicated that changes in the percent distribution of different phases of cell cycle were also unchanged (data not shown). We also showed that these AhR agonists did not affect pERbB2 expression (Figure 20B) or induction of apoptosis as indicated by the failure to observed caspase-dependent PARP cleavage (Figure 20C).

Thus although TCDD, DIM and 6-MCDF inhibit growth of BT474 and MDA-MB 453 breast cancer cells, their effects on constitutively active kinase pathways, cell cycle progression and cell cycle genes are minimal to non-detectable (Figures 16-20). A recent study reported that Hes-1 is induced by TCDD in T47D breast cancer cells (379) and this protein has been linked to antiproliferative activity in several cell line (379-383). Treatment of MDA-MB 453 cells with TCDD, DIM and 6-MCDF induced Hes-1 protein expression (Figure 21) and this was consistent with results reported in other cell lines and represents a potential mechanism for the growth inhibitory action of AhR agonists in ER-negative Ah-responsive breast cancer cell lines. Current studies

are focused on fully characterizing Hes-1 induction in these cells and the contributions of this protein to the growth inhibitory activity of AhR agonists.

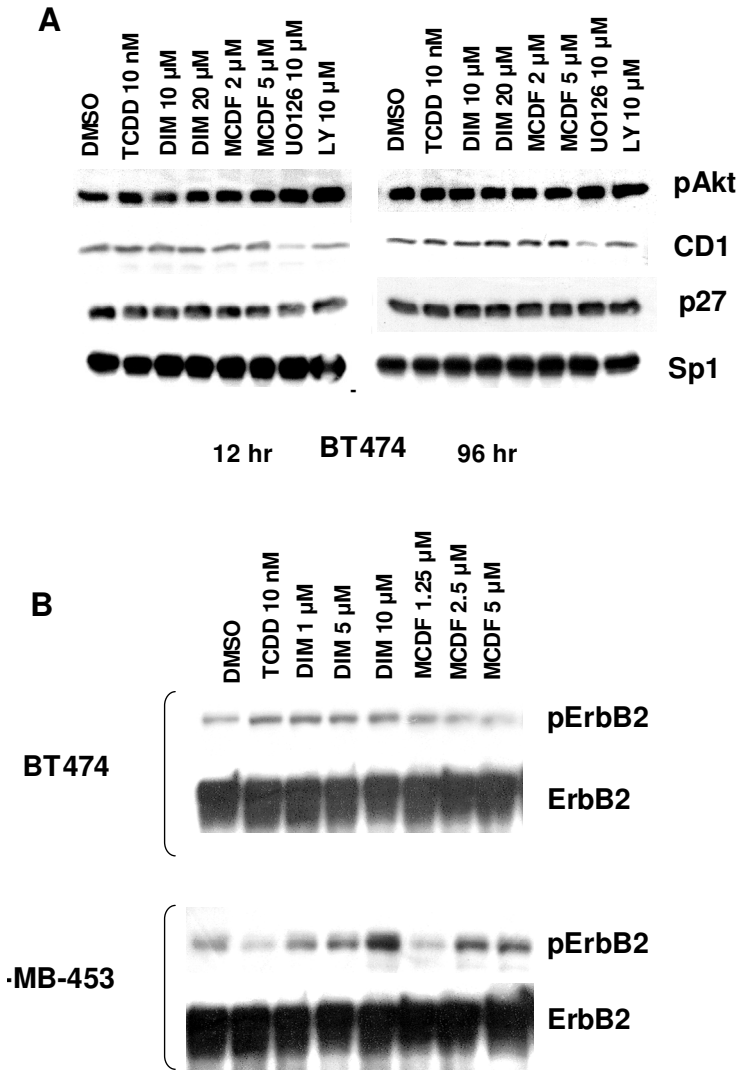


Figure 20. Cellular kinase expression and phosphorylation. (A) Analysis of inhibitory cell cycle genes p27, pAKT, CD1. BT474 cells were treated with different concentrations of compounds for 12 or 48 hr and whole cell lysates were analyzed by western blot analysis. (B) Phosphorylation of ErbB2 in BT474 and MDA-MB-453 cells. Cells were treated with different concentrations of compounds for 48 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods.

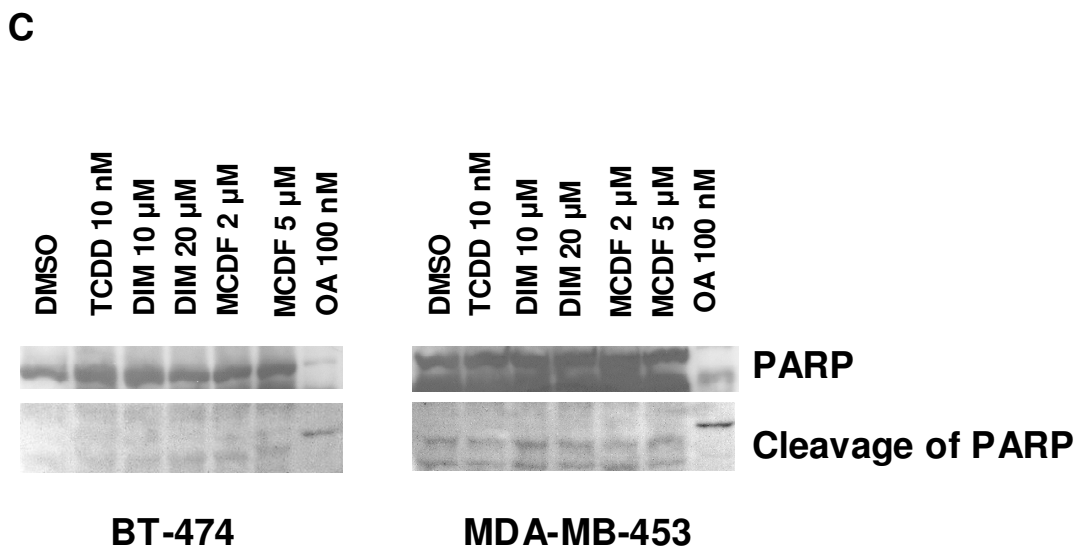


Figure 20. (continued) (C). Induction of apoptosis. BT474 and MDA-MB-453 cells were treated with different concentration of compounds for 36 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods Sp1 was used as loading control for these experiments.

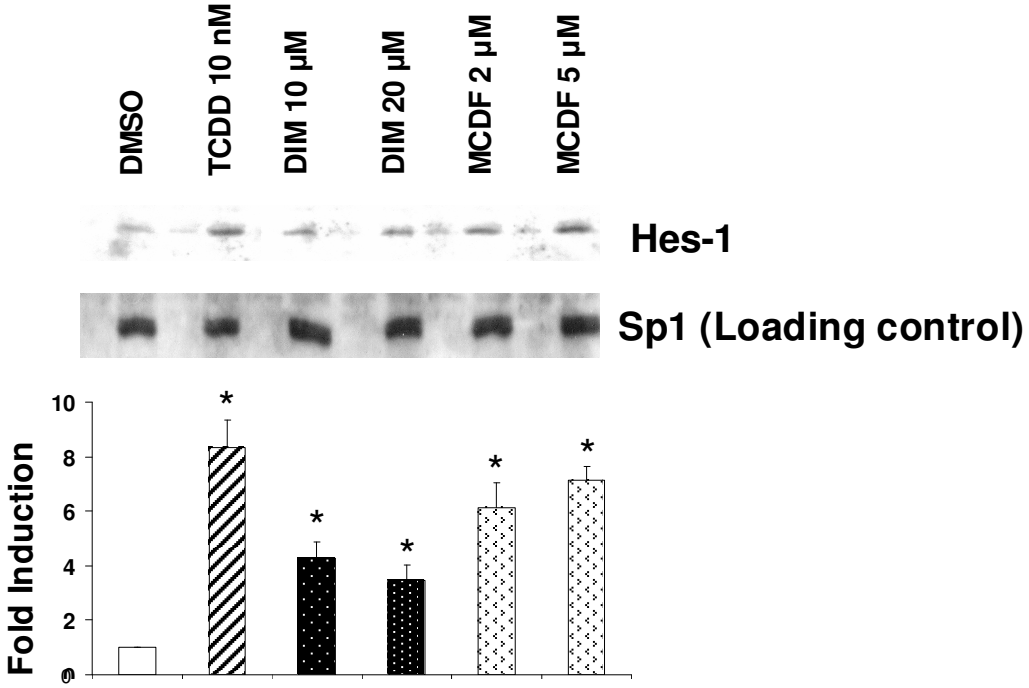


Figure 21. Effects of DIM and 6-MCDF on Hes-1 protein levels. MDA-MB-453 cells were treated with different concentration of compounds for 96 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. Sp1 was used as a loading control for these experiments.

2.4. Discussion

Selective receptor modulators have been developed for steroid hormone receptors as a new class of mechanism-based drugs for treatment of hormone-related diseases. For example tamoxifen is a selective ER modulator (SERM) that exhibits tissue-selective ER agonist and antagonist activities and because of its antiestrogenic effect in breast tumors, tamoxifen has been used as the major drug of choice for treating early stage ER-positive breast cancer in women.

Currently several newer SERMs such a raloxifene are also being developed for endocrine therapies including treatment of breast cancer (384-387) Research in this laboratory has been investigating an alternative mechanism-based strategy for treating breast cancer with SAhRMs (183, 349-351). AhR agonists such as TCDD inhibit E2 dependent gene expression, proliferation of breast cancer cell growth and mammary tumor growth in rodent models (143, 145, 146, 148, 150-152, 154, 155, 157-162, 167, 169, 170, 172, 352-367, 369) . The relatively non-toxic SAhRMs 6-MCDF and DIM also inhibit hormone-dependent mammary cell tumor growth and 6-MCDF is slightly more potent than tamoxifen in the carcinogen-induced rat mammary tumor model (168, 184, 376-378). Moreover treatment with 6-MCDF plus tamoxifen synergistically inhibited mammary tumor growth (184) and this combination therapy is a highly promising new approach for treating early stage breast cancer.

Vickers and coworkers initially showed that ER-positive breast cancer cells were Ah-responsive whereas ER-negative cells were non-responsive to the effects of TCDD and related compounds (365). However, one study reported that MDA-MB-468 cells express a functional AhR and TCDD inhibited cell growth through an unusual mechanism which involved induction of (TGFα which acted as growth inhibitory peptide in this cell line (369). In this study, we now show that two ErbB2 over-expressing breast cancer cell lines BT474 and MDA-MB-453 cells also express the AhR (Figure 16C) and TCDD induced CYP1A1-dependent EROD activity in both cells (Figures 16A and 16B). Not surprisingly

DIM and 6-MCDF exhibited minimal activity as CYP1A1 inducers (Figures 16 and 17) and this correlates to similar results previously observed in ER-positive breast cancer cell lines (376-378). In addition, both TCDD and the SAhRMs DIM and 6-MCDF also inhibited BT-474 and MDA-MB-453 cell proliferation (Figures 14 and 15) suggesting that ER-negative cell lines may also respond to AhR agonists as a new class of drugs for treating these highly resistant later stage tumors.

The mechanism of AhR-mediated inhibition of ER-negative or ER-independent cancer cell growth has not been extensively investigated and the induction of TGFα by TCDD in MDA-MB-468 cells is the only prior report in ER-negative breast cancer cells (369). AhR agonists also inhibited proliferation of pancreatic cancer cells and this was associated with induction of p21 expression (388). TCDD and the SAhRMs did not affect p27 or cyclin D1 expression in BT 474 or MDA-MB-453 cells (Figure 20A). We also investigated induction of other growth inhibitory genes including p21, ATF, NAG1 and KLF 6 and expression of these proteins was also unaffected by SAhRMs or TCDD (data not shown).

MDA-MB-453 and BT-474 cells over-express the oncogene ErbB2 and many ER-negative breast cancer cells also over-express kinases that contribute to their highly proliferative and invasive properties (141, 370-375). Therefore we investigated the effects of TCDD and the SAhRMs as potential inhibitors of constitutively active kinases. However, treatment of BT474 cells with these AhR agonists did not affect several kinase activities including ErbB2 (Figures 18-20).

Recent studies in T47D cells showed that E2 downregulated the HES-1 protein and TCDD induced HES-1 expression in the same cell lines (379-381) . HES-1 is an antiproliferative basic-helix-loop-helix transcription factor and we therefore investigated expression of this gene in BT474 and MDA-MB-453 cells. The results (Figure 21) show that HES-1 protein is induced by TCDD, DIM and 6-MCDF and this represents a possible mechanism for mediating the growth inhibitory effects observed for these compounds. Current studies are further investigating the mechanism of HES-1 induction by AhR agonists in ER-negative breast cancer cells and determining the role of HES-1 and other proteins in mediating the antiproliferative effects of these compounds in these highly proliferative ER-negative cells.

CHAPTER III

STRUCTURE-DEPENDENT ANDROGEN RECEPTOR AGONIST/ANTAGONIST ACTIVITIES AND AR DOWN-REGULATION BY DICHLORO AND DIBROMO-SUBSTITUTED 1, 1'-BIS (3-INDOLYL METHANES)

3.1 Introduction

Epidemiological studies have demonstrated that high consumption of cruciferous vegetables such as cauliflower, broccoli and Brussels sprouts are associated with decreased risks for several cancers (185, 188, 190, 331-333). Indole-3-carbinol (I3C) glucosinolates are expressed in high levels in *Brassica* vegetables, and the anticarcinogenic activity of these vegetables or their extracts in laboratory animal studies is associated with I3C and related chemoprotective phytochemicals (196, 199, 389-394). I3C conjugates are rapidly hydrolyzed in the acidic environment of the gut and converted into structurally diverse condensation products including 1,1-bis(3'-indolyl)methane (DIM) (140, 334). At low pH, the percentage conversion of I3C into DIM is minimal; however, in cell culture studies at pH 6.6 - 7.5, I3C is primarily converted into DIM (194) and both compounds induce many of the same responses *in vitro*.

I3C and DIM exhibit anticarcinogenic activity in several animal models (196, 199, 378, 389-395). For example, in mice bearing mouse TRAMP-C2

prostate cancer cells as xenografts, 2.5, 5.0, and 10.0 mg/kg (3X weekly) DIM significantly decreased tumor weight/volume and induced apoptosis in the prostate tumors (395), and *in vitro* studies also show that DIM induced apoptosis in prostate cancer cells (396). Results from several laboratories show that decreased cancer cell survival after treatment with DIM or I3C may be related to activation of multiple and possibly overlapping pathways including modulation of cell cycle regulatory proteins, induction of apoptosis and ER stress, and decreased mitochondrial membrane potential (193, 201, 202, 396-402).

Le and coworkers (403) reported that DIM is a potent androgen receptor (AR) antagonist in LNCaP prostate cancer cells and inhibits dihydrotestosterone (DHT)-induced cell proliferation and gene/reporter gene expression. Their results showed that the antiandrogenic activity of DIM was associated with the inhibition of DHT-induced AR nuclear translocation, whereas DIM alone did not induce accumulation of nuclear AR. These results were unique for DIM since other structural classes of antiandrogens such as casodex induce accumulation of nuclear AR, which is transcriptionally inactive.

