ROLE OF ESTROGEN RECEPTOR α (ERα) IN INSULIN-LIKE GROWTH FACTOR (IGF)-I-INDUCED RESPONSES IN MCF-7 BREAST CANCER CELLS

A Dissertation

by

SHU ZHANG

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 2007

Major Subject: Toxicology

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Approved by:

Chair of Committee,	Stephen H. Safe
Committee Members,	Robert C. Burghardt
	Timothy D. Phillips
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ABSTRACT

Role of Estrogen Receptor α (ER α) in Insulin-like Growth Factor (IGF)-I-induced Responses in MCF-7 Breast Cancer Cells. (December 2007)

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Insulin-like growth factor-I (IGF-I) is a mitogenic polypeptide that induces proliferation and activation of kinase pathways in MCF-7 breast cancer cells. The role of estrogen receptor α (ERα) in mediating responses induced by IGF-I was investigated in cells transfected with small inhibitory RNA for ERα (iERα) or cotreated with the pure antiestrogen ICI 182780. The results showed that IGF-I-dependent phosphorylation of Akt and MAPK, induction of G1–S-phase progression and enhanced expression of cyclin D1 and cyclin E were dependent on ERα. Moreover, these IGF-I-induced responses were also inhibited by the antiestrogen ICI 182780, suggesting that the effects of ICI 182780 as an inhibitor of IGF-I induced responses in breast cancer cells are primarily related to downregulation of ERα.

Chemoprotective phytochemicals exhibit multiple activities and interact with several cellular receptors, including the aryl hydrocarbon receptor (AhR). We investigated the AhR agonist/antagonist activities of the following flavonoids:

chrysin, phloretin, kaempferol, galangin, naringenin, genistein, quercetin, myricetin, luteolin, baicalein, daidzein, apigenin, and diosmin, in MCF-7 breast cancer cells, HepG2 human liver cells and mouse Hepa-1 cells. The dietary phytochemicals exhibited substantial cell context–dependent AhR agonist as well as antagonist activities, and these are factors that must be considered in risk assessment of overall exposures to AhR agonists.

Halogenated aromatic hydrocarbons (HAHs) such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8- pentachlorodibenzo-p-dioxin (PeCDD), 3,3',4,4',5-pentachlorobiphenyl (PCBP), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) bind and activate the aryl hydrocarbon receptor (AhR). It has been assumed that these compounds only differ in their potencies. The SAhRM-like activity of the 5 HAHs was investigated by determining ligand structure dependent differences in their induction of CYP1A1 and interactions of the AhR with a series of coactivators in a mammalian two-hybrid assay in three AhR-responsive cell lines, including mouse Hepa-1, Human HEK293 and human Panc1 cells. There were multiple structure-dependent differences in activation of luciferase activity in these cell lines transfected with VP-AhR and six different GAL4-coactivator chimeras and a GAL4-response element-luciferase promoter construct. The results show that HAHs selectively interact with coactivators and these interactions are dependent on cell-context, and even among HAHs, these compounds exhibit selective receptor modulator activity.

To my parents, Hong Fan and Fengyou Zhang

谨以此文献给我的父母，樊红和张丰友

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I would like to thank my committee chair, Dr. Safe, and my committee members, Dr. Phillips, Dr. Burghardt and Dr. Donnelly, for their guidance and support throughout the course of this research.

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CHAPTER I

INTRODUCTION

1.1. Breast cancer

1.1.1. Incidence and risk factors

1.1.1.1. Incidence

Breast cancer is the most common cancer for women in the United States. It is estimated that a woman in the United States has a 1 in 8 chance of developing invasive breast cancer during her lifetime (Draper, 2006). There are more than 200,000 new cases diagnosed every year and 12% of the women diagnosed with this disease die within five years (Howe et al., 2006). Although mortality has declined in recent years due to early diagnosis and improved treatments, the incidence of breast cancer is still increasing (Feig, 2006).

Epidemiological studies suggest that the incidence of breast cancer can vary greatly in different geographic regions (Kamangar et al., 2006). In general, women in industrialized countries have higher risk of breast cancer than women

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in third world countries (Esteve, 2007). For example, the incidence rate of breast cancer in North America and Northern Europe is one out of eight, while in Africa and Asia the incidence is approximately 10 times lower (Sasco, 2003).

1.1.1.2. Risk factor

1.1.1.2.1. Hormones

Breast cancer is largely a disease in women. Male breast cancer cases are extremely rare, with less than 2000 new cases reported in 2006 in the United States (NCCDPHP, 2006). The cause for this drastic gender difference has been primarily attributed to higher lifetime exposures of women to the hormones, 17β-estradiol and progesterone.

There is a strong association between the cumulative lifetime exposure to estrogens and the risk of breast cancer (Missmer et al., 2004). Estrogen induces cell proliferation in both normal breast tissue and malignant breast cells. Women who have early menarche and/or late menopause are exposed to estrogen for a prolonged period, and are at higher risk for breast cancer. The overall estrogen burden can also be increased by oral contraceptives and hormone replacement therapy (HRT), and it is not surprising that both the use of oral contraceptives and HRT have been linked with increased breast cancer incidence (Enserink, 2002; Ursin et al., 1998).

Although the connection between high circulating estrogen levels and increased risk of breast cancer is well established and supported by most epidemiological studies and animal models, there are some exceptions. One study indicates that higher body weights for 7-year-old girls have been associated with a lower lifetime risk for breast cancer (Magnusson et al., 1998). Since adipocytes produce a significant portion of the total estrogen body burden in women before puberty, a high body fat content is a marker for high levels of circulating estrogens. Therefore, it is an interesting phenomenon that pre-pubertal exposure to estrogens decreased the lifetime breast cancer risk, since this is contradictory to the effects of adult exposure to estrogens. Another study has corroborated this finding by showing that girls with a low body mass index (BMI) between the ages of 7 and 15 have a significantly increased breast cancer risk. Thus, it has been proposed that the weight of a girl at puberty is negatively correlated with her risk of developing breast cancer later in life (Hilakivi-Clarke et al., 2001). These results suggest that timing of estrogen exposure, not just the level of estrogen exposure, may be important in determining breast cancer risk. Early exposure to estrogens may be protective against breast cancer by inducing cellular differentiation and decreasing proliferative potential, whereas later exposure to estrogens increase breast cancer risk by promoting cell proliferation. This observation also explains, in part, why there is a historically low incidence of breast cancer in women living in soy food-consuming countries

where there is a high intake of phytoestrogen-rich soy products early in life (Messina et al., 2006).

Early pregnancy also has a protective effect on breast cancer incidence. Compared to women giving birth before age 20, nulliparous women and women giving birth at or after age 30 have a relative risk of 1.67 and 2.23, respectively (Brinton et al., 1983a; Brinton et al., 1983b). During full-term pregnancy and lactation, estrogen and progestin exposure is significantly increased. Thus, these studies highlight the importance of timing of hormonal exposure in determining breast cancer risk.

Some synthetic progestins, combined with estrogen are used in hormone replacement therapy (HRT), and breast cancer risk is higher for combined exposure than in women using estrogen alone. Although prolonged exposure to estrogen and progestin combined is associated with an increased risk for breast cancer, shorter exposures to exogenous estrogen plus progesterone together mimics the protective effects of pregnancy and decreases the breast cancer risk. Studies in mice confirm that estrogen and progestin in combination have a protective effect on mammary tumors initiated by a spectrum of oncogenic alterations that are common in breast cancers (Rajkumar et al., 2004).

One controversial issue regarding exogenous estrogen exposure is the effects of synthetic or “xenoestrogens”, which are a broad spectrum of

compounds that exhibit weak estrogenic activities. Because of their widespread occurrence in the environment, there are concerns that these compounds may increase the overall estrogen burden in humans and elevate the incidence of breast cancer. Although there have been reports of negative impacts of xenoestrogens on wildlife, human exposure to these chemicals is relatively low and it has been difficult to assess their risk to humans. Safe (Safe, 2004) argued that the estrogen burden caused by xenoestrogen is negligible compared to endogenous estrogens and it was suggested that exposure to xenoestrogens does not constitute a significant risk factor for breast cancer. This conclusion is controversial and requires further evaluation.

1.1.1.2.2. Age

Age is one of the most well-established risk factors for breast cancer. The incidence of breast cancer increases sharply after age 30 (Figure1). However, the early onset breast cancer (developed before age 35) is more aggressive and usually associated with a genetic predisposition to this disease (Winchester et al., 1996).

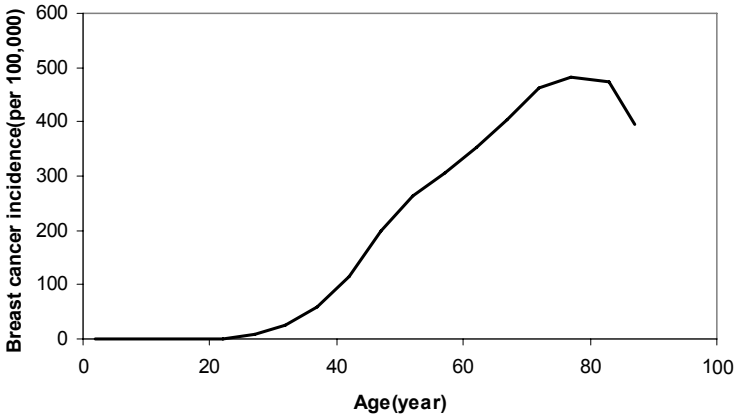

Figure 1 Breast cancer incidence (per 100,000) as a function of age (Adapted from rating the risk factors for breast caner, S. E. Singletary, 2003).

1.1.1.2.3. Life style

Lifestyle has a significant impact on breast cancer risk. Women in East Asia have a much lower breast cancer risk than their counterparts in Western society. Genetic factors cannot account for this difference because women who emigrate from lower to higher risk areas will have the same risk as women in the high risk area after only one generation (Stanford et al., 1995).

Diet is an important component of lifestyle and appears to be a significant modifying factor for breast cancer risk. An East Asian diet, which is high in vegetables and low in fat, is protective against breast cancer. However, the reasons for this protective effect are unknown, even though many hypotheses

have been proposed. Some believe that plant consumption increases the intake of antioxidants or other anti-cancer phytochemicals; others suggest that exposure to phytoestrogens in plant-based diets before puberty accelerates the differentiation of breast tissue and decreases breast cancer risk later in life.

A sedentary lifestyle, lack of exercise, increased body weight and alcohol consumption, have all been associated with elevated levels of circulating estrogens and may increase the incidence of breast cancer (Okobia and Bunker, 2005).

1.1.1.2.4. Genetic background

1.1.1.2.4.1. Family history

Women with a family history of breast and/or ovarian cancer are at an increased risk for breast cancer. It is estimated that hereditary breast cancer accounts for approximately 5-10% of all breast cancer cases and as many as 25% of early-onset cases (Weber and Garber, 1993). Compared to women with no family history of breast cancer, those with a first degree relative with breast cancer at 50 years of age or older have a relative risk of 1.8; those with a first degree relative who develop breast cancer younger than 50 years of age have a relative risk of 3.3. The relative risk is decreased to 1.5 in women with only a second degree relative affected with this disease (Singletary, 2003).

1.1.1.2.4.2. Genetic mutation

BRCA1 and BRCA2 are very important breast cancer susceptibility genes. BRCA genes have prominent roles in maintaining genome integrity, thus loss-of –function mutations lead to an increased cancer risk. BRCA1 has been identified as an essential player in the early cellular response to DNA damage. Within minutes of DNA damage, H2AX, a member of histone H2A family, becomes extensively phosphorylated, forms foci at break sites and recruits BRCA1 to these foci. BRCA1 appears to be involved in the initiation of DNA repair by modifying chromatin structure and facilitating access of DNA repair enzymes to damaged genomic sites. Furthermore, some experiments suggest that BRCA1 has a physical association with SWI/SNF and forms a complex, which is essential for chromatin remodeling. In response to DNA damage, BRCA2 binds to BRCA1 and RAD51, indicating that BRCA2 also plays a crucial role in repairing double stranded breaks and maintaining the integrity of chromosome. Also, BRCA1 and BRCA2 function as transcriptional co-regulators by interacting directly with some components of the transcriptional machinery, and thereby regulate gene transcription.

Thus, BRCA1 and BRCA2 mutations result in chromosome instability, which may be an important factor in development of breast cancer. It is reported that breast cancer risk for carriers of BRCA mutations is very high, with an

expected lifetime incidence greater than 80% by the age of 70 (Welcsh and King, 2001).

Although BRCA1 and BRCA2 mutations lead to a drastic elevated risk for developing breast cancer, due to the low frequency of these mutations in the general population, a significant portion of inherited breast cancer cases cannot be attributed to BRCA1/2 mutations. The effort to identify another predisposing gene, called BRCA3, has been pursued for a long time but without success. Some recent genetic epidemiology studies show non-Mendelian inheritance of congenital breast cancer. The familial residual risk independent of BRCA1 or BRCA2 genes could be explained by a polygenic model, corresponding to the multiplicative effects of several low pentrance genes. Although mutations of each of these genes confer only a moderate risk for breast cancer compared to high pentrance genes, such as BRCA1 and BRCA2, due to their greater frequency in the general population, these genes may account for a high proportion of inherited breast cancer cases. Mutations of five low penetrance genes, including ATM, PTEN, TP53, LKB1 and CHEK2, have been established and are associated with increased risk of inherited breast cancer (Table1).

Table 1 Summary of genes associated with hereditary breast cancer

	Frequency of mutant allele	Breast cancer risk	Major associated cancers	Possible associated cancers
BRCA1	≈0.1% (?)* (2.5% in Ashkenazim)	High	Breast, ovarian	Colon, prostate
BRCA2	≈0.1% (?)* (2.5% in Ashkenazim)	High	Breast, ovarian, male breast	Pancreatic, prostate
p53	rare	High	Sarcoma, breast, brain, adrenal, leukemia	
PTEN	rare	Intermediate-high	Breast, thyroid	Brain
ATM	0.7%	high	Lymphoma, leukemia (in homozygotes), breast	
CHEK2	Rare (1.3-1.6% in Finland)	high	breast	Prostate, colon
LKB1	rare	high	Breast, intestinal, extra-intestinal	

*The frequency of BRCA1 and BRCA2 mutations in the general population is unknown.

1.1.2. Classification and stage of breast cancer

1.1.2.1. Mammary gland

The mammary gland developed in the ancestors of mammals as organs that secrete milk in order to nurture young or newborns. Both males and females have mammary glands, but only female mammary glands will develop and be functional. Before puberty, mammary glands in males and females are virtually

identical in structure, and are comprised of simple ductal systems. During puberty, under the stimulation of ovarian hormones, female mammary glands begin to develop, whereas male mammary glands remain rudimentary. Female mammary glands extend their ductal structures, undergo repeated branching and develop numerous alveolar buds. Alveolar buds are the basic components of mammary glands in adult women. Alveoli join to form groups known as lobules, and each lobule links to a lactiferous duct, which drains into the nipple (Figure 2). The alveolar cells are surrounded with myoepithelial cells, which can contract and push the milk from the alveoli through the lactiferous ducts towards the nipple. This is a period when females' breasts are especially vulnerable to carcinogens.

Full development of mammary glands is not complete until the first full-term pregnancy and following lactation. When a woman becomes pregnant, her mammary glands undergo drastic growth of ductal and lobular structures and development of milk-secreting acini. This process will continue until the peak of lactation. After lactation, epithelial cells undergo apoptosis or become inactive, thus alveoli and ducts regress back to a resting state. During this process, the structure of the mammary gland has become different from that of the pre-pregnancy state, since they have completed the developmental stage and have become well-differentiated. Later in a woman's life, with the onset of menopause, the mammary gland undergoes its involution. Under the influence of decreased levels of circulating ovarian hormones, the ductal elements degenerate and the

loose connective tissues are replaced by dense connective tissue. Figure 3 illustrates these changes in a mouse model of mammary gland development, which is similar to that observed in humans.

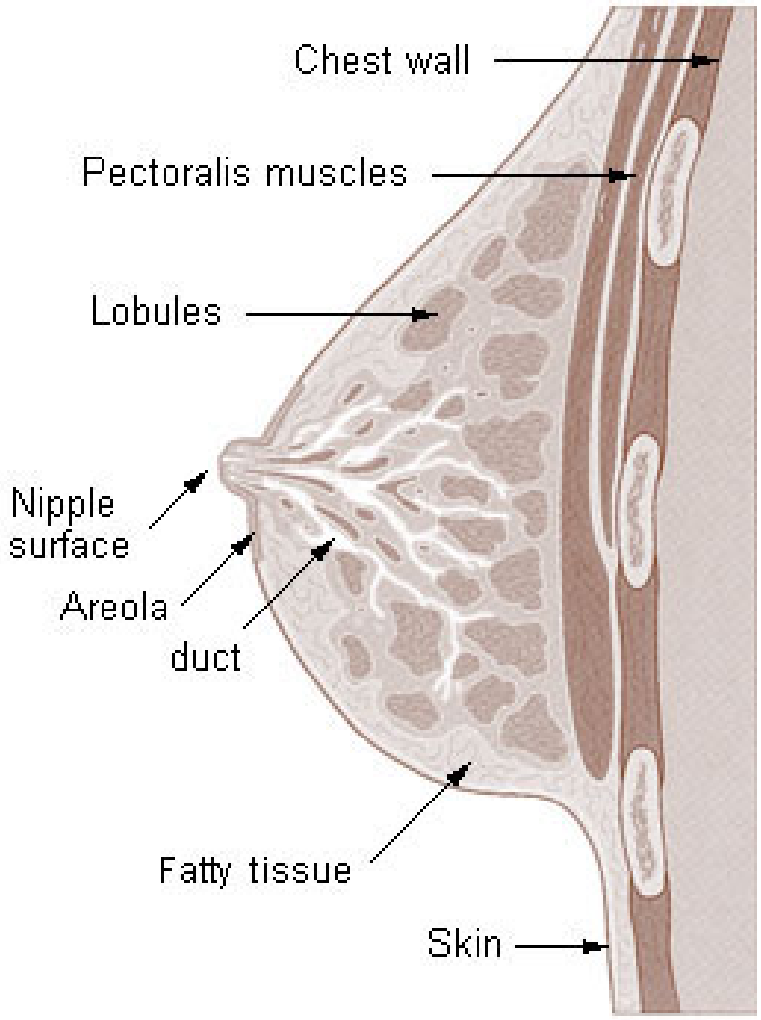

Figure 2 Breast anatomy (en.wikipedia.org).

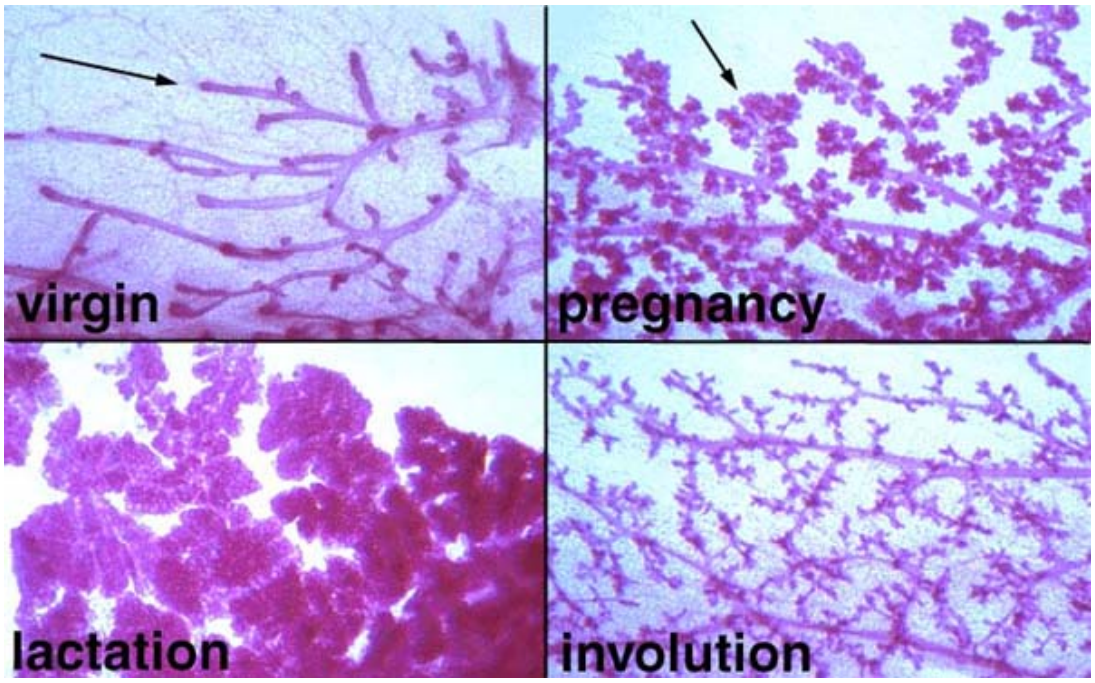

Figure 3 Development of mammary gland – mouse model (Hennighausen).

1.1.2.2. Classification and stage of breast cancer

Breast cancer can be divided to three major groups: Noninvasive carcinoma (in situ carcinoma), invasive (infiltrating) carcinoma and Paget's disease.

Noninvasive carcinoma has two subtypes: intraductal carcinoma (Ductal Carcinoma In Situ: DICS) and lobular carcinoma in situ (LICS). DICS is the most common noninvasive carcinoma in women. The malignant epithelial cells of DICS are originally from the ductal system, and have not moved out of the duct

into surrounding tissues. With the widespread use of mammography screening, DCIS has become one of the most commonly diagnosed breast conditions. An excision treatment, which excises all the abnormal duct elements, is the most common treatment, and this is usually combined with local radiation therapy. LCIS is generally considered to be a pre-cancerous condition, which is confined to the lobules and has not moved out of the lobules. LCIS does not show up on a mammogram, and is usually discovered as a chance finding, upon biopsy of breast tissues or lumps. Although LCIS is not considered cancerous and most women with LCIS will not develop breast cancer and will not require treatment, LCIS is a breast cancer risk factor (Chun et al., 2006). Frequent monitoring every 6-12 months is recommended as a precaution for LCIS-positive women.

Invasive carcinoma or infiltrating carcinoma also has two subtypes originating from ducts and lobules, respectively, named invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). IDC is the most common type of breast cancer and accounts for about 80% of all breast cancers, whereas ILC is less common. Although these two invasive carcinomas have different sites of origin, they act in a similar way. The malignant cells break free of their original sites and invade surrounding breast tissue. The cancer cells may remain localized or they can spread throughout the patient's body in the bloodstream or lymphatic system. For treatment of invasive carcinomas, it is important to determine the status of the metastasis and choose from a combination of several treatments, including surgery, chemotherapy, hormonal therapy and

radiotherapy. This issue will be further explored under the “prevention and treatment” section in this dissertation.

Paget’s disease is an uncommon type of breast cancer, but more than 95% patients with Paget’s disease have underlying breast cancer, which may be an invasive breast cancer or a DCIS**.** It is characterized by the presence of large neoplastic cells in the epidermis of the nipple-areola complex. Symptoms include eczema-like rash, inflamed, crusting, bleeding, ulceration and discharge around nipples.

Chemotherapeutic regimens are based on the type of cancer and therefore it is important to have a widely accepted standard for categorizing these tumors. TNM staging is a well-established system adopted by the AJCC (American Joint Committee on Cancer), and the definition of each stage is summarized below:

STAGE 0. In Situ ("in place") disease in which the cancerous cells are in their original location within normal breast tissue. Known as either DCIS or LCIS depending on the type of cells involved and the location, this is a pre-cancerous condition, and only a small percentage of DCIS tumors progress to invasive cancers. There is some controversy within the medical community on how to best treat DCIS.

STAGE I. The tumor is less than 2 cm in diameter and has not spread beyond the breast.

STAGE IIA. The tumor is 2 to 5 cm and has not spread to axillary (armpit) lymph nodes or the tumor is less than 2 cm in size with some spreading to the axillary lymph nodes.

STAGE IIB. The tumor is greater than 5 cm in size and has not spread to the axillary lymph nodes or the tumor is 2 to 5 cm in size with some spreading to the axillary lymph nodes.

STAGE IIIA. The tumor is smaller than 5 cm in size with spreading to the axillary lymph nodes which are attached to each other or to other structures, or the tumor is larger than 5 cm in size and has spread to the axillary lymph nodes.

STAGE IIIB. The tumor has penetrated outside the breast to the skin of the breast or chest wall or has spread to lymph nodes inside the chest wall along the sternum.

STAGE IV. A tumor of any size that has spread beyond the region of the breast and chest wall to organs, such as the liver, bone, or lungs.

1.1.3. Prevention and treatment

1.1.3.1. Prevention

Although many risk factors for breast cancer cannot be controlled, epidemiology and animal studies suggest that changes in diet and lifestyle may

reduce the incidence of breast cancer. Since patients diagnosed with cancer at an early stage have much better prognosis that those with later stage cancers, routine checkups can be a proactive approach to minimize the risk. The National Cancer Institute (NCI) recommends a mammogram screening every 1-2 years for women over the age of 40. However, questions have been raised about the values of routine mammogram screening in improving prognosis and the use of frequent mammography may be decreasing (Breen et al., 2007; Dilhuydy and Barreau, 1997).

Tamoxifen has already been widely used to prevent recurrence of tumors in women who have been surgically treated for breast cancer. Studies are also underway to investigate the efficacy of tamoxifen in reducing the risk of breast cancer among high risk women. Preventive mastectomy is believed to be the most effective way to prevent hereditary breast cancer. Results of several studies (Hartmann et al., 1999; Hartmann et al., 2001; Klaren et al., 2003; Meijers-Heijboer et al., 2001; Rebbeck et al., 2004) clearly show that mastectomy results in an 85-100%decrease in relative risk among women with BRCA mutations.

Alternative ways to prevent breast cancer in populations at high risk are being investigated and these include the use of hormone-induced differentiation and estrogen deprivation (Howell et al., 2007; Russo and Russo, 2007).

1.1.3.2. Treatment

Many factors affect the choice of treatment for breast cancer and these include TNM staging, the type of the cancer, the estrogen receptor and progesterone receptor status of the tumor, age, menopausal status and whether the tumor is new or recurring.

For treatment of Stage I, II or III breast cancers, the goal is to treat the cancer and prevent recurrence at the original site and metastasis to other parts of the body. In most cases, stage IV cancer cannot be cured and the main consideration for these patients is to prolong survival and improve their quality of life by treating the symptoms.

Thirty years ago, if a woman was diagnosed with breast cancer, there were no other choices but a radical mastectomy. Today with the increased understanding of the nature of breast cancer, many treatment options are now available. The primary treatment in most operable cases is usually surgery, combined with some pre-surgical and/or post-surgical treatments, such as radiation, chemotherapy and endocrine therapy, to achieve the goal of local control and disease-free survival. Given the patients' age, menopausal status, TNM staging and types of the cancer, the estrogen receptor and progesterone receptor status of the cancer cells, treatments can be optimized for each individual.

1.1.3.2.1. Surgery

In order to provide local control and prevent cancer cells from metastasizing to other parts of body, extensive surgery has been performed and this includes removal of the breast, the pectoralis major muscle, and axillary lymph node (ALN) levels 1, 2, and 3. This operation, called radical mastectomy, was developed by William Halsted in 1882. It has served as a standard procedure for breast cancer treatment for three quarters of a century and 90% breast cancer patients during that period in US underwent this procedure. Although it significantly reduces local recurrence rate, these patients suffer from disfiguring and disabling conditions after the operation, and the morbidity is still high. In 1970s, radical mastectomy was abandoned and more conservative operations were developed to replace it.

Today, surgical removal is no longer the only way to treat breast cancer, but in most cases, it is the primary treatment for this disease. Fortunately, current surgical treatments are less invasive and there are several options including modified radical mastectomy (MRM), breast conservation therapy (BCT), total mastectomy (TM), axillary lymph node dissection (ALND) versus sentinel lymph node biopsy (SLNB). For noninvasive breast cancer, usually a total mastectomy or a breast conservation therapy is adopted, the latter includes lumpectomy, wide excision, partial or segmental mastectomy, and quadrantectomy; for invasive breast cancer, it is necessary for patients to

undergo a breast conservation with sentinel lymph node biopsy and axillary lymph node dissection or a total mastectomy with sentinel lymph node biopsy or a modified radical mastectomy.

1.1.3.2.2. Radiation therapy

For Stage I and II breast cancer, BCT is a widely accepted treatment option and this consists of whole breast radiation therapy after surgery. Radiation therapy usually begins 4 weeks after surgery and a typical radiation requires treatment every day for 5 weeks. Since radiation can eliminate microscopic tumors that cannot be removed by surgery, the local control rate of BCT is comparable to total mastectomy. Accelerated, partial breast irradiation (APBI) is a potentially important alternative for incorporating radiotherapy in BCT. This treatment is more feasible than the typical approach since it shortens the treatment time for 1 to 5 days and decreasing the treatment area from the entire breast to the area of the breast immediately around the lumpectomy site. Several clinical trails over the last 10 years show that as a less invasive and more focused treatment, the efficacy of APBI is comparable to whole breast radiotherapy (Taghian and Recht, 2006).

1.1.3.2.3. Chemotherapy

Chemotherapy for breast cancer is used to destroy cancer cells with cytotoxic drugs, such as cyclophosphamide, epirubicin and 5-fluorouracil (5 FU).

Although both normal and malignant cells can be killed by these cytotoxic drugs, and malignant cells are more sensitive to these drugs because of their rapid proliferation and decreased ability for self-repairing. Chemotherapy can be employed before surgery, after surgery or as a primary treatment for metastatic cancer. Neoadjuvant therapy, which is given before surgery, can shrink a tumor so that less surgery will be needed. For example, a mastectomy can be replaced by BCT with a pre-treatment of cytotoxic drugs. Chemotherapy after surgery is called adjuvant therapy, which is employed usually when the patients are at higher risk because cancer cells have already spread to other parts of body and cannot be completely removed by surgery or when patients have estrogen receptor negative breast cancer and do not respond to hormone therapy. The primary use of chemotherapy is for the patients with metastatic cancers, which are unlikely to be removed by surgery. The goal of chemotherapy for these patients is to treat the symptoms and prolong survival.

Usually, a treatment is given daily for 5 days, and is followed by a break for 3 to 4 weeks; this is called a treatment cycle. Depending on the drugs used, cancer type and response of the cancer cells to the drugs, the length of the treatment is variable and can be up to 8 months in duration.

1.1.3.2.4. Endocrine therapy

Chemotherapy has serious side effects due to its relatively indiscriminate nature, and is considered for patients with a poor prognosis. With our rapidly expanding knowledge of breast cancer, it became clear that as cancer cells arise from endocrine-responsive tissues, a major portion of breast cancer can be treated by manipulating endocrine factors. It has been estimated that 60-65% of total primary tumors are ER-positive and respond to endocrine intervention (Putti et al., 2004). Since endocrine therapy specifically targets breast cancer cells, positive therapeutic outcomes can be achieved with minimal side effects.

Since estrogen exposure is a critical risk factor for breast cancer, blocking the effects of estrogen is a viable strategy for treating ER positive hormone sensitive breast cancer. Before effective ER antagonists were introduced into clinical practice, endocrine therapy constituted either a surgical removal of the ovaries or ovarian irradiation, in order to eliminate the major source of estrogen production. Since this process is invasive and irreversible, it is understandable that many patients were hesitant to undertake this treatment. Furthermore, because estrogen is important not only for mammary glands, but also for ovary, bone, cardiovascular and nervous systems, elimination of the ovaries causes undesirable side-effects within these systems. Thus the concept of a selective estrogen receptor modulator (SERM) was introduced and that these compounds would exhibit tissue-specific ER agonist or ER antagonist activity. An ideal

SERM for treating breast cancer would be an ER antagonist in breast tissue to inhibit cancer growth, but an ER agonist in skeleton, cardiovascular, nervous systems to maintain the beneficial effects of estrogen.

In the 1970s, the first clinically proven SERM, tamoxifen, was introduced for breast cancer treatment. Tamoxifen is antiestrogenic in mammary glands and inhibits ER-positive breast cancer growth. At the same time, tamoxifen is estrogenic in bone and cardiovascular systems and may offer some benefits in preventing osteoporosis and cardiovascular diseases (Love et al., 1992; McDonald et al., 1995). One concern for tamoxifen is that it is estrogenic in the uterus and increases the risk of endometrial cancer (Bernstein et al., 1999; Group, 1998). Generally, tamoxifen is prescribed for 5 years for ER-positive patients when used in adjuvant therapy and this decreases the risk of breast cancer recurrence at least 30-40% after 10 years and also decreases the mortality rate by approximately 30%. However, no further benefits were observed for prolonged treatment beyond 10 years. The success of tamoxifen serves as a proof of principle for SERMs, showing that it is possible to achieve tissue-specific estrogenic /antiestrogenic activities with a single compound.

