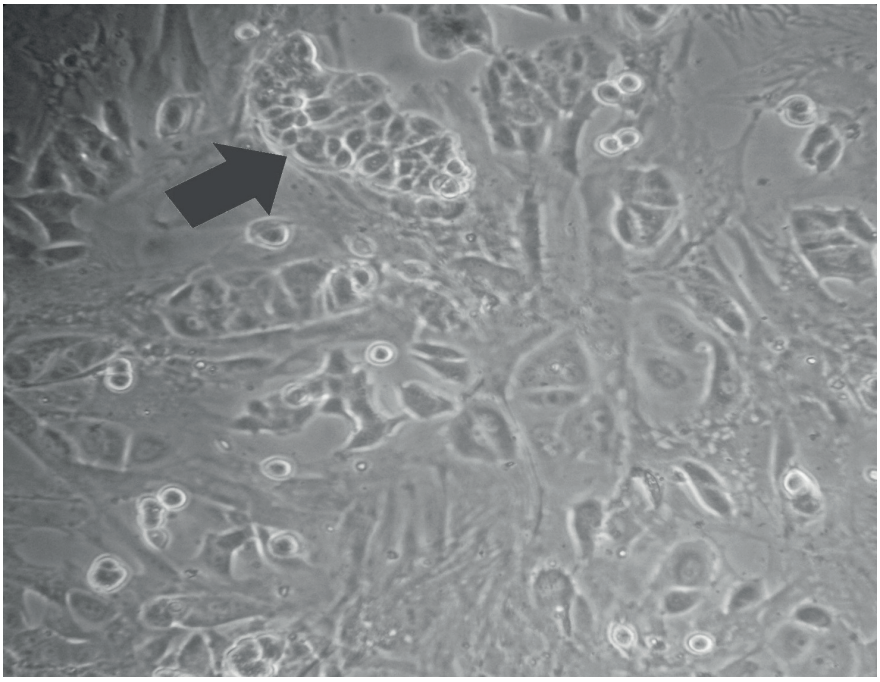
The possible role of Androgen and selected natural chemicals effect on human breast cancer cells and the co-culture system



Suthat Chottanapund

The possible role of Androgen and selected natural chemicals effect on human breast cancer cells and the co-culture system

De mogelijke rol van Androgen en geselecteerde natuurlijke chemische stoffen effect op menselijke borstkankercellen en het co-cultuur systeem
(met een samenvatting in het Nederlands)

Proefschrift
ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 27 november 2018 des middags te 2.30 uur

door
Suthat Chottanapund
geboren op 9 januari 1973 te Bangkok, Thailand

The possible role of Androgen and selected natural chemicals effect on human breast cancer cells and the co-culture system.

Suthat Chottanapund, Utrecht University, Institute for Risk Assessment Sciences (IRAS), 2018.

ISBN 978-90-393-7062-9

Promotoren: Prof. dr. M. van den Berg
Prof dr. M. Ruchirawat

Copromotoren: Prof. dr. M.B.M. Van Duursen
Dr. P. Navasumrit

This thesis was accomplished with financial support from Chulabhorn Graduate Institute, Chulabhorn Research Institute (Thailand)

Index

Chapter 1

General Introduction

1. Overview of breast cancer.

Infiltrating or invasive ductal carcinoma is the most common type of female breast cancer. Approximately 80% of all breast cancers are invasive ductal carcinomas. The origin of the cancer begins in the milk ducts and spreads to surrounding breast tissue. Over time, invasive ductal carcinoma can spread to the lymph nodes and other areas of the body.

Breast cancer is the most frequent cancer among women. Approximately 1.38 million new cases were diagnosed in 2008 (1) worldwide. Of those 1.38 million cases, the approximate number of deaths from breast cancer was about one third (458,000 cases). The treatment of breast cancer needs a multimodality approach to eradicate residual cancer and prevent recurrence.

1.1. Signs and symptoms of breast cancer.

At the early stage, invasive ductal carcinoma may not cause any symptoms. The patients usually have an abnormal finding on their screening mammogram (x-ray of the breast), which leads to further investigation. For the later stage, patients usually come with a new lump or mass in the breast. The American Cancer Society defines the following signs as unusual changes, which may be signs of breast cancers; swelling of all or part of the breast, skin irritation or dimpling, nipple pain or the nipple turning inward, inflammation of the nipple or breast skin, a nipple discharge other than breast milk, and a lump in the underarm area (lymphadenitis)

1.2. Diagnosis and treatment of breast cancer. [2, 3]

Diagnosis of invasive ductal carcinoma usually involves a combination of procedures, including a physical examination and imaging tests. Nowadays, triple assessment for breast cancer is a standard approach that includes physical examination, mammography and tissue diagnosis (either fine needle aspiration or core needle biopsy). Invasive ductal carcinoma is usually found by mammography, a test that obtains x-ray images of the breast. Mammograms are used to screen apparently healthy women for early signs of breast cancer. One key feature of an invasive breast cancer is speculated margins, which means that on the mammography film, the doctor sees an abnormality with finger-like projections coming out of it. These projections show the “invasion” of the cancer into other tissues.

If a patient has a suspicious mammogram or other imaging test result, the physician will ask for tissue diagnosis. A biopsy involves taking out some or all of the abnormal-looking tissue for examination by a pathologist. The physician may use less invasive approaches such as fine needle aspiration biopsy or core needle biopsy. Both methods involve inserting a larger needle into the breast to remove samples of breast tissue.

Treatment of breast cancer is usually a combination of surgery, radiation and (adjuvant) chemotherapy (include hormonal therapy) (4). Treatments depend on the age of the patient, and the stage and hormonal status of the cancer. Hormone receptor assay is a test to determine whether a breast cancer has receptors for the hormones estrogen and progesterone. A positive result means that the breast tumor can use either estrogens or progesterone to drive cell proliferation. Physicians use hormonal therapies aimed at blocking the effects of (low) estrogen levels in the body in order to shrink such tumors. The hormonal tests described above can be done either on tissue removed during biopsy, or after the surgery that removed the tumor (5).

1.3. Molecular biology of breast cancer.

Breast cancer has a pathogenesis like other somatic genetic diseases. Breast epithelial cells suffer irreversible alterations of their genomes. As the changes are largely random and part of a multistep process, the natural history of any single cancer may be due to processes that are partly determined by stochastic events. This means that each cancer has a uniqueness, which makes it difficult to forecast the prognosis in individual cases. It follows that the effects of any particular intervention can be hard to predict. It is customary to divide the carcinogenetic process into 2 phases: induction and progression (6). After full development in situ, a breast cancer will develop metastatic ability and will emerge fully developed and invasive.

Because of the complexity of the tumor development processes, breast cancer becomes a heterogeneous disease characterized by distinct pathological types with different outcomes. Breast cancers have diverse genetic mutations that affect a variety of signaling pathways. The tumors can be classified into different subgroups according to their molecular expression profiles. It is not clear whether specific molecular pathways establish the subtype of breast cancer or whether different cell types become transformed and give rise to each tumor subtype. Patients would benefit from individual therapeutic regimens designed to treat their particular type of cancer.

Classically, human breast carcinoma has been classified for therapeutic purposes in two distinct categories: one hormone-correlated, the other hormone-uncorrelated. However, recent advancements in the technology applied to molecular biology by genomic and proteomic analyses suggest that many more factors are involved in the growth and progression of these cancers. That is, some clusters, point to subgroups of patients with different prognoses. Successful distinction between a tumor and a normal tissue of origin is the key to identifying novel targets for new selective therapeutic strategies.

1.4. Some molecular target of breast cancer (other than hormones). [7]

In breast cancer, there is evidence of over-expression of epidermal growth factor receptors (EGFR, HER1) and HER-2/neu proteins. Both are transmembrane tyrosine kinase cell surface growth receptors. The EGFR and HER-2/neu oncoproteins are composed of three membrane portions: an internal tyrosine kinase which is responsible for signal transduction; a short transmembrane, and an extracellular domain (ECD) which is the binding site for ligand growth factors. One promising new anticancer therapeutic approach is the development of EGFR and HER-2/neu antagonists. EGFR and HER-2/neu are overexpressed or dysregulated in approximately 50% and 25%, respectively, of breast cancer cases. Their activation is associated with increased cell proliferation, tumor cell motility and invasiveness, angiogenesis, and inhibition of apoptosis. Overexpression of HER-2/neu characterizes a subgroup of patients with aggressive tumors, frequent negative hormone receptors and a poor prognosis. Furthermore, tumor amplification of the HER-2 gene has been associated with resistance to a variety of cytotoxic agents and tamoxifen. Trastuzumab is one of the drugs developed from this molecular target. Trastuzumab is a humanized monoclonal antibody with high specificity for the HER-2 protein. The positive results from clinical research of trastuzumab alone or in combination with cytotoxic agents provide a rationale for its use in the adjuvant setting to treat breast cancer. Signaling pathways activated by HER-2/neu include;

- mitogen-activated protein kinase (MAPK)
- phosphoinositide 3-kinase (PI3K/Akt)
- phospholipase C γ
- protein kinase C (PKC)
- Signal transducer and activator of transcription (STAT)

The primary mechanism of HER-2/neu signaling is through ErbB family receptors. Because these receptors promote cell proliferation and oppose apoptosis and therefore must be tightly regulated to prevent uncontrolled cell growth. (Figure. 1)

The second most frequently observed molecular pathway in breast cancer is via angiogenesis, the process by which new capillaries are formed from pre-existing vessels. This process is necessary for tumor growth and metastasis. The initial phase of angiogenesis requires the acquisition of angiogenic phenotype through a series of molecular events leading to increased expression of angiogenic factors and/or down-regulation of naturally occurring inhibitors. Vascular endothelial growth factor (VEGF) is the most specific and powerful angiogenic factor. Angiopoietin-2, transforming growth factor-b1 (TGF-b1), basic fibroblast growth factor (bFGF) and matrix metalloproteinases (MMPs) also play a major role in angiogenesis. The biological effects of VEGF are mediated as it is bound to three specific endothelial surface cell receptors VEGF-R1

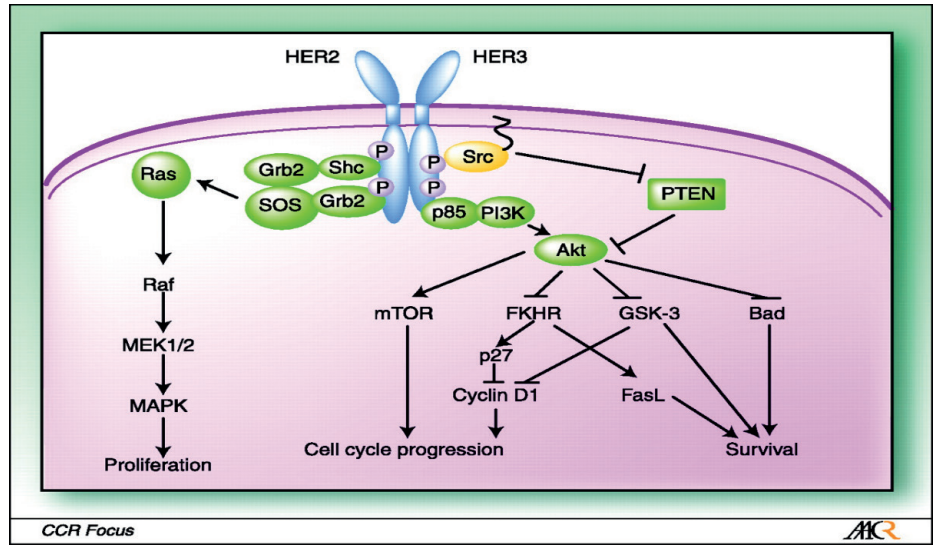


Figure 1. Signaling pathways activated by HER-2/neu. (8)

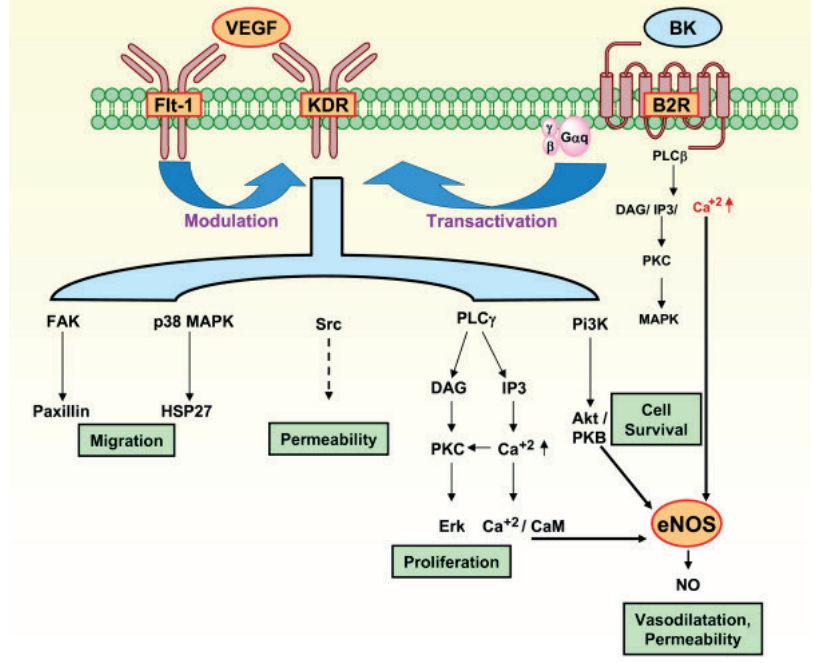


Figure 2. VEGF pathway. (10)

(flt-1), VEGF-R2 (flk-1/kdr), and VEGF-R3. VEGF-R1 promotes differentiation and vascular maintenance, VEGF-R2 induces endothelial cell mitogenesis and vascular permeability, and VEGF-R3 stimulates lymphangiogenesis. VEGF gene expression may be upregulated by a number of stimuli, including: hypoxia, nitric oxide, various growth factors, estrogens, progestins, loss of p53, activation ras, v-src, and HER2/neu. A number of antiangiogenic agents are being tested in Phase I/II clinical trials for the treatment of breast cancer, either alone or in combination with other therapies, including carboxyamidotriazole, interleukin-12, thalidomide, celecoxib, soy isoflavone, anti avb3 integrin monoclonal antibody and MMPs inhibitors (9). (Figure. 2)

2. Hormones and breast cancer.

Hormone receptors are special proteins found on the surface and in the cytoplasm of breast cells that travel into the nucleus after dimerization. These receptor proteins act like an on-off switch for particular activities in the cell. If the right substance comes along that fits, like a key fitting into a lock, into the binding pocket of the receptor, the switch is turned on and a particular activity in the cell begins. Female breast development occurs at puberty. Complex hormonal interplay occurs, resulting in growth and maturation of the adult female breast. In early fetal life, epithelial cells, derived from the epidermis of the area programmed to later become the areola, proliferate into ducts, which connect to the nipple at the skin's surface. The blind ends of these ducts bud to form alveolar structures in later gestation. With the decline in fetal prolactin, placental estrogen and progesterone at birth, the infantile breast regresses until puberty. In girls as young as nine or ten, the initial clinical appearance of the breast bud and growth and division of the ducts occur, giving rise to club-shaped terminal end buds, which then form alveolar buds. The entire differentiation process takes years after the onset of puberty. If there is no pregnancy, the process may never be completed. Hormonal regulation, initiation and progression of breast development involves a coordinated effort of pituitary and ovarian hormones, as well as local mediators. Estrogen and progesterone act in an integrative fashion to stimulate normal adult female breast development. Estrogen, acting through its receptor, promotes duct growth, while progesterone, also acting through its receptor, supports alveolar development. This is demonstrated by experiments in estrogen receptor knockout mice, which display grossly impaired ductal development. By contrast, progesterone receptor knockout mice possess significant ductal development, but lack alveolar differentiation. Androgens' actions are more complicated than estrogen, because some androgens can be converted by aromatase enzyme to estrogen. Mostly, androgens act as inhibitors of breast tissue development. (Figure. 3)

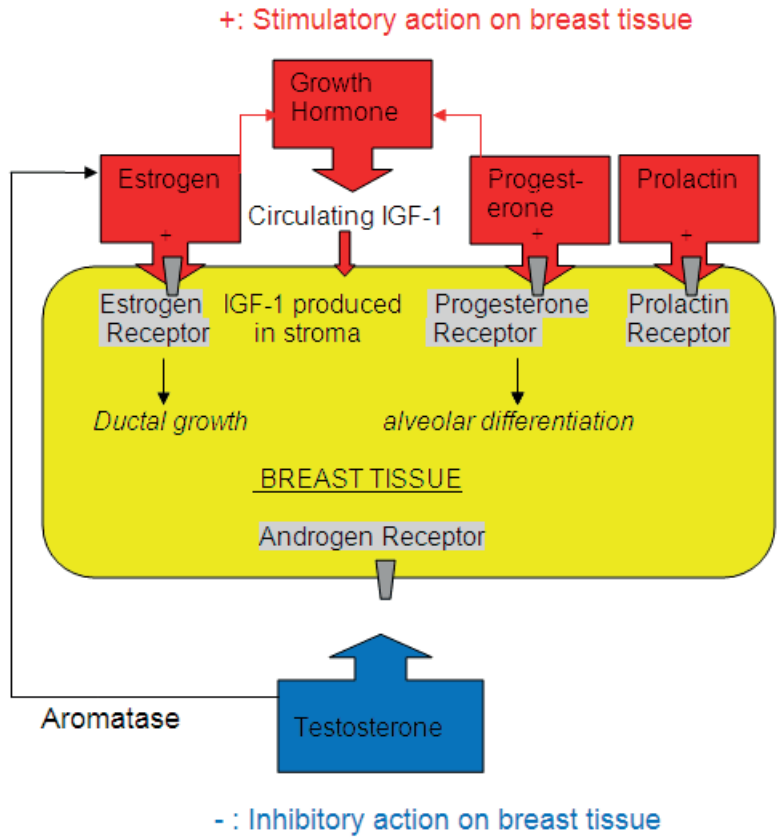


Figure 3. Hormones and breast development. [11]

In breast cancer patients, estrogen and/or progesterone can attach to those receptors and tell the cell to grow and divide. This means that when these hormones are present, the cells receive a strong message to keep on growing and dividing. On the other hands, the same mechanism of cancer development is used as a target of breast cancer treatment. Hormonal therapy usually starts after other treatments such as chemotherapy or radiation therapy have stopped. In some cases of advanced invasive ductal carcinoma, hormonal therapy is given before surgery to help shrink the tumors (neo-adjuvant hormonal therapy) (12). Hormonal therapy, also called anti-estrogen therapy, works by lowering the amount of estrogen in the body or blocking the estrogen from signaling breast cancer cells to grow (13-15). Currently, there are two types of hormonal therapy that are most frequently used, as described below.

Of the selective estrogen-receptor response modulators (SERMs), the best known is tamoxifen. Tamoxifen "pretends" to be estrogen and attaches to the receptors on the

breast cancer cells, taking the place of real estrogen. As a result, the cells do not receive the signal to grow. Raloxifene is another SERM. Tamoxifen is usually recommended for women who have not yet gone through menopause, although it can be used after menopause as well.

Aromatase inhibitors (AIs) are medications which include Arimidex (chemical name: anastrozole), Aromasin (chemical name: exemestane), and Femara (chemical name: letrozole). AIs reduce the amount of estrogen produced in women. They have side effects, however, most importantly loss of bone density, which can be especially hazardous for women already at risk for osteoporosis. Therefore, tamoxifen may still provide a more favorable risk/benefit ratio for some subgroups of women, which need to be identified. In addition, the role of AIs in premenopausal patients is still uncertain. AIs alone may not be effective in premenopausal women, because the ovaries can override the inhibition by producing a large amount of aromatase. However, clinical trials are now testing AIs in premenopausal women in combination with ovarian suppression drugs such as goserelin, which suppress ovarian function. AIs are recommended as a treatment for women in menopause, because the main sources of estrogen for post-menopausal women are the adrenal glands and fat tissue, not the ovaries.

Other types of hormonal therapy: Estrogen-receptor down regulators (ERDs): ERDs destroy the estrogen receptors in the cells, which prevents the estrogen from getting its message through. Faslodex (chemical name: fulvestrant) is an ERD approved for use in postmenopausal women with advanced breast cancer. Faslodex is an estrogen receptor antagonist without known agonist effects. The chemical name is 7-alpha-[9-(4,4,5,5,5-penta fluoropentylsulphinyl) nonyl]estra-1,3,5-(10)- triene-3,17-beta-diol. The molecular formula is $C_{32}H_{47}F_5O_3S$ and its structural formula is:

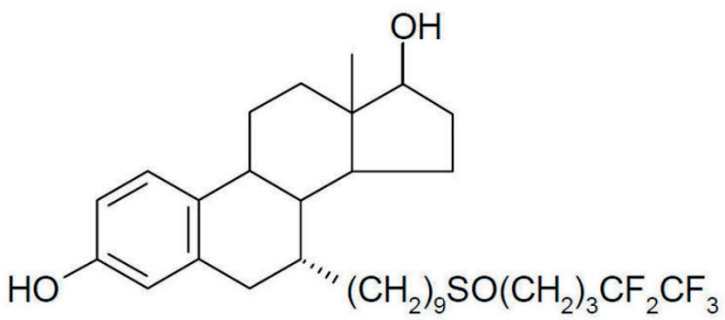


Figure 4. Structural formula of Faslodex. (16)

Ovarian shutdown or removal: The ovaries are the main source of estrogen in women before menopause. They can be shut down permanently or temporarily. For a temporary shutdown, Zoladex (chemical name: goserelin) and other drugs in the same class can be used. After administration of Zoladex, peak serum concentrations are reached in about two hours. This drug rapidly binds to the LHRH receptor cells in the pituitary gland, thus leading to an initial increase in production of luteinizing hormone and leading to an initial increase in the production of corresponding sex hormones. Eventually, after a period of about 14-21 days, production of LH is greatly reduced due to receptor down regulation. Sex hormones are generally reduced to castration levels. Permanent shut down of ovarian function, can be done by a surgical procedure, called oophorectomy (cutting out the ovaries).

3. Estrogen. [17]

Estrogens are a group of sex steroid compounds. Estrogens are synthesized in all vertebrates as well as in some insects. Estrogens are sex hormones and are used as part of some oral contraceptives and replacement therapy for postmenopausal women. Like all steroid hormones, estrogens readily diffuse across the cell membrane. Once inside the cell, they bind to and activate estrogen receptors which, in turn, up-regulate the expression of many genes. Additionally, estrogens have been shown to activate a G protein-coupled receptor, GPR30 (18). Estrogens are produced primarily by developing follicles in the ovaries, the corpus luteum, and the placenta. Luteinizing hormone (LH) stimulates the production of estrogen in the ovaries. Some estrogens are also produced in smaller amounts by other tissues such as the liver, adrenal glands, and breasts. These secondary sources of estrogens are especially important in postmenopausal women. Fat cells also produce estrogen(19). In females, synthesis of estrogens starts in theca interna cells in the ovaries, by the synthesis of androstenedione from cholesterol. Androstenedione is a substance of moderate androgenic activity. This compound crosses the basal membrane into the surrounding granulosa cells, where it is converted to estrone or estradiol, either immediately or through testosterone. The conversion of testosterone to estradiol, and of androstenedione to estrone, is catalyzed by the enzyme aromatase.

While estrogens are present in both men and women, they are usually present at significantly higher levels in women of reproductive age. They promote the development of female secondary sexual characteristics, such as breasts, and are also involved in the thickening of the endometrium and other aspects of regulating the menstrual cycle. In males, estrogen regulates certain functions of the reproductive system important to the maturation of sperm.

4. Estrogen receptor. [20]

Estrogen receptors can be activated by the hormone 17β-estradiol (estrogen). There are two types of estrogen receptors: a family of intracellular receptors or nuclear hormone receptors and the estrogen G protein-coupled receptor GPR30 (GPER). The main function of the estrogen receptor is as a DNA-binding transcription factor that regulates gene expression. However, the estrogen receptor has additional functions independent of DNA binding, through the G protein coupled receptor.

Estrogen receptors (ER) have two isoforms, ER-α and ER-β. These two isoforms are genomic estrogen receptors which can act as transcription factors. An estrogen receptor consists of six structural domains A-F with a total of 595 amino acids. Three functional domains are ligand-independent activation (AF-1) domain, DNA binding domain (DBD) and ligand-binding domain. After the ligand binds to the ligand-dependent domain (AF-2), the dimerization domain will initiate the dimerization of the receptor. Once activated, ER dimerizes, binds to estrogen response element (ERE) via two zinc-finger motifs, and then recruits coactivator complex (CoA) to activate gene promotors. (Figure. 5)

5. Androgens. [16]

Androgens are other sex steroid hormones that can act as a source of estrogens when metabolized by aromatase. Androgens also stimulate or control the development of male characteristics through androgen receptors, including the activity of the accessory male sex organs and development of male secondary sex characteristics. Androgens were first discovered in 1936. There were the original anabolic steroid and the precursor of all estrogens, the female sex hormones (Figure. 6). The primary and best known androgen is testosterone. Androgen ablation can be used as an effective therapy in prostate cancer.

A subset of (weak) androgens includes any of the 19-carbon steroids synthesized by the adrenal cortex, the outer portion of the adrenal gland. These can function as weak steroids, including dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S), and androstenedione. Besides testosterone, other androgens include the following:

Dehydroepiandrosterone (DHEA): a steroid hormone produced in the adrenal cortex from cholesterol. It is the primary precursor of natural estrogens (Figure. 7). DHEA is also called dehydroisoandrosterone or dehydroandrosterone.

Androstenedione (Andro): an androgenic steroid produced by the testes, adrenal cortex, and ovaries. While androstenediones are converted metabolically to testosterone and other androgens, they are also the parent structure of estrone. Androstenedione is used

as an athletic or body building supplement. Androstenediol is the steroid metabolite. It is thought to act as the main regulator of gonadotropin secretion.

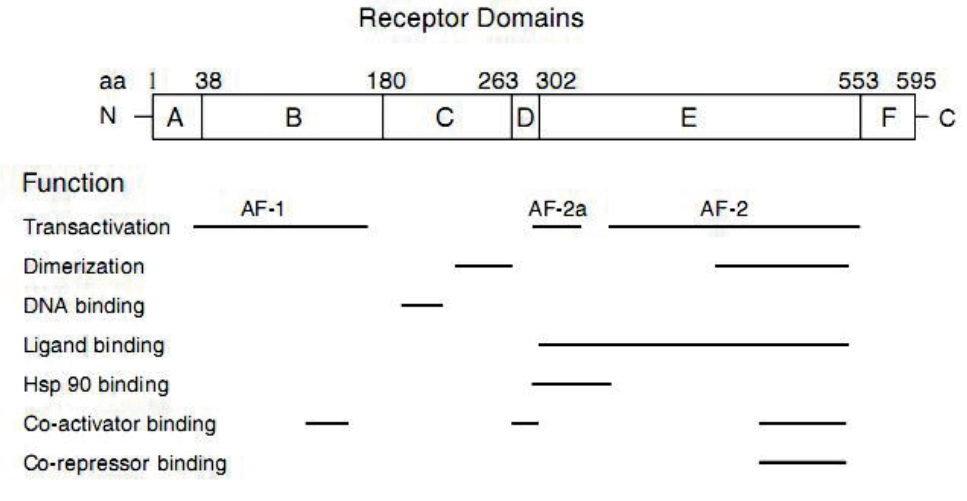


Figure 5. Schematic diagram of ER structural functional domains. (20)

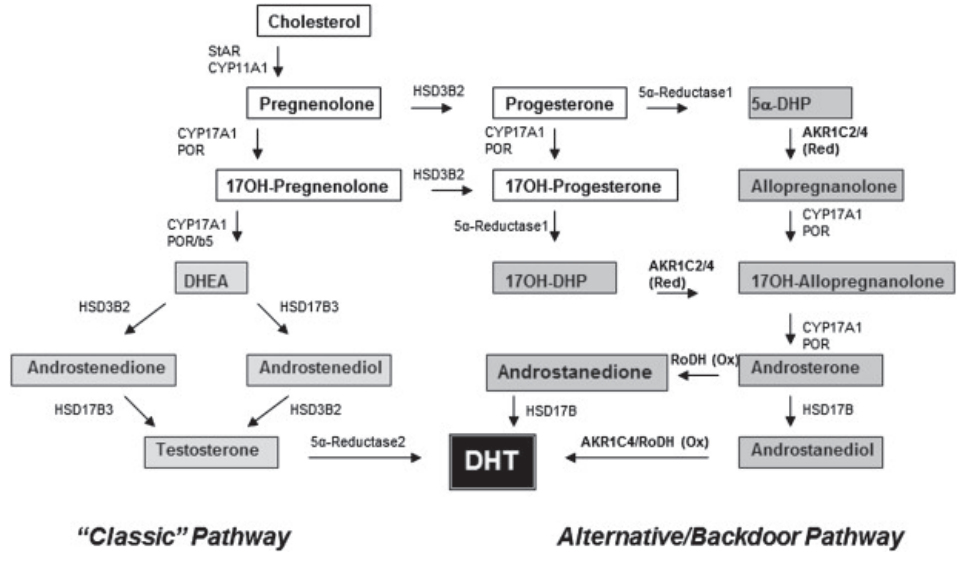


Figure 6. Human steroidogenesis pathways. (16)

Androsterone: a chemical by-product created during the breakdown of androgens, or derived from progesterone. It exerts minor effects on masculinization, but with one-seventh the potency of testosterone. It is found in approximately equal amounts in the plasma and urine of both males and females.

Dihydrotestosterone (DHT): a metabolite of testosterone. It is a more potent androgen than testosterone in that it binds more strongly to androgen receptors. It is produced in the adrenal cortex, prostate, and testes

6. Androgen receptor. [22]

The androgen receptor (AR) is a type of nuclear receptor that is activated by binding either of the androgenic hormones testosterone or dihydrotestosterone in the cytoplasm and then translocating into the nucleus. The androgen receptor is most closely related to the progesterone receptor. Progestins in higher dosages can block the androgen receptor (23). The main function of the androgen receptor is as a DNA binding transcription factor which regulates gene expression. However, the androgen receptor has other functions as well. Androgen regulated genes are critical for the development and maintenance of the male sexual phenotype. The primary mechanism of action for androgen receptors is direct regulation of gene transcription. The binding of an androgen to the androgen receptor results in a conformational change in the receptor which in turn causes dissociation of heat shock proteins, transport from the cytosol into the cell nucleus, and dimerization. The androgen receptor dimer binds to a specific sequence of DNA known as a hormone response element. Androgen receptors interact with other proteins in the nucleus resulting in up or down regulation of specific gene transcription.

Two isoforms of the androgen receptor (A and B) have been identified (24); AR-A and AR-B. AR-A has a truncated N-terminus (it lacks the first 187 amino acids; 87 kDa). AR-B is a full length protein of 110 kDa. Like other nuclear receptors, the androgen receptor is modular in structure and is composed of six functional domains labeled A through F. (Figure. 8)

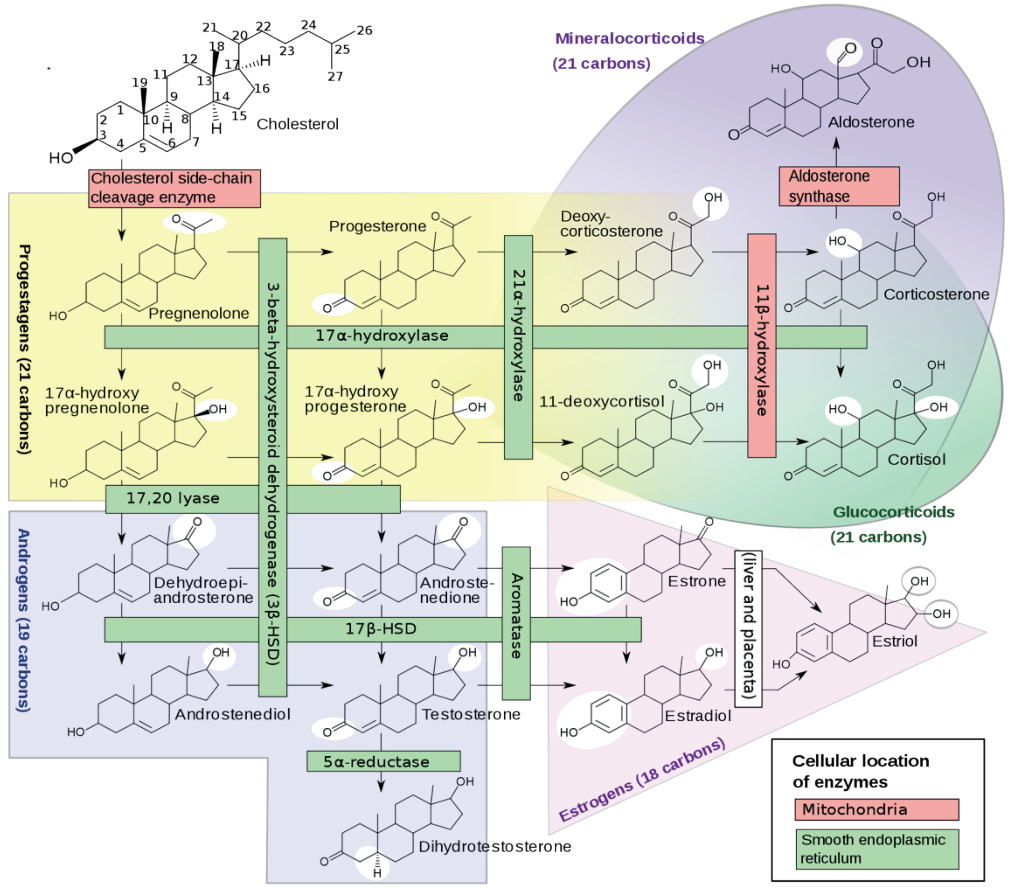


Figure 7. Enzymes in human steroidogenesis pathways. (21)

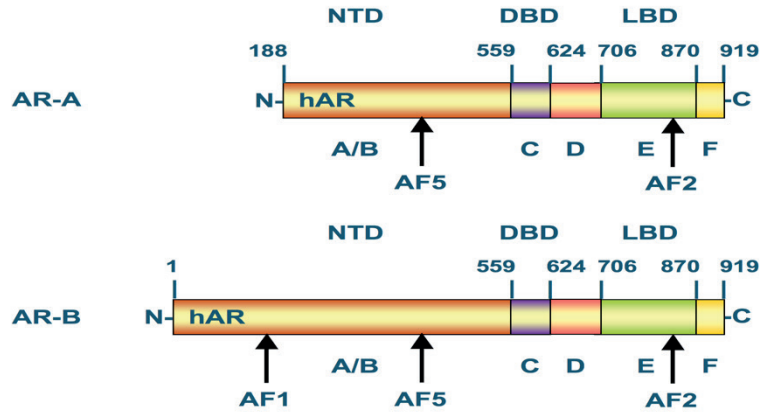


Figure 8. Structural domains of the human androgen receptor. (24)

7. Triple negative breast cancer.

Breast cancer is a heterogeneous disease exhibiting substantial differences with regard to biological behavior and requiring distinct therapeutic interventions. Steroid hormone receptors (HR) such as estrogen receptor (ER) and progesterone receptor (PgR) in concert with the oncogene ErbB-2/human epidermal growth factor receptor 2 (HER-2) are critical determinants of breast cancer subtypes. While HR is thought to mirror a good prognosis (25-27), expression of HER-2 has long been understood as an unfavorable prognostic feature (28). However, since the introduction of trastuzumab as a potent therapeutic approach in HER-2-positive breast cancer, HER-2 expression is perceived as a favorable rather than a negative prognostic factor (29). Trastuzumab (Herceptin) is a monoclonal antibody. Monoclonal antibodies are made in the laboratory. Some are designed to attack specific cancer cells. Trastuzumab targets cancer cells that "overexpress," or make too much of, a protein called HER–2 or *erb* B2, which is found on the surface of some cancer cells. Trastuzumab attaches to the HER–2 positive cancer cells and slows or stops their growth. Trastuzumab is used only to treat breast cancers that are HER–2 positive. HER–2 positive cancers overexpress the HER–2 protein or have amplification (too many copies) of the HER–2 gene. These tumors tend to grow faster and are generally more likely to recur than tumors that do not overproduce HER–2.

Triple-negative breast cancer (TNBC) is characterized by a lack of expression of ER, PgR and HER-2. To date, chemotherapy remains the only possible therapeutic option in the adjuvant or metastatic setting in the TNBC. A recent analysis indicates that TNBC carries a distinct molecular profile when compared with hormonal receptors positive breast cancer. Compared with hormonal positive breast cancer, triple negative breast cancer usually has a worse prognosis and outcome (30).

Triple negative breast cancers with their aggressive clinical course and unresponsiveness to anti-estrogens represent approximately 30% of all breast cancers. Typically, triple negative breast cancer is poorly differentiated, has a higher histological grade, and is associated with a higher recurrence rate. Androgen receptor (AR) expression has been reported in over 70% of breast cancer and in 45–50% of patients with triple negative breast cancer(31). There is emerging evidence that the androgen signaling pathway may also play a critical role in breast carcinogenesis, independent of ER and PR. Preclinical data have suggested the inhibitory role of adrenal steroids, such as dehydroepiandosterone (DHEA) and its sulfate on the growth of human ER-negative breast cancer cell lines, when these demonstrate a strong expression of AR (32). This means that Dehydroepiandosterone (DHEA) and its sulfate may potentially decrease AR gene expression. However, as DHEA has also been shown to stimulate growth in breast cancer cells when an ER is expressed in ER-positive breast cancer cells, the effects of androgens in breast cancer need to be further explored. Apparently, the cancer prevention aspect of chemical compounds can vary depending on the type of tumors.

8. Cis-stilbene. [33, 34]

Phenolic phytoalexin or stilbenes which are found in a variety of fruits such as grapes, blueberries, cranberries, and strawberries as well as in many fruit products such as wine are said to have benefits to human health. Two main metabolites, Resveratrol (3, 40, 5-trihydroxystilbene) and piceid (3, 40, 5-trihydroxystilbene-3-b-D-glucoside), are also said to protect the heart and neurons, and to ward off anti-leukemic cancer in humans and rats.

Resveratrol (35); one of the stilbene metabolites, found in the skin of red grapes, protects the fruit from many pathogens such as bacteria and fungi. Many studies have found that, as with other stilbenes, it can protect human from cancer cells. Recently a study from Sangjun (36) found that resveratrol may also inhibit breast cancer proliferation in MCF-7 breast cancer cell line. (Figure. 9.1, 9.2)

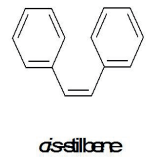


Figure 9.1. cis-stilbene. (32)

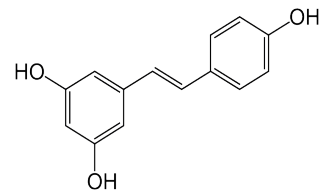


Figure 9.2. Resveratrol. (33)

Several epidemiological studies have also indicated that high resveratrol intake may reduce breast cancer risk (37). Multiple mechanisms have been identified that could explain the anti-carcinogenic properties of resveratrol, which include its role as a SERM, an aromatase inhibitor and/or an anti-oxidant (38-40). With respect to its properties as an aromatase (*CYP19A1*) inhibitor, several in vivo and in vitro studies have confirmed the modularity function of resveratrol in this key step in sex steroidogenesis (41-44). It has been suggested that the anti-aromatase activity of resveratrol is its major property with respect to chemoprevention of breast cancer (42). In a 28-day repeated dose study, the no observed adverse effect level (NOEAL) was 300 mg/kg/day in rats (45). This low toxicity of resveratrol could make it a good candidate for adjuvant breast cancer treatment.