Previous studies in this laboratory have shown that several ring-substituted DIMs were more potent than DIM as inhibitors of carcinogen-induced mammary tumor growth in Sprague-Dawley rat (330, 404). The major objective of this study was to determine the structure-dependent antiandrogenic activities of a series of ring-substituted DIMs and identify potential new antiandrogens for treatment of prostate cancer. A series of symmetrical dichloro- and dibromo

DIM isomers were investigated as antiandrogens and androgens in AR-responsive LNCaP and 22Rv1 prostate cancer cells transfected with the androgen-responsive pPB construct, which contains the -288 to +28 region of the probasin gene promoter linked to the luciferase gene (405, 406). Most of the ring-substituted DIMs exhibited antiandrogenic activity; 7,7'-dichloro- and 7,7'-dibromo DIMs were only partial AR antagonists, and the compounds alone exhibited partial androgenic activity in the transactivation assay. The antiandrogenic/androgenic responses of 4,4'- and 7,7'-dihalo DIMs were further investigated by determining their effects on AR levels and expression of the androgen-responsive FKBP51 protein in LNCaP cells (335). The antiandrogenic activities of 4,4'-dihalo DIMs were confirmed in these assays; however, both 7,7'-dibromo- and 7,7'-dichloro DIM uniquely downregulated AR mRNA and protein levels after treatment for 48 hr.

3.2 Materials and methods

3.2.1 Cell lines and reagents.

Human prostate carcinoma cell lines LNCaP and 22Rv1 were obtained from American Type Culture Collection (Manassas, VA). Fetal bovine serum was obtained from JRH Biosciences, Lenexa, KS. Cells were maintained in RPMI 1640 (Sigma Chemical St. Louis, MO) supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% FBS, and 10 mL/L of 100x antibiotic antimycotic solution (Sigma). Cells were

maintained at 37°C in the presence of 5% CO_2. Antibodies for AR (sc- 816), Sp1 (sc-59), and FKBP 51(sc-11514) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega (Madison, WI). β-Galactosidase (β-Gal) reagent was obtained from Tropix (Bedford, MA). Lipofectamine reagents were supplied by Invitrogen (Carlsbad, CA). Western Lightning chemiluminescence reagents were from Perkin-Elmer Life Sciences (Boston, MA). Dihydrotestosterone was purchased from Sigma. MG132 was obtained from Calbiochem (San Diego, CA) and gliotoxin was kindly provided by Dr. Alan Taylor, Atlantic Regional Laboratory, National Research Council (Halifax, Canada). The ring-substituted DIMs were prepared in this laboratory by condensation of ring-substituted indoles and formaldehyde or by self-condensation of ring-substituted indole-3-carbinols; compounds were >95% pure by gas chromatography-mass spectrometry as previously described (330, 404). All substitute indoles were purchased from the Aldrich Chemical Co. (Milwaukee, WI).

3.2.2 Plasmids

The pPB reporter containing -288 to +28 region of the probasin gene promoter was kindly provided by Dr. Robert Matusik (Vanderbilt University Medical Center).

3.2.3 Transfection and luciferase assay

Prostate cancer cells were plated in 12-well plates at 2.5 x 10^5 cells/well in DMEM:Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS. After overnight attachment, cells were transfected with 400 ng of pPB and 50 ng of β-galactosidase reporter plasmid, using Lipofectamine reagent (Invitrogen) according to the manufacturer's recommended protocol. Cells were transfected and, after 8 - 9 hr, the transfection mix was replaced with 5% charcoal-stripped FBS media containing either vehicle (DMSO) or the indicated DIM-isomer and incubated for 36 - 38 hr. Based on results of preliminary studies showing that responses were not observed at concentrations ≤ 1.0 μM, concentrations ranging from 5 - 15 (for 6,6'-dichloro DIM) or 5 - 20 μM were used. Cytotoxicity was observed at higher doses. Cells were then lysed with 100 mL of 1x reporter lysis buffer, and 30 μL of cell extract were used for luciferase and β-galactosidase assays. Lumicount (Parkard, Meriden, CT) was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-gal activity.

3.2.4 Western blot analysis

LNCaP, and 22Rv1 prostate cells were seeded in 100 mm plates in DMEM:Ham's F-12 media containing 2.5% charcoal-stripped FBS and allowed to attach overnight, followed by treatment with either the vehicle (DMSO) or the indicated compounds for the desired time points. Nuclear and cytoplasmic

fractionating kit (Pierce Biotechnology, Rockford, IL) was used to obtain the nuclear and cytoplasmic lysates. For whole cell lysates cells were scraped in 500 µL of lysis buffer [50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% v/v glycerol, 1% Triton X, and 5 µL/ml of Protease Inhibitor Cocktail (Sigma)]. The lysates were incubated on ice for 1 - 1.5 hr with intermittent vortexing followed by centrifugation at 40,000 *g* for 10 min at 4°C. Protein levels were estimated using Bradford reagent; equal amounts of protein were diluted with loading buffer and boiled for 4 min, and loaded onto 10% SDS-polyacrylamide gel. After electrophoresis, gels were transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) using an electroblotting apparatus overnight at 4°C in transfer buffer containing 48 mM Tris-Cl, 29 mM glycine, and 0.025% SDS. The membranes were blocked with TBS [10 mM Tris-HCl (pH 8) and 150 mM sodium chloride] plus 5% milk (blotto-buffer) for 1 hr, and then incubated in primary antibody at 1:1000 dilution in blotto buffer at 4°C overnight, followed by one min washes (2X) and incubation with secondary antibody for 3 - 5 hr at 4°C. Membranes were then rinsed with water and incubated in enhanced chemiluminescence (ECL) reagents (Perkin Elmer, Boston, MA) for 1 min, removing excess ECL with paper towelette. The membrane was sealed in plastic wrap and photographed for immunoreactive bands using ECL hyperfilm.

3.2.5 RNA extraction and cDNA synthesis

Total RNA was extracted using Quiagen RNeasy Protect (Quiagen, Valencia, CA) according to the manufacturer's instruction. RNA was quantitated and 5 μg was used for reverse transcription using the SuperScript II reverse transcriptase (Invitrogen).

3.2.6 Real-time PCR.

The cDNA was amplified in a real-time PCR using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and 10 pM primers for AR. The reactions were performed in an ABI PRISM model 7700 sequence detector (Applied Biosciences, Foster City, CA). The PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 min, and 60°C for 1 min. The sequences for the AR primers were as follows: forward primer, 5' gta ccc tgg cgg cat ggt 3'; and reverse primer, 5' ccc att tcg ctt ttg aca ca 3'. The TATA binding protein (TBP) was used as a reference standard for quantitating AR mRNA. The primers for TBP are as follows: forward primer, 5' tgc aca gga gcc aag agt gaa 3', and reverse primer, 5' cac atc aca gct ccc cac ca 3'.

3.2.7 Statistical analysis

Statistical differences between different groups were determined by ANOVA and Scheffe's test for significance. The data are presented as mean ± SE for at least three separate determinations for each treatment.

3.3 Results

3.3.1 DIM and ring-substituted DIMs: AR agonist and antagonist activities in transactivation assays

Previous studies show that DIM exhibits AR antagonist activity in LNCaP cells and inhibits DHT-induced PSA protein and reporter gene activity in cells transfected with androgen-responsive constructs (403). In this study, the antiandrogenic activity of DIM has been investigated in LNCaP and 22Rv1 prostate cancer cells transfected with the androgen-responsive pPB construct (Figure 22). Both cell lines express mutant forms of the AR; however, DHT activates the receptor in LNCaP and 22Rv1 cells (407, 408). The results show that DHT but not DIM alone significantly induced luciferase activity in LNCaP and 22Rv1 cells transfected with pPB. In cells cotreated with DHT plus DIM and transfected with pPB, hormone-induced luciferase activity was significantly decreased at DIM concentrations of 5, 10 and 20 μM. These results confirm that DIM inhibits activation of the androgen responsive pPB construct by DHT in both LNCaP and 22Rv1 cells and complements results of a previous report on the antiandrogenic activity of DIM (403).

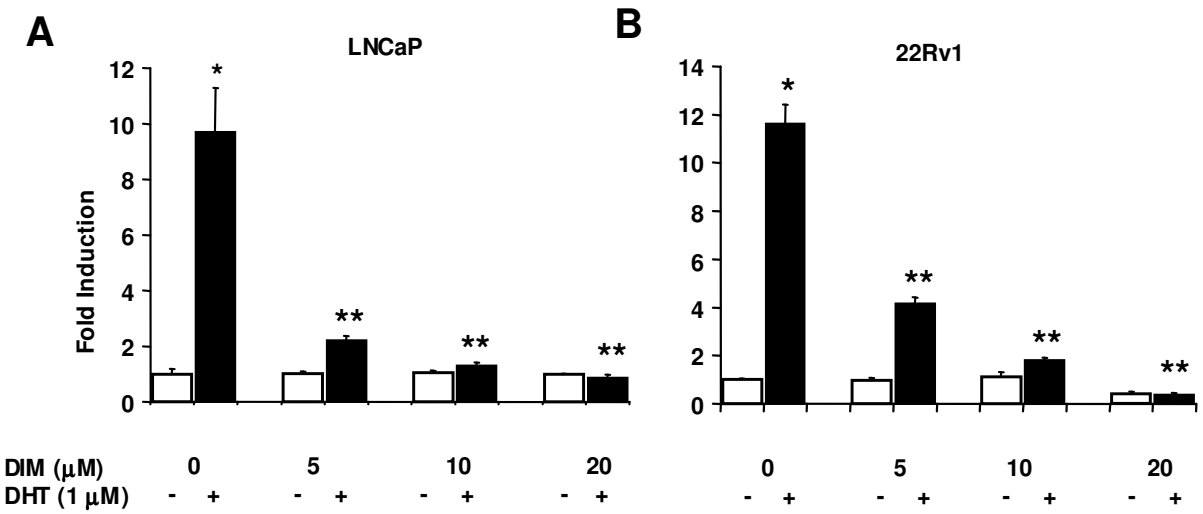


Figure 22. Antiandrogenic activity of DIM.
LNCaP (A) or 22Rv1 (B) cells were transfected with pPB, treated with DHT, 5 - 20 μM DIM alone or in combination with DHT, and luciferase activity determined as described in the Materials and methods. Results are expressed as means ± SE for at least 3 determinations per treatment group, and significant ($p < 0.05$) induction (*) or inhibition (**) in the cotreatment groups are indicated.

Ring-substituted DIMs also exhibit potent anticancer activities (330, 404); however, the structure-dependent effects of these compounds as antiandrogens have not been reported. This study investigates the AR antagonist and agonist activities of symmetrical dihalo DIMs containing substituents in the 4, 5, 6, and 7 positions of the benzene ring. The structure-dependent AR antagonist/agonist activities of 4,4'-, 5,5'-, 6,6'-, and 7,7'-dichloro- and -dibromo DIMs were investigated in LNCaP and 22Rv1 cells transfected with the PB construct

(Figures 23 and 24). The dichloro- and dibromo DIM isomers induce similar structure-dependent responses in both cell lines. The results obtained for the dichloro DIM isomers show that 4,4'-, 5,5'-, and 6,6'-dichloro DIM were AR antagonists in LNCaP and 22Rv1 cells (Figures 23A and 24B), although their AR antagonist activity was more pronounced in the latter cell line. 7,7'-Dichloro DIM was a partial AR agonist/antagonist in both cell lines (Figures 23C and 23D), whereas the other isomers did not exhibit AR agonist activities. The pattern of antiandrogenic/androgenic activities for the isomeric dichloro DIMs (Figure 23) was similar to that observed for the brominated analogs (Figure 24). 4,4'-, 5,5'- and 6,6'-Dibromo DIM primarily exhibited antiandrogenic activities in LNCaP and 22Rv1 cells (Figures 24A and 24B), and 7,7'-dibromo DIM was a partial AR agonist/antagonist in both cell lines (Figures 24C and 24D).

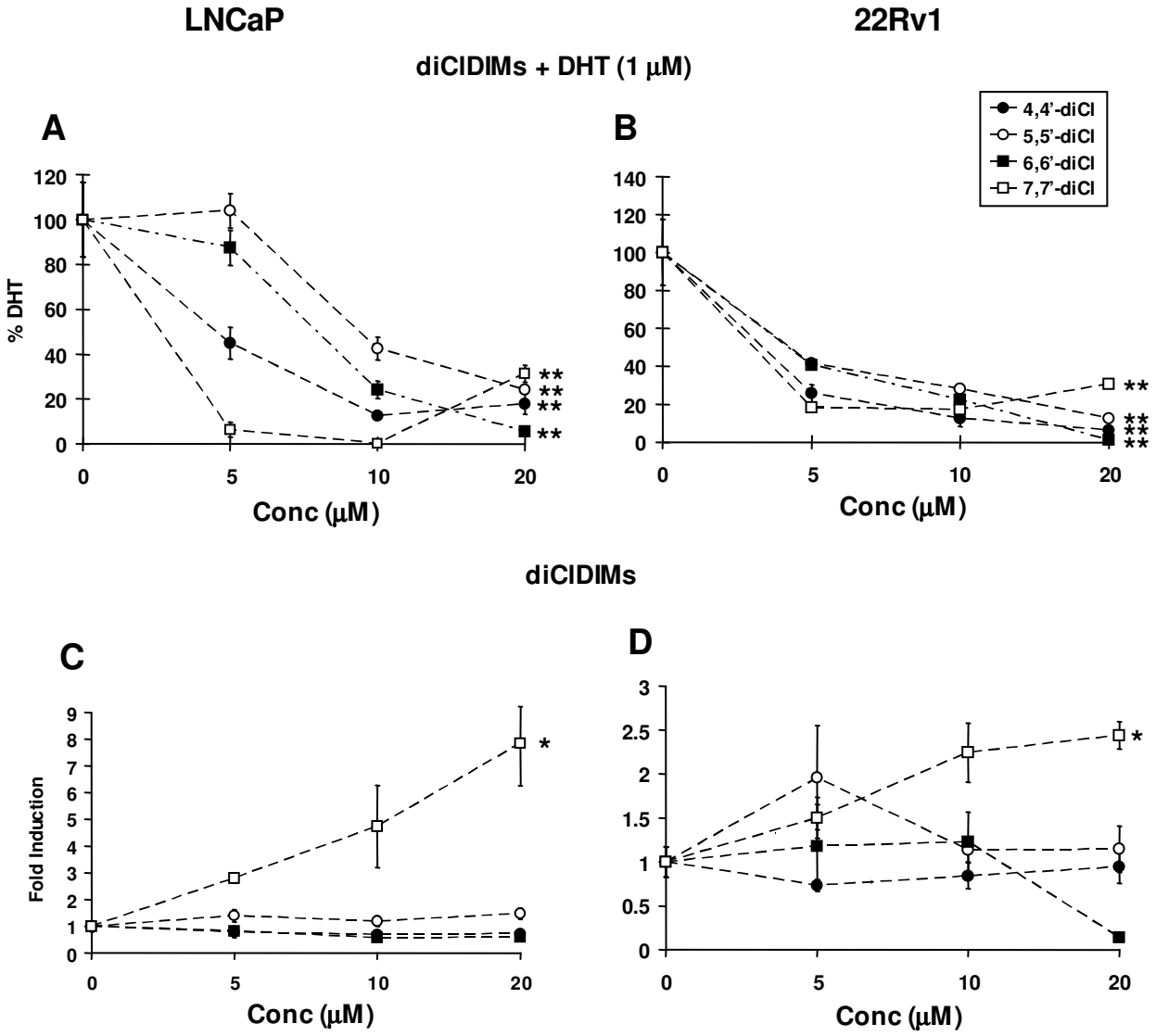


Figure 23. Antiandrogenic/androgenic activity of isomeric dichloro DIMs in prostate cancer cells. (A, C) LNCaP and 22Rv1 (B, D) cells. Cells were transfected with pPB, treated with DHT, 5 - 20 μM dichloro DIMs alone or in combination with DHT, and luciferase activity determined as described in the Materials and methods. Results are expressed as means ± SE for at least 3 determinations per treatment group, and significant ($p < 0.05$) induction (*) or inhibition (**) in the 20 μM cotreatment groups are indicated.