Today, four classes of endocrine agents are clinically available: (i) tamoxifen and other SERMs, (ii)aromatase inhibitors (AIs), (iii)progestins and (iv)pure antiestrogens (Figure 4). These agents manipulate hormonal pathways

and serve as a first or second line of endocrine therapy according to the type of the breast cancer and menopausal status of the patients.

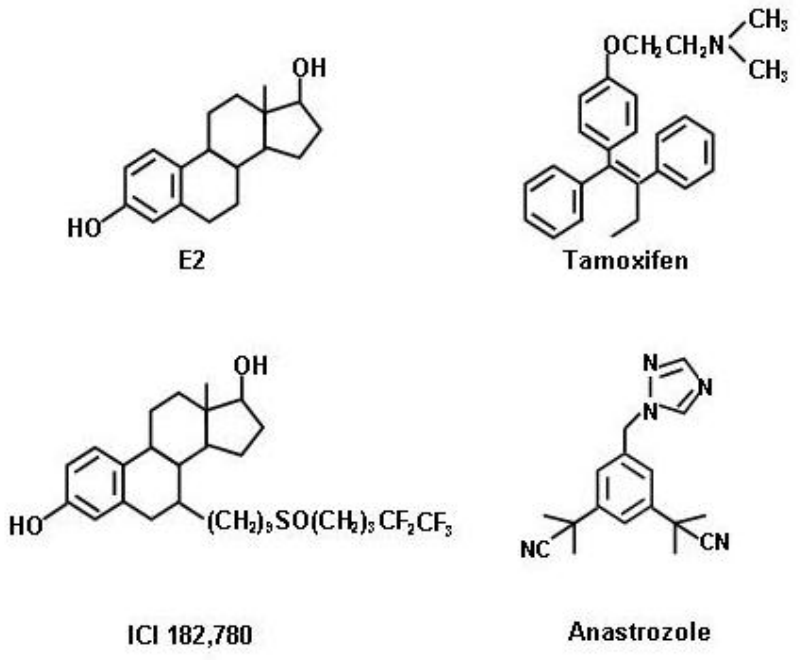

Figure 4 Structure of E2, tamoxifen (SERM), ICI 182,780 (pure antiestrogen) and anastrozole (AI).

Recently, AIs have received attention and have become a first-line treatment in adjuvant therapy for post-menopausal women with endocrine-responsive breast cancers (Goss and von Eichel, 2007). In post-menopausal women, circulating estrogens are mainly derived from the aromatization of adrenal-derived androstendione and testosterone into estrone and estradiol in peripheral tissues. CYP19 or aromatase a cytochrome P450 (CYP) enzyme is

critical for catalyzing conversion androgens to estrogens; thus, AIs can decrease levels of circulating estrogens in post-menopausal women by abolishing the synthesis of estrogens. Third generation AIs, including anastrozole, letrozole, and exemestane, cause less undesirable side-effects than unselective AIs, while providing an advantage over tamoxifen for treating post-menopausal women with ER positive breast cancer. Because AIs disrupt estrogen signaling in a way that is distinct from tamoxifen, they can be effective for treating tamoxifen-resistant breast cancers. AIs have been recommended as a second-line treatment after tamoxifen treatment fails (Coombes et al., 2004).

Selective estrogen receptor down-regulators (SERDs), also termed “pure antiestrogens”, are a novel class of endocrine therapeutic agents. SERDs are distinguished from tamoxifen as well as SERMs both in their pharmaceutical effects and their molecular activity. Because SERDs are considered “pure antiestrogens” and are devoid of ER agonist activity, they will not cause the uterotrophic effects observed for tamoxifen. The unique effects of SERDs are due to their binding to ER which differs from the binding of E2 and SERMs and their subsequent down-stream effects. Table 2 summarizes the three different modes of ER binding by 17β-estradiol (E2), tamoxifen and fulvestrant.

Table 2 Actions of estradiol, tamoxifen and fulvestrant to ER

	Affinity of binding to ER	Interaction with ER	Actions in nucleus	E2-responive genes
estradiol	high	Forms complex, activates AF-1 &AF-2	Complex binds to ERE	Activates transcription
tamoxifen	low	Forms complex, only activates AF-1	Complex binds to ERE	Partially activates transcription
fulvestant	high	Rapid degrades ER	Binding of complex to ERE is reduced	No gene transcription

Fulvestrant, also known as ICI 182780, is the only pure antiestrogen treatment proven in clinical trials to be effective against breast cancer. Since fulvestrant does not have estrogen-like effects, it has potential advantages over tamoxifen. Moreover, since the binding affinity of fulvestrant-ER is approximately 100 times higher than tamoxifen-ER, it can antagonize the uterotrophic effects caused by tamoxifen. Tamoxifen resistance develops during therapy and other SERMs exhibit different levels of cross resistance to tamoxifen, however, in vitro and in vivo studies show that tamoxifen-resistant tumors remain sensitive to fulvestrant, demonstrating that this antiestrogen has a different mode of action than tamoxifen. Thus, fulvestrant has become a second-line endocrine agent, which serves as an alternative treatment for tamoxifen-resistant breast cancers (Howell et al., 1996; Lee et al., 2000).

Progestational agents such as progestins, megestrol acetate and medroxyprogesterone are effective in endocrine therapy for breast cancer and have comparable response rates to that of tamoxifen. However, this hormone can cause some side-effects such as significant weight gain and fluid retention and this is unacceptable for many patients (Sedlacek, 1988). Therefore, progestins are usually considered as second- or third-line endocrine agents for treatment of advanced breast cancer.

1.2. Insulin-like growth factor system

1.2.1. Overview

The insulin-like growth factor (IGF) family is a network of proteins responsible for transducing and regulating IGF-induced signals. It includes two ligands, IGF-I and IGF-II, three cell membrane receptors, IGF-IR, IGF-IIR and the insulin receptor (IR) and six IGF binding proteins designated from IGFBP1 to IGFBP6 (Hwa et al., 1999). IGF-I and II are small polypeptide molecules that play important physiological roles, including their regulation of cell proliferation, differentiation, apoptosis and transformation (Jones et al., 1995). IGF receptors are located on the cell membrane and responsible for sensing extracellular IGFs and transmitting the IGF signals into cells. The IGF binding proteins (IGFBPs) bind to circulating IGFs in the bloodstream to modulate their bioavailability and half-life, thus inhibiting or enhancing the actions of IGFs. The activities of IGFBPs can be further modulated by IGFBP proteases, which hydrolyze IGFBPs

and release IGFs (Hwa et al., 1999). Together, these components of the IGF family form a tightly-regulated signal transduction system.

1.2.2. Insulin-like growth factors

1.2.2.1. Historical discovery

The discovery of IGFs can be traced back to the 1950s. The first existence of IGFs came when Salmon and Daughaday (1957) observed that serum from normal rats stimulates the uptake of sulfate into cartilage, whereas serum from hypophysectomized rats lacks this activity. Growth hormone (GH) treatment, although in itself cannot directly stimulate the uptake of sulfate, can restore the ability to induce the uptake of sulfate in serum from hypophysectomized rats. Based on this observation, it was suggested that there must be a substance which is downstream of GH that mediates promotion of growth. This substance was named “sulfation factor activity” (SFA) in their classic paper (Salmon and Daughaday, 1957). By employing a newly-developed radioimmunoassay for insulin, (Froesch et al., 1963) found that insulin-like activity in serum cannot be completely accounted for only by insulin, indicating that some other factors in serum can induce activities similar to insulin. In the 1960s, studies by Temin and others found that specific factors in serum play critical roles in cell proliferation in some cell lines. These factors may originate in the liver because cells derived from rat liver can produce their own growth-

promoting substances. Dulak and Temin named this kind of activity multiplication-stimulating activity (MSA) (Dulak and Temin, 1973).

Later, it was realized that these activities described by different research teams in fact represented a group of GH-inducible substances that mediate the action of GH. Thus, in 1972, a paper published in *Nature* introduced the term somatomedin (Daughaday et al., 1972) to refer to this group of substances.

In late 1970s, two biologically active polypeptides were identified from human plasma to induce insulin-like activities. Subsequently, these two polypeptides were named IGF-I and IGF-II (Rinderknecht and Humbel, 1976; Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). With the rapid development of molecular biology methods in the 1980s, studies confirmed that IGF-I, IGF-II or variant forms of these two polypeptides are important contributors to the proliferative activity of serum factors (Baxter, 1986; Hall and Sara, 1983). In 1987, Daughaday et al recommended that the term IGF be used to refer to this family of peptides (Daughaday et al., 1987).

1.2.2.2. Structure

Insulin-like growth factors (IGFs) are polypeptide hormones that are structurally homologous to proinsulin. Two IGFs have been identified, namely IGF-I and IGF-II, which are both single-chain polypeptides of approximate 7 Kda with 62% homology in their amino acid sequences (Yu and Rohan, 2000). IGFs

contain a C peptide bridge between A- and B- chains which is cleaved in insulin (Figure5).

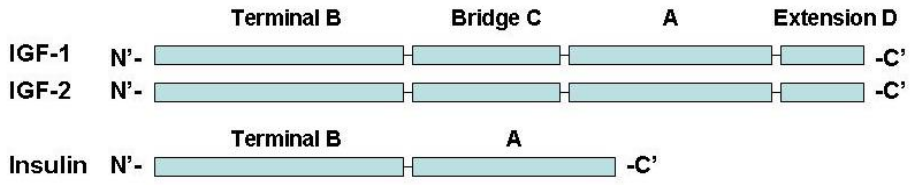

Figure 5 Structure of IGF-I, IGF-II and insulin.

1.2.2.3. Biosynthesis and regulation of expression

Both IGF-I and IGF-II are expressed during embryogenesis, with IGF-II more widely expressed in embryos (Bhaumick and Bala, 1987). Both IGF-I and IGF-II are important for the fetal development. IGF-I null mice usually suffer severe muscle dystrophy and most die at birth (Powell-Braxton et al., 1993). IGF-II null mice were less than 60% of the size of their wild-type littermates (DeChiara et al., 1990). After birth, the major organ for production of IGF-I and IGF-II is the liver; however, many cells can synthesize IGF-I locally in an autocrine or paracrine manner (Daughaday and Rotwein, 1989).

The genes encoding IGF-I and IGF-II are located on chromosome 12 and 11, respectively. Although IGFs are simple peptides, their gene structures and

patterns of expression are complicated. For example, the IGF-I gene is composed of six exons and five introns and utilizes two promoter sites (Rotwein, 1991). Expression of IGF-I is mainly regulated by growth hormone (GH) and circulating levels of IGF-I generally correlate with GH secretion. Moreover, local tissue levels of IGF-I also can be upregulated upon GH secretion by autocrine or paracrine actions (Jones and Clemmons, 1995). However, in some tissues, such as the uterus, expression of IGF-I is independent of GH; instead, it is regulated by 17β-estradiol (E2). Some other hormones, including adrenocorticotropic hormone, thyrotropin, luteinizing hormone, follicle-stimulating hormones (FSH), as well as other growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), also have influence on IGF-I production (Yu and Rohan, 2000).

Despite its major impact on IGF-I expression, GH has no regulatory effects on IGF-II expression. The main factors regulating expression of IGF-II remain unknown. IGF-II is believed to play a less important role in postnatal growth than does IGF-I, and circulating levels of IGF-II are relatively stable after puberty and independent of GH secretion (Stewart and Rotwein, 1996).

1.2.3. IGF-binding proteins (IGFBPs)

IGF-binding proteins (IGFBPs) are a family of homologous proteins that play modulating roles in the IGF system. IGFBPs can regulate the interaction

between IGFs and IGF-Rs by binding these ligands, and thereby inhibiting the mitogenic effects of IGFs. There are six proteins (IGFBP1-6) with high affinity for IGFs and several IGFBP-related proteins with lower affinity in the IGFBP family. IGFBPs, therefore, regulate the bioavailability of IGFs by acting as a circulating reservoir of bound IGFs.

Among the IGFBPs, IGFBP-3 carries >90% of the circulating IGF in adult serum by forming a ternary complex with IGF and a bound “acid-labile subunit” (ALS) (Baxter, 1994). The half life of IGF in the ternary complex can be as long as 12 hours in the circulating system, whereas the half life of unbound IGF is less than 10 minutes in circulation (Guler et al., 1989).

Although it is well established that the concentrations of IGFBP-3 are inversely associated with the risk of developing of prostate cancer (Chan et al., 1998), the evidence for the association between IGFBP-3 and breast cancer risk has been inconsistent. Three epidemiology studies suggest that IGFBP-3 concentration is positively associated with pre-menopausal but not post-menopausal breast cancer (Fletcher et al., 2005). However, a European study (Rinaldi et al., 2006) suggested that there is an association between IGFBP-3 expression and breast cancer risk in women after the age of 50 but not in younger women. The reason for these discrepancies is unknown, however, one possible explanation is that the study by Rinaldi et al (2006) used age 50 as a

divider to group the samples, instead of menopausal status which was used in other studies. This issue is still controversial and being further investigated.

IGFBPs can be secreted by many cell types, and the expression of IGFBPs is regulated by steroid hormones, growth factors and cytokines in a cell- and tissue-dependent manner. There are also some post-transcriptional modifications that are involved in regulation of IGFBP functions, such as phosphorylation, glycosylation and proteolytic cleavage (Clemmons, 1997). For example, a circulating protease can specifically hydrolyze IGFBP-3 and limited cleavage of IGFBP-3, lowers its affinity for binding IGFs (Maile and Holly, 1999).

1.2.4. IGF-IR signaling

1.2.4.1. IGF receptors (IGF-R)

Two main types of IGF receptors have been identified, namely IGF type I receptor (IGF-IR) and IGF type II receptor (IGF-IIR). IGF receptors are receptor tyrosine kinases that serve as the intermediary between the circulating IGFs outside of cells and signaling systems within cells. IGF-IR is structurally similar to the insulin receptor (IR), and consists of 2α and 2β subunits of 125-135 and 90-95 kDa, respectively. Several disulfide bonds bundle 2α and 2β subunits together to form a α2β2-heterotetrameric receptor (Figure 6). The α-subunit is entirely extracellular and forms a ligand-binding domain, whereas the β-subunit is composed of a short extracellular domain, a transmembrane domain and an

intracellular domain. The β-subunit has tyrosine kinase activity and is responsible for autophosphorlation upon ligand binding. Unlike IGF-IR, IGF-IIR is a single-chain polypeptide of molecular weight approximately 250 KDa, which consists of a large extracellular domain, a single transmembrane region and a small cytoplasmic domain.

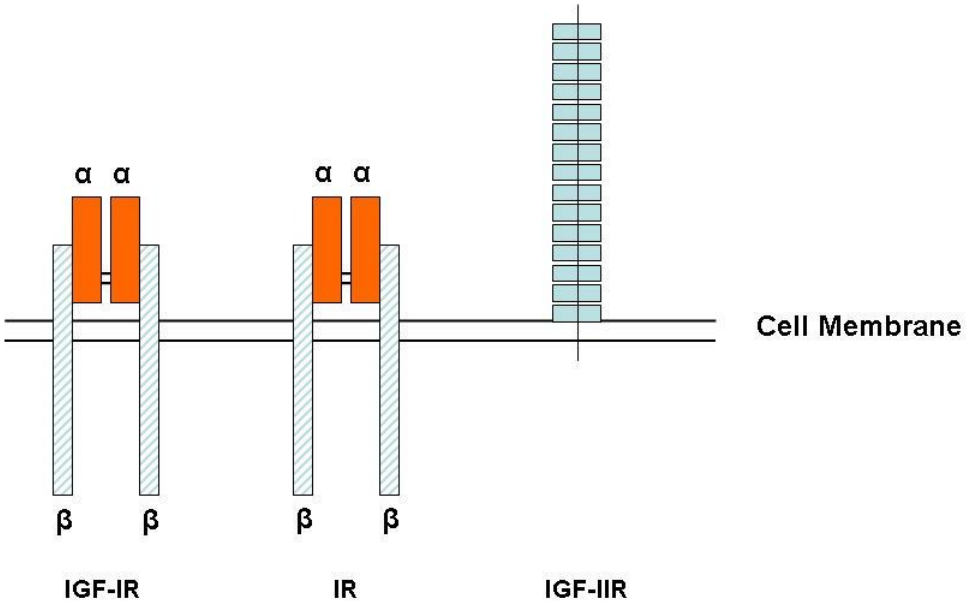

Figure 6 Structure of IGF-I receptor, IGF-II receptor and insulin receptor.

Whereas IGF type II receptor (IGF-IIR) exclusively binds to IGF-II, IGF type I receptor (IGF-IR) binds both IGF-I and IGF-II, although IGF-I binding is stronger than IGF-II binding. Upon IGF binding, IGF-IR dimerizes and undergoes

autophosphorylation of multiple tyrosine residues, which is followed by activation of several intracellular targets, such as insulin receptor substrate (IRS) proteins and Shc (Kim et al., 2001; Sasaoka et al., 2001). Phosphorylated IRS and Shc initiate various kinase cascades, including MAPK and PI3K (Jackson et al., 1998) (Figure 7).

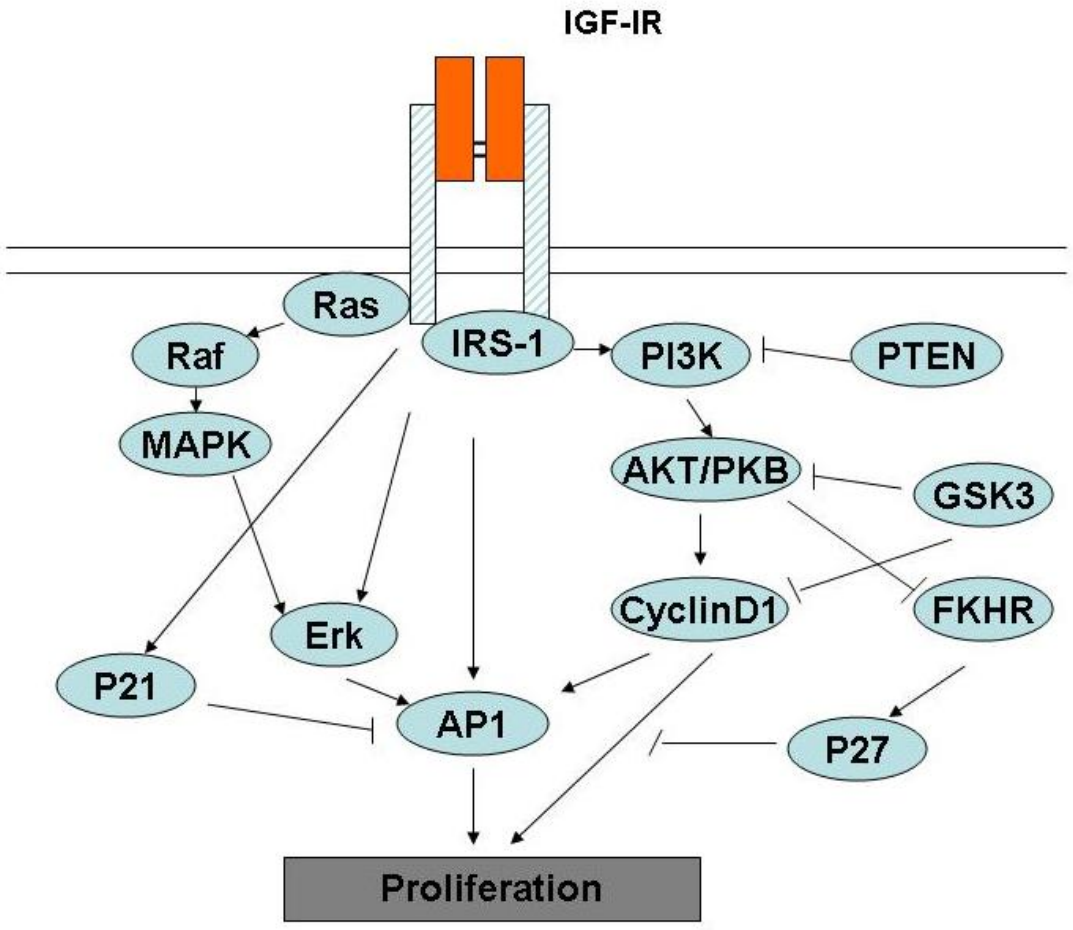

Figure 7 IGF-IR signaling for proliferation, adapted from Gray et al (2003).

The IGF-I/IGF-IR pathway is important for mammary carcinogenesis. In clinical studies, elevated IGF-1 levels have been observed in premenopausal breast cancer patients (Sugumar et al., 2004; Toniolo et al., 2000). Caloric restriction, a regimen that greatly decreases cancer risk in animal models, has been proposed to exert its cancer-preventive effects by decreasing circulating IGF-1 levels (Dunn et al., 1997). The carcinogenic effects of IGF-1 can be attributed to its proliferative and anti-apoptotic activities. Overexpression of IGF-1R in ER-positive MCF-7 breast cancer cells enhances cell proliferation and survival (Guvakova and Surmacz, 1997). Inhibition of the IGF-I/IGF-IR pathway resulted in reduced breast cancer cell proliferation and metastasis (Dunn et al., 1998; Neuenschwander et al., 1995).

In addition, subunits of the IGF-IR and IR can form a hybrid heterodimeric receptor (Hybrid-R), which could play a role in receptor signaling in normal or abnormal tissues. A study (Pandini et al., 1999) examined 8 human breast cancer cell lines and 39 human breast cancer specimens, and found that Hybrid-R content exceeded the pure IGF-IR content in >75% of breast cancer cells and specimens. Furthermore, Hybrid-R undergoes autophosphorylation in response to IGF-1 but not to insulin, suggesting an essential role for Hybrid-R in mediating IGF-1 signaling.

1.2.4.2. Phosphoinositol-3 kinase/AKT

IGF-IR-dependent cell survival is primarily mediated through activation of phosphoinositol-3 kinase (PI3K). PI3Ks are a family of lipid kinases that include three distinct classes: Class I, II and III. Class I PI3Ks are the most well characterized class and can be further divided into two subclasses, Class IA and IB. Both subclasses are heterodimers consisting of a 110 kDa catalytic subunit and an adaptor/regulatory subunit. Class IA PI3Ks are activated by receptor tyrosine kinases and catalyze the phosphorylation of phosphatidylinositol-4,5-diphosphosphate (PIP[4,5]2) to PI[3,4,5]P3 (Czupalla et al., 2003). IGF1 is an important activator of PI3K. With the activation of IGF-IR, the Src-homology 2 (SH2) domain of regulatory subunit of PI3K binds to IGF-IR by docking to the associated IRS-1, which in turn activates the p110 catalytic subunit, leading to increased PIP3 production.

PIP3 is located on the cytosolic side of the cell membrane and recruits AKT (also called PKB, protein kinase B) and phosphoinositide-dependent kinase 1 (PDK1) from the cytosol to the plasma membrane by interacting with the Pleckstrin homology (PH) domain of AKT and PDK1. PDK1 is responsible for phosphorylating one of two conserved residues of AKT, Thr508, and once the other residue, Ser175 is phosphorylated by another enzyme PDK2, AKT becomes fully activated and moves to the cytoplasm and nucleus, and in turn activates downstream targets (Figure 8).

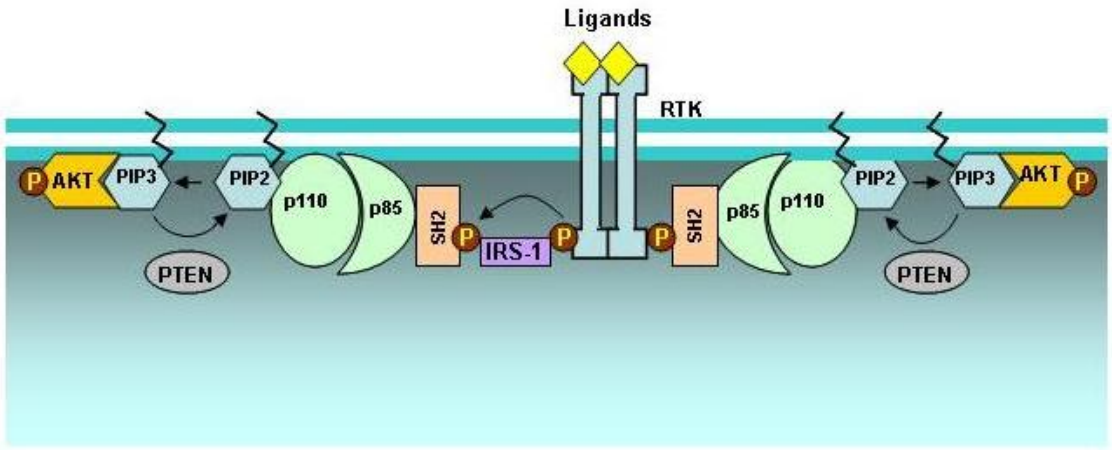

Figure 8 Model of PI3K activation, adapted from Vivanco and Sawyers (2002).

The AKT family are the major downstream mediators of PI3K. AKT is ubiquitously expressed in all cell types and tissues, including normal and cancer cells. Increasing evidence shows that AKT regulates both growth and survival mechanisms. For example, AKT not only increases the translation and stabilization of cyclin D1, but also phosphorylates and prevents the cell cycle inhibitors p21 and p27 from entering nucleus and inhibiting the cell cycle (Diehl et al., 1998). Some studies demonstrated that many AKT signaling mediators or AKT activity regulators, such as MDM2 and PTEN, are frequently mutated in cancer cells (Li et al., 1997; Zhou et al., 2001). Furthermore, the PI3K gene itself is mutated with high frequency in human breast, colon, brain and lung cancers, suggesting that PI3K plays a crucial role in tumorigenesis (Bachman et al., 2004;

Samuels and Velculescu, 2004). Additionally, some research groups reported that AKT increased cell mobility and migration in some cell lines thus demonstrating a role for AKT in cancer cell migration and invasion (Kim et al., 2001; Park et al., 2001).

There are three AKT isoforms, AKT1/PKBα, AKT2/PKBβ and ATK3/PKBγ. The functions of three Akt isoforms are not identical for regulation of cell migration and invasion (Yoeli-Lerner et al., 2005). They are all activated through PI3K pathway; however, shRNA (short hairpin RNA) knockdown studies indicate that downregulation of AKT1 enhanced cell migration induced by IGF-I and EGF, while downregulation of AKT2 reversed the mobility enhancement (Irie et al., 2005).

1.2.4.3. Mitogen activated protein kinase (MAPK)

Mitogen activated protein kinases (MAPKs) are important enzymes in signal transduction and are sensitive to diverse extracellular stimuli, such as mitogens, growth factors and stress signals. MAPK pathways are evolutionarily conserved and responsible for regulation of some essential cellular processes such as proliferation, differentiation and apoptosis. The MAPK pathway consists of three kinases modules, which are sequentially activated upon phosphorylation in sequence. The MAPK kinase kinase (MAPKKK/MEKK) phosphorylates serine/threonine residues on MAPK kinase (MAPKK/MEK). The latter in turn

activates MAPK through phosphorylation (Chang and Karin, 2001) (Figure 21). The full activity of MAPK requires both threonine and tyrosine phosphorylation in a dual-phosphorylation motif Thr-X-Tyr (Kyriakis and Avruch, 2001).

MAPKs include extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), p38 kinase (CSBP) and big MAP kinase-1(ERK5/BMK-1). Among these MAPK pathways, the ERK pathway has been the most thoroughly studied. In this pathway, ligand-mediated activation of receptor tyrosine kinases triggers the accumulation of GTP, leading to activation of Ras GTPase, which in turn recruits and phosphorylates a MAPKKK named Raf, Upon phosphorylation, Raf gains kinase-activity and phosphorylates its corresponding MAPKK, namely MEK1/2. Like Raf, MEK1/2 is activated by phosphorylation and proceeds to phosphorylate its own downstream MAPK target ERK1/2, Through these sequential phosphorylation and activation steps, an extracellular stimulus results in increased MAPK activity (Otsu et al., 1993).

Activation of ERK signaling elicits a wide array of downstream effectors, including kinases, phosphatases, transcription factors, apoptosis-related proteins and cytoskeletal and scaffold proteins (Yoon and Seger, 2006). One of the best studied nuclear substrates of ERK is Elk1, which belongs to the ternary complex factors (TCFs) subfamily of ETS-domain transcription factors that are phosphorylated by ERK via direct interaction (Sharrocks et al., 2000). Stimulation with growth factors or other ligands can induce rapid phosphorylation

at multiple sites on Elk1, and this facilitates recruitment of coactivators such as Srb and p300, resulting in increased Elk-dependent transcriptional activity.

Other nucleus transcriptional factors, such as Fos, Jun, Myc and Egr-1 as well as some cytoplasmic substrates, such as RSK and Paxillin, can also be activated by ERK signaling. The mobilization of this wide variety of proteins underscores the essential roles of ERK signaling in multiple cellular processes (Figure 9).

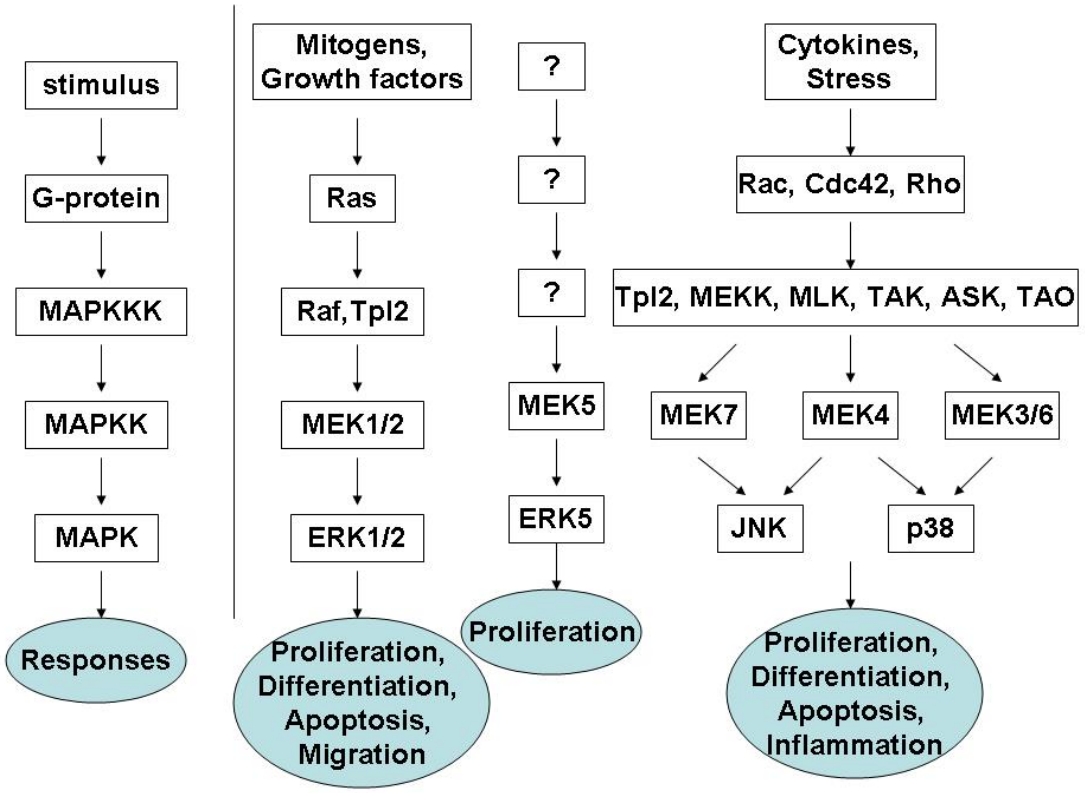

Figure 9 MAPKs, MAPKKs and MAPKKKs, adapted from Dhillon et al (2007).