9. Melatonin.

Melatonin (N-acetyl-5-methoxytryptamine) is an indolic and endogenous compound that is naturally produced by the pineal gland in the human body (Figure. 10). Melatonin plays a primary role in the circadian pattern and is regulated by the hypothalamic suprachiasmatic nucleus (SCN). In addition, melatonin is believed to have oncostatic

properties against many forms of cancer such as breast, colorectal and prostate cancer (46-49). Multiple mechanisms have been proposed to explain the chemopreventive properties of melatonin against breast cancer, (50-53) of which two are especially important with respect to our present study. Firstly, melatonin can act as SERM by reducing estrogen binding to ERα receptors and inhibiting binding of the E_2-ERα complex to the DNA. The proposed mechanism of this anti-estrogenic effect of melatonin does not depend on its binding to the ER but depends on its high affinity for binding to membrane melatonin receptors (MT1). This complex will interfere with the estrogen-binding activity of ERα without changing its affinity and will reduce the ligand-receptors transactivation (53-55). Another important mechanism of melatonin with respect to prevention of breast cancer may be its role as an aromatase inhibitor. Melatonin could decrease cAMP formation and down-regulate expression of promoter regions pII, pI.3 and p1.4-regulated *CYP19A1* gene in MCF-7 cells (56).

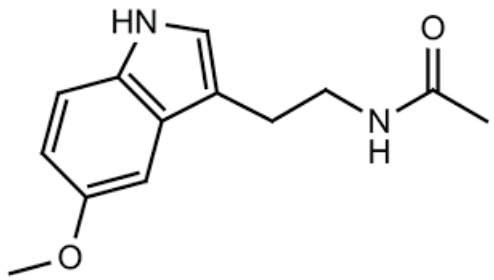


Figure 10. Melatonin.

10. Depsidone.

Depsidones are compounds isolated from Aspergillus unguis CRI282-03, a fungi isolated from marine organisms and environments. From these fungi several classes of compounds, like protubonines, cerebroside analogues and depsidones, with a variety of interesting anti-tumor activities have been extracted. One of the promising anti-tumor activities is an anti-aromatase effect. (Figures. 11.1, 11..2)

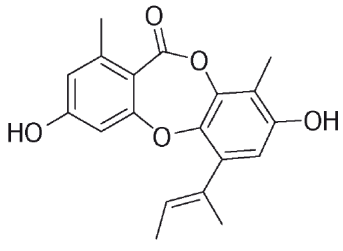


Figure 11.1. Unguinol.

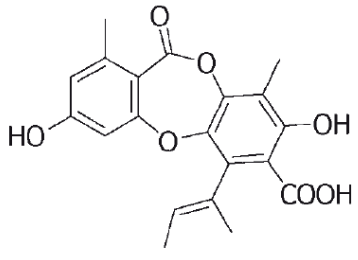


Figure 11.2. Aspergillusidone A.

11. Co-cultured breast cancer cell lines and breast stromal fibroblasts.

Most breast tumors are classified as invasive ductal carcinomas, malignant growths of the ductal epithelial cells of breast tissue (57-59). The remaining breast stromal comprise fibroblasts, adipocytes and endothelial cells. There is increasing evidence that tumor-associated stromal cells promote the development of epithelial malignancies. For example, in vitro premalignant mammary epithelial cells adopt a malignant growth pattern if cultured with carcinoma derived adipose fibroblasts (CAFs) (60). In addition, a study by Heneweer, et al. found that primary breast adipose fibroblasts (BAFs) could have an indirect effect on the proliferation of breast tumor cells in a co-culture system via modulation of cytokine-regulated estrogen receptors (61). The presence of a feedback loop for intercellular communication between epithelial MCF-7 cells and fibroblasts was proved by consistently higher levels of pS2 gene transcription after exposure of the co-culture to estrogenic compounds in the presence of testosterone compared to the same estrogen treatment without addition of testosterone (61). The efficacy of a co-culture system in converting testosterone to estrogen can be used to prove the actual role of androgens in breast cancer cell proliferation as in the experiment. The co-culture system showed the different effect of aromatizing androgens; with testosterone, with non-aromatizing androgens; and with DHT to breast cancer cells.

11.1. Fibroblast isolation.

Primary breast tissue fibroblasts were obtained from breast cancer patients who attended Bamrasnaradura Infectious Institute for modified radical mastectomies. All patients freely gave their informed consent for the surgery and the research. Approximately 5-10 g of normal breast tissue were set aside for this study. The rest of the tissue was sent for routine pathological analysis. The isolated tissue was immediately transported to laboratory for fibroblasts isolation processes, as described here in the section on methodology. Three series of breast cancer fibroblast cells were isolated from three patients who had undergone a modified radical mastectomy**.** All of them were successfully proliferated, frozen and thawed up to more than 20 passages. These isolated fibroblasts were used for all the experiments in this study (Fig. 12.1 and 12.2).

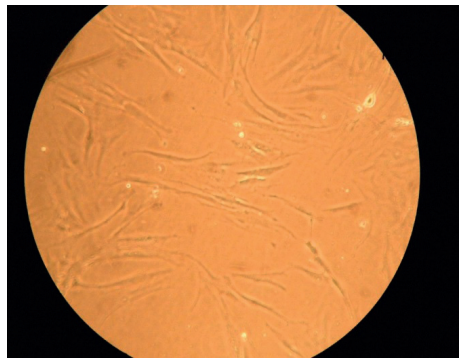


Figure 12. 1 Fibroblast before freezing.

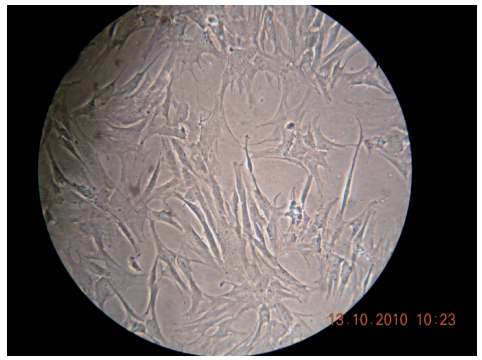


Figure 12. 2 Fibroblast after freezing and thawing.

11.2. Establishment of co-culture system.

The isolated fibroblasts were used to establish all the co-culture system with MCF-7 or T47D or MDA-MB-231 breast cancer cell lines. These co-culture systems were employed for experiments throughout the study. (Fig. 13.1-13.4)

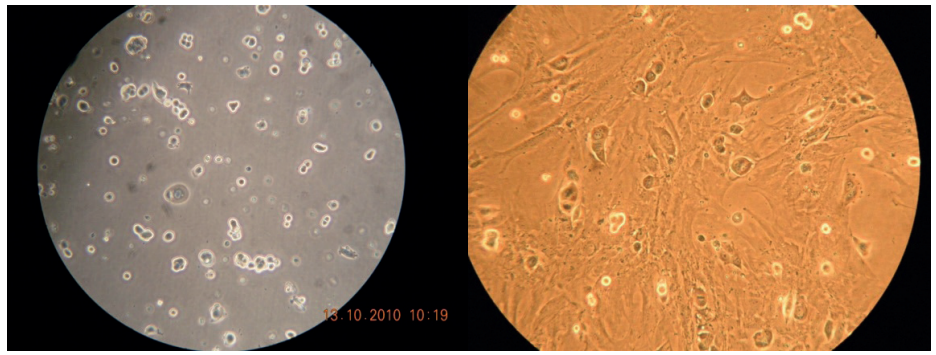


Figure 13. 1. MCF-7.

Figure 13. 2. Co-culture fibroblast+MCF-7.

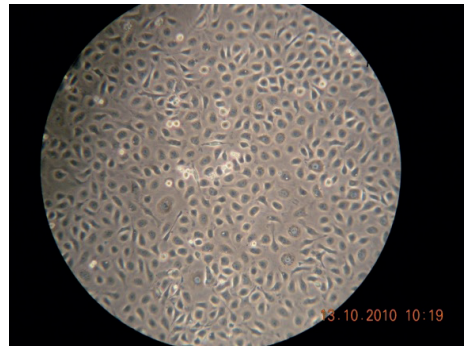


Figure 13.3. MDA-MB-231.

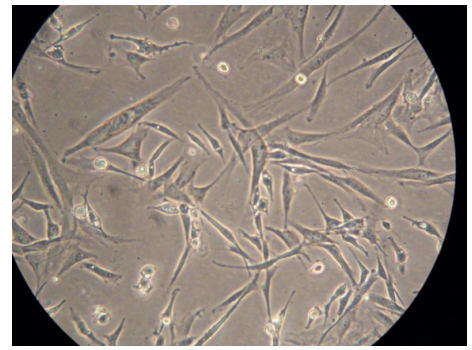


Figure 13.4. Co-culture fibroblast+MDA-MB-231.

12. Rationale for this thesis.

Breast cancer is one of the most frequent cancers among women. Approximately 1.38 million new cases were diagnosed in 2008 (1). Of those 1.38 million cases, the approximate number of deaths from breast cancer was about one third (458,000cases). The treatment of breast cancer requires a multimodality approach in order to eradicate residual cancer and prevent recurrent disease. Targeting the growth and invasion pathways of breast cancer is critical to effective treatment (62-64). The estrogen receptor pathway is a well-known route for breast cancer development and progression. This kind of cancer is diagnosed more frequently in postmenopausal women. The incidence of hormone sensitive tumors is higher in postmenopausal women (60%) than in premenopausal women (65, 66). Many estrogen receptor antagonists and aromatase inhibitors have been successfully developed for breast cancer treatment. Nonetheless, patients resist hormonal therapy. These are the triple negative breast cancers; negative for estrogen receptors, progesterone receptors, and human epidermal growth factor receptors (HER2-receptor). Their cancers are resistant both to hormonal treatment and to chemotherapy. Consequently, there is a high morbidity rate among these patients. Recent research has found that more than 70 % of breast cancer cases, including triple negative breast cancer, express androgen receptors in the malignant tissue (31, 67, 68). This makes the androgen receptor may be another possible target in cases of hormonal resistance or triple negative breast cancer. There is much clinical evidence suggesting that androgens normally inhibit mammary epithelial proliferation and breast growth. Consequently, androgens have been hypothesized to influence the risk of breast cancer by many mechanisms, including their conversion to estradiol or their ability to bind estrogen receptors and/or androgen receptors (AR) in the breast (32, 69).

Preclinical data suggests that dehydroepiandosterone (DHEA) stimulates growth in hormonal positive breast cancer cells. However, in some studies DHEA appears rather to inhibit the growth of human ER-negative breast cancer cell lines, when they demonstrate

a strong expression of AR. This suggest that DHEA could lead to decreased AR gene expression (32). These apparent contradictions may be due to the complexity of the breast cancer ontogenesis

Breast cancer usually refers to invasive ductal carcinoma, the abnormal growth of the ductal epithelium which accounts for 20 % of the breast tissue. The rest of breast stroma comprises fibroblasts, adipocytes and endothelial cells. There is some evidence that tumor-associated stromal cells promote the development of epithelial malignancy. For example, *in vitro*, premalignant mammary epithelial cells adopt a malignant growth pattern when cultured with tumor-derived stromal cells (60). A study by Heneweer, et al. found that fibroblasts could have an indirect effect on the proliferation of breast cancer cell lines in a co-culture system via cytokine regulated estrogen receptor modulation (61).

The aim of this doctoral research was to test the effect of estrogen, aromatase inhibitor (letrazole), androgens (testosterone and dihydrotestosterone) and synthetic derivatives of phytochemicals in both single cell lines and co-culture cell systems. The co-culture systems comprised a hormonal positive breast cancer cell line such as MCF-7 cells or T47D; a hormonal negative breast cancer cell line such as MDA-MB-231; and both hormonal positive and negative primary human breast cancer cells co-culture with isolated human fibroblasts. We compared the direct and indirect effects of estrogen, aromatase inhibitors, androgens and synthetic derivatives of phytochemicals in these cell cultures.

13. Statement of the problem.

One of the well-known pathways for the development and progress of breast cancer is mediated through interaction with the estrogen receptor alpha (ERα), leading to enhanced proliferation of the tumor cell (70). In addition, breast cancer is more frequently diagnosed in postmenopausal women. The ERα positive tumor (ER^+) incidence is also higher in this group (60%) than in premenopausal women (65, 66). Consequently, many selective estrogen receptor modulators (SERMs) that compete with endogenous estrogens on the ERα in tumor cells, or selective estrogen enzyme modulators (SEEMs) that target enzymes involved in the biosynthesis of steroid hormones have been developed for breast cancer treatment. In this way, the risk of recurrence and mortality has been significantly decreased (71, 72). However, there is a group of breast cancer patients who do not respond to hormonal therapies. Often, their breast tumors do not express the hormonal receptors ERα, progesterone receptors (PR) or human epidermal growth factor receptors (HER-2). They are so-called triple negative breast tumors (73). These cancers are resistant not only to various hormonal treatments, but also to chemotherapy, resulting in a high morbidity and mortality rate (74).

Recent studies have found that up to seventy percent of all breast tumors, including triple negative breast tumors, express the androgen receptor (AR) in the malignant tissue (67, 68). However, the available information is both limited and conflicting regarding the role of androgens or the AR in the etiology of breast cancer. Some studies report an association between a low affinity androgen receptor that displays a CAG repeated polymorphism and breast tumor formation (69). At the same time, *in vitro* experiments have shown both proliferative and anti-proliferative actions of androgens in breast cancer cells. Preclinical data has suggested that dehydroepiandosterone (DHEA) can stimulate growth in AR positive breast cancer cells (75), but other studies indicate that this weak androgen can inhibit the growth of human ER negative (ER^-) breast cancer tumor cells, if there is a strong expression of the AR (32). In addition, there is clinical evidence suggesting that androgens normally inhibit mammary epithelial proliferation and breast growth (11, 76). By contrast, there is also evidence suggesting that androgens increase the risk of breast cancer (77). The peripheral conversion of androgens to estradiol by aromatase (Cytochrome P450 19A1, CYP19A1) has been proposed to contribute to breast tumor cell growth (32, 69). Despite the contradictory results obtained so far, androgens and the AR are often suggested as possible targets for hormonal treatment in breast cancer patients, especially in the case of triple negative breast cancer (32)

In addition to several pharmaceutical SERMs and SEEMs that have been developed over the last decades to treat or prevent breast cancer, a variety of naturally occurring, biologically active compounds have been identified that may be useful as chemopreventive agents for breast cancer. Three biological agents that are often suggested to have such cancer chemopreventive actions are resveratrol, melatonin, and depsidones.

Androgens and synthetic derivatives of phytochemicals may have a role for the hormonal treatment in breast cancer, especially for breast cancer and especially triple negative breast cancer.

14. Hypotheses.

Our main hypothesis is that non-aromatized androgen could be a hormonal treatment for breast cancer especially triple negative ontogenesis. This hypothesis takes into consideration with the fact that surrounding fibroblast cells can modify the effects of androgens in breast cancer. A secondary consideration is the possibility that synthetic derivatives of phytochemicals like resveratrol and melatonin may have a role in breast cancer treatment.

14.1. Non-aromatized androgen might be enlisted as hormonal treatment for breast cancer especially triple negative ontogenesis.

14.2 Resveratrol, melatonin, and depsidones may also have a role in breast cancer treatment.

15. Objectives of the thesis.

To demonstrate the effects of androgens and the synthetic derivatives of phytochemicals on both hormonal positive and triple negative breast cancer cells in co-cultures with isolated primary human breast fibroblast cells.

The specific objectives are as follows:

Chapter II: To find the prevalence of AR expression in Thai patients

15.1. To find the prevalence of AR expression and some factors related to AR expression in Thai breast cancer patients.

Chapter III: To study the effects of estrogen and androgens on breast cancer cell lines

15.2. To establish a breast co-culture system; isolated primary fibroblast cells from breast tissue and co-cultures with breast cancer cell lines; MDA-MB-231, MCF-7 and T47D

15.3. To measure the proliferative effect on breast cancer cell lines of estrogen and androgens

15.4. To measure the proliferative effect on co-culture systems of fibroblasts and breast cancer cell lines of estrogen and androgens

15.5. To measure estrogen and androgen related gene expression in both single cell and co-culture systems

15.6. Compare the different effects of estrogen and androgens on single systems and co-culture systems

Chapter IV and V: To assess the effects of synthetic derivatives of phytochemicals (resveratrol, melatonin, and depsidones) on breast cancer cell lines

15.7. To test the effects of synthetic derivatives of phytochemicals (melatonin and cis-stilbenes) in both single cell lines and in the co-culture cell systems of both hormonal positive and triple negative breast cancer cell lines.

15.8. To test the effect of depsidones in both single cell lines and in the co-culture cell systems of hormonally positive breast cancer cell lines.

Chapter VI: General discussion and summary.

References

1. Breast Cancer Incidence and Mortality Worldwide in 2008 Summary [Internet]. IARC. 2008 [cited 27/4/2010].
2. Trop I, Lalonde L, Mayrand MH, David J, Larouche N, Provencher D. Multimodality breast cancer screening in women with a familial or genetic predisposition. Curr Oncol.17(3):28-36.
3. McPherson K. Screening for breast cancer--balancing the debate. BMJ.340:c3106.
4. Hickey M, Emery LI, Gregson J, Doherty DA, Saunders CM. The multidisciplinary management of menopausal symptoms after breast cancer: a unique model of care. Menopause.17(4):727-33.
5. Tamaki K, Sasano H, Ishida T, Miyashita M, Takeda M, Amari M, et al. Comparison of core needle biopsy (CNB) and surgical specimens for accurate preoperative evaluation of ER, PgR and HER2 status of breast cancer patients. Cancer Sci.
6. Holmberg L, Ponten J, Adami HO. The biology and natural history of breast cancer from the screening perspective. World J Surg. 1989;13(1):25-30.
7. Gasparini G, Longo R, Torino F, Morabito A. Therapy of breast cancer with molecular targeting agents. Ann Oncol. 2005;16 Suppl 4:iv28-36.
8. Nancy U. Lin EPW. Brain Metastases: The HER2 Paradigm. Clinical Cancer Research. 2007;13(6):8.
9. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature. 1993;362(6423):841-4.
10. Valdes G, Erices R, Chacon C, Corthorn J. Angiogenic, hyperpermeability and vasodilator network in utero-placental units along pregnancy in the guinea-pig (Cavia porcellus). Reproductive biology and endocrinology : RB&E. 2008;6:13.
11. Dimitrakakis C, Bondy C. Androgens and the breast. Breast Cancer Res. 2009;11(5):212.
12. Bear HD. Neoadjuvant chemotherapy for operable breast cancer: individualizing locoregional and systemic therapy. Surg Oncol Clin N Am.19(3):607-26.
13. Goldhirsch A, Price KN. Adjuvant endocrine therapy in premenopausal women with operable breast cancer: understanding the issues. Oncology (Williston Park).24(4):331-2.
14. Burstein HJ, Griggs JJ. Adjuvant hormonal therapy for early-stage breast cancer. Surg Oncol Clin N Am.19(3):639-47.
15. Nasim S, Pritchard KI. Adjuvant hormonal therapy for premenopausal breast cancer: incorporating clinical experience. Oncology (Williston Park).24(4):332, 7, 40.
16. IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). The nomenclature of steroids. Recommendations 1989. Eur J Biochem. 1989;186(3):429-58.
17. Ryan KJ. Biochemistry of aromatase: significance to female reproductive physiology. Cancer Res. 1982;42(8 Suppl):3342s-4s.
18. Prossnitz ER, Arterburn JB, Sklar LA. GPR30: A G protein-coupled receptor for estrogen. Mol Cell Endocrinol. 2007;265-266:138-42.
19. Nelson LR, Bulun SE. Estrogen production and action. J Am Acad Dermatol. 2001;45(3 Suppl):S116-24.
20. Sommer S, Fuqua SA. Estrogen receptor and breast cancer. Semin Cancer Biol. 2001;11(5):339-52.
21. Boron WF BE. Medical Physiology: A Cellular And Molecular Approach: Elsevier/Saunders; 2003.
22. Lu NZ, Wardell SE, Burnstein KL, Defranco D, Fuller PJ, Giguere V, et al. International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol Rev. 2006;58(4):782-97.
23. Bardin CW, Brown T, Isomaa VV, Janne OA. Progestins can mimic, inhibit and potentiate the actions of androgens. Pharmacol Ther. 1983;23(3):443-59.
24. Heemers HV, Tindall DJ. Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. Endocr Rev. 2007;28(7):778-808.
25. Burness ML, Grushko TA, Olopade OI. Epidermal growth factor receptor in triple-negative and basal-like breast cancer: promising clinical target or only a marker? Cancer J.16(1):23-32.
26. Jung SY, Kim HY, Nam BH, Min SY, Lee SJ, Park C, et al. Worse prognosis of metaplastic breast cancer patients than other patients with triple-negative breast cancer. Breast Cancer Res Treat.120(3):627-37.
27. Isakoff SJ. Triple-negative breast cancer: role of specific chemotherapy agents. Cancer J. 2010;16(1):53-61.
28. Venkitaraman R. Triple-negative/basal-like breast cancer: clinical, pathologic and molecular features. Expert Rev Anticancer Ther.10(2):199-207.
29. Ryden L, Jirstrom K, Haglund M, Stal O, Ferno M. Epidermal growth factor receptor and vascular endothelial growth factor receptor 2 are specific biomarkers in triple-negative breast cancer. Results from a controlled randomized trial with long-term follow-up. Breast Cancer Res Treat.120(2):491-8.
30. Jung SY, Kim HY, Nam BH, Min SY, Lee SJ, Park C, et al. Worse prognosis of metaplastic breast cancer patients than other patients with triple-negative breast cancer. Breast Cancer Res Treat. 2010;120(3):627-37.
31. Luo X, Shi YX, Li ZM, Jiang WQ. Expression and clinical significance of androgen receptor in triple negative breast cancer. Chin J Cancer. 2010;29(6):585-90.
32. Gucalp A, Traina TA. Triple-negative breast cancer: role of the androgen receptor. Cancer J. 2010;16(1):62-5.
33. Paul S, Mizuno CS, Lee HJ, Zheng X, Chajkowisk S, Rimoldi JM, et al. In vitro and in vivo studies on stilbene analogs as potential treatment agents for colon cancer. Eur J Med Chem. 2010;45(9):3702-8.
34. Hasiah AH, Ghazali AR, Weber JF, Velu S, Thomas NF, Inayat Hussain SH. Cytotoxic and antioxidant effects of methoxylated stilbene analogues on HepG2 hepatoma and Chang liver cells: Implications for structure activity relationship. Hum Exp Toxicol. 2010;30(2):138-44.
35. Park JW, Woo KJ, Lee JT, Lim JH, Lee TJ, Kim SH, et al. Resveratrol induces pro-apoptotic endoplasmic reticulum stress in human colon cancer cells. Oncol Rep. 2007;18(5):1269-73.
36. Sangjun S, de Jong E, Nijmeijer S, Mutarapat T, Ruchirawat S, van den Berg M, et al. Induction of cell cycle arrest in human MCF-7 breast cancer cells by cis-stilbene derivatives related to VIOXX. Toxicol Lett. 2009;186(2):115-22.
37. Levi F, Pasche C, Lucchini F, Ghidoni R, Ferraroni M, La Vecchia C. Resveratrol and breast cancer risk. Eur J Cancer Prev. 2005;14(2):139-42.
38. Alkhalaf M, El-Mowafy A, Renno W, Rachid O, Ali A, Al-Attyiah R. Resveratrol-induced apoptosis in human breast cancer cells is mediated primarily through the caspase-3-dependent pathway. Arch Med Res. 2008;39(2):162-8.
39. Lee HS, Ha AW, Kim WK. Effect of resveratrol on the metastasis of 4T1 mouse breast cancer cells in vitro and in vivo. Nutr Res Pract. 2012;6(4):294-300.
40. Leon-Galicia I, Diaz-Chavez J, Garcia-Villa E, Uribe-Figueroa L, Hidalgo-Miranda A, Herrera LA, et al. Resveratrol induces downregulation of DNA repair genes in MCF-7 human breast cancer cells. Eur J Cancer Prev. 2012;22(1):11-20.
41. Lee JE, Safe S. Involvement of a post-transcriptional mechanism in the inhibition of CYP1A1 expression by resveratrol in breast cancer cells. Biochem Pharmacol. 2001;62(8):1113-24.
42. Le Corre L, Chalabi N, Delort L, Bignon YJ, Bernard-Gallon DJ. Resveratrol and breast cancer chemoprevention: molecular mechanisms. Mol Nutr Food Res. 2005;49(5):462-71.
43. Zhang S, Cao HJ, Davis FB, Tang HY, Davis PJ, Lin HY. Oestrogen inhibits resveratrol-induced post-translational modification of p53 and apoptosis in breast cancer cells. Br J Cancer. 2004;91(1):178-85.
44. Wang Y, Lee KW, Chan FL, Chen S, Leung LK. The red wine polyphenol resveratrol displays bilevel inhibition on aromatase in breast cancer cells. Toxicol Sci. 2006;92(1):71-7.
45. Crowell JA, Korytko PJ, Morrissey RL, Booth TD, Levine BS. Resveratrol-associated renal toxicity. Toxicol Sci. 2004;82(2):614-9.

46. Mediavilla MD, Sanchez-Barcelo EJ, Tan DX, Manchester L, Reiter RJ. Basic mechanisms involved in the anti-cancer effects of melatonin. Curr Med Chem.17(36):4462-81.
47. Srinivasan V, Spence DW, Pandi-Perumal SR, Trakht I, Cardinali DP. Therapeutic actions of melatonin in cancer: possible mechanisms. Integr Cancer Ther. 2008;7(3):189-203.
48. Dai M, Cui P, Yu M, Han J, Li H, Xiu R. Melatonin modulates the expression of VEGF and HIF-1 alpha induced by CoCl2 in cultured cancer cells. J Pineal Res. 2008;44(2):121-6.
49. Mills E, Wu P, Seely D, Guyatt G. Melatonin in the treatment of cancer: a systematic review of randomized controlled trials and meta-analysis. J Pineal Res. 2005;39(4):360-6.
50. Grant SG, Melan MA, Latimer JJ, Witt-Enderby PA. Melatonin and breast cancer: cellular mechanisms, clinical studies and future perspectives. Expert Rev Mol Med. 2009;11:e5.
51. Korkmaz A, Sanchez-Barcelo EJ, Tan DX, Reiter RJ. Role of melatonin in the epigenetic regulation of breast cancer. Breast Cancer Res Treat. 2009;115(1):13-27.
52. Blask DE, Brainard GC, Dauchy RT, Hanifin JP, Davidson LK, Krause JA, et al. Melatonin-depleted blood from premenopausal women exposed to light at night stimulates growth of human breast cancer xenografts in nude rats. Cancer Res. 2005;65(23):11174-84.
53. Sanchez-Barcelo EJ, Cos S, Mediavilla D, Martinez-Campa C, Gonzalez A, Alonso-Gonzalez C. Melatonin-estrogen interactions in breast cancer. J Pineal Res. 2005;38(4):217-22.
54. Treeck O, Haldar C, Ortmann O. Antiestrogens modulate MT1 melatonin receptor expression in breast and ovarian cancer cell lines. Oncol Rep. 2006;15(1):231-5.
55. Yuan L, Collins AR, Dai J, Dubocovich ML, Hill SM. MT(1) melatonin receptor overexpression enhances the growth suppressive effect of melatonin in human breast cancer cells. Mol Cell Endocrinol. 2002;192(1-2):147-56.
56. Martinez-Campa C, Gonzalez A, Mediavilla MD, Alonso-Gonzalez C, Alvarez-Garcia V, Sanchez-Barcelo EJ, et al. Melatonin inhibits aromatase promoter expression by regulating cyclooxygenases expression and activity in breast cancer cells. Br J Cancer. 2009;101(9):1613-9.
57. Li CI, Anderson BO, Daling JR, Moe RE. Trends in incidence rates of invasive lobular and ductal breast carcinoma. JAMA. 2003;289(11):1421-4.
58. Wellings SR. A hypothesis of the origin of human breast cancer from the terminal ductal lobular unit. Pathol Res Pract. 1980;166(4):515-35.
59. Wren BG. The origin of breast cancer. Menopause. 2007;14(6):1060-8.
60. Shekhar MP, Werdell J, Santner SJ, Pauley RJ, Tait L. Breast stroma plays a dominant regulatory role in breast epithelial growth and differentiation: implications for tumor development and progression. Cancer Res. 2001;61(4):1320-6.
61. Heneweer M, Muusse M, Dingemans M, de Jong PC, van den Berg M, Sanderson JT. Co-culture of primary human mammary fibroblasts and MCF-7 cells as an in vitro breast cancer model. Toxicol Sci. 2005;83(2):257-63.
62. Tagliabue E, Balsari A, Campiglio M, Pupa SM. HER2 as a target for breast cancer therapy. Expert Opin Biol Ther. 2010;10(5):711-24.
63. Guth U, Huang DJ, Schotzau A, Zanetti-Dallenbach R, Holzgreve W, Bitzer J, et al. Target and reality of adjuvant endocrine therapy in postmenopausal patients with invasive breast cancer. Br J Cancer. 2008;99(3):428-33.
64. Miles DW. Recent advances in systemic therapy. When HER2 is not the target: advances in the treatment of HER2-negative metastatic breast cancer. Breast Cancer Res. 2009;11(4):208.
65. Brody JG, Rudel R, Maxwell NI, Swedis SR. Mapping out a search for environmental causes of breast cancer. Public Health Rep. 1996;111(6):494-507.
66. Brody JG, Tickner J, Rudel RA. Community-initiated breast cancer and environment studies and the precautionary principle. Environ Health Perspect. 2005;113(8):920-5.
67. Higgins MJ, Wolff AC. The androgen receptor in breast cancer: learning from the past. Breast Cancer Res Treat. 2010;124(3):607-17.

68. Park S, Koo J, Park HS, Kim JH, Choi SY, Lee JH, et al. Expression of androgen receptors in primary breast cancer. Ann Oncol. 2009;21(3):488-92.
69. Hao Y, Montiel R, Li B, Huang E, Zeng L, Huang Y. Association between androgen receptor gene CAG repeat polymorphism and breast cancer risk: a meta-analysis. Breast Cancer Res Treat. 2010.
70. Russo J, Snider K, Pereira JS, Russo IH. Estrogen induced breast cancer is the result in the disruption of the asymmetric cell division of the stem cell. Horm Mol Biol Clin Investig. 2011;1(2):53-65.
71. Dixon JM. Prospects of neoadjuvant aromatase inhibitor therapy in breast cancer. Expert Rev Anticancer Ther. 2008;8(3):453-63.
72. Howell A, Howell SJ, Clarke R, Anderson E. Where do selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) now fit into breast cancer treatment algorithms? J Steroid Biochem Mol Biol. 2001;79(1-5):227-37.
73. Griffiths CL, Olin JL. Triple negative breast cancer: a brief review of its characteristics and treatment options. J Pharm Pract. 2012;25(3):319-23.
74. Amos KD, Adamo B, Anders CK. Triple-negative breast cancer: an update on neoadjuvant clinical trials. Int J Breast Cancer. 2012;2012:385978.
75. Nahleh Z. Androgen receptor as a target for the treatment of hormone receptor-negative breast cancer: an unchartered territory. Future Oncol. 2008;4(1):15-21.
76. Dimitrakakis C. Androgens and breast cancer in men and women. Endocrinol Metab Clin North Am. 2011;40(3):533-47, viii.
77. Howard JM. Testosterone may cause breast cancer. Int J Cancer. 2005;115(3):497; author reply 8.

Chapter 2

Androgen Receptor Expression in Thai Breast Cancer Patients

Suthat Chottanapund [1,2,3,4,*], Majorie B. M. Van Duursen [5], Kumpol Ratchaworapong [6], Panida Navasumrit [1,2,3], Mathuros Ruchirawat [1,2,3] and Martin Van den Berg [5]

[1] Division of Environmental Toxicology, Chulabhorn Graduate Institute, Bangkok, Thailand, 10210; panida@cri.or.th (P.N.); mathuros@cri.or.th (M.R.)
[2] Laboratory of Environmental Toxicology, Chulabhorn Research Institute, Bangkok, Thailand, 10210.
[3] Center of Excellence on Environmental Health, Toxicology and Management of Chemicals, Bangkok, Thailand, 10400.
[4] Bamrasnaradura Infectious Diseases Institute, Ministry of Public Health, Nontaburi, Thailand, 11000.
[5] Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands, NL-3508; m.vanduursen@uu.nl (M.B.M.V.D.); m.vandenberg@uu.nl (M.V.d.B.)
[6] Department of Surgery, Charoenkrung Pracharuk Hospital, Bangkok, Thailand, 10120; siajeab@hotmail.com (K.R.)

* **Correspondence:** suthat.c@bidi.mail.go.th; Tel: +662-590-3408; Fax: +662-590-3411

Keywords: breast cancer; androgens; androgen receptors

Published in Med. Sci. 2016, 4(3), 15; doi:10.3390/medsci4030015

Abstract.

The aim of this study was to investigate prevalence and related factors of androgen receptor (AR) expression in Thai breast cancer patients. A descriptive study was done in 95 patients, who were admitted to Charoenkrung Pracharak Hospital, Bangkok (2011–2013). Statistical relationships were examined between AR protein expression, tumor status, and patient characteristics. Compared with those from Western countries, ethnic Thai patients were younger at age of diagnosis and had a higher proliferative index (high Ki-67 expression), which indicates unfavorable prognosis. In addition, 91% of the Thai breast tumors that were positive for any of the following receptors, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) also expressed the AR protein, while in triple negative breast tumors only 33% were AR positive. ER and PR expression was positively related with AR expression, while AR expression was inversely correlated to Ki-67 expression. AR status was strongly correlated with ER and PR status in Thai patients. There is an inverse relationship between Ki-67 and AR, which suggests that AR may be a prognostic factor for breast cancer.

1. Introduction.

Breast cancer is by far the most common type of cancer among women around the world. In 2012, there were about 1,671,000 new cases of female breast cancer worldwide (1). According to the American Cancer Society, there still is an increasing trend of female breast cancer incidence. The number of female breast cancer cases in the US in 2015 was 231,840, which accounts for 122.8 per 100,000 persons/year of all female cancers (2). The number of new breast cancer cases in Thailand in 2012 was about 12,000 cases, which accounts for 137.5 per 100,000 persons/year (3). Interestingly, the mortality of breast cancer patients is strongly dependent on region and ranges from 6 to 20 per 100,000 persons/year (1).

There is scientific consensus that breast cancer is a systemic disease and multimodality treatment is needed to cure or prevent residual cancer after surgery (4,5). The three well-established systemic treatments for breast cancer are chemotherapy, hormonal therapy and biological targeted therapy. Systemic chemotherapy is generally considered to have the highest efficacy against breast cancer, but it can cause serious side effects for the patient (6,7). Compared to chemotherapy, hormonal treatment has fewer side effects, but it can only be used if the breast tumor cells express the estrogen receptor (ER) (4,8). Currently, there are two types of hormonal treatments available for ER responsive breast cancer, which use either an anti-ER drug (e.g., tamoxifen) (9) or an aromatase inhibitor (e.g., letrozole) (10). In addition, biological targeted therapies are a relatively new way to fight breast cancer and they target a specific protein in the tumor cell. However, the costs of these treatments are very high and often tumor specific, which makes them less affordable for developing countries and emerging economies. Despite the availability of these different systemic treatment options, breast cancer continues to have a high case-fatality of about one-fifth of all cases (2).

Mortality and morbidity are higher for patients with triple negative breast cancer, which has a negative ontology for hormonal receptors (11,12). At present, chemotherapy is the only systemic treatment for triple negative breast cancer, but the results on overall survival still remain poor (13,14). Most of the patients with a triple negative breast tumor will develop brain metastasis in the terminal phase of the disease (14). Therefore, there is an urgent need to find systemic treatment therapies that can increase overall survival for these patients.

There is growing evidence that the androgen receptor (AR) might be a new target for systemic breast cancer treatment (15,16). Several studies show that androgens can inhibit mammary epithelial proliferation and breast tumor growth. Conversely, some androgens may actually increase tumor size due to their conversion into estrogens by aromatase and their subsequent ability to bind to the ER, which leads to increased proliferation

of the ER-responsive breast tumor cells (16). We previously showed, using a co-culture model of human breast tumor cells with primary breast fibroblasts, that some androgens can indeed inhibit breast cancer cell growth via the AR (17). However, the positive or negative effects depend on the type of androgen used, e.g., aromatizable testosterone (T) or non-aromatizable dihydrotestosterone (DHT), but also on the ERα and AR status of the breast tumor cell and aromatase activity in the surrounding breast fibroblasts (17). Our present study was aimed to determine the prevalence of AR expression and its relationship with some clinical and pathological parameters in Thai breast cancer patients**.**

2. Methods.

2.1. Thai Breast Cancer Patient and Tumor Characteristics.

This exploratory study enrolled a total of 95 Thai breast cancer patients that were admitted in the Charoenkrung Pracharuk Hospital (Bangkok, Thailand) between October 2011 and October 2013. Clinical and pathological data were collected from the patients' records. These data included age, menstrual status, body mass index (BMI), pre-operative pathology, previous diagnostic method, tumor size, tumor location, clinical nodal status, type of surgery, tumor stage, final pathology, pathological nodal metastasis, nuclear grade, margin of lymphovascular invasion (LVI), neural invasion, proliferative index (Ki-67), status of the ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2) responsiveness. The immunohistochemistry staining process was done with a Bench Mark GX automatic machine (Ventana, Roche, Tucson, AZ, USA). The level of receptor expression was reported by a Thai board qualified pathologist. Rabbit monoclonal primary antibodies (clone SP1), rabbit monoclonal primary antibodies (clone 1E2), anti-HER-2/neu (4B5) rabbit monoclonal primary antibodies, and rabbit monoclonal primary antibodies (clone 30-9) (Ventana, Roche) were used to stain ER, PR, HER-2 and Ki-67, respectively.

2.2. Ethical Considerations.

All patients who enrolled in the study gave a written informed consent. In addition, these patients received full information and explanation about the study objectives and rights that the patient has before enrolling in the study. The study was approved by the Ethics Committee for Researches Involving Human Subjects at the Bangkok Metropolitan Administration (Charoenkrung Pracharuk Hospital), on 21 October 2011, with the project identification number 073.54. This research has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.3. Androgen Receptor Protein Expression.

The presence of the androgen receptor protein was tested in the remaining pathological specimens of the breast tumor tissue by using an anti-androgen receptor (SP107) rabbit monoclonal primary antibody (Cell-Marque, Roche, CA, USA), which was stained with

the immunoperoxidase method (18). The immunohistochemistry staining process was done with a Bench Mark GX automatic machine (Ventana, Roche). The level of androgen receptor expression was reported by a Thai board qualified pathologist. While immunohistochemistry is a common method for protein detection, there is no standard cutoff point for androgen expression in breast cancer (19,20). Therefore, we adopted the AR staining procedure that is used for prostate cancer and the H-score method (21), which is similar to the immunohistochemical scoring methods that are used for other hormone receptors (21–23). This score was given as multiplication of the intensity level of staining (0 = none, 1 = weak, 2 = moderate, and 3 = strong) by the percent of staining (the total score is 3 × 100% = 300) (21–23). Figures 1A and 1B show typical examples of AR^+ and AR^- breast tumor tissue from Thai patients in this study.