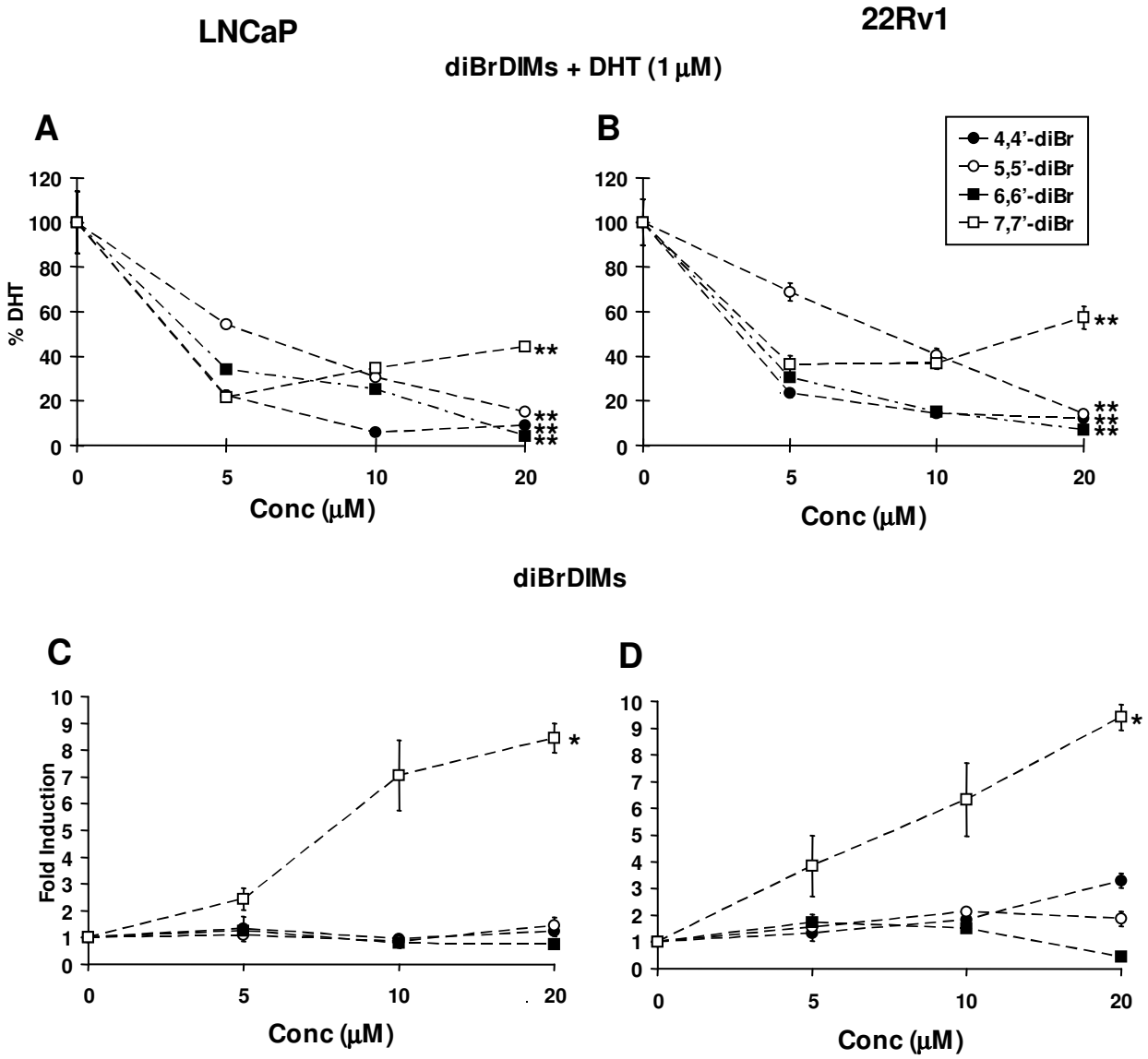


Figure 24. Antiandrogenic and androgenic activity of isomeric dibromo DIMs in prostate cancer cells.
(A, C) LNCaP and 22Rv1 (B, D cells. Cells were transfected with pPB, treated with DHT, 5 - 20 μM dibromo DIMs alone or in combination with DHT, and luciferase activity determined as described in the Materials and methods. Results are expressed as means ± SE for at least 3 determinations per treatment group, and significant ($p < 0.05$) induction (*) or inhibition (**) in the 20 μM cotreatment groups are indicated.

3.3.2 Structure-dependent effects of isomeric dihalo DIMs on AR protein expression

The antiandrogenic activity of DIM was associated with inhibition of DHT-induced formation of nuclear AR (403); however, other compounds such as tea polyphenols and emodin inhibit androgen responsiveness through downregulation of AR protein (409, 410). We therefore investigated the effects of the dichloro DIM (Figure 25A) and dibromo DIM (Figure 25B) isomers on AR protein expression in LNCaP cells. Cells were treated with different concentrations of the individual compounds for 24 hr and whole cell lysates were analyzed for AR protein by Western blot analysis. 4,4'- and 5,5'-Dichloro DIM (up to 20 μM) did not affect AR protein levels; however, AR protein expression was decreased by both 6,6'- and 7,7'-dichloro DIM. Results are shown only for 15 μM 6,6'-dichloro DIM due to the high cytoxicity of this compound. Results in Figure 25B for the dibromo DIMs gave a similar pattern of isomer-dependent responses, namely 4,4'-dibromo DIM had minimal effects on levels of AR protein, whereas both 6,6'- and 7,7'-dibromo DIM decreased AR levels. 5,5'-Dibromo DIM also decreased expression of AR protein but only at the 20 μM concentration. These results demonstrate remarkable differences in the effects of 4,4'-/5,5'-dihlo DIMs and 6,6'-/7,7'-dihalo DIMs on AR protein expression in LNCaP cells, and these differences were further investigated using the 4,4' and 7,7'-dihalo DIM as prototypes.

Previous studies showed that nuclear levels of AR increased after treatment of LNCaP cells with DHT for 24 hr, and DHT-induced nuclear translocation of AR was inhibited after cotreatment with DIM (403). Figure 26A summarizes the effects of 20 μM 4,4'- and 7,7'-dichloro DIM, 20 μM DIM and 10 nM DHT on cytosolic and nuclear AR levels after treatment for 1 and 24 hr. Minimal changes in cytosolic (c) and nuclear (n)

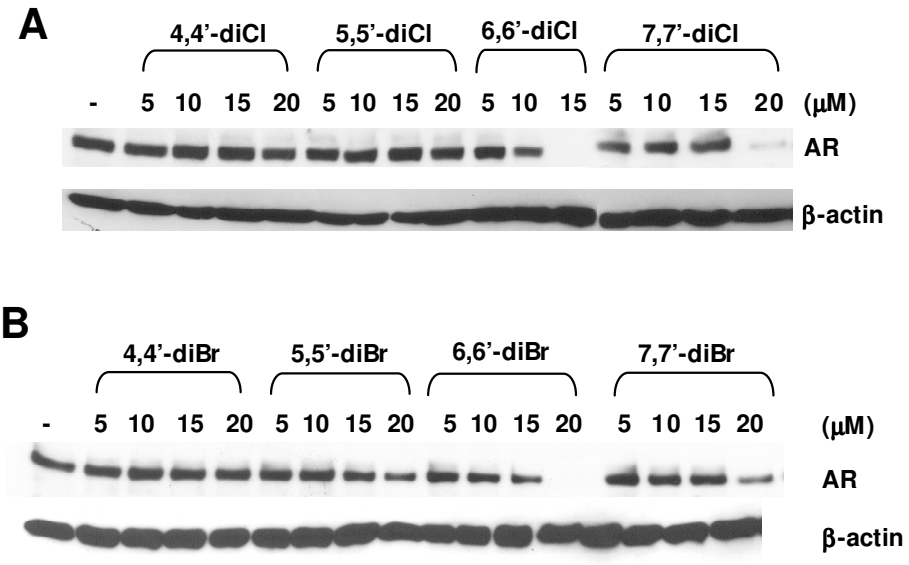


Figure 25. Structure-dependent effects of isomeric dihalo DIMs on AR protein levels.
LNCaP cells were treated with different concentrations of isomeric dichloro DIMs (A) and dibromo DIMs (B) for 24 hr, and whole cell lysates were analyzed for AR and β-actin (loading control) protein by Western blot analysis as described in the Materials and methods.

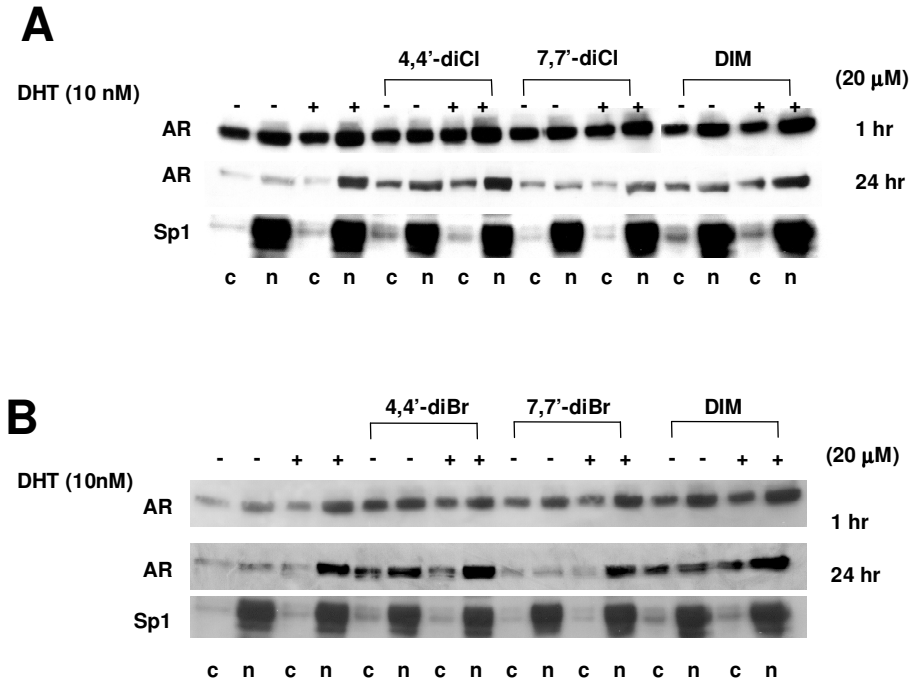


Figure 26. Cytosolic (c) and nuclear (n) AR protein in LNCaP cells treated with DIM and dihalo DIMs.
(A) Treatment with DIM, 4,4'- and 7,7'-dichloro DIM. Cells were treated with DMSO, DHT, 4,4'- and 7,7'-dichloro DIM or DIM alone or in combination with DHT for 1 or 24 hr, and cytosolic or nuclear fractions were obtained and analyzed by Western blot analysis as described in the Materials and methods. The nuclear Sp1 protein was determined as a loading control and to determine the efficiency of the isolation of the cytosolic and nuclear fractions. (B) Treatment with DIM, 4,4'- and 7,7'-dibromo DIM. Cells treated with DMSO, DHT, 4,4' or 7,7'-dibromo DIM alone or in combination with DHT for 48 hr, and nuclear and cytosolic fractions were analyzed by Western blot analysis as described in the Materials and methods. Nuclear Sp1 protein serves as a control for determining the efficiency of the isolated cytosolic and nuclear fractions.

AR levels were observed in all treatment groups (compared to DMSO) after 1 hr, and no major changes in AR protein staining in the cytosolic or

nuclear fractions were observed. In cells treated for 24 hr, DHT induced a more intense staining of AR in the nuclear fraction and enhanced overall AR staining (c+n) compared to cells treated with DMSO alone (c+n). This was observed in replicate experiments and is consistent with results of previous studies showing that DHT enhanced AR expression in LNCaP cells (405, 411, 412). Both DIM and 4,4'-dichloro DIM alone also enhanced AR levels in both the cytosolic and nuclear fractions; however, in combination with DHT, these compounds did not block DHT-induced formation of nuclear AR, and this was in contrast to a previous report showing that DIM inhibited this response (403). In contrast, 7,7'-dichloro DIM alone decreased nuclear and cytosolic AR levels after treatment for 24 hr and, in combination (DHT + 7,7'-dichloro DIM), the DHT-induced nuclear AR levels were only slightly decreased. The observed downregulation of AR protein in both the cytosolic and nuclear fractions (Figure 26A) complements the results observed for AR levels in whole cell lysates from cells treated with 7,7'-dichloro DIM (Figure 26A). Sp1 protein served as a loading control for this study, and the identification of Sp1 only in the nuclear fraction confirms the efficiency of the separation of nuclear and cytosolic fractions.