1.2.4.4. The crosstalk between IGF-I and Estrogen Receptor α (ERα)

The mitogenic activity of IGF-I and interactions between growth factors and estrogen receptor (ER) signaling has been extensively investigated in breast cancer cells, and estradiol (E2) and IGF-I induce many of the same responses in these cell lines (Dickson & Lippman 1995, Smith 1998, Weigel & Zhang 1998, Kato *et al.* 2000, Yee & Lee 2000, Luconi *et al.* 2002, Segars & Driggers 2002) (Figure 10). For example, both IGF-I and E_2 induce the proliferation of ER-positive breast cancer cells and many E2-responsive genes, including pS2, cathepsin D, cyclin D1, progesterone receptor and IGF-IR, are also induced IGF-I (Cavailles *et al.* 1989, Wakeling *et al.* 1989, Katzenellenbogen & Norman 1990, Chalbos *et al.* 1993, Ignar-Trowbridge *et al.* 1993, Westley & May 1994, Martin *et al.* 2000, Oesterreich *et al.* 2001, Varma & Conrad 2002). The estrogen-like activity of IGF-I, transforming growth factor α (TGF α) and epidermal growth factor (EGF) are due, in part, to growth factor-induced phosphorylation of ER, which can become activated independent of ligands and activates ER-dependent genes in the absence of E2 (Ali *et al.* 1993, Kato *et al.* 1995, 2000, Bunone *et al.* 1996, Ignar-Trowbridge *et al.* 1996, Joel *et al.* 1998, Smith 1998, Weigel & Zhang 1998, Luconi *et al.* 2002). Conversely, E2 also exhibits growth factor-like activity and elicit rapid kinase activation through non-genomic mechanisms (Watson *et al.* 1998, 2002, Levin 2002). At least part of the nongenomic activities of E2 are dependent on extranuclear ER and its association with several proteins involved in kinase signaling pathways. For

example, ERα directly interacts with the SH2 region of Src, the p85α regulatory subunit of phosphoinositide 3-kinase (PI3K), caveolins, GαI (a G protein), Shc and IGF-IR (Kahlert *et al.* 2000, Migliaccio *et al.* 2000, Simoncini *et al.* 2000, Sun *et al.* 2001, Wyckoff *et al.* 2001, Razandi *et al.* 2002, Song *et al.* 2002). The functional significance of these interactions may be cell-context-dependent. For example, antibodies that can block IGF-IR signaling by IGF-I did not inhibit the activation of the MAPK pathway by E2 in MCF-7 cells (Duan *et al.* 2001).

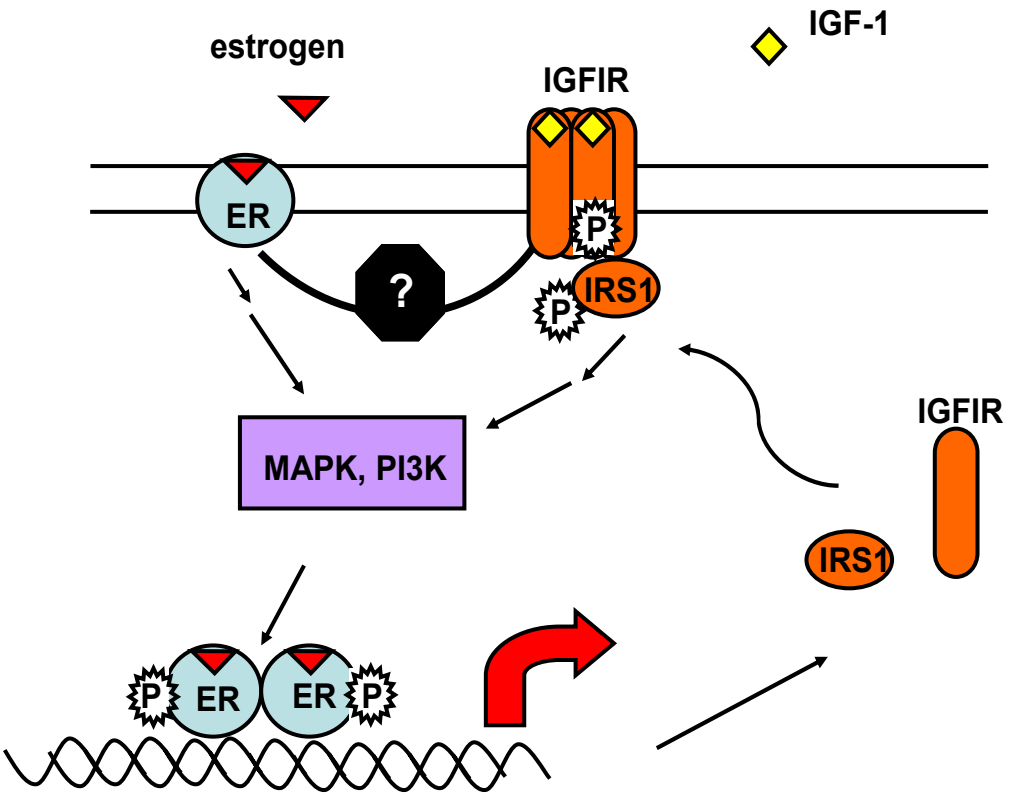

Figure 10 Crosstalk between IGF-I and ERα.

The close relationship between IGF-I and ER pathways reaches a new level in ER-positive breast cancer cells. In these cells, the IGF-I and ER nongenomic pathways are so intertwined that the existence of ERα is essential for IGF-I-dependent response. Transfection of small interfering RNA for ERα (iERα) into ER-positive MCF-7 breast cancer cells not only resulted in decreased ERα levels, and but also led to inhibition of IGF-1-dependent MAPK and PI3K activities. In addition, loss of ERα also resulted in decreased IGF-I-induced G_1–S-phase progression and expression of proteins associated with cell-cycle progression such as cyclin D1 and cyclin E (Zhang et al., 2005). The exact nature of this reliance on ER for IGF-1 signaling has not been identified.

1.2.5. IGF-I and cancer

IGF-I induces cell proliferation by regulating the cell cycle machinery. On one hand, IGF-I signaling targets multiple cell cycle components involved in proliferation in different types of cells. For example, IGF-I can affect the activity of cyclin A, cyclin E/CDK2 and pRb via the PI3K pathway and facilitate the transition from G1 to S phase (Chakravarthy et al., 2000; Takahashi et al., 1997). On the other hand, IGF-I can upregulate expression of important cell cycle proteins, such as cyclin D1 and CDK4 (Dufourny et al., 1997; Rosenthal and Cheng, 1995) (Figure 11).

Tumor suppressor p53 functions as the guardian for genome integrity by halting cell cycle progression and inducing apoptosis in response to DNA damage. IGF-1R expression is down-regulated by p53 transcriptionally, indicating that IGF-1 signaling can be antagonized by p53 (Werner et al., 1996). Conversely, IGF-I induced the expression of MDM2, an E3 ubiquitin ligase and a well-known repressor of p53 (Leri et al., 1999).

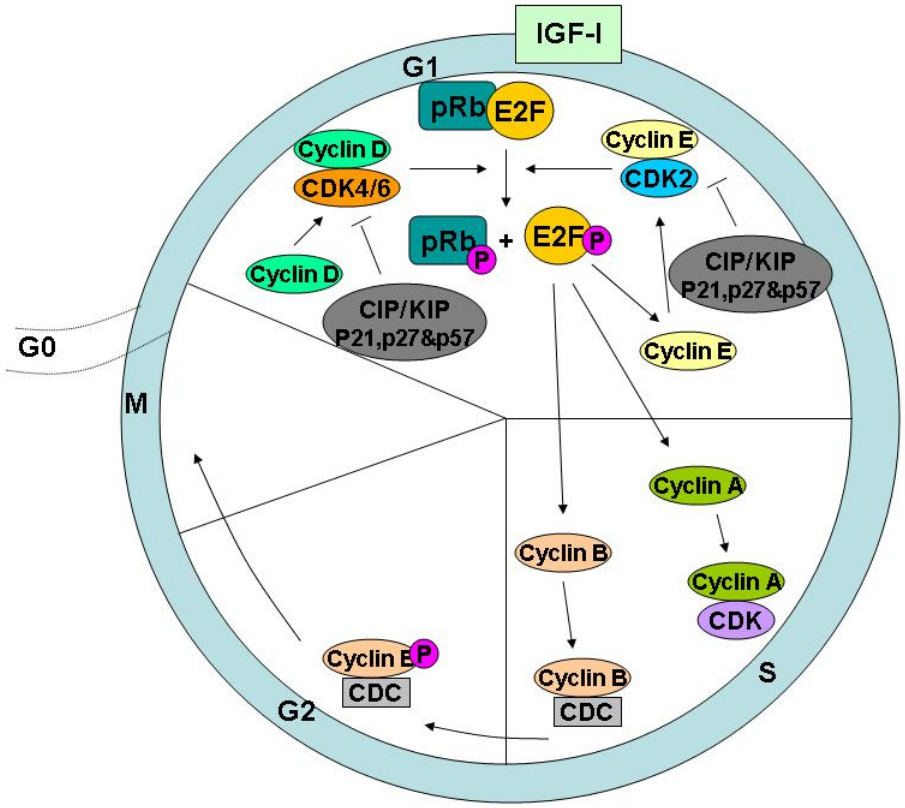

Figure 11 IGF-I promotes cell progression, adapted from (Dupont and Holzenberger, 2003).

Due to its active roles in cell cycle regulation, insulin-like growth factor I (IGF-I) has been well-characterized as a potent Mitogen. Multiple epidemiological studies have found that elevated circulating IGF-I levels are associated with increased risk of certain malignancies, such as prostate, colorectal and premenopausal breast cancers (Chan et al., 1998; Hankinson et al., 1998; Postma et al., 1999). While circulating IGF levels may provide valuable information regarding cancer risk, caution must be exercised in interpreting the data. First, circulating IGF only represents the systemic IGF-I activities and may not accurately reflect IGF-1 activities in local tissues. Locally-produced IGFs can act in an autocrine or paracrine fashion and this may be important for carcinogenesis in localized microenvironments. Second, IGF-dependent signaling is subject to modulation by numerous factors, especially the types and serum levels of IGF-binding proteins (Grimberg & Cohen 2000).

IGF-I/IGF-IR pathways are crucial for mammary carcinogenesis. Primary breast cancers usually exhibit elevated levels of IGF-IR compared to normal tissues (Resnik et al., 1998). Genetically modified mice with low IGF levels are more resistant to the effects of the mammary carcinogen, 7, 12-dimethybenz (a)anthracene or SV40 large T-antigen (Wu et al., 2003). However, there is a tendency for the loss of IGF-IR during the course of cancer development (Schnarr et al., 2000). Overexpression of IGF-1R in ER-positive MCF-7 breast cancer cells enhanced cell proliferation and survival (Guvakova and Surmacz, 1997). Inhibition of the IGF-I/IGF-IR pathway result in reduced breast cancer cell

proliferation and metastasis in several in vitro and in vivo model systems (Dunn et al., 1998; Neuenschwander et al., 1995). The IGF-I pathway is also a potential target for novel breast cancer therapies, and some encouraging results have been obtained with anti-IGF-IR therapies in pre-clinical studies, which have resulted in the first Phase I human clinical trial (Cohen et al., 2005; Hopfner et al., 2006a; Hopfner et al., 2006b; Hopfner et al., 2006c; Menu et al., 2006). As the importance of the other components in the IGF axis is discovered, other targeted therapies will also be developed.

1.2.6. IGF-I and aging

The IGF-I signaling pathway has important regulatory roles in the aging processes. Aging had once been thought to be an inevitable body breakdown, which is dependent on time, and beyond human intervention. However, in recent decades, it has become clear that the aging process is regulated by genes that can be altered by genetic manipulation. Although the underlying molecular physiology of aging has yet to be determined, it appears that elevation in calorie intake, the load of reactive oxygen species (ROS), and body weight accelerate the aging process. These three issues are not independent of one another, because high calorie intake is associated with heavy ROS load and high body weight. IGF-I/IGF-IR also appears to have a significant role in regulating lifespan. Decreased IGF-I levels, achieved by caloric restriction or genetic manipulation, has been associated with increased life span in animal models (Anisimov et al.,

2005). The deleterious effects of depressed IGF levels appeared to be minor. A 75% decrease in circulating IGF-I levels in transgenic mice was achieved by specifically disrupting the *IGF-I* gene in liver, however, this had no apparent impact on growth and development (Wu et al., 2003). Complete elimination of IGF-IR carries significant deficiencies, and IGF-IR knockout mice are only half the normal size at birth, they suffer generalized organ hypoplasia, including the muscles, and developmental delays in ossification and die at birth due to respiratory failure (Liu et al., 1993). However, heterozygous IGF-IR -/+ mice with less than 50% of normal IGF-IR activities are healthy with normal energy metabolism and physical activity. When compared to their wild-type littermates, these mice have a significant 26% increased lifespan (Holzenberger et al., 2003). Similar result can be obtained by disrupting the corresponding genes in Drosophila. Mutations in single genes encoding components in insulin/IGF-I signaling pathway also results in an increase in lifespan (Clancy et al., 2001; Tatar et al., 2001), suggesting a potential role for IGF-IR in regulating human longevity and alterations of IGF-I signaling may be a possible strategy for lifespan extension.

1.3. Aryl hydrocarbon receptor (AhR)

1.3.1. AhR structure and functions

Our initial understanding of the function of Ah receptor (aryl hydrocarbon receptor, AhR) began with research on the toxicities of polycyclic aromatic

hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs). In modern society, it is virtually inevitable to be exposed to PAHs/HAHs from the air, soil and food. The detrimental health effects of PAHs/HAHs had been well documented in the 1960s-1970s; however, the underlying molecular mechanism of these compounds remained elusive. The first clue came in the late 1960s, when it was noticed that there was a significant variability in the induction of AHH (aryl hydrocarbon hydroxylase) activity (now identified as CYP1-dependent activity) by aromatic hydrocarbons among different mouse strains (Nebert et al., 1969). Further studies showed that the variability could be attributed to a single locus, which was later identified to encode a receptor protein that binds aromatic hydrocarbons (Nebert et al., 1975; Thomas and Hutton, 1973). Soon the existence of what is now called AhR was confirmed and this receptor protein exhibited stereospecific, high-affinity and saturable binding to radiolabeled TCDD (Poland et al., 1976). Later the AhR was identified as an intracellular receptor for a plethora of structurally diverse compounds, produced by either chemical or biological processes (Denison et al., 2002).

Like many other receptor proteins, the AhR has a modular structure that consists of relatively independent domains. The N-terminal domain of the AhR contains a bHLH (basic-helix-loop-helix) motif, the DNA binding domain and a nuclear localization signal. The C-terminal of the bHLH has a region of approximately 200 amino acids, which is a highly conserved domain that exists in many proteins involved in environmental sensory and developmental

processes. This domain was named PAS (Per-Arnt-Sim), after three proteins containing the domain namely, the Drosophila periodicity (Per), AhR nuclear translocator (Arnt) and Drosophila singleminded (Sim) (Gu et al., 2000). The C-terminal of the AhR contains a Q-rich region that is important for its transactivation function (Burbach et al., 1992) (Figure 12).

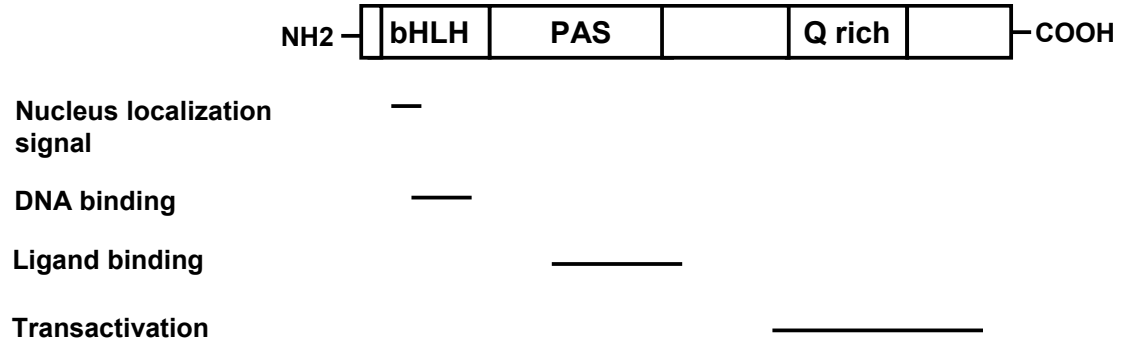

Figure 12 Structure of the AhR and various functional domains.

The molecular mechanism of AhR activation has been the subject of intensive studies. The unliganded AhR resides in the cytoplasm as a complex with heat shock protein 90 (hsp90), which help stabilize AhR and retain it in cytoplasm (Perdew, 1988; Pongratz et al., 1992). Both the bHLH and PAS domains of AhR are involved in hsp90 binding, and this association keeps the receptor in the cytoplasm by masking the NLS (nuclear localization sequence) of AhR (Ikuta et al., 1998), Two other proteins, AIP (also know as XAP2 or Ara9) and p23, are also parts of the AhR cytosolic complex (Kazlauskas et al., 1999;

Ma and Whitlock, 1997; Meyer et al., 1998). Most AhR ligands are highly hydrophobic molecules and can easily enter cells through passive diffusion. Upon ligand binding, the AhR dissociates from hsp90s and other associated proteins, translocates to the nucleus and forms a heterodimers with its dimerization partner, another bHLH/PAS protein named Arnt (Ah receptor nuclear translocator protein) (Elferink et al., 1990; Reyes et al., 1992). The AhR-Arnt heterodimer recognizes and binds a consensus DNA motif, the DRE (Dioxin Response Element), also known as XRE (Xenobiotic Response Element). The DNA-bound AhR-Arnt recruits coactivators, DNA polymerase II and other nuclear accessory factors, leading to transcriptional activation of a diverse group of target genes (Fig 13). One of these target genes is a bHLH/PAS protein named aryl hydrocarbon receptor repressor (AhRR), which shares significant homology with AhR/Arnt at the N-terminal region and functions as a repressor of the AhR pathway (Baba et al., 2001). AhRR upregulation may be a negative-feedback mechanism to keep activation of the AhR at the appropriate level to maintain homeostasis.

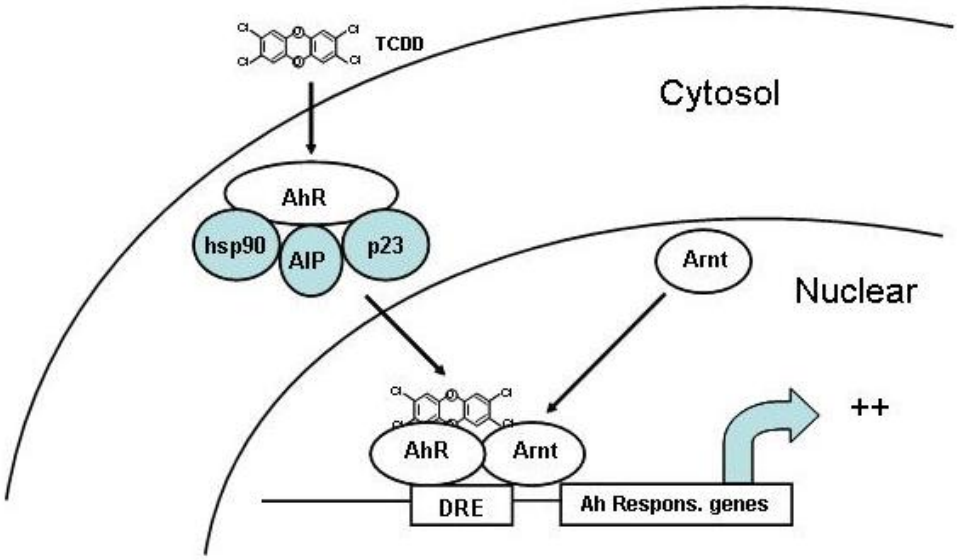

Figure 13 Mechanism of AhR action involved in activation of several Ah-responsive genes including CYP1A1, CYP1A2, CYP1B1, and HGT.

1.3.2. The physiological functions of the AhR

Among the genes upregulated by the AhR-Arnt complex are several drug-metabolizing enzymes, including CYP1A1, CYP1A2, glutathione S-transferase Ya subunit, NAD(P)H:quinone reductase 1 and 2, and class 3 aldehyde dehydrogenase (Schmidt and Bradfield, 1996). Not coincidently, many of these enzymes are responsible for the metabolism of AhR ligands. These observations support the notion that AhR pathway is an important part of a cellular defense mechanism against the toxic compounds by inducing metabolizing enzymes responsible for their subsequent detoxification (Schrenk et al., 1999).

Although the AhR is undoubtedly important for inducing drug metabolizing enzymes, it would be an over-simplification to view xenobiotic metabolism as the sole function of the AhR. The list of AhR-regulated genes includes a diverse spectrum of genes involved in various physiological functions. The AhR has been implicated in regulation of neuronal differentiation (Akahoshi et al., 2006), liver development (Harstad et al., 2006; Walisser et al., 2004), cardiac function (Lund et al., 2006) and the immune system (Rodriguez-Sosa et al., 2005).

The diverse functions of the AhR can be illustrated by the broad spectrum of toxicities, induced by TCDD and these include tumor promotion, teratogenesis, epithelial hyperplasia, immunosuppression, and a severe wasting syndrome (Poland and Knutson, 1982; Schmidt and Bradfield, 1996). Many of the symptoms require several days or even weeks to manifest themselves (Poland and Knutson, 1982). Most, if not all, of these toxicities are AhR-mediated, because AhR knockout mice are unresponsive to the toxic effects of TCDD (Fernandez-Salguero et al., 1995; Schmidt et al., 1996).

The Ah receptor regulates a broad spectrum of responses and the role of AhR in xenobiotic metabolism represent only a small part of its activities. The AhR may also be an important regulator of cell proliferation under some circumstances. The Hepa1c1c7 mouse liver cancer cell line with a non-functional mutant AhR exhibits a slower rate of proliferation and transfection of the AhR into these cells restores the normal growth rate (Ma and Whitlock,

1996). The G1/S phase progression of HepG2 liver cancer cells can be inhibited by downregulation of AhR through transfection of AhR siRNA (Abdelrahim et al., 2003). However, in contrast to HepG2 cells, in MCF-7 breast cancer cells, repression of AhR leads to accelerated cell proliferation (Abdelrahim et al., 2003). These results suggesting the roles of AhR in cell proliferation are complex and cell-context dependent.

The AhR has also been implicated in cellular mobility. In rat liver epithelial cells, AhR activation relieves intercellular contact inhibition and accelerates cell proliferation (Andrysik et al., 2007). In zebrafish where regeneration of tail fins after a partial amputation TCDD treatment causes disorganization of extracellular matrix (ECM) in newly generated tissues indicating a role for the AhR in extracellular matrix modeling (Andreasen et al., 2007). These findings provide further evidence of the roles for the AhR in development and suggest that dysregulation of AhR signaling may also occur during cancer metastasis.

By inducing CYP enzymes, AhR activation can greatly boost oxidative stress, a major trigger for apoptosis (Nebert et al., 2000). Some precarcinogens can be metabolized into DNA-damaging carcinogens by induced CYP enzymes. MCF-7 cancer cells with diminished CYP1A1 expression are more resistant to apoptosis induced by the AhR ligands, implicating a role for cytochrome P450s in mediating apoptosis in response to AhR agonists (Ciolino et al., 2002). AhR knockout female mice are resistant to the PAH-induced apoptotic effects in

oocytes within immature follicles (Robles et al., 2000). TCDD induces apoptosis of thymic T cells in wildtype mice but not in AhR knockout mice. This activity is mediated through AhR by stimulated production of FasL in thymic stromal cells (Camacho et al., 2005). AhR agonists also cause apoptosis in midbrain of zebrafish embryo through a mechanism that involves AhR activation, CYP1A1 induction, and oxidative stress (Dong et al., 2002). However, it must be noted that the roles of the AhR in apoptosis are cell context dependent. Under certain circumstances, AhR activity can lead to inhibition of apoptosis. For example, in AhR knockout mice, there is an increased rate of apoptosis in hepatocytes (Gonzalez and Fernandez-Salguero, 1998).

Another function of the AhR may be its role in regulating circadian rhythm. One prominent structural feature of the AhR is the two PAS domains, which are shared by many other genes involved in circadian rhythm, such as per-1/2 (period-1/2) (Shearman et al., 1997) and bmal1(brain and muscle ARNT-like) (Yu et al., 1999). In female Sprague-Dawley rats, both AhR and Arnt exhibit a circadian pattern of expression in liver and lungs (Richardson et al., 1998). The cycling of AhR activities may be an important adaptive mechanisms for dealing with the different rates of food intake during different times of the day, and activation by putative endogenous AhR ligands, such as 6-formylindolo[3,2-b]carbazole (FICZ), a the light-induced photoproduct of tryptophan (Mukai and Tischkau, 2007; Wei et al., 1998).

AhR homologs have been found in species across vertebrate classes, including mammals, birds, amphibians and fish. They share strong conservation in sequence and functions, suggesting that AhR pathway had already been well-established when vertebrates originated (Hahn, 2002). AhR homologs have also been identified in phylogenetically diverse invertebrates, from nematode (*Caenorhabditis elegans*) (Huang et al., 2004) to soft shell clam (*Mya arenaria*) (Butler et al., 2001) and fruitfly (*Drosophila melanogaster*) (Emmons et al., 1999), suggesting that AhR signaling is an ancient pathway and plays a role in some fundamental cellular functions. Interestingly, the invertebrate AhR homologs lack TCDD-binding capacity and are incapable of inducing xenobiotic metabolizing enzymes (Butler et al., 2001). Instead, these AhR homologs play an important role in regulating embryonic development. For example, the ahr-1, the C. elegans AhR homolog, is crucial for the normal differentiation of touch receptor neuron AVM and the interneuron SDQR (Qin and Powell-Coffman, 2004). The spineless, the AhR homolog in Drosophila, is important in defining the distal antennal and tarsal identity (Duncan et al., 1998). Based on these findings, the ancient AhR probably existed long before the rise of vertebrates, and it functioned primarily as a developmental regulator. The role of the AhR as a master regulator of induction of drug metabolizing enzymes is most likely a function acquired later by cells to adapt to an increasingly complex chemical environment.

1.3.3. AhR ligands

The AhR is extremely promiscuous and binds to a wide variety of structurally diverse compounds (Figure 14). This lack of specificity is probably important for the AhR to respond to challenges from a bewildering number of endogenous and exogenous chemicals. In general, AhR ligands are loosely divided into two groups: the industrial compounds and naturally occurring compounds.

The industrial compounds group includes some of the most potent and persistent AhR ligands. They are planar, hydrophobic molecules that represent the best characterized group of AhR ligands and the most important groups in this category are polycyclic aromatic hydrocarbons (PAHs) and Halogenated aromatic hydrocarbons (HAHs). PAHs exist naturally in crude oil and petroleum products and include compounds such as benzo(a)pyrene, 3-methylcholanthrene and benzoflavone. In modern society, the predominant sources of PAHs are the incomplete combustion of organic substances, including wood, coal, petroleum products and tobacco. While occupational exposure can occur at high levels in some industries, dietary intake is the major route of exposure to PAHs for the general public (Unwin et al., 2006).

HAHs are a group of compounds that include polychlorinated dibenzo-p-dioxin (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated

biphenyls (PCBs). They exist ubiquitously in our environment and represent a significant portion of industrial pollutants. Tremendous public concerns have been raised about their potential detrimental effects on both the ecosystem and human health. In general, HAHs are several order of magnitude more potent than PAHs in activating AhR signaling (Bohonowych and Denison, 2007). HAHs are remarkably stable and can persist in biological tissues for decades. Due to this combination of high potency and long half-life, HAHs can exert their toxicities long after initial exposures. In contrast, other AhR agonists, including PAHs, are much more readily metabolized and generally only induce transient TCDD-like responses (Denison and Nagy, 2003). This by no means indicates that PAHs are less harmlful than HAHs. Many PAHs, such as BaP, are well recognized procarcinognens, which can be metabolically converted to carcinogens and increase cancer risk in some tissues (Li et al., 1996). In fact, induced mammary carcinogenesis by a prototypic PAH 7,12-dimethylbenz(a)anthracene (DMBA) in female Sprague-Dawley rats has long served as a model for studies on breast cancer (Isogai et al., 1998; Klurfeld et al., 1989; Moore et al., 1988).

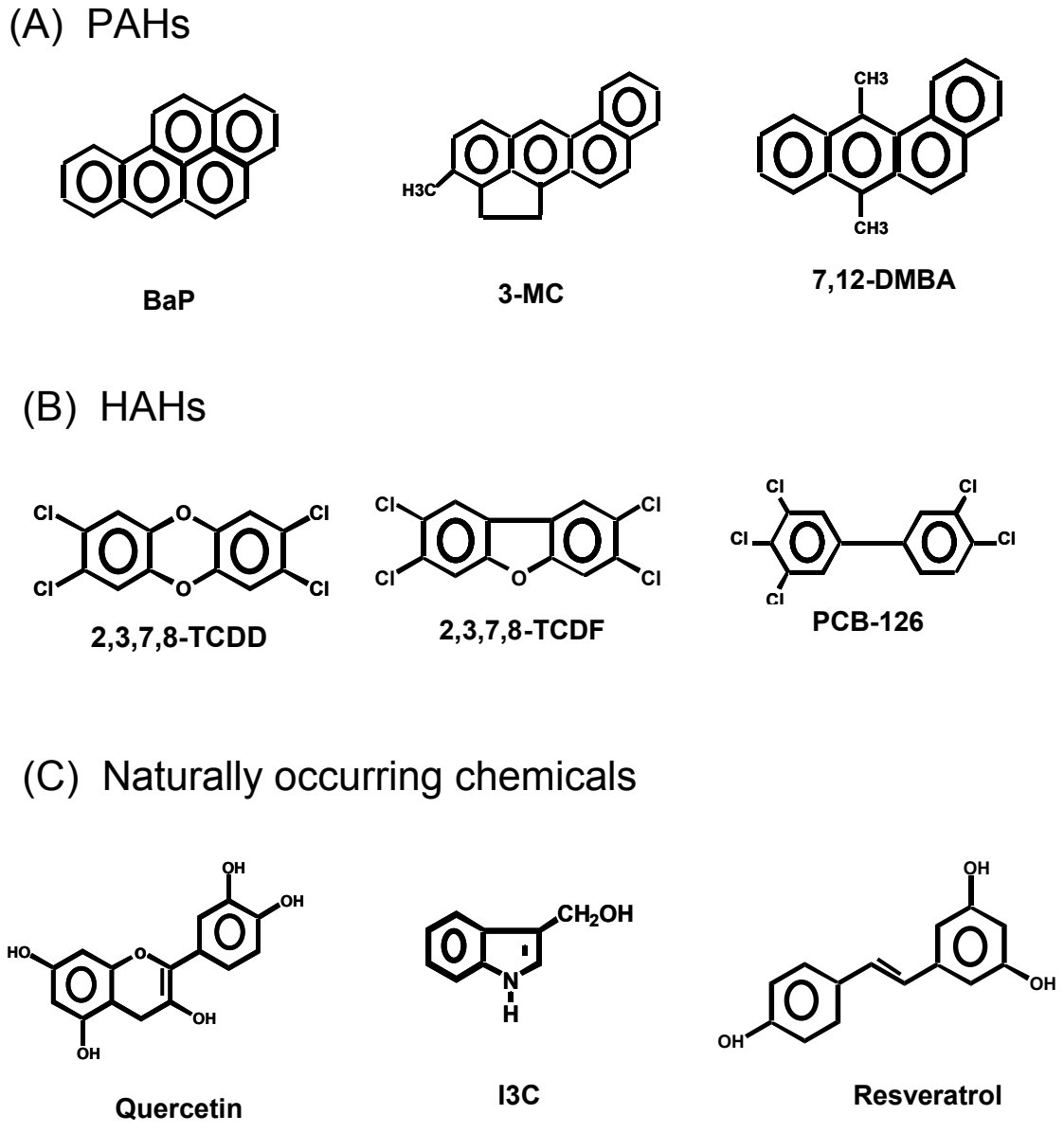

Figure 14 Structures of different structural classes of AhR agonists.

Naturally occurring AhR agonists include many secondary metabolites from plants, including quercetin, I3C and resveratrol. In general, these dietary

compounds are less potent and less persistent than the industrial ligands. However, since potential dietary exposure to these compounds is much higher, and over long time period their impact on human health cannot be underestimated (Denison et al., 2002).

Environmental pollutants and phytochemicals are well-characterized as exogeneous AhR ligands; however, there is some evidence suggesting that endogenous AhR ligands may also exist. Searching the answer to this question will provide us with a deeper understanding of the physiological roles of the AhR.