(a) (b)

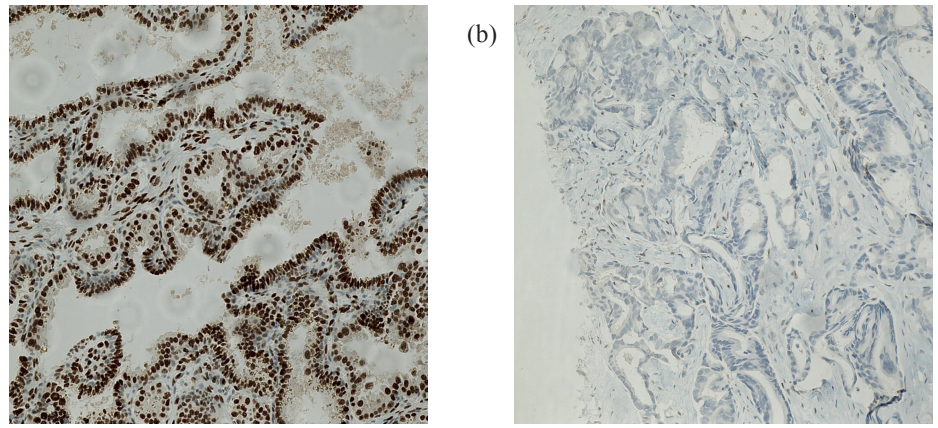


Figure 1. (**a**) Androgen receptor positive breast cancer. Stained androgen receptor in breast cancer tissue (brown color); and (**b**) androgen receptor negative breast cancer. There is no brown color staining in androgen receptor negative breast cancer tissue.

2.4. Sample Size Calculation for Detecting the Prevalence of AR Expression in Thai Patients.

To achieve our primary research objective, the following sample size formula for single proportion was applied:

$$n = \frac{z^2\ (p)\,(1\ -\ p)}{d^2}$$

z = 1.96 at alpha 0.05% or 95% confidence interval
p = Proportion of androgen receptor positive; 70%, according to Ren et al. (24)
d = Acceptable error of 10% = 0.01
$n = (1.96)^2 \cdot (0.7) \cdot (1-0.7)/(0.1) \cdot 2 = 81$ cases

This calculation indicated that at least 81 patients had to enroll in this study in order to achieve enough statistical power.

2.5. Data Analysis.

The statistical analysis of the clinical and pathological data was done with descriptive and inferential statistics. For descriptive analysis, the total values and percentages were used as categorical variables and the mean value with standard error of the mean for the numerical values. In addition, a comparative analysis was carried out using bivariate and multivariate analysis; the χ^2 test was used for categorical variables and Student's *t* test was used for numerical variables with normal distribution. A p value <0.05 was considered statistically significant. Logistic binary regression was used for the multivariate analysis.

3. Results.

3.1. Patient and Tumor Characteristics and AR Expression.

A total of 95 ethnic Thai breast cancer patients were enrolled in this study. Their mean age was 51.37 ± 1.17 (mean ± standard error). Mean body surface area (BSA) and BMI were 1.56 ± 0.01 and 24.38 ± 0.46, respectively. Forty-one cases (43.16%) were in natural post-menopausal state. Axillary nodes were clinically palpable in twelve cases (12.63%), while 46 cases (48%) were pathologically node positive.

Invasive ductal carcinoma was the most common diagnosis in these patients (95.79%) and about 56% of the cases had lympho-vascular invasion. About 70% of the patients had a high proliferative index (Ki-67 expressed ≥20%). The pathological staging at the time of treatment was equally distributed with about 30% of the patients in a late stage of disease (Stage 3).

Approximately 60% of the malignant breast tumors were ER and PR positive, 40% had HER-2 receptor expression (HER-2 positive 2+ and 3+), while only 19% were of triple negative ontology. Of all tumors, 80% (76/95) expressed the AR protein. For more detailed information, see Figures 2 and 3.

3.2. Association of Tumor Characteristics with AR Expression.

91% of the breast tumors with positive expression of ER, PR, or HER-2 also expressed the AR. In contrast, only one-third (6/18) of the triple negative tumors showed an expression of the AR. The factors (including baseline characteristics, pathological and hormonal factors) related to AR expression were statistically analyzed. The expression of the AR was strongly related to ER expression. In addition, it was found that a triple negative tumor status was inversely related to the expression of the AR (p value <0.001) (Table 1).

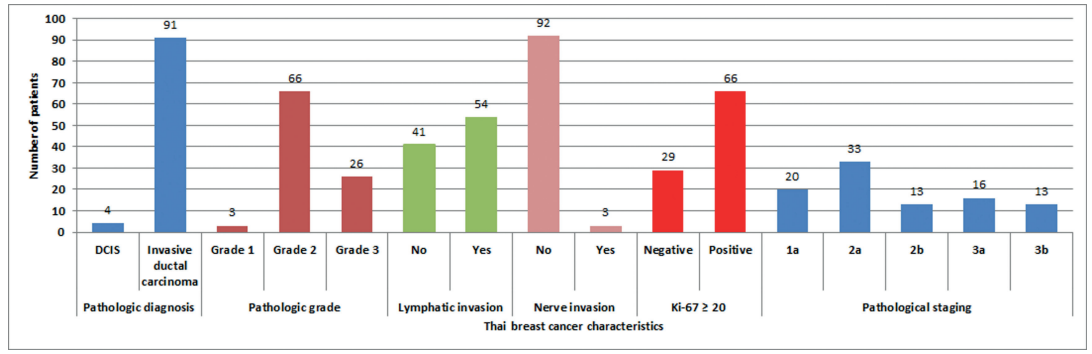


Figure 2. Thai breast cancer patients' characteristics and staging in this study. DCIS: ductal carcinoma in situ.

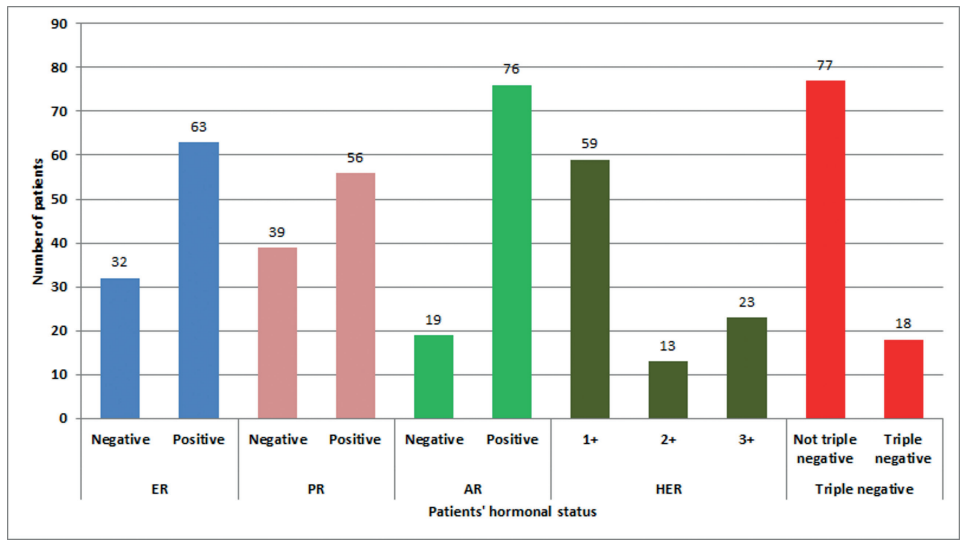


Figure 3. Hormonal status of Thai breast cancer patients in this study. ER: estrogen receptor; PR: progesterone receptor; AR: androgen receptor; HER: human epidermal growth factor receptor.

Table 1. Univariate analysis of factors related to androgen receptor (AR) expression.

Factors		Androgen Status		*p* Value
		Negative ($n = 19$)	Positive ($n = 76$)	
Menstrual status	Pre-menopause	13	30	0.092
	Peri-menopause	1	10	
	Menopause	5	36	
ER	Negative	15	17	<0.001
	Positive	4	59	
PR	Negative	14	25	<0.001
	Positive	5	51	
HER-2	1+	17	42	0.022
	2+	1	12	
	3+	1	22	
Ki-67 ≥20	Negative	2	27	0.034
	Positive	17	49	
Hormonal status	Not triple negative	7	70	<0.001
	Triple negative	12	6	

ER: estrogen receptor; PR: progesterone receptor; HER: human epidermal growth factor receptor.

3.3. Relationship between AR Expression (H-Score) and Other Hormonal Receptors and Ki-67.

The H-score for AR expression was positively correlated with expression of the ER, PR and HER-2 ($p < 0.001$, 0.014, and 0.006, respectively). However, in the triple negative tumors, the H-score of the AR was approximately threefold lower than in the hormonal positive tumors ($p < 0.001$). These quantitative differences in the H-score of AR expression with expression of other (hormonal) receptors are shown in Figure 4a–d. The relationship between AR^+ and AR^- tumors with the cell proliferation marker Ki-67 was also analyzed, and it was found that AR^- tumors had an approximate 50% higher Ki-67 expression than AR+ tumors ($p < 0.01$) (Figure 5).

4. Discussion.

This study is the first to describe AR protein expression in Thai breast cancer patients. Demographic data of Thai breast cancer patients are clearly different from those of Western patients. Unlike those from Western countries, breast cancer patients in Thailand generally come to the hospital with a more advanced, late stage of the disease. This is illustrated by our data in which our patients had a higher breast cancer stage than that generally observed in studies from Western countries. In fact, 15 cases in our study needed chemotherapy before surgery (neoadjuvant chemotherapy; all pathological data of this group of patients were obtained from core needle biopsy), representing 16% of

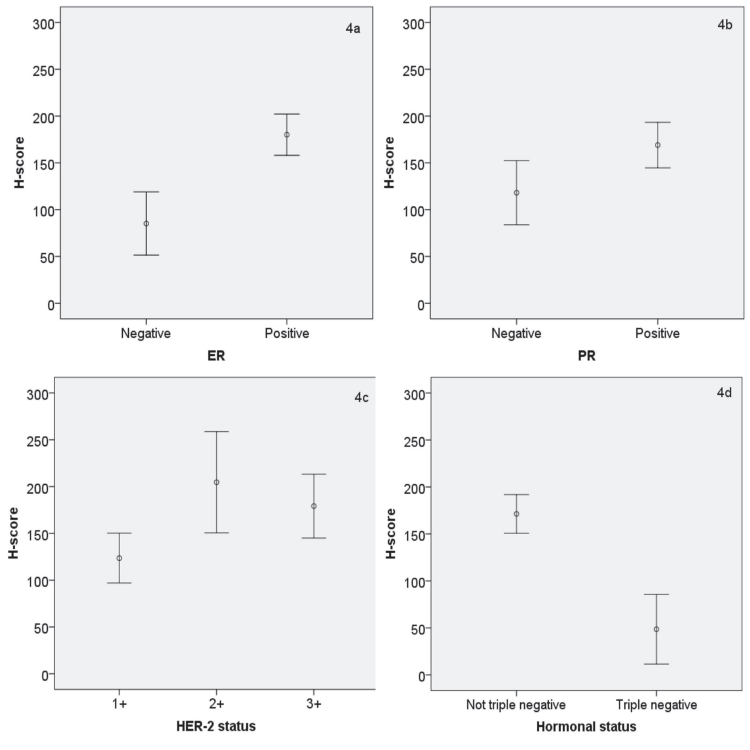


Figure 4. (**a**–**d**) Error bar graph showing the mean ± standard error (S.E.) of androgen H-score by ER, PR, HER-2 and hormonal status. The means ± S.E. were as follows: 85.2 ± 16.6 in ER^- and 180.1 ± 11.0 in ER^+; $p < 0.001$; 118.1 ± 16.9 in PR^- and 169.0 ± 12.1 in PR^+; $p = 0.014$; 123.6 ± 13.0 in HER-2 1+, 204.6 ± 24.8 in HER-2 2+ and 179.1 ± 16.5 in HER-2 3+; $p = 0.006$; 171.4 ± 10.3 in any hormone receptor positive group and 48.6 ± 17.6 in hormone receptor triple negative group; $p < 0.001$.

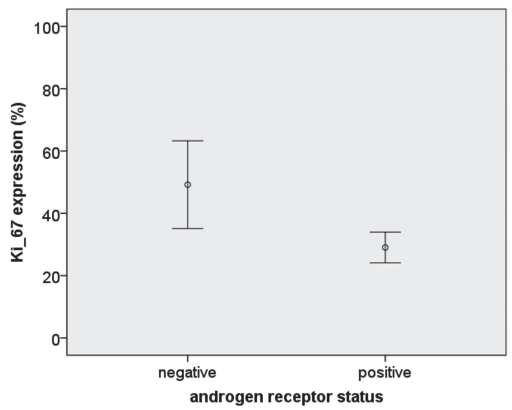


Figure 5. Error bar graph showing the mean ± S.E. of Ki-67 expression by androgen receptor status (49.21 ± 6.70 in AR negative group and 29.03 ± 2.48 in AR positive group; $p = 0.014$).

the patients. The incidence of neoadjuvant chemotherapy in Western countries is usually less than 10% (3,25). In our study, Thai breast cancer patients were younger at the age of diagnosis (90% were diagnosed at an age below 65, compared to only 50% of UK cases diagnosed at an age below 65. In addition, they are more often in premenopausal state (2). The mean age of our patients was 52 years old; half of them were still in the pre-menopausal phase. The most common histological type of breast cancer in our study group was invasive ductal carcinoma and most of our patients were undergoing simple mastectomy (81%). Two-thirds of the tumors expressed hormonal receptors (estrogen and/or progesterone), which is a similar number to that reported from Western countries (26). In our study, 40% of the tumors also expressed the HER2/neu receptor, which is a much higher proportion than that reported from Western countries (19%) (27,28). Approximately 19% of our patients had a triple negative breast tumor, which is comparable to the occurrence reported in Western countries (15%) (29,30). Most of the Thai patients in this study had a high expression of the proliferative index Ki-67 (68%), which indicates that these patients have a less favorable prognostic histology (31). These data clearly demonstrate the geographic variation in breast cancer characteristics, which may strongly influence therapeutic success. Clearly, more research is needed to provide better breast cancer treatment options in non-Western countries. This is also reflected in the relatively high mortality rate for breast cancer in Asian countries as compared to Western countries (1). Here, genetic variations might also play a role. It should be noted that Thailand's population is relatively heterogeneous culturally, but genetically consists of only a few ethnic groups of which some have Chinese, Vietnamese or Malaysian backgrounds. In this study, we did not establish genetic descent, but considered all women of Thai ethnicity as "Thai".

There is emerging evidence that the androgen receptor might be an additional target for systemic breast cancer treatment (15,16); however, the role of androgens and the AR in breast cancer is still controversial. Androgens can either induce or inhibit proliferation of breast cancer. Some clinical studies and in vitro experiments support the view that a low dose of androgens can increase the risk of breast tumor formation and growth (16, 32). In contrast, there are studies that indicate androgens can inhibit the growth of hormonal negative breast cancer cells both in vitro and in vivo, if a strong expression of the AR is present (33,34). The underlying mechanism for this phenomenon may partly be explained by the conversion of some androgens to estrogens by the aromatase enzyme, which can subsequently stimulate breast tumor cell proliferation (17). In a clinical study done by Key et al. and our previous in vitro studies with a co-culture of human breast fibroblasts and tumor cells, indications were found that aromatase can play a key role in the indirect stimulation of androgens on cell proliferation (17,35). However, the latter study also indicated that non-aromatizable androgens can inhibit the growth of breast cancer cells that have AR expression. These androgens can also induce apoptosis regardless of the cells' ER/PR status. Furthermore, androgens may exert their effects via two other pathways; direct stimulation by binding to AR (AR-positive/ER-negative

tumors) or increased synthesis of growth factors, such as epidermal growth factor (EGF) (ER-negative/AR-negative tumors). A more recent study demonstrated that the AR/ER ratio influences breast tumor responses to hormonal treatment (36). Moreover, it was concluded that the AR antagonist enzalutamide could be used for treatment of AR^+ tumors regardless of ER status, since it can block both AR- and ER-mediated tumor growth. Clearly, AR and ER status of a breast tumor will determine the effect of an (anti) androgen on breast tumor cell proliferation.

The AR expression in our Thai patients was about 80%, which is comparable to previous reports from Western countries (24,37). Noticeably, the Thai triple negative breast cancer patients had a lower expression of the AR (33%) compared to Western patients (40%–50%) (15), but AR expression observed in our study was comparable with that reported for Chinese breast cancer patients (28%) (38). These differential findings between breast cancer types in Asian and Western countries may result from population stratification bias and the difference of demographic data (39,40). Some studies have reported that the AR expression is also related to the size of the tumor, the spread to lymph nodes in the armpit, certain kinds of histology, the stage of breast cancer and the status of the ER and PR (41,42). However, these relationships are equivocal, as another study did not detect an association between the AR and any clinical pathological characteristics (43). In our study, the expression of the AR was significantly associated with the concurrent expression of the ER and PR. Our observation is in agreement with that of Soreide et al. who also reported a high AR expression in hormonal positive breast tumors (44). Another Asian study done by Ogawa et al. reported that the menstrual status was related to the AR expression (42). However, we did not observe this in our study population (data not shown).

Another interesting finding in our study was the fact that the expression of the AR was inversely correlated with the non-ER-dependent proliferation index Ki-67. This is an essential protein for progression through the cell cycle (45) and is considered to be a hormone-independent prognostic factor for disease-free and overall survival in breast cancer patients (46). Based on our observations, there is a possibility that AR expression in hormone-dependent breast tumors may also be a prognostic factor for a favorable outcome of the disease. However, considering the limited time that has passed since the collection of the tissue samples in this study, no conclusions can yet be drawn on clinical outcomes and prognosis in our study population based on AR status. Still, a study by Gasparini et al. showed that AR^+ breast cancer patients had a better survival prognosis than those who were AR^- (19). In line with these observations, it should be noted that, in the past, androgens have been used to treat advanced stage breast cancer as reported by de Matteis et al. and Scholl et al. (47,48). In addition, such a treatment was also effectively used to treat skin metastasis of male breast cancer patients by Battelli et al. (49). However, treatment with androgens was abolished in the past because of serious side effects such as hirsutism and aggressive behavior (50). Moreover, the success of anti-

estrogens (51) and aromatase inhibitors to treat hormonal positive breast cancer patients (52,53) made the use androgen therapy less needed. Nevertheless, more recent results, including those from our study, again indicate a possible role of the AR in inhibiting breast tumor progression. However, more experimental and human studies are needed to define more clearly the relevant target group of patients and tumor characteristics needed for a positive response.

5. Conclusions.

In our study, most Thai breast cancer patients had tumors that expressed the AR. These tumors were mostly hormonal and HER-2 positive in nature. Expression of the AR in triple negative breast tumors was less common. When compared with data from Western studies, the Thai patients show distinct differences in AR expression and the ratio between hormonal/HER-2 responsive and triple negative breast tumors. An inverse relationship between the non-hormone-dependent proliferation index Ki-67 and AR expression was found in this study. It is suggested that AR expression may be a prognostic factor for a more favorable outcome of the disease. Further experimental and human studies are needed to establish whether the AR is also a useful therapeutic target in a specific group of patients besides already available adjuvant systemic treatments with ER antagonists and aromatase inhibitors.

Acknowledgments: This work was supported by the Chulabhorn Graduate Institute, Chulabhorn Research Institute (Bangkok, Thailand), and the Institute for Risk Assessment Sciences at the Utrecht University (The Netherlands). The pathological reports were reported by pathologist Chanokpan Chotirat, Division of Pathology, Professional Laboratory Management Corp Co., Ltd., Bangkok, Thailand.

Author Contributions: Suthat Chottanapund participated in the study design, collection of data, analysis of results and writing the manuscript; Kumpol Ratchaworapong participated in the collection of data; M.B.M. Van Duursen, Panida Navasumrit, Mathuros Ruchirawat, and Martin Van den Berg participated in the analysis of results and writing of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on 04/02/2014.
2. The American Cancer Society. *Cancer Facts & Figures 2013*; The American Cancer Society: Atlanta, Georgia, 2015; p. 52.
3. Attasara, P.B.R. *Hospital-Based Cancer Registry*; National Cancer Institute: Bangkok, Thailand, 2007.
4. Tung, N. What is the optimal endocrine therapy for postmenopausal women with hormone receptor-positive early breast cancer? *J. Clin. Oncol.* 2013, *31*, 1391–1397.
5. Goldhirsch, A.; Winer, E.P.; Coates, A.S.; Gelber, R.D.; Piccart-Gebhart, M.; Thurlimann, B.; Senn, H.J. Personalizing the treatment of women with early breast cancer: Highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer. *Ann. Oncol.* 2013, *24*, 2206–2223.
6. Lee, M.C.; Newman, L.A. Management of patients with locally advanced breast cancer. *Surg. Clin. N. Am.* 2007, *87*, 379–398.
7. Ma, C.D.; Chen, C.M.; Chen, X.S.; Liu, G.Y.; Di, G.H.; Wu, J.; Lu, J.S.; Yang, W.T.; Chen, J.Y.; Shao, Z.M.; et al. Neoadjuvant chemotherapy with vinorelbine-containing regimens in elderly patients with locally advanced breast cancer. *Anticancer Res.* 2008, *28*, 3093–3097.
8. McGuire, W.L. Estrogen receptors in human breast cancer. *J. Clin. Investig.* 1973, *52*, 73–77.
9. Regan, M.M.; Neven, P.; Giobbie-Hurder, A.; Goldhirsch, A.; Ejlertsen, B.; Mauriac, L.; Forbes, J.F.; Smith, I.; Lang, I.; Wardley, A.; et al. Assessment of letrozole and tamoxifen alone and in sequence for postmenopausal women with steroid hormone receptor-positive breast cancer: The BIG 1–98 randomised clinical trial at 8·1 years median follow-up. *Lancet Oncol.* 2011, *12*, 1101–1108.
10. Ortmann, O.; Pagani, O.; Jones, A.; Maass, N.; Noss, D.; Rugo, H.; van de Velde, C.; Aapro, M.; Coleman, R. Which factors should be taken into account in perimenopausal women with early breast cancer who may become eligible for an aromatase inhibitor? Recommendations of an expert panel. *Cancer Treat. Rev.* 2010, *37*, 97–104.
11. Yuan, Z.Y.; Wang, S.S.; Gao, Y.; Su, Z.Y.; Luo, W.B.; Guan, Z.Z. Clinical characteristics and prognosis of triple-negative breast cancer: A report of 305 cases. *Ai Zheng* 2008, *27*, 561–565.
12. Kaplan, H.G.; Malmgren, J.A. Impact of triple negative phenotype on breast cancer prognosis. *Breast J.* 2008, *14*, 456–463.
13. Lara-Medina, F.; Perez-Sanchez, V.; Saavedra-Perez, D.; Blake-Cerda, M.; Arce, C.; Motola-Kuba, D.; Villarreal-Garza, C.; Gonzalez-Angulo, A.M.; Bargallo, E.; Aguilar, J.L.; et al. Triple-negative breast cancer in Hispanic patients: High prevalence, poor prognosis, and association with menopausal status, body mass index, and parity. *Cancer* 2011, *117*, 3658–3669.
14. Venkitaraman, R.; Joseph, T.; Dhadda, A.; Chaturvedi, A.; Upadhyay, S. Prognosis of patients with triple-negative breast cancer and brain metastasis. *Clin. Oncol. (R. Coll. Radiol.)* 2009, *21*, 729–730.
15. Nahleh, Z. Androgen receptor as a target for the treatment of hormone receptor-negative breast cancer: An unchartered territory. *Future Oncol.* 2008, *4*, 15–21.
16. Hao, Y.; Montiel, R.; Li, B.; Huang, E.; Zeng, L.; Huang, Y. Association between androgen receptor gene CAG repeat polymorphism and breast cancer risk: A meta-analysis. *Breast Cancer Res. Treat.* 2010, *124*, 815–820.
17. Chottanapund, S.; Van Duursen, M.B.; Navasumrit, P.; Hunsonti, P.; Timtavorn, S.; Ruchirawat, M.; Van den Berg, M. Effect of androgens on different breast cancer cells co-cultured with or without breast adipose fibroblasts. *J. Steroid Biochem. Mol. Biol.* 2013, *138*, 54–62.

18. Gonzalez, L.O.; Corte, M.D.; Junquera, S.; Bongera, M.; Rodriguez, J.C.; Vizoso, F.J. Expression of androgen receptor and two androgen-induced proteins (apolipoprotein D and pepsinogen C) in ductal carcinoma in situ of the breast. *Histopathology* 2007, *50*, 866–874.
19. Gasparini, P.; Fassan, M.; Cascione, L.; Guler, G.; Balci, S.; Irkkan, C.; Paisie, C.; Lovat, F.; Morrison, C.; Zhang, J.; et al. Androgen receptor status is a prognostic marker in non-basal triple negative breast cancers and determines novel therapeutic options. *PLoS ONE* 2014, *9*, e88525.
20. Niemeier, L.A.; Dabbs, D.J.; Beriwal, S.; Striebel, J.M.; Bhargava, R. Androgen receptor in breast cancer: Expression in estrogen receptor-positive tumors and in estrogen receptor-negative tumors with apocrine differentiation. *Mod. Pathol.* 2009, *23*, 205–212.
21. Cohen, D.A.; Dabbs, D.J.; Cooper, K.L.; Amin, M.; Jones, T.E.; Jones, M.W.; Chivukula, M.; Trucco, G.A.; Bhargava, R. Interobserver agreement among pathologists for semiquantitative hormone receptor scoring in breast carcinoma. *Am. J. Clin. Pathol.* 2012, *138*, 796–802.
22. Choudhury, K.R.; Yagle, K.J.; Swanson, P.E.; Krohn, K.A.; Rajendran, J.G. A robust automated measure of average antibody staining in immunohistochemistry images. *J. Histochem. Cytochem*. 2009, *58*, 95–107.
23. Rizzardi, A.E.; Johnson, A.T.; Vogel, R.I.; Pambuccian, S.E.; Henriksen, J.; Skubitz, A.P.; Metzger, G.J.; Schmechel, S.C. Quantitative comparison of immunohistochemical staining measured by digital image analysis versus pathologist visual scoring. *Diagn Pathol.* 2012, *7*, 42.
24. Ren, Q.; Zhang, L.; Ruoff, R.; Ha, S.; Wang, J.; Jain, S.; Reuter, V.; Gerald, W.; Giri, D.D.; Melamed, J.; et al. Expression of androgen receptor and its phosphorylated forms in breast cancer progression. *Cancer* 2013, *119*, 2532–2540.
25. The American Cancer Society. *Cancer Facts & Figures*; The American Cancer Society: Atlanta, Georgia, 2013.
26. Li, C.I.; Daling, J.R.; Malone, K.E., Incidence of invasive breast cancer by hormone receptor status from 1992 to 1998. *J. Clin. Oncol.* 2003, *21*, 28–34.
27. Cronin, K.A.; Harlan, L.C.; Dodd, K.W.; Abrams, J.S.; Ballard-Barbash, R. Population-based estimate of the prevalence of HER-2 positive breast cancer tumors for early stage patients in the US. *Cancer Investig.* 2010, *28*, 963–968.
28. Chan, A.; McGregor, S.R. Prevalence and management of HER2/neu-positive early breast cancer in a single institution following availability of adjuvant trastuzumab. *Intern. Med. J.* 2011, *42*, 267–274.
29. Ismail-Khan, R.; Bui, M.M. A review of triple-negative breast cancer. *Cancer Control* 2010, *17*, 173–176.
30. Swede, H.; Gregorio, D.I.; Tannenbaum, S.H.; Brockmeyer, J.A.; Ambrosone, C.; Wilson, L.L.; Pensa, M.A.; Gonsalves, L.; Stevens, R.G.; Runowicz, C.D. Prevalence and prognostic role of triple-negative breast cancer by race: A surveillance study. *Clin. Breast Cancer* 2011, *11*, 332–341.
31. Li, H.; Han, X.; Liu, Y.; Liu, G.; Dong, G. Ki67 as a predictor of poor prognosis in patients with triple-negative breast cancer. *Oncol. Lett.* 2014, *9*, 149–152.
32. Dreyer, G.; Vandorpe, T.; Smeets, A.; Forceville, K.; Brouwers, B.; Neven, P.; Janssens, H.; Deraedt, K.; Moerman, P.; Van Calster, B.; et al. Triple negative breast cancer: Clinical characteristics in the different histological subtypes. *Breast* 2013, *22*, 761–766.
33. Gucalp, A.; Traina, T.A. Triple-negative breast cancer: Role of the androgen receptor. *Cancer J.* 2010, *16*, 62–65.
34. Narayanan, R.; Ahn, S.; Cheney, M.D.; Yepuru, M.; Miller, D.D.; Steiner, M.S.; Dalton, J.T. Selective androgen receptor modulators (SARMs) negatively regulate triple-negative breast cancer growth and epithelial: Mesenchymal stem cell signaling. *PLoS ONE* 2014, *9*, e103202.
35. Key, T.; Appleby, P.; Barnes, I.; Reeves, G. Endogenous sex hormones and breast cancer in postmenopausal women: Reanalysis of nine prospective studies. *J. Natl. Cancer Inst.* 2002, *94*, 606–616.
36. Cochrane, D.R.; Bernales, S.; Jacobsen, B.M.; Cittelly, D.M.; Howe, E.N.; D'Amato, N.C.; Spoelstra, N.S.; Edgerton, S.M.; Jean, A.; Guerrero, J.; et al. Role of the androgen receptor in breast cancer and preclinical analysis of enzalutamide. *Breast Cancer Res.* 2014, *16*, R7.

37. Hu, R.; Dawood, S.; Holmes, M.D.; Collins, L.C.; Schnitt, S.J.; Cole, K.; Marotti, J.D.; Hankinson, S.E.; Colditz, G.A.; Tamimi, R.M. Androgen receptor expression and breast cancer survival in postmenopausal women. *Clin. Cancer Res.* 2011, *17*, 1867–1874.
38. Luo, X.; Shi, Y.X.; Li, Z.M.; Jiang, W.Q. Expression and clinical significance of androgen receptor in triple negative breast cancer. *Chin. J. Cancer* 2010, *29*, 585–590.
39. Wang, Y.; Localio, R.; Rebbeck, T.R. Evaluating bias due to population stratification in epidemiologic studies of gene-gene or gene-environment interactions. *Cancer Epidemiol. Biomark. Prev.* 2006, *15*, 124–132.
40. Millikan, R.C. Re: Population stratification in epidemiologic studies of common genetic variants and cancer: Quantification of bias. *J. Natl. Cancer Inst.* 2001, *93*, 156–158.
41. Riva, C.; Dainese, E.; Caprara, G.; Rocca, P.C.; Massarelli, G.; Tot, T.; Capella, C.; Eusebi, V. Immunohistochemical study of androgen receptors in breast carcinoma. Evidence of their frequent expression in lobular carcinoma. *Virchows Arch.* 2005, *447*, 695–700.
42. Ogawa, Y.; Hai, E.; Matsumoto, K.; Ikeda, K.; Tokunaga, S.; Nagahara, H.; Sakurai, K.; Inoue, T.; Nishiguchi, Y. Androgen receptor expression in breast cancer: Relationship with clinicopathological factors and biomarkers. *Int. J. Clin. Oncol.* 2008, *13*, 431–435.
43. Gonzalez, L.O.; Corte, M.D.; Vazquez, J.; Junquera, S.; Sanchez, R.; Alvarez, A.C.; Rodriguez, J.C.; Lamelas, M.L.; Vizoso, F.J. Androgen receptor expression in breast cancer: Relationship with clinicopathological characteristics of the tumors, prognosis, and expression of metalloproteases and their inhibitors. *BMC Cancer* 2008, *8*, 149.
44. Soreide, J.A.; Lea, O.A.; Varhaug, J.E.; Skarstein, A.; Kvinnsland, S. Androgen receptors in operable breast cancer: Relation to other steroid hormone receptors, correlations to prognostic factors and predictive value for effect of adjuvant tamoxifen treatment. *Eur. J. Surg. Oncol.* 1992, *18*, 112–118.
45. Scholzen, T.; Gerdes, J. The Ki-67 protein: From the known and the unknown. *J. Cell. Physiol.* 2000, *182*, 311–322.
46. Inwald, E.C.; Klinkhammer-Schalke, M.; Hofstadter, F.; Zeman, F.; Koller, M.; Gerstenhauer, M.; Ortmann, O. Ki-67 is a prognostic parameter in breast cancer patients: Results of a large population-based cohort of a cancer registry. *Breast Cancer Res. Treat.* 2013, *139*, 539–552.
47. De Matteis, A.; Perrone, V.; Marone, A. Clinical considerations on the use of a new synthetic androgen, Calusterone, in the treatment of advanced breast cancer. *Miner. Med.* 1977, *68*, 2499–2504.
48. Scholl, A. Therapeutic results of androgen treatment and ovariectomy in breast cancer. *Norm Pathol. Anat. (Stuttg.)* 1975, 30, 1–46.
49. Battelli, T.; Bonsignori, M.; Manocchi, P.; Perilli, A. Skin metastases of male breast cancer improved after androgen treatment. *Miner. Med.* 1977, *68*, 1663–1666.
50. Kennedy, B.J. Fluoxymesterone therapy in advanced breast cancer. *N. Engl. J. Med.* 1958, *259*, 673–675.
51. Jordan, V.C. Tamoxifen as the first targeted long-term adjuvant therapy for breast cancer. *Endocr. Relat. Cancer* 2014, *21*, R235–R246.
52. Garay, J.P.; Park, B.H. Androgen receptor as a targeted therapy for breast cancer. *Am. J. Cancer Res*. 2012, *2*, 434–435.
53. Dubsky, P.C.; Jakesz, R.; Mlineritsch, B.; Postlberger, S.; Samonigg, H.; Kwasny, W.; Tausch, C.; Stoger, H.; Haider, K.; Fitzal, F.; et al. Tamoxifen and anastrozole as a sequencing strategy: A randomized controlled trial in postmenopausal patients with endocrine-responsive early breast cancer from the Austrian Breast and Colorectal Cancer Study Group. *J. Clin. Oncol.* 2012, *30*, 722–728.

Chapter 3

Effect of androgens on different breast cancer cells co-cultured with or without breast adipose fibroblasts

Suthat Chottanapund[1,2,3,4,5] , Majorie B.M. Van Duursen[4], Panida Navasumrit[1,2,3], Potchanee Hunsonti[2], Supatchaya Timtavorn[2], Mathuros Ruchirawat[1,2,3] and Martin Van den Berg[4]

[1] Division of Environmental Toxicology, Chulabhorn Graduate Institute, Bangkok, Thailand
[2] Laboratory of Environmental Toxicology, Chulabhorn Research Institute, Bangkok, Thailand
[3] Center of Excellence on Environmental Health, Toxicology and management of Chemicals, Bangkok, Thailand
[4] Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
[5] Bamrasnaradura Infectious Diseases Institute, Ministry of Public Health, Thailand

Corresponding author: Dr. Suthat Chottanapund, MD. 126 Bamrasnaradura Infectious Disease Institute 4th floor, 2nd building, Soi Tiwanon 14, Tiwanon Rd., Nontaburi, Thailand 11000

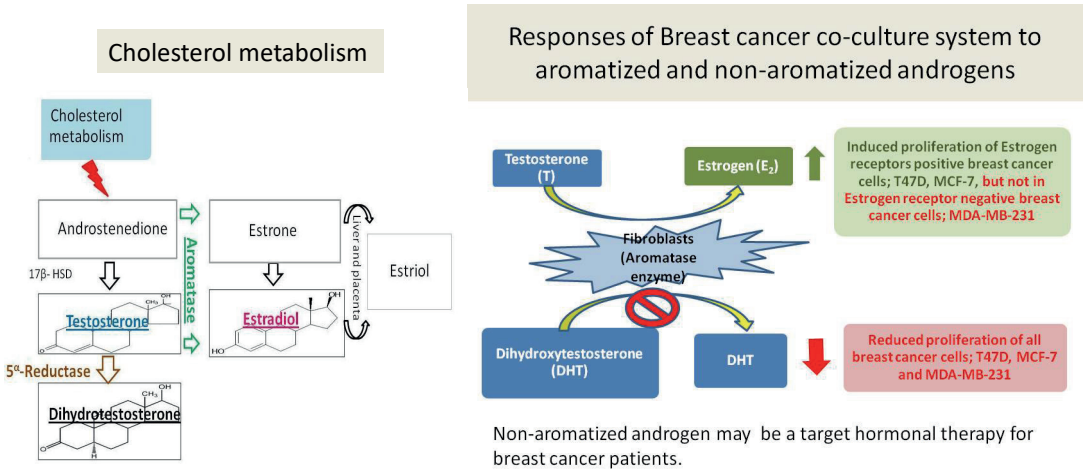
Key words: breast adipose fibroblasts; breast tumor cells, co-culture, estrogen receptor; Androgens; cell proliferation; *pS2; Ki-67;* Aromatase

Published in Journal of Steroid Biochemistry & Molecular Biology 138 (2013) 54– 62

Abstract

About 70% of breast tumors express androgen receptors. In addition, there is clinical evidence suggesting that androgens can inhibit mammary epithelial proliferation. Vice versa, there is also significant evidence indicating that androgens can increase the risk of breast cancer via multiple mechanisms, e.g. direct conversion to estrogens that can bind to the estrogen receptor and thereby stimulate cell proliferation. We examined the effect of testosterone (T) and dihydroxytestosterone (DHT) on cell proliferation, *pS2* and *Ki-67* expression in three different breast cancer cell lines alone or in co-culture with primary human breast adipose fibroblasts (BAFs) obtained from breast cancer patients. In the co-cultures, T induced cell proliferation, *pS2* and *Ki-67* expression in the estrogen receptor positive (ER^+) MCF-7 and T47D cells. This was not observed in the (ER^-) MDA-MB-231 cells. The differences might be explained by the high expression of aromatase, which converts androgens to estrogens in BAFs followed by ER-mediated cell proliferation. In line with this absence of increased cell proliferation, *pS2 and Ki-67* expression was observed in the presence of DHT, which is not a substrate for aromatase. In contrast, DHT caused a significant suppression of cell proliferation (68 % and 38%), *pS2* and *Ki-67* expression in the (ER^+) MCF-7 and T47D cells. More importantly, DHT decreased cell proliferation in (ER^-) MDA-MB-231 cells by 38%. The results suggest that androgens that cannot be aromatized, like DHT, may provide a perspective for treatment of breast cancer patients, especially those with triple negative breast cancer

Graphical Abstract



1. Introduction.

Breast cancer is one of the most frequent cancers among women. In 2008, approximately 1.38 million new cases were diagnosed worldwide**.** Of those 1.38 million cases, the number of fatalities by breast cancer was about one third (approximately 458,000 women) (1). There is general consensus that treatment of breast cancer needs a multimodality approach to eradicate residual cancer cells and prevent recurrent disease. In this respect, targeting cell growth and invasion pathways of the breast tumor is critical for an effective treatment of breast cancer (2-4).

One of the well-known pathways for development and progression of breast cancer is mediated through interaction with the estrogen receptor alpha (ERα) leading to enhanced proliferation of the tumor cell (5-8). In addition, breast cancer is more frequently diagnosed in postmenopausal women and the ERα positive tumor (ER^+) incidence is higher in this group (60%) than in premenopausal women (9, 10). Consequently, many selective estrogen receptor modulators (SERMs) that compete with endogenous estrogens on the ERα in tumor cells or selective estrogen enzyme modulators (SEEMs) that target enzymes involved in the biosynthesis of steroid hormones have been developed for breast cancer treatment, thereby significantly decreasing the risk of recurrence and mortality (11-14). However, there is a group of breast cancer patients who do not respond to hormonal therapies. Often, these breast tumors do not express the hormonal receptors ERα, progesterone receptor (PR) and human epidermal growth factor receptor (HER-2), the so-called triple negative breast tumors (15, 16). Not only are these patients resistant to various hormonal treatments, but these tumors are also often resistant to chemotherapy, resulting in a high morbidity and mortality rate (17-20).