In two separate experiments (1 and 24 hr), the effects of DMSO, DHT, 4,4'- and 7,7'-dibromo DIM on AR levels were determined in LNCaP cells (Figure 26 A and B). The pattern of effects for the dibromo DIMs alone, and in combination with DHT was similar to those observed for the dichloro DIM isomers. DHT and 4,4'-dibromo DIM induced a time-dependent increase in AR

levels (compared to DMSO). Interaction of 4,4'-dibromo DIM with DHT decreased the ratio of nuclear/cytosolic levels of AR; however, this could be an additive effect since the former compounds alone induced higher cytosolic AR levels. In contrast, cytosolic and nuclear AR protein levels were decreased after treatment with 7,7'-dibromo DIM alone for 24 hr. In cells cotreated with 7,7'-dibromo DIM plus DHT, AR levels and their distribution were similar to those observed for DHT alone. Both 7,7'-dibromo- and 7,7'-dichloro DIM appeared to induce a time-dependent decrease in AR protein, whereas DHT, DIM and 4,4'-dichloro-, and 4,4'-dibromo DIM increased or stabilized AR protein in LNCaP cells. This was further investigated in LNCaP cells treated for 48 hr with DHT, 4,4'- and 7,7'-dihalo DIMs followed by Western blot analysis of whole cell lysates. Results in Figure 27A confirm that 7,7'-dichloro- and 7,7'-dibromo DIM decreased AR protein expression (compared to DHT), whereas AR levels after treatment with the corresponding 4,4'-dihalo DIMs (Figure 27B) were significantly higher than observed in cells treated with the 7,7'-dihalo DIMs (Figure 27A). We also observed that Sp1 protein was also slightly decreased only after prolonged treatment with the 7,7'-dihalo DIMs.

3.3.3 Effects of dihalo DIM isomers on AR protein and mRNA levels and their antiandrogenic activities in LNCaP cells

The potential role of proteasome activation in mediating downregulation of AR was investigated. LNCaP cells were treated with 7,7'-dihalo DIMs for 48

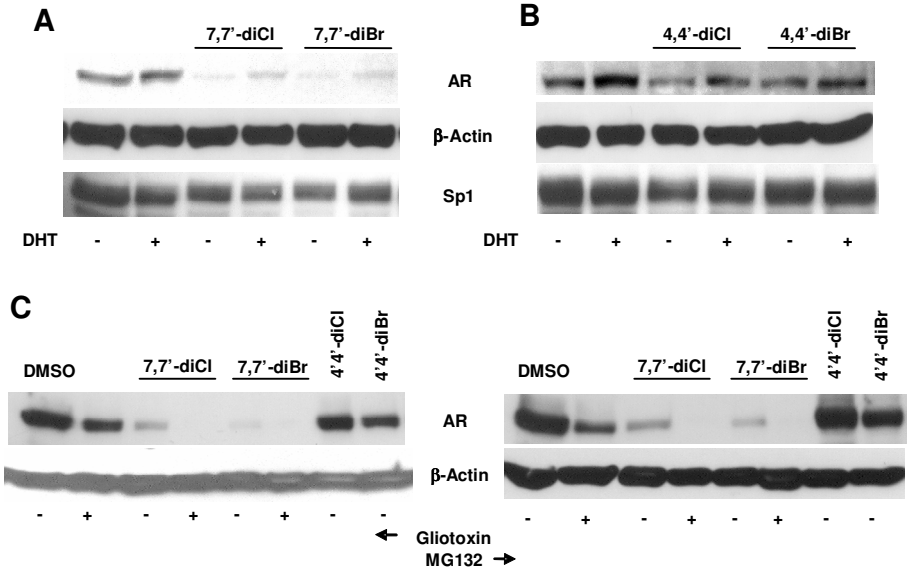


Figure 27. Effects of 4,4'- or 7,7'-dihalo DIM on AR expression and androgen responsiveness.
Effects of 7,7'-dihalo DIMs (A) and 4,4'-dihalo DIMs (B) on AR protein levels. LNCaP cells were treated with DMSO,10 nM DHT, 20 μM dihalo DIMs for 48 hr, and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. AR, β-actin (loading control) and Sp1 protein were determined. (C) Effects of proteasome inhibitors on AR protein levels. LNCaP cells were treated with 20 μM 7,7'-dichloro- or 7,7'-dibromo DIM alone or in combination with the proteasome inhibitor gliotoxin (3 μM) or MG132 (10 μM) for 48 hr, and AR protein levels were determined by Western blot analysis as described in Materials and methods. β-Actin served as a loading control.

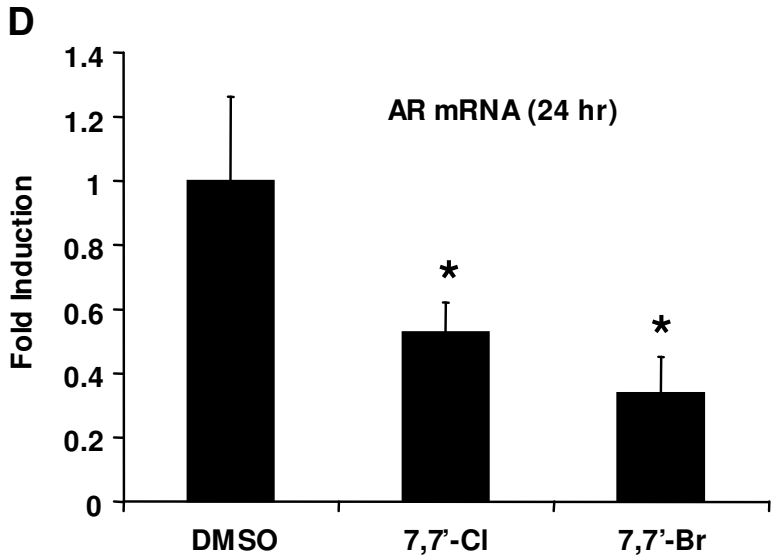


Figure 27. (continued) (D)Time-dependent effects of 7,7'-dihalo DIMs on AR mRNA protein levels. LNCaP cells were treated with 7,7'-dihalo DIMs for 24 hr, and AR mRNA was determined by real-time PCR as described in the Materials and methods. The experiments were carried out in triplicate. Results are expressed as means ± SE, and significantly ($p < 0.05$) decreased AR mRNA is indicated by an "*". TBP mRNA was also determined and used to normalize the AF mRNA levels.

hr in the presence or absence of the proteasome inhibitors gliotoxin or MG132 (Figure 27C). Western blot analysis of whole cell lysates showed that 7,7'-dichloro- and 7,7'-dibromo DIM significantly decreased AR protein compared to levels observed in solvent-treated cells, and cotreatment with the proteasome inhibitors further increased AR degradation. The proteasome inhibitors alone also decreased AR protein, whereas 4,4'-dichloro- and 4,4'-dibromo DIM did not affect AR protein. The data indicated that decreased AR protein in LNCaP cells treated with 7,7'-dihalo DIMs is not due to activation of the proteasome pathway.

The time-dependent effects of 7,7'-dihalo DIMs on AR mRNA levels was determined (Figure 27D), and the results show that mRNA levels are significantly decreased within 24 and 48 hr (data not shown). We also investigated the effects of 7,7'-dichloro DIM on AR mRNA stability by pretreating cells with DMSO or 20 μM 7,7'-dichloro DIM for 12 hr prior addition of actinomycin D. The results showed an initial 6 - 12 hr increase in AR mRNA levels after addition of actinomycin D; however, the subsequent rates of degradation of AR mRNA in the DMSO and 7,7'-dichloro DIM treatment groups were comparable (data not shown). These data indicate that 7,7'-dihalo DIMs decrease both transcriptional and translational regulation of the AR.

Results of transient transfection studies showed that both 7,7'-dibromo- and 7,7'-dichloro DIM were partial AR agonists and AR antagonists (Figures 23 and 24), and the former was observed after treatment for 36 hr. This AR agonist activity of 7,7'-dihalo DIMs is inconsistent with their effects on AR and it is possible that 7,7'-dihalo DIM-induced androgenic activity after 36 hr (Figures 23 and 24) may be due to the relatively slow rate of AR degradation. We therefore investigated the time-dependent effects of 7,7'-dihalo DIMs on androgen-responsiveness in LNCaP cells transfected with pPB (for 9 hr), and then treated with different concentrations of 7,7'-dihalo DIMs for 24, 36 or 48 hr (Figure 28A). The results indicated that after 36 hr, 5 - 20 μM 7,7'-dichloro DIM significantly induced luciferase activity; however, this response was significantly decreased after treatment for 24 or 48 hr and similar results were observed for 7,7'-dibromo

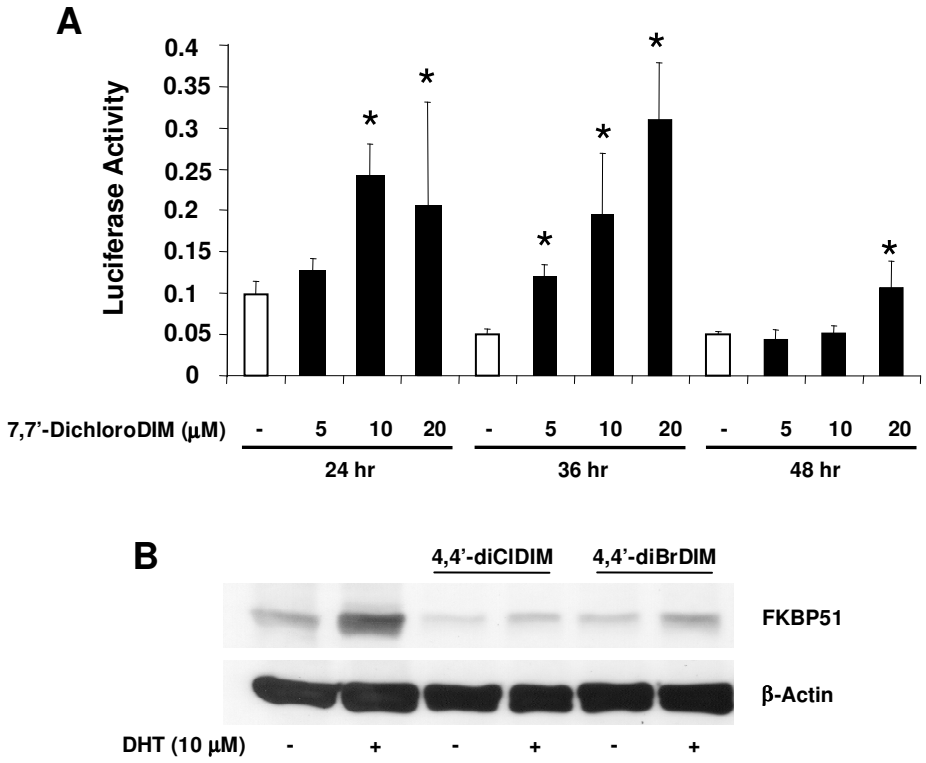


Figure 28. AR agonist/antagonist activities of dihalo DIMs.
(A) Time-dependent effects of 7,7'-dichloro DIM on transactivation. LNCaP cells were transfected with pPB and, after 9 hr, were treated with 7,7'-dichloro DIM (5 - 20 μM) for 24, 36 and 48 hr, and luciferase activity was determined as described in Materials and methods. Results are expressed as means ± SE for at least 3 determinations for each treatment group and significant ($p < 0.05$) induction (*) is indicated. (B) Regulation of FKBP51 protein expression. LNCaP cells were treated with DMSO, 10 nM DHT, 4,4'-dichloro- or 4,4'-dibromo DIM alone or in combination with DHT for 48 hr, and whole cell lysates were analyzed by Western blot analysis for FKBP and β-actin (loading control) as described in Materials and methods.

DIM (data not shown). The decreased AR agonist activity of 7,7'-dichloro DIM after 48 hr is consistent with the effects of this compound on AR protein degradation (Figure 27A).

A recent study identified a 51 kDa progesterone receptor-associated immunophilin, FKBP51, as an androgen-responsive gene in prostate cancer cells (335), and the effects of 4,4'-dichloro- and 4,4'-dibromo DIM alone and in combination with DHT were investigated in LNCaP cells 48 hr after treatment (Figure 28B). DHT alone enhanced FKBP51 protein expression, whereas 4,4'-dichloro- and 4,4'-dibromo DIM did not affect levels of FKBP51. In cells cotreated with DHT plus 4,4'-dichloro- or 4,4'-dibromo DIM, the hormone-induced response was inhibited by both DIM compounds and this was consistent with their antiandrogenic activity in transactivation assays (Figures 23 and 24). Minimal induction of FKBP51 was observed after treatment with 7,7'-dichloro- or 7,7'-dibromo DIM (data not shown) and this may be due, in part, to the low levels of AR expression in LNCaP cells treatment with these compounds for 48 hr. In summary, these results indicate that ring-substituted DIMs and DIM differentially modulate androgenic responses in prostate cancer cells and subtle changes in the position of the ring substituents of the dihalo DIMs (i.e. 25 vs. 28) can modulate their mechanisms of antiandrogenic action and effects on AR expression.

3.3 Discussion

I3C and DIM inhibit growth of several cancer cell lines through multiple mechanisms and these compounds also directly activate receptors. I3C and/or DIM interact with the AhR, the fish ER and AR (140, 334), and ring-substituted DIMs also activated the AhR (330, 404). A recent study showed that DIM competitively bound the AR and decreased DHT-induced transactivation/gene expression in LNCaP cells (403). The mechanism of the antiandrogenic activity of DIM was novel since this compound inhibited DHT-induced nuclear translocation of the cytosolic AR (403), whereas other antiandrogens do not block nuclear translocation of the AR but form transcriptionally inactive nuclear AR complexes (413, 414). This study reports the androgenic/antiandrogenic activity of a series of symmetrical ring-substituted DIMs to delineate possible structure-dependent effects that modulate their activities.

Results in Figures 23 and 24 demonstrated that like DIM (Figure 22), the symmetrically substituted dichloro- and dibromo DIM isomers also inhibited DHT-induced transactivation in LNCaP and 22Rv1 prostate cancer cells. The assay system used the androgen responsive pPB construct, which contains the -288 to +28 region of the probasin reporter linked to firefly luciferase (405, 406). The antiandrogenic activities of the compounds were similar in both cell lines; however, with few exceptions their overall potencies were higher in LNCaP than 22Rv1 cells. We also investigated the structure-dependent AR agonist activities

of ring-substituted DIMs in cells transfected with pPB. 7,7'-Dichloro- and 7,7'-dibromo DIM were partial AR agonists and partial antagonists in both cell lines (Figures 23 and 24), and their androgenic potencies were at least three orders of magnitude lower than that of DHT. In contrast, symmetrical 4,4'-, 5,5'-, 6,6'-dichloro- and -dibromo DIMs primarily exhibited antiandrogenic activity in this transactivation assay in both cell lines. Other symmetrical ring-substituted methyl, methoxy and fluoro DIM analogs also exhibited antiandrogenic activity and a few of these isomers were also partial AR agonists; however, these activities also depended on cell context and were not further investigated (data not shown).