The Indole structure is an integral part of the essential amino acid tryptophan and indole-containing compounds are ubiquitous in human body. Some exogenous indoles, such as indolo[3,2-b]carbazole (ICZ) (Bjeldanes et al., 1991), have been identified as AhR ligands, and these observations raise the possibility that some endogenous indole compounds are capable of activating the AhR. Indirubin, a naturally occurring indole-containing compound that is found in human urine, induces CYP1A1 and CYP1B1 in MCF-7 breast cancer cells with potency comparable to TCDD but with a much shorter duration (Spink et al., 2003). Interestingly, Indirubin can be synthesized by human cytochrome P450-catalyzed metabolism of indole, suggesting that it may be part of endogenous AhR signaling modulator (Gillam et al., 2000). Tryptamine (TA), indole acetic acid (IAA), and 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid

methyl ester (ITE) have also been proposed to be the endogenous AhR ligands (Heath-Pagliuso et al., 1998; Henry et al., 2006).

Indole-containing compounds are not the only candidates as endogenous AhR agonists. Lipoxin A4, a metabolite of arachidonic acid, binds and activates the AhR (Schaldach et al., 1999). Several prostaglandins, including PGB_3, PGD_3, $PGF_{3\alpha}$, PGG_2, PGH_1, and PGH_2, elicit AhR responses in a mouse hepatoma cell culture model (Seidel et al., 2001). Given the promiscuous nature of the AhR, it is not impossible that some or all of the above-mentioned compounds are the physiologically relevant endogenous AhR ligands.

1.3.4. SAhRMs

As a prototypical AhR ligand, TCDD can induce a wide array of physiological activities. However, some other AhR ligands selectively induce some but not all AhR activities, and under some circumstances they may antagonize AhR-mediated responses. This type of AhR ligands, such as DIM and 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF), has been classified as selective AhR modulators (SAhRM) (Chen et al., 1998; Hestermann and Brown, 2003; McDougal et al., 2001a; Safe, 2001). The mechanistic basis of SAhRMs has not been completely elucidated; however, current evidence suggests that an important component of this activity may be the ligand-dependent differential recruitment of coactivators by the bound AhR.

Transcription factors, such as AhR-Arnt, regulate their target genes by binding to a specific DNA sequence and this mode of regulation also requires recruitment of transcriptional coactivators and other nuclear cofactors, which serve as part of a bridging complex between specific transcription factors, the basal transcription machinery and DNA polymerase II. These transcriptional coactivators, which often exhibit intrinsic enzymatic activities, generally increase transcriptional activity without directly binding to DNA. Moreover, some coactivators can be recruited in response to different signals in different organs and provide ligand or tissue specificity for transcriptional activation. Therefore the responses to multiple signals in different organ can be controlled, in part at the coactivator level.

Several studies report that nuclear receptor coactivators and corepressors and other nuclear cofactors can interact with and modulate ligand-dependent AhR-mediated transactivation (Rowlands et al., 1996; Kobayashi et al., 1997; Kumar and Perdew, 1999; Kumar et al., 1999; Nguyen et al., 1999; Antenos et al., 2002; Beischlag et al., 2002; Wang and Hankinson, 2002; Beischlag et al., 2004; Kim and Stallcup, 2004; Wang et al., 2004; Chen et al., 2006; Kim et al., 2006). For example, a study shows that SRC-1, SRC-2 (TIFII) and SRC-3 (AIB1) enhance TCDD-induced transactivation of a luciferase reporter in Hepa-1 cells, and this induction can be abolished by injection of anti-SRC-1 and anti-SRC-3 immunoglobulin G into the cells (Beischlag et al., 2002); Depleting endogenous TRAP220 by RNA interference decreased TCDD-

induced CYP1A1 transcription in human HepG2 cells (Wang et al., 2004) and CARM-1 also enhance AhR-mediated gene expression (Yanan Tian, personal communication).

Ligand-dependent activation of the AhR and members of the nuclear receptor (NR) superfamily is complex and studies on NRs have defined some of the elements which contribute to this complexity (Katzenellenbogen and Katzenellenbogen, 1996; Katzenellenbogen et al., 1996; Smith et al., 1994). Activation of NRs depends on the structure of the NR agonist, tissue/cell-specific expression of nuclear cofactors including coactivator proteins and gene promoter context which can also influence modulation of gene expression. Because of the multiple factors that are required for ligand-activation or repression of a nuclear receptor, structurally diverse compounds that activate NRs can be referred to as selective receptor modulators (SRMs), which exhibit tissue-specific NR agonist or antagonist activity (Klinge, 2000; Krishnan et al., 2000).

The concept of selective receptor modulator is important for developing mechanism-based drugs. Since it is rare to find that all the effects associated with a specific receptor-mediated pathway are desirable, the ability of employing selective receptor modulators to tease out the undesirable responses while retaining the therapeutic effects is crucial for successful drug development.

1.3.5. The antiestrogenicity of AhR signaling

Many AhR agonists demonstrate antiestrogenic activities in some experimental systems (Safe, 1995). This observation raises the prospect of utilizing AhR agonists for treatment of estrogen-dependent cancers, including breast and endometrial cancer. Epidemiological studies corroborate the validity of this concept in humans. In a population study on the residents of Seveso, Italy, who were accidentally exposed to high levels of TCDD, a reduction in breast and endometrial cancer incidence has been reported (Bertazzi et al., 1993).

The antiestrogenicity of AhR ligands is mediated through multiple mechanisms (Figure 15). First, AhR signaling enhances oxidative metabolism of estrogen by inducing drug metabolizing enzymes. Several enzymes involved in the metabolism of estrogen, includes CYP1A1 and CYP1B1, are induced by TCDD (Spink et al., 2003). AhR agonists also downregulate ER levels (Safe et al., 1991). This suppression appears to take place at both the transcriptional and post-transcriptional levels. TCDD treatment decreases ERα mRNA in AhR-responsive but not in AhR non-responsive mice, suggesting an essential role of the AhR in the TCDD-dependent suppression of the ER (Tian et al., 1998). TCDD also increase degradation of ERα by a proteasome-dependent, post-transcriptional pathway in breast cancer cells (Wormke et al., 2003). AhR agonists also antagonize estrogen-induced responses by inhibiting the

transactivation of ER-dependent genes. For example, the E2-responsive cathepsin D gene contains an inhibitory DRE (iDRE) motif located within close proximity to ER/Sp1 binding site in the 5'-regulatory promoter region. This iDRE binds to the liganded AhR-Arnt complex in the proximity to an E2-responsive GC rish ERα/Sp binding site, causing interference with ER/Sp1-DNA complex formation and thereby inhibiting estrogen-dependent activation of cathepsin D (Krishnan et al., 1995).

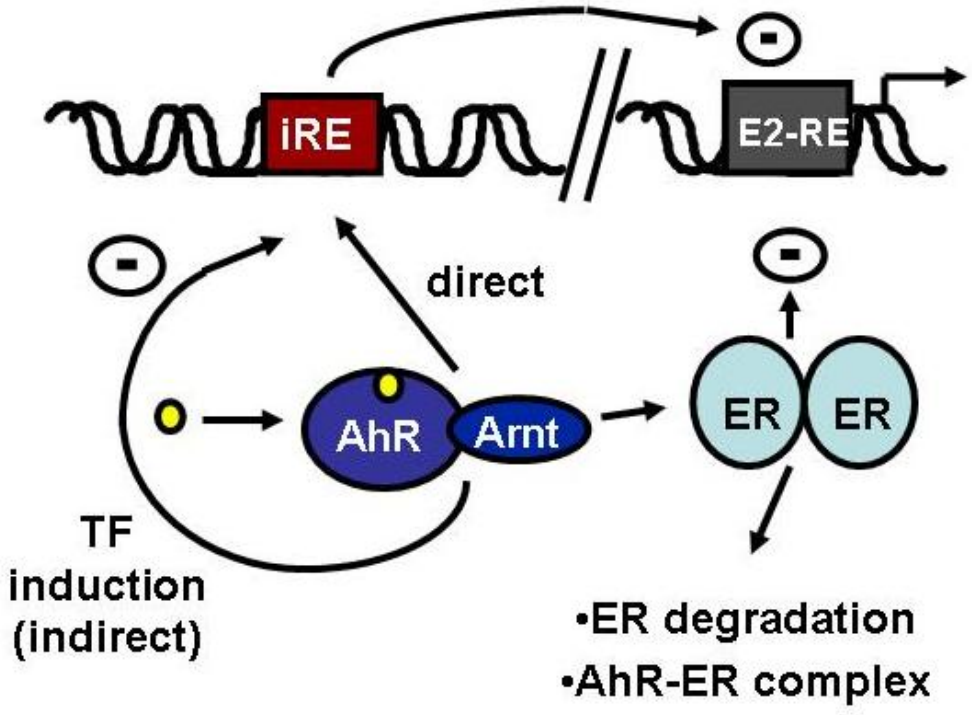

Figure 15 Mechanisms of the antiestrogenicity of AhR (Pearce et al., 2004).

The therapeutic applications of AhR agonists for breast cancer treatment are limited by their toxicities. Many AhR ligands, such as TCDD, are carcinogens in animal models and therefore, clinical applications of these compounds are not possible. Fortunately, certain AhR ligands are SAhRMs, making it possible to develop AhR compounds that retain their antiestrogenic activity but not the prototypical TCDD-like toxic effects.

MCDF (6-Methyl-1,3,8-trichlorodibenzofuran) was initially developed as an AhR antagonist, and MCDF inhibits TCDD-induced CYP1A1 induction, immunotoxicity, teratogenicity and porphyria in mice (Astroff et al., 1988). In addition, MCDF alone exhibits weak activity for these toxic responses. In contrast, MCDF (like TCDD) is a highly effective inhibitor of E2-induced gene expression and growth in breast cancer cells and also inhibits mammary tumor growth in vivo without causing significant toxicity (McDougal et al., 1997; Safe, 2001). A study in rats showed that MCDF prevented E2-induced increases in ER and progesterone receptor in the uterus and liver, as well as uterine wet weight increases (Astroff and Safe, 1988). MCDF can also act synergistically in combination with tamoxifen to suppress the DMBA-induced mammary tumors in Sprague-Dawley rats, and protect against the estrogenic effects of tamoxifen on the uterus in these rats (McDougal et al., 2001b). Moreover, MCDF inhibits growth of LNCaP prostate cancer cells and blocks hormone-induced upregulation of AR protein (Morrow et al., 2004).

Certain plant metabolites also show great promise as AhR agonists that inhibit mammary carcinogenesis. Cruciferous vegetables, such as cabbage, radishes, broccoli, and cauliflower, contain high levels of indole-3-carbinol (I3C). I3C and its related metabolites such as DIM (diindolylmethane) and indolo[3,2-b]carbazole (ICZ) have all been identified as AhR ligands (Bjeldanes et al., 1991; Chen et al., 1996). I3C and DIM inhibit the growth of MCF-7 breast cancer cell in cell culture and mammary tumors in rodents (Chen et al., 1998). High consumption of cruciferous vegetables has been associated with a decreased risk of lung, colorectal, prostate and breast cancer, and it is believed that a component of these protective effects can be attributed to I3C and its various derivatives (Higdon et al., 2007). Research in this area holds promise for development of novel therapeutic agents for cancer chemotherapy.

CHAPTER II

ROLE OF ESTROGEN RECEPTOR α (ER α) IN INSULIN-LIKE GROWTH FACTOR (IGF)-I-INDUCED RESPONSES IN MCF-7 BREAST CANCER CELLS*

2.1. Overview

Insulin-like growth factor-I (IGF-I) is a mitogenic polypeptide that induces proliferation of MCF-7 breast cancer cells, and cotreatment with the phosphoinositide 3-kinase (PI3-K) inhibitor LY294002 and the antiestrogen ICI 182780 inhibits IGF-I-induced growth. The role of estrogen receptor α (ERα) in mediating responses induced by IGF-I was investigated in cells transfected with small inhibitory RNA for ERα (iERα). The results showed that IGF-I-dependent phosphorylation of Akt and mitogen-activated protein kinase, induction of G_1–S-phase progression and enhanced expression of cyclin D1 and cyclin E were dependent on ERα. Moreover, these same IGF-I-induced responses were also inhibited by the antiestrogen ICI 182780 and this was in contrast to a previous

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*Part of the data reported in this chapter is reprinted with permission from Zhang S, Li X, Burghardt R, Smith R III, Safe SH. Role of estrogen receptor (ER) alpha in insulin-like growth factor (IGF)-I-induced responses in MCF-7 breast cancer cells. J Mol Endocrinol. 2005; 35(3):433-47, © Society for Endocrinology (2005).

report suggesting that ICI 182780 did not affect IGF-I-dependent activation of PI3-K or induction of cyclin D1 expression. ICI 182780 exhibits antimitogenic activity and iERα inhibits G_1–S-phase progression and proliferation of MCF-7 cells treated with IGF-I, suggesting that the effects of the antiestrogen are primarily related to downregulation of ERα.

2.2. Materials and methods

2.2.1. Chemicals, cells, antibodies, oligonucleotides, and plasmids

MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (DMEM/F-12) with and without Phenol Red, 100x antibiotic/antimycotic solution, propidium idodide, E_2 and human recombinant IGF-I were purchased from Sigma (St Louis, MO, USA). Fetal bovine serum was purchased from Intergen (Purchase, NY, USA). [γ-^{32}P]ATP (300 Ci/mmol) was obtained from Perkin-Elmer Life Sciences. Poly(dI-dC) and T4 polynucleotide kinase were purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). Antibodies for lamin A/C, ERα, pAKT, and pERK proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for GAPDH were obtained from Ambion (Austin, TX, USA). Human ERα expression plasmid was provided by Dr Ming-Jer Tsai (Baylor College of Medicine, Houston, TX, USA). Lysis buffer, luciferase reagent and RNase were obtained from Promega Corp. (Madision, WI, USA). PD98059 and LY294002 were purchased from

Calbiochem (San Diego, CA, USA). ICI 182780 was provided by Dr Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD;>98%) was prepared in this laboratory. The pSRF construct containing five tandem serum-response factor (SRF) elements linked to a luciferase reporter gene was purchased from Stratagene (La Jolla, CA, USA), and the GAL4-ElkC plasmid was provided by Roger Treisman (Imperial Cancer Research Centre, London, UK). The $pDRE_3$-luc construct containing three tandem consensus dioxin-responsive elements (DREs) was prepared in this laboratory and the E_2-responsive pC3-luc construct was provided by Dr Donald McDonnell (Duke University, Durham, NC, USA). DMSO was used as solvent for E_2 and the antiestrogens. Acetic acid (0.1 M) was used as solvent for IGF-I. The siRNAs for GL2, lamin A/C and ERα were prepared by Xeragon (Germantown, MD, USA), and the sequences of iRNA duplexes were indicated as follows: GL2, 5'-CGU ACG CGG AAU ACU UCG ATT-3', 3'-TTG CAU GCG CCU UAU GAA GCU-5'; lamin A/C, 5'-CUG GAC UUC CAG AAG AAC ATT-3', 3'-TTG ACC UGA AGG UCU UCU UGU-5'; ERα, 5'-AGG CUC AUU CCA GCC ACA GTT-3', 3'-TTU CCG AGU AAG GUC GGU GUC-5'. The siRNA for IRS-I was comprised of four pooled siRNA duplexes (catalogue number M-003015) from Dharmacon (Lafayette, CO, USA).

2.2.2. Cell-proliferation assay

MCF-7 cells were seeded in DMEM/F-12 with 2.5% stripped fetal bovine serum and treated with different factors for 9 days. Cell numbers were determined using a Coulter Z1 counter, and results for each treatment are given as means ± S.D. from at least three different determinations for each treatment group. For the cell-proliferation study involving siRNA, cells were transfected with iERα or siRNA for GL2 (iGL2) and, after 24 h, treated with 10 nM IGF-I for 48 h. Cell numbers were then determined.

2.2.3. Transfection of MCF-7 cells

Cells were cultured in six-well plates in 2 ml DMEM/F-12 supplemented with 5% fetal bovine serum. After 16–20 h when cells were 30–50% confluent, siRNA duplexes and/or reporter gene constructs were transfected using Oligofectamine Reagent (Invitrogen, Carlsbad, CA, USA). For each well of a six-well plate, 0.2 μmol iRNA duplex was transfected. Cells were harvested 36–44 h after transfection by manual scraping in 1 x lysis buffer (Promega). For whole-cell lysates, cells were frozen and thawed in liquid nitrogen, vortexed for 30 s, and centrifuged at 12 000 *g* for 1 min. Lysates were assayed for luciferase activity using luciferase assay reagent (Promega). ß-Galactosidase activity was measured using Tropix Galacto-Light Plus assay system (Tropix, Bedford, MA, USA) in a Lumi-count microwell plate reader (Packard Instrument Co.).

2.2.4. Preparation of nuclear extracts

MCF-7 cells were seeded in a 100 mm tissue culture dish (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in DMEM/F-12 with 2.5% dextran/charcoal-stripped fetal bovine serum. After 24 h, cells were washed twice in PBS, scraped in 1 ml HEGD buffer (25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol and 10% (v/v) glycerol, pH 7.6) and homogenized. The cellular homogenate was centrifuged for 5 min at 1000 *g*. The supernatant was discarded and the pellet was suspended in 200 ml HEGDK (HEGD with 0.5 M KCl) and incubated on ice for 15 min with frequent vortexing. Samples were centrifuged at 14 000 *g* for 1 min, and nuclear protein concentration in the supernatant was determined by the Bradford assay (Bradford 1976). The supernatant was stored in small aliquots at –80 °C for further use.

2.2.5. Western immunoblot analysis

An aliquot of whole-cell lysate containing 30 µg proteins was diluted with loading buffer, boiled and loaded on a 10% SDS/polyacrylamide gel. Samples were electrophoresed at 150–180 V for 3–4 h, and separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) in buffer containing 48 mM Tris/HCl, 29 mM glycine and 0.025% SDS. Proteins were detected by incubation with polyclonal primary antibodies for lamin A/C, ERα-H184, GAPDH, p-AKT1/2/3-Ser473 and p-ERK (1:1000 dilution) followed by blotting with horseradish peroxidase-conjugated anti-rabbit (for ERα

and p-AKT), anti-goat (for lamin A/C) or anti-mouse (for GAPDH and p-ERK) secondary antibody (1:5000 dilution). Blots were then exposed to chemiluminescent substrate (PerkinElmer Life Sciences) and placed in Kodak X-Omat AR autoradiography film. Band intensities were determined by scanning laser densitometer (Sharp Electronics Corp., Mahwah, NJ, USA) using Zero-D Scanalytics software (Scanalytics Corp., Billerica, MA, USA).

2.2.6. FACS analysis

Cells were transfected with iERα or iGL2. After 20–24 h cells were treated with 0.1 M acetic acid or 10 nM IGF (acetic acid as a vehicle) for 18–20 h in serum-free medium. Cells were then trypsinized, and ~2 x 10^6 cells were centrifuged and resuspended in 1 ml staining solution (50 μg/ml propidium iodide (PI), 4 mM sodium citrate, 30 units/ml RNase and 0.1% Triton X-100, pH 7.8). Cells were then incubated at 37 °C for 10 min, and prior to FACS analysis, NaCl was added to give a final concentration of 0.15 M. Cells were analyzed on a FACS Calibur flow cytometer (BD PharMingen) using CellQuest acquisition software (BD PharMingen). PI fluorescence was collected through a 585/542 nm bandpass filter, and list mode data were acquired on a minimum of 12 000 single cells defined by a dot plot of PI width versus PI area. Data analysis was performed in ModFit LT (Verity Software House, Topsham, ME, USA) using PI width versus PI area to exclude cell aggregates. FlowJo (Treestar, Palo Alto, CA, USA) was used to generate plots shown in the figures.

2.2.7. Gel electrophoretic mobility shift assay (EMSA)

The probe containing a consensus estrogen-responsive element (ERE; 5'-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3') was synthesized, annealed and ^{32}P-labeled at the 5'-end using T4 polynucleotide kinase (Roche) and [γ-^{32}P]ATP. The binding reactions were performed on ice. For each lane, an appropriate amount of HEGDK buffer was added to 5 μg MCF-7 cell nuclear extracts to bring the total volume to 5 μl. HEGD buffer (15 μl) was added to dilute the salt concentration and 1 μg poly(dI-dC) was used to block the non-specific binding. After incubation for 5 min, 0.01 pmol radiolabeled probe was added and incubated for 10 min. ER antibody (sc-7202; Santa Cruz Biotechnology) was then added to the mixture and incubated for another 5 min if applicable. The mixture was resolved on 5% non-denaturing PAGE and protein–DNA complexes were visualized using Storm Imager system (Molecular Dynamics, Sunnyvale, CA, USA).

2.2.8. Immunostaining

MCF-7 cells were seeded in DMEM/F-12 with 2.5% dextran/charcoal-stripped fetal bovine serum in two-well Lab-Tek chamber slides (Nalge Nunc International Corp., Naperville, IL, USA). In experiments involving siRNAs, after 24 h cells were transfected with siRNA for lamin A/C (iLMN) or iERα Oligofectamine according to manufacturer's recommendation and incubated for 48 h before methanol fixation. Otherwise, cells were directly treated and fixed.

Cells were fixed in methanol at –20 °C for 10 min. Slides were air-dried, washed in PBS/0.3% Tween-20 for 10 min, and blocked with 1:20 goat serum in antibody dilution buffer (1% BSA, PBS, 0.3% Tween-20 and 31% glycerol with 0.5 M Na_2CO_3, pH 9.5) for 1 h in a humidified chamber. ER antibody (sc-7202) in antibody dilution buffer (1:100 dilution) was added and incubated in a humidified chamber at 4 °C for overnight. Slides were washed three times, every 20 min in PBS/0.3%Tween-20, and then probed with FITC-conjugated anti-rabbit IgG antibody (sc-2012; Santa Cruz Biotechnology) in antibody dilution buffer (1:1000 dilution) for 2 h. Slides were then washed for 30 min (three times) in PBS/0.3%Tween-20 and then in deionized water for 15 min and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Immunostaining was observed with a Zeiss Axioplan2 microscope fitted with a Hamamatsu-C5810 chilled 3 CCD color camera.

2.2.9. Statistical analysis

Statistical significance was determined by analysis of variance and Scheffe's test, and the levels of probability are noted. The results are expressed as means ± S.D. from at least three separate (replicate) experiments for each treatment group.

2.3. Results

2.3.1. Mitogenic activity of IGF-I

The mitogenic activity of IGF-I was initially investigated in MCF-7 cells and the results in (Figure 16) show that treatment with 10 nM IGF significantly induced proliferation of these cells. Treatment of MCF-7 cells with 5 μM LY294009, 15 μM PD98059, and 1 μM ICI 182780 also significantly inhibited MCF-7 cell growth compared with solvent (DMSO)-treated controls. However, in cells cotreated with IGF-I plus these inhibitors, growth factor-induced proliferation of MCF-7 cells was inhibited by LY294002 and ICI 182780 but not PD98059. These results are consistent with previous reports on the effects of LY294002 and ICI 182780 on IGF-I induced growth (Dufourny et al., 1997; Oesterreich et al., 2001; Varma and Conrad, 2002; Wakeling et al., 1989) and suggest a role for PI3-K and ERα in mediating the mitogenic activity of IGF-I.

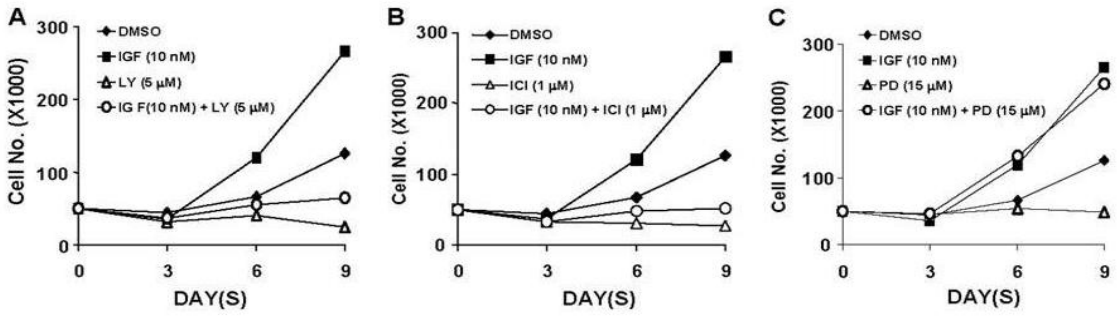

Figure 16 IGF-I-induced proliferation of MCF-7 cells and interactions with LY294002 (LY; A), ICI 182780 (ICI; B), and PD98059 (PD; C). MCF-7 cells were seeded in six-well plates and treated with 10 nM IGF-I and/or 1, 5, or 15 μM of the different chemicals for 9 days, and cell numbers were determined as described in the Materials and methods. IGF-I significantly ($P < 0.05$) increased growth (compared with solvent control) and only ICI 182780 and LY294002 significantly decreased IGF-I-induced proliferation.

2.3.2. IGF-I-induced responses are dependent on ERα: effects of iERα

The role of ERα in mediating IGF-I-induced proliferation and gene activation was further investigated in MCF-7 cells using RNA interference with iERα. Whole-cell lysates from MCF-7 cells transfected with iERα or iLMN (non-specific control) and untransfected cells were analyzed by western blot analysis (Figure 17A). iERα specifically induced downregulation of ER but not LMN (non-specific) protein, whereas iLMN decreased iLMN but not ERα protein. Over several studies, transfected iERα decreased ERα protein by >50% in whole-cell lysates and this is consistent with the high transfection efficiencies observed in MCF-7 cells. iERα-mediated decreases in nuclear ER were confirmed in a gel mobility shift assay with nuclear extracts from MCF-7 cells and [32P]ERE (Figure 17B). The intensity of the specifically bound retarded ERα-ERE band (Figure 17B, lane 3), was decreased in cells transfected with iERα (Figure 17B, lane 4) or after incubation with excess unlabeled ERE (Figure 17B, lane 2). Extracts from cells transfected with iLMN did not affect retarded band intensity (Figure 17B, lane 5) and ERα antibodies super-shifted the specifically bound band (Figure 17B, lane 1). iERα-mediated decreases in ERα protein is also evident in MCF-7 cells immunostained for ERα (Figure 17C). Compared with control (untreated) cells or cells transfected with iLMN, ERα protein was significantly decreased in MCF-7 cells transfected with iERα.

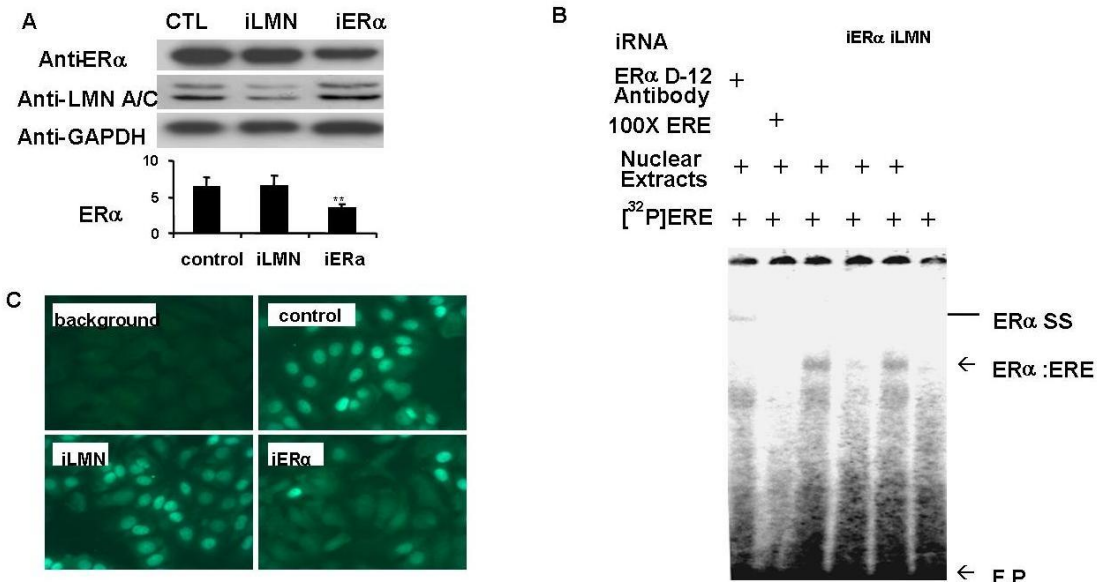

Figure 17 RNA interference with iERα. (A) Effects of iERα on ERα protein expression in MCF-7 cells. Cells were transfected with iERα and iLMN (as control siRNA), and whole-cell extracts were analyzed for ERα, lamin A/C (LMN) and GAPDH proteins by Western blot analysis as described in the Materials and Methods section. Results are expressed means ± S.D. from three replicate determinations for each treatment group, and a significant ($P < 0.05$) decrease in ERα (compared with solvent control or iLMN) protein levels was observed (**). CTL, control. (B) Gel mobility shift assay. MCF-7 cells were treated with solvent, iERα, or iLMN, and binding of nuclear extracts to [^{32}P]ERE was determined in gel mobility shift assays as described in the Materials and methods section. Specifically bound bands and supershifted complexes are indicated by arrows. (C) Immunocytochemistry. MCF-7 cells were transfected with iERα or iLMN, and stained with ERα or lamin A/C antibodies. Immunofluorescence was determined as described in the Materials and methods section.

2.3.3. iERα specifically targets ERα

The specificity of the RNA interference assay was further investigated in MCF-7 cells transfected with an E_2-responsive pC3-luc construct which contains the human complement C3 gene promoter insert. E2-induced luciferase activity

in cells transfected with pC3-luc and iLMN, whereas hormone-induced activity was decreased in cells transfected with iGL2 (which targets bacterial luciferase) or iERα (Figure 18A). As a negative control, we show that iERα does not affect TCDD-induced transactivation in MCF-7 cells transfected with an aryl hydrocarbon-responsive construct (pDRE$_3$-luc; Figure 18B). These results are consistent with previous studies showing that IGF-I induced E_2-responsive genes through ligand-independent activation of nuclear ERα (Stoica et al., 2000; Wang et al., 2000; Xie et al., 2001). IGF-I also activates MAPK- and PI3-K-dependent pathways including phosphorylation-dependent activation of Elk-1 and SRF, which are serum-response element (SRE)-dependent genes (Duan et al., 2001b; Duan et al., 2002a). Compared with the solvent control, using acetic acid, IGF-I induced transactivation in MCF-7 cells transfected with iLMN, a GAL4-Elk-1 expression plasmid, and a pGAL4 reporter construct (Figure 18C; containing five tandem GAL4-response elements), or an SRF-luc construct (Figure 18D; containing three tandem SRF motifs). In contrast, activation of both constructs was significantly inhibited by transfection with iGL2 or iERα demonstrating that ERα plays a role in IGF-I-induced transactivation in MCF-7 cells.