Recent studies have found that up to seventy percent of all breast tumors, including triple negative breast tumors, express the androgen receptor (AR) in the malignant tissue (21-23). However, limited and conflicting information is available regarding the role of androgens or the AR in the etiology of breast cancer. Some studies report an association between a low affinity androgen receptor that displays a CAG repeated polymorphism and breast tumor formation (24). Furthermore, *in vitro* experiments have shown both proliferative and anti-proliferative actions of androgens in breast cancer cells. Preclinical data suggested that dehydroepiandosterone (DHEA) can stimulate growth in AR positive breast cancer cells (25), but other studies indicated that this weak androgen can inhibit the growth of human ER negative (ER^-) breast cancer tumor cells, if there is a strong expression of the AR (26). In addition, there is clinical evidence that suggests that androgens normally inhibit mammary epithelial proliferation and breast growth (27, 28). In contrast, there is also evidence suggesting that androgens may increase the risk of breast cancer (29, 30). Also, the peripheral conversion of androgens to estradiol by aromatase (Cytochrome P450 19A1, CYP19A1) has been proposed to contribute

to breast tumor cell growth (24, 26). Despite the contradictory results obtained so far, androgens and the AR are often suggested as possible targets for hormonal treatment in breast cancer patients, especially in the case of triple negative breast cancer (26, 31, 32).

Most breast tumors are classified as invasive ductal carcinomas, which are the malignant growth of the ductal epithelial cells of breast tissue (33-35). The remaining breast stroma comprises fibroblasts, adipocytes and endothelial cells. There is increasing evidence that tumor-associated stromal cells promote the development of epithelial malignancies. For example, *in vitro* premalignant mammary epithelial cells adopt a malignant growth pattern if cultured with carcinoma derived adipose fibroblasts (CAFs) (36). In addition, a study by Heneweer, et al. found that primary breast adipose fibroblasts (BAFs) could have an indirect effect on the proliferation of breast tumor cells in a co-culture system via modulation of cytokine-regulated estrogen receptors (37).

The main objective of the present study was to demonstrate the effects of testosterone (T) and dihydrotestosterone (DHT) on cell proliferation and *pS2* expression of three different breast tumor cell lines MCF-7 (ER^+, PR^+, $HER2^+$), T47D (ER^+, PR^+, $HER2^-$), and MDA-MB-231 (ER^-, PR^-, $HER2^-$). DHT was chosen because it is a potent androgen, but unlike T cannot be converted to estradiol by aromatase. Experiments were performed either as mono-cultures of these three breast tumor cell lines or in co-cultures with primary human BAFs. The latter *in vitro* model has been shown to better reflect the *in vivo* situation, as it takes into consideration intra-endocrine effects between the breast tumor cells and surrounding fibroblasts (Figure. 1).

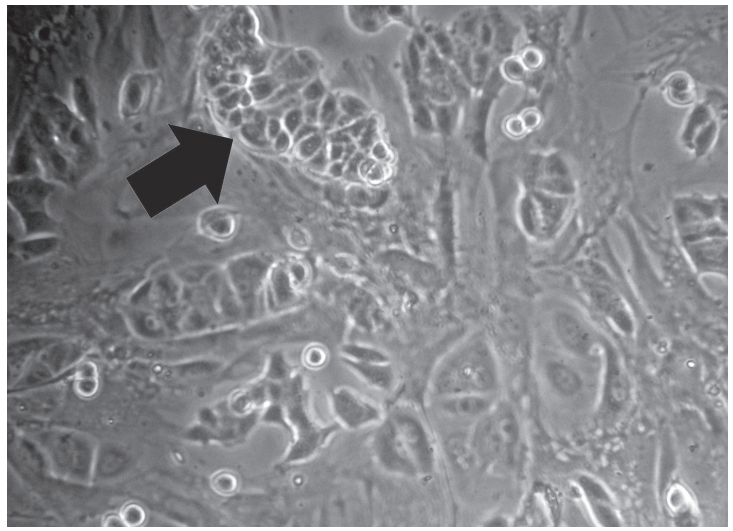


Figure 1. The ductal formation of MCF-7 cells with surrounding human breast adipose fibroblasts in a co-culture (arrow).

2. Materials and methods.

2.1. Breast cancer cell culture and incubation.

The three human breast cancer cell lines MCF-7, T47D and MDA-MB-231 were obtained from ATCC (Rockville, MD, USA). MCF-7 cells were grown in culture medium comprising Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% l-Glutamine at seeding concentration of 5×10^4 cells/ml. T47D cells were grown in culture medium comprising RPMI 1640, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% l-Glutamine, 1% glucose, 1% sodium pyruvate, and 0.08% insulin solution at seeding concentration of 5×10^4 cells/ml. MDA-MB-231 cells were grown in culture medium comprising Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% l-Glutamine, and 1% non-essential amino acids (NEAA) at seeding concentration of 5×10^4 cells/ml. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Primary breast adipose fibroblasts (BAFs).

Primary breast tissue fibroblasts were obtained from three breast cancer patients who attended the Bamrasnaradura Infectious Disease Institute (Nonthaburi, Thailand) for modified radical mastectomy, after informed consent. The research protocol was approved by the Medical Ethical Committee, the Bamrasnaradura Infectious Disease Institute, Thailand. About 5-10 g of macroscopically normal breast tissue was collected for this study and the remaining tissue was sent for routine pathological examination. After the tissue was obtained, it was stored in 4°C saline solution (0.9% NaCl) directly after surgery and immediately transported to the laboratory. BAFs were isolated from breast tissue following the method described earlier (37, 38). Human BAFs were cultured as adherent cultures in RPMI 1640-medium w/o phenol red (Gibco/Invitrogen 11835) supplemented with Pen/Strep (Invitrogen 15140), FCS (Invitrogen 10270) and Insulin 10^{-3} M (144 mg/25 ml) (sigma I-5500) at a seeding concentration of 5×10^4 cells/ml. The cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2. The BAFs were sub-cultured when the bottles were confluent, which was usually once a week.

2.3. Breast cancer cells in co-culture with BAFs.

Approximately three weeks after isolation, fibroblasts were used to establish a co-culture together with the human mammary carcinoma MCF-7, T47D or MDA-MB-231 cells (37, 39). On day 1, BAFs were plated at a density of approximately 4×10^3 cells/well in a 96-well plate (for proliferation) or 5×10^4 cells/ml in a 25 cm^2 flask (for gene expression) in culture medium. The day after, mono-cultures of breast cancer cells and plated primary BAFs were washed with phosphate buffered saline (PBS) and placed on the assay medium in which heat inactivated FCS was replaced with heat-inactivated, charcoal/dextran-treated FCS (Hyclone, SH30068.03). On day 4, BAFs were washed with PBS and breast cancer cells were trypsinized and seeded on top of the BAFs at a density of

4×10^3 cells/well (for proliferation) or 2.5×10^5 cells/flask (for RNA expression). On day 5, fresh assay medium was added to the co-cultures after which the cells were exposed to the test compounds for 96 h (40). Final solvent concentration was 0.1% v/v in the medium and concentrations of steroid hormones were 1, 5, and 10 nM for T and DHT, and 1, 5, and 10 pM for E_2.

2.4. Cell proliferation.

Cell proliferation was determined after treatment with steroid hormones by performing an MTT assay as described earlier (Heneweer et al., 2005) After exposure to the test compounds, 1 ml of MTT (5 mg MTT/ ml in PBS) was added for 4 hours. Next, the medium was aspirated and 100 µl of DMSO was added to dissolve the accumulated formazan crystals. The absorbance was measured at 550 nm using the 96-well microplate reader (Spectramax plus 384, Molecular Devices, California, USA).

2.5. Analysis of gene expression.

After the cells were treated with the different steroid hormones, total RNA was extracted. RNA was reverse-transcribed into cDNA using the Qiagen high capacity cDNA reverse-transcription kit (Qiagen, Texas, USA). The gene expression of *aromatase*, *pS2* and *Ki-67* was performed using a Roche-Light-cycler-480 one step RT PCR kit (Roche, Indiana, USA). Relative quantification of gene expression was expressed as the ratio of the intensity of the target gene to the housekeeping gene *beta-actin*. The conditions for RT-PCR of each gene are described below.

2.6. RNA isolation and gene expression.

Total RNA was isolated using RNeasy® mini kit (Qiagen, Texas, USA). Cells were centrifuged and lysed with RLT buffer and β-mercaptoethanol. One volume of 70% ethanol was added and this was vigorously mixed. The sample was transferred to RNeasy spin column, centrifuged at 12,000 g for 15 seconds, and then washing buffer RW1 (350 µl) was added into the column and centrifuged at 12,000 g for 15 seconds. The 80 ul of DNase I reaction mixture was directly added onto the membrane of the column, and incubated for 15 minutes. The column was washed by RW1 buffer and RPE buffer twice. The bound RNA was eluted with 40 ul of RNase-free water and centrifuged at 12,000 g for 1 minute. The purification and concentration of total RNA were measured by the ratio of absorbance at 260/280 and 260/230 NM using Nanodrop (Nanodrop Technologies, Inc., Delaware, USA).

Obtained RNA was stored at -20°C in aliquots of 10 ng/µl. The reaction mixture contained 0.1µM of primers, 1X Quantitect SYBR Green RT-PCR Master mix, 0.2 µl QuantiTect RT mix and 2 µl template RNA (10 ng/µl) in a total volume of 20 µl. The mixture was reverse transcribed to cDNA at 50°C for 20 minutes. After reverse transcription, PCR reaction was initiated by heating at 95°C for 15 minutes, then followed by denaturation at 95°C for 10 seconds, annealing at 57°C for 25 seconds, an extension

at 68°C for 30 seconds and acquisition at 82°C for 5 seconds for 45 cycles. Primers for aromatase mRNA amplification were used as described previously by Sanderson (41). Primers coding for the estrogen-responsive *pS2* gene were used as described by Lee (42). Primers coding for the androgen receptor gene were used as described by Bieche (43). Primers for *Ki-67* amplification were designed in our laboratory and were forward: 5'-CGGACTTTGGGTGCGACTT -3' and reverse: 5'-GTCGACCCCGCTCCTTTT -3'. Expression of *β-ACTIN* (forward: 5'-TCTACAATGAGCTGCG-3' and reverse: 5'-AGGTAGTCAGCTAGGT-3') was used as a reference housekeeping gene. All primers were run through the National Center for Biotechnology Information (NCBI) blast (nucleotide nonredundant database) to confirm specificity. The efficiency was determined for a dilution range of cDNA and new primers were sought if the efficiency was below 95% or higher than 105%. After each RT-qPCR, a melt curve was run to ensure that primer-dimers and other non-specific products were omitted.

2.7. Data analysis.

Each experiment was performed 3 times, and in each experiment every concentration was tested in triplicate. Means and standard deviations were calculated of all nine values (n=9). The statistical significance of differences of the means were calculated using the Student's t-test or one-way ANOVA. Differences were considered statistically significant if $P < 0.05$.

3. Results.

3.1. Aromatase expression in different tissue types.

Gene expression of *pS2* and aromatase (*CYP19A1*) was determined in the three breast cancer cell lines (T47D, MCF-7 and MDA-MB-231) and primary BAFs obtained from three women. All three types of breast cancer cells and BAFs expressed aromatase, but the aromatase expression in the tumor cells was lower than the breast fibroblasts (Figure. 2). This suggest that indeed BAFs are a major source of peripheral estrogen synthesis in postmenopausal women. In order to reduce the biological variation in the co-culture experiments, only the BAFs from the individual patient with the highest aromatase expression (F3) were used for the co-culture experiments described below.

3.2. Exposure of mono and co-cultures to 17β-estradiol.

In mono-cultures of the $ER\alpha^+$ breast cancer cell lines MCF-7 and T47D, a significant increase in cell proliferation was observed after a 96-hour exposure to 17β-estradiol (E2, 1-10 pM). Cell proliferation was 120% and 150% for mono-cultured MCF-7 and T47D cells respectively at 10 pM E2 compared to vehicle control treated cells (Figures. 3a and 3b). In co-culture with BAFs, some cell proliferation of MCF-7 or T47D cells could still be observed. However, this proliferation was much less than in the mono-cultures with maximum proliferation being 110% and 113% at 10 pM E2 for MCF-7 and T47D co-

cultures, respectively (Figures. 3a and 3b). In both mono- and co-cultured ERα- breast cancer MDA-MB-231 cells, E2 did not cause any significant cell proliferation after a 96-hour incubation (Figure.3c). In the presence of BAFs the MDA-MB-231 showed a slightly higher cell proliferation than in the mono-culture, but this seemed largely independent of the E2 concentration that was added.

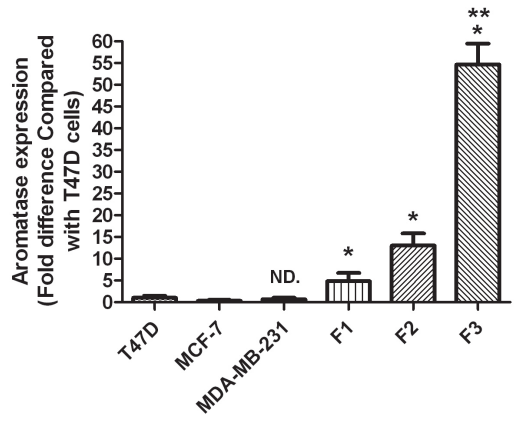


Figure 2. Differences of aromatase gene expression in breast cancer cell lines and primary BAFs of three patients (F1, F2, and F3). * Significantly different from three breast cancer cell lines (Mann-Whitney test, $p < 0.01$). ** Significantly different from patients F1 and F2 (Mann-Whitney test, $p < 0.01$).

3.3. Exposure of mono and co-cultures to testosterone.

In the mono-cultured breast tumor cells, testosterone caused a significant reduction in cell growth in MCF-7 (40%) and T47D cells (31%) as well as in the ERα- MDA-MB-231 cells (15%) at 10 nM of testosterone after 96 hours. In order to investigate whether the observed effects on proliferation by testosterone were caused by interaction with the AR, monocultures of the three breast tumor cells were simultaneously exposed to the potent AR antagonist bicalutamide (10 nM). In combination with bicalutamide, the growth inhibiting effect of testosterone was abolished in all three cell types (Figures. 4a-c).

In all co-cultures, proliferation was higher than in the mono-cultured breast tumor cells at all testosterone concentrations tested. In the co-cultures of BAFs with MCF-7 cells and T47D cells, testosterone caused a biphasic effect, with an initial increased proliferation at 1 and 5 nM followed by a slight decrease at 10 nM. In the co-culture with MCF-7 cells, growth was even significantly lower than vehicle control-treated cells (9%). In contrast, testosterone caused a monotonic reduction in cell growth in the MDA-MB-231cells co-cultured with BAFs compared to vehicle control-treated co-cultured cells. Cell proliferation in the MDA-MB-231/BAF co-culture was 36% of the vehicle control-treated co-cultured cells at 10 nM testosterone.

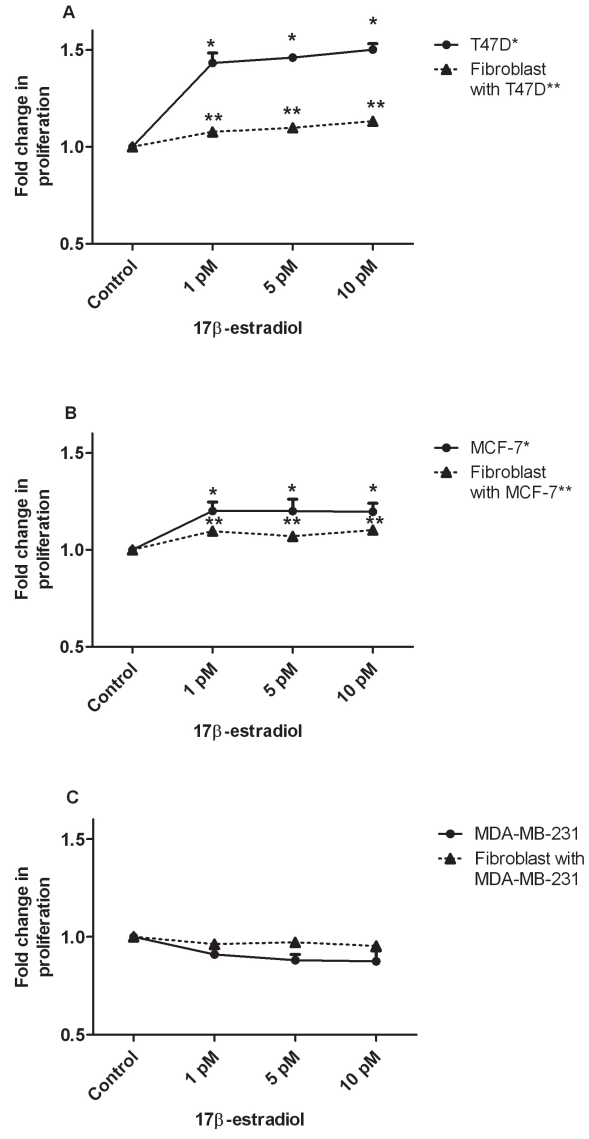


Figure 3. Increased proliferation of ER+ breast cancer cells alone or in co-culture with BAFs when exposed to 17β-estradiol. (A) T47D and (B) MCF-7 cells alone or in co-culture with BAFs. Cell cultures were exposed to vehicle control or 17β-estradiol (1 pM, 5 pM or 10 pM) for 96 hours. Data are expressed as means ± SD (n=9). *, ** Significantly different from vehicle control-treated mono-and co-cultured breast cancer cells (one-way ANOVA, $p < 0.001$). There were no statistically significant differences in proliferation of (C) MDA-MB-231 cells alone or in co-culture with BAFs, when compared with vehicle control-treated cells.

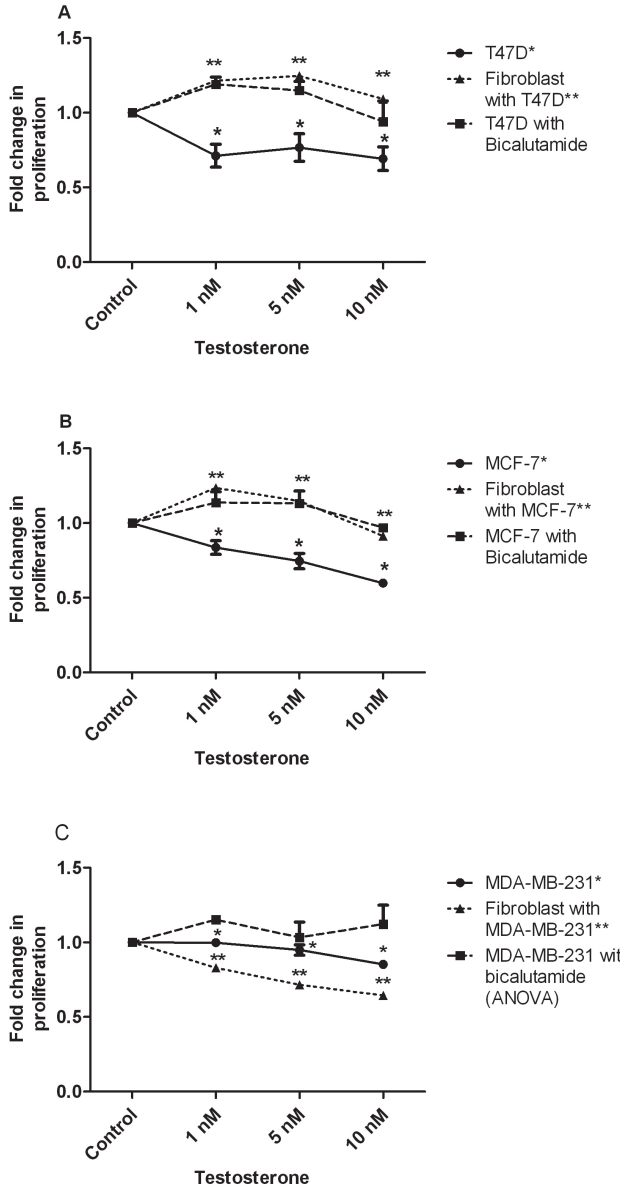


Figure 4. Increased proliferation of ER^+ breast cancer cells in co-culture with BAFs when exposed to testosterone. (A) T47D (B) MCF-7 and (C) MDA-MB-231 cells alone or in co-culture with BAFs. Cell cultures were exposed to vehicle control or testosterone (1 nM, 5 nM or 10 pM) for 96 hours. Data are expressed as means ± SD (n=9). *, ** Significantly different from vehicle control- treated mono-cultured and co-culture breast cancer cells (one-way ANOVA, $p < 0.001$). There is no significant difference in cell proliferation between vehicle control and treated mono-cultured breast cancer cells when treated with the bicalutamide.

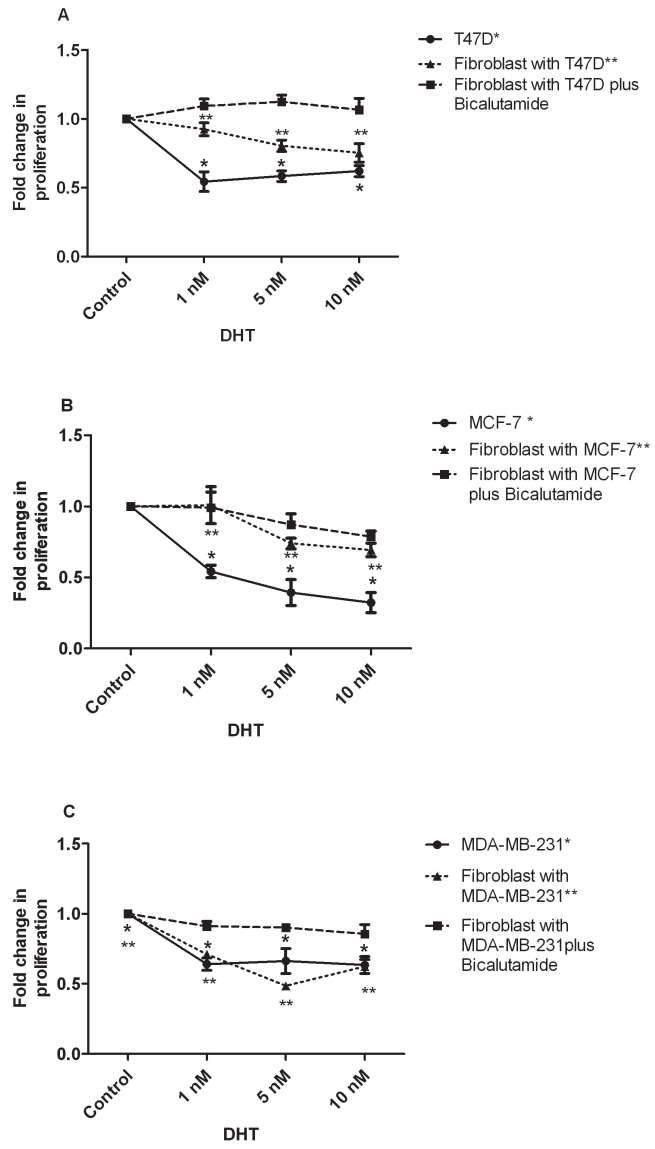


Figure 5. Decreased proliferation of both ER^+ and ER^- breast cancer cells alone or in co-culture with BAFs when exposed to dihydrotestosterone. (A) T47D (B) MCF-7 and (C) MDA-MB-231 cells alone or in co-culture with BAFs. Cell cultures were exposed to vehicle control or dihydrotestosterone (1 nM, 5 nM or 10 nM) for 96 hours. Data are expressed as means ± SD (n=9). *, ** Significantly different proliferation from vehicle control-treated cells in mono- or co-cultured breast cancer cells (one-way ANOVA, $*p < 0.001$ and $**p < 0.02$, respectively). The effects of reduction by DHT were diminished by adding bicalutamide into the co-culture system.

3.4. Exposure of mono and co-cultures to dihydrotestosterone.

Ninety-six hours after exposure with dihydrotestosterone (DHT), a statistically significant concentration-dependent reduction in cell growth was observed in both the mono and co-cultures (Figures. 5a-c). At 10 nM DHT, cell growth was reduced to 68%, 38%, and 38% of the vehicle control-treated cells for mono-cultures of MCF-7, T47D and MDA-MB-231, respectively. Cell growth was less strongly reduced for the ER^+ dependent MCF-7 and T47D cells when combined with BAFs and exposed to DHT, but this "co-culture" effect was not observed with the MDA-MB-231 cells. Adding 10 nM bicalutamide abolished or strongly reduced the growth reducing effect of DHT in co-cultures of the breast tumor cells (Figures 5a-c).

3.5. pS2 and Ki-67 gene expression in mono and co-cultures exposed to testosterone.

In order to elucidate a possible interaction between testosterone and ERα-mediated processes, *pS2* gene expression was determined. BAFs do not express *pS2* (data not shown). In the monocultures of the three breast tumor cells, *pS2* expression was not significantly increased after exposure to testosterone (5-10 nM) (Figures. 6a-c). In contrast, a strong induction of *pS2* expression was observed in the $ER\alpha^+$ tumor cells MCF-7 and T47D when co-cultured with breast fibroblasts (Figures. 6a, b). The induction by testosterone (10 nM) was 8-fold in BAFs co-cultured with MCF-7 and up to 32-fold in BAFs co-cultured with T47D. No effect of co-culturing with BAFs was observed on *pS2* expression in the $ER\alpha^-$ MDA-MB-231 tumor cells (Figure. 6c). To further confirm the effects on cell proliferation as described above, mRNA expression of *Ki-67*, which is a proliferative index gene, was determined in mono and co-culture system of T47D and MCF-7 cells (Figures.7a and 7b). In our lab, *Ki-67* gene expression could not be determined in MDA-MB-231 cells (data not shown). *Ki-67* expression was significantly increased after exposure to testosterone (1-10 nM) in co-culture system, but the proliferative index declined at the highest testosterone concentration (10 nM) in co-culture system (Figures.7a and 7b).

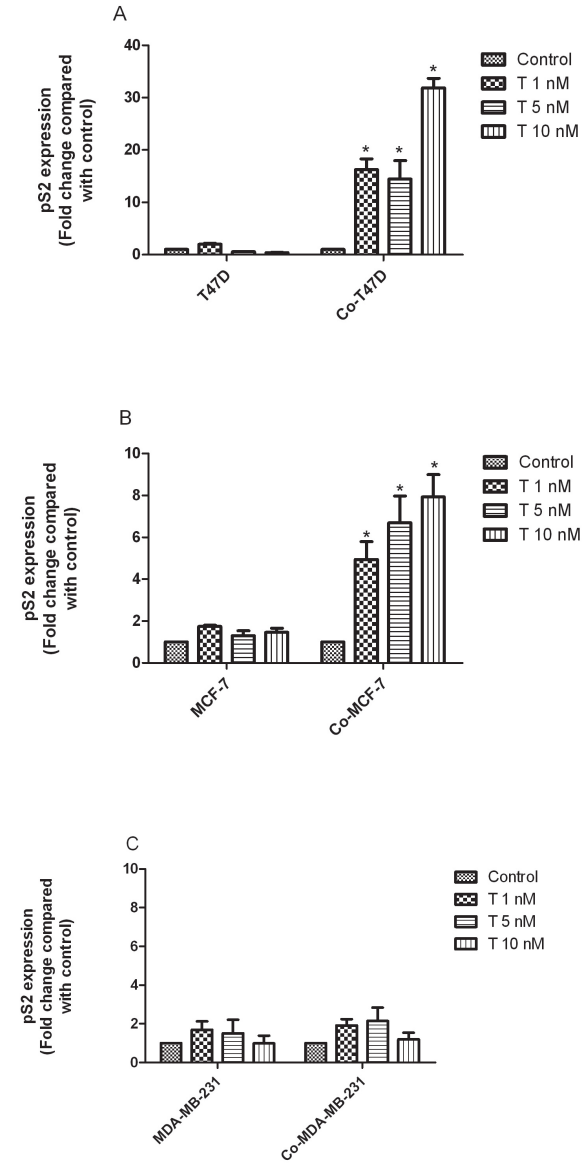


Figure 6. Increased of *pS2* expression in ER^+ breast cancer cells in co-culture with BAFs when exposed to testosterone. *pS2* expression in (A) T47D (B) MCF-7 and (C) MDA-MB-231 cells alone or in co-culture with BAFs. Cell cultures were exposed to vehicle control or testosterone (1 nM, 5 nM or 10 nM) for 96 hours. Data are expressed as means ± SD (n=9). * Significantly different from vehicle control-treated co-cultured breast cancer cells with BAFs (one-way ANOVA, $p < 0.001$).

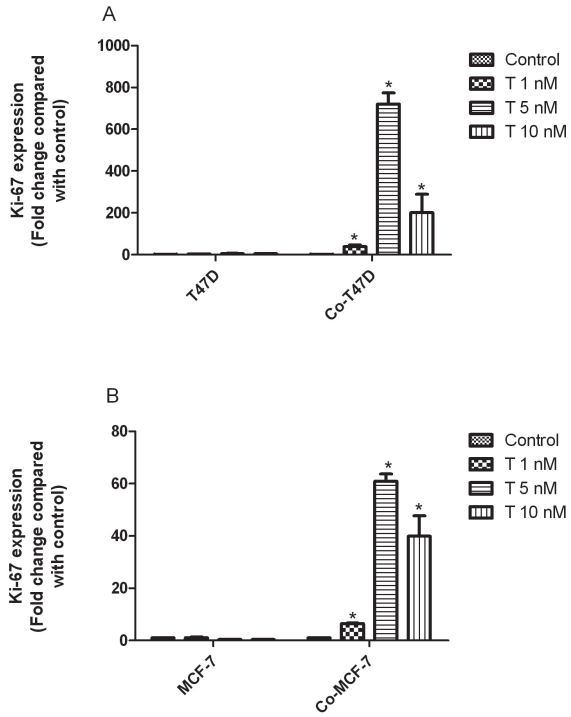


Figure 7. Increased of *Ki-67* expression in ER$^+$ breast cancer cells in co-culture with BAFs when exposed to testosterone. *Ki-67* expression of (A) T47D and (B) MCF-7 cells alone or in co-culture with BAFs. Cell cultures were exposed to vehicle control or testosterone (T; 1 nM, 5 nM or 10 nM) for 96 hours. Data are expressed as means ± SD (n=9). * Significantly different from vehicle control-treated co-cultured breast cancer cells (one-way ANOVA, $p < 0.001$).

3.6. Expose of co-culture with T47D cells to testosterone in the presence of letrozole (aromatase inhibitor).

When a co-culture of BAFs with T47D cells was exposed to testosterone (1, 5, 10 nM) only, the cell proliferation of T47D cells and expressions of *pS2* and *Ki-67* mRNA were significantly increased. The differences between mono- and co-cultures in *pS2* and *Ki-67* gene expression responses for the ER-positive cell lines are most likely due to the efficient conversion of testosterone to 17β-estradiol by aromatase in the BAFs. To confirm this, a T47D co-culture was exposed to 1, 5 or 10 nM testosterone in combination with the aromatase inhibitor letrozole (30 nM). Adding letrozole completely negated cell proliferation and induction of *pS2* and *ki-67* mRNA expression (Figures. 8a-c).

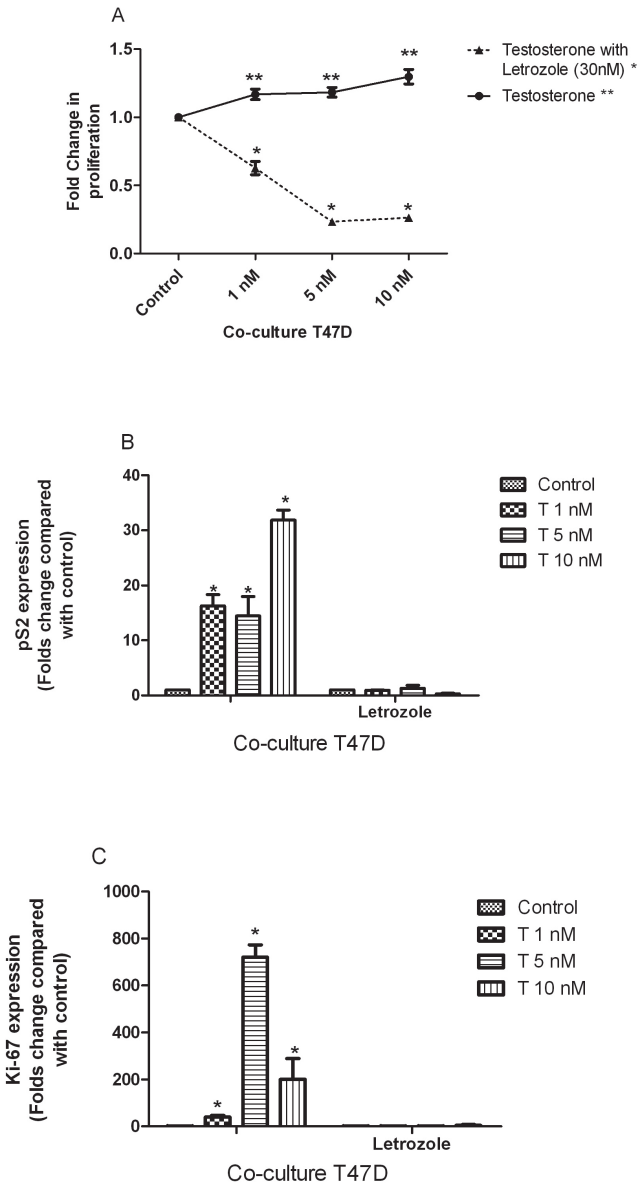


Figure 8. The increased in proliferation, *pS2* and *Ki-67* expression in testosterone exposed co-culture T47D were diminished by adding aromatase inhibitor. (A) Proliferation, (B) *pS2* expression, and (C) *Ki-67* expression of T47D co-cultured with BAFs. Co-cultures were exposed to vehicle control or testosterone (1 nM, 5 nM or 10 pM) for 96 hours alone or in combination with the aromatase inhibitor, letrozole (30nM). Data are expressed as means ± SD (n=9). *, ** Significantly different from vehicle control-treated of co-cultured breast cancer cells (one-way ANOVA, $p < 0.001$).

4. Discussion.

4.1. Relevance of co-culture system.

It is widely accepted that (mammary) carcinomas should be considered as complex organs consisting of tumor epithelial cells interacting with surrounding cell types via intercellular communication (37, 39, 44). Therefore, an *in vitro* co-culture model combining breast tumor cells and primary BAFs creates a more realistic micro-environment to study hormonal interactions in breast cancer compared to mono-cultures of breast tumor cell lines only (44). There are two possible techniques suggested for establishing co-culture systems; a direct (one-compartment) co-culture system or layering technique, and an indirect (two-compartment) co-culture system. The benefit of a direct co-culture system is direct cell-cell contact causing paracrine interactions, which is important for the induction of aromatase in fibroblasts and absent in an indirect co-culture system (37). This difference between both co-culture systems is most clearly illustrated by the absence of MCF-7 cell proliferation in the presence of breast fibroblasts and dexamethasone (aromatase inducer) in the indirect co-culture system (37, 39).

Furthermore, the direct co-culture system, as used in our study, also shows the ductal formation in breast tissues (Fig. 1). However, the direct co-culture system has some technical limitations as well, such as quantifying the number of cells and separation of different cell types after exposure (44). Yet, studies with mono-cultured BAFs exposed to estradiol, testosterone, and dihydrotestosterone showed that proliferation of BAFs was not affected by either of these steroid hormones (Figure. 9).

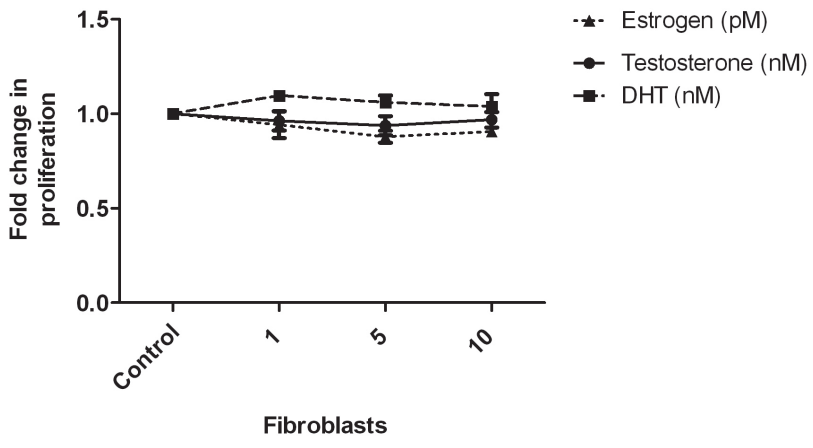


Figure 9. Proliferation of mono-culture BAFs was not affected by exposure to estrogen, testoterone, or dihydrotestosterone.

In the present study, three different breast cancer cell types were combined with primary BAFs. The MCF-7 cell line was chosen because it is worldwide one of the most commonly used cell lines for breast cancer studies on tumor proliferation, estrogen receptor binding and activation, e.g. by various environmental chemicals with estrogenic properties (45) or hormonal active pharmaceuticals in breast cancer studies (46). For comparison in the present study, another hormone receptor positive breast cancer cell line, T47D (47), and the ER^- cell line MDA-MB-231 were used (48). Although they differ in expression of ERα and ERβ, all three breast cancer cell lines have been described to express the androgen receptor (49). To confirm this, we performed RT-PCR on all three breast tumor cell lines, indeed showing expression of mRNA of androgen receptors (43). (Figure. 10)

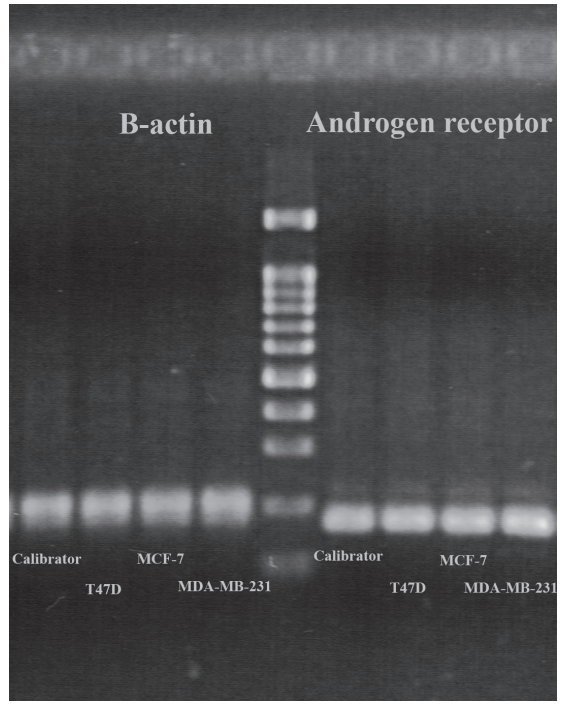


Figure 10. Gel electrophoresis of mRNA products from RT-PCR for the androgen receptor gene in T47D, MCF-7 and MDA-MB231 cell lines.