The structure-dependent effects of dihalo DIMs on AR expression clearly differentiated between the 4,4'-/5,5'- and 6,6'-/7,7'-dihalo DIMs since the latter compounds downregulated AR protein, whereas minimal effects were observed for 4,4'- and 5,5'-dihalo DIMs (Figure 25). 6,6'-Dichloro DIM exhibited significant cytotoxicity at concentrations > 10 μM and, therefore, structure-dependent differences in the activities of the dihalo DIM isomers were further investigated using 4,4'- and 7,7'-dichloro- and -dibromo DIMs as models in LNCaP cells. Previous studies show that DHT stabilizes the AR (compared to DMSO) resulting in increased AR expression for at least 24 hr after treatment (405, 411, 412), and results of this study also showed that treatment with DHT increased AR protein (Figures 26 and 27). We directly compared AR protein levels in LNCaP cells treated with DMSO (control), DHT, 4,4'-dichloro-, 7,7'-dichloro-,

4,4'-dibromo-, 7,7'-dibromo DIM, DIM alone, and DHT plus the DIMs (in combination) for 1 and 24 hr (Figure 26). The most pronounced changes were observed in the 24 hr treatment group, where DHT, DIM, 4,4'-dichloro- and 4,4'-dibromo DIM stabilized the AR (compared to DMSO). DHT alone induced nuclear translocation of the AR; however, in contrast to a previous report with DIM (403), results of this study showed that DIM and 4,4'-dihalo DIMs did not markedly inhibit DHT-induced AR translocation and the combined treatments tended to give additive effects (Figure 26). 7,7'-Dichloro DIM also had minimal effects on DHT-induced translocation of the AR; however, both 7,7'-dichloro- and 7,7'-dibromo DIM alone did not stabilize the AR protein after treatment for 24 hr.

Compared to treatment with DHT, DIM or the 4,4'-dihalo-DIMs, 7,7'-dichloro-, and 7,7'-dibromo DIM significantly decreased AR protein and mRNA levels in LNCaP cells (Figure 27). Down-regulation of AR by these compounds was similar to the reported time-dependent decrease of AR protein in LNCaP cells after treatment with tea polyphenol epigallocatechin (ECCG) (409). It was hypothesized that the effects of ECCG were due to downregulation of Sp1 protein, which also plays a role in regulating AR expression. We also observed that the 7,7'-dihalo DIMs slightly decreased Sp1 protein expression after treatment for 48 hr (Figures 27A and 27B). This may contribute, in part, to the lower expression of AR in these cells but cannot fully explain the dramatic drop in AR protein. A recent study reported that the phytochemical emodin inhibited

AR-dependent transactivation in prostate cancer cells, and this was associated with inhibition of AR nuclear translocation and activation of proteasome-dependent degradation of AR protein (410). The effects of emodin on AR protein in LNCaP cells were blocked by the proteasome inhibitor MG132, and emodin did not affect AR mRNA levels. In contrast 7,7'-dihalo DIM-induced degradation of AR protein was not blocked by the proteasome inhibitors gliotoxin or MG132 (Figure 27C), and these compounds induced a time-dependent decrease in both AR mRNA and protein levels. We did not observe any compound-induced changes in AR mRNA stability (data not shown), suggesting a transcriptional mechanism of action which is being currently investigated. These results clearly distinguished between the effects of 7,7'-dihalo DIMs with other compounds such as emodin and ECCG which also affect AR mRNA and protein expression in LNCaP cells.

Although the initial *in vitro* screening assays showed that 7,7'-dihalo DIMs exhibited potential AR agonist activity in transactivation assays, we further investigated the effects of duration of treatment on their androgenic activity. 7,7'-Dihalo DIMs induced transactivation in LNCaP cells transfected with pPB and treated for 36 hr (Figure 28A). However, after 48 hr, this response was significantly decreased and this was consistent with decreased AR expression at this time point, suggesting that the partial androgenic activity of these compounds is reversed after longer periods of exposure due to AR downregulation.

The AR agonist activity of the 7,7'-dihalo DIMs was also affected by lower AR expression since these compounds did not significantly induce FKBP protein in LNCaP cells (data not shown). The androgen-responsive FKBP protein is maximally induced by DHT only after treatment for 48 hr, and 4,4'-dichloro- and 4,4'-dibromo DIM inhibited this response (Figure 28B).

In summary, results of this study confirm that DIM and several symmetrical ring-substituted DIM isomers exhibit antiandrogenic activity. In addition, some isomers, notably 7,7'-dichloro- and 7,7'-dibromo DIM also exhibit partial time-dependent androgenic activity in transfection assays, and these results illustrate that subtle changes in the phenyl ring substitution pattern have marked effects on the androgenic activity of the dihalo DIMs. At the concentrations used in this study, the antiandrogenic activity of the 4,4'-dihalo DIMs was not related to inhibition of DHT-induced nuclear translocation of AR. Our results suggest that the antiandrogenic activity of DIM and 4,4'-dihalo DIMs may be complex and involve multiple pathways including inhibition of nuclear AR-dependent transactivation. We also observed that 6,6'-dihalo DIMs and 7,7'-dihalo DIMs decreased AR expression in LNCaP cells, and current studies are investigating the potential clinical importance of these and other effects of ring-substituted DIMs on the growth of prostate cancer cells/tumors in both *in vitro* and *in vivo* models.

CHAPTER IV

INHIBITION OF PROSTATE CANCER CELL GROWTH BY ACTIVATION OF PRO-APOPTOTIC PROTEINS NAG-1 AND ATF3 BY SYMMETRICALLY SUBSTITUTED DIBROMO AND DICHLORO 1,1'-BIS(3'-INDOLYLMETHANES)

4.1 Introduction

Indole-3-carbinol (I3C) is a phytochemical that is found as an isothiocyanate conjugate (glucobrassican) in cruciferous vegetables such as cauliflower, brussel's sprouts and broccoli (333). Epidemiological studies have demonstrated that for several cancers there are lower incidence rates associated with high consumption of cruciferous vegetables (185, 188, 190, 331, 332). Moreover, there is also evidence in laboratory animal feeding studies that rodents consuming diets containing cruciferous vegetables or I3C are more resistant to carcinogen-induced tumor formation (196, 199, 389-394). Although the identities of anticarcinogenic compounds in cruciferous extracts have not been determined unequivocally, most studies have focused on either I3C or sulfurophane, an allylisothiocyanate that exhibits multiple activities (415, 416). The induction of glutathione-S-transferase by sulfurophane has been extensively investigated and has been shown to play a role in the anticarcinogenic activity of this compound due to the enhanced detoxification of toxicants and carcinogens

through enhanced formation of glutathione conjugates which are readily excreted.

I3C has been extensively investigated in laboratory animals and in vitro cell culture studies and this compound exhibits multiple activities that are associated with the antitumorigenic properties of I3C (193, 397, 398, 417-422). For example I3C inhibits several different cell cycle enzymes, induces apoptotic pathways and inhibits invasion and migration of cancer cell lines.

A major problem in interpreting results from I3C studies is due to its instability and rapid formation of various metabolites and rearrangement products. In acidic conditions I3C forms multiple linear and cyclic condensation products including 3,3' DIM and in vivo studies show that in the acidic environment of the gut a wide variety of metabolites are formed (140, 334). In cell culture studies carried out at or near physiological pH I3C is converted to DIM and it is therefore difficult to distinguish between the effects of both compounds (194). Research in this laboratory has focused on using DIM as a building block for synthesis of both ring- and methylene-substituted DIMs (C-DIMs) as a novel class of anticancer drugs (330, 404, 423-431). The selection of DIM as a template is due to the relative stability of this compound under acidic and basic conditions thereby abrogating the instability problems associated with I3C.

DIM, C-DIM and ring substituted DIMs exhibit some common properties including the induction of endoplasmic reticulum (ER) stress and induction of

apoptosis in cancer cell lines, however the synthetic analogs were invariably more potent than DIM (430). C-DIMs are triaryl methane analogs that activate several orphan receptors including peroxisome proliferator-activated receptor γ (PPAR γ) and nerve growth factor induced-B γ (NGFI-B γ, Nur77)(424-429, 432) whereas the diarylmethane DIM analogs activate or deactivate the aryl hydrocarbon receptor (AhR) (ER) and androgen receptor (AR) (330, 404, 423, 433). Recent studies in this laboratory show that symmetrically substituted dihalo DIMs exhibit androgenic or antiandrogenic activities in AR-positive LNCaP and 22Rv1 prostate cancer cells (433). Moreover, the 7,7' dichloro/dibromo DIMs but not the corresponding 4,4'dihalo DIM isomers preferentially downregulated AR protein expression. This study further investigates the effects of dihalo DIMs on the growth of prostate cancer cells. IC_{50} values for growth inhibition in LNCaP and 22Rv1 cells were ≤5 μM for the 4,4'- 5,5'- 6,6'- and 7,7'-dihalo DIMS and the 4,4'- and 7,7'-dihalo DIMs (chloro and bromo) induced caspase-dependent apoptosis in LNCaP cells. The dihalo DIMs did not activate ER stress, however these compounds induced both NAG-1 and activating transcription factor 3 (ATF3), two antiproliferative/proapoptotic genes which are responsible in part for the effects of these compounds on the proliferation of prostate cancer cells.

4.2 Materials and methods

4.2.1 Cell lines and reagents

Human prostate carcinoma cell lines LNCaP and 22Rv1 were obtained from American Type Culture Collection (Manassas, VA). Fetal bovine serum was obtained from JRH Biosciences, Lenexa, KS. Cells were maintained in RPMI 1640 (Sigma Chemical St. Louis, MO) supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% FBS, and 10 mL/L of 100x antibiotic antimycotic solution (Sigma). Cells were maintained at 37°C in the presence of 5% CO_2. Antibodies for PARP, GRP-78, and ATF-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for NAG-1 was purchased from Upstate Biotechnologies (Charlottesville VA). Monoclonal antibody for β-actin was purchased from Sigma-Aldrich. Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega (Madison, WI). β-Galactosidase (β-Gal) reagent was obtained from Tropix (Bedford, MA). Lipofectamine reagents were supplied by Invitrogen (Carlsbad, CA). Western Lightning chemiluminescence reagents were from Perkin-Elmer Life Sciences (Boston, MA). Dihydrotestosterone was purchased from Sigma. MG132 was obtained from Calbiochem (San Diego, CA). Kinase inhibitors LY294002, PD98059, SB203580, SP600125 were obtained from Sigma. The ring-substituted DIMs were prepared in this laboratory by condensation of ring-substituted indoles and formaldehyde or by self-

condensation of ring-substituted indole-3-carbinols; compounds were >95% pure by gas chromatography-mass spectrometry.

4.2.2 Plasmids

The pNAG-1 reporter containing -1085/+41 was generated previously (336, 434).

4.2.3 Cell proliferation assays

LNCaP prostate cancer cells were maintained in RPMI media with phenol red and were seeded into 6-well plates at a density of 50,000 cells/well in DME-F12 plus 5% FBS stripped with dextran-treated charcoal. After allowing 24 hr to attach, cell were treated with test compounds in DMSO (1% final volume) or DMSO alone every 48 hr in fresh media. Cells were harvested every 48 hr by trypsinization and counted using a Coulter Z1 cell counter (Coulter Electronics, Hialeah, FL).

4.2.4 Transfection and luciferase assay

Prostate cancer cells were plated in 12-well plates at 2.5 x 10^5 cells/well in DMEM:Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS. After overnight attachment, cells were transfected with 100 ng of pNAG-1 and 50 ng of β-galactosidase reporter plasmid, using Lipofectamine reagent (Invitrogen) according to the manufacturer's recommended protocol. Cells were transfected

and, after 8 - 9 hr, the transfection mix was replaced with 5% charcoal-stripped FBS media containing either vehicle (DMSO) or the indicated DIM-isomer and incubated for 22-24 hr. Cells were then lysed with 100 mL of 1x reporter lysis buffer, and 30 μL of cell extract were used for luciferase and β-galactosidase assays. Lumicount (Parkard, Meriden, CT) was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-gal activity.

4.2.5 Western blot analysis

LNCaP prostate cells were seeded in 100 mm plates in DMEM:Ham's F-12 media containing 2.5% charcoal-stripped FBS and allowed to attach overnight, followed by treatment with either the vehicle (DMSO) or the indicated compounds for the desired time points. For whole cell lysates cells were scraped in 500 μL of lysis buffer [50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% v/v glycerol, 1% Triton X, and 5 μL/ml of Protease Inhibitor Cocktail (Sigma)]. The lysates were incubated on ice for 1 - 1.5 hr with intermittent vortexing followed by centrifugation at 40,000 *g* for 10 min at 4°C. Protein levels were estimated using Bradford reagent; equal amounts of protein were diluted with loading buffer and boiled for 4 min, and loaded onto 10% SDS-polyacrylamide gel. After electrophoresis, gels were transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) using an electroblotting apparatus overnight at 4°C in transfer buffer containing 48 mM

Tris-Cl, 29 mM glycine, and 0.025% SDS. The membranes were blocked with TBS (10 mM Tris-HCl (pH 8) and 150 mM sodium chloride] plus 5% milk (blotto-buffer) for 1 hr, and then incubated in primary antibody at 1:1000 dilution in blotto buffer at 4°C overnight, followed by one min washes (2X) and incubation with secondary antibody for 3 - 5 hr at 4°C. Membranes were then rinsed with water and incubated in enhanced chemiluminescence (ECL) reagents (Perkin Elmer, Boston, MA) for 1 min, removing excess ECL with paper towelette. The membrane was sealed in plastic wrap and photographed for immunoreactive bands using ECL hyperfilm.

4.2.6 Statistical analysis

Statistical differences between different groups were determined by ANOVA and Scheffe's test for significance. The data are presented as mean ± SE for at least three separate determinations for each treatment.

4.3 Results

4.3.1 Effects of symmetrical dihalo DIM isomers on LNCaP and 22Rv1 prostate cancer cell proliferation.

Previous studies in this laboratory showed that ring substituted DIMs inhibited growth of ER-positive and ER-negative breast cancer cells and this was due, in part, to induction of apoptosis (330, 404, 423). We also investigated the antiproliferative activity of a series of symmetrical ring-substituted DIMs in AR-

positive LNCaP prostate cancer cells and the results in Figure 29 illustrate the effects of 5, 10 and 20 μM concentrations of 4,4'-, 5,5', 6,6'-, and 7,7'-, dichloro DIMs on LNCaP cell growth. All symmetrical dichloro DIMs inhibited LNCaP cell growth and the lowest concentration and IC_{50} values were ≤ 5 μM for all isomers. A comparable study was carried out for a series of 4,4'-, 5,5'-, 6,6'-, and 7,7'-, dibromo DIM isomers and their IC_{50} values for cell growth inhibition were also ≤ 5 μM (Figure 30). Based on direct comparison of their growth inhibitory activity at the 5 μM concentration the, 6,6'-, and 7,7'-, dihalo DIMS were slightly more potent than the corresponding 4,4'-, 5,5'-dihalo DIMs. We also investigated the growth inhibitory effects of these compounds in AR-positive 22Rv1 prostate cancer cells and the results were similar to those observed in LNCaP cells with some isomer-specific differences. Among the symmetrical dichloro DIMs the IC_{50} values for growth inhibition was higher for 5,5'- dichloro DIM (≈20 μM) and ≤ 5 μM for the other isomers (Figure 31). Using lower concentrations of 5,5'-dichloro DIM (0.1-1 μM) in 22Rv1 cells the IC_{50} value was approximately 1 μM . The pattern of growth inhibition for the dibromo DIM isomers in 22Rv1 cells (Figure 32) was similar to that observed in LNCaP cells.