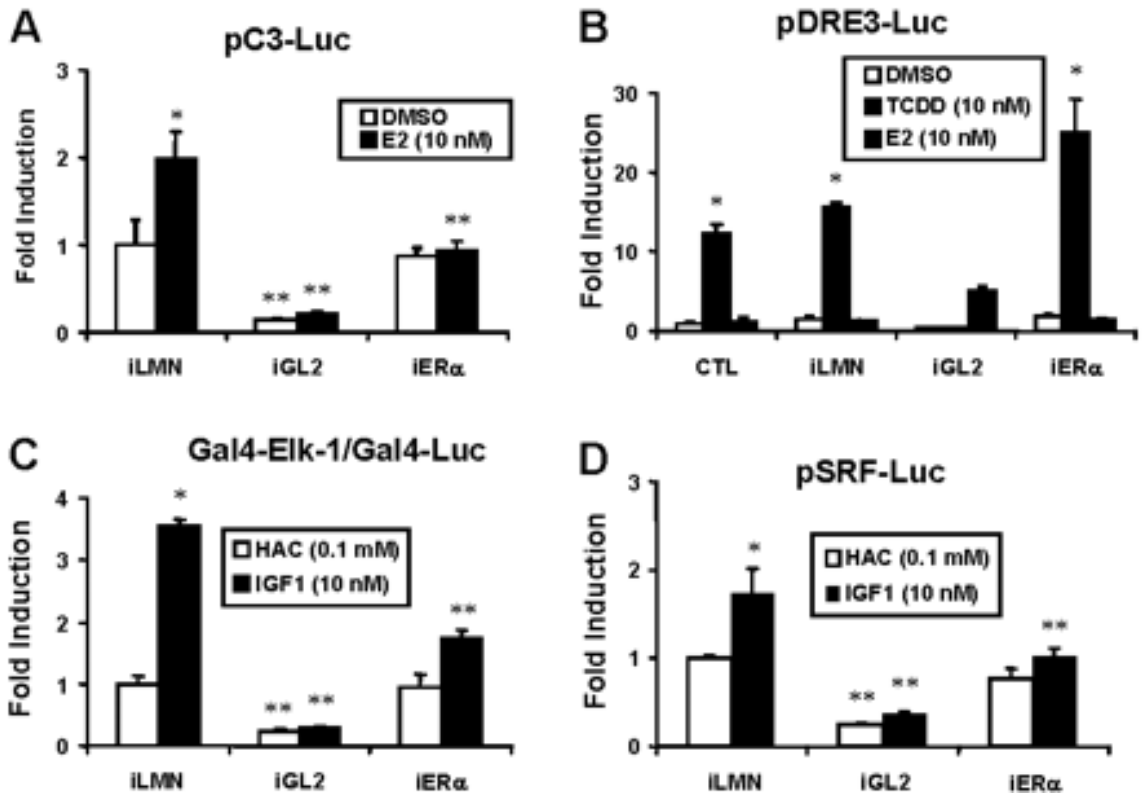

Figure 18 Effects of iERα on transactivation in MCF-7 cells transfected with several ligand-activated constructs. Cells were transfected with iLMN, iGL2, or iERα, and the E_2-responsive pC3-Luc (A), aryl hydrocarbon-responsive pDRE$_3$-Luc (B), MAPK-regulated Gal4-Elk-1/Gal4-Luc (C), and a PI3-K-regulated pSRF-Luc (D) constructs. Cells were treated with DMSO, 10 nM E_2, 10 nM TCDD, or 10 nM IGF-I, and luciferase activity was determined as described in the Materials and methods section. Results summarized in A–D are means ± S.D. from three replicate determinations for each treatment group, and significantly ($P < 0.05$) increased activities (compared with solvent control) are indicated by * and significant inhibition by siRNAs is indicated by **. CTL, control; HAC, acetic acid vehicle.

2.3.4. RNA interference inhibits IGF-I-induced phosphorylation of AKT and MAPK

We further investigated the role of ERα in mediating IGF-I-induced gene expression by investigating phosphorylation of AKT or MAPK (Figure 19A and B). Figure 19A shows that, compared with untransfected cells or cells transfected with iLMN (a control; Figure 19A, lanes 1 and 2), iERα significantly decreased ERα protein levels in the presence or absence of IGF-I (Figure 19A, lanes 3 and 6). IGF-I treatment did not affect levels of ERα protein but induced AKT phosphorylation (Figure 19A, lanes 4–6); however, in cells cotransfected with iERα there was a significant decrease in IGF-I-induced AKT phosphorylation. In a separate experiment using a comparable approach, it was also apparent that iERα also decreased MAPK phosphorylation (Figure 19B), and this was consistent with decreased Elk-1 and SRF activation by IGF-I in cells transfected with iERα (Figure 18C and D). These results suggest that ERα is required for activation of MAPK/PI3-K-dependent kinases by IGF-I. IGF-I-induced signaling in the mouse uterus (Klotz et al., 2002) suggested that activation of PI3-K required IRS-I, whereas activation of MAPK was IRS-I-dependent. The results in Figure 19C show that transfection of iIRS-I in MCF-7 cells decreases IRS-I protein expression but does not significantly decrease phosphorylation of AKT or MAPK. These results suggest that IRS-I is not required for IGF-I-induced activation of PI3-K or MAPK in MCF-7 cells, and differs from the activation pathway in the mouse uterus (Klotz et al., 2002).

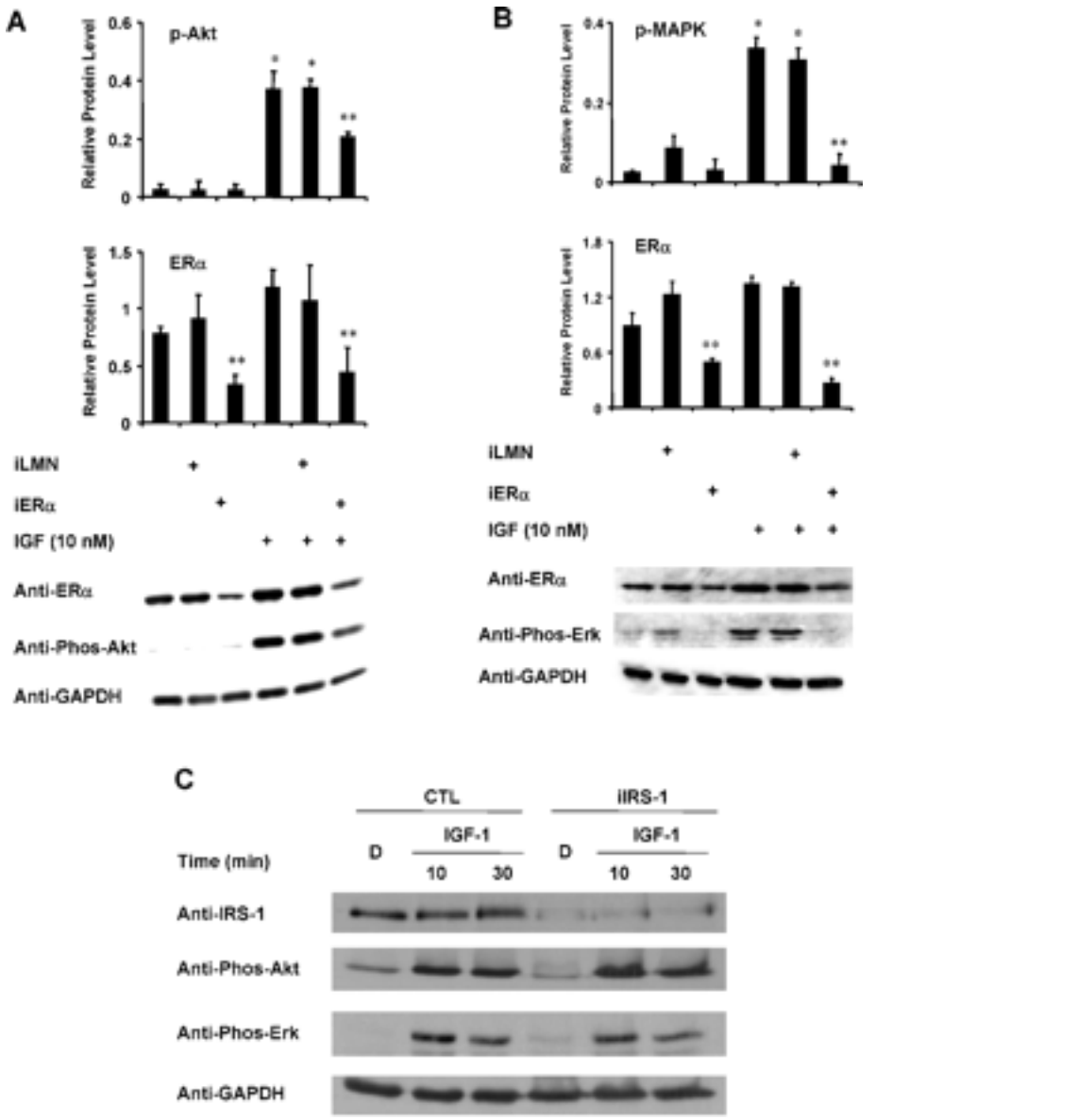

Figure 19 Effects of iERα and iIRS-I on IGF-I-dependent kinase activation. Effects of iERα on phosphorylation of AKT (A) and MAPK (B). MCF-7 cells were transfected with siRNAs as described in the Materials and methods section, and phosphoproteins were determined by Western blot analysis of whole-cell lysates. The intensity of the different bands were determined by densitometry and plotted as means ± S.D. from three replicates in each treatment group. Significant ($P < 0.05$) increases in band intensity (compared with solvent control) are indicated by * and significant inhibition by siRNAs is indicated by **. (C) Effects of iIRS-I on phosphorylation of AKT and MAPK. The experiments were carried essentially as described in (A) and (B) except the phosphorylation levels were determined in DMSO (D; solvent control) and IGF-I-treated cells after treatment for 10 or 30 min. CTL, control.

2.3.5. Effects of iERα on IGF-I-induced cell-cycle progression and proliferation

Previous studies (Dufourny et al., 1997; Oesterreich et al., 2001; Varma and Conrad, 2002; Wakeling et al., 1989) and results in Figure 1 suggest that IGF-I-induced growth is dependent on activation of the PI3-K pathway, and therefore the effects of iERα on cell-cycle progression were investigated. The distribution of MCF-7 cells in G_0/G_1, S, and G_2/M phases was 82.4, 9.5, and 8.1%, respectively (Figure 20A). Similar results (83.6, 9.9, and 6.5%) were observed in cells transfected with iGL2, a non-specific inhibitor of bacterial luciferase. In cells transfected with iERα, there was a further increase in the percentage of cells in G_0/G_1 phase (86.5%) and a decrease of those in S phase (5.8%), suggesting that ERα contributes, in part, to the G_0/G_1–S-phase progression of untreated (control) MCF-7 cells. Treatment of MCF-7 cells with 10 nM IGF-I for 18–20 h significantly decreased (25–28%) the percentage of cells in G_0/G_1 and a comparable increase in the percentage of cells in S phase was observed. However, in MCF-7 cells transfected with iERα, IGF-I-induced G_1–S-phase progression was partially reversed, demonstrating a role for ERα in mediating IGF-I-dependent effects on this specific phase of the cell cycle. In addition, ERα knockdown in MCF-7 cells decreased IGF-I-induced cell proliferation (Figure 20B). It is possible that some component of IGF-I-induced G_1–S-phase progression and cell proliferation may be ERα-independent.

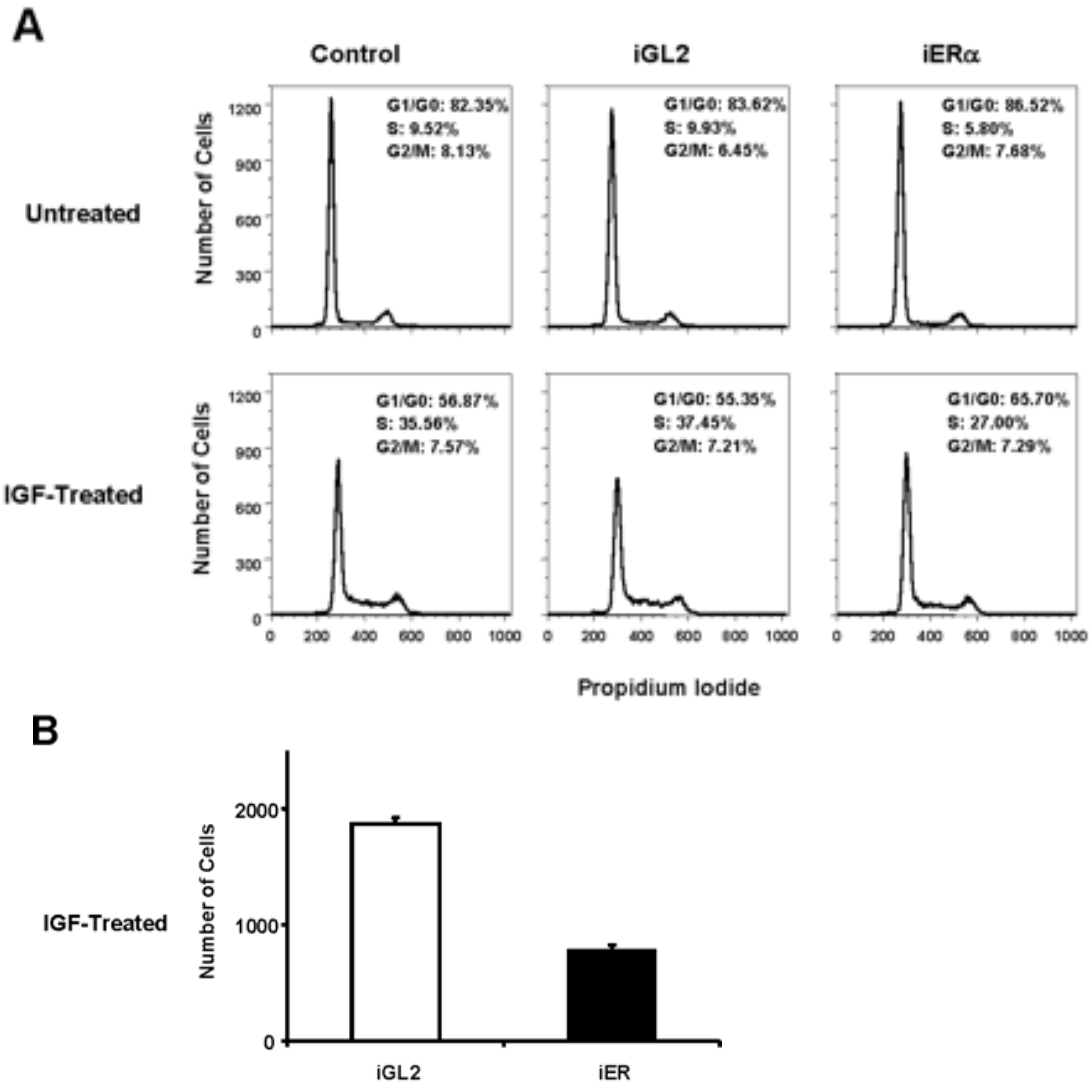

Figure 20 Effects of iERα on IGF-I-induced cell-cycle progression and proliferation in MCF-7 cells. (A) FACS analysis. Serum-starved MCF-7 cells were treated with DMSO or 10 nM IGF-I alone or cotransfected with iGL2 and iERα, and the percentage of distribution of cells in G_1/G_0, S, and G_2/M were determined by FACS analysis as described in the Materials and methods section. The results of three separate FACS experiments showed that after transfection with iERα there was a 13.0±1.44% increase in G_0/G_1 and a 9.27±3.47% decrease in S-phase distribution of the cells. Results are expressed as means ± S.D. (B) Cell proliferation. MCF-7 cells were transfected with iERα or iGL2 and, after 24 h, cells were treated with 10 nM IGF-I for 48 h, and cell numbers were determined as described in the Materials and methods section. Results are expressed as means ± S.D. for three replicate determinations for each treatment group, and a significant ($P < 0.05$) decrease in cell number was observed in cells transfected with iERα compared with cells transfected with iGL2.

2.3.6. Effects of iERα on IGF-I-induced cell-cycle proteins

The potential role of cell-cycle-regulatory proteins associated with ERα-dependent actions of IGF-I were further investigated by western blot analysis (Figure 21). Untreated (Figure 21, lanes 1–3) and IGF-I-treated (Figure 21, lanes 4–6) cells were untransfected (Figure 21, lanes 1 and 4) or transfected with iLMN (Figure 21, lanes 2 and 4) or iERα (Figure 21, lanes 3 and 6), and whole-cell lysates were analyzed by western blot analysis. The patterns of ERα expression were similar to those illustrated in Figs 19A and B, and the various treatments did not affect levels of cdk2 or cdk4 or phospho-Rb proteins, and levels of IGF-I receptor were also unchanged (data not shown). In contrast, IGF-I induced cyclin D1 and cyclin E protein levels and cotransfection with iERα decreased induction of both proteins. The results indicate that ERα plays a role in endogenous expression of both cyclin D1 and cyclin E since levels of these proteins were significantly decreased in untreated cells transfected with iERα. These data suggest that induction of cyclin D1 and cyclin E by IGF-I is an important response associated with G_1–S-phase progression. Previous studies also show that cyclin D1 and cyclin E were induced by IGF-I in MCF-7 cells (Dufourny et al., 1997; Dupont et al., 2000) and this is consistent with the induction of G_1–S-phase progression (Figure 20) which is also dependent, in part, on expression of ERα.

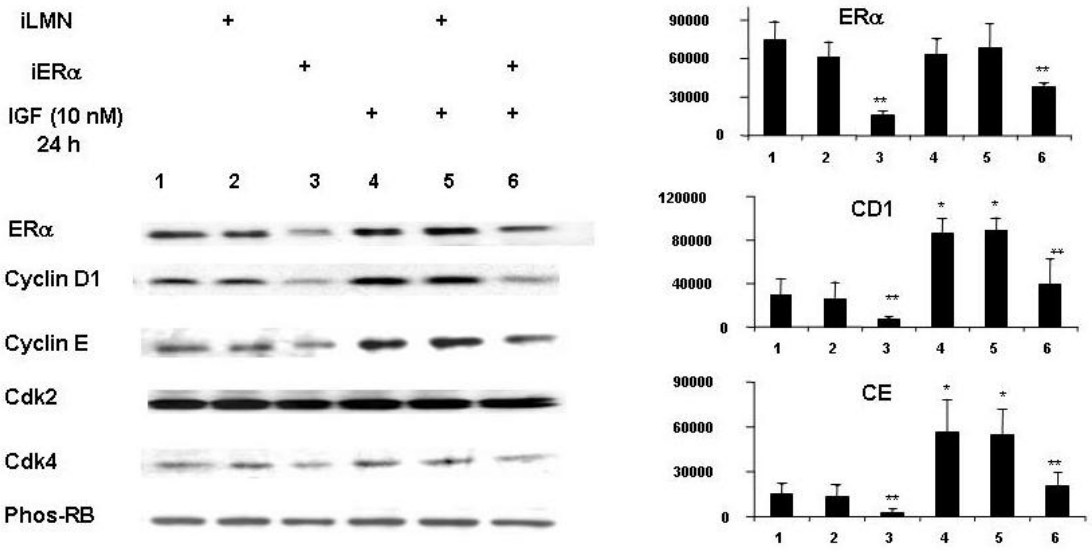

Figure 21 Effects of iERα on IGF-I-induced cell-cycle proteins. MCF-7 cells were transfected with iLMN (as a control siRNA) or iER and treated with either (10 nM) IGF-I or solvent vehicle for 24 h. The expression of cyclin D1 (CD1) and cyclin E (CE) proteins were determined by Western blot analysis as described in the Materials and methods section, and intensities were determined and plotted as means ± S.D. from three replicate for each treatment group. Significant ($P < 0.05$) increases in band intensities (compared with solvent control) are indicated by * and significant inhibition by siRNA is indicated by **.

2.3.7. Effects of ICI 182780 on IGF-I-induced responses

A previous study reported that although ICI 182780 inhibited IGF-I-induced growth of MCF-7 cells (Varma and Conrad, 2002), the antiestrogen did not completely block IGF-I signaling including phosphorylation of AKT and IRS-I or induction of cyclin D1. These results are surprising since ICI 182780 induces proteasome-dependent downregulation of ERα in breast cancer cells (Fan et al., 2003; Stenoien et al., 2001; Wijayaratne and McDonnell, 2001; Wormke et al.,

2003). Based on the results obtained in the siRNA knockdown studies with iERα (Figs 17–21), we further investigated the effects of ICI 182780 on IGF-I-induced pathways in MCF-7 cells. Results of initial studies showed that cotreatment of MCF-7 cells with 1 μM ICI 182780 and 10 nM IGF-I (8 min) only slightly decreased MAPK and AKT phosphorylation (Figure 22A). Since ICI 182780 induces a time-dependent decrease in levels of ERα (Fan et al., 2003; Stenoien et al., 2001; Wijayaratne and McDonnell, 2001; Wormke et al., 2003), we therefore determined the effects of 1 μM ICI 182780 on IGF-I-induced phosphorylation for up to 4 h after treatment with the antiestrogen (Figure 22B). IGF-I (10 min) alone induced phosphorylation of AKT and MAPK. After cotreatment with ICI 182780 for 20 min, there was a significant decrease of ERα protein, and phosphorylation of AKT and MAPK was also decreased compared to treatment with IGF-I alone. Quantitation of ERα protein levels and kinase phosphorylation in cells cotreated with IGF-I plus ICI 182780 showed a time-dependent decrease in these parameters and decreased AKT phosphorylation was the most pronounced response at the 20-min time point. Decreased ERα protein in MCF-7 cells treated with 1 μM ICI 182780 for 2 h was also confirmed by immunocytochemical analysis (Figure 22C) and the results were similar to those observed in MCF-7 cells transfected with iERα (Figure 17C). Moreover, ICI 182780 also inhibited IGF-I-induced transactivation in cells transfected with PI3-K- and MAPK-dependent constructs (data not shown) as observed in MCF-7 cells transfected with iERα (Figure 18C and D).

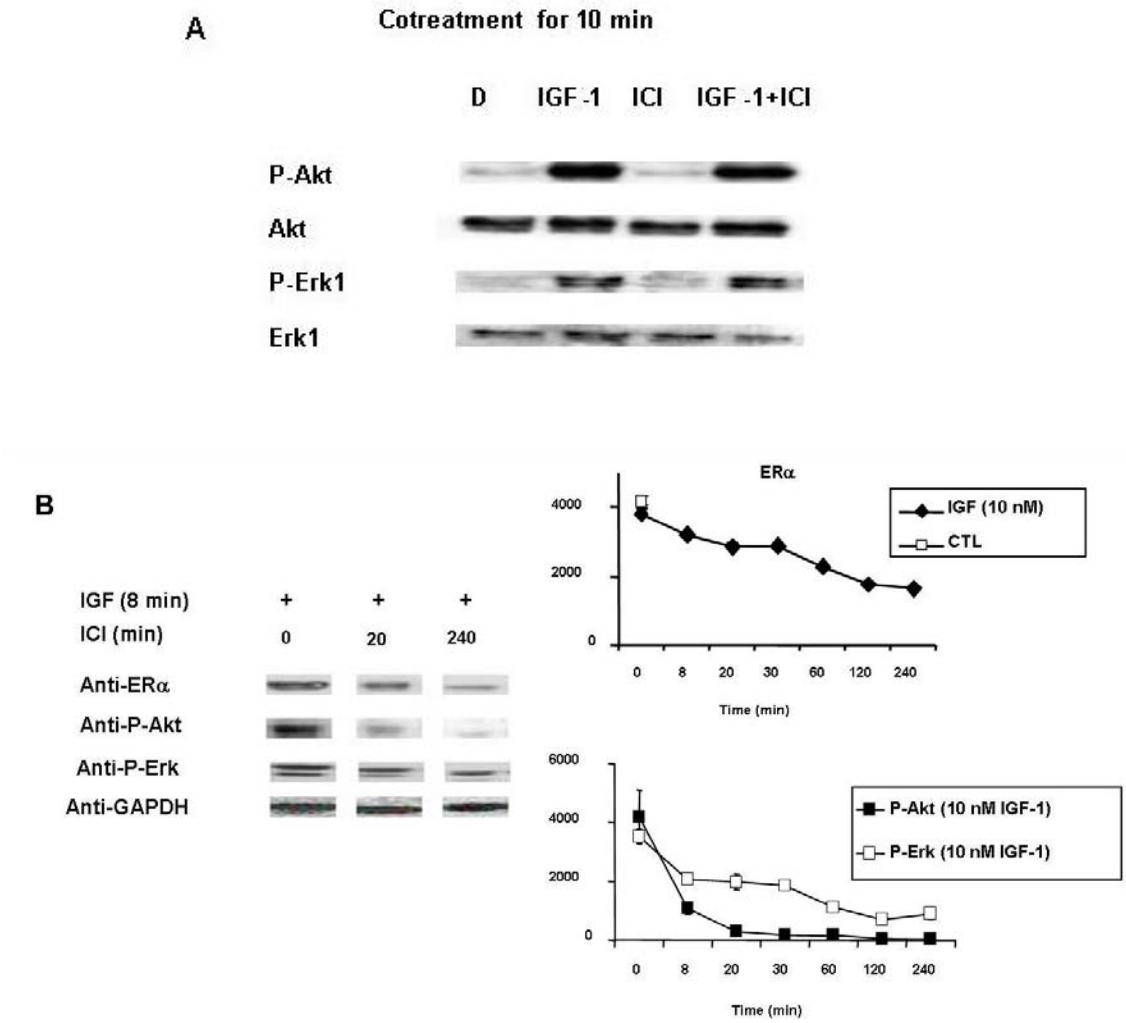

Figure 22 Inhibition of IGF-I-induced responses by ICI 182780. (A) Activation of kinases (short term). Cells were treated with 10 nM IGF-I alone or in combination with 1 μM ICI 182780 for 10 min, and whole-cell lysates were analyzed by Western blot analysis for AKT, p-AKT, Erk-1, and pErk-1 as described in the Materials and methods section. (B) Time-dependent inhibition on IGF-I-induced kinases. MCF-7 cells were pretreated with ICI 182780 for different periods of time and then treated with IGF-I for 10 min, and whole-cell lysates were analyzed by Western blot analysis as described in the Materials and methods section. Band intensities were determined by densitometry as described in the Materials and methods section. Each sample was determined in triplicate, and the data shown are from a representative experiment, and expressed in the bar graphs as means ± S.D. CTL, control.

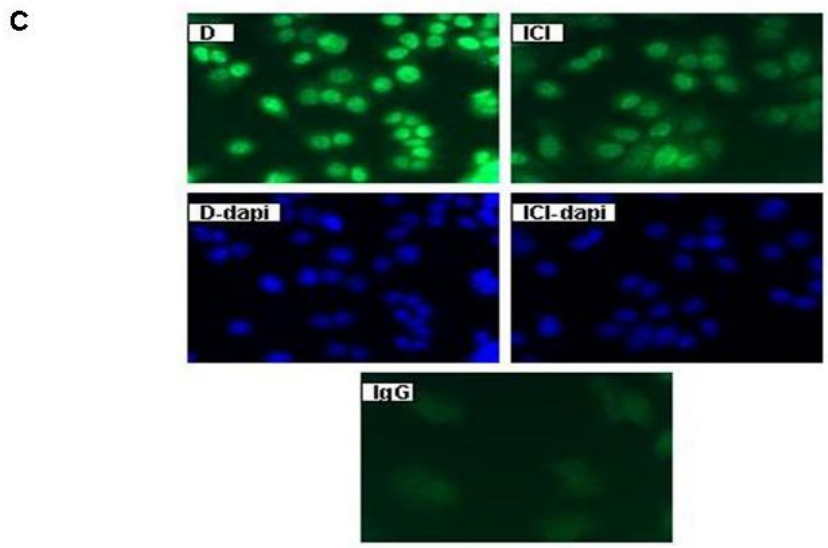

Figure 23 (Continued)

(C) Immunocytochemical analysis of ERα. MCF-7 cells were untreated (DMSO; D) or treated with ICI 182780 (ICI) for 2 h, and stained with ERα antibody or IgG. Immunofluorescence was determined as described in the Materials and methods section. DAPI (4,6-diamidino-2-phenylindole) staining was shown to demonstrate nuclear staining.

2.3.8. ICI 182780 inhibits IGF-I-induced cell-cycle proteins

The effects of ICI 182780 on induction of cyclins D1, A and E by IGF-I were also investigated in MCF-7 cells treated with 10 nM IGF-I (24 h), 1 μM ICI 182,780 (24 h), or their combination (Figure 23). ICI 182780 alone decreased levels of ERα protein but did not significantly affect cyclins D1, A and E, and this was in contrast to the decrease in cyclin D1 and A protein levels in cells transfected with iERα (Figure 21). This suggests that other activities of ICI 182780 may prevent degradation of these proteins. IGF-I alone induced cyclin

D1, A and E proteins; however, in the combined treatment (ICI 182780 plus IGF-I), the antiestrogen significantly inhibited induction of the cyclins by IGF-I. ERα protein was not affected by IGF-I but decreased after combined treatment with ICI 182780. Thus, degradation of ERα by iERα or ICI 182780 is linked to inhibition of IGF-I signaling in MCF- cells, indicating that extranuclear ERα plays an essential role in IGF-I-induced cell proliferation, cell-cycle progression, MAPK and PI3-K activation and gene expression.

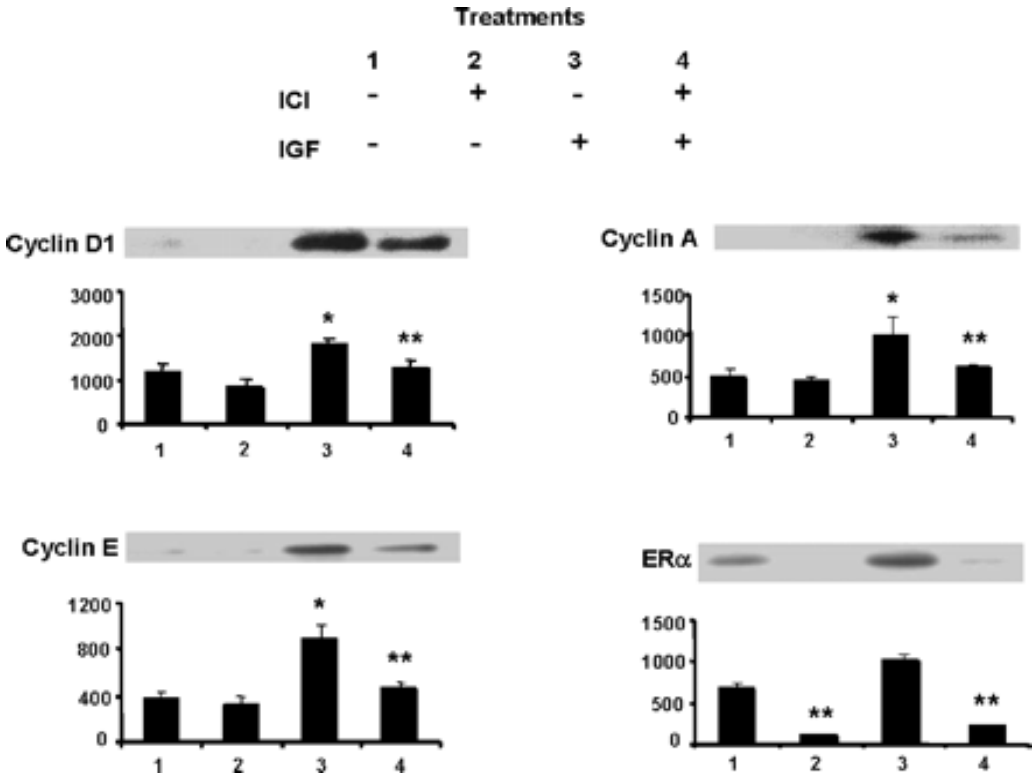

Figure 24 Inhibition of IGF-I-induced cell-cycle proteins by ICI 182780. MCF-7 cells were treated with 10 nM IGF-I and/or 1 μM ICI for 24 h, and whole-cell lysates were analyzed by western blot analysis as described in the Materials and Methods section. The experiment was carried out in triplicate and results from densitometric analysis are plotted as means ± S.D. from three replicates for each treatment group. Significant ($P < 0.05$) increases in band intensity (compared with solvent control) are indicated by * and significant inhibition by ICI 182,780 is indicated by **.

2.4. Discussion

Growth factors play an important role in mammary tumor growth and induce breast cancer cell proliferation through both endocrine and paracrine pathways. IGF-I and other polypeptide growth factors interact with their cognate membrane-bound receptors, which have tyrosine kinase activities, and these receptor–ligand interactions initiate activation of kinase cascades and their downstream nuclear genomic targets. Although growth factors induce mitogenic responses and gene expression in cells/tissues that do not express the ER, in E_2-responsive cells/tissues growth factor-induced responses are tightly coupled with ER expression. For example, in ERα-knockout (ERKO) mice, the effects of EGF and IGF-I are attenuated or suppressed (Curtis et al., 1996; Klotz et al., 2002). EGF induces uterine EGF receptor autophosphorylation and c-Fos expression in ERKO mice, whereas induction of uterine progesterone receptor mRNA and DNA synthesis by EGF were not observed (Curtis et al., 1996; Klotz et al., 2002). IGF-I induces uterine DNA synthesis in wild-type but not ERKO mice; in contrast, IGF-I receptor-dependent activation of PI3-K and MAPK is induced by IGF-I in both wild-type and ERKO mice, suggesting that kinase activation in the absence of ERα is insufficient for uterine proliferation (Klotz et al., 2002). IGF-I induces proliferation of MCF-7 cells (Figure 16) and this response is inhibited by antiestrogens. Moreover, IGF-I does not induce growth of an ERα-negative MCF-7 cell subline; however, re-expression of ERα in these cells restores IGF-I responsiveness (Oesterreich et al., 2001).