4.2. Aromatase in BAFs.

An earlier study has shown that *pS2* gene transcription can be induced in a co-culture of MCF-7 cells and primary breast fibroblasts (BAFs) when exposed to testosterone. This observation was explained by the ability of BAFs to convert testosterone to estrogen by aromatase (37). In our present study, we observed only minor aromatase expression in all three breast tumor cell lines, while the BAFs had on average a 10-fold higher expression of

aromatase. Expression of aromatase is tissue-specific and controlled by alternate splicing of promoter regions with at least four promoter regions, PI.4, PI.3, PII and PI.7 that have an association with breast cancer (50). Aromatase expression in healthy BAFs is mainly driven by promoter I.4 and regulated by class I cytokines, tumor necrosis factor alpha and glucocorticoids (51). In BAFs surrounding a tumor, aromatase expression is elevated 3-4-fold (52, 53) and a promoter switch to PI.3 and PII takes place. This promoter switch is associated with the increased secretion of prostaglandin E2 (PGE2) by breast tumor cells and subsequent activation of cAMP/PKA- and PKC signaling (54). In our present study we used BAFs from three different patients with a breast tumor, which actually may represent a more realistic situation for intercellular communication with the tumor cells compared to co-cultures using BAFs from healthy women. However, our study did not include the identification of active aromatase promotor regions or expression of proteins relevant for intercellular communication between both cell types. Such aspects should clearly be topics for further research in order to establish possible differences in co-culture responses when BAFs are either derived from breast cancer patients or healthy individuals.

4.3. Androgens and breast cancer.

From clinical and epidemiological studies, there is evidence suggesting that androgens inhibit mammary epithelial proliferation and breast growth. Consequently, androgens have been hypothesized to influence the risk of breast cancer by several mechanisms, including their conversion to estradiol and subsequent binding to the estrogen receptor (ER) or direct binding to the androgen receptor (AR) (55, 56). The co-culture systems used in this study showed that the role of androgens in responses of breast cancer cells depend strongly on the conversion into estrogens by aromatase. Firstly, aromatase in BAFs allows aromatizing testosterone into 17β-estradiol followed by proliferation of ER^+ breast cancer cells, the latter process being absent in ER^- breast tumor cells in co-culture. Earlier studies from our laboratory demonstrated a reduction of *pS2* expression in a co-culture of MCF-7 cells and BAFs after combined exposure to testosterone and an aromatase inhibitor, which was confirmed by the results of our present study of the BAFs co - culture of T47D in section 3.6 (37). We found that the non-aromatizable androgen DHT again inhibited significantly cell proliferation not only in the two ER^+ breast tumor cell, but also in the ER^- MDA-MB-231 breast tumor cells. The latter observation concurs with results from another study showing that the major circulating androgen dehydroepiandosterone (DHEA) also inhibits the growth of human ER^- breast cancer cell lines, when having a strong expression of the AR (56). The results from our study indicate a role of androgens and possibly the AR in growth inhibition of breast tumor cells, irrespective of their ER+ or ER- status. On the other hand, some studies indicate that DHT may be converted via multiple enzymes including 3αHSD, 17αHSD, and 3βHSD, to 5α- androstane 3α, 17β-diol (3β-diol) that has little activity at the AR, but displays a moderate ability to bind and activate ERβ (57). Several *in vitro* studies suggest that ERβ may function as a tumor suppressor upon estrogen exposure, while overwhelming

evidence shows that exposure of ERα expressing breast cancer cells to estrogen results in increased rates of proliferation (58). Some suggest that under prolonged estrogen restriction, 3β-diol can stimulate proliferation of ER+ breast cancer cells via the ERα and that this metabolite is responsible for aromatase inhibitor resistance (59, 60). However it must be noted that in humans DHT metabolites mostly occur in e.g. the liver and prostate (61), and proved undetectable in breast cancer tissues (59). Clearly, the relevance of DHT metabolites and their potential role via ERs in the etiology of breast cancer needs to be clarified further, but this is beyond the scope of our study.

4.4. Triple negative breast cancer.

The treatment of breast cancer needs a multimodality approach to eradicate residual cancer cells and prevent recurrence of the disease. Targeting the growth and invasion pathways of breast cancer is critical to effective treatment of breast cancer (2, 3). The estrogen receptor pathway is a well-known pathway for breast cancer development and progression. Breast cancer is diagnosed more frequently in postmenopausal women and adjuvant hormonal treatment is more effective in postmenopausal women than premenopausal women (9, 10). Many estrogen receptor modulators and aromatase inhibitors have been successfully developed for breast cancer treatment and prevention of hormone-responsive breast cancer. However, patients with triple negative breast cancer (ER^-, PR^-, and $HER2^-$) are unresponsive to hormonal therapies. Not only is this specific group of patients resistant to hormonal treatment, many of these breast cancer patients have no or limited response to chemotherapy, resulting in a high morbidity rate due to limited treatment options and frequent tumor aggressiveness. Recent research found that up to 70% of breast cancer cases, including triple negative breast tumors, express androgen receptors in the malignant tissue (21-23). Thus, the androgen receptor may be a suitable target for hormonal treatment in hormonal resistance or triple negative breast cancer (26). The results of our present study showed that Testosterone only slightly reduced proliferation of MDA-MB-231 cells when co-cultured with BAFs, which is most likely caused by aromatization to 17β-estradiol via aromatase in the latter cells. However, DHT that could not be metabolized to an estrogen inhibited the growth of both the ER^+ (MCF-7 and T47D) and the ER^- MDA-MB-231 breast cancer cells. Our results may imply that an androgen like DHT that cannot be aromatized to an estrogenic agonist can offer a new perspective for possible hormonal treatment of triple negative breast cancer patients, who express androgen receptors in their tumor cells. However, it must be noted that although cell lines are classified as ER^+ or ER^-, this concerns expression of ERα and not ERβ, while MDA-MB-231 is categorized as ER^-, it does express low levels of ERβ (62). Therefore, it cannot be excluded that some of the observed effects in the present study might be partly attribute to interaction of an androgen metabolite with ERβ, as described above.

5. Conclusion.

Our results again demonstrated the relevance of using a co-culture system of breast tumor cells and BAFs for breast cancer research. Using this co-culture model, it was shown that androgens can inhibit breast cancer cell growth directly via the AR, but the effect will be modified depending on the type of androgen (aromatizing T or non-aromatizing DHT), the ERα and AR status of the breast tumor cell and aromatase activity in the surrounding breast fibroblasts. Taken together, the results suggest that androgens that cannot be aromatized, like DHT, may provide a perspective for treatment of breast cancer patients with triple negative breast cancer tumors.

Acknowledgements.

This work was supported by the Chulabhorn Graduate Institute, Chulabhorn Research Institute (Thailand), and The Institute for Risk Assessment Sciences at the Utrecht University (The Netherlands).

References.

1. Breast Cancer Incidence and Mortality Worldwide in 2008 [Internet]. International Agency for Research on Cancer. 2008. Available from: http://globocan.iarc.fr/factsheets/cancers/breast.asp.
2. Guth U, Huang DJ, Schotzau A, Zanetti-Dallenbach R, Holzgreve W, Bitzer J, et al. Target and reality of adjuvant endocrine therapy in postmenopausal patients with invasive breast cancer. Br J Cancer. 2008;99(3):428-33.
3. Miles DW. Recent advances in systemic therapy. When HER2 is not the target: advances in the treatment of HER2-negative metastatic breast cancer. Breast Cancer Res. 2009;11(4):208.
4. Conlin AK, Seidman AD. Beyond cytotoxic chemotherapy for the first-line treatment of HER2-negative, hormone-insensitive metastatic breast cancer: current status and future opportunities. Clin Breast Cancer. 2008;8(3):215-23.
5. Russo J, Snider K, Pereira JS, Russo IH. Estrogen induced breast cancer is the result in the disruption of the asymmetric cell division of the stem cell. Horm Mol Biol Clin Investig. 2011;1(2):53-65.
6. Noh EM, Lee YR, Chay KO, Chung EY, Jung SH, Kim JS, et al. Estrogen receptor alpha induces down-regulation of PTEN through PI3-kinase activation in breast cancer cells. Mol Med Report. 2011;4(2):215-9.
7. Goto N, Hiyoshi H, Ito I, Tsuchiya M, Nakajima Y, Yanagisawa J. Estrogen and antiestrogens alter breast cancer invasiveness by modulating the transforming growth factor-beta signaling pathway. Cancer Sci. 2011;102(8):1501-8.
8. Ma CX. Estrogen and progesterone receptors in breast cancer. Clin Adv Hematol Oncol. 2011;9(5):385-6.
9. Brody JG, Rudel R, Maxwell NI, Swedis SR. Mapping out a search for environmental causes of breast cancer. Public Health Rep. 1996;111(6):494-507.
10. Brody JG, Tickner J, Rudel RA. Community-initiated breast cancer and environment studies and the precautionary principle. Environ Health Perspect. 2005;113(8):920-5.
11. Yu KD, Zhou Y, Liu GY, Li B, He PQ, Zhang HW, et al. A prospective, multicenter, controlled, observational study to evaluate the efficacy of a patient support program in improving patients' persistence to adjuvant aromatase inhibitor medication for postmenopausal, early stage breast cancer. Breast Cancer Res Treat. 2012.
12. Paepke S, Jacobs VR, Ohlinger R, Warm M, Kummel S, Thomas A, et al. Treatment strategies that effectively reduce early recurrence risk in postmenopausal women with endocrine-sensitive breast cancer: AIs upfront vs. switching. J Cancer Res Clin Oncol. 2007;133(12):905-16.
13. Howell A, Howell SJ, Clarke R, Anderson E. Where do selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) now fit into breast cancer treatment algorithms? J Steroid Biochem Mol Biol. 2001;79(1-5):227-37.
14. Dixon JM. Prospects of neoadjuvant aromatase inhibitor therapy in breast cancer. Expert Rev Anticancer Ther. 2008;8(3):453-63.
15. Griffiths CL, Olin JL. Triple negative breast cancer: a brief review of its characteristics and treatment options. J Pharm Pract.25(3):319-23.
16. Reddy KB. Triple-negative breast cancers: an updated review on treatment options. Curr Oncol.18(4):e173-9.
17. Brady-West DC, McGrowder DA. Triple negative breast cancer: therapeutic and prognostic implications. Asian Pac J Cancer Prev. 2012;12(8):2139-43.
18. Amos KD, Adamo B, Anders CK. Triple-negative breast cancer: an update on neoadjuvant clinical trials. Int J Breast Cancer. 2012;2012:385978.
19. Dreyer G, Vandorpe T, Smeets A, Forceville K, Brouwers B, Neven P, et al. Triple negative breast cancer: Clinical characteristics in the different histological subtypes. Breast. 2013.

20. O'Shaughnessy JA. Breast cancer in focus: treatment options for triple-negative metastatic breast cancer. Clin Adv Hematol Oncol. 2012;10(1):43-5.
21. Luo X, Shi YX, Li ZM, Jiang WQ. Expression and clinical significance of androgen receptor in triple negative breast cancer. Chin J Cancer. 2010;29(6):585-90.
22. Higgins MJ, Wolff AC. The androgen receptor in breast cancer: learning from the past. Breast Cancer Res Treat. 2010;124(3):619-21.
23. Park S, Koo J, Park HS, Kim JH, Choi SY, Lee JH, et al. Expression of androgen receptors in primary breast cancer. Ann Oncol. 2009;21(3):488-92.
24. Hao Y, Montiel R, Li B, Huang E, Zeng L, Huang Y. Association between androgen receptor gene CAG repeat polymorphism and breast cancer risk: a meta-analysis. Breast Cancer Res Treat. 2010;124(3):815-20.
25. Nahleh Z. Androgen receptor as a target for the treatment of hormone receptor-negative breast cancer: an unchartered territory. Future Oncol. 2008;4(1):15-21.
26. Gucalp A, Traina TA. Triple-negative breast cancer: role of the androgen receptor. Cancer J. 2010;16(1):62-5.
27. Dimitrakakis C. Androgens and breast cancer in men and women. Endocrinol Metab Clin North Am. 2011;40(3):533-47, viii.
28. Dimitrakakis C, Bondy C. Androgens and the breast. Breast Cancer Res. 2009;11(5):212.
29. Cauley JA, Lucas FL, Kuller LH, Stone K, Browner W, Cummings SR. Elevated serum estradiol and testosterone concentrations are associated with a high risk for breast cancer. Study of Osteoporotic Fractures Research Group. Ann Intern Med. 1999;130(4 Pt 1):270-7.
30. Howard JM. Testosterone may cause breast cancer. Int J Cancer. 2005;115(3):497; author reply 8.
31. He J, Peng R, Yuan Z, Wang S, Peng J, Lin G, et al. Prognostic value of androgen receptor expression in operable triple-negative breast cancer: a retrospective analysis based on a tissue microarray. Med Oncol. 2011;29(2):406-10.
32. Tang D, Xu S, Zhang Q, Zhao W. The expression and clinical significance of the androgen receptor and E-cadherin in triple-negative breast cancer. Med Oncol. 2011;29(2):526-33.
33. Li CI, Anderson BO, Daling JR, Moe RE. Trends in incidence rates of invasive lobular and ductal breast carcinoma. JAMA. 2003;289(11):1421-4.
34. Wellings SR. A hypothesis of the origin of human breast cancer from the terminal ductal lobular unit. Pathol Res Pract. 1980;166(4):515-35.
35. Wren BG. The origin of breast cancer. Menopause. 2007;14(6):1060-8.
36. Shekhar MP, Werdell J, Santner SJ, Pauley RJ, Tait L. Breast stroma plays a dominant regulatory role in breast epithelial growth and differentiation: implications for tumor development and progression. Cancer Res. 2001;61(4):1320-6.
37. Heneweer M, Muusse M, Dingemans M, de Jong PC, van den Berg M, Sanderson JT. Co-culture of primary human mammary fibroblasts and MCF-7 cells as an in vitro breast cancer model. Toxicol Sci. 2005;83(2):257-63.
38. van Meeuwen JA, Korthagen N, de Jong PC, Piersma AH, van den Berg M. (Anti)estrogenic effects of phytochemicals on human primary mammary fibroblasts, MCF-7 cells and their co-culture. Toxicol Appl Pharmacol. 2007;221(3):372-83.
39. Heneweer M, Muusse M, Dingemans M, de Jong PC, van den Berg M, Sanderson JT. Direct and indirect endocrine disruption: Co-culture of primary human mammary fibroblasts and MCF-7 cells as an *in vitro* breast cancer model. Institute for Risk Assessment Sciences (IRAS). Ph.D. Utrecht Utrecht University; 2005. p. 175.
40. Sonne-Hansen K, Lykkesfeldt AE. Endogenous aromatization of testosterone results in growth stimulation of the human MCF-7 breast cancer cell line. J Steroid Biochem Mol Biol. 2005;93(1):25-34.
41. Sanderson JT, Letcher RJ, Heneweer M, Giesy JP, van den Berg M. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environ Health Perspect. 2001;109(10):1027-31.
42. Lee Y, Jin Y, Lim W, Ji S, Choi S, Jang S, et al. A ginsenoside-Rh1, a component of ginseng saponin, activates estrogen receptor in human breast carcinoma MCF-7 cells. J Steroid Biochem Mol Biol. 2003;84(4):463-8.
43. Bieche I, Parfait B, Tozlu S, Lidereau R, Vidaud M. Quantitation of androgen receptor gene expression in sporadic breast tumors by real-time RT-PCR: evidence that MYC is an AR-regulated gene. Carcinogenesis. 2001;22(9):1521-6.
44. Miki Y, Ono K, Hata S, Suzuki T, Kumamoto H, Sasano H. The advantages of co-culture over mono cell culture in simulating in vivo environment. J Steroid Biochem Mol Biol. 2012;131(3-5):68-75.
45. Worth AP, Balls M. Alternative (non-animal) methods for chemical testing: Current status and future prospects. Altern Lab Anim. 2002;30:103-13.
46. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat. 2004;83(3):249-89.
47. Lu M, Mira-y-Lopez R, Nakajo S, Nakaya K, Jing Y. Expression of estrogen receptor alpha, retinoic acid receptor alpha and cellular retinoic acid binding protein II genes is coordinately regulated in human breast cancer cells. Oncogene. 2005;24(27):4362-9.
48. Mur C, Martinez-Carpio PA, Fernandez-Montoli ME, Ramon JM, Rosel P, Navarro MA. Growth of MDA-MB-231 cell line: different effects of TGF-beta(1), EGF and estradiol depending on the length of exposure. Cell Biol Int. 1998;22(9-10):679-84.
49. Subik K, Lee JF, Baxter L, Strzepek T, Costello D, Crowley P, et al. The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines. Breast Cancer (Auckl). 2010;4:35-41.
50. Irahara N, Miyoshi Y, Taguchi T, Tamaki Y, Noguchi S. Quantitative analysis of aromatase mRNA expression derived from various promoters (I.4, I.3, PII and I.7) and its association with expression of TNF-alpha, IL-6 and COX-2 mRNAs in human breast cancer. Int J Cancer. 2006;118(8):1915-21.
51. Purohit A, Reed MJ. Regulation of estrogen synthesis in postmenopausal women. Steroids. 2002;67(12):979-83.
52. Bulun SE, Lin Z, Zhao H, Lu M, Amin S, Reierstad S, et al. Regulation of aromatase expression in breast cancer tissue. Ann N Y Acad Sci. 2009;1155:121-31.
53. Harada N. Aberrant expression of aromatase in breast cancer tissues. J Steroid Biochem Mol Biol. 1997;61(3-6):175-84.
54. Brueggemeier RW, Quinn AL, Parrett ML, Joarder FS, Harris RE, Robertson FM. Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens. Cancer Lett. 1999;140(1-2):27-35.
55. Hao Y, Montiel R, Li B, Huang E, Zeng L, Huang Y. Association between androgen receptor gene CAG repeat polymorphism and breast cancer risk: a meta-analysis. Breast Cancer Res Treat.
56. Gucalp A, Traina TA. Triple-negative breast cancer: role of the androgen receptor. Cancer J.16(1):62-5.
57. Weihua Z, Makela S, Andersson LC, Salmi S, Saji S, Webster JI, et al. A role for estrogen receptor beta in the regulation of growth of the ventral prostate. Proc Natl Acad Sci U S A. 2001;98(11):6330-5.
58. Gustafsson JA, Warner M. Estrogen receptor beta in the breast: role in estrogen responsiveness and development of breast cancer. J Steroid Biochem Mol Biol. 2000;74(5):245-8.
59. Honma N, Saji S, Hirose M, Horiguchi S, Kuroi K, Hayashi S, et al. Sex steroid hormones in pairs of tumor and serum from breast cancer patients and pathobiological role of androstene-3beta, 17beta-diol. Cancer Sci. 2011;102(10):1848-54.
60. Sikora MJ, Cordero KE, Larios JM, Johnson MD, Lippman ME, Rae JM. The androgen metabolite 5alpha-androstane-3beta,17beta-diol (3betaAdiol) induces breast cancer growth via estrogen receptor: implications for aromatase inhibitor resistance. Breast Cancer Res Treat. 2009;115(2):289-96.
61. Pirog EC, Collins DC. Metabolism of dihydrotestosterone in human liver: importance of 3alpha- and 3beta-hydroxysteroid dehydrogenase. J Clin Endocrinol Metab. 1999;84(9):3217-21.
62. Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lakins J, Lupu R. Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. Oncol Rep. 2000;7(1):157-67.

Chapter 4

ANTI-AROMATASE EFFECT of Resveratrol and Melatonin on Hormonal positive breast cancer cells co-culturED with BREAsT ADIPOSE fibroblasts

Suthat Chottanapund[1,2,3,5], Majorie B.M. Van Duursen[4], Panida Navasumrit[1,2,3], Potchanee Hunsonti[2], Supatchaya Timtavorn[2], Mathuros Ruchirawat[1,2,3] and Martin Van den Berg[4]

[1] Division of Environmental Toxicology, Chulabhorn Graduate Institute, Bangkok, Thailand
[2] Laboratory of Environmental Toxicology, Chulabhorn Research Institute, Bangkok, Thailand
[3] Center of Excellence on Environmental Health, Toxicology and management of Chemicals, Bangkok, Thailand
[4] Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
[5] Bamrasnaradura Infectious Diseases Institute, Ministry of Public Health, Thailand

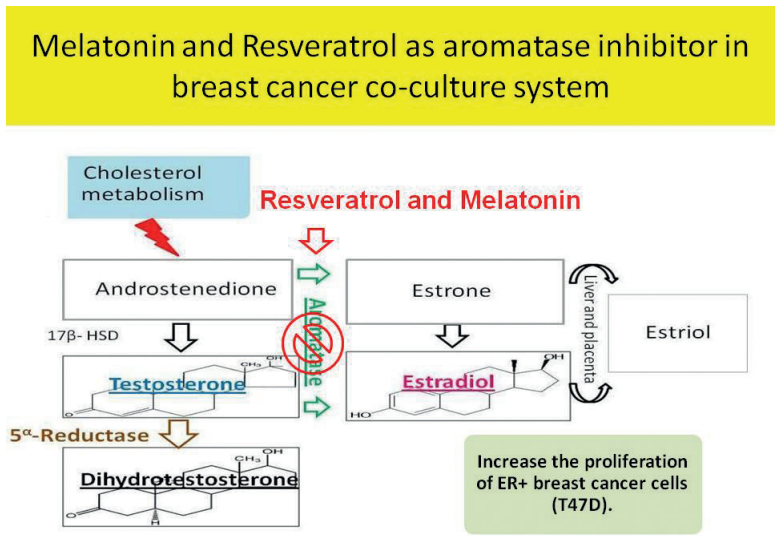
Key words: co-culture; human fibroblasts; Breast cancer; Resveratrol; Melatonin.

Published in Toxicology in vitro 2014 OCT;28(7):1215-21. DOI: 10.1016/J TIV.2014.05.015.

Abstract

Targeting the estrogen pathway has been proven effective in the treatment of estrogen receptor positive breast cancer. There are currently two common groups of anti-estrogenic compounds used in the clinic; Selective Estrogen Receptor Modulators (SERMs, e.g. tamoxifen) and Selective Estrogen Enzyme Modulators (SEEMs e.g. letrozole). Among various naturally occurring, biologically active compounds, resveratrol and melatonin have been suggested to act as aromatase inhibitors, which makes them potential candidates in hormonal treatment of breast cancer. Here we used a co-culture model in which we previously demonstrated that primary human breast adipose fibroblasts (BAFs) can convert testosterone to estradiol, which subsequently results in estrogen receptor-mediated breast cancer T47D cell proliferation. In the presence of testosterone in this model, we examined the effect of letrozole, resveratrol and melatonin on cell proliferation, estradiol (E_2) production and gene expression of *CYP19A1, pS2* and *Ki-67*. Both melatonin and resveratrol were found to be aromatase inhibitors in this co-culture system, albeit at significantly different concentrations. Our co-culture model did not provide any indications that melatonin is also a selective estrogen receptor modulator. In the T47D-BAF co-culture, a melatonin concentration of 20 nM and resveratrol concentration of 20 μM have aromatase inhibitory effect as potent as 20 nM letrozole, which is a clinically used anti-aromatase drug in breast cancer treatment. The combination of both SERM and SEEM mechanisms of action of especially melatonin clearly offers potential advantages for breast cancer treatment.

Graphical Abstract



1. Introduction.

Adjuvant hormonal therapy is an effective treatment to reduce or prevent the recurrence of hormone receptor positive breast cancer (1-5). Here, two common groups of pharmaceuticals are used for clinical hormonal therapy; Selective Estrogen Receptor Modulators (SERMs) e.g. tamoxifen (6, 7) or Selective Estrogen Enzyme Modulators (SEEMs) such as the aromatase inhibitor letrozole (8-11). SERMs have anti-estrogenic properties because of antagonistic actions on estrogen receptor α (ERα), which reduces the proliferation of estrogen responsive breast tumor cells. SEEMs reduce the synthesis of estrogens from androgens that results in lower circulating levels of estrogens and also reduced estrogen production in peripheral tissues, which results in reduction of cell proliferation of estrogen receptor (ER)-positive breast tumor cells. In this approach the estrogen stimulated pathways are among the most important targets for adjuvant breast cancer therapy (12, 13).

Beside several pharmaceutical SERMs and SEEMs that have been developed over the last decades to treat or prevent breast cancer, a variety of naturally occurring, biologically active compounds have been identified that may be useful as chemopreventive agents for breast cancer. Two biological agents that are often suggested to have such cancer chemopreventive actions are resveratrol and melatonin. Resveratrol is a non-flavonoid phytoestrogen found in grapes and several epidemiological studies indicated that high resveratrol intake could reduce breast cancer risk (14). Multiple mechanisms of action have been identified that could explain the anti-carcinogenic properties of resveratrol, which include its role as a SERMs, aromatase inhibitor and/or anti-oxidant (15-17). With respect to its properties as an aromatase (*CYP19A1*) inhibitor, several *in vivo* and *in vitro* studies have confirmed the modularity function of resveratrol in this key step in sex steroidogenesis (18-21). It has been suggested that the anti-aromatase activity of resveratrol is its major property with respect to chemoprevention of breast cancer (19). In a 28-day repeated dose study, the no observed adverse effect level (NOEAL) was 300 mg/kg/day in rats (22). This low toxicity of resveratrol could make it a good candidate for adjuvant breast cancer treatment.

Melatonin (N-acetyl-5-methoxytryptamine) is an indolic and endogenous compound that is naturally produced by the pineal gland in the human body. Melatonin plays a primary role in the circadian pattern and is regulated by the hypothalamic suprachiasmatic nucleus (SCN). In addition, melatonin is believed to have oncostatic properties against many forms of cancers such as leukemia, breast, colorectal and prostate cancer (23-26). Multiple mechanisms have been proposed to explain the chemopreventive properties of melatonin for breast cancer (27-30) from which two are especially important with respect to our present study. Firstly, melatonin can act as SERM by reducing estrogen binding to ERα receptors and inhibiting binding of the E_2-ERα complex to the DNA.

The proposed mechanism of this anti-estrogenic effect of melatonin does not depend on its binding to the ER but depend on the high affinity binding to membrane melatonin receptors (MT1) and this complex will interfere with the estrogen-binding activity of ERα without changing its affinity and will reduce the ligand-receptors transactivation (30-32). Another important mechanism of action of melatonin with respect to prevention of breast cancer may be its role as aromatase inhibitor. Melatonin could decrease cAMP formation and down-regulate expression of promoter regions pII, pI.3 and p1.4-regulated *CYP19A1* gene in MCF-7 cells (33).

Previous studies from our laboratories have demonstrated the added value of using co-cultures from breast tumor cells and human primary breast fibroblasts (BAFs) in *in vitro* breast cancer studies (34). In contrast with mono-cultures of breast tumor cell lines, these co-cultures have paracrine interactions between both cell types and therefore represent a more realistic approach to the actual *in vivo* situation of a breast tumor (35). In our present study we examined the breast cancer chemopreventive properties of resveratrol and melatonin in co-cultures of ERα positive T47D cells and BAFs with an emphasis on the modulating effects on tumor cell proliferation and aromatase activity.

2. Materials and methods.

2.1. Breast cancer cell culture and incubation.

The human breast cancer cell line T47D was obtained from ATCC (Rockville, MD, USA). T47D cells were grown in culture medium comprising of RPMI 1640, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1% glucose, 1% sodium pyruvate, and 0.08% insulin solution at a seeding concentration of 5×10^4 cells/ml. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Primary breast adipose fibroblasts (BAFs).

Primary breast tissue fibroblasts were obtained after informed consent from three breast cancer patients who attended the Bamrasnaradura Infectious Institute (Nontaburi, Thailand) for modified radical mastectomy. The research protocol was approved by the Medical Ethical Committee of the Bamrasnaradura Infectious Diseases Institute. About 5 to 10 g of macroscopically normal breast tissue was collected for this study. The remaining tissue was used for routine pathological examination. After the tissue was obtained, it was stored directly at 4°C in a saline solution (0.9%NaCl) and immediately transported to the laboratory. BAFs were isolated from breast tissue following the method described earlier (34) and cultured, as adherent cultures in RPMI 1640-medium w/o phenol red (Gibco/Invitrogen 11835) supplemented with Pen/Strep (invitrogen 15140), FCS (invitrogen 10270) and Insulin 10^{-3} M (144 mg/25 ml) (sigma I-5500) at a seeding concentration of 5 x 10^4cells/ml. The cells were maintained in a humidified atmosphere of 37 °C with 5% CO_2. The BAFs were sub-cultured when the bottles were confluent,

which was usually after one week (34, 35). The BAFs were kept for subculture. We isolated three primary BAFs from three patients. All were named F1-3. In order to reduce the biological variation in the co-culture experiments, the BAFs from the patient with the highest aromatase expression (F3) (data from our previous experiments) were used for the co-culture in this study (36).

2.3. Co-cultures of T47D breast cancer cells with BAFs.

Approximately three weeks after isolation, the fibroblasts were used to establish a co-culture with T47D cells. On day 1, BAFs were plated at a density of approximately 5×10^3 cells/well in a 96-wells plate (for proliferation) or at 5×10^4 cells/ml in a 25 cm^2 flask (for gene expression) in culture medium. At day 2, mono-cultures of breast cancer cells and plated primary BAFs were washed with phosphate buffered saline (PBS) and placed on assay medium in which heat inactivated FCS was replaced with heat-inactivated, charcoal/dextran-treated FCS (Hyclone, SH30068.03). On day 4, BAFs were washed with PBS and breast cancer cells were trypsinized and seeded on top of the BAFs at a density of 5×10^3 cells/well (for proliferation) or 2.5×10^5 cells/flask (for RNA expression). On day 5, fresh assay medium was added to the co-cultures after which the cells were exposed to the test compounds for 120 h (37-39). Final solvent (ethanol) concentration was 0.1% w/v in the medium and concentrations of testing chemicals were respectively, 1 nM, 5 nM and 10 nM for testosterone (Sigma-Aldrich, Saint Louis, USA), 1 pM, 5 pM and 10 pM for 17β-estradiol (E_2) (Sigma-Aldrich, Saint Louis, USA), 10 nM, 20 nM and 30 nM for letrozole (Sigma-Aldrich, Saint Louis, USA), 5 μM, 10 μM and 20 μM for resveratrol (Sigma-Aldrich, Saint Louis, USA), and 1 nM, 5 nM, 10 nM and 20 nM for melatonin (Sigma-Aldrich, Saint Louis, USA).

2.4. Cell proliferation.

Cell proliferation was determined after treatment with the test compounds by performing an MTT assay as described earlier (34). The optimal cell number was established based on results from a cell viability assay of vehicle-control treated cells and visual inspection of the cells. After exposure to the test compounds, 1 ml of MTT (5 mg MTT/ ml in PBS) was added for 4 hours. Next, the medium was aspirated and 100 μl of DMSO was added to dissolve the accumulated formazan crystals. The absorbance was measured at 550 nm using the 96-wells microplate reader (Spectramax plus 384, Molecular Devices, California, USA).

2.5. Analysis of gene expression

After the cells were treated with the test compounds, total RNA was extracted using RNeasy® mini kit (Qiagen, Texas, USA). The purification and concentration of total RNA were measured by the ratio of absorbance at 260/280 and 260/230 NM using Nanodrop (Nanodrop Technologies, Inc., Delaware, USA). Obtained RNA was stored at -20°C in aliquots of 10 ng/μl. Next, total RNA was reverse-transcribed into cDNA using the Qiagen high capacity cDNA reverse-transcription kit (Qiagen, Texas, USA). Gene

expression of *aromatase (CYP19A1)*, *pS2* and *Ki-67* was performed using a Roche-Light-cycler-480 one step RT PCR kit (Roche, Indiana, USA). The reaction mixture contained 0.1µM of primers, 1X Quantitect SYBR Green RT-PCR Master mix, 0.2 µl QuantiTect RT mix and 2 µl template RNA (10 ng/µl) in a total volume of 20 µl. The mixture was reverse transcribed to cDNA at 50°C for 20 minutes. After reverse transcription, the PCR reaction was initiated by heating at 95°C for 15 minutes, then followed by denaturation at 95°C for 10 seconds, annealing at 57°C for 25 seconds, extended at 68°C for 30 seconds and acquisition at 82°C for 5 seconds for 45 cycles. Relative quantification of gene expression was expressed as the ratio of the intensity of the target gene to the housekeeping gene *beta-actin*. Primers for aromatase mRNA amplification were used as described previously by Sanderson *et al*. (40). Primers coding for the estrogen-responsive *pS2* gene were used after Lee *et al*. (41). *Ki-67* mRNA amplification was done in T47D cell lines as described by Chottanapund *et al*. (36). Amplification of *β-ACTIN* (forward: 5'-TCTACAATGAGCTGCG-3' and reverse: 5'-AGGTAGTCAGCTAGGT-3') was used as a reference housekeeping gene. All primers were run through the National Center for Biotechnology Information (NCBI) blast (nucleotide nonredundant database) to confirm specificity. The efficiency was determined for a dilution range of cDNA and new primers were sought if the efficiency was below 95% or higher than 105%. After each RT-qPCR, a melt curve was run to ensure that primer-dimers and other non-specific products were omitted.

2.6. Measurement of Estradiol (E_2).

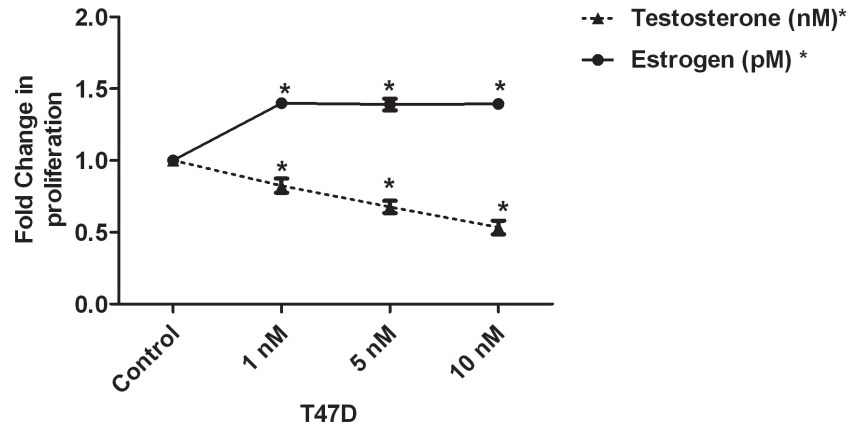
After the cell proliferation assay with the test compounds, 50 µL assay medium of each treated co-cultured system was dispensed to measure the amount E_2 production using a commercially available ELISA Kit for human Estradiol according to the manufacturer's instruction (Invitrogen, CA, USA)

2.7. Statistical analysis.

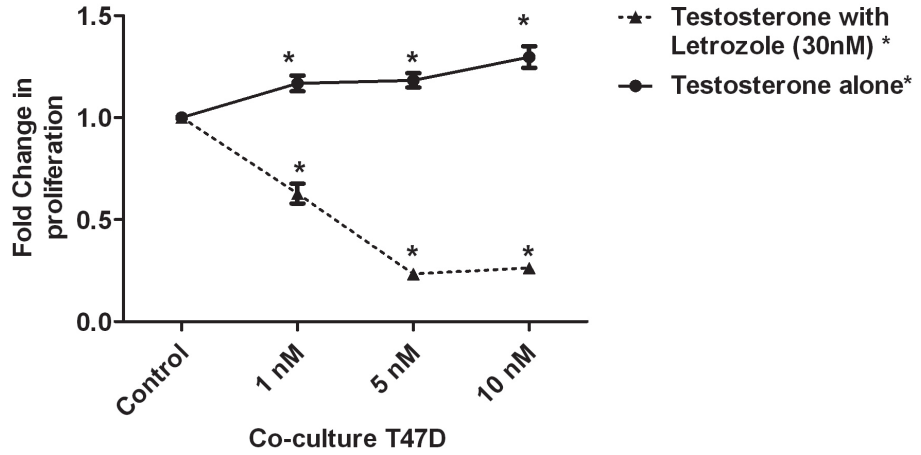
Each experiment was performed in three times and in each experiment, every concentration was tested in triplicate, except for the estradiol production which was measured in duplicate as recommended by the manufacturer of the ELISA kit. Means and standard deviations were calculated of all nine values (n=9). The statistical significance of differences of the means was calculated using the student t-test or one-way ANOVA. Differences were considered statistically significant if $P < 0.05$.

3. Results.

Previous experiments in our laboratory showed that in the mono-cultured T47D breast tumor cells, testosterone caused a significant reduction (69%) in cell growth at 10 nM of testosterone (36). In contrast, estradiol induced a significant proliferation of T47D cells (150%) at 1 pM after 120 hours (Supplementary Figure. S1). Conversely, 1 nM

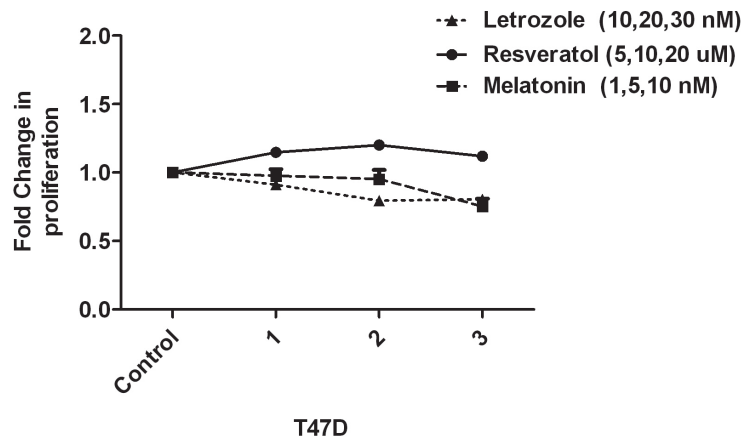


Supplementary Figure S1. Cell proliferation of T47D, cell cultures were exposed to vehicle control or testosterone (1 nM, 5 nM or 10 nM) and estrogen (1 pM, 5 pM, or 10 pM) for 120 hours. Data are expressed as means ± SD (n=9). There are significantly decrease cells proliferation from vehicle control to treated mono-cultured T47D breast cancer cells with testosterone (one-way ANOVA, *p < 0.01). There are significantly increase cells proliferation from vehicle control to treated mono-cultured T47D breast cancer cells with estrogen (one-way ANOVA, **p <0.01)



Supplementary Figure S2. Cell proliferation of co-culture T47D, cell cultures were exposed to vehicle control or testosterone (1 nM, 5 nM or 10 nM) with or without letrozole (30 nM) for 120 hours. Data are expressed as means ± SD (n=9). There are significantly increase cells proliferation from vehicle control to treated co-cultured T47D breast cancer cells with testosterone (one-way ANOVA, **p < 0.01). There are significantly decrease cells proliferation from control to treated co-cultured T47D breast cancer cells with testosterone in presented of letrozole 30 nM (one-way ANOVA, **p <0.01)

testosterone induced significant proliferation of T47D cells (130%) when co-cultured in the presence of BAFs. This proliferative effect of testosterone declined significantly after the aromatase inhibitor letrozole (30 nM) was added to the co-cultured system with BAFs. A maximum reduction of 25% cell growth was obtained at 10 nM testosterone, in the presence of letrozole (30 nM) (Supplementary Figure. S2). Exposure of mono-cultured T47D cells to letrozole, resveratol and melatonin did not induce any significant cell proliferation (Supplementary Figure. S3)



Supplementary Figure S3. Cell proliferation of T47D, cell mono-cultures were exposed to vehicle control or letrozole (10 nM, 20 nM or 30 nM) or resveratrol (5 μM, 10 μM, or 20 μM) or melatonin (1 nM, 5nM, 10 nM) for 120 hours. No proliferation response of T47D cells to letrozole, resveratrol and melatonin. Data are expressed as means ± SD (n=9).

3.1. Expression of Aromatase gene in a co-culture of T47D with BAFs when exposed to testosterone combined with Letrozole.