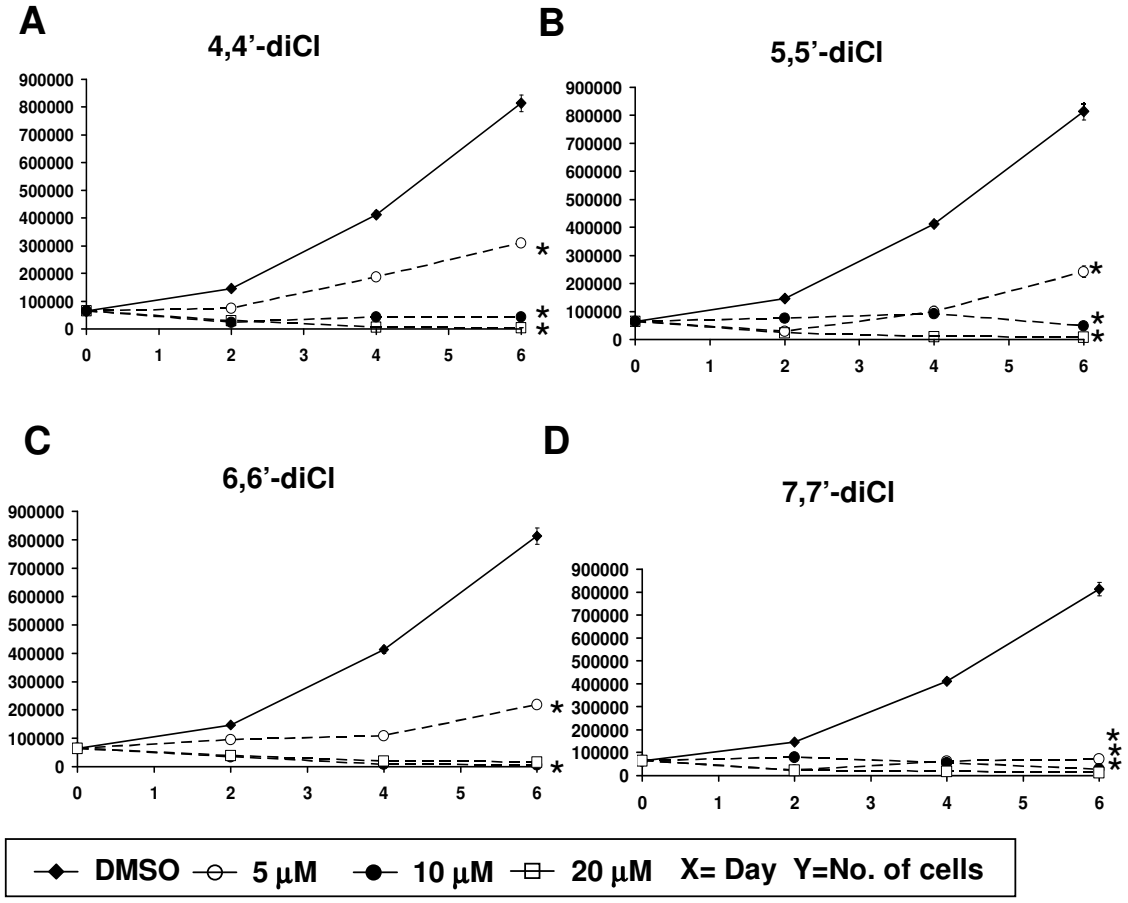


Figure 29. Cell proliferation assays in LNCaP cells with dichloro DIMs. **LNCaP cells were treated with DMSO or (A) 4,4'-dichloro DIM (B) 5,5' dichloro DIM or (C) 6,6'-dichloro DIM or (D) 7,7'dichloro DIM and cell numbers were determined after 2, 4 and 6 days as described in Materials and methods. Data presented as means of three replicate determinations for each treatment group. Significant inhibition ($p<0.05$) of cell proliferation ± SE (to DMSO) is indicated as "*" in figures (6 day point).**

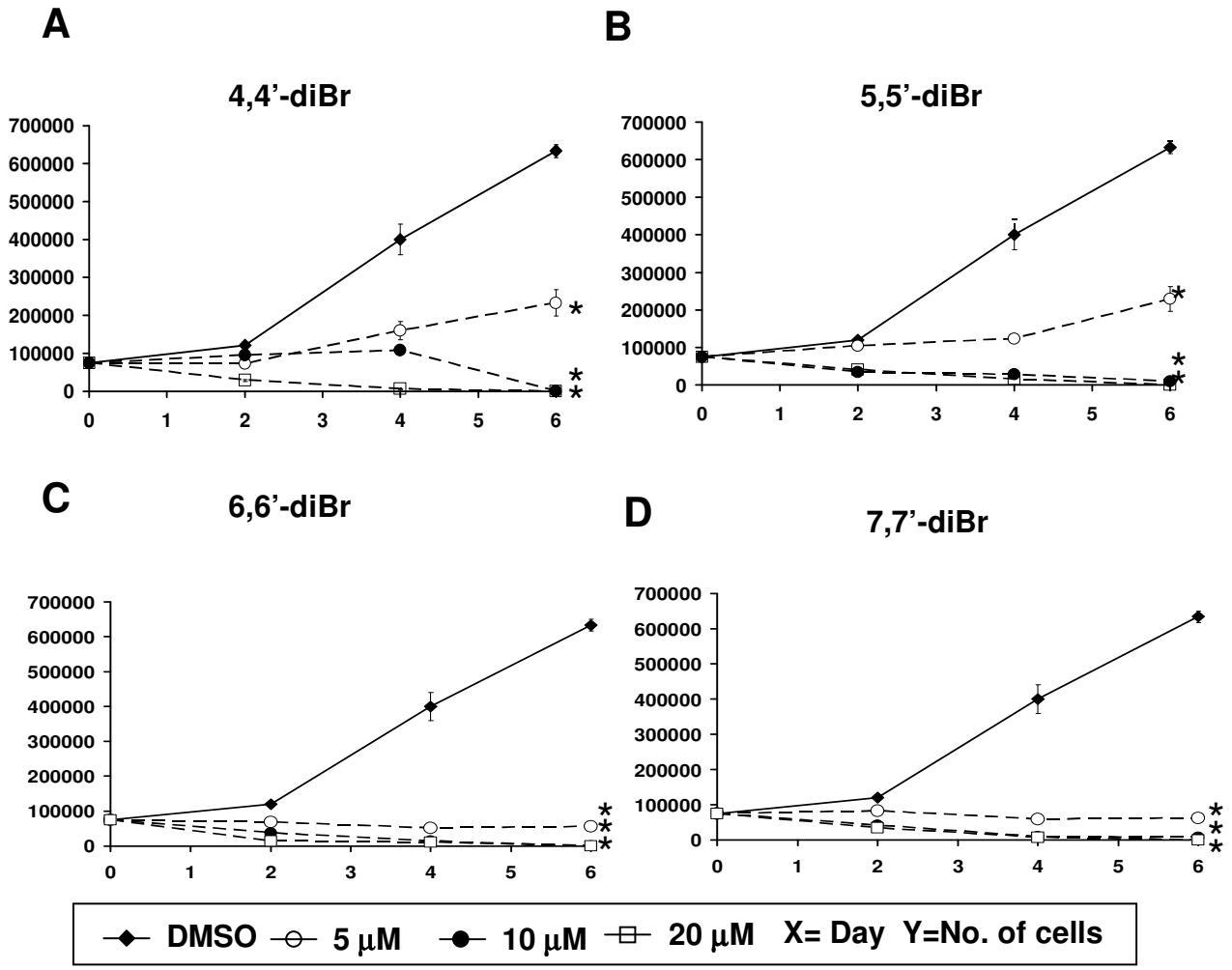


Figure 30. Cell proliferation assays in LNCaP cells with dibromo DIMs. LNCaP cells were treated with DMSO or (A) 4,4'-dibromo DIM (B) 5,5' dibromo DIM or (C) 6,6'-dibromo DIM or (D) 7,7'dibromo DIM and cell numbers were determined after 2, 4 and 6 days as described in Materials and methods. Data presented as means of three replicate determinations for each treatment group. Significant inhibition ($p<0.05$) of cell proliferation ± SE (to DMSO) is indicated as "*" in figures (6 day point).

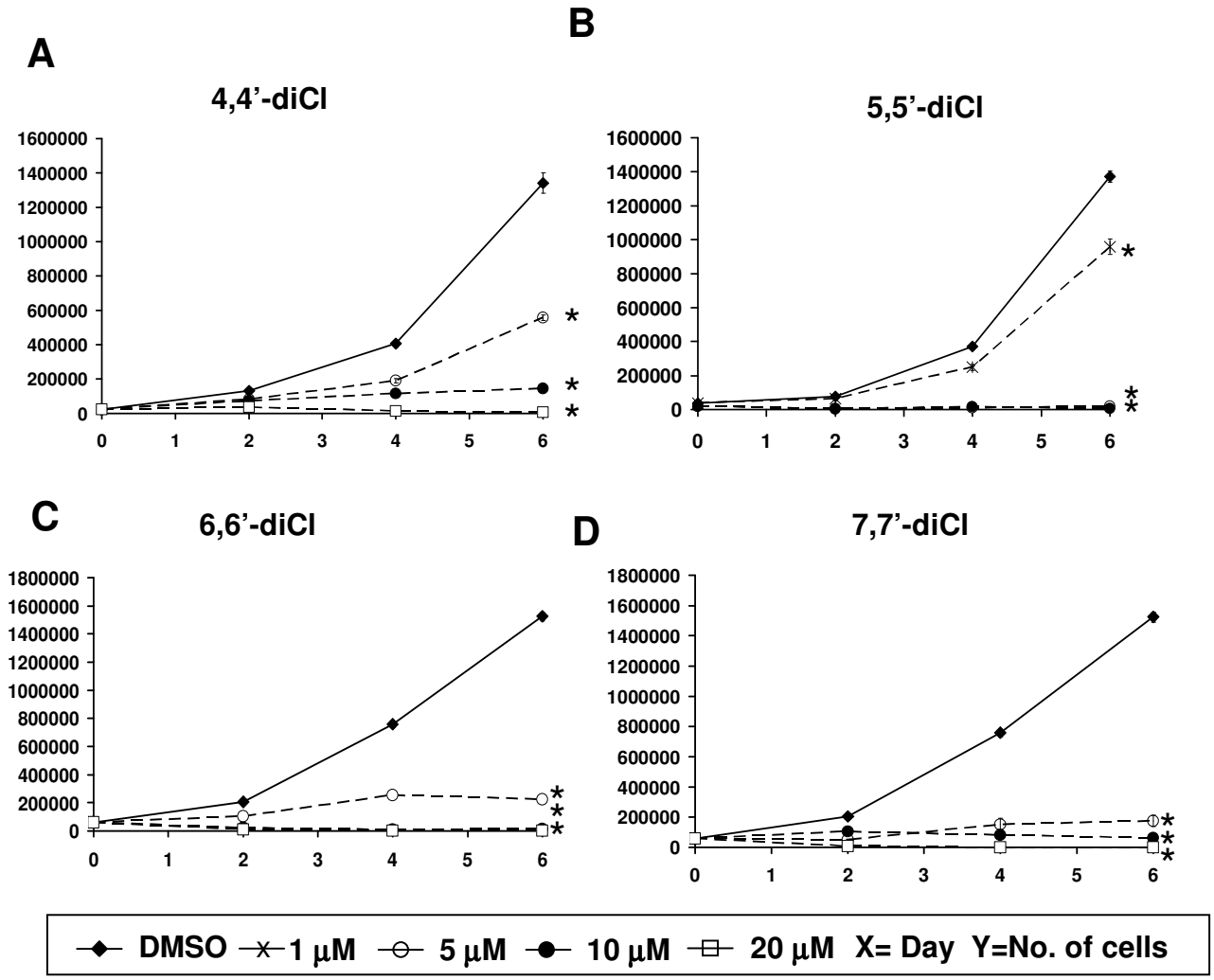


Figure 31. Cell proliferation assays in 22Rv1 cells with dichloro DIMs. 22Rv1 cells were treated with DMSO or (A) 4,4'-dichloro DIM (B) 5,5' dichloro DIM or (C) 6,6'-dichloro DIM or (D) 7,7'dichloro DIM and cell numbers were determined after 2, 4 and 6 days as described in Materials and methods. Data presented as means of three replicate determinations for each treatment group. Significant inhibition ($p<0.05$) of cell proliferation ± SE (to DMSO) is indicated as "*" in figures (6 day point).

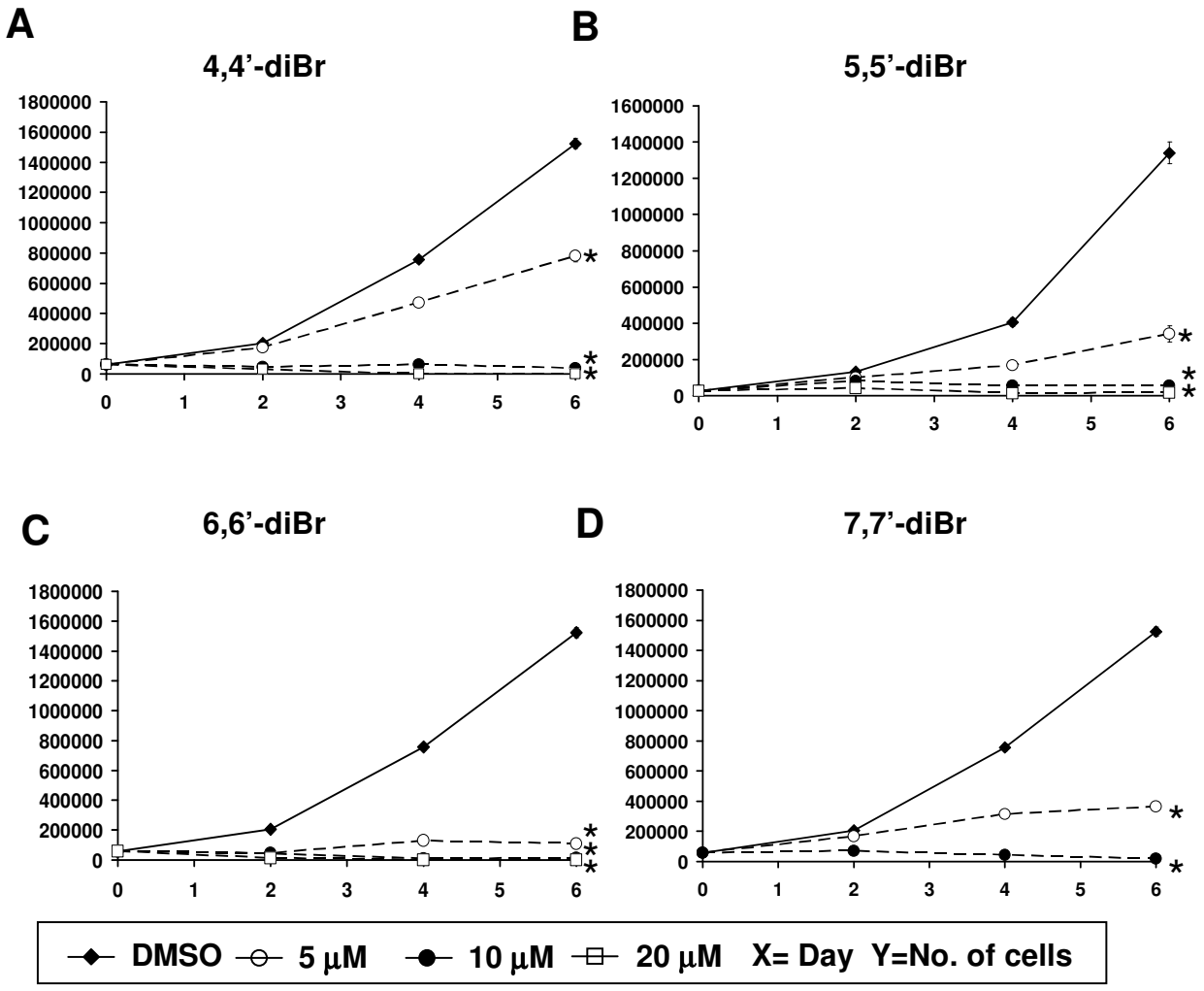


Figure 32. Cell proliferation assays in 22Rv1 cells with dibromo DIMs. 22Rv1 cells were treated with DMSO or (A) 4,4'-dibromo DIM (B) 5,5' dibromo DIM or (C) 6,6'-dibromo DIM or (D) 7,7'dibromo DIM and cell numbers were determined after 2, 4 and 6 days as described in Materials and methods. Data presented as means of three replicate determinations for each treatment group. Significant inhibition ($p<0.05$) of cell proliferation ± SE (to DMSO) is indicated as "*" in figures (6 day point).