One level of IGF–ERα crosstalk involves ligand-independent activation of E_2-responsive genes through selective phosphorylation of ERα (Ali et al., 1993; Bunone et al., 1996; Ignar-Trowbridge et al., 1996; Joel et al., 1998; Kato et al., 1995). This pathway involves genomic or nuclear pathways of ERα action in which growth factor-dependent phosphorylation of the receptor is sufficient for induction of genes that require nuclear ERα–DNA (promoter) or ERα–protein–DNA interactions (Cavailles et al., 1989; Wakeling et al., 1989; Katzenellenbogen and Norman, 1990; Chalbos et al., 1993; Ignar-Trowbridge et al., 1993; Westley and May, 1994; Wang et al., 2000; Xie et al., 2001). IGF-I-induced responses may also involve extranuclear ERα which associates with several key proteins involved in kinase pathways, including the IGF-IR, p85α (PI3-K regulatory subunit), G proteins, Src, and Shc (Kahlert et al., 2000; Migliaccio et al., 2000; Razandi et al., 2002; Simoncini et al., 2000; Song et al., 2002b; Sun et al., 2001; Wyckoff et al., 2001). Inhibition of IGF-I-induced cell proliferation by ICI 182780 and LY294002 (Figure 16) implies that both ERα and PI3-K are necessary for this response. The role of ERα in mediating IGF-I-induced gene expression, kinase activation, and cell-cycle progression was extensively investigated using iERα to efficiently knockdown ERα protein (Figure 17). This approach was then used to demonstrate that ERα was required for IGF-I-dependent activation of MAPK and PI3-K signaling pathways (Figure 19) and subsequent transactivation of SRE- and SRF-dependent promoter–reporter constructs (Figure 18). These results are in contrast to the effects of IGF-I in the

uterus of wild-type and ERKO mice where both Akt and MAPK are phosphorylated after treatment with IGF-I (Klotz et al., 2002). This report (Klotz et al., 2002) also suggested that IRS-I may be important for IGF-I-induced activation of PI3-K, and another study demonstrated that re-expression of ERα in ER-negative MCF-7 cells not only restored IGF-I responsiveness but was accompanied by increased expression of IRS-I and IGF-IR (Ignar-Trowbridge et al., 1996). However, using iIRS-I to knockdown the protein (Figure 19C) it was apparent that induction of MAPK and Akt phosphorylation by IGF-I were unaffected, whereas knockdown of ERα clearly decreased kinase-dependent phosphorylation (Figure 19A and B). Thus, although IRS-I is an important signaling molecule activated by IGF-I in MCF-7 cells (Jackson et al., 1998; Lee et al., 1999; Nolan et al., 1997), our results suggest that IRS-I does not directly regulate IGF-I-dependent activation of MAPK or PI3-K.

The critical role of ERα in mediating the mitogenic activity of IGF-I in MCF-7 cells was also confirmed in ERα-knockdown experiments, which demonstrated that IGF-I-induced G_1–S-phase progression and cell proliferation (Figure 20) and cyclin D1 and E protein expression were dependent on ERα (Figure 21). While these results were consistent with previous reports on the mitogenic activity of IGF-I (Dufourny et al., 1997; Dufourny et al., 2000), these data are in contrast to a study showing that ICI 182780 inhibited IGF-dependent growth of MCF-7 cells but not IGF-I-dependent kinase activation (Varma and Conrad, 2002). For example, ICI 182780 did not inhibit IRS-I, MAPK, or Akt

phosphorylation or cyclin D1 induction in MCF-7 cells treated with 10 nM IGF-I (Varma and Conrad, 2002). These results are surprising since ICI 182780 induces proteasome-dependent down-regulation of ERα in MCF-7 cells (Fan et al., 2003; Varma and Conrad, 2002; Wormke et al., 2003) and RNA-interference studies (Figs 17 –21) clearly demonstrate an essential role for ERα in IGF-I signaling. Moreover, Lee and coworkers (1999) also reported that ICI 182780 inhibited IGF-I-induced phosphorylation of MAPK, IRS-I, and IGF-IR. We therefore further investigated the time-dependent effects of ICI 182780 on IGF-I-induced activation of kinases, and cyclins (Figs 22 and 23). Preliminary studies showed that short-term cotreatment of MCF-7 cells with IGF-I plus ICI 182780 did not decrease IGF-I-induced phosphorylation of MAPK or PI3-K (Figure 22A) as previously reported (Varma and Conrad, 2002). However, longer-term studies with ICI 182780 showed that the inhibitory effects of ICI 182780 were time-dependent. The results clearly show that ICI 182780 inhibits IGF-I-induced cyclin D1, E, and A protein expression (Figure 23), and this was paralleled by inhibition of IGF-I induced phosphorylation of Akt and MAPK (Figure 22B) and IRS-I (data not shown). These data, coupled with ICI 182780-induced degradation of ERα (with or without IGF-I; Figure 22B and C), complement the results of RNA-interference studies with iERα showing that IGF-I action in MCF-7 cells is dependent on crosstalk with extranuclear ERα (Figs 17–21). Although ICI 182780 did not inhibit some IGF-I-induced cell-cycle proteins or Akt/MAPK phosphorylation in the study by Varma and Conrad (2002), they reported that

proliferation of MCF-7 cells induced by IGF-I was inhibited by ICI and this corresponded to results of this study (Figure 16). It was also shown that although ICI 182780 did not affect IGF-I-induced cyclin D1 protein, the antiestrogen inhibited growth factor-dependent upregulation of both cyclin D1 mRNA and cyclin A protein levels (Varma and Conrad, 2002), and the latter response was also observed in this study (Figure 23). The remaining differences between our results and the previous study could also be due to variations in MCF-7 cell passage and origin, serum lot, and the different concentrations of ICI 182780 (1 μM (this study) versus 100 nM (Varma and Conrad, 2002).

In summary, results from this study demonstrate the important role of ERα in mediating the mitogenic activity of IGF-I in MCF-7 cells and demonstrate that there were clear differences between the mechanisms of IGF-I-induced signaling in breast cancer cells and in the mouse uterus (Klotz et al., 2002). Both E_2 and IGF-I induce many comparable responses in MCF-7 and other breast cancer cell lines, and the activities of both mitogens are ER-dependent. However, there are also mechanistic differences in the actions of E_2 and IGF-I. For example, IGF-I-induced activation of an SRE (PI3-K/MAPK) was inhibited by the H1356 polypeptide, which is an IGF-I receptor antagonist, whereas E_2-induced activation of this promoter was unaffected (Duan et al., 2001a; Duan et al., 2002b). A recent study showed that E_2-induced formation of an ERα –Shc–IGF-IR complex in MCF-7 cells, whereas treatment with IGF-I did not recruit ERα to this complex (Song et al., 2002a). Current studies are focused on further

delineating the mechanisms of IGF-I/E_2-induced responses in breast cancer cells and determining the differential role of ERα and its interactions with extranuclear factors.

CHAPTER III

FLAVONOIDS AS ARYL HYDROCARBON RECEPTOR AGONISTS/ANTAGONISTS: EFFECTS OF STRUCTURE AND CELL CONTEXT*

3.1. Overview

Chemoprotective phytochemicals exhibit multiple activities and interact with several cellular receptors, including the aryl hydrocarbon (Ah) receptor (AhR). In this study we investigated the AhR agonist/antagonist activities of the following flavonoids: chrysin, phloretin, kaempferol, galangin, naringenin, genistein, quercetin, myricetin, luteolin, baicalein, daidzein, apigenin, and diosmin. We also investigated the AhR-dependent activities of cantharidin and emodin (in herbal extracts) in Ah-responsive MCF-7 human breast cells, HepG2 human liver cancer cells, and mouse Hepa-1 cells transiently or stably transfected with plasmids expressing a luciferase reporter gene linked to multiple copies of a consensus dioxin-responsive element. The AhR agonist

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*Part of the data reported in this chapter is reprinted with permission from Zhang S, Qin C, and Safe SH. Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell context. Environ Health Perspect. 2003 Dec;111(16):1877-82.

activities of the compounds (1 and 10 μM) were as high as 25% of the maximal response induced by 5 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and their potencies were dependent on cell context. Galangin, genistein, daidzein, and diosmin were active only in Hepa-1 cells, and cantharidin induced activity only in human HepG2 and MCF-7 cells. Western blot analysis confirmed that baicalein and emodin also induced CYP1A1 protein in the human cancer cell lines. The AhR antagonist activities of four compounds inactive as agonists in MCF-7 and HepG2 cells (kaempferol, quercetin, myricetin, and luteolin) were also investigated. Luteolin was an AhR antagonist in both cell lines, and the inhibitory effects of the other compound were dependent on cell context. These data suggest that dietary phytochemicals exhibit substantial cell context–dependent AhR agonist as well as antagonist activities. Moreover, because phytochemicals and other AhR-active compounds in food are present in the diet at relatively high concentrations, risk assessment of dietary toxic equivalents of TCDD and related compounds should also take into account AhR agonist/antagonist activities of phytochemicals.

3.2. Materials and methods

3.2.1. Chemicals, biochemicals, and cells

The compounds used in this study were purchased from Sigma-Aldrich (Milwaukee, WI) and include chrysin (purity > 97%), phloretin (> 95%), kaempferol (> 95%), galangin (95%), naringenin (95%), genistein (98%),

quercetin (99%), myricetin (95%), cantharidin (98%), luteolin (> 90%), baicalein (98%), daidzein (> 95%), emodin (> 90%), apigenin (> 90%), and diosmin (95%). These compounds were used without further purification. All compounds were dissolved in dimethyl sulfoxide (DMSO; 10^{-2} M). Human MCF-7 breast cancer cells and HepG2 liver cancer cells were purchased from the American Type Culture Collection (Manassas, VA). M. Denison (University of California, Davis, CA) kindly provided the mouse Hepa-1 cells stably transfected with a dioxin-responsive element (DRE) promoter derived from the CYP1A1 gene (Garrison et al. 1996). The transient transfection studies used a $pDRE_3$ construct, which contained three tandem consensus DREs (TCT TCT CAC GCA ACT CCG A--a single DRE sequence). The modified pGL2 vector contains a minimal TATA sequence between BglII and HindIII. We synthesized TCDD (purity > 98%) in this laboratory.

3.2.2. DRE-dependent activation by 5 nM TCDD, flavonoids, cantharidin, and emodin

Human MCF-7 cells, HepG2 cells, and stably transfected mouse Hepa-1 cells were maintained in Dulbecco modified Eagle medium (DME) supplemented with 5% fetal bovine serum (FBS), 2.2 g/L sodium bicarbonate, and 10 mL/L antibiotic/antimycotic solution. Cells for transient transfection assays were seeded in DME-F12 medium without phenol red and supplemented with 5% dextran-charcoal-stripped FBS, 2.2 g/L sodium bicarbonate, and 10 mL/L

antibiotic/antimycotic solution. One day after seeding in DME-F12 and 5% stripped FBS, 1.5 μg $pDRE_3$ was transfected into MCF-7 or HepG2 cells by calcium phosphate precipitation. Cells were also cotransfected with pCDNA3.1 ß-galactosidase (ß-gal; 250 ng) (Invitrogen, Carlsbad, CA), which served as a control for transfection efficiency. Sixteen hours after transfection, media were removed, and fresh media containing the appropriate chemicals were added. Cells were grown for an additional 24 hr before harvesting with 200 μL/well of reporter lysis buffer. Lysates were centrifuged at 40,000 ×g, and luciferase and ß-gal activities were determined with 30 μL of the supernatant. Luciferase activity was determined using the luciferase assay system with reporter lysis buffer from Promega Corp. (Madison, WI). ß-Gal activity was determined using the luminescent Galaction-Plus assay system from Tropix (Bedford, MA). The intensity of light emission from assays of cell extracts was determined using a lumicount luminometer (Perkin-Elmer, Boston, MA). Luciferase activity was normalized to ß-gal activity for each treatment. Results are expressed as mean ± SE for at least three determinations for each treatment group, and the fold induction (over DMSO) is shown in the figures.

3.2.3. Western blot analysis

We extracted whole-cell lysates using 1 × Western sampling buffer. Protein samples were heated at 100°C for 5 min, separated on 8% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane (Amersham,

Piscataway, NJ). The PVDF membrane was blocked for 30 min and incubated with 1:1,000 CYP1A1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr at room temperature or with 1:1,000 AhR (Santa Cruz Biotechnology) overnight at 4°C. After vigorous washing for 20 min, 1:3,000 secondary antibody (Santa Cruz Biotechnology) was added, and the membrane was incubated with shaking for 45 min. After washing for 20 min, the membrane was incubated with ECL chemiluminescent substrate (NEN Life Science Products, Inc., Boston, MA) for 1 min, and exposed to Kodak X-Omat AR autoradiography film (Kodak, Rochester, NY). The membrane was reused and probed with the other antibody as indicated.

3.2.4. Statistics

All quantitative data were analyzed by analysis of variance followed by Fisher's protected least-significant-difference test for significance ($p < 0.05$). Data from the transfection studies are expressed as mean ± SE ($n \geq 3$) for each treatment group.

3.3. Results

AhR-mediated induction of CYP1A1 is a sensitive measure of Ah responsiveness. However, many phytochemicals interact with and inhibit CYP1A1 protein catalytic activity (Chen et al. 1996; Shertzer et al. 1999). Therefore, in this study we used a highly sensitive AhR-responsive assay

(Denison et al. 1998) in which ligands activate the bacterial luciferase reporter gene activity in cells transfected with constructs containing multiple DRE promoter elements. Figure 24 illustrates structures of the 15 compounds used in this study; these include 12 flavonoids with different hydroxyl substitution patterns, plus the chemicals phloretin (a dihydrochalcone), cantharidin (a lactone), and emodin (an herbal laxative). Based on results of preliminary studies, we used 5 nM TCDD as a standard that induced maximal luciferase activity in stably transfected Hepa-1 cells (Figure 25) or in transiently transfected MCF-7 (Figure 26) or HepG2 cells (Figure 27). Results from the stably transfected Hepa-1 cells demonstrate their sensitivity to 5 nM TCDD, with a 124-fold inducibility, whereas lower but significant induction was observed for chrysin, galangin, genistein, baicalein, daidzein, emodin, apigenin, and diosmin. Previous studies have also reported that emodin induced AhR-dependent CYP1A1 in human lung adenocarcinoma CL5 cells (Wang et al. 2001), and diosmin was also an AhR agonist in MCF-7 cells (Ciolino et al. 1998b). In contrast, the reported AhR agonist activity of quercetin in MCF-7 cells (Ciolino et al. 1999) was not observed in stably transfected Hepa-1 cells (Figure 24). Galangin exhibited AhR antagonist activity in BU-11, a murine B cell line (Quadri et al. 2000), but AhR agonist activity was observed in stably transfected Hepa-1 cells (Figure 24), and agonist activity of 60 μM galangin has also been observed in Hepa-1 cells (Wang et al. 2001).

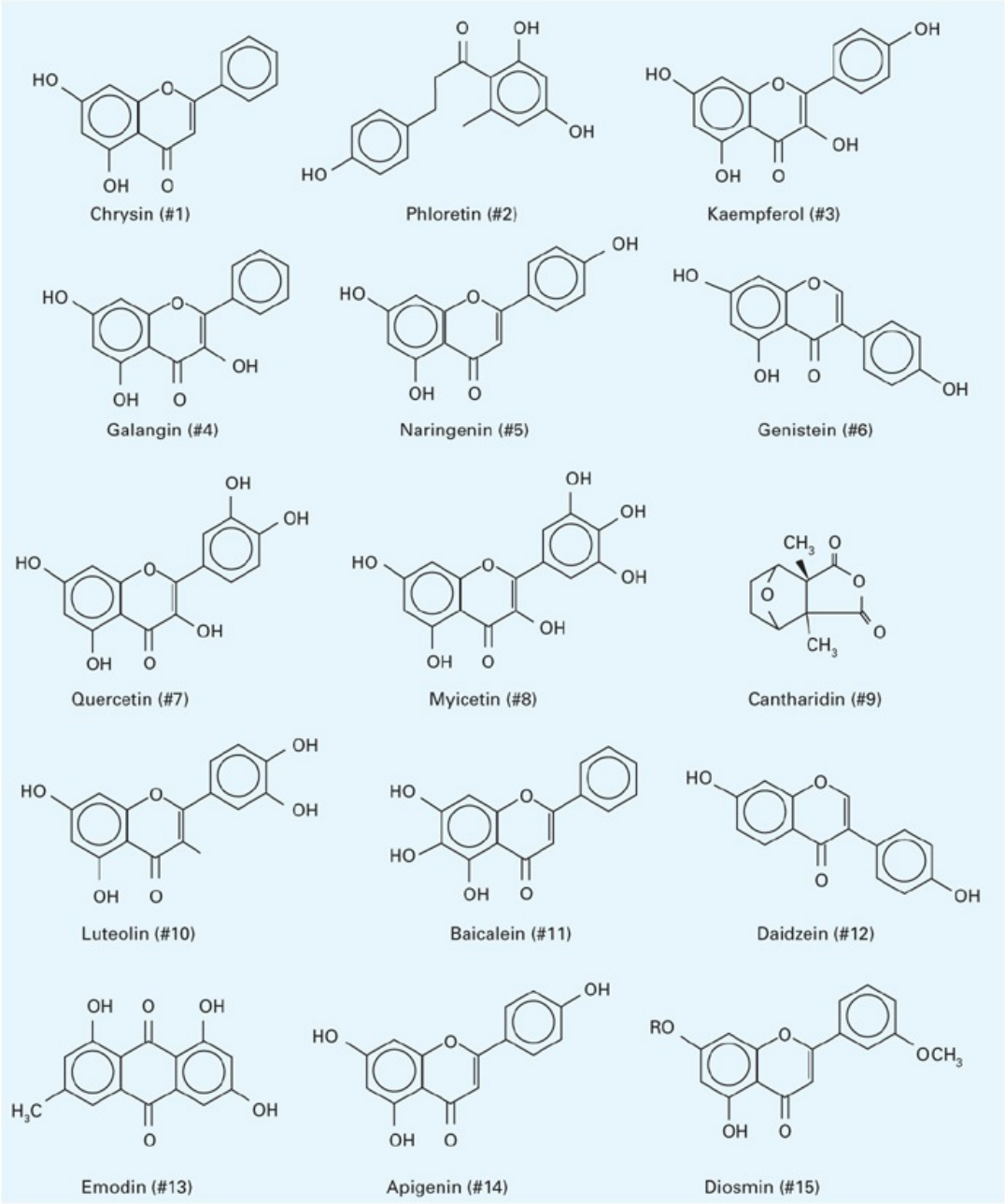

Figure 25 Structures of compounds used in this study.

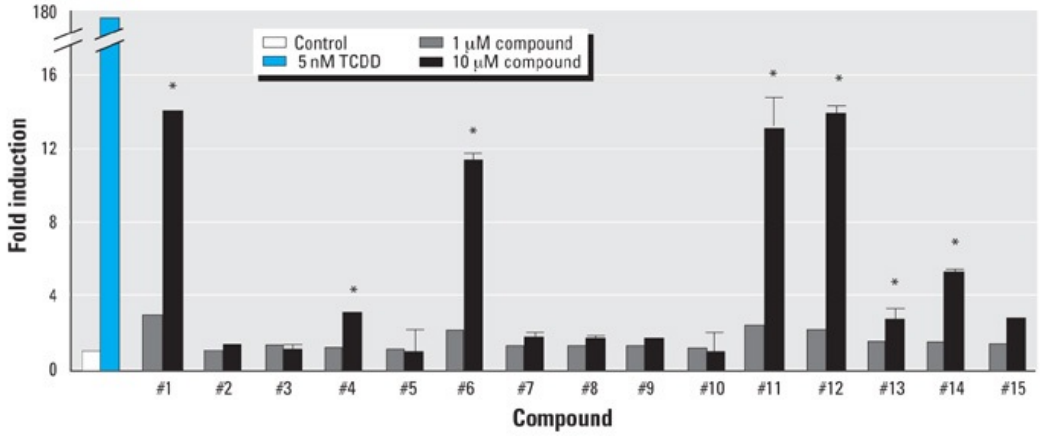

Figure 26 AhR-mediated transactivation in stably-transfected Hepa-1 cells. Cells were treated with DMSO (solvent), 5 nM TCDD, 1 or 10 μM of the test compound for 24 h and luciferase activity was determined as described in the Materials and Methods. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant ($p < 0.05$) induction is indicated with an asterisk. The different compounds #1 - #15 are indicated in Figure 24.

We further investigated the role of cell context in activation of transiently transfected $pDRE_3$ in human MCF-7 and HepG2 cell lines. At concentrations of 1 or 10 μM, only chrysin, cantharidin, baicalein, and emodin activated luciferase activity in MCF-7 cells (Figure 26). With the exception of cantharidin, these compounds were also AhR agonists in stably transfected Hepa-1 cells, and compounds such as galangin, genistein, daidzein, apigenin, and diosmin that were active in Hepa-1 cells did not induce a response in MCF-7 cells. The pattern of induction responses in HepG2 cells was similar to that observed in MCF-7 cells in that chrysin, cantharidin, and baicalein activated gene expression, whereas (10 μM) emodin was not active in this cell line (Figure 27). These data

demonstrate that the AhR agonist activities of structurally diverse phytochemicals and cantharidin, which is derived from insect extract, are highly variable among different cell lines, and that their fold inducibility compared with TCDD is also dependent on cell context. The stably transfected Hepa-1 cells are more highly sensitive to the induction of luciferase activity by TCDD (5 nM) than to the other compounds. TCDD at 5 nM induced a 124-fold increase in luciferase activity, whereas only a 14-fold induction response was observed for 10 μM chrysin. In contrast, 5 nM TCDD and 10 μM chrysin, respectively, induced a 20- and 5.5-fold increase in luciferase activity in MCF-7 cells (Figure 26), and the potency of chrysin relative to TCDD was clearly higher in MCF-7 and HepG2 cells compared with stably transfected Hepa-1 cells.

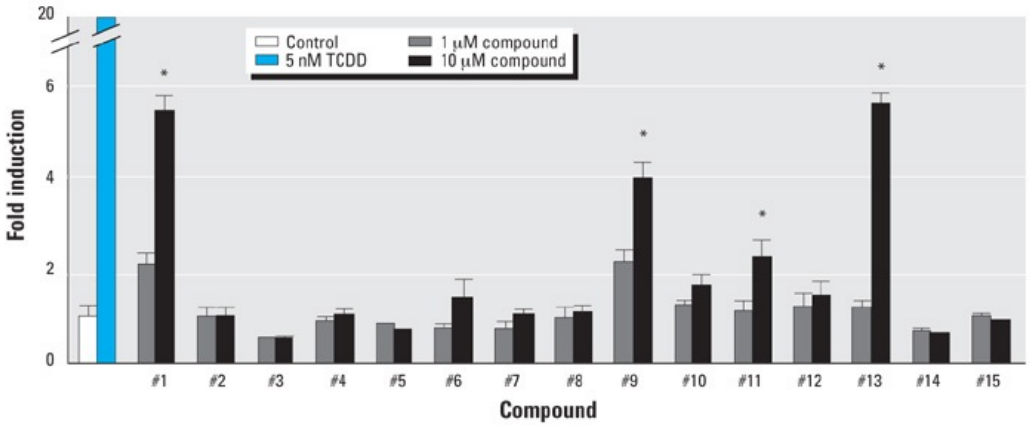

Figure 27 AhR-mediated transactivation in MCF-7 cells. Cells were transfected with $pDRE_3$ treated with DMSO, 5 nM TCDD, 1 or 10 μM of the test compounds, and luciferase activity was determined as described in the Materials and Methods. Results are expressed as means ± SE for each treatment group (3 replicate determinations) and significant ($p < 0.05$) induction is indicated with an asterisk. The different compounds #1 - #15 are indicated in Figure 24.

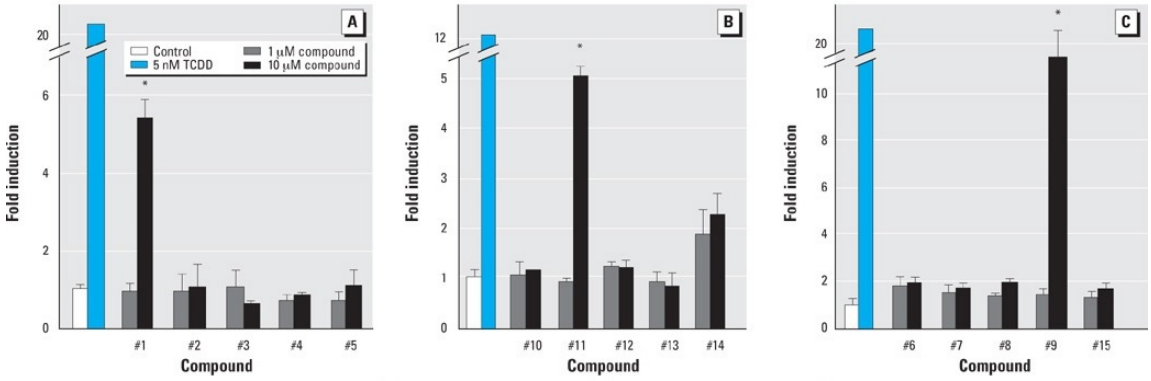

Figure 28 AhR-mediated transactivation in HepG2 cells. The effects of the various compounds as inducers of luciferase activity were determined essentially as described for MCF-7 cells (Figure 26). Results are expressed as means ± SE for 3 separate determinations for each treatment group and significant ($p < 0.05$) induction is indicated with an asterisk. The different compounds #1 - #15 are indicated in Figure 24.

The four compounds that activated luciferase activity in MCF-7 and HepG2 cells (chrysin, cantharidin, baicalein, and emodin) were also investigated as inducers of CYP1A1 protein in these cell lines (Figure 28). The highest nontoxic concentrations of each compound were used in the CYP1A1 protein induction assay because of the decreased sensitivity of this response compared with activation of luciferase activity in the transfected cells. With the exception of cantharidin, higher concentrations could be used because of the short duration (6 hr) of the experiment. Both baicalein and emodin increased CYP1A1 protein at concentrations of 100 μM (MCF-7) or 50 μM (HepG2), whereas chrysin was inactive at the same concentrations (Figure 28). In the nontransfected cells, cantharidin exhibited high cytotoxicity, and CYP1A1 protein was induced only in

MCF-7 cells (Figure 28B). In MCF-7 or HepG2 cells treated with 5 nM TCDD, there was a decrease in AhR protein levels as previously reported (Davarinos and Pollenz 1999; Ma and Baldwin 2000; Roberts and Whitelaw 1999; Wormke et al. 2000). In contrast, treatment with baicalein and cantharidin increased levels of the AhR protein, whereas no effects were observed after treatment with emodin or chrysin (Figure 28).

We also investigated the AhR antagonist activities of four compounds that were inactive in all three cell lines: kaempferol, quercetin, myricetin, and luteolin. Previous studies showed that quercetin was an AhR agonist and kaempferol was an AhR antagonist for induction of AhR-mediated CYP1A1 and DRE-dependent reporter gene activity in MCF-7 cells (Ciolino et al. 1999). However, in this study, cotreatment of MCF-7 cells with kaempferol or quercetin plus 5 nM TCDD resulted in significant inhibition of TCDD-induced luciferase activity at both concentrations (1 and 10 μM) of flavone (Figure 29A). Myricetin (10 μM) slightly decreased activity, whereas luteolin was a potent AhR antagonist. In contrast, 1 or 10 μM quercetin, kaempferol, and myricetin did not affect induction of luciferase activity by TCDD, whereas luteolin was an AhR antagonist in HepG2 cells (Figure 29B, C). These results demonstrate that AhR antagonist activities of these phytochemicals are also dependent on cell context.

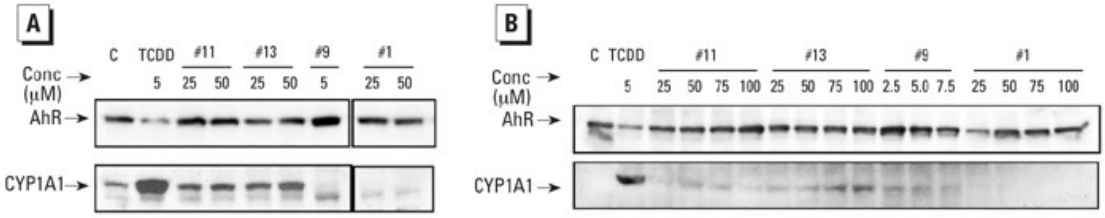

Figure 29 CYP1A1 protein induction by chrysin (#1), cantharidin (#9), baicalein (#11), and emodin (#13) in (A) HepG2 cells or (B) MCF-7 cells. Abbreviations: C, control; Conc, concentration. Cells were treated with DMSO (control), 5 nM TCDD, or different concentrations of the flavonoids for 6 hr. Whole-cell lysates were then prepared, and CYP1A1 and AhR proteins were detected by Western blot analysis as described in "Materials and Methods". These experiments were determined at least two times for each cell line. Comparable results were obtained showing increased CYP1A1 protein after treatment with baicalein (#11; MCF-7/HepG2), emodin (#13; MCF-7/HepG2), and cantharidin (#9; MCF-7). CYP1A1 protein induction was not observed for chrysin (#1).

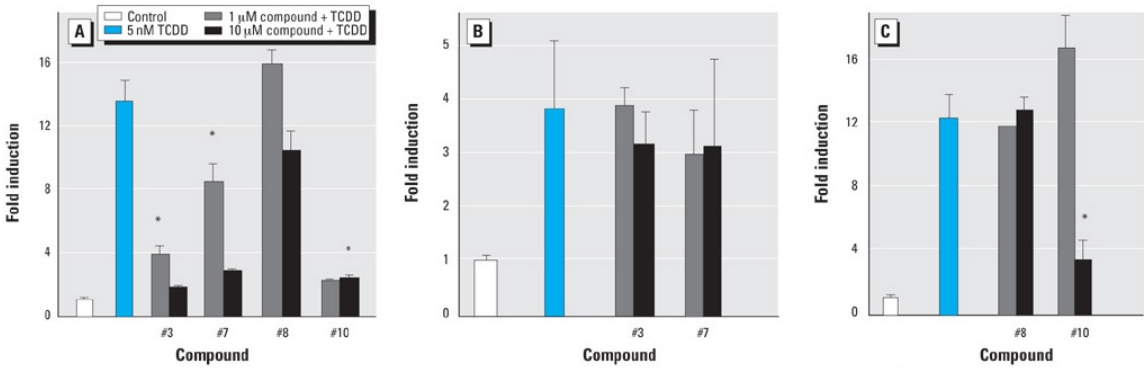

Figure 30 AhR antagonist activities of phytochemicals in MCF-7 and HepG2 cells. [A] MCF-7, [B] and [C] HepG2 cells. Cells were transfected with pDRE3 and treated with DMSO, 5 nM TCDD alone or in combination with 1 or 10 μM concentrations of kaempferol, quercetin, myricetin and luteolin (#3, #7 #8 and #10; Figure 24), and luciferase activities were determined as described in the Materials and Methods. Results are expressed as means ± SE for 3 separate determinations for each treatment group and significant ($p < 0.05$) inhibition in the cotreated groups is indicated with an asterisk.

3.4. Discussion

Results of this study demonstrate that several structurally diverse phytochemicals and cantharidin activate DRE-dependent luciferase (reporter gene) activity in cancer cell lines derived from mouse and human liver and human breast tumors. There are both similarities and differences in the AhR agonist activities of these compounds that are dependent on both structure and cell context. Our results show that TCDD, chrysin, and baicalein induced luciferase activity in all three cell lines. Cantharidin induced luciferase activity only in the human cells (MCF-7 cells, HepG2 cells), emodin was active in Hepa-1 and MCF-7 cells, and galangin, genistein, daidzein, apigenin, and diosmin were active only in stably transfected Hepa-1 cells. Previous studies have demonstrated that many of these compounds exhibit weak AhR agonist and/or partial antagonist activities in transactivation or receptor transformation assays (Ashida et al. 2000; Chun et al. 2001; Ciolino et al. 1998b, 1999; Quadri et al. 2000). However, it is apparent that there were some differences between this and other studies on the AhR agonist or antagonist activities of individual phytochemicals. For example, Ciolino et al. (1999) reported that quercetin and kaempferol exhibited AhR agonist and antagonist activities, respectively, in MCF-7 cells, whereas these compounds exhibited minimal AhR agonist activity in our studies in the same cell line (Figure 26).