The *CYP19A1* gene is encoding for the aromatase enzyme. The aromatase enzyme has been found to be inducible by androgen-like compound in co-cultured BAFs with human breast cancer cells (34, 35). In this study, aromatase gene expression was concentration-dependently upregulated by testosterone in T47D-BAF co-cultures compared to vehicle control (Fig 1). Testosterone at 1 nM induced aromatase expression upto 6.5 fold compared with control cells. Maximal induction of aromatase gene expression was 15.40 fold with 10 nM testosterone. Additionally, we observed that aromatase gene expression could still be significantly induced by testosterone in the presence of the aromatase inhibitor (30 nM of letrozole) (Figure 1). Maximal induction of aromatase gene expression was 10.25 fold with 10 nM testosterone in combination with 30 nM letrozole, which was lower than aromatase expression using testosterone only.

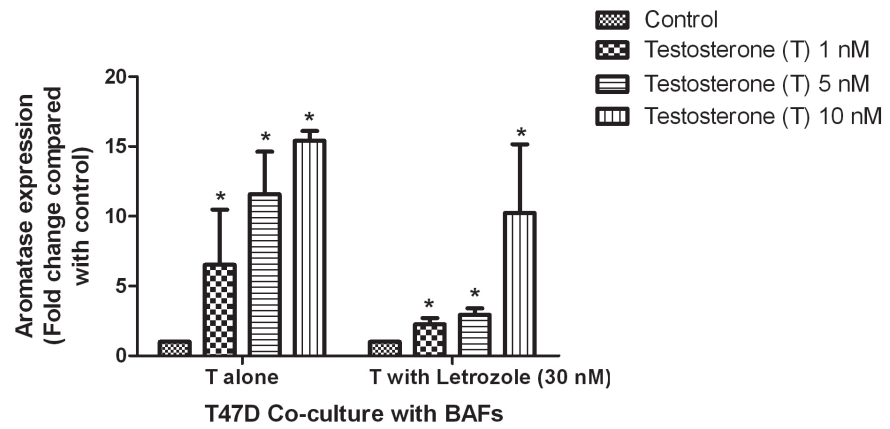
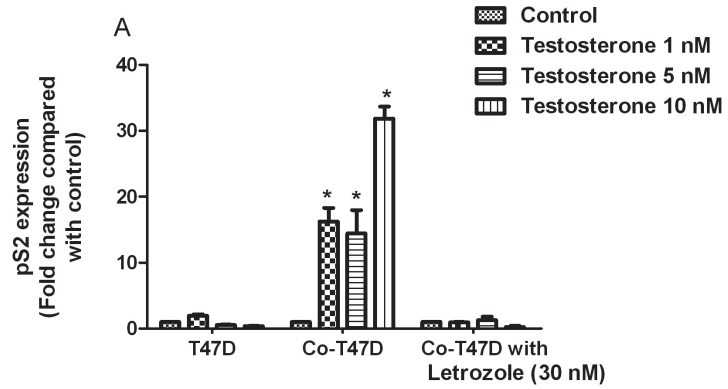


Figure 1. On the left, *Aromatase* expression in T47D cells co-culture with BAFs treated with testosterone 1, 5 and 10 nM comparing to the vehicle- control. On the right, *Aromatase* expression in T47D cells co-culture with BAFs treated with testosterone 1, 5 and 10 nM in the presence of letrozole 30 nM comparing to the control. *Aromatase* expression in T47D cells co-culture with BAFs increased in the presence of testosterone or even combined with letrozole. Cell cultures were exposed to the tested chemicals for 120 hours. Data are expressed as means ± SD (n=9). * Significantly different from control - treated co-cultured breast cancer cells with BAFs (one-way ANOVA, P< 0.01).

3.2. Expression of pS2 and ki-67 genes in mono- and co-cultures of T47D cells with BAFs when exposed to testosterone with or without Letrozole.

The *pS2* gene is an E_2-responsive gene that can be easily up-regulated in estrogen receptor positive breast tumor cells such as the T47D cells. In mono-cultured T47D cells, testosterone did not statistically significantly affect *pS2* expression (data not shown). However, in a T47D-BAF co-culture, testosterone increased *pS2* gene expression already 16.24 fold at 1 nM and even up to 31.85 fold at 10 nM compared with vehicle-treated control cells (Figure. 2a). Also, gene expression of the cell proliferation marker *Ki-67* was increased by testosterone only in the co-culture with a maximum induction of 720 fold at 5nM (Figure. 2a). Co-incubation of the T47D-BAF co-culture with testosterone and letrozole (30 nM) completely negated the induction of *pS2* and *ki-67* gene expression by testosterone alone. (Figure. 2a and b)



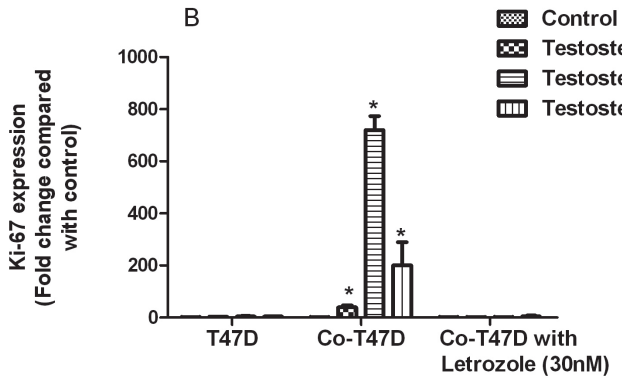


Figure 2. (A) *pS2* and (B) *ki-67*expression in mono-culture T47D (on the left), co-cultured of T47D with BAFs (in the middle) or co-cultured T47D with BAFs in the presence of letrozole 30 nM (on th right) treated with testosterone 1, 5 and 10 nM comparing to the control. (A) *pS2* and (B) *ki-67*expression were not induced by testosterone in mono-culture of T47D cells, but induced by testosterone in co-culture with BAFs. Cell cultures were exposed to the tested chemicals for 120 hours. Data are expressed as means ± SD (n=9). * Significantly different from control - treated co-cultured breast cancer cells with BAFs (one-way ANOVA, P< 0.01). The expression was diminished by adding letrozole (30 nM) into the system.

3.3. Exposure of co-cultures of T47D cells and BAFs to testosterone combined with melatonin or resveratrol.

Next, we studied the influence of melatonin and resveratrol on testosterone-induced cell proliferation of T47D-BAF co-culture. Melatonin reduced the testosterone induced cell proliferation in a concentration-dependent manner. At 20 nM melatonin, the proliferative

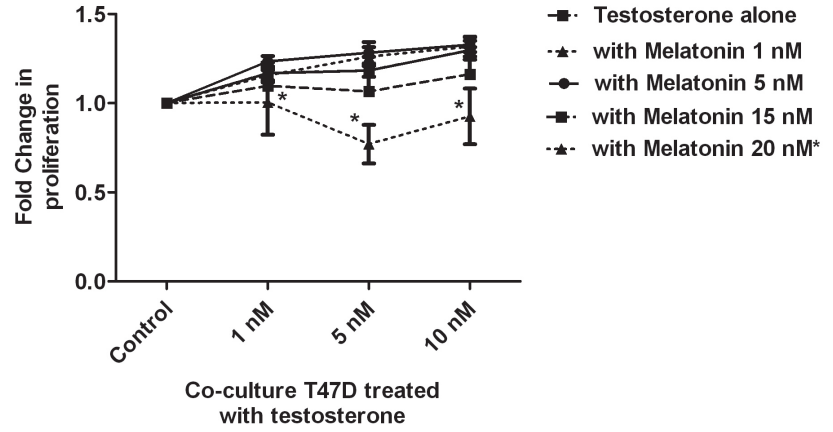


Figure 3. Cell proliferation of T47D breast cancer cells and BAFs in co-culture with testosterone (1 nM, 5 nM or 10 nM) with or without melatonin (1, 5, 15, 20 nM) compared to the control after 120 hours. There was significantly increased in cell proliferation from control to treated co-cultured T47D breast cancer cells with testosterone alone. The proliferation is significantly decreased in the presence of melatonin in the system. The maximum reduction is at 20 nM of melatonin. Data are expressed as means ± SD (n=9). * Significantly different from control to treated co-cultured breast cancer cells with BAFs (one-way ANOVA, *p < 0.01).

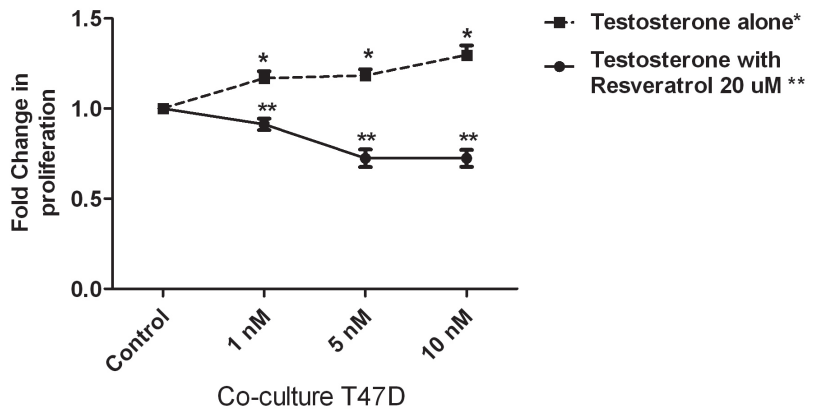


Figure 4. Cell proliferation of T47D breast cancer cells and BAFs in co-culture with testosterone (1 nM, 5 nM or 10 nM) with or without resveratrol (20 μM) compared to the control after 120 hours. There was significantly increased in cell proliferation from control to treated co-cultured T47D breast cancer cells with testosterone alone. The proliferation was significantly decreased in the presence of resveratrol (20 μM) in the system. Data are expressed as means ± SD (n=9). *,** Significantly different from control to treated co-cultured breast cancer cells with BAFs (one-way ANOVA, p < 0.01).

effect of testosterone on the T47D cells in the co-culture system was fully abolished (Figure. 3). Also, cell proliferation in T47D-BAF co-culture was significantly reduced by resveratrol albeit at a much higher concentration. In the presence of 20 μM resveratrol, the maximum reduction of T47D cell growth in theT47D-BAF co-culture was 27.62 (±13.94) % at 10 nM of testosterone (Figure. 4).

3.4. Gene expression of aromatase, pS2 and ki-67 genes in co-cultures of T47D with BAFs exposed to testosterone combined with resveratrol or melatonin.

The induced gene expression of aromatase by testosterone in the co-cultures was not influenced by the presence of 20 μM reseveratrol or 20 nM melatonin (Figure. 5). Interestingly, both resveratrol and melatonin at these concentrations had a significant reductive effect on the expression of *pS2*; (98.04±1.42%, 75.75±19.42%, respectively) and *ki-67* in T47D cells; (97.83±0.58%, 89.19±4.95%, respectively, respectively), which indicates a chemopreventive effect of both these compounds in the co-culture with BAFs (Figures. 6a and b).

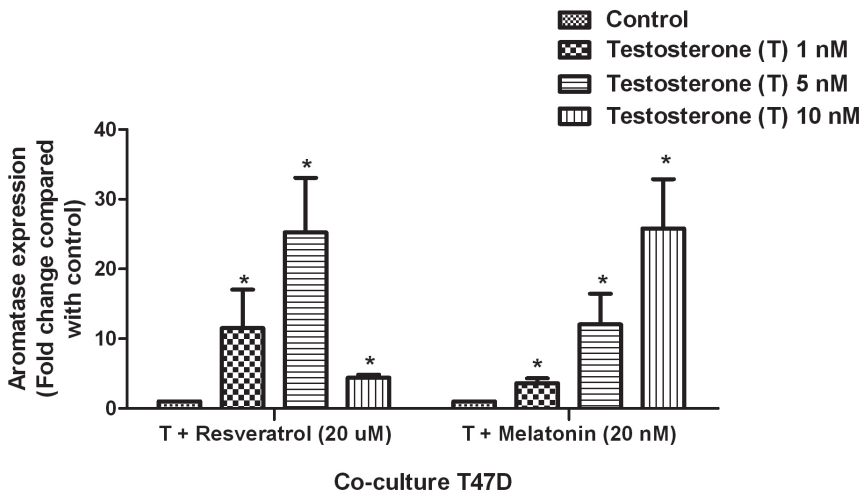
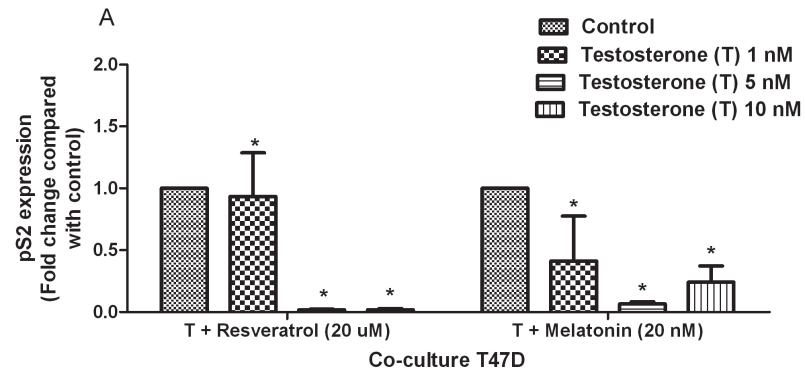


Figure 5. On the left, *Aromatase* expression in T47D cells co-culture with BAFs treated with testosterone 1, 5 and 10 nM in the presence of resveratrol (20 μM) comparing to the control. On the right, *Aromatase* expression in T47D cells co-culture with BAFs treated with testosterone 1, 5 and 10 nM in the presence of melatonin (20nM) comparing to the control. Induction of *Aromatase* expression by testosterone in T47D cells co-cultured with BAFs in the presence of resveratrol or melatonin. Cell cultures were exposed to the tested chemicals for 120 hours. Data are expressed as means ± SD (n=9). * Significantly different from control to treated co-cultured breast cancer cells with BAFs (one-way ANOVA, P< 0.01).



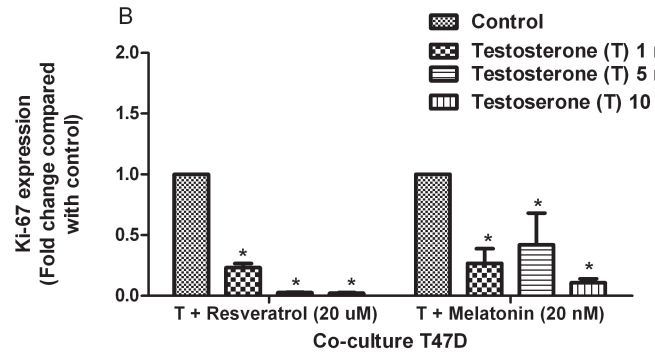


Figure 6. (A) *pS2* and (B) *ki-67*expression in co-cultured of T47D with BAFs in the presence of testosterone (1 nM, 5 nM or 10 nM) with resveratrol (20 μM) (on th left) or testosterone (1 nM, 5 nM or 10 nM) with melatonin (20 nM) (on the right) comparing to the control. Induced expression of (A) *pS2* and (B) *ki-67* by testosterone were suppressed in the presence of resveratrol and melatonin in co-culture T47D with BAFs. Cell cultures were exposed to the tested chemicals for 120 hours. Data are expressed as means ± SD (n=9). * Significantly different from control to treated co-cultured breast cancer cells with BAFs (one-way ANOVA, P< 0.01).

3.5. Estradiol production in co-cultures of T47D with BAFs exposed testosterone alone or combined with letrozole, resveratrol or melatonin.

To determine if the increase in aromatase gene expression also results in higher aromatase activity and thus estradiol levels, the levels of E_2 in the medium of T47D-BAF co-cultures was measured using a commercially available RIA. Indeed, E_2 concentrations in the medium increased with increasing concentrations of testosterone. At 10 nM testosterone, E_2 concentration was increased 2.4 fold compared with vehicle-treated control T47D-BAF co-cultures. Letrozole (30 nM), resveratrol (20 µM) and melatonin (20 nM) all statistically significantly reduced E_2 production in the co-cultures at 5 and 10 nM of testosterone. Interestingly, melatonin was approximately equally effective as the pharmacological aromatase inhibitor letrozole (Figure. 7).

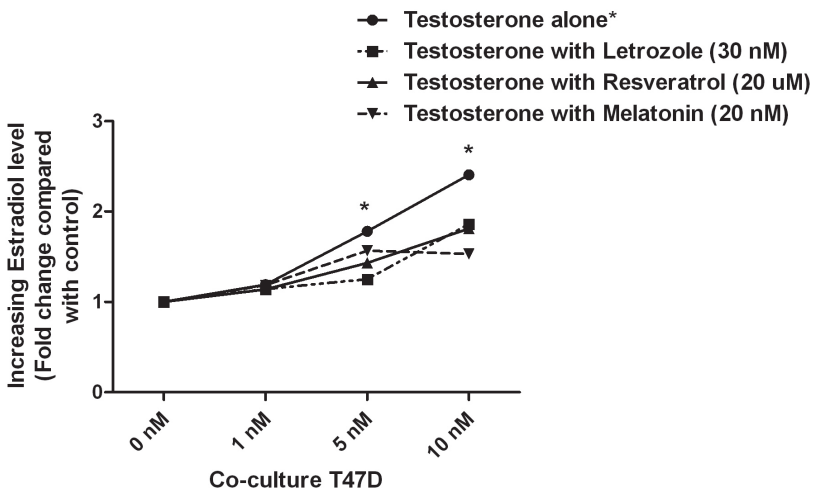


Figure 7. Co-cultures T47D with BAFs were exposed to vehicle control or testosterone (1 nM, 5 nM or 10 nM) alone or in the presence of letrozole (30 nM) or resveratrol (20 µM) or melatonin (20 nM) for 120 hours. The levels of E_2 in the medium of T47D-BAF co-cultures was measured using a commercially available RIA. There was an increasing of estrodiol convertion according to the doses of testosterone**.** Data are expressed as means ± SD (n=6). * Significantly different from control to treated co-cultured breast cancer cells with BAFs (one-way ANOVA, P< 0.01). The highest expression was in testosterone treated co-cultured breast cancer cells ( ). ** Significantly different from letrozole, resveratrol and melatonin treated co-cultured breast cancer cells (one-way ANOVA, P< 0.01).

4. Discussion.

4.1. suitability/relevance of co-culture system for breast cancer study.

There is evidence that stromal cells surrounding epithelial breast tumor cells play an important role in tumor cell behavior (38). In order to mimic the human vivo situation more closely, the tumor microenvironment should be considered in the experiment. There are two techniques for establishing co-culture systems; a direct (one-compartment) co-culture system or layering technique, and an indirect (two-compartment) co-culture system. Heneweer *et al*. previously described the paracrine interactions between breast carcinoma MCF7 cells (34, 35) and human primary BAFs in a direct cell-cell contact in vitro co-culture system, while the effect was not observed in two-compartment system. The modulation of aromatase in BAFs was found to be an important aspect in these paracrine interactions as stimulation of aromatase activity in BAF subsequently stimulated cell proliferation of the tumor cells (34). The results of our present study clearly demonstrate the relevance of a co-culture system, as testosterone induced tumor-cell derived, estrogen-dependent pS2 gene expression in a T47D-BAF co-culture, but not in a T47D mono-culture. This clearly demonstrates that in our co-culture system, testosterone is converted into E_2, which subsequently induced proliferation of T47D tumor cells and increase ER-dependent pS2 expression. Our results concur with the conclusion of Miki *et al*., as described in their recent review (42).

In view of the estrogen receptor independent reduction of ki-67 expression in the presence of testosterone in our co-culture observed this and our previous study indicated a possible role of testosterone, and the androgen receptor (AR), in growth inhibition of these breast tumor cells (36). However, it can not be excluded that testosterone binds to ERβ that is also expressed in T47D cells and reduces cell proliferation via this pathway (43). In contrast with ERα, activation of ERβ can have an anti-cell proliferative effect on tumor cells (43). However, it should also be noted that the expression of ERβ is much lower in T47D cells than that of the ERα and AR (43), which may suggest a dominant role in our experiments of the AR.

4.2. aromatase inhibition by melatonin and resveratrol.

In our present study, we used the T47D-BAF co-culture system to further study the suggested breast cancer chemopreventive effects of resveratrol and melatonin. These compounds have been suggested to act as inhibitors of aromatase and therefore can reduce breast tumor cell proliferation. We have previously explored two estrogen receptor (ERα)-positive breast cancer cell lines MCF-7 and T47D to use for co-culture studies. Both cell lines express the ERα and are also positive for the androgen receptor (AR) (43). Theoretically, both these cell lines could be used for our co-culture studies, because conversion of testosterone by BAFs to E_2 should induce cell proliferation in the MCF-7 as well as T47D breast tumor cells. (36). We decided to use the T47D cell line

in this study, because there is evidence that it has a higher androgen receptor expression than MCF-7 cells (43). Moreover, T47D cells display lower aromatase expression and activity than MCF-7 cells. This means that the BAFs will be the major source of E2 synthesis in our co-culture system, which better reflects the in vivo situation (43).

Firstly, we studied the role of the aromatase enzyme in BAFs and subsequent ER-dependent cell proliferation of T47D cells in our co-culture system. Indeed, testosterone increased cell proliferation and pS2 and ki-67 gene expression, while adding the aromatase inhibitor letrozole reduced this testosterone -dependent cell proliferation and gene expression. Next, we tested resveratrol and melatonin for their chemopreventive properties with respect to inhibition of aromatase and cell proliferation. As a natural product, resveratrol has very few reports on toxicity at very high dose, which makes resveratrol a good candidate for breast cancer treatment or prevention (22). We have tested only one, high concentration of resveratrol (20 μM), which was based on an earlier study done by Wang *et al*. in SK-BR-3 cell line. Their study found that resveratrol suppressed the I.1 promotor region of *CYP19A1* gene (44). At this concentration, proliferation of T47D cells in co-culture was reduced in the presence of testosterone. Together with the observations that resveratrol reduced estrogen levels in testosterone -exposed T47D-BAF co-cultures, these data suggest that resveratrol is indeed an aromatase inhibitor albeit at a high concentration (20 μM) (18, 19, 21, 44).

Melatonin is an indolic compound secreted by the pineal gland, which plays a physiological role in regulation of the sleep cycle. Melatonin may have anti-tumoral actions including breast cancer, because of its function as the modulator hormone in the neuroendocrine-reproductive axis (45, 46). There are two proposed mechanisms of melatonin as a chemopreventive agent against breast cancer; its can act as a SERM and/or a SEEM. The SERM mechanism of action was suggested to be mediated by the MT1 melatonin receptor, but not via the ERα (47). Melatonin can bind with high affinity to MT1 receptors and this complex will interfere with the estrogen-binding activity of ERα without changing its affinity and will reduce the ligand-receptors transactivation (48). However, with respect to SERM action of melatonin, there are controversial results in literature. Studies with the human MCF-7 breast carcinoma cell line indicate a decrease in cell proliferation due to the action of melatonin as a SERM at the normal physiologic dose of 1 nM (30, 49). In contrast, other studies with both the estrogen responsive MCF-7 and T47D breast carcinoma cell lines showed no influence of melatonin on cell proliferation up to 0.1 mM concentration (50, 51). The latter results are in agreement with our present study with mono-cultured T47D cells. However, we additionally showed in our T47D-BAF co-culture that melatonin has a concentration-dependent anti-aromatase. Melatonin has previously been shown to act like a SEEM by inhibiting the expression and activity of aromatase (*CYP19A1*), estrogen sulfatase, and type 1, 17β- hydroxysteroid dehydrogenase in MCF-7 cell line (47). In our study, the most effective concentration of melatonin as an aromatase inhibitor was 20 nM, which is about one order of magnitude

higher than physiologically relevant concentrations that lie between 45-200 pM (39). However, upon oral intake of melatonin supplements (2 mg), melatonin plasma levels in the nM range can easily be reached without any side effect (52). Also, in our study the effective concentration of melatonin as a SEEM was in the same concentration range as the well known potent pharmaceutical aromatase inhibitor letrozole. Our results are in agreement with a previous study done by Grant *et al*. that melatonin works as SEEM (27) and melatonin may be one of the target for breast cancer therapy.

5. Conclusion.

Our study again shows that the co-culture of the T47D breast tumor cells combined with breast fibroblast appears to be a good model to study effects on aromatase, the estrogen receptor as well as the role of androgens in the paracrine interactions between both cell types. Both melatonin and resveratrol were found to be aromatase inhibitors in this co-culture system, albeit at significantly different concentrations. Our co-culture model did not provide any indications that melatonin is also a selective estrogen receptor modulator. The melatonin concentration of 20 nM and resveratrol concentration of 20 μM have aromatase inhibitory effect as potent as 20 nM of letrozole, which is a clinically used anti-aromatase drug in breast cancer treatment. The combination of both SERM and SEEM mechanisms of action of especially melatonin may offer potential advantages for breast cancer treatment.

Declaration of interest.

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgements.

This work was supported by the Chulabhorn Graduate Institute, Chulabhorn Research Institute, and The Institute for Risk Assessment Sciences at Utrecht University.

References.

1. Bando H. [Adjuvant endocrine therapy in premenopausal breast cancer]. Nihon Rinsho. 2013;70 Suppl 7:627-35.
2. Bell RJ, Schwarz M, Fradkin P, Robinson PJ, Davis SR. Patterns of use of oral adjuvant endocrine therapy in Australian breast cancer survivors 5 years from diagnosis. Menopause. 2013.
3. Higgins MJ, Liedke PE, Goss PE. Extended adjuvant endocrine therapy in hormone dependent breast cancer: The paradigm of the NCIC-CTG MA.17/BIG 1-97 trial. Crit Rev Oncol Hematol. 2012.
4. Yoshinami T, Yagi T, Ishitobi M. [Endocrine therapy for advanced breast cancer]. Nihon Rinsho. 2013;70 Suppl 7:641-4.
5. Rao RD, Cobleigh MA. Adjuvant endocrine therapy for breast cancer. Oncology (Williston Park).26(6):541-7, 50, 52 passim.
6. Balkenende EM, Dahhan T, Linn SC, Jager NG, Beijnen JH, Goddijn M. A prospective case series of women with estrogen receptor-positive breast cancer: levels of tamoxifen metabolites in controlled ovarian stimulation with high-dose tamoxifen. Hum Reprod. 2013.
7. Yang LH, Tseng HS, Lin C, Chen LS, Chen ST, Kuo SJ, et al. Survival benefit of tamoxifen in estrogen receptor-negative and progesterone receptor-positive low grade breast cancer patients. J Breast Cancer. 2012;15(3):288-95.
8. Thurlimann B, Keshaviah A, Coates AS, Mouridsen H, Mauriac L, Forbes JF, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med. 2005;353(26):2747-57.
9. Doughty JC. A review of the BIG results: the Breast International Group 1-98 trial analyses. Breast. 2008;17 Suppl 1:S9-S14.
10. Joerger M, Thurlimann B. Update of the BIG 1-98 Trial: where do we stand? Breast. 2009;18 Suppl 3:S78-82.
11. Mouridsen H, Giobbie-Hurder A, Goldhirsch A, Thurlimann B, Paridaens R, Smith I, et al. Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer. N Engl J Med. 2009;361(8):766-76.
12. Lumachi F, Brunello A, Maruzzo M, Basso U, Basso SM. Treatment of Estrogen Receptor-Positive Breast Cancer. Curr Med Chem. 2013.
13. Yue W, Yager JD, Wang JP, Jupe ER, Santen RJ. Estrogen receptor-dependent and independent mechanisms of breast cancer carcinogenesis. Steroids. 2012;78(2):161-70.
14. Levi F, Pasche C, Lucchini F, Ghidoni R, Ferraroni M, La Vecchia C. Resveratrol and breast cancer risk. Eur J Cancer Prev. 2005;14(2):139-42.
15. Alkhalaf M, El-Mowafy A, Renno W, Rachid O, Ali A, Al-Attyiah R. Resveratrol-induced apoptosis in human breast cancer cells is mediated primarily through the caspase-3-dependent pathway. Arch Med Res. 2008;39(2):162-8.
16. Lee HS, Ha AW, Kim WK. Effect of resveratrol on the metastasis of 4T1 mouse breast cancer cells in vitro and in vivo. Nutr Res Pract. 2012;6(4):294-300.
17. Leon-Galicia I, Diaz-Chavez J, Garcia-Villa E, Uribe-Figueroa L, Hidalgo-Miranda A, Herrera LA, et al. Resveratrol induces downregulation of DNA repair genes in MCF-7 human breast cancer cells. Eur J Cancer Prev. 2012;22(1):11-20.
18. Lee JE, Safe S. Involvement of a post-transcriptional mechanism in the inhibition of CYP1A1 expression by resveratrol in breast cancer cells. Biochem Pharmacol. 2001;62(8):1113-24.
19. Le Corre L, Chalabi N, Delort L, Bignon YJ, Bernard-Gallon DJ. Resveratrol and breast cancer chemoprevention: molecular mechanisms. Mol Nutr Food Res. 2005;49(5):462-71.
20. Zhang S, Cao HJ, Davis FB, Tang HY, Davis PJ, Lin HY. Oestrogen inhibits resveratrol-induced post-translational modification of p53 and apoptosis in breast cancer cells. Br J Cancer. 2004;91(1):178-85.

21. Wang Y, Lee KW, Chan FL, Chen S, Leung LK. The red wine polyphenol resveratrol displays bilevel inhibition on aromatase in breast cancer cells. Toxicol Sci. 2006;92(1):71-7.
22. Crowell JA, Korytko PJ, Morrissey RL, Booth TD, Levine BS. Resveratrol-associated renal toxicity. Toxicol Sci. 2004;82(2):614-9.
23. Mediavilla MD, Sanchez-Barcelo EJ, Tan DX, Manchester L, Reiter RJ. Basic mechanisms involved in the anti-cancer effects of melatonin. Curr Med Chem.17(36):4462-81.
24. Srinivasan V, Spence DW, Pandi-Perumal SR, Trakht I, Cardinali DP. Therapeutic actions of melatonin in cancer: possible mechanisms. Integr Cancer Ther. 2008;7(3):189-203.
25. Dai M, Cui P, Yu M, Han J, Li H, Xiu R. Melatonin modulates the expression of VEGF and HIF-1 alpha induced by CoCl2 in cultured cancer cells. J Pineal Res. 2008;44(2):121-6.
26. Mills E, Wu P, Seely D, Guyatt G. Melatonin in the treatment of cancer: a systematic review of randomized controlled trials and meta-analysis. J Pineal Res. 2005;39(4):360-6.
27. Grant SG, Melan MA, Latimer JJ, Witt-Enderby PA. Melatonin and breast cancer: cellular mechanisms, clinical studies and future perspectives. Expert Rev Mol Med. 2009;11:e5.
28. Korkmaz A, Sanchez-Barcelo EJ, Tan DX, Reiter RJ. Role of melatonin in the epigenetic regulation of breast cancer. Breast Cancer Res Treat. 2009;115(1):13-27.
29. Blask DE, Brainard GC, Dauchy RT, Hanifin JP, Davidson LK, Krause JA, et al. Melatonin-depleted blood from premenopausal women exposed to light at night stimulates growth of human breast cancer xenografts in nude rats. Cancer Res. 2005;65(23):11174-84.
30. Sanchez-Barcelo EJ, Cos S, Mediavilla D, Martinez-Campa C, Gonzalez A, Alonso-Gonzalez C. Melatonin-estrogen interactions in breast cancer. J Pineal Res. 2005;38(4):217-22.
31. Treeck O, Haldar C, Ortmann O. Antiestrogens modulate MT1 melatonin receptor expression in breast and ovarian cancer cell lines. Oncol Rep. 2006;15(1):231-5.
32. Yuan L, Collins AR, Dai J, Dubocovich ML, Hill SM. MT(1) melatonin receptor overexpression enhances the growth suppressive effect of melatonin in human breast cancer cells. Mol Cell Endocrinol. 2002;192(1-2):147-56.
33. Martinez-Campa C, Gonzalez A, Mediavilla MD, Alonso-Gonzalez C, Alvarez-Garcia V, Sanchez-Barcelo EJ, et al. Melatonin inhibits aromatase promoter expression by regulating cyclooxygenases expression and activity in breast cancer cells. Br J Cancer. 2009;101(9):1613-9.
34. Heneweer M, Muusse M, Dingemans M, de Jong PC, van den Berg M, Sanderson JT. Co-culture of primary human mammary fibroblasts and MCF-7 cells as an in vitro breast cancer model. Toxicol Sci. 2005;83(2):257-63.
35. Heneweer M, Muusse M, Dingemans M, de Jong PC, van den Berg M, Sanderson JT. Direct and indirect endocrine disruption: Co-culture of primary human mammary fibroblasts and MCF-7 cells as an *in vitro* breast cancer model. Institute for Risk Assessment Sciences (IRAS). Ph.D. Utrecht Utrecht University; 2005. p. 175.
36. Chottanapund S, Van Duursen MB, Navasumrit P, Hunsonti P, Timtavorn S, Ruchirawat M, et al. Effect of androgens on different breast cancer cells co-cultured with or without breast adipose fibroblasts. J Steroid Biochem Mol Biol. 2013;138C:54-62.
37. Sonne-Hansen K, Lykkesfeldt AE. Endogenous aromatization of testosterone results in growth stimulation of the human MCF-7 breast cancer cell line. J Steroid Biochem Mol Biol. 2005;93(1):25-34.
38. Birrell SN, Bentel JM, Hickey TE, Ricciardelli C, Weger MA, Horsfall DJ, et al. Androgens induce divergent proliferative responses in human breast cancer cell lines. J Steroid Biochem Mol Biol. 1995;52(5):459-67.
39. Cops EJ, Bianco-Miotto T, Moore NL, Clarke CL, Birrell SN, Butler LM, et al. Antiproliferative actions of the synthetic androgen, mibolerone, in breast cancer cells are mediated by both androgen and progesterone receptors. J Steroid Biochem Mol Biol. 2008;110(3-5):236-43.
40. Sanderson JT, Letcher RJ, Heneweer M, Giesy JP, van den Berg M. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environ Health Perspect. 2001;109(10):1027-31.

41. Lee Y, Jin Y, Lim W, Ji S, Choi S, Jang S, et al. A ginsenoside-Rh1, a component of ginseng saponin, activates estrogen receptor in human breast carcinoma MCF-7 cells. J Steroid Biochem Mol Biol. 2003;84(4):463-8.
42. Miki Y, Ono K, Hata S, Suzuki T, Kumamoto H, Sasano H. The advantages of co-culture over mono cell culture in simulating in vivo environment. J Steroid Biochem Mol Biol. 2012;131(3-5):68-75.
43. Aka JA, Lin SX. Comparison of functional proteomic analyses of human breast cancer cell lines T47D and MCF7. PLoS One. 2012;7(2):e31532.
44. Wang Y, Ye L, Leung LK. A positive feedback pathway of estrogen biosynthesis in breast cancer cells is contained by resveratrol. Toxicology. 2008;248(2-3):130-5.
45. Cos S, Sanchez-Barcelo EJ. Melatonin and mammary pathological growth. Front Neuroendocrinol. 2000;21(2):133-70.
46. Cos S, Sanchez-Barcelo EJ. Melatonin, experimental basis for a possible application in breast cancer prevention and treatment. Histol Histopathol. 2000;15(2):637-47.
47. Sanchez-Barcelo EJ, Mediavilla MD, Alonso-Gonzalez C, Rueda N. Breast cancer therapy based on melatonin. Recent Pat Endocr Metab Immune Drug Discov. 2012;6(2):108-16.
48. Sanchez-Barcelo EJ, Mediavilla MD, Alonso-Gonzalez C, Reiter RJ. Melatonin uses in oncology: breast cancer prevention and reduction of the side effects of chemotherapy and radiation. Expert Opin Investig Drugs. 2012;21(6):819-31.
49. Cos S, Sanchez-Barcelo EJ. Melatonin inhibition of MCF-7 human breast-cancer cells growth: influence of cell proliferation rate. Cancer Lett. 1995;93(2):207-12.
50. Eck KM, Yuan L, Duffy L, Ram PT, Ayettey S, Chen I, et al. A sequential treatment regimen with melatonin and all-trans retinoic acid induces apoptosis in MCF-7 tumour cells. Br J Cancer. 1998;77(12):2129-37.
51. Papazisis KT, Kouretas D, Geromichalos GD, Sivridis E, Tsekreli OK, Dimitriadis KA, et al. Effects of melatonin on proliferation of cancer cell lines. J Pineal Res. 1998;25(4):211-8.
52. Pink JJ, Bilimoria MM, Assikis J, Jordan VC. Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. Br J Cancer. 1996;74(8):1227-36.

Chapter 5

DEPSIDONES INHIBIT AROMATASE ACTIVITY AND TUMOR CELL PROLIFERATION IN A CO-CULTURE OF HUMAN PRIMARY BREAST ADIPOSE FIBROBLASTS AND T47D BREAST TUMOR CELLS

Suthat Chottanapund[1,2,3,4,5], Majorie B.M. Van Duursen[5], Anne Zwartsen[2,5], Supatchaya Timtavorn[2], Panida Navasumrit[1,2,3], Prasat Kittakoop[1,2,3], Sanya Sureram[2], Mathuros Ruchirawat[1,2,3], Martin Van den Berg[5]

[1] Chulabhorn Graduate Institute, Kamphaeng Phet 6 Road, Laksi, Bangkok 10210, Thailand
[2] Chulabhorn Research Institute, Kamphaeng Phet 6 Road, Laksi, Bangkok 10210, Thailand
[3] Center of Excellence on Environmental Health and Toxicology (EHT), CHE, Ministry of Education, Bangkok, Thailand
[4] Bamrasnaradura Infectious Diseases Institute, Ministry of Public Health, Thailand
[5] Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

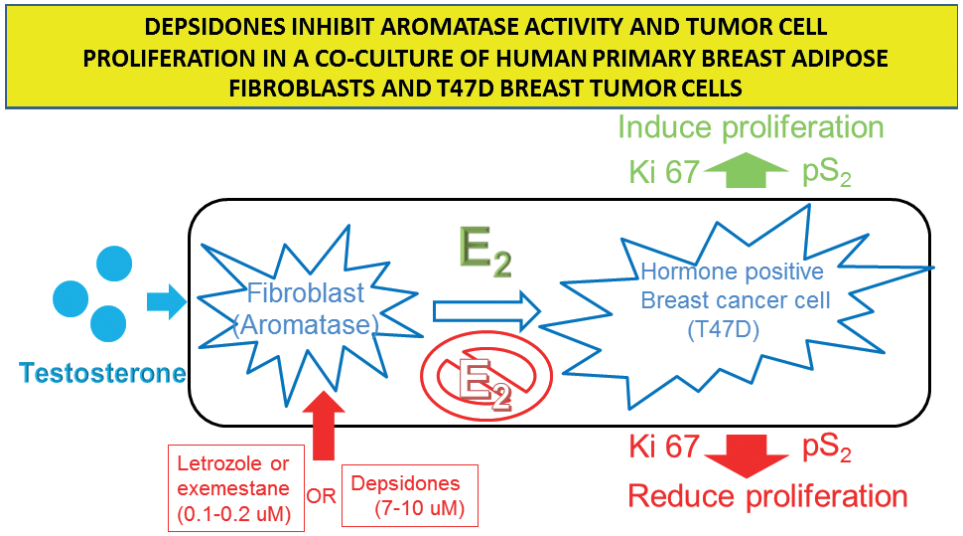
Key words: Depsidones; Aromatase; Breast cancer; Co-culture

Published in Toxicology Reports 2017, Mar 19;4:165-171. doi:10.1016/j.toxrep.2017.03.004.