We also investigated a comparable series of isomeric dimethoxy and dimethyl DIMs and their growth inhibitory IC_{50} values in LNCaP and 22Rv1 cells were in the 5-10 μM range (data not shown). The mechanisms of growth inhibition of prostate cancer cells by symmetrical ring substituted DIMs was further investigated using the dihalo DIMs as models in LNCaP cells.

4.3.2 Mechanism of action of dihalo DIMs- ER stress

Recent studies in this laboratory have demonstrated that DIM, ring substituted DIMs and methylene substituted DIMs (C-DIMs) induced apoptosis in pancreatic cancer cells through induction of endoplasmic reticulum (ER) stress and activation of the extrinsic apoptotic pathways (430). Using the 4,4'-, and 7,7'-, dihalo DIMs as models, treatment of LNCaP cells for 24 hr with 20 μM 4,4'-, and 7,7'-, dichloro DIMs and 4,4'-, and 7,7'-dibromo DIMs resulted in the induction of PARP cleavage which is a hallmark of caspase-dependent apoptosis (Figure 33A). Treatment with 5 μM MG132 also induced apoptosis in LNCaP cells and this compound served as a positive control for this experiment. Since 5,5'-dibromo DIM induced apoptosis through activation of ER stress in

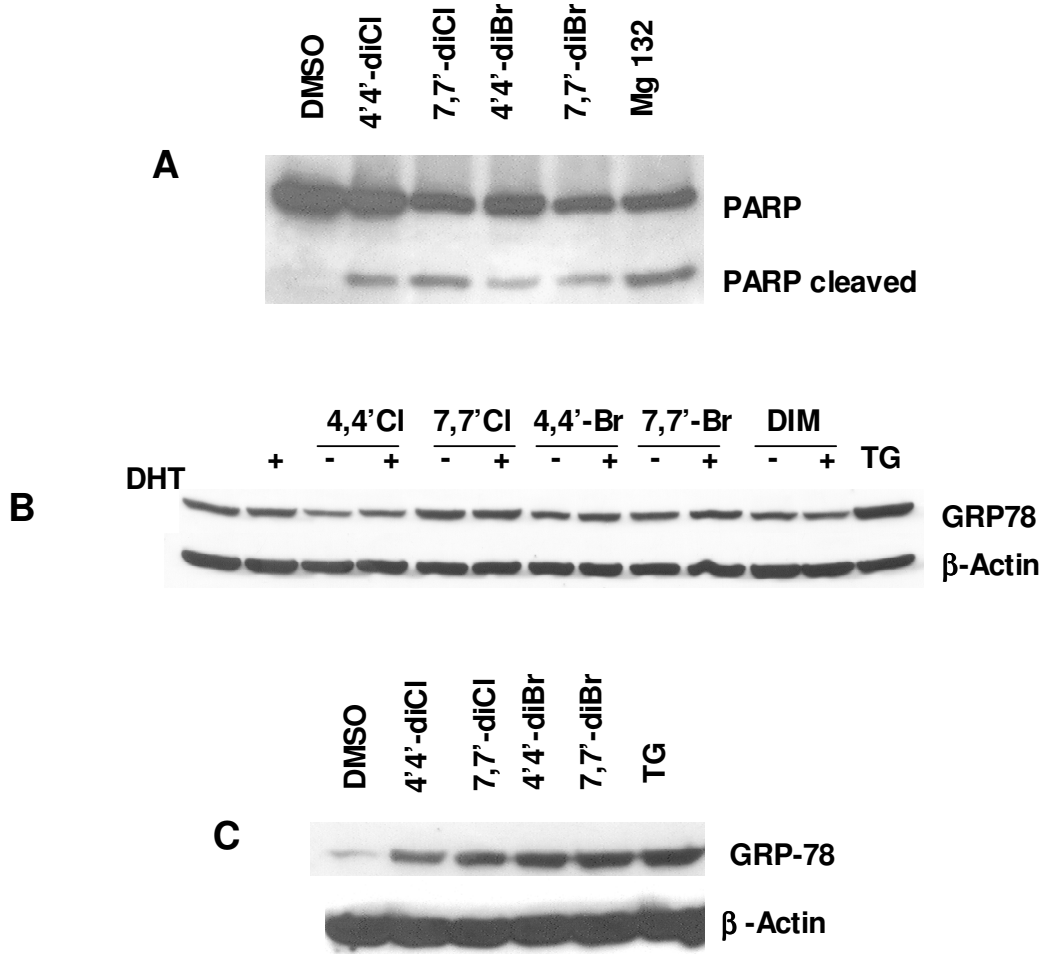


Figure 33. Induction of apoptosis and ER stress by dihalo DIMs. (A) Induction of apoptosis. LNCaP cells were treated with 20 μM of 4,4'-, 7,7'-dihalo DIMs and 5 μM MG132 as positive control for 24 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. (B) Activation of ER stress protein GRP78 in LNCaP cells. Cells were treated with 4,4'-, 7,7'-dihalo DIMs (± 10 μM DHT) and 1 μM TG as positive control for 48 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. (C) Activation of ER stress protein GRP78 in Panc-1 cells. Cells were treated with 4,4'-, 7,7'-dihalo DIMs and 1 μM TG as positive control for 48 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. β-actin was used as loading control for these experiments.

pancreatic cancer cells (430) we examined the effects of the 4,4'- and 7,7'-dihalo compounds on induction of GRP78, a well known marker of ER stress. The experiment was carried out in the presence or absence of DHT and the results (Figure 33B) show that 20 μM concentrations of these dihalo DIMs did not significantly induce ER stress in LNCaP cells. In contrast, thapsigargin (TG), a well characterized inducer of ER stress induced approximately a 2-fold increase in GRP78 protein. Thus the dihalo DIMs did not induce ER stress in LNCaP cells. As an additional positive control for this experiment Panc-1 cells were treated with 20 μM of the dihalo DIMs and 1 μMTG and Western blot analysis of whole cell lysates shows that GRP78 was induced in all treatment groups (Figure 33C) Thus the differences in the effects of the 4,4'-, and 7,7'-dihalo DIMs on induction of ER stress were cell context dependent and this pathway was not activated in LNCaP cells.

4.3.3 Mechanism of action of dihalo DIMs. Activation of NAG-1 and ATF3

DIM and C-DIMs induced the TGFβ-like peptide NAG-1 and ATF3 in colon cancer cells and both factors have been linked to growth inhibition and apoptosis (428, 435). The results in Figure 34 show that 4,4'-, 5,5'-, 6,6'- and 7,7'-dichloro DIM induce both NAG-1 and ATF3 proteins in LNCaP cells after treatment for 24 hr. Both protein are induced in parallel, however at higher concentration of the more toxic 6,6'-dichloro DIM (15 μM) and 7,7'-dichloro DIM

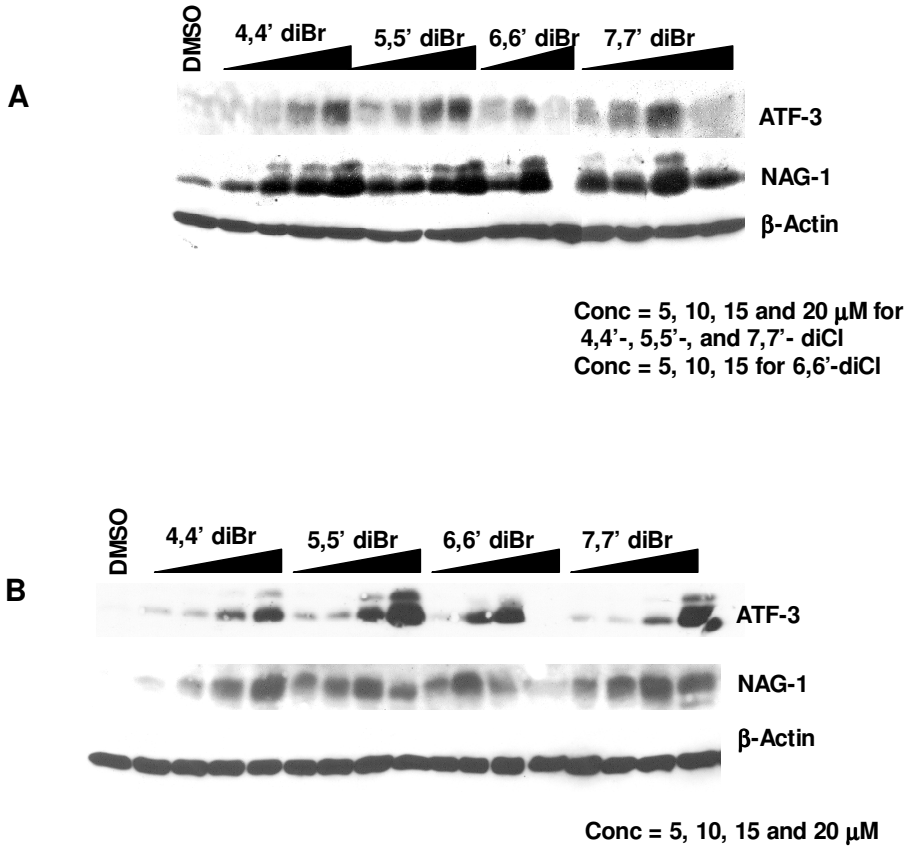


Figure 34. Activation of NAG-1 and ATF3.
(A) Effects of isomeric dichloro DIMs on induction of NAG-1 and ATF3. LNCaP cells were treated with isomeric dichloro DIMs for 24 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. (B) Effects of isomeric dibromo DIMs on induction of NAG-1 and ATF3. LNCaP cells were treated with isomeric dichloro DIMs for 24 hr and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods.

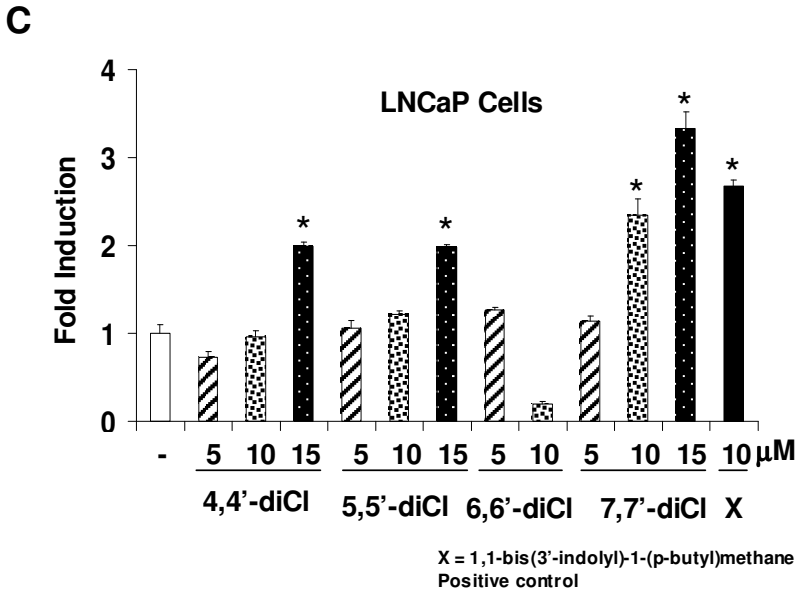


Figure 34 (continued) (C)Transactivation of pNAG-1. LNCaP cells were transfected with pNAG-1luc and treated with the isomeric dichloro DIMs and luciferase activity was determined as described in Materials and methods. Results are expressed as means of three replicate determinations for each treatment groups Significant induction ($p<0.05$) of pNAG-1 ± SE (to DMSO) is indicated by "*" in figure.

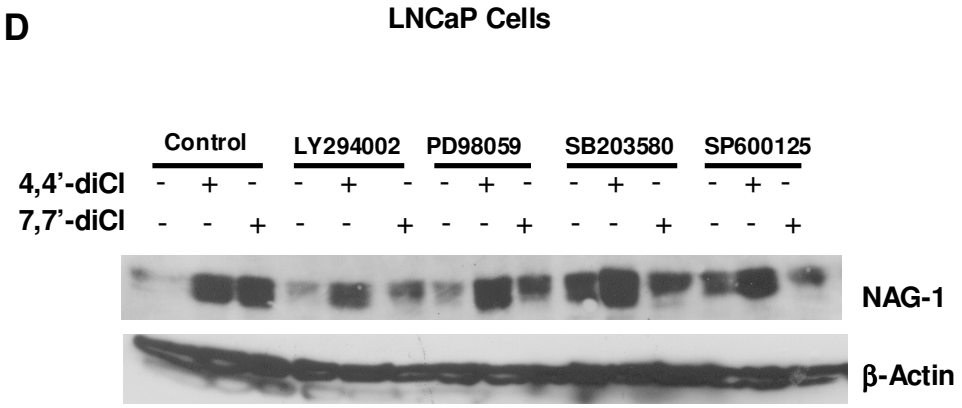


Figure 34. (continued) (D) Effects of kinase inhibitors on induction of NAG-1. LNCaP cells were treated with various kinase inhibitors alone or in combination with the 4,4'-, or 7,7'-dichloro DIMs for 24 hr (in case of kinase inhibitors cells were pretreated with kinase inhibitors for 2 hr before adding the isomeric DIMs) and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. β-actin was used as loading control for the Western blot experiments.