There could be several explanations for differences in Ah responsiveness of phytochemicals in the Hepa-1, MCF-7, and HepG2 cells. The stably transfected mouse Hepa-1 cell line was more sensitive than the transiently transfected human MCF-7 and HepG2 cells to TCDD and to most of the phytochemicals. This could due to the stable integration of the construct and the presence of four DREs compared with three DREs in the transiently transfected $pDRE_3$ used in the HepG2 and MCF-7 cell studies (Figures 26 and 27). In addition, the mouse AhR expressed in Hepa-1 cells exhibits higher binding affinity for TCDD than does the human AhR (Ema et al. 1994), and structural differences in the mouse and human AhR may also affect the binding and transactivation activities of the phytochemicals. Chrysin (10 μM) was the most consistent inducer in the reporter gene assays in the three cell lines (Figures 25-27). However, at concentrations as high as 100 and 50 μM in MCF-7 and HepG2 cells, respectively, induction of CYP1A1 protein was not observed (Figure 28). This illustrates the high sensitivity of the reporter gene assays for detecting AhR agonists and suggests that relative compound potencies in this assay may be different for other AhR-mediated responses (Figure 28). This has been observed for TCDD and related compounds that also exhibit species- and response-specific potency differences (Safe 1995). Like the nuclear hormone receptors, ligand-induced activation of the AhR is dependent on interactions with nuclear coregulatory proteins (Beischlag et al. 2002; Kumar et al. 1999; Nguyen

et al. 1999). Nevertheless, results of this and other studies clearly demonstrate that structurally diverse phytochemicals exhibit AhR agonist activities.

We have also investigated interactions of kaempferol, quercetin, myricetin, and luteolin as AhR antagonists in MCF-7 and HepG2 cells (Figure 29) because these compounds alone at concentrations of 1 or 10 μM did not induce luciferase activity in these cell lines (Figures 26 and 27). The results showed that luteolin blocked TCDD-induced luciferase activity in both cell lines, and these results were comparable with the inhibition of TCDD-induced transformation of the rodent cytosolic AhR as previously reported (Ashida et al. 2000; Thenot et al. 1999). The AhR antagonist activities of kaempferol, quercetin, and myricetin were dependent on the cell context (Figure 29). Myricetin exhibited weak (but not significant) antagonist activity only in MCF-7 cells, and both kaempferol and quercetin were also antagonists in MCF-7 but not HepG2 cells. Because many flavonoids activate the estrogen receptor (ER), it is possible that inhibitory ER-AhR crosstalk that has previously been reported (Jeong and Lee 1998; Ricci et al. 1999) may contribute to AhR antagonist activities observed in MCF-7 cells (Figure 29). It is possible that higher concentrations of compounds 1-15 (Figure 24) may exhibit AhR agonist/antagonist activities. However, higher concentrations were not investigated because of cytotoxicity.

Several studies show that phytochemicals weakly activate the AhR in one or more assays and also act as AhR antagonists. These compounds include

kaempferol (Ciolino et al. 1999), resveratrol (Casper et al. 1999; Ciolino and Yeh 1999), galangin (Quadri et al. 2000), rhapontigenin (Chun et al. 2001), indole-3-carbinol (Chen et al. 1996), and diindolylmethane (Chen et al. 1996). Ashida et al. (2000) also showed that ≤25 μM concentrations of various phytochemicals block TCDD-induced transformation of rat liver cytosolic AhR, and these include chrysin, baicalein, apigenin, luteolin, tangeretin, galangin, kaempferol, fisetin, morin, quercetin, myricetin, tamarixetin, isorhamnetin, naringenin, eriodictyol, and hesperitin. Total daily intakes of dietary flavonoids may be as high as 1 g (Verdeal and Ryan 1979), and serum levels of some flavonoids such as quercetin and genistein can be in the nanomolar to low micromolar range. The overall serum concentrations of most phytochemicals in humans is unknown. However, levels are probably in the nanomolar to micromolar range and are dependent on the food product and clearance times for individual compounds. 7-Ketocholesterol is also an AhR antagonist with a competitive binding IC_{50} value (concentration that inhibits 50%) of 500 nM (Savouret et al. 2001), and plasma concentrations of this compound range from 20 to 200 nM in healthy humans (Dzeletovic et al. 1995). This would suggest that many phytochemicals and endogenous compounds with AhR agonist/antagonist activities are present in human serum.

Risk assessment of HA compounds uses the TEF/TEQ approach. For example, daily TEQ intakes of TCDD and related compounds are 50-200 pg in most countries, and these values have substantially decreased over the past 10

years (van Leeuwen et al. 2000). Serum TEQ values are < 5 ppt (lipid weight) or approximately 0.1 pM for TCDD and related compounds, whereas serum levels of some "natural" AhR agonists are in the nanomolar to low micromolar range. Thus, the serum ratios of flavonoids/TCDD TEQs are 10^4 to 10^6, and these ratios are similar to those required for inhibition of TCDD-induced responses by some phytochemicals (Ashida et al. 2000; Chun et al. 2001; Ciolino et al. 1998b, 1999; Quadri et al. 2000). Results shown in Figure 28 demonstrate that 1 μM luteolin inhibited (> 90%) TCDD-induced transactivation in MCF-7 cells at flavonoid/TCDD ratios as low as 200/1. Moreover, ratios of PCB 153/TCDD TEQs in human tissues are also > 10^4, which is comparable with ratios required for PCB 153-mediated inhibition of several TCDD-induced biochemical and toxic responses (Safe 1998a, 1998b). It is likely that dietary intakes of most phytochemicals would be below levels required for an AhR agonist response based on results from cell culture studies. The potential chemoprotective effects of the expanding list of AhR-active phytochemicals and related compounds on TCDD-TEQ-mediated adverse responses should be further investigated in *in vivo* models. These results can then be used for development of recommended dietary TCDD-TEQ values that reflect the combined intake of HA compounds plus high levels of "natural/phytochemical" AhR antagonists/agonists.

CHAPTER IV

LIGAND-DEPENDENT INTERACTIONS OF THE AH RECEPTOR WITH COACTIVATORS IN A MAMMALIAN TWO-HYBRID ASSAY

4.1. Overview

Halogenated aromatic hydrocarbons (HAHs) such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8- pentachlorodibenzo-p-dioxin (PeCDD), 3,3',4,4',5-pentachlorobiphenyl (PCBP), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) bind and activate the aryl hydrocarbon receptor (AhR). HAHs and many other structurally-diverse AhR agonists including chemoprotective phytochemicals induce CYP1A1, however, induction of many other responses are ligand structure and cell context-dependent and several selective AhR modulators (SAhRMs) have been identified. We hypothesize that HAHs may also exhibit SAhRM-like activity, and this was investigated by determining ligand structure-dependent differences in the interaction of AhR with a series of coactivators in mammalian two-hybrid assays. TCDD, PeCDD, TCDF, PeCDF and PCBP induced CYP1A1 in several Ah-responsive cancer cell lines including mouse Hepa-1, human Panc1 and human HEK293 cells. However, there was structure-dependent activation of luciferase activity in these same cells transfected with VP-AhR and GAL4-coactivator (SRC-1, SRC-2, SRC-3, CARM-1, PGC-1 and TRAP220) chimeras and a GAL4-response element-luciferase promoter

construct. In Panc1 cells, TCDD and PeCDD induced transactivation in cells transfected with GAL4-TRAP220 and VP-AhR constructs; and TCDD but not PeCDD also induced activity in cells transfected with GAL4-CARM-1. In contrast, ligand induced AhR-coactivator interactions were not observed for any other coactivators in a mammalian two-hybrid assay in Panc1 cells. The most striking difference in ligand-induced AhR-coactivator interactions in this assay was observed in HEK293 cells where TCDD induced activity only in cells transfected with the GAL4-CARM-1 chimera. In contrast, the other HAHs did not induce AhR interactions with any of the coactivators. Current studies are evaluating the ligand-dependent activity of CARM-1 as a coactivator of AhR-mediated responses. These results suggest that HAHs selectively interact with coactivators and these interactions are dependent on cell-context.

4.2. Materials and methods

4.2.1. Cell lines, constructs and antibodies

Panc1 and HEK293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Hepa1c1c7 cells were kindly provided by Dr. Yanan Tian (Texas A&M University, TX). The $pDRE_3$-luciferase reporter plasmid was constructed in this laboratory and contains three tandem consensus dioxin response elements (DRE) (TCT TCT CAC GCA ACT CCG A—a single DRE sequence). The Gal4 reporter containing 5x Gal4DBD (Gal4Luc) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). Gal4-

coactivator fusion plasmids pM-SRC-1, pM-AIBI, pM-TIFII, pM-DRIP205, pM-TRAP220, and pM-CARM-1 were obtained from by Dr. Shigeaki Kato (University of Tokyo, Tokyo, Japan). VP-AhR was provided by Dr. Gary Perdew (Penn State Univerisity, PA). Antibodies for CYP1A1, AhR and Arnt proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for β-actin was obtained from Sigma (St. Louis, MO).

4.2.2. Transient transfection assays

Cells were cultured in 12-well plates in 1 ml of DME/F12 medium supplemented with 2.5% fetal bovine serum. After 16-20 h when cells were 30-50% confluent, the pDRE-luc(0.4 ug) or GAL4-coactivator/GAL4-luc(0.4 ug), β-galactosidase(0.1 ug) constructs were transfected using lipfectamine2000 Reagent (Invitrogen, Carlsbad, CA). Cells were harvested 36-44 h after transfection by manual scraping in 1X lysis buffer (Promega, Madison, WI). For whole-cell lysates, cells were frozen and thawed in liquid nitrogen, vortexed for 30 s, and centrifuged at 12 000 ***g*** for 1 min. Lysates were assayed for luciferase activity using luciferase assay reagent (Promega, Madison, WI). β-galactosidase activity was measured using Tropix Galacto-Light Plus assay system (Tropix, Bedford, MA, USA) in a Lumicount microwell plate reader (Packard Instrument Co.).

4.2.3. Western immunoblot analysis

Cells were seeded into 35-mm six-well tissue culture plates in phenol red-free DME/F12 medium supplemented with 2.5% dextran/charcoal-stripped fetal bovine serum. After 24 h, cells were treated with 5 HAHs and DMSO control for 24 h, respectively, and harvested in ice-cold high salt lysis buffer (50 mM HEPES, 500 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, pH 7.5) supplemented with protease inhibitor cocktail (Sigma). An aliquot of whole cell lysates containing 30 μg protein was diluted with loading buffer, boiled, and loaded on a 10% SDS-polyacrylamide gel. Samples were electrophoresed at 150-180 V for 3-4 h, and separated proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Proteins were detected by incubation with polyclonal primary antibodies CYP1A1, AhR, Arnt and β-actin (1:1000 dilution), respectively, followed by blotting with horseradish peroxidase-conjugated anti-rabbit (for CYP1A1, AhR and Arnt) or anti-mouse (for β-actin) secondary antibody (1:5000 dilution).

4.2.4. Quantitative real-time PCR

Cells were seeded into 35-mm six-well tissue culture plates in phenol red-free DME/F12 medium supplemented with 2.5% dextran/charcoal-stripped fetal bovine serum. After 24 h, cells were treated with 5 HAHs and DMSO control respectively for 24 h, and then cells were collected and total RNA was isolated using the RNeasy Protect Mini kit (QIAGEN, Valencia, CA) according to the

manufacturer's protocol. RNA was eluted with 30 µl of RNasefree water and stored at -80°C. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA obtained from reverse transcription using a combination of oligodeoxythymidylic acid and dNTP mix (Applied Biosystems, Foster City, CA) and Superscript II (Invitrogen). Each PCR was carried out in triplicate in a 25-µl volume using SYBR Green Master mix (Applied Biosystems) for 15 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min in the ABI Prism 7700 sequence detection system (Applied Biosystems). The ABI Dissociation Curves software was used after a brief thermal protocol (95°C 15 s and 60°C 20 s, followed by a slow ramp to 95°C) to control for multiple species in each PCR amplification. Primers used in the experiment are human CYP1A1 (forward: 5'-TGG TCT CCC TTC TCT ACA CTC TTG T-3; reverse: 5'-ATT TTC CCT ATT ACA TTA AAT CAA TGG-3'), mouse CYP1A1 (forward: 5'-CAC CAT CCC CCA CAG CAC-3'; reverse: 5'-ACA AAG ACA CAG CAC CCC TT-3') and TBP (forward: 5'-TGC ACA GGA GCC AAG AGT GAA-3'; reverse: 5'-CAC ATC ACA GCT CCC CAC CA-3'). Primers were purchased from Integrated DNA Technologies (Coralville, IA).

4.2.5. Statistical analysis

Statistical significance was determined by analysis of variance and Scheffe's test, and the levels of probability are noted. The results are expressed

as means ± SD for at least three separate (replicate) experiments for each treatment group.

4.3. Results

2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF and PCB126 are structurally-related HAHs that binds the AhR and induce AhR-mediated biochemical and toxic responses in different tissues/cell lines. Relative potency factors for all 5 compounds have been established, however, for each compound there is a range of potency values (relative to 2,3,7,8-TCDD), which is species, age, sex and tissue/cell-specific (Van den Berg et al., 1998; Van den Berg et al., 2006). Results in Figure 30 summarize the concentration dependent induction of CYP1A1 protein by the 5 HAHs in human Panc1 pancreatic cancer cells, human HEK293 embryonic kidney cells and Hepa1c1c7 mouse hepatoma cells. Similar patterns of CYP1A1 protein induction were observed in all 3 cell lines and these results are consistent with expression of both AhR and Arnt in these cells. In addition, we also observed ligand-dependent repression of AhR in Panc1 cells whereas this response was less obvious in Hepa1c1c7 and HEK293 cells. Using concentrations that maximally induced CYP1A1 and correlated with relative potency factors, we also examined ligand-dependent induction of CYP1A1 mRNA levels by quantitative real-time PCR (Figs 31A-C). In panc1 and HEK293 cells a similar fold-induction response was observed for 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF whereas ≤50% of this response

was induced by 2,3,7,8-TCDF and PCB126. This pattern of results was replicated in both cell lines and similar results were observed for lower concentrations of both compounds (data not shown). Higher concentrations of 2,3,7,8-TCDF and PCB126 were cytotoxic to these cells. In contrast, all 5 compounds induced maximal CYP1A1 mRNA levels in Hepa1c1c7 cells (Figure 31C) demonstrating some cell context dependent differences in the efficacy of these HAHs as inducers of CYP1A1 mRNA.

We also used a third “induction” response to compare the 5 HAHs and cells were transiently transfected with a pDRE3-luc construct which contains three tandem consensus human DREs linked to luciferase gene. The concentration-dependent induction of luciferase activity by the 5 HAH congeners in Panc1 and HEK293 cells (Figure 32A and 32B) showed that the fold-induction response for 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF was higher than observed for 2,3,7,8-TCDF and PCB126. In contrast, comparable fold-induction responses were observed for all 5 HAHs in Hepa1c1c7 cells (Figure 32C) and the ligand structure- and cell context-dependent differences observed for induction of luciferase activity was similar to that observed for induction of CYP1A1 mRNA levels (Figure 31).

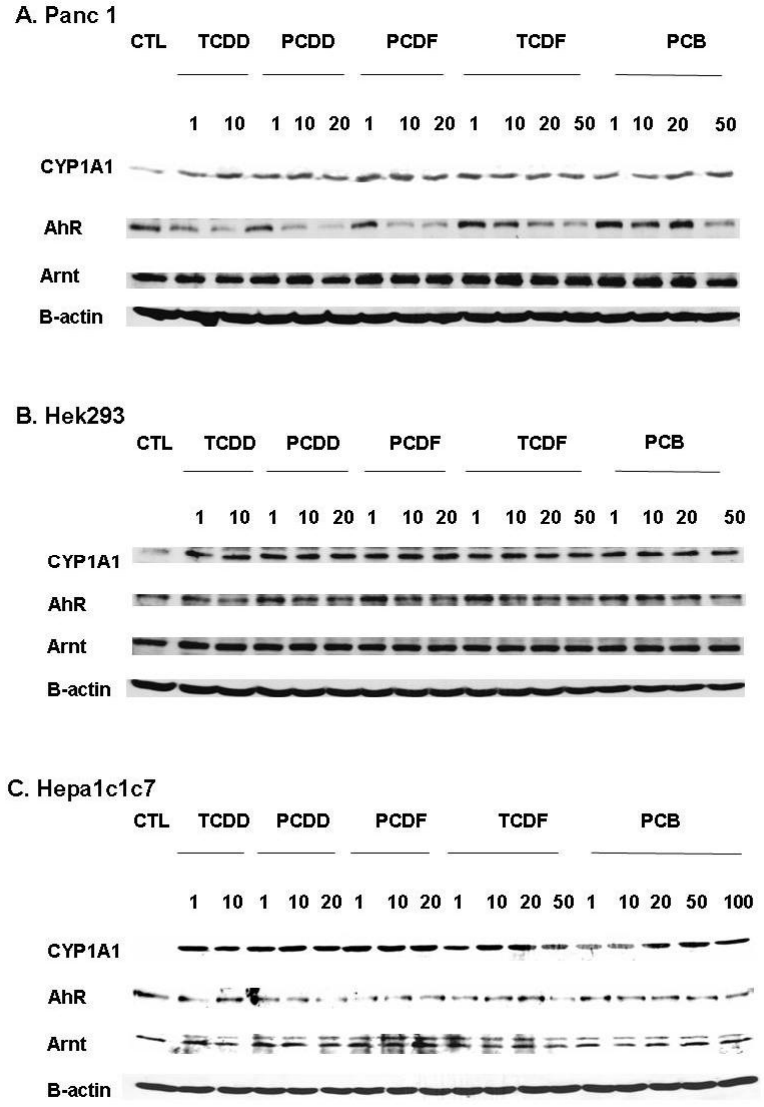

Figure 31 Effects of HAHs on induction of CYP1A1 protein. Panc1 (A), Hek293 (B) and Hepa1c1c7 (C) cells were treated with different concentrations of the five compounds for 24 h, and CYP1A1 protein levels were determined by Western blot analysis of whole cell lysates as decribed in the *Materials and Methods.*

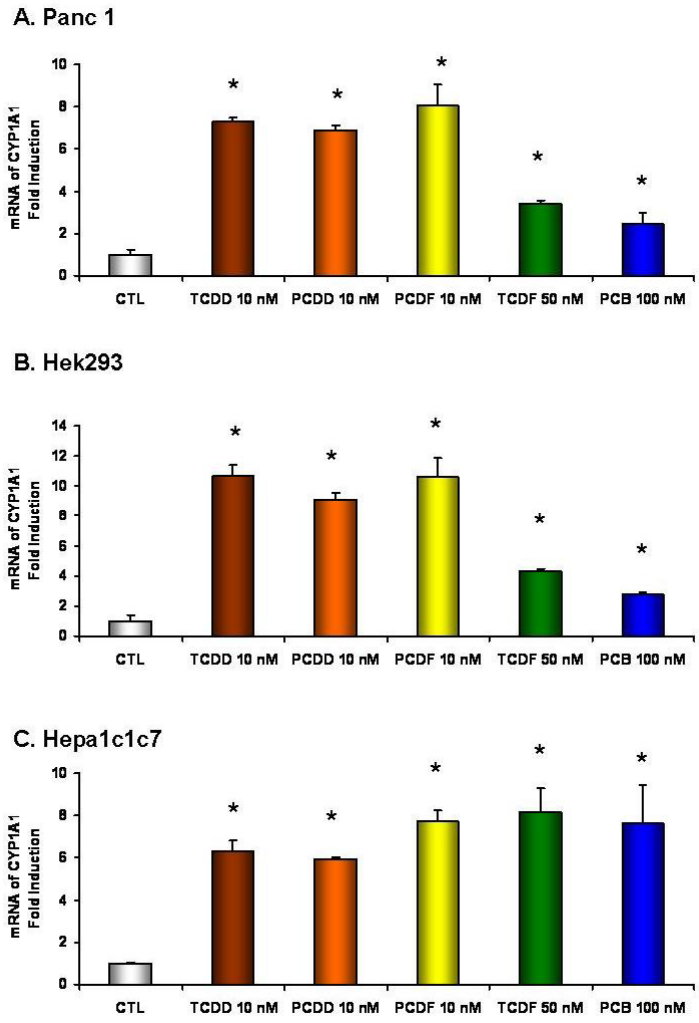

Figure 32 TCDD and structurally related four HAHs induce CYP1A1 transcription. Panc1 (A), Hek293 (B) and Hepa1c1c7 (C) cells were treated with the five compounds for 24 h, respectively, and mRNA levels were determined by real-time PCR described under *Materials and Methods*. Results are given as means ± S.E. for three replicate determinations for each treatment group, and all of the five compounds significantly ($p < 0.05$) increased activities (compared to solvent control) and are indicated by an *asterisk*. The concentration for each compound used in this study was not cytotoxic and maximally induced CYP1A1 (Figure 30).

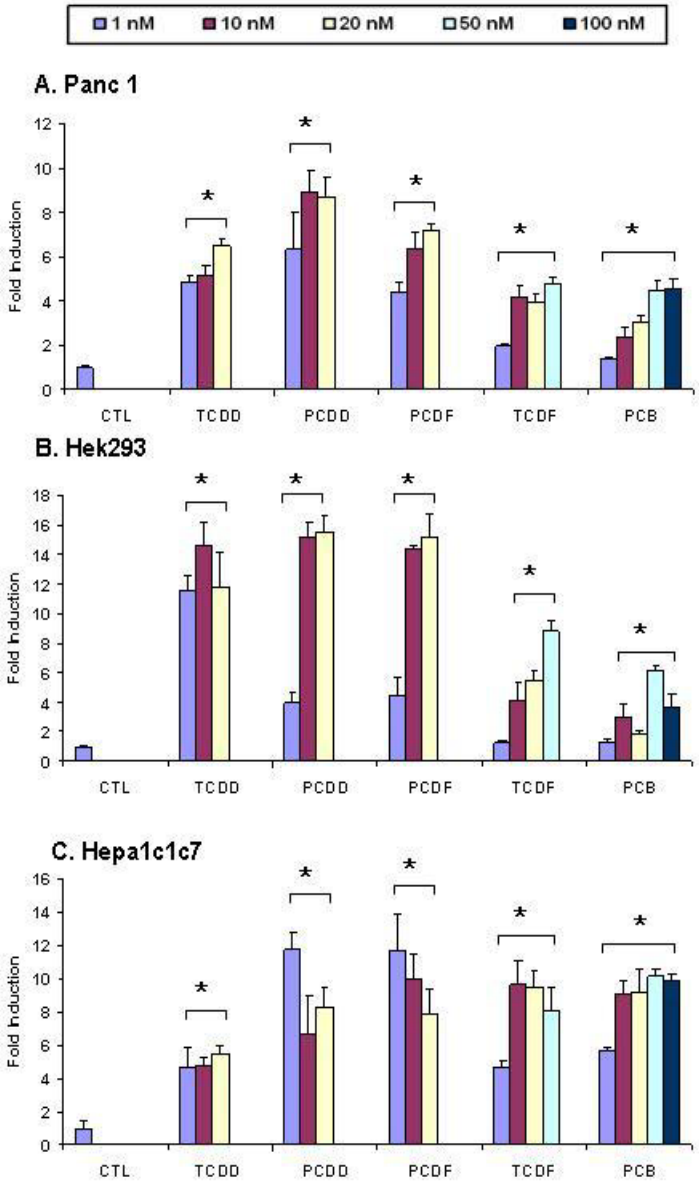

Figure 33 Effects of TCDD and other HAHs on activation of the Ah-responsive pDRE3-Luc. iPanc1(A), Hek293(B) and Hepa1c1c7(C) cells were transfected with Ah-responsive pDRE3-Luc construct and treated with DMSO, TCDD and four other HAHs at different concentration for 24 h, and luciferase activity was determined as described under the Materials and Methods. Results summarized in A, B and C are means ± SD for three replicate determinations for each treatment group, and all the five compounds significantly ($p < 0.05$) increased luciferase activity (compared to solvent control) and are indicated by an *asterisk*.

A mammalian two hybrid assay in cells transfected with GAL4-coactivator and AhR-VP16 chimeras was used to investigate structure-dependent interactions of these chimeras in Panc1, HEK293 and Hepa1c1c7 cells. The reporter construct, pGAL4-luc contains 5 tandem GAL4 response elements linked to a luciferase gene and the GAL4-coactivator chimeras contain the DNA binding domain of the yeast GAL4 gene linked to the full length coactivator. The VP16-AhR chimera contains the powerful C-terminal 80 amino acid transactivator from herpes simplex virus protein 16 (VP16) and the ligand binding domain of the AhR (Ko et al., 1997). Results in Figure 33 illustrate the concentration-dependent induction responses in HEK293 cells treated with the 5 HAHs and transfected with the 6 different GAL4-coactivator chimeras used in this study. The range of HAH concentrations used in this assay was similar to those used for the studies illustrated in Figure 30-32.

The approach illustrated in figure 33 was employed for all 3 cell lines and Figure 34 summarizes the ligand structure-dependent induction responses observed in these cells transfected with GAL4-SRC-1, GAL4-CARM-1, GAL4-TRAP220 and GAL4-TIFII. The HAH congeners did not induce transactivation in any of the cells line transfected with the GAL4-PGC-1 or GAL4-SRC3 chimeras. In Panc1 cells, increased activity (with variable significance) was observed for both 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD in cells transfected with all 4 GAL4-chimeras, whereas among the 2 PCDF congeners and PCB126, only the latter compound induced transactivation and this was observed for GAL4-TRAP220

and not the other GAL4-coactivation chimeras. A different pattern of ligand-dependent transactivation was observed in HEK293 cells (Figure 34B), 2,3,7,8-TCDD induced luciferase activity in cells transfected with GAL4-SRC-1, GAL4-CARM-1, GAL4-TRAP220 but not GAL4-TIFII; 2,3,4,7,8-PeCDF activated only GAL4-SRC-1 and GAL4-TIFII and the remaining compounds were inactive in cells transfected with all 4 GAL4-coactivator chimeras. Structure-dependent transactivation in Hepa1c1c7 was also highly variable; 2,3,7,8-TCDD, 2,3,7,8-TCDF and PCB126 activated GAL4-SRC-1; only PCB126 activated GAL4-CARM-1 and 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF activated GAL4-TIFII whereas none of the HAHs activated GAL4-TRAP220. These results clearly demonstrated that activation of pGAL4-luc in cells transfected with VP-AhR and GAL4-coactivator chimeras is dependent on the structure of the HAH congener, cell context and coactivator suggesting that the prototypical HAH congeners used in this study exhibit selective AhR modulation activity.

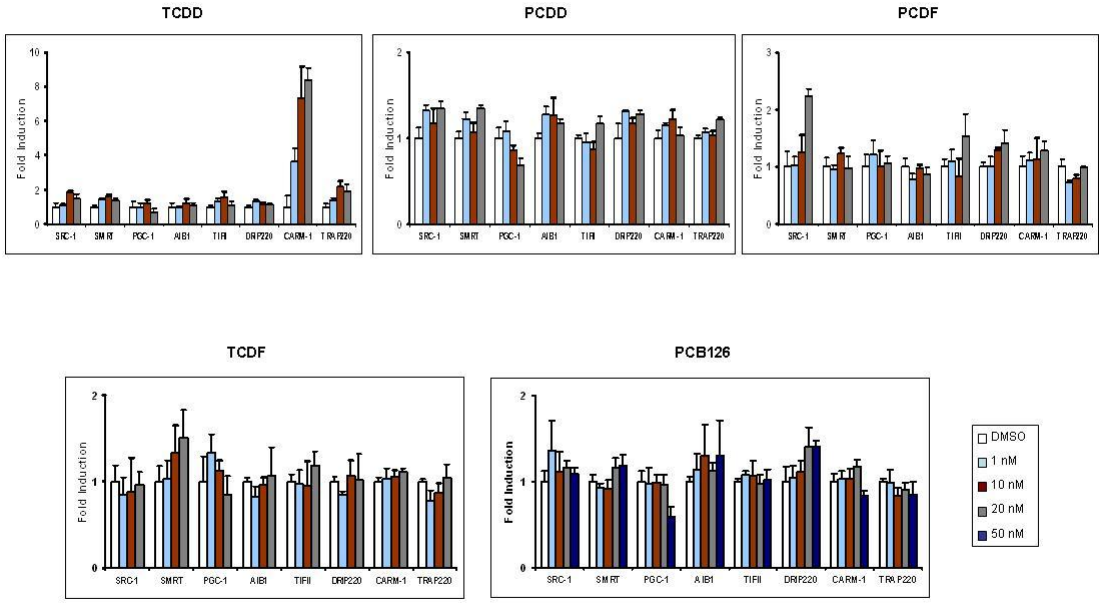

Figure 34 Structure dependent induction of luciferase activity in a mammalian two-hybrid assay in Hek293 cells. Cells were transfected with Gal4luc, Gal4-coactivators and AhR-VP16 constructs and treated with DMSO and different concentration of TCDD(A), PCDD(B), PCDF(C), TCDF(D) or PCB(E) for 24 h, and luciferase activity was determined as described under the Materials and Methods. Results are expressed as means±SD for 3 separate determinations for each treatment group and significant ($p < 0.05$) induction compared to solvent control is indicated (*).

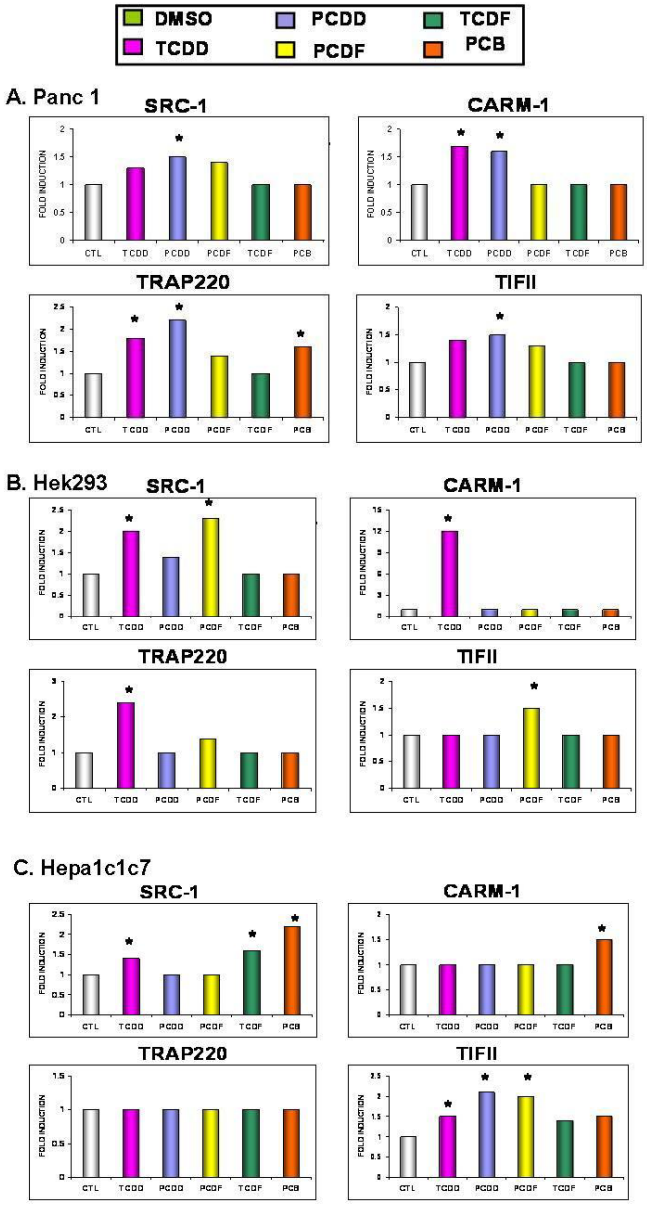

Figure 35 Structure dependent activation of luciferase activity in a mammalian two-hybrid assay. Panc1 (A), Hek293 (B) andHepa1c1c7 (C) cells were transfected with Gal4luc, Gal4-coactivators and AhR-VP16 constructs and treated with DMSO, different concentration of TCDD and four other HAHs for 24 h, and luciferase activity was determined as described under the Materials and Methods. Significant ($p < 0.05$) induction (*) was only observed at the maximal concentration of TCDD (10 nM), PCDD (20 nM), PCDF (20 nM), TCDF (20 nM) and PCB (50 nM) used in this study.