Abstract

Naturally occurring depsidones from the marine fungus *Aspergillus unguis* are known to have substantial anti-cancer activity, but their mechanism of action remains elusive. The purpose of this study was to examine the anti-aromatase activity of two common depsidones, unguinol and aspergillusidone A, in a co-culture system of human primary breast adipose fibroblasts and hormonal responsive T47D breast tumor cells. Using this *in vitro* model it was shown that these depsidones inhibit the growth of T47D tumor cells most likely via inhibition of aromatase (CYP19) activity. The IC_{50} values of these depisidones were compared with the aromatase inhibitors letrozole and exemestane. Letrozole and exemestane had IC_{50} values of respectively, 0.19 and 0.14 µM, while those for Unguinol and Aspergillusidone A were respectively, 9.7 and 7.3 µM. Our results indicate that among the depsidones there maybe aromatase inhibitors with possible pharmacotherapeutical relevance.

Graphical Abstract



1. Introduction.

Depsidones are secondary metabolites mostly found in lichens, but also in some higher plants, where they play a role in the protection against insects and microbes or sun light. Depsidones are esters which compose of polyphenolic depsides and cyclic ethers, but the chemical structure of the depsidones formed is highly dependent on the type of fungus and its environment (1). Generally, the chemical structure of depsidones resembles prostaglandins and leukotrienes in humans, which makes these compounds may have beneficial health effects in humans. Indeed, several studies have already reported biological activities of these naturally occurring depsidones like anti-proliferative actions (2-4), antimalarial and cytotoxic properties (5), antibacterial activity against the multidrug-resistant *Staphylococcus aureus* (6), radical scavenging, antioxidant activities and antifungal activities (7-9). On the other hand, because of their structural similarity, leukotriene depsidones may induce allergic reactions in humans (10). Also, apoptosis or inflammatory processes that involve free oxygen radicals by depsidones have been reported to cause cytotoxic in rat hepatocytes (11) and rat thymocytes (12). More recently, *in vitro* studies from our laboratory indicate that some depsidones are potent inhibitors of the aromatase enzyme (CYP 19) (7, 9). The aromatase enzyme is responsible for the conversion of androgens into estrogens and is used as a therapeutic target for breast cancer treatment (13, 14). In view of the above biological properties of these depsidones novel cancer treatments may arise. In this respect, the inhibition of aromatase activity may be especially relevant for treatment of estrogen-dependent tumors, such as breast tumors.

Aromatase inhibitors, such as exemestane and letrozole, are currently used as hormonal therapy in estrogen positive postmenopausal breast cancer patients (15). To detect inhibitory properties of aromatase, studies are frequently performed using relatively simple *in vitro* models such as human placental tissue in which radiolabeled androgens are used as substrates (16). Other methods such as HPLC separation with UV detection (17) and recombinant enzyme systems are often less sensitive and laborious (18). Stresser *et al.* developed a high throughput screening method using the fluorometric substrate O-benzyl fuorescein benzyl ester (DBF) as a substrate for microsomal aromatase activity (19). This method was also found suitable for detecting aromatase inhibition in our present co-culture study. In our previous study, we used a more realistic *in vitro* breast cancer model that consists of a co-culture of primary human breast fibroblasts with hormonal positive T47D breast cancer cells to study effects on aromatase activity (20). In this co-culture model, paracrine interactions between various cell types are implemented leading to a more physiologically relevant test model to assess local effects of compounds on aromatase activity and subsequent (anti)tumor effects.

The purpose of this co-culture study was to examine the inhibitory properties of the common depsidones unguinol and aspergillusidone A, which are isolated from the marine-derived fungus *A. unguis* CRI282-03, on aromatase activity in primary human breast fibroblasts and its subsequent effect on the proliferation of T47D breast tumor cells. The results were compared with two clinical relevant aromatase inhibitors, letrozole and exemestane.

2. Materials and methods.

2.1. Depsidones.

Depsidones were isolated from *Aspergillus unguis CRI282-03,* a fungus isolated from the marine environment by the laboratory of natural products from the Chulaborn Research Institute (Bangkok, Thailand) (2, 7). For our present study two depsidones, unguinol (UNG) and aspergillusidone A (ASP-A), were selected. This was based on a preliminary study using the microsomal placental assay, which indicated inhibitory properties for aromatase (7). Structures of UNG and ASP-A are shown in figures 1 A and B. Range finding studies in our laboratory indicated cytotoxicity of UNG and ASP-A in the mM range(2, 7), which is significantly higher than concentrations used in our present co-culture study. For comparison the IC_{50} values of letrozole and exemestane were also determined under similar experimental conditions (See figures 1 C and D). Cytotoxic effects of letrozole and exemestane have been reported earlier to be in the μM range and therefore concentrations in our co-culture experiments did not exceed 0.25 μM.

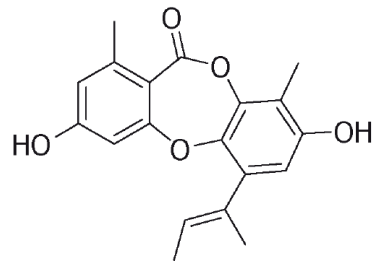
2.2. Breast cancer cell culture and incubation.

The T47D cell line was obtained from ATCC (Rockville, MD, USA). These cells were grown in culture medium comprising of RPMI 1640, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% l-Glutamine, 1% glucose, 1% sodium pyruvate, and 0.08% insulin solution. Seeding concentration was 5×10^4 cells/ml and cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 until 80-90% confluent.

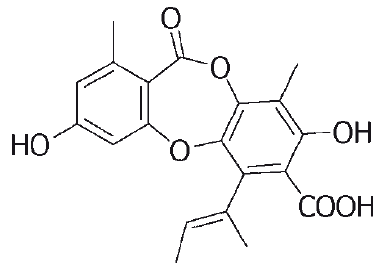
2.3. Primary breast adipose fibroblasts (BAFs).

After written informed consent, BAFs were obtained from three breast cancer patients, who attended the Bamrasnaradura Infectious Disease Institute (Nonthaburi, Thailand) for modified radical mastectomy. The research protocol was approved by the Medical Ethical Committee of the Bamrasnaradura Infectious Disease Institute. About 5 to 10 g of macroscopically normal breast tissue was collected, while the remaining tissue was sent for routine pathological examination. Directly after the tissue was obtained via surgery, it was stored in a 4°C saline solution (0.9% NaCL) and immediately transported to our laboratory. BAFs were isolated from this breast tissue as described earlier (21, 22). These BAFs were cultured as adherent cultures in RPMI 1640-medium w/o phenol red (Gibco/ Invitrogen 11835) supplemented with Pen/Strep (Invitrogen 15140), FCS (Invitrogen

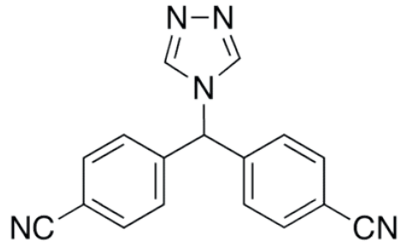
10270) and Insulin 10^{-3} M (144 mg/25 ml) (sigma I-5500) at a seeding concentration of 5×10^4 cells/ml. The cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2. BAFs were sub-cultured if cell cultures in the bottles were confluent, which was usually once a week.



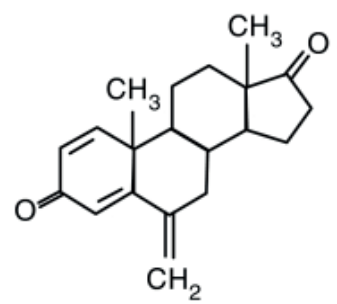
1A. Unguinol



1B. Aspergillusidone A



1C. Letrozole
(Reference compound)



1D. Exemestane
(Reference compound)

Fig. 1. Chemical structures of a) unguinol (UNG), b) asperigillusidone A (ASP-A), c) letrozole and d) exemestane.

2.4. Breast cancer cells in co-culture with BAFs.

Approximately three weeks after isolation, fibroblasts were used to establish a co-culture together with the T47D tumor cells (20, 22, 23). On day one, BAFs were plated at a density of approximately 4×10^3 cells/well in a 96-well plate for proliferation or 5×10^4 cells/ml in a 25 cm^2 flask for gene expression and grown in culture medium as described above. The day after, mono-cultures of breast cancer cells and plated primary BAFs were washed with phosphate buffered saline (PBS) and placed on the assay medium in which heat inactivated FCS was replaced with heat-inactivated, charcoal/dextran-treated FCS (Hyclone, SH30068.03). On day 4, BAFs were washed with PBS and the T47D cells were trypsinized and seeded on top of the BAFs at a density of 4×10^3 cells/well for proliferation or 2.5×10^5 cells/flask for gene expression. On day 5, fresh assay medium was added to the co-cultures after which the cells were exposed to the test compounds for 96 hrs (24). Final solvent concentration was 0.1% v/v in the medium. Initial concentrations were 1, 5, and 10 nM for testosterone, 50 nM for exemestane, 30 nM for letrozole, and 2 µM for depsidones to screen for aromatase inhibition in the presence of testosterone. In this co-culture model testosterone is converted by aromatase in the BAFs to estradiol, which in turn induces cell proliferation in the estrogen positive T47D tumor cells.

2.5. Cell proliferation.

Cell proliferation was determined after treatment with testosterone by performing an MTT assay as described earlier (21). After 96 hours of exposure to the test compounds, 1 ml of MTT (5 mg MTT/ ml in PBS) was added for 4 hours. Next, the medium was aspirated and 100 µl of DMSO was added to dissolve the accumulated formazan crystals. The absorbance was measured at 550 nm using 96-well plates in a Spectramax plus 384 microplate reader (Molecular Devices, California, USA).

2.6. Modified high-throughput screen to identify inhibitors of Aromatase (CYP19). (19)

Aromatase activity was measured by the conversion of the fluorometric substrate O-benzylfluorescein benzyl ester (DBP) as described earlier (19) with the following minor modifications. Assays were conducted in 96-well microtiter plates (Catalog No. 3915, Corning Costar, Cambridge, MA) using a 200 µl volume containing a serially dilution of 1:2 of UNG, ASP-A, exemestane or letrozole. DBF was obtained from Gentest Corporation (Woburn, MA). In this method the metabolite production of DBP by aromatase is linear in time up to 30 min and 2 µM DBP, which was used to determine IC_{50} values of the test compounds. The enzymatic reaction was terminated by adding 40 µl 2 N NaOH. To develop adequate signal to background ratio, the 96-well plates (with lid) were incubated for 2 hrs at 37°C. Fluorescence in each individual well was measured in top-read mode using a FLUOstar Model 403 fluorescence plate reader (BMG Lab Technologies, Inc., Durham, NC). DBF metabolite concentrations were measured using an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

2.7. Analysis of gene expression.

After the cells were treated total RNA was extracted. RNA was reverse-transcribed into cDNA using the Qiagen high capacity cDNA reverse-transcription kit (Qiagen, Texas, USA). The gene expression of *pS2* and *Ki-67* was performed using a Roche-Light-cycler-480 one step RT PCR kit (Roche, Indiana, USA). Relative quantification of gene expression was expressed as the ratio of the intensity of the target gene to the housekeeping gene *beta-actin*. The RT-PCR conditions for each gene are described below.

2.8. RNA isolation and gene expression.

Total RNA was isolated using RNeasy® mini kit (Qiagen, Texas, USA) according to the manufacturer's instruction. Cells were centrifuged and lysed with RLT buffer and β-mercaptoethanol. One volume of 70% ethanol was added, and this was vigorously mixed. The sample was transferred to a RNeasy spin column, centrifuged at 12,000 g for 15 seconds, and then 350 µl washing buffer RW1 was added on the column and again centrifuged at 12,000 g for 15 seconds. The 80 ul of DNase I reaction mixture was directly added on the membrane of the column and incubated for 15 minutes. Thereafter, the column was washed with RW1 buffer and RPE buffer twice. Bound RNA was eluted with 40 ul of RNase-free water and centrifuged at 12,000 g for 1 minute. The purification and concentration of total RNA were measured by the ratio of absorbance at 260/280 and 260/230 NM using Nanodrop (Nanodrop Technologies, Inc., Delaware, USA).

Obtained RNA was stored at -20°C in aliquots of 10 ng/µl. The reaction mixture contained 0.1 µM of primers, 1X Quantitect SYBR Green RT-PCR Master mix, 0.2 µl QuantiTect RT mix and 2 µl template RNA (10 ng/µl) in a total volume of 20 µl. The mixture was reverse transcribed to cDNA at 50°C for 20 minutes. After reverse transcription, the PCR reaction was initiated by heating at 95°C for 15 minutes, then followed by denaturation at 95°C for 10 seconds, annealing at 57°C for 25 seconds, an extension at 68°C for 30 seconds and acquisition at 82°C for 5 seconds for 45 cycles. Primers coding for the estrogen-responsive *pS2* gene were used as described earlier by Lee et al. (25, 26). Primers for *Ki-67* amplification were designed in our laboratory as described previously (23). Expression of *β-ACTIN* (forward: 5'-TCTACAATGAGCTGCG-3' and reverse: 5'-AGGTAGTCAGCTAGGT-3') was used as a reference housekeeping gene. All primers were run through the National Center for Biotechnology Information (NCBI) blast (nucleotide nonredundant database) to confirm specificity. The efficiency was determined for a dilution range of cDNA and primers were only used with an efficiency between 95% and 105%. After each RT-qPCR, a melt curve was run to ensure that primer-dimers and other non-specific products were omitted.

2.9. Data analysis.

Each experiment and each concentration were performed in triplicate. Data were analyzed using Microsoft Excel and IC_{50} values calculated by interpolation. Means and standard deviations were calculated of all nine values (n=9). The statistical significance of differences of the means were calculated using the Student's t-test or one-way ANOVA. Differences were considered statistically significant if $P < 0.05$.

3. Results.

3.1. Exposure of mono and co-cultured T47D cells with BAFs to testosterone.

Firstly, aromatase activity and cell proliferation were determined in a co-culture of BAFs and T47D cells to validate the performance of the co-culture model. BAFs were obtained from one patient, since it has been established that aromatase activity can vary largely between individuals(23). As a result, this variation may influence the sensitivity and functionality of this co-culture system significantly(21, 23). Adding testosterone upto 10 nM increased proliferation of T47D cells in the co-culture up to 30%. In contrast, adding testosterone to a mono-culture of T47D cells caused a maximum reduction in cell growth of 50% at 10 nM ($p<0.01$) (See figure 2). These observations confirm the aromatization of testosterone to estradiol in the co-culture of T47D and BAFs, which is followed by a subsequent increase in estrogen-dependent cell proliferation of the tumor cells. Furthermore, the reduction of cell proliferation in the mono culture of T47D cells by testosterone confirms our earlier observations that androgens can reduce the proliferation of androgen receptor positive breast cancer cell lines like that of the T47D cells (23).

The inhibition of cell proliferation of T47D cells in co-culture with BAFs by the different test compounds at different concentrations of testosterone was also studied. As could be expected the aromatase inhibitors letrozole and exemestane inhibited T47D cell proliferation at concentrations of respectively 30 and 50 nM (See figures 3 A and B).

At concentrations of 2 μM UNG and ASP-A inhibition of T47D cell proliferation in these co-cultures was observed, albeit at approximately two orders of magnitude higher concentrations than those of letrozole and exemestane (see figures 3 C and D). These results indicate that UNG and ASP-A may act as aromatase inhibitors in these T47D and BAF co-cultures. In the subsequent experiments the relative potency of these compounds as aromatase inhibitors were studied in further detail for confirmation.

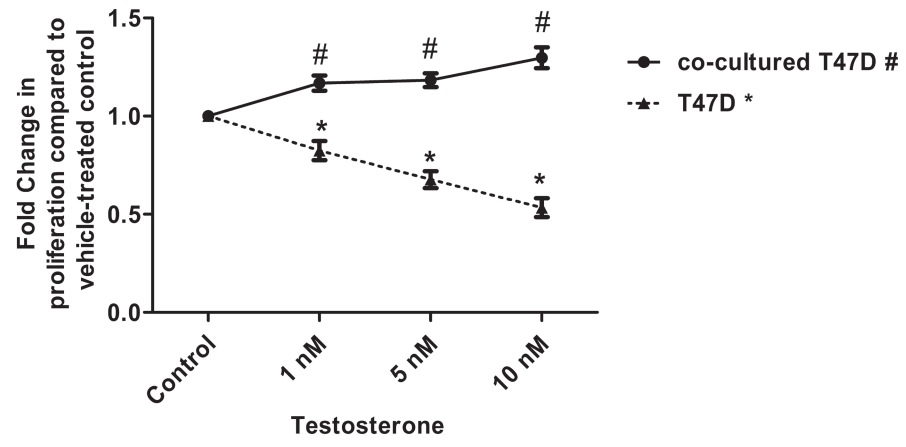


Fig. 2. Effect of testosterone on the proliferation of T47D cells in a mono- or co-culture with human breast adipose fibroblasts (BAFs). Data are expressed as mean proliferation ± SD (N=3). Statistical significance compared with vehicle-treated cells in the same experimental model is indicated as follows: reduced proliferation in T47D mono-culture: * = $p<0.01$; increased proliferation in co-culture T47D and BAFs: # = $p<0.01$.

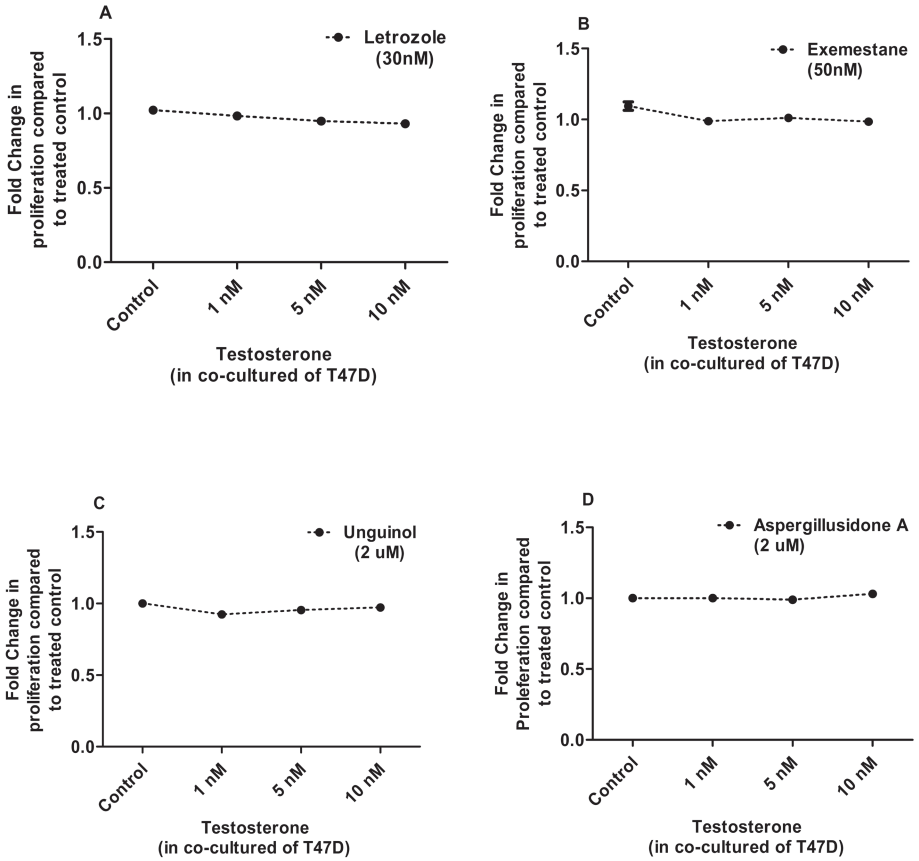


Fig. 3. Inhibition of T47D cell proliferation by letrozole (a), exemestane (b), UNG (c) and ASP-A (d) when co-cultured with human breast adipose fibroblasts at different testosterone concentrations. In the presence of the compounds tested, testosterone-induced proliferation (see Fig. 2) could no longer be observed in the T47D-BAF co-culture. Data are expressed as mean proliferation (N=3).

3.2 Aromatase inhibition in co-cultures of T47D cells and BAFs.

The inhibitory potency of letrozole, exemestane, UNG and ASP-A on aromatase activity was determined at a fixed DBP concentration of 2 µM. A concentration-dependent inhibition of aromatase was observed for all four compounds. IC_{50} values for letrozole, exemestane, UNG and ASP-A were 0.19 µM (95%CI; 0.13-0.28 µM), 0.14 µM (95%CI; 0.11-0.18 µM), 9.73 µM (95%CI; 7.31-12.96 µM) and 7.26 µM (95%CI; 4.99-10.57 µM), respectively. Concentration – response relationships for these compounds are shown in figure 4 A to D.

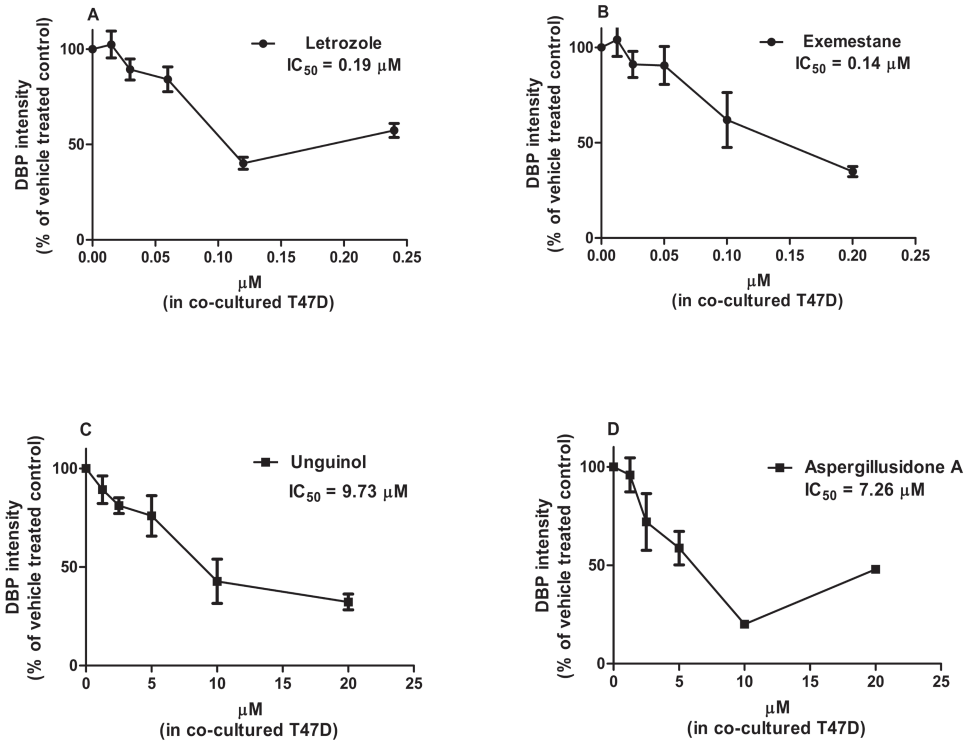


Fig. 4. Inhibition of aromatase activity in co-cultures of T47D cells and human breast adipose fibroblasts by letrozole (a), exemestane (b), UNG (c) and ASP-A (d). Data are expressed as mean DBP intensity as marker for aromatase activity compared to vehicle-treated control cells ± SD (N=3).

3.3 pS2 and Ki67 gene expression in co-cultures of T47D cells and BAFs.

When a co-culture of T47D cells and BAFs was exposed to testosterone only (1, 5, 10 nM), a concentration dependent increase in the expression of *pS2* and *Ki67* mRNA was observed ($p<0.01$) (figure 5 A and B). The induction of both cell proliferation markers was considered to be induced by estradiol production via aromatase in the BAFs. To confirm this, the T47D and BAF co-cultures were exposed to 1, 5 or 10 nM testosterone in combination with the aromatase inhibitor exemestane (50 nM). Adding exemestane completely negated the induction of *pS2* and *Ki67* mRNA expression, which confirmed the functional role of aromatase in this estradiol production. Similarly, UNG and ASP-A (2 µM) fully negated the testosterone-induced expression of *pS2* and *Ki67* mRNA, which again confirms their role as aromatase inhibitors.

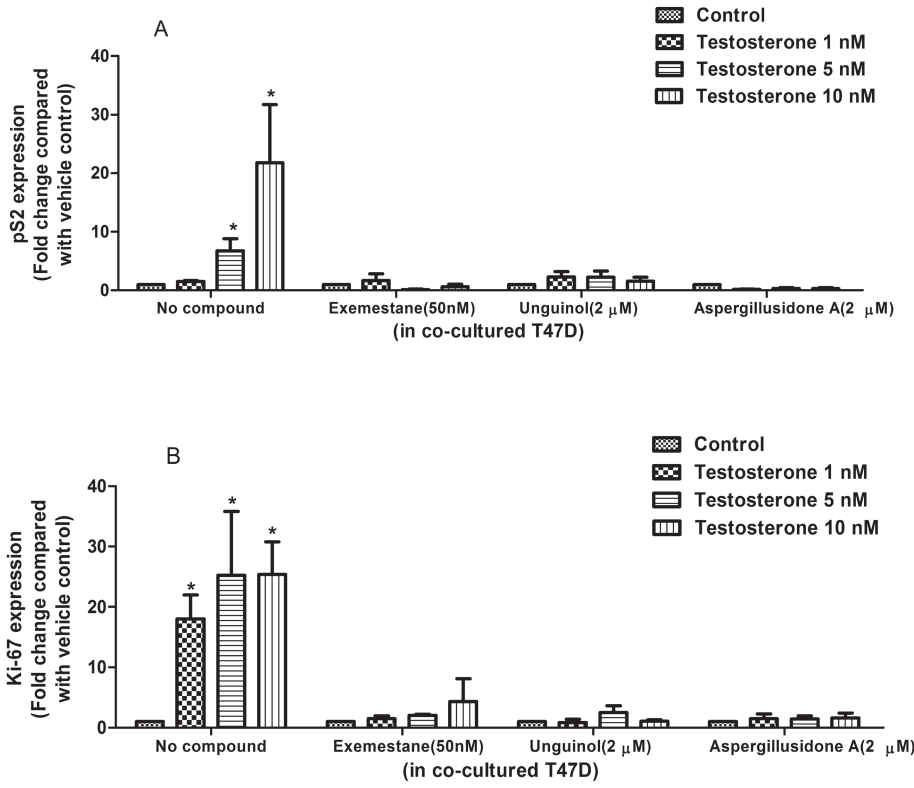


Fig. 5. Effect of testosterone, exemestane, UNG and ASP-A on pS2 (A) and Ki67 (B) mRNA expression in co-cultures of T47D cells and human breast adipose fibroblasts. Data are expressed as means ± SD (n=9). * Statistically significantly different from vehicle-treated control co-cultures ($p < 0.001$).

4. Discussion.

In this study, we clearly show that the naturally occurring despidones unguinol and aspergillusidone A can effectively inhibit aromatase activity and subsequently reduce estrogen-dependent breast cancer cell proliferation in an *in vitro* co-culture model with T47D breast cancer cells and primary BAFs.

4.1. Physiological Relevance of the co-culture system.

This co-culture system consists of T47D breast tumor cells in combination with breast adipose tissue fibroblasts (BAFs). In our earlier studies, we established that this T47D cell line expresses both the estrogen α and androgen receptors and is very suitable for use in a co-culture with BAFs to mimic the in vivo breast cancer situation (23, 27).

The role of steroid hormones in breast tumors is complex due to the various paracrine interactions between different cell types in the breast tissue. We and others have previously shown that (part of) the intercellular communication between the tumor cells and surrounding BAFs remains operational in our co-culture model (21, 28, 29). Therefore, the effects studied in the co-culture model used in present study have more physiological relevance and predictive value compared to effects a monoculture of breast tumor cells *in vitro*. For estrogen dependent breast tumors, the presence of surrounding BAFs is especially relevant, as the local estrogen production is situated in BAFs due to the presence of the aromatase enzyme. In several earlier studies with these breast co-cultures we have shown their relevance for studying natural compounds like androgens, melatonin and reseveratrol (20-23). Most co-culture studies have focused on estrogen dependent tumors. However, a substantial number of breast tumors are not hormonal responsive and for these clinical treatment options are very limited (30-33). At present, little information is available about the paracrine interactions and intercellular communications between non-hormonal responsive (triple negative) breast tumor cells and BAFs. In the quest for finding new compounds with anti-tumor properties for these non-hormonal responsive breast tumors these co-cultures may offer a more advanced *in vitro* testing system.

4.2. Depsidones as aromatase inhibitors.

Our institute has already published several studies on depsidones that were isolated from *A. unguis CRI282-03*, which had anti-aromatase activity (2, 9, 34). These previous studies with *A. unguis*-derived ASP-F, D, E and Unguinol showed that inhibition of aromatase activity by these depsidones was in the same order of magnitude, ranging between 0.5 and 1 μM (7). This suggests that the differences in chemical structure with respect to halogen substitution and hydroxyl placement, have no apparent effect on the aromatase inhibitory potency of the depsidones tested. Although ASP-A was not tested in that study, the IC50 for aromatase inhibition for unguinol was 0.6 μM compared with 9.7 μM in present study. This difference can most likely be attributed to the difference in experimental set-up; the study of Sureram et al. used recombinant human aromatase enzyme, while in this study primary human breast cells were used. In present study, the bioavailability of the tested depsidones might have been affected by metabolism or active excretion from the cells. Moreover, in present study, the aromatase inhibiting potencies of UNG and ASP-A were two orders of magnitude lower than the clinically used therapeutic exemestane. Yet, the aromatase inhibiting properties occurred at non-cytotoxic concentrations, which is relevant to assess with respect to breast cancer therapeutic. Other depsidones; e.g. fungal-derived norstictic acid and plant-derived phomopsidone A and atrovirisidone B have been shown to cause cytotoxic in breast cancer cell lines. Norstictic acid inhibited MDA-MB-231 proliferation by inhibition of proto-oncogenic tyrosine kinase (c-Met) activity (35). Phomopsidone A, which has the structure similar to breast cancer drug named Taxol, also inhibited the proliferation of breast cancer cell line *in vitro* (36). Atrovirisodone B displayed cytotoxicity in MCF-7

cells with an IC50 value of approximately 23 μM (37). Clearly, there are more types of depsidones with bioactive properties that might be relevant for breast cancer research. To determine the clinical relevance of our findings with respect to aromatase inhibition, clearly a more toxicological and pharmacological comparison between these depsidone and standard anti-aromatase is needed. These studies should then also address possible adverse effects of depsidones; e.g. caused by cytotoxicity at the higher dose levels.

5. Conclusion.

Our results demonstrate that in a co-culture system of estrogen-dependent T47D breast tumor cells and primary breast adipose fibroblasts two depsidones, unguinol and aspergillusidone A, can act as aromatase inhibitors leading to inhibition of tumor cell proliferation. The multiple anti-cancer activities of the depsidones warrant further studies for their potential clinical applications.

Acknowledgements.

This work was supported by the Chulabhorn Graduate Institute, Chulabhorn Research Institute (Thailand), and The Institute for Risk Assessment Sciences at the Utrecht University (The Netherlands).

Conflict of interest statement.

All authors declared no financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

References

1. Hauck M, Jürgens S-R, Leuschner C. Norstictic acid: Correlations between its physico-chemical characteristics and ecological preferences of lichens producing this depsidone. Environmental and Experimental Botany. 2010;68(3):309-13.
2. Sureram S, Kesornpun C, Mahidol C, Ruchirawat S, Kittakoop P. Directed biosynthesis through biohalogenation of secondary metabolites of the marine-derived fungus Aspergillus unguis. RSC Advances. 2013;3:1781-8.
3. Lee SU, Asami Y, Lee D, Jang JH, Ahn JS, Oh H. Protuboxepins A and B and protubonines A and B from the marine-derived fungus Aspergillus sp. SF-5044. J Nat Prod. 2011;74(5):1284-7.
4. Jiang T, Li T, Li J, Fu HZ, Pei YH, Lin WH. Cerebroside analogues from marine-derived fungus Aspergillus flavipes. J Asian Nat Prod Res. 2004;6(4):249-57.
5. Khumkomkhet P, Kanokmedhakul S, Kanokmedhakul K, Hahnvajanawong C, Soytong K. Antimalarial and cytotoxic depsidones from the fungus Chaetomium brasiliense. J Nat Prod. 2009;72(8):1487-91.
6. Kokubun T, Shiu WK, Gibbons S. Inhibitory activities of lichen-derived compounds against methicillin- and multidrug-resistant Staphylococcus aureus. Planta Med. 2007;73(2):176-9.
7. Sureram S, Wiyakrutta S, Ngamrojanavanich N, Mahidol C, Ruchirawat S, Kittakoop P. Depsidones, aromatase inhibitors and radical scavenging agents from the marine-derived fungus Aspergillus unguis CRI282-03. Planta Med. 2012;78(6):582-8.
8. Abdou R, Scherlach K, Dahse HM, Sattler I, Hertweck C. Botryorhodines A-D, antifungal and cytotoxic depsidones from Botryosphaeria rhodina, an endophyte of the medicinal plant Bidens pilosa. Phytochemistry. 2009;71(1):110-6.
9. Chomcheon P, Wiyakrutta S, Sriubolmas N, Ngamrojanavanich N, Kengtong S, Mahidol C, et al. Aromatase inhibitory, radical scavenging, and antioxidant activities of depsidones and diaryl ethers from the endophytic fungus Corynespora cassiicola L36. Phytochemistry. 2009;70(3):407-13.
10. Zheng Z, Zhang S, Lu X, Ma Y, Fan Y, Shi Y, et al. Trivaric acid, a potent depside human leukocyte elastase inhibitor. Biological & pharmaceutical bulletin. 2012;35(12):2247-51.
11. Correche ER, Enriz RD, Piovano M, Garbarino J, Gomez-Lechon MJ. Cytotoxic and apoptotic effects on hepatocytes of secondary metabolites obtained from lichens. Altern Lab Anim. 2004;32(6):605-15.
12. Pavlovic V, Stojanovic I, Jadranin M, Vajs V, Djordjevic I, Smelcerovic A, et al. Effect of four lichen acids isolated from Hypogymnia physodes on viability of rat thymocytes. Food Chem Toxicol. 2012;51:160-4.
13. Thompson EA, Jr., Siiteri PK. The involvement of human placental microsomal cytochrome P-450 in aromatization. J Biol Chem. 1974;249(17):5373-8.
14. Toma Y, Higashiyama T, Yarborough C, Osawa Y. Diverse functions of aromatase: O-deethylation of 7-ethoxycoumarin. Endocrinology. 1996;137(9):3791-6.
15. Brodie A, Lu Q, Long B. Aromatase and its inhibitors. J Steroid Biochem Mol Biol. 1999;69(1-6):205-10.
16. Mak P, Cruz FD, Chen S. A yeast screen system for aromatase inhibitors and ligands for androgen receptor: yeast cells transformed with aromatase and androgen receptor. Environ Health Perspect. 1999;107(11):855-60.
17. Fauss J, Pyerin W. Rapid assay of aromatase activity by fast liquid chromatography: spectroscopic evaluation of metabolite profiles indicate unexpected pitfalls. Anal Biochem. 1993;210(2):421-3.
18. Zhou DJ, Pompon D, Chen SA. Stable expression of human aromatase complementary DNA in mammalian cells: a useful system for aromatase inhibitor screening. Cancer Res. 1990;50(21):6949-54.
19. Stresser DM, Turner SD, McNamara J, Stocker P, Miller VP, Crespi CL, et al. A high-throughput screen to identify inhibitors of aromatase (CYP19). Anal Biochem. 2000;284(2):427-30.

20. Chottanapund S, Van Duursen MB, Navasumrit P, Hunsonti P, Timtavorn S, Ruchirawat M, et al. Anti-aromatase effect of resveratrol and melatonin on hormonal positive breast cancer cells co-cultured with breast adipose fibroblasts. Toxicol In Vitro. 2014;28(7):1215-21.
21. Heneweer M, Muusse M, Dingemans M, de Jong PC, van den Berg M, Sanderson JT. Co-culture of primary human mammary fibroblasts and MCF-7 cells as an in vitro breast cancer model. Toxicol Sci. 2005;83(2):257-63.
22. van Meeuwen JA, Korthagen N, de Jong PC, Piersma AH, van den Berg M. (Anti)estrogenic effects of phytochemicals on human primary mammary fibroblasts, MCF-7 cells and their co-culture. Toxicol Appl Pharmacol. 2007;221(3):372-83.
23. Chottanapund S, Van Duursen MB, Navasumrit P, Hunsonti P, Timtavorn S, Ruchirawat M, et al. Effect of androgens on different breast cancer cells co-cultured with or without breast adipose fibroblasts. J Steroid Biochem Mol Biol. 2013;138:54-62.
24. Sonne-Hansen K, Lykkesfeldt AE. Endogenous aromatization of testosterone results in growth stimulation of the human MCF-7 breast cancer cell line. J Steroid Biochem Mol Biol. 2005;93(1):25-34.
25. Lee Y, Jin Y, Lim W, Ji S, Choi S, Jang S, et al. A ginsenoside-Rh1, a component of ginseng saponin, activates estrogen receptor in human breast carcinoma MCF-7 cells. J Steroid Biochem Mol Biol. 2003;84(4):463-8.
26. Lu D, Kiriyama Y, Lee KY, Giguere V. Transcriptional regulation of the estrogen-inducible pS2 breast cancer marker gene by the ERR family of orphan nuclear receptors. Cancer Res. 2001;61(18):6755-61.
27. Aka JA, Lin SX. Comparison of functional proteomic analyses of human breast cancer cell lines T47D and MCF7. PLoS One. 2012;7(2):e31532.
28. Heneweer M. Direct and indirect endocrine disruption: Co-culture of primary human mammary fibroblasts and MCF-7 cells as an in vitro breast cancer model. Utrecht: Utrecht University; 2005. 175 p.
29. Miki Y, Ono K, Hata S, Suzuki T, Kumamoto H, Sasano H. The advantages of co-culture over mono cell culture in simulating in vivo environment. J Steroid Biochem Mol Biol. 2012;131(3-5):68-75.
30. Ogawa Y, Hai E, Matsumoto K, Ikeda K, Tokunaga S, Nagahara H, et al. Androgen receptor expression in breast cancer: relationship with clinicopathological factors and biomarkers. Int J Clin Oncol. 2008;13(5):431-5.
31. Bosch A, Eroles P, Zaragoza R, Vina JR, Lluch A. Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research. Cancer Treat Rev. 2010;36(3):206-15.
32. Akiyama F, Iwase H. Triple negative breast cancer: clinicopathological characteristics and treatment strategies. Breast Cancer. 2009;16(4):252-3.
33. Rodler E, Korde L, Gralow J. Current treatment options in triple negative breast cancer. Breast Dis.32(1-2):99-122.
34. Sultana N, Afolayan AJ. A new depsidone and antibacterial activities of compounds from Usnea undulata Stirton. J Asian Nat Prod Res. 2011;13(12):1158-64.
35. Ebrahim HY, Elsayed HE, Mohyeldin MM, Akl MR, Bhattacharjee J, Egbert S, et al. Norstictic Acid Inhibits Breast Cancer Cell Proliferation, Migration, Invasion, and In Vivo Invasive Growth Through Targeting C-Met. Phytotherapy research : PTR. 2016;30(4):557-66.
36. Zhang W, Xu L, Yang L, Huang Y, Li S, Shen Y. Phomopsidone A, a novel depsidone metabolite from the mangrove endophytic fungus Phomopsis sp. A123. Fitoterapia. 2014;96:146-51.
37. Permanaa D, Abas F, Maulidiani, Shaari K, Stanslas J, Ali AM, et al. Atrovirisidone B, a new prenylated depsidone with cytotoxic property from the roots of Garcinia atroviridis. Z Naturforsch C. 2005;60(7-8):523-6.