(20 μM) there was a subsequent decrease in NAG-1 protein expression and ATF3 was also decreased. Figure 34B summarizes the effects of 4,4'-, 5,5'-, 6,6'- and 7,7' dibromo DIM on induction of NAG-1 and ATF3 and the results show that like the dichloro compounds all the isomeric dibromo DIMs enhanced expression of both the proteins. Moreover NAG-1 and ATF3 expression decreased at the highest concentration of the toxic 6,6'-dibromo DIM isomer. The induction of p53 or Egr-1 was not observed in these studies (data not

shown). The induction of NAG-1 protein by the dichloro DIMs was also paralleled by induction of reporter gene (luciferase) activity in LNCaP cells transfected with pNAG-1 construct which contains the -1084 to +41 NAG-1 gene promoter insert (428). The results showed that 4,4'-, 5,5'-, and 7,7'-dichloro DIM compounds significantly induced pNAG-1 whereas the 6,6'-dichloro DIM did not induce activity and 1,1-bis (3'indolyl)-1-(p-butylphenyl) methane was used as a positive control since this compound has previously been shown induce NAG-1 in cancer cell lines (428). Interestingly the only inactive compound was the most toxic 6,6'-dichloro DIM isomer which decreased luciferase activity (Figure 34C) and NAG-1 protein (Figure 34A). Using 4,4'- and 7,7'-dichloro DIM as models we investigated the effects of kinase inhibitors on induction of NAG-1 since previous studies have shown that C-DIMs and other compounds induce NAG-1 through activation of kinases (428). Results in Figure 34D show that NAG-1 protein is induced after treatment of LNCaP cells for 24 hr with 15 □□ 4,4'- and 7,7'-dichloro DIM. After co-treatment with MAPK (PD98059), PI3K (LY294002), JNK SP600125) and p38MAPK (SB203580) inhibitors there was some inhibition of 7,7'-dichloro DIM-induced NAG-1 expression in cells co-treated with LY294002 and SP600125 and to a lesser extent with the other inhibitors. In contrast, induction of NAG-1 by 4,4'-dichloro DIM was not affected in LNCaP cells co treated with these inhibitors. These results suggest that dihalo DIM-dependent induction of NAG-1 through kinase pathways is complex and the

mechanism of induction may be dependent on the structure of the compounds and this is currently being investigated.

4.4 Discussion

DIM is a phytochemical metabolite that inhibits cancer cell growth and induces apoptosis in cells derived from multiple tumors, however the mechanisms of this response are complex and dependent on cell context (193, 202, 378, 395, 396, 399, 422). Previous studies in this laboratory have described the antitumorigenic effects of symmetrical ring substituted DIM derivatives and the results show both some similarities and differences (330, 404, 423, 430). DIM and ring-substituted DIMs inhibit growth of breast cancer cells and the latter compounds were more active (330, 404, 423). Results of in vivo studies showed that doses of DIM as low as 5 mg/kg/48 hr inhibit growth of carcinogen-induced rat mammary tumors (378). In contrast, several ring substituted DIMs, including 4,4'-dichloro-, 6,6'-dichloro-, 5,5'-dibromo-, 1,1'-dimethyl-, 5,5'-dimethyl and 1,1',2,2'-tetramethyl DIMs inhibited rat mammary tumor growth at doses of ≤ 1mg/kg/48 hr and were at least 5 times more potent than DIM (330, 404). Similar differences in potency were observed for induction of ER stress in pancreatic cancer cells where 5,5'-dibromo DIM was more potent than DIM (430).

DIM exhibits antiandrogenic activity in LNCaP prostate cancer cells and this was associated with inhibition of dihydrotestosterone (DHT)-induced

accumulation of nuclear AR (403). In contrast, results from this laboratory using DIM and a series of ring substituted isomeric dihalo DIMs gave different results in LNCaP and 22Rv1 cells (433). For example, DIM did not block DHT-induced nuclear uptake of AR and the effects of the isomeric dihalo DIMs were structure-dependent. In transient transfection studies using an androgen-responsive construct containing a probasin promoter insert the 7,7'-dichloro and 7,7'-dibromo DIMs were partial AR agonists whereas like DIM, the 4,4'-, 5,5'- and 6,6'-dihalo DIMs were antiandrogens. We did not observe any effects of DIM or dihalo DIMs on DHT induced nuclear uptake of the AR however, 6,6'- and 7,7'-dihalo DIMs decreased AR expression (protein and mRNA). However, these effects were not observed for DIM or 4,4'- and 5,5'-dihalo DIMS.

Results in Figures 29-32 show that 4,4'-, 5,5'-, 6,6'- and 7,7'- dihalo DIMs all inhibited growth of LNCaP and 22 Rv1 prostate cancer cells. Over the concentrations used in this study (5,10 and 20) the IC_{50} (growth inhibition) values were ≤ 5 μM and using lower concentrations the IC_{50} value for 5,5'-dichloro DIM was approximately 1 μM in 22Rv1 cells (Figure 31). Thus despite the structure-dependent AR expression in LNCaP cells all of these compounds were potent inhibitors of LNCaP and 22Rv1 cell proliferation. It was apparent from the cell proliferation that at doses ≥5 μM there was considerable induction of cell death and results in Figure 33A show that treatment of LNCaP cells with 20 μM 4,4'-dichloro, 4,4'-dibromo, 7,7'-dichloro and 7,7'-dibromo DIM induce caspase-dependent apoptosis. Similar results were observed in LNCaP cells

treated with 5 μM MG132, a proteasome inhibitor and know inducer of apoptosis. Since previous studies showed that DIM and ring substituted DIMs induce ER stress which leads to activation of apoptosis in pancreatic cancer cells (430), we also investigated activation of ER stress by dihalo DIMs in LNCaP cells after treatment for 48 hr. Treatment with the ring substituted DIMs did not enhance expression of the stress protein GRP78, and similar results were observed in the co-treatment studies with DHT, whereas TG a known inducer of ER stress enhanced GRP78 protein expression. These experiments were also repeated in the presence of DHT and similar results were obtained suggesting that stress pathways were not associated with the proapoptotic effects of the dihalo DIMs in prostate cancer cells. In contrast, as a positive control for this experiment we showed that like TG, the dihalo DIMs induced ER stress in Panc-1 cells (Figure 33C) and this clearly demonstrates the cell context dependent differences in the mechanisms of action of these compounds.

Previous studies reported that DIM and selected PPARγ-active C-DIMs induced the TFGβ-like peptide NAG-1 in colon cancer cells and the latter compounds also induced ATF3 (428, 435). NAG-1 is induced by NSAIDS in some colon cancer cell lines and this gene is also enhanced by different drugs, PPARγ-agonists, other chemoprotective agents such as genistein, polyphenolics and DIM (336-341, 434-438). Moreover, overexpression of NAG-1 induced apoptosis (436). ATF-3 is often co-induced with NAG-1 and this gene is also associated with growth inhibitory/proapoptotic pathways (439-441). Results in

Figure 34A show that treatment of LNCaP cells with 5-20 μM 4,4'-, 5,5'-, 6,6'- and 7,7'dichloro DIM induced both NAG-1 and ATF3. The dose response curves were variable particularly with respect to higher concentrations of 6,6'-dichloro DIM (15 μM) and 7,7'-dichlro DIM (20 μM) where the induction of NAG-1 and ATF3 observed at lower concentrations were decreased. The reason for these concentration-dependent responses are unknown but have previously been observed for other compounds that induce NAG-1 (428). The effects of 4,4'-, 5,5'-, 6,6'- and 7,7'-dibromo DIM on NAG-1 and ATF3 expression were similar to that observed for the dichloro DIM isomers and expression of both proteins was enhanced (Figure 34B). Moreover, induction of luciferase activity was also observed in LNCaP cells transfected with pNAG-1 and treated with the isomeric dichloro DIMs (Figure 34C) and similar effects have been observed for C-DIMs (428).

Previous studies with C-DIMs showed that these compounds rapidly induced PI3K dependent activation of early growth response gene-1 (Egr-1) which in turn activated NAG-1 through the proximal Egr-1 site in the NAG-1 promoter (428). Results in Figure 34D show that both 4,4' and 7,7' dichloro DIM induce NAG-1 expression and these effects are differentially modulated by kinase inhibitors. For example, induction of NAG-1 by 4,4'-dichloro DIM was inhibited by LY294002 (PI3K inhibitor) and partially inhibited by PD98059 (MAPK inhibitor) and SP600125 (JNK inhibitor). SB203580 (p38 MAPK inhibitor) alone induced NAG-1 but also inhibited induction of NAG-1 by 4,4'-dichloro DIM. In

contrast induction of NAG-1 by 7,7'-dichlor DIM was not affected by these inhibitors suggesting that although both compounds induce NAG-1 (and ATF3) and their mechanism of induction are structure dependent.

In summary this study shows that symmetrical ring substituted 4,4'-, 5,5'-, 6,6'- and 7,7'-dichloro and dibromo DIMs inhibit growth of LNCaP cells and also induce caspase dependent apoptosis in this cell line. The dihalo DIMs induce NAG-1 and ATF3 and these responses are related in part to their apoptotic/antiproliferative effects. However mechanistic studies suggest that activation of protein kinases by dihalo DIMs may play a role in the activity of these compounds, moreover, these effects may be structure-dependent and are currently being investigated.

CHAPTER V

SUMMARY AND CONCLUSIONS

5.1 Inhibition of ER-negative breast cancer cell growth by SAhRMs by induction of pro-apoptotic protein HES-1

Selective receptor modulators have been developed for therapeutic uses in hormone related diseases. Our laboratory is investigating new mechanism-based/receptor modulating drugs for the treatment of advanced stage breast cancer where cytotoxic chemotherapy is the only current available treatment.

It has been reported that SAhRMs are effective for treatment of ER+ breast cancers (168, 349) and in this study their effects on ER-negative breast cancer cells were investigated. MDA-MB-453 and BT-474 are ER negative breast cancer cell lines that over express epidermal growth factor receptor 2 EGFR2, erbB2 and exhibit high constitutively active kinase activities. Our results showed that treatment of both cell lines with TCDD induced CYP1A1 expression and proteasome-dependent degradation of the AhR. Low but significant induction of CYP1A1 was also observed for the SAhRMs 6-MCDF and DIM. However these compounds did not downregulate AhR levels. TCDD (10 nM), MCDF (2 and 5 μM), and DIM (10 and 20 μM) all significantly inhibited MDA-MB-453 and BT-474 cell proliferation but did not significantly affect the percent distribution of the cells in G0/G1, S or G2/M phases of the cell cycle. TCDD and

the SAhRMs had minimal effects on the expression of ErbB2 and pErbB2, mitogen activated protein kinase 1/2 (MAPK1/2) or phospho-MAPK1/2, whereas the MAPK inhibitor UO126 inhibited cell proliferation and phosphorylation of MAPK. These data coupled with results obtained for other activated kinase pathways demonstrate that TCDD and SAhRMs uniquely inhibit growth of ER-negative MDA-MB 453 and BT-474 breast cancer cells through kinase-independent pathways. Our study showed that the pro-apoptotic protein HES-1 is induced by TCDD, DIM and 6-MCDF and this represents a possible mechanism for mediating the growth inhibitory effects observed for these compounds. Current studies are further investigating the mechanism of HES-1 induction by AhR agonists in ER-negative breast cancer cells and determining the role of HES-1 and other proteins in mediating the antiproliferative effects of these compounds in these highly aggressive ER-negative cells.

5.2 Structure activities of symmetrically substituted DIMs in prostate cancer cells

Ring-substituted DIMs exhibit potent anticancer activities (330, 404), however the structure-dependent effects of these compounds as antiandrogens have not been reported. This study investigated the structure-dependent androgenic/antiandrogenic activity of several symmetrical dichloro- and dibromo DIM isomers. Initial transactivation studies in LNCaP and 22Rv1 cells transfected with an androgen responsive construct (pPB) containing a probasin

promoter insert showed that both 7,7'-dichloro- and 7,7'-dibromo DIMs exhibited partial androgenic activity. Most of the other isomeric substituted DIMs, including 4,4'-dichloro DIM and 4,4'-dibromo DIM, exhibited antiandrogenic activity in the transactivation assay. Structure-dependent differences were also observed for the effects of 4,4'- and 7,7'-dihalo DIMs on AR expression in LNCaP cells. Like DIM, 4,4'-dichloroDIM and 4,4'-dibromo DIM did not affect AR protein levels for up to 48 hr and inhibited dihydrotestosterone (DHT)-induced responses without affecting cytosolic or nuclear AR distribution. In contrast, the AR agonist activity of 7,7'-dihalo DIMs was significantly decreased after 48 hr, and this was due to decreased AR mRNA and protein levels, and the latter response was proteasome-independent. Results of this study demonstrate that the antiandrogenic activity of symmetrical dihalo DIMs was structure-dependent and the 7,7'-dihalo DIMs exhibited partial AR agonist activity, whereas 4,4'-, 5,5'- and 6,6'-dihaloDIMs and DIM were antiandrogens in transactivation assays. The mechanisms of action of ring-substituted DIMs were also structure-dependent since 4,4'- and 5,5'-dihalo DIMs and DIM did not affect AR expression, and 6,6'- and 7,7'-dihaloDIMs induced degradation of AR protein and AR mRNA levels.

5.3 Mechanism of action for the antiproliferative effects of symmetrically substituted dibromo and dichloro DIMs

Preliminary results with the dihalo DIMs showed that all the dihalo series of compounds inhibited growth of LNCaP and 22Rv1 prostate cancer cells. Thus despite the structure dependent effects on AR expression in LNCaP and 22Rv1 cells all of the compounds were potent inhibitors of cell proliferation. Treatment of LNCaP cells with 20 μM 4,4'- and 7,7'-dichloro and dibromo DIMs induced caspase dependent apoptosis. Since previous results showed that DIM and ring substituted DIMs induce ER stress which leads to activation of apoptosis in pancreatic cells (430) we also investigated the activation of ER stress by dihalo DIMs in LNCaP cells after treatment for 48 hr. However, treatment with the ring substituted DIMs did not enhance expression of the stress protein GRP78 and this clearly demonstrates the cell context dependent differences in the mechanism of action of these compounds. However, the dihalo DIMS induced NAG-1 and ATF3, two proapoptotic proteins which could in part be responsible for the antiproliferative and pro-apoptotic effects that were observed.

In summary, this study describes development of new mechanism based pharmaceutical agents for treatment of various cancers. The search for new and improved drugs is essential since many of the cancer become drug-resistant after prolonged used of a specific compound and development of new agents is essential. Moreover for ER-negative breast cancer, it is very important to find

new mechanism based drugs for their treatment since current chemotherapies are not highly effective.

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