4.4. Discussion

Coactivators and other nuclear cofactors play a critical role in activation of gene expression by serving as part of a budging complex between specific transcription factors and the basal transcription machinery and DNA polymerase II. Many coactivator interactions with NRs are ligand-dependent and the interactions of steroid receptor coactivators (SRCs) through their LXXLL (NR-box) motifs with the ER and other steroid hormone receptors have been extensively investigated (Hall et al., 2002; Roeder, 2005). Several assay methods have been used to demonstrate differences and similarities between diverse structural classes of estrogenic compounds. Some of these assays include activation of wild-type and variant forms of ERα and ERβ, activation of different E2-responsive promoter constructs and the differential ligand-dependent effects of various LXXLL peptides on ER-coactivator interactions (Hall et al., 2002; Matthews et al., 2002; Routledge et al., 2000; Schaufele et al., 2000). In addition, a mammalian two-hybrid assay using GAL4-coactivator and VP-ER chimeras have also been using to show ligand-dependent ER-coactivator interactions (Mueller et al., 2004; Nishikawa et al., 1999).

The advantages of a mammalian two-hybrid assay is that this system is highly flexible and can be transfected into different cell lines and is not dependent on over or underexpression of an endogenous coactivator since the GAL4-coactivator chimera is transfected directly into the cells. In addition,

promoter complexities are minimized since the GAL4-coactivator activates a yeast pGAL4-luc promoter construct in a mammalian cell context. However, an important feature of this assay is that the effects of cell context on transactivation can be determined in this assay since ligand-dependent GAL4-coactivator-VP-receptor interactions also rely on endogenous cofactors for transactivation.

Several studies report the NR receptor coactivators and corepressors and other nuclear cofactors also interact with and modulate ligand-dependent AhR-mediated transactivation (Rowlands et al., 1996; Kobayashi et al., 1997; Kumar and Perdew, 1999; Kumar et al., 1999; Nguyen et al., 1999; Antenos et al., 2002; Beischlag et al., 2002; Wang and Hankinson, 2002; Beischlag et al., 2004; Kim and Stallcup, 2004; Wang et al., 2004; Chen et al., 2006; Kim et al., 2006). For example SRC-1, SRC-2 (TIFII) and SRC-3 (AIB1) enhance TCDD-induced transactivation in Hepa-1 cells (Beischlag et al., 2002); knockdown of TRAP220 by RNA interference decreased TCDD-induced transactivation in human HepG2 cells (Wang et al., 2004) and CARM-1 also enhance AhR-mediated gene expression (Yanan, Tian, personal communication).

In this study we used a mammalian two-hybrid assay in Panc1, HEK293 and Hepa1c1c7 cells to investigate possible ligand structure- and cell context-dependent differences in GAL4-coactivator-VP-AhR interactions using five coactivators (SRC-1, SRC-2, SRC-3, TRAP220 and CARM-1) known to

enhance AhR-mediated gene expression. We also selected PGC-1 for these studies since PGC-1 coactivates gene expression mediated by several NRs.

The five HAH ligands induced CYP1A1 protein and mRNA levels in all these cell lines and also induced luciferase activity in cells transfected with pDRE3 (Figures 30-32). Inducibility of CYP1A1 by HAHs and other AhR agonists is probably the most characteristic response observed for these compounds in multiple tissues/cell-types. The only major differences between these compounds was observed between 2,3,7,8-TCDD, 12,3,7,8-PeCDD, 2,3,4,7,8-PeCDF which induced maximal CYP1A1 mRNA/luciferase activity and 2,3,7,8-TCDF and PCB126 which induced submaximal responses in Panc1 and HEK293 cells (Figures 31 and 32). Higher concentrations of the latter two congeners were cytotoxic and did not enhance activity and lower concentrations also did not induce CYP1A1 mRNA or luciferase activity in any of the cell lines. Previous studies with HAHs and PAHs have also observed structure-dependent differences in maximal induction of CYP1A1 mRNA and/or CYP1A1-dependent activity, however, these effects are variable and cell context-dependent. For example, both 2,3,7,8-TCDD and PCB126 induced similar maximal-induction responses in rat H4IIE cells whereas in human HepG2 cells differences were similar to those illustrated in Figures 31 and 32 (Zeiger et al., 2001). Moreover, in COS7 cells transfected with a DRE promoter and AhR/Arnt from different species the maximal was highly variable and depended on the source species of the AhR (Abnet et al., 1999; Yang et al., 2006).

Results of the mammalian two-hybrid assay with the 5 HAHs (Figs 33 and 34) clearly show that HAH-induced GAL4-coactivators-VP-AhR interactions were both structure and cell context dependent. One common feature for all 5 HAHs in the three cell lines was that interactions of VP-AhR with GAL4-PGC-1 or GAL4-SRC-3 were not observed. This was somewhat surprising for GAL4-SRC3 since this coactivator interacts with AhR and 2,3,7,8-TCDD induces recruitment of SRC-3 to the DRE enhance region of the mouse CYP1A1 promoter in Hepa1c1c7 cells (Kumar et al., 1999). Nevertheless in this study significant HAH-induced GAL4-SRC-3-AhR-VP interactions were not observed in Hepa1c1c7, HEK293 or Panc1 cells. Examination of the transactivation results (Figure 34) clearly illustrates marked structure-dependent differences of the HAHs as inducers of transactivation in the 3 cell lines. 2,3,7,8-TCDD, which is the most avid ligand for the AhR does not uniformly exhibit the highest potency for inducing GAL4-coactivator-AhR-VP interactions in the various cell lines and in no cell line does TCDD induce transactivation in cells transfected with all four GAL4-coactivator chimeras. Figure 35 illustrates some of the cell context-dependent differences and similarities between the 5 HAHs in activation of specific coactivator-AhR interactions. For example, only 2,3,7,8-TCDD induces transactivation in HEK293 cells transfected with GAL4-TRAP220 whereas none of the HAHs induced transactivation in Hepa1c1c7 cells transfected with the same construct (Figure 35A). 2.3.7.8-TCDD but not the 4 other HAHs induced transactivation in HEK293 cells transfected with Gal4-CARM-1 whereas in

Hepa1c1c7 cells only PCB126 induced luciferase activity (Figure 35B). In contrast, a similar structure-dependent induction of transactivation by the HAHs was observed in Hepa1c1c7 and Panc1 cells transfected with GAL4-TIFII (Figure 35C).

In summary, results of this study demonstrate that 5 HAHs induce CYP1A1 or DRE-dependent gene expression in HEK293, Panc1 and Hepa1c1c7 cells with some cell context dependent differences observed only in maximally-induced activity. However, in a mammalian two-hybrid assay in the same cell lines, induction of transactivation was ligand structure-, coactivator- and cell context-dependent. These results suggest that the 5 structurally-related HAHs exhibit selective receptor modulator activity and this could result in tissue/cell-specific differences in their AhR agonist or antagonist activity at the gene or response level. Current studies are focused on several aspects of this intriguing problem including the identity of HAH-specific nuclear coactivators/cofactors in cell lines/tissues that may dictate structure-dependent differences in HAH action.

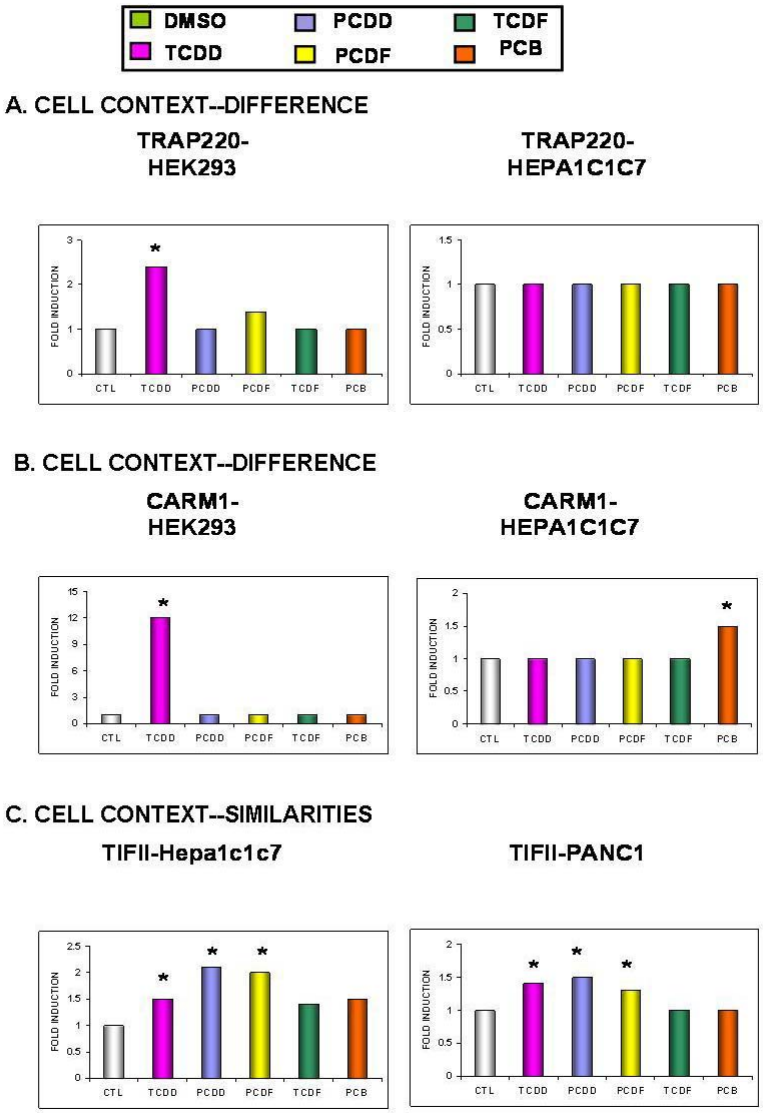

Figure 36 Cell context-dependent differences and similarities between the 5 HAHs in activation of specific coactivator-AhR interactions in a mammalian two-hybrid assay. Cell context-dependent differences between the 5 HAHs in activation of GAL4-TRAP220 was observed in HEK293 and Hepa1c1c7 cells (A). Cell context-dependent differences between the 5 HAHs in activation of GAL4-CARM1 was observed in HEK293 and Hepa1c1c7 cells (B). In contrast, a similar structure-dependent induction of transactivation by the HAHs was observed in Hepa1c1c7 and Panc1 cells transfected with GAL4-TIFII (C). Significant ($p < 0.05$) induction (*) was only observed at the maximal concentration of TCDD (10 nM), PCDD (20 nM), PCDF (20 nM), TCDF (20 nM) and PCB (50 nM) used in this study.

CHAPTER V

CONCLUSIONS

5.1. Role of estrogen receptor α (ERα) in insulin-like growth factor-I (IGF-I)-induced responses in MCF-7 breast cancer cells

Insulin-like growth factor-I (IGF-I) is a mitogenic polypeptide that induces proliferation in MCF-7 breast cancer cells and cotreatment with IGF-I plus the phosphoinositide 3-kinase (PI3-K) inhibitor LY 294002 (5 μM) or the antiestrogen ICI 182780 (1 μM) inhibited the IGF-I-induced cell growth. The MAPK inhibitor PD 98059 (15 μM) did not affect IGF-I-induced proliferation, suggesting that PI3-K and ERα but not MAPK are essential in mediating the mitogenic activity of IGF-I in breast cancer cells. The role of ERα in mediating IGF-I-induced proliferation and gene activation was further investigated in MCF-7 cells using RNA interference with a small inhibitory RNA for ERα (iERα). Western blots confirmed that ERα protein was decreased by >50% in whole-cell lysates from cells transfrected with iERα, and iERα-mediated decreases in nuclear ERα were confirmed in a gel mobility shift assays with nuclear extracts from MCF-7 cells. The intensity of the specifically bound retarded ERα-ERE band was decreased in cells transfected with iERα or after incubation with excess unlabeled ERE. The specificity of the RNA interference assay was further investigated in MCF-7 cells transfected with an E2-responsive pC3-luc construct which contains the human complement C3 gene promoter insert. E2

induced luciferase activity in cells transfected with pC3-luc and iLMN (an iRNA targeting Lamin), whereas hormone-induced activity was decreased in cells transfected iERα. As a negative control, we showed that iERα does not affect TCDD-induced transactivation in MCF-7 cells transfected with an aryl hydrocarbon-responsive construct (pDRE3-luc). IGF-I also activates MAPK- and PI3-K-dependent pathways including phosphorylation dependent activation of Elk-1 and SRF, which are serum-response element (SRE)-dependent genes (Duan et al., 2001b; Duan et al., 2002a). Compared with the solvent control (aqueous acetic acid), IGF-I induced transactivation in MCF-7 cells transfected with iLMN, a GAL4-Elk-1 expression plasmid, and a pGAL4 reporter construct or an SRF-luc construct. In contrast, activation of both constructs was significantly inhibited in cells transfected with iER demonstrating that ERα plays a role in IGF-I-induced transactivation in MCF-7 cells. We further investigated the role of ERα in mediating IGF-I-induced phosphorylation. Compared to solvent control, IGF-I significantly induced both Akt and MAPK phosphorylation without affect ing ERα protein levels, and this induction was inhibited in cells transfected with iERα. IGF-I-induced signaling in the mouse uterus (Klotz et al., 2002) suggests that activation of PI3-K required IRS-I, whereas activation of MAPK was IRS-I dependent. Our results show that transfection of a small inhibitory RNA for IRS-I (iIRS-I) in MCF-7 cells decreases IRS-I protein expression but does not significantly decrease phosphorylation, suggesting that IRS-I is not required for IGF-I-induced activation of PI3-K or MAPK in MCF-7 cells. Furthermore, IGF-I-

induced G1-S-phase progression was partially reversed in the cells transfected with iERα compared to the cells transfected with control iRNA, demonstrating a role for ERα in mediating IGF-I-dependent effects on this specific phase of the cell cycle. The cell-cycle-regulatory proteins associated with ERα-dependent actions of IGF-I was also investigated by western blot analysis. The various treatments did not affect levels of cdk2 or cdk4 or phospho-Rb proteins, and levels of IGF-I receptor were also unchanged. In contrast, in cells transfected with iERα, IGF-I induced cyclin D1 and cyclin E protein levels were decreased, suggesting that induction of cyclin D1 and cyclin E by IGF-I is an important response associated with G_1–S-phase progression which is also dependent, in part, on expression of ERα. As Figure 36 shows, iERα, which can efficiently knockdown ERα protein, significantly downregulates IGF-I-induced gene expression, kinase activation, and cell-cycle progression.

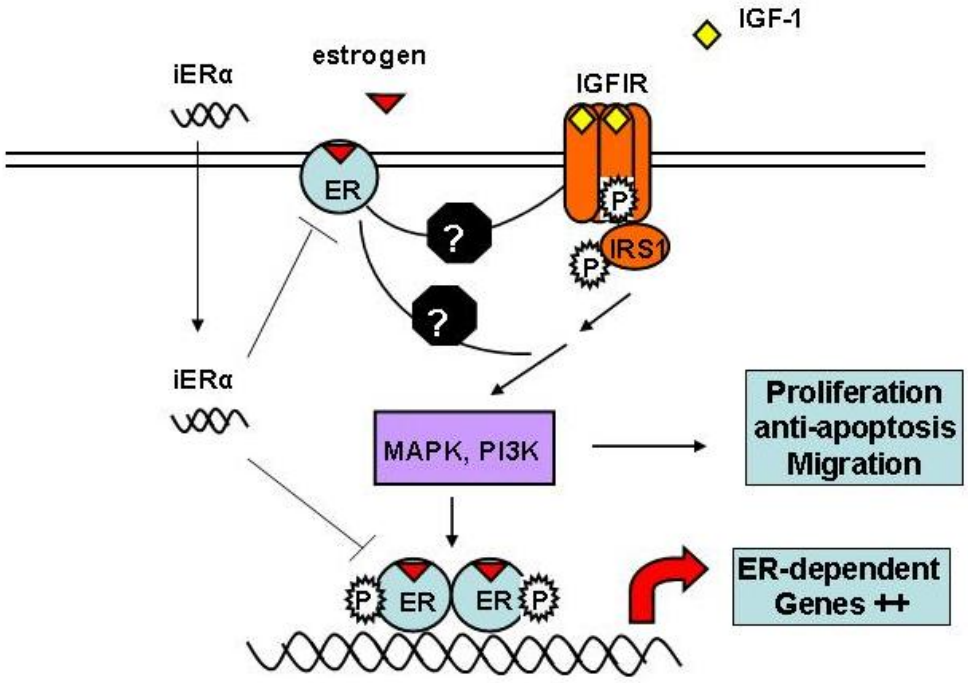

Figure 37 iERα downregulates IGF-I-induced gene expression, kinase activation, and cell-cycle progression.

We further investigated the effects of ICI 182780 on IGF-I-induced pathways in MCF-7 cells. Quantitation of ERα protein levels and kinase phosphorylation in cells cotreated with IGF-I plus ICI 182780 showed a time-dependent decrease in these parameters; both Akt and MAPK phosphorylation were decreased and the most pronounced responses were observed after treatment for 20 and 60 min, respectively. Moreover, ICI 182780 also inhibited IGF-I-induced transactivation in cells transfected with PI3-K- and MAPK-

dependent constructs and this paralleled effects observed in MCF-7 cells transfected with iERα. The effects of ICI 182780 on induction of cyclins D1, A and E by IGF-I were also investigated in MCF-7 cells. ICI 182780 alone decreased levels of ERα protein but did not significantly affect cyclins D1, A and E, and this was in contrast to the decrease in cyclin D1 and A protein levels in cells transfected with iERα. This suggests that other activities of ICI 182780 may prevent degradation of these proteins. IGF-I alone induced cyclin D1, A and E proteins; however, in the combined treatment (ICI 182780 plus IGF-I), the antiestrogen significantly inhibited induction of the cyclins by IGF-I. ERα protein was not affected by IGF-I but decreased after combined treatment with ICI 182780. Thus, degradation of ERα by iERα or ICI 182780 is linked to inhibition of IGF-I signaling in MCF-7 cells, indicating that extranuclear ERα plays an essential role in IGF-I-induced cell proliferation, cell-cycle progression, MAPK and PI3-K activation and gene expression. Figure 37 summarizes the effects of ICI 182780 on IGF-I-induced responses in this study.

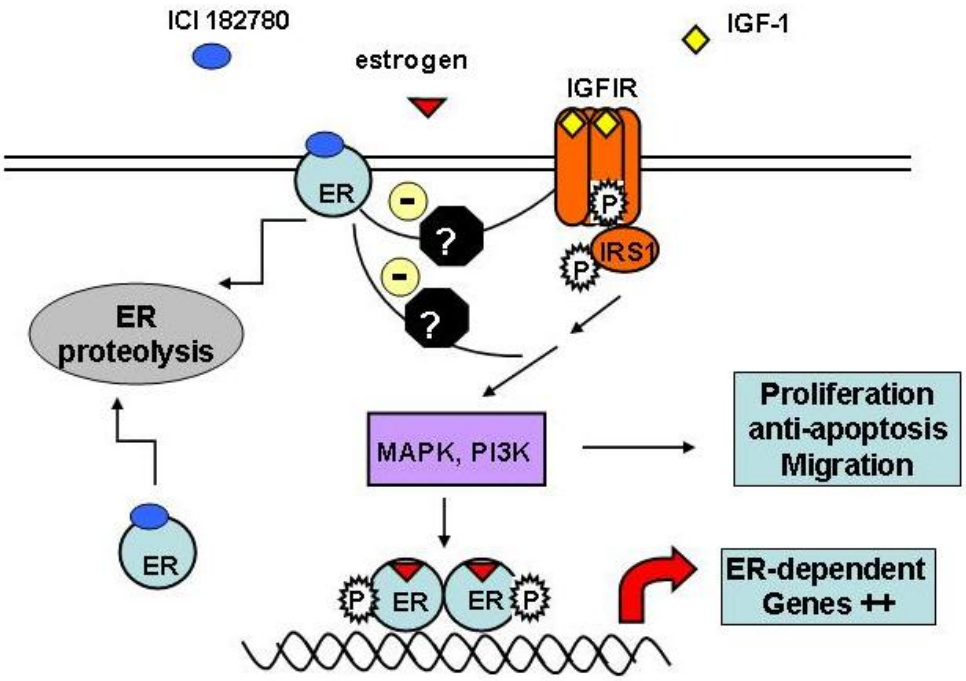

Figure 38 Effects of ICI 182780 on IGF-I-induced responses.

5.2. Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell context

AhR-mediated induction of CYP1A1 is a sensitive measure of Ah responsiveness. However, many phytochemicals interact with and inhibit CYP1A1 protein catalytic activity (Chen et al. 1996; Shertzer et al. 1999). Therefore, in this study we used a highly sensitive AhR-responsive assay (Denison et al. 1998) in which ligands activate the luciferase reporter gene activity in cells transfected with constructs containing multiple DRE promoter

elements. The 15 compounds used in this study including 12 flavonoids with different hydroxyl substitution patterns, plus the chemicals phloretin (a dihydrochalcone), cantharidin (a lactone), and emodin (an herbal laxative). We used 5 nM TCDD as a standard that induced maximal luciferase activity in stably transfected Hepa-1 cells or in transiently transfected MCF-7 or HepG2 cells. Results from the stably transfected Hepa-1 cells demonstrate their sensitivity to 5 nM TCDD, with a 124-fold induction of activities, whereas lower but significant induction was observed for chrysin, galangin, genistein, baicalein, daidzein, emodin, apigenin, and diosmin.

We further investigated the role of cell context in activation of transiently transfected $pDRE_3$ in human MCF-7 and HepG2 cell lines. At concentrations of 1 or 10 μM, only chrysin, cantharidin, baicalein, and emodin activated luciferase activity in MCF-7 cells. With the exception of cantharidin, these compounds were also AhR agonists in stably transfected Hepa-1 cells, and compounds such as galangin, genistein, daidzein, apigenin, and diosmin that were active in Hepa-1 cells did not induce a response in MCF-7 cells. The pattern of induction responses in HepG2 cells was similar to that observed in MCF-7 cells in that chrysin, cantharidin, and baicalein activated gene expression, whereas (10 μM) emodin was not active in this cell line. These data demonstrate that the AhR agonist activities of structurally diverse phytochemicals and cantharidin, which is derived from insect extracts, are highly variable among different cell lines, and that their fold inducibility compared with TCDD is also dependent on cell context.

The stably transfected Hepa-1 cells are more highly sensitive to the induction of luciferase activity by TCDD (5 nM) than the other compounds. Five nM TCDD induced a 124-fold increase in luciferase activity, whereas only a 14-fold induction response was observed for 10 μM chrysin. In contrast, 5 nM TCDD and 10 μM chrysin, respectively, induced a 20- and 5.5-fold increase in luciferase activity in MCF-7 cells, and the potency of chrysin relative to TCDD was clearly higher in MCF-7 and HepG2 cells compared with stably transfected Hepa-1 cells.

The four compounds that activated luciferase activity in MCF-7 and HepG2 cells (chrysin, cantharidin, baicalein, and emodin) were also investigated as inducers of CYP1A1 protein in these cell lines. With the exception of cantharidin, higher concentrations could be used because of the short duration (6 hr) of the experiment. Both baicalein and emodin increased CYP1A1 protein at concentrations of 100 μM (MCF-7) or 50 μM (HepG2), whereas chrysin was inactive at the same concentrations. In the nontransfected cells, cantharidin exhibited high cytotoxicity, and CYP1A1 protein was induced only in MCF-7 cells. In MCF-7 or HepG2 cells treated with 5 nM TCDD, there was a decrease in AhR protein levels. In contrast, treatment with baicalein and cantharidin increased levels of the AhR protein, whereas no effects were observed after treatment with emodin or chrysin. We also investigated the AhR antagonist activities of four compounds that were inactive in all three cell lines: kaempferol, quercetin, myricetin, and luteolin, and results show that cotreatment of MCF-7 cells with

kaempferol or quercetin plus 5 nM TCDD resulted in significant inhibition of TCDD-induced luciferase activity at both concentrations (1 and 10 μM) of flavone. Myricetin (10 μM) slightly decreased activity, whereas luteolin was a potent AhR antagonist. In contrast, 1 or 10 μM quercetin, kaempferol, and myricetin did not affect induction of luciferase activity by TCDD, whereas luteolin was an AhR antagonist in HepG2 cells. These results demonstrate that AhR antagonist activities of these phytochemicals are also dependent on cell context. These data suggest that dietary phytochemicals exhibit substantial cell context–dependent AhR agonist as well as antagonist activities (Figure 38). Moreover, because phytochemicals and other AhR-active compounds in food are present in the diet at relatively high concentrations, risk assessment of dietary toxic equivalents of TCDD and related compounds should also take into account AhR agonist/antagonist activities of phytochemicals.

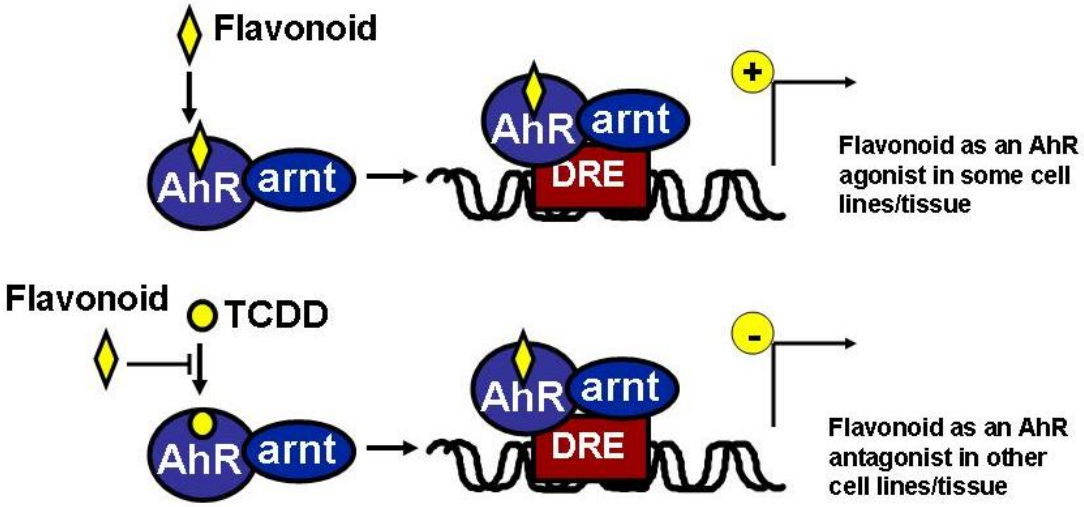

Figure 39 Flavonoid exhibits AhR agonist/antagonist activities in different cell lines/tissue.

5.3. Ligand-dependent interactions of the Ah receptor with coactivators in a mammalian two-hybrid assay

2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF and PCB126 are structurally-related HAHs that bind the AhR and induce AhR-mediated biochemical and toxic responses in different tissues/cell lines. Relative potency factors for all 5 compounds have been established, however, for each compound there is a range of potency values (relative to 2,3,7,8-TCDD), which is species, age, sex and tissue/cell-specific (Van den Berg et al., 1998; Van den Berg et al., 2006). Concentration dependent induction of CYP1A1 protein and mRNA levels by the 5 HAHs was observed in human Panc1 pancreatic cancer cells, human HEK293 embryonic kidney cells and Hepa1c1c7 mouse hepatoma

cells. We also used a third “induction” response to compare the 5 HAHs and cells were transiently transfected with a pDRE3-luc construct which contains three tandem consensus human DREs linked to the luciferase gene. The concentration-dependent induction of luciferase activity by the 5 HAH congeners in Panc1 and HEK293 cells showed that the fold-induction response for 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF was higher than observed for 2,3,7,8-TCDF and PCB126. In contrast, comparable fold-induction responses were observed for all 5 HAHs in Hepa1c1c7 cells and the ligand structure- and cell context-dependent differences observed for induction of luciferase activity were similar to that observed for induction of CYP1A1 mRNA levels. A mammalian two hybrid assay in cells transfected with GAL4-coactivator and AhR-VP16 chimeras was used to investigate structure-dependent interactions of these chimeras in Panc1, HEK293 and Hepa1c1c7 cells. The reporter construct, pGAL4-luc contains 5 tandem GAL4 response elements linked to a luciferase gene and the GAL4-coactivator chimeras contain the DNA binding domain of the yeast GAL4 gene linked to the full length coactivator. The VP16-AhR chimera contains the powerful C-terminal 80 amino acid transactivator from herpes simplex virus protein 16 (VP16) and the ligand binding domain of the AhR (Ko et al., 1997). The approach was employed for all 3 cell lines and the ligand structure-dependent induction responses was observed in these cells transfected with GAL4-SRC-1, GAL4-CARM-1, GAL4-TRAP220 and GAL4-TIFII. The HAH congeners did not induce transactivation in any of the cells line

transfected with the GAL4-PGC-1 or GAL4-SRC3 chimeras. In Panc1 cells, increased activity (with variable significance) was observed for both 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD in cells transfected with all 4 GAL4- chimeras, whereas among the 2 PCDF congeners and PCB126, only the latter compound induced transactivation and this was observed for GAL4-TRAP220 and not the other GAL4-coactivation chimeras. A different pattern of ligand-dependent transactivation was observed in HEK293 cells, 2,3,7,8-TCDD induced luciferase activity in cells transfected with GAL4-SRC-1, GAL4-CARM-1, GAL4-TRAP220 but not GAL4-TIFII; 2,3,4,7,8-PeCDF activated only GAL4-SRC-1 and GAL4-TIFII and the remaining compounds were inactive in cells transfected with all 4 GAL4-coactivator chimeras. Structure-dependent transactivation in Hepa1c1c7 was also highly variable; 2,3,7,8-TCDD, 2,3,7,8-TCDF and PCB126 activated GAL4-SRC-1; only PCB126 activated GAL4-CARM-1 and 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF activated GAL4-TIFII whereas none of the HAHs activated GAL4-TRAP220. These results clearly demonstrated that activation of pGAL4-luc in cells transfected with VP-AhR and GAL4-coactivator chimeras is dependent on the structure of the HAH congener, cell context and coactivator suggesting that the prototypical HAH congeners used in this study exhibit selective AhR modulation activity and this could result in tissue/cell-specific differences in their AhR agonist or antagonist activity at the gene or response level (Figure 39),These observations suggest that the effects of the

HAHs used in this study may not be additive and this is contrary to one of the major assumption used in the TEF approach for rish assessment of HAHs.

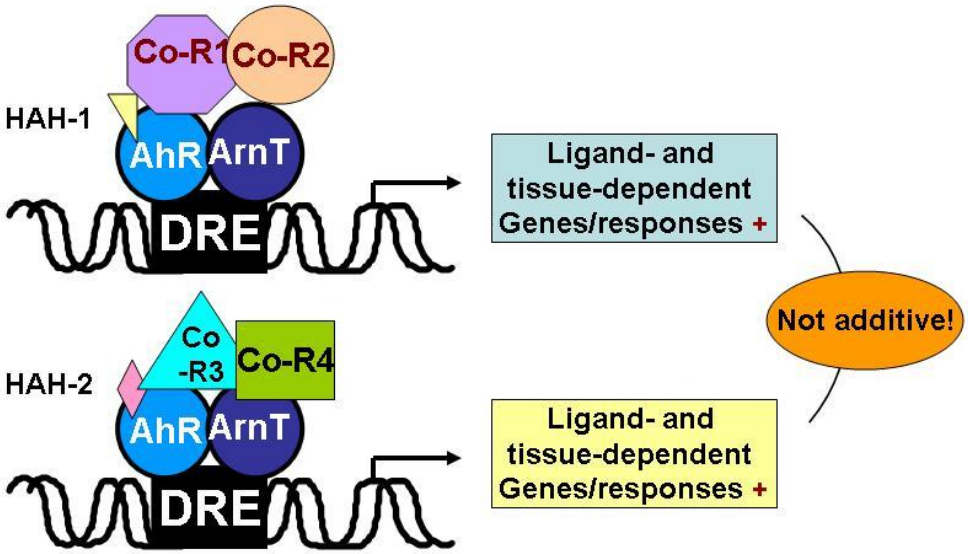

Figure 40 HAHs exhibit selective AhR modulator activity.

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