Chapter 6

Evaluation of anti-tumor properties of two depsidones - Unguinol and Aspergillusidone D in triple hormone negative MDA-MB-231 breast tumor cells

Anne Zwartsen[1,2], Suthat Chottanapund[2,3,4,5], Prasat Kittakoop[2,3,4], Panida Navasumrit[2,3,4], Mathuros Ruchirawat[2,3,4], Majorie B. M. Van Duursen[1], Martin Van den Berg[1*]

[1] Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
[2] Chulabhorn Research Institute, Kamphaeng Phet 6 Road, Laksi, Bangkok 10210, Thailand
[3] Chulabhorn Graduate Institute, Kamphaeng Phet 6 Road, Laksi, Bangkok 10210, Thailand
[4] Center of Excellence on Environmental Health and Toxicology (EHT), CHE, Ministry of Education, Bangkok, Thailand
[5] Bamrasnaradura Infectious Diseases Institute, Ministry of Public Health, Thailand

*** Corresponding author:** M. Van den Berg

Key words: cell cycle arrest; apoptosis; hormone negative breast cancer; depsidones; in vitro.

Submitted to Toxicology in vitro

Abstract

There is an ongoing search for new compounds to lower the mortality and recurrence of breast cancer. Naturally occurring depsidones, extracted from the fungus *Aspergillus*, are known for their wide range of biological activities such as cytotoxicity, aromatase inhibition, radical scavenging and antioxidant properties. This study investigated the possibility of two depsidones (Unguinol and Aspergillusidone D) to induce apoptosis and cell cycle arrest and reduce cell proliferation and cell viability in the triple-negative MDA-MB-231 breast cancer cell line. Results were compared with the effects of doxorubicin, 17β-estradiol, testosterone and dihydrotestosterone. At sub μM levels, Unguinol and Aspergillusidone D did not influence cell proliferation. In addition, cell viability reduction was not observed below 25 μM. Unguinol initiated both cell cycle arrest and apoptosis at 100 μM, however the latter was not proven to be significant. Aspergillusidone D showed comparable results on apoptosis at 60 μM, however, Aspergillusidone D did not induce cell cycle arrest at this concentration.

Even though these results are found on relatively high doses, it shows the possibility of depsidones as an anti-triple-negative breast cancer treatment. Further research should include structural analogues, which might exhibit similar effects, yet at lower concentrations.

1. Introduction.

Even though breast cancer has a high priority in cancer research, it remains one of the leading causes of cancer deaths in woman worldwide (1). 10 to 20% of all breast cancers are diagnosed as being hormone (triple) negative, which means that they do not express the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 receptor (HER2). These three receptors are main targets for adjuvant hormonal treatment, such as estrogen receptor antagonists and aromatase inhibitors, in breast cancer therapy. Triple-negative breast tumors obviously do not respond to hormonal therapies, but are usually treated with cytotoxic drugs like paclitaxel, doxorubicin or cyclophosphamide. Due to these limited treatment options as well as the aggressive nature of triple-negative breast tumors, the 5-year survival for triple receptor-negative breast cancer patients is with 65% significantly lower compared to hormone positive breast cancers with 86% 5-year survival (2). For hormone negative breast tumors, the general consensus is that these tumors need to be treated via a multi-target approach to reduce mortality and lethality, remove residual cancer cells and prevent recurrence (3).

During the last decades, the anti-tumor properties of naturally occurring compounds has been of increasing interest. Among these are natural compounds that have been isolated from *Aspergillus unguis CRI282-03*, a fungus found in marine organisms and their environment. From this fungus, several classes of compounds, like protubonines, cerebroside analogues and depsidones have been extracted.

Depsidones are organic compounds comprising of depside-like esters and cyclic ethers. These naturally occurring depsidones are known to have a variety of biological activities that may be therapeutically relevant (4) including anti-tumor activity in triple hormone negative cell line MDA-MB-231 (5) and human epidermoid KB cell line (6). In addition, antimalarial activity against *Plasmodium falciparum* and cytotoxicity of human breast cancer (BC1) cell line (7), antibacterial activity against the multidrug-resistant *Staphylococcus aureus* (8), cytotoxicity of MDA-MB-231, inhibition of aromatase, radical scavenging and antioxidant activities (the latter three tested without cell systems) (9), antiviral (HIV-1-IN) and antifungal (*Aspergillus terreus* and *Fusarium osysporum*) activity (10, 11) have been reported. Apoptotic properties of depsidones have also been reported in rat thymocytes (12). Recently, Chottanapund, et al. identified two depsidones, Unguinol and Aspergillusidone A, that reduce cell growth and inhibit aromatase activity in the hormone positive breast cancer cell line T47D that was co-cultured with primary human breast fibroblasts (13). To determine the clinical relevance of these findings with respect to aromatase inhibition, clearly a more toxicological examination of these depsidones is needed. Moreover, in view of these biological activities of these depsidones, they may present potential as novel cancer treatments for triple-negative breast cancer patients.

Therefore, the purpose of this study was to investigate whether two specific depsidones; Unguinol and Aspergillusidone D (Figure. 1.) affected cell proliferation, cell viability, cell cycle arrest and apoptosis in the triple-negative breast cancer cell line MDA-MB-231. These compounds were compared to 17β-estradiol (E_2), testosterone (T) and dihydrotestosterone (DHT) and doxorubicin, which is a common treatment option for triple-negative breast cancer.

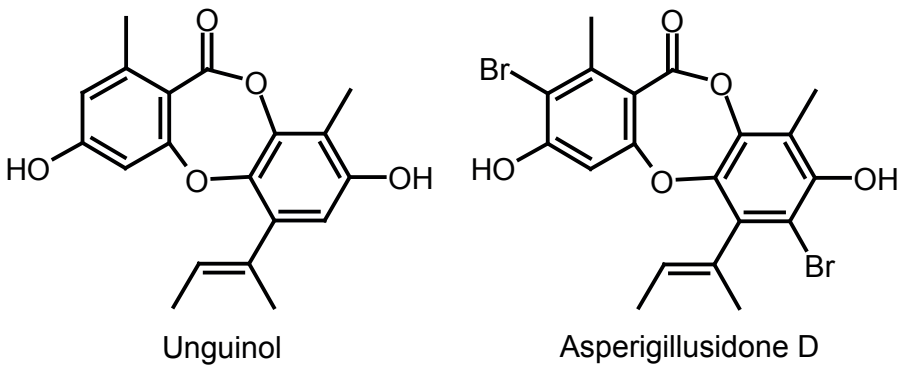


Figure. 1. Chemical structures of Uguinol and Asperigillusidone D.

2. Materials and methods.

2.1 Chemicals.

The depsidones Unguinol and Aspergillusidone D were provided by the Chulabhorn Research Institute and have been biosynthesized according to Sureram et al. (14). The other compounds tested - 17β-estradiol (E_2; purity >98%), testosterone C-III (T; purity >98%), 5α-androstan-17β-ol-one C-III or dihydrotestosterone (DHT; purity 97.5%), colchicine (purity ±95%) and doxorubicin (purity >98%) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). These compounds were dissolved and diluted in dimethyl sulphoxide (DMSO) Hybri-max from Sigma-Aldrich (St. Louis, MO, USA) and added to the exposure medium at <0.5% (v/v).

2.2 Breast cancer cell culture and incubation.

The human triple-negative breast cancer cell line MDA-MB-231 was obtained from ATCC (Rockville, MD, USA). Cells were grown under sterile conditions in culture medium comprising of Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine at seeding concentration of 5×10^4 cells/mL. All chemicals were obtained from Gibco, Thermo Fisher Scientific, Waltham, MA, USA. The cells were kept at 37°C in a humidified atmosphere with 5% CO_2, unless otherwise stated, in 25 or 75 cm^2 culture

flasks (Thermo Fisher Scientific, Waltham, MA, USA). The cells were subcultured when they reached 80% confluence, which was typically after 2-3 days.

At the start of the experiments, cells were plated at a density needed corresponding to the different experimental endpoints. In doing this, the medium was changed into plating medium: DMEM- low glucose medium (Sigma-Aldrich, St. Louis, MO, USA) without phenol red containing10% FBS, 1% penicillin/streptomycin and 1% L-glutamine. Cells were kept overnight to adhere to the plate. After 24 hours, plating medium was removed, and assay medium was added. Assay medium consisted of DMEM- low glucose, 10% dextran/charcoal-stripped serum, 1% penicillin/streptomycin and 1% L-glutamine, spiked with the test compounds. The cells were exposed to the test compounds for 1, 3 or 5 days, depending on type of experiment.

2.3. Cell proliferation.

To test for cell proliferation, 100 µL of MDA-MB-231 cells were plated in a 96 well plate at a concentration of 20,000 cells/mL. After 24 hours, cells were exposed to 200 µL 5-20 pM E_2, 5-20 µM T or DHT, or 0.1% DMSO (negative control). Other cells were exposed to 0.01-5 µM Unguinol or Aspergillusidone D. After 5 days of exposure, 20 µL of MTT (5 mg MTT/ mL in PBS) was added to each well for 4 hours (13, 15). Next, the medium was aspirated and 100 µL DMSO was added to dissolve the accumulated formazan crystals. The absorbance was measured at 560/670 nm using the 96-wells microplate reader (Spectramax plus 384, Molecular Devices, California, USA).

2.4 Cell viability testing.

To determine the effects of E_2, T, DHT, Unguinol, Aspergillusidone D and doxorubicin (positive control) on cell viability, a MTT assay was performed (14). 100 µL of MDA-MB-231 cells were plated in a 96 well plate at a concentration of 60,000 cells/mL. After 24 hours, the cells were exposed for 3 days to 200 µL assay medium containing 0-100 µM E_2, 0-500 µM T of DHT, 0-10 µM doxorubicin (positive control) or 0.1% DMSO (negative control). Unguinol and Aspergillusidone D were tested at 1-200 µM for 3 days, before a MTT assay was performed as stated above.

2.5. Cell cycle arrest.

Cell cycle arrest was studied in MDA-MB-231 cells that were plated in 6-well culture plates with 2.5 mL of 400,000 cells/mL per well. After 24 hours, the cells were exposed to assay medium containing 50 µM E_2, 50 µM T, 50 µM DHT, 3 µM colchicine (positive control) or 0.05% (v/v) DMSO (negative control). Unguinol and Aspergillusidone D were tested at concentrations of 100 and 60 µM respectively. These concentrations were chosen based on cell viability assays done in this study.

After 24 hours of exposure, assay medium and the cells were harvested and centrifuged (5 min, 2000 rpm, 4°C), after which the supernatant was removed. The cells were re-

suspended in 1000 µL PBS and transferred to an Eppendorf tube before centrifugation (5 min, 5000 rpm, 4°C). Again, the supernatant was removed and 300 µL of PBS was used to re-suspend the cells before adding 700 µL of 70% ice-cold ethanol. After mixing, this suspension was incubated for 40 min at 4°C to fix the cells. This was again followed by centrifugation (5 min, 5000 rpm, 4°C), removing supernatant, re-suspending in 1000 µL PBS and centrifugation (5 min, 5000 rpm, 4°C). After centrifugation, the supernatant was removed and 500 µL propidium iodine solution (PIS; PBS with a final concentration of 0.05 mg/mL propidium iodine + final concentration $5.0x10^{-4}$ mg/mL RNAse stock solution (DNAse free)) was added to each sample. These samples were incubated for 15 min at room temperature and transferred to FACs tubes before the cell cycle phase distributions were measured on the DB FACSCanto ™ flow cytometer (BD Biosciences, San Diego, CA, USA). 30,000 cells were analysed per sample at a middle flow rate, as described previously (16). Duplicates and triplicates were tested at 3 or 4 independent occasions. Cells were sorted in the cell cycle phases gated as G0/G1, S and G2/M. Data was analysed using ModFit LT ™ software.

2.6. Apoptosis study.

To determine the percentage of apoptotic cell populations induced after exposure to the test compounds, the cells were treated following the above described method for the cell cycle arrest assay until cells were harvested. Next, apoptosis was determined using the Muse™ Annexin V & Dead Cell Assay kit (Merck MCH100105) according to the manufacturer's description. Briefly, after 24 hours of exposure, the cells were harvested and centrifuged (5 min, 2000 rpm, 4°C) after which the supernatant was removed. The cells were re-suspended in 1000 µL DMEM with 10% FBS. 100 µL of this suspension was moved to an Eppendorf tube containing 100 µL of Muse™ Annexin V & Dead Cell Reagent (7-aminoactinomycin-D (7-AAD) and Annexin V dyes) following an incubation for 20 min at room temperature in the dark. After this incubation period, the amount of phosphatidylserine (PS) molecules translocated to the outer membrane and the membrane structural integrity were measured on a Muse® Cell Analyser (Merck Millipore, Millipore Sigma, Merck, Darmstadt, Germany).

2.7 Data analysis.

Data on cell viability and cell proliferation were background corrected before exposed wells were normalized per plate. Averages per concentration per plate were combined with other plates. Values were excluded when > mean ± 2x S.D. Out of all substances, only 1 out of 6 values for 40 uM estradiol on cell viability was excluded. No values/ plates were excluded for apoptosis and cell proliferation experiments. 1 out of 10 values were considered outliers for DMSO, testosterone and colchicine in cell cycle arrest. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, CA, USA). Concentration-response curves were calculated using the GraphPad Prism nonlinear regression sigmoidal dose-response curve fitting module. To test statistical significance one-way and two-way ANOVA's followed by Bonferroni's multiple comparisons test were used with $p<0.05$. All data in figures expressed as mean ±SEM for n wells of N plates. Biological variation is shown as 1xSD. Cell viability curves were fitted using non-linear regressions, and a bottom=0 constraint for all compounds who did not reach 0% viability, using GraphPad Prism 7.0. IC_{50} values are shown with 95% confidence intervals [CI].

3. Results and discussion.

3.1 Proliferation.

3.1.1 Steroid hormones.

To determine whether the cell proliferation of the MDA-MB-231 cell line was influenced by the steroid hormones E_2, T and DHT, cells were exposed for 5 days to low concentrations of steroids; 5-20 pM (E_2) or 5-20 nM (T and DHT). E_2 did not cause any effect on the cell proliferation of these cells, as could be expected as MDA-MB-231 cells have been reported to lack expression of the ERα protein (17) (Figures. 2A-C). The ERβ acts independently and opposite from ERα and its expression in MDA-MB-231 cells is considered not to be sufficient to mediate estrogenic effects (18). The androgen T induced a slight, but statistically significant decrease in cell proliferation at 5 nM, however, this was not seen at 10 nM or 20 nM. No effects of DHT were seen on cell proliferation. An earlier study by Chottanapund et al. (15) showed a minor reduction in cell population for T and a more pronounced decrease for DHT. One possible reason for this observed discrepancy between both studies could be the normal variability between batches of MDA-MB-231 cells (19).

3.1.2 Depsidones.

Unguinol and Aspergillusidone D caused a slight increase in cell proliferation. However, this is only proven to differ significantly from control when cells were exposed to 0.1 µM Aspergillusidone D for 5 days (Figures. 2D-E). As this was not seen at higher concentrations, this effect might not be meaningful. Based on these observations it can be argued that low µM concentration can be used a clinical setting, as tumor growth is not induced. Norstictic acid, a different depsidone, was shown to inhibit proliferation of 6 different breast cancer cell lines, including two triple negative breast cancer cell lines (MDA-MB-231 and MDA-MB-468), starting at concentrations of 10 µM (20).

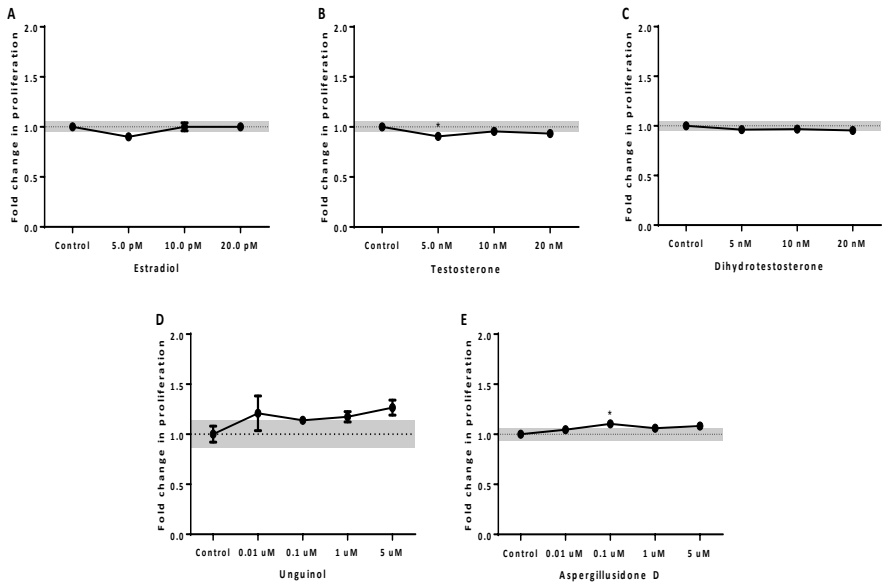


Figure. 2. Cell proliferation of MDA-MB-231 triple-negative breast tumor cells exposed for five days to low concentrations of 17β-estradiol (A), testosterone (B), dihydrotestosterone (C) and the depsidones Unguinol (D) and Aspergillusidone D (E)(data are represented as means ± SEM; n=12-40, N=3-4). Biological variation is shown by the grey blocks (±1xSD) (* statistically significant when $p<0.05$).

3.2 Cell viability.

Steroid hormones, Figures. 3A an 3B show a decrease in cell viability of MDA-MB-231 cells after 3 days of exposure to E_2, T, and DHT (IC_{50} values of 42 [37-47], 203 [145-286] and 92 [79-106] µM, respectively). These results are in agreement with those of Kvasnica et al. (21), who showed a decrease in cell viability by E_2 in a similar concentration range in two ER- breast cancer cell lines. Reduced cell viability can be due E_2 induced inhibition of microtubule assembly by modifying the microtubule polymerization process (22). Chottanapund et al. showed that MDA-MB-231 cells have limited aromatase activity (15). Theoretically, it may be argued that T could, to some extent, be conversed to E_2 by the aromatase activity in these cells. T was therefor expected to reduce cell viability at lower concentrations compared to DHT, however this was not observed. This observation is in line with a study from Wang et al. (23). In addition, Kipp and Ramirez (2003) showed that T inhibited the depolymerisation process of microtubule, even when depolymerisation was stimulated by colchicine, possibly explaining our findings.

Concurrently, we showed that MDA-MB-231 cells are highly sensitive to doxorubicin, 3 days after exposure (IC_{50}: 0.02 [0.01-0.05] µM). Other studies with MDA-MB-231 cells show that the time of incubation influences the toxicity of doxorubicin, as only 1 day exposure results in a IC_{50} value of 1.3 µM and 2 days exposure results in a IC_{50} value of 0.6 µM (24, 25).

Depsidones, Figure. 3C shows, the decrease in cell viability of MDA-MB-231 cells after exposure to Unguinol and Aspergillusidone D. The IC_{50} values for Unguinol and Aspergillusidone D were in the same range as the tested androgens (81 [64-104] µM and 50 [39-63] µM, respectively). Even though both depsidones have a similar molecular core structure (see Figure 1), the observed difference between IC_{50} values might be explained by the presence of two bromine atoms in the Aspergillusidone D molecule.

3.3 Cell cycle arrest.

3.3.1 Hormones.

Cell cycle arrest in the MDA-MB-231 was only seen when exposed to colchicine (3 µM) and E_2 (50 µM) (Figure. 4A). Both compounds showed a statistically significant reduction in number of cells in the G0/G1-phase and an increase in number of cells in the G2/M-phase. Because MDA-MB-231 cells do not have ERα expression, the observed cell cycle arrest caused by E_2 suggests an ERα- independent pathway. As mentioned before, E_2 causes microtubule depolymerisation (22), which is also the mechanism of action for colchicine cell cycle arrest (26). No cell cycle arrest was observed after exposure with T and DHT (50 µM). Results for E_2, T and DHT were in accordance with findings on hormone negative cell line MDA-MB-230 (27).

3.3.2. Depsidones.

The possible occurrence of cell cycle arrest in hormone negative breast cancer cells after exposure to Unguinol (100 µM) and Aspergillusidone D (60 µM) was tested against DMSO as a control. The number of cells in different cell cycle phases was not significantly influenced by Aspergillusidone D (Figure. 4B). Unguinol, however, did significantly increase the number of cells in the G2/M-phase ($p<0.05$) and decreased the number of cells in the S-phase significantly after 24 hours of exposure ($p<0.0001$). It could be argued that the observed cell cycle arrest will lead to cell death (see cell viability IC_{50}: 81 µM), which could make Unguinol of potential clinical relevance. However, it should be noted that the exposure concentration of this depsidone for the MDA-MB-231 cells was in the high µM, which might be relatively high for systemic levels in the clinical setting.

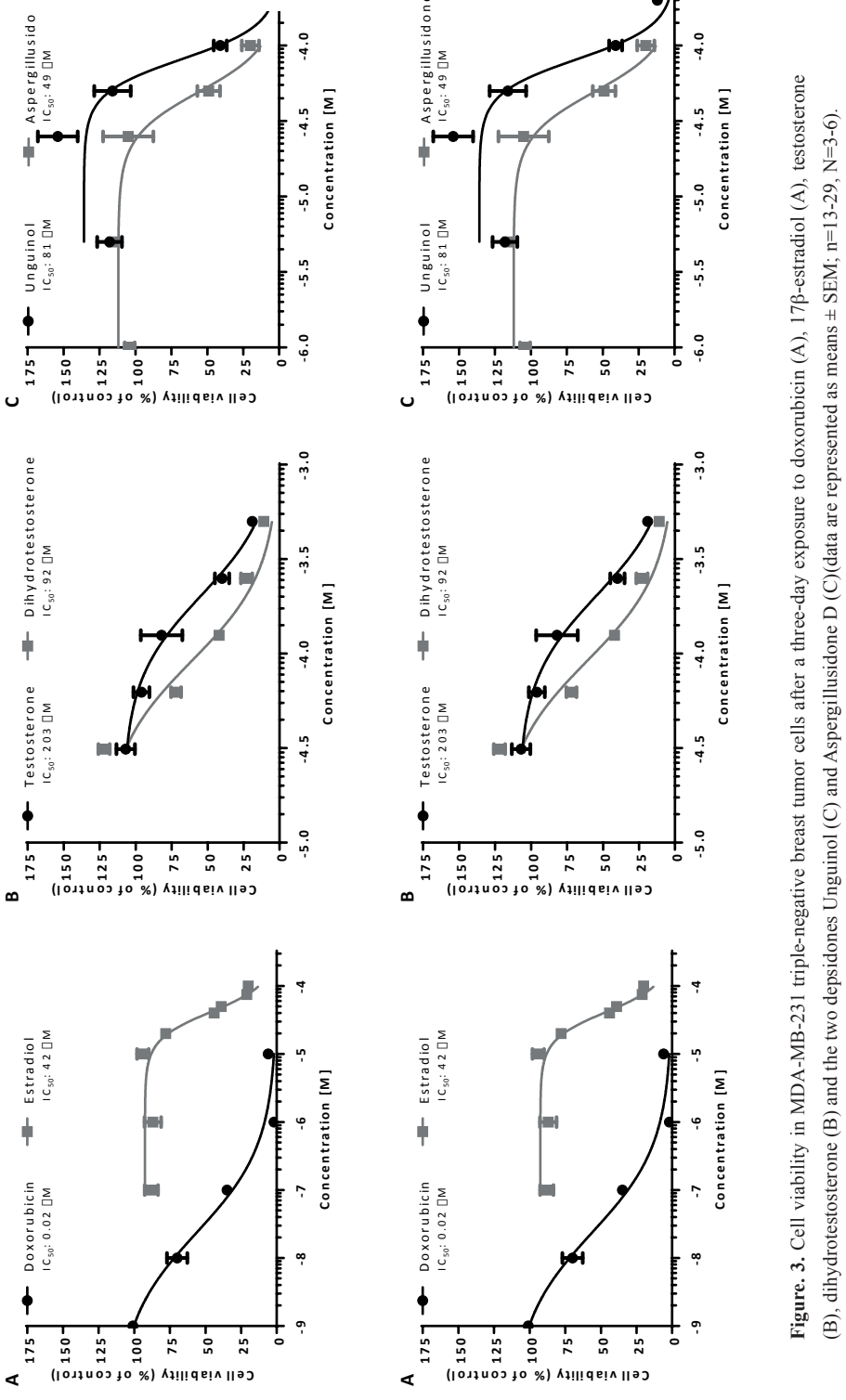


Figure. 3. Cell viability in MDA-MB-231 triple-negative breast tumor cells after a three-day exposure to doxorubicin (A), 17β-estradiol (A), testosterone (B), dihydrotestosterone (B) and the two depsidones Unguinol (C) and Aspergillusidone D (C)(data are represented as means ± SEM; n=13-29, N=3-6).

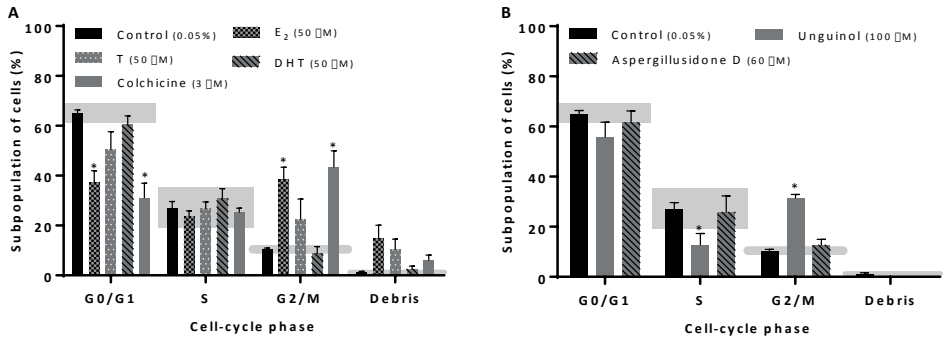


Figure. 4. Cell cycle arrest in MDA-MB-231 triple-negative breast tumor cells induced by (A) DMSO (control), colchicine, 17β-estradiol (E_2), testosterone (T), dihydrotestosterone (DHT) and (B) the two depsidones Unguinol and Aspergillusidone D (data are represented as means ± SEM; n=9-19, N=3-10) Biological variation is shown by the grey blocks (±1xSD) (* statistically significant when $p<0.05$).

3.4 Apoptosis.

3.4.1. Hormones.

To determine if the steroid hormones can induce apoptosis in MDA-MB-231 cells, cells were exposed for 24 hours to 50 μM of E_2, T or DHT. Next, apoptosis was determined using the Muse™ Annexin V & Dead Cell Assay kit. At these concentrations none of these steroid hormones statistically significantly induced apoptosis (Figure 5A), which corresponds with literature (28). As a positive control doxorubicin was used, which caused a significant apoptotic effect at 0.4 μM ($p<0.05$).

Further, a small but not significant increase in apoptotic cells after E_2 exposure was seen. This is in agreement with our cell cycle data showing a small portion of debris after similar exposure.

3.4.2. Depsidones.

Unguinol and Aspergillusidone D both induced apoptosis at concentrations similar to the IC_{50} values determined for cell viability (100 and 60 μM, respectively) (Figure 5B). However, apoptosis induced by Aspergillusidone D was not found to be statistically significant due to high (biological) variation in responses. Both compounds show the same potential to induce apoptosis, however this even at a rather high concentration not significant.

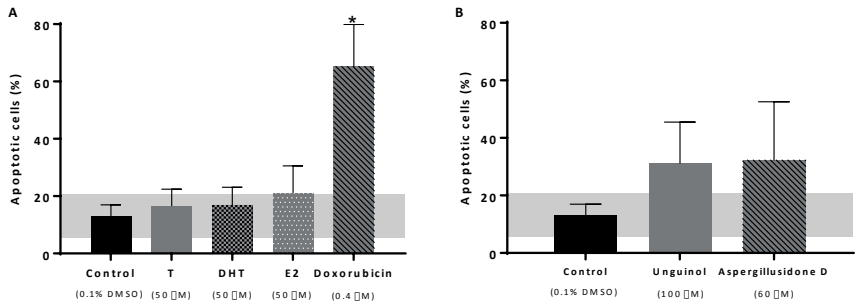


Figure. 5. Apoptosis in MDA-MB-231 triple-negative breast tumor cells induced by (A) doxorubicin, 17β-estradiol, testosterone, dihydrotestosterone and (B) the two depsidones Unguinol and Aspergillusidone D (data are represented as mean ± SEM; n=6-11, N=2-4). Biological variation is shown by the grey blocks (±1xSD) (* significant when $p<0.05$).

4. Conclusion.

The depsidones, Unguinol and Aspergillusidone D, were investigated for potential chemotherapeutic or preventive properties in the triple-negative MDA-MB-231 breast tumor cells. Both compounds did not show the ability to influence cell proliferation, but minor reduction in cell viability was seen at concentrations over 25 µM. Both compound increased the number of apoptotic cells, and Unguinol in addition induced cell cycle arrest. However, the concentration at which apoptosis and cell cycle arrest were induced, is high for systemic treatment. That said, these results show the ability of depsidones to influence several targets capable of reducing hormone (triple) negative breast cancer cells. This knowledge can be used in the search towards new cancer treatments, especially when looking at structurally similar depsidones.

Acknowledgements.

This work was supported by the Chulabhorn Graduate Institute, Chulabhorn Research Institute (Thailand), and The Institute for Risk Assessment Sciences at the Utrecht University (The Netherlands).

Conflict of interest statement.

All authors declared no financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

References.

1. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012.* Int J Cancer, 2015. 136(5): p. E359-86.
2. Aghili, M., et al., *Triple-negative breast cancer survival in Iranian patients.* Acta Med Iran, 2013. 51(8): p. 560-6.
3. Sathish Kumar, B., et al., *Synthesis of 2-alkoxy and 2-benzyloxy analogues of estradiol as anti-breast cancer agents through microtubule stabilization.* European Journal of Medicinal Chemistry, 2014. 86: p. 740-751.
4. Sureram, S., et al., *Depsidones, aromatase inhibitors and radical scavenging agents from the marine-derived fungus Aspergillus unguis CRI282-03.* Planta Med, 2012. 78(6): p. 582-8.
5. Lee, S.U., et al., *Protuboxepins A and B and protubonines A and B from the marine-derived fungus Aspergillus sp. SF-5044.* J Nat Prod, 2011. 74(5): p. 1284-7.
6. Jiang, T., et al., *Cerebroside analogues from marine-derived fungus Aspergillus flavipes.* J Asian Nat Prod Res, 2004. 6(4): p. 249-57.
7. Khumkomkhet, P., et al., *Antimalarial and cytotoxic depsidones from the fungus Chaetomium brasiliense.* J Nat Prod, 2009. 72(8): p. 1487-91.
8. Kokubun, T., W.K. Shiu, and S. Gibbons, *Inhibitory activities of lichen-derived compounds against methicillin- and multidrug-resistant Staphylococcus aureus.* Planta Med, 2007. 73(2): p. 176-9.
9. Chomcheon, P., et al., *Aromatase inhibitory, radical scavenging, and antioxidant activities of depsidones and diaryl ethers from the endophytic fungus Corynespora cassiicola L36.* Phytochemistry, 2009. 70(3): p. 407-413.
10. Vlietinck, A.J., et al., *Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection.* Planta Med, 1998. 64(2): p. 97-109.
11. Abdou, R., et al., *Botryorhodines A-D, antifungal and cytotoxic depsidones from Botryosphaeria rhodina, an endophyte of the medicinal plant Bidens pilosa.* Phytochemistry, 2010. 71(1): p. 110-6.
12. Pavlovic, V., et al., *Effect of four lichen acids isolated from Hypogymnia physodes on viability of rat thymocytes.* Food Chem Toxicol, 2013. 51: p. 160-4.
13. Chottanapund, S., et al., *Depsidones inhibit aromatase activity and tumor cell proliferation in a co-culture of human primary breast adipose fibroblasts and T47D breast tumor cells.* Toxicology Reports, 2017. 4: p. 165-171.
14. Sanya Sureram, et al., *Directed biosynthesis through biohalogenation of secondary metabolites of the marine-derived fungus Aspergillus unguis.* Royal Society of Chemistry, 2013(6): p. 8.
15. Chottanapund, S., et al., *Effect of androgens on different breast cancer cells co-cultured with or without breast adipose fibroblasts.* J Steroid Biochem Mol Biol, 2013. 138: p. 54-62.
16. Sangjun, S., et al., *Induction of cell cycle arrest in human MCF-7 breast cancer cells by cis-stilbene derivatives related to VIOXX.* Toxicol Lett, 2009. 186(2): p. 115-22.
17. Subik, K., et al., *The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines.* Breast Cancer (Auckl), 2010. 4: p. 35-41.
18. Treeck, O., et al., *Novel estrogen receptor beta transcript variants identified in human breast cancer cells affect cell growth and apoptosis of COS-1 cells.* Molecular and Cellular Endocrinology, 2007. 264(1–2): p. 50-60.
19. Nguyen, A., et al., *Highly variable cancer subpopulations that exhibit enhanced transcriptome variability and metastatic fitness.* Nat Commun, 2016. 7: p. 11246.
20. Ebrahim, H.Y., et al., *Norstictic Acid Inhibits Breast Cancer Cell Proliferation, Migration, Invasion, and In Vivo Invasive Growth Through Targeting C-Met.* Phytother Res, 2016. 30(4): p. 557-66.
21. Kvasnica, M., et al., *Synthesis and cytotoxic activities of estrone and estradiol cis-dichloroplatinum(II) complexes.* Bioorg Med Chem, 2012. 20(24): p. 6969-78.

22. Kipp, J.L., et al., *Estradiol and testosterone have opposite effects on microtubule polymerization*. Neuroendocrinology, 2003. 77(4): p. 258-272.
23. Wang, Y., et al., *The plant polyphenol butein inhibits testosterone-induced proliferation in breast cancer cells expressing aromatase.* Life Sci, 2005. 77(1): p. 39-51.
24. Abu, N., et al., *3-Bromo-1-hydroxy-9,10-anthraquinone (BHAQ) inhibits growth and migration of the human breast cancer cell lines MCF-7 and MDA-MB231.* Molecules, 2013. 18(9): p. 10367-77.
25. Costantini, D.L., et al., *Methotrexate, paclitaxel, and doxorubicin radiosensitize HER2-amplified human breast cancer cells to the Auger electron-emitting radiotherapeutic agent (111)In-NLS-trastuzumab.* J Nucl Med, 2010. 51(3): p. 477-83.
26. Blajeski, A.L., et al., *G1 and G2 cell-cycle arrest following mecrotubule depolymerization in human breast cancer cells*. J. Clin. Invest., 2002. 110: p. 91-99.
27. Reddel, R.R., et al., *Effects of pharmacological concentrations of estrogens on proliferation and cell cycle kinetics of human breast cancer cell lines in vitro.* Cancer Research, 1987. 47: p. 5323-5329.
28. Rajah, T.T., et al., *Physiological concentrations of genistein and 17beta-estradiol inhibit MDA-MB-231 breast cancer cell growth by increasing BAX/BCL-2 and reducing pERK1/2.* Anticancer Res, 2012. 32(4): p. 1181-91.

Chapter 7

Discussion and summary

Hormones and breast cancer.

In practice, adjuvant breast cancer treatment depends largely on the hormone receptor status of the breast tumor. The hormone responsive group (estrogen receptor (ER) / progesterone receptor (PR) / HER-2 positive) makes up 50-80% of the breast cancer patients (1). This group has a good survival chance, because there are several chemo-treatments that target those receptors. The other group, triple negative or hormone non-responsive tumors, does not express these markers and therefore the strategies for treatment are limited (2-5).

Estrogen related mechanisms and therapies.

For the hormone responsive group, tamoxifen is a preferred treatment. Tamoxifen is classified as a selective estrogen receptor modulator (SERM). Tamoxifen antagonizes the ER, which means that there is no space for E2 to bind and activate the ER. It is used to treat metastatic breast cancer, after surgery and radiation. It can be used for a longer period (5-10 years) after surgery in order to reduce the risk of recurrence. However, tamoxifen can cause serious side effects like thromboembolic events, endometrial cancer or endometrial hyperplasia (6). Another method to interfere with the ER and inhibit breast cancer cell growth is by down regulating the ER with a SERD (selective estrogen receptor down-regulator). An example of medication that reduces the amount of ERs is fulvestrant (7).

In recent years, there has also been a shift towards the use of selective estrogen enzyme modulator (SEEM) such as aromatase inhibitors. Aromatase inhibitors reduce the local production of estrogens in breast tissue, thereby inhibiting the growth stimulus of ER-dependent breast cancer cells. There is evidence that overall patients who receive aromatase inhibitors survive longer and are more disease-free (8, 9). Compared to aromatase inhibitors, results with SERDs are not inferior. However, fulvestrant is more expensive and only available by injection. In addition, according to Clemens and Goss (2001), estrogen metabolites as well as estrogens can initiate breast cancer (10). Therefore, aromatase inhibitors have dual effects on breast health by blocking the local formation of estrogens and formation of potentially carcinogenic metabolites (10-12). In patients with advanced breast cancer, tumors can develop resistance towards aromatase inhibitors and recur. According to Fabian et al (7) resistance can occur by development of hypersensitivity of the ER to very low levels of estrogen and up-regulation of growth factor receptors and/or associated signaling pathways (HER-2, EGFR and insulin growth factor receptor (IGFR)) (7, 13). However, there are two classes of third generation aromatase inhibitors; the steroidal and non-steroidal inhibitors. They can bind to aromatase enzyme either reversibly (e.g. anastrozole and letrozole) or irreversible (e.g. exemestane) (7). Research shows that there is no cross-resistance between these two classes, which means that women who develop resistance to one of the two groups of SEEMs still have a chance to use AI before going into chemotherapy or to another method (7). Yet, despite the multitude of treatment options, breast cancer is still a leading cause of death among women and new treatment options remain of utmost importance.

Androgen related mechanisms

There is emerging evidence that the androgen receptor (AR) might be an additional target for systemic breast cancer treatment (14, 15). However, the role of androgens and the AR in breast cancer is still controversial. Androgens can induce or inhibit the proliferation of breast tumor cells. Some clinical studies and *in vitro* experiments support the view that a low dose of androgens can increase the risk of breast tumor formation and growth (15-17). By contrast, some other studies indicate that androgens can inhibit the growth of triple negative tumor cells both *in vitro* and *in vivo*, if there is a strong expression of the AR (15, 18-20). Androgens may exert their effects via other two pathways; direct stimulation by binding to AR (AR-positive/ER-negative tumors) or increased synthesis of growth factors, such as EGF, (ER-negative/AR-negative tumors). A recent cohort study demonstrated that the AR/ER ratio influences breast tumor responses to hormonal treatment (21).

Moreover, it was concluded that the AR antagonist enzalutamide could be used for treatment of AR+ tumors regardless of ER status, since it blocks both AR- and ER-mediated tumor growth in preclinical models (21). Based on the effects of these studies it has become clear that both the AR and ER status of a breast tumor will determine the effect of an (anti)androgen on cell proliferation. In this thesis it is shown in Chapter 2 that the majority (80%) of Thai breast cancer patients have breast tumors that express the AR. These tumors were mostly hormonal and HER-2 positive in nature. Expression of the AR in triple negative breast tumors is much less common; 33%, (see chapter 2). Another, recent study in Western patients has also demonstrated that up to seventy percent of all malignant breast tumors, including triple negative breast tumors, express the androgen receptor (AR) (15). However, the information is still limited and conflicting regarding the role of androgens or the AR in the etiology of breast cancer. With respect to breast tumor cell proliferation under the influence of androgens a mechanistic explanation may be found by the conversion of some androgens to estrogens by the aromatase enzyme (22). To investigate this further, we performed *in vitro* studies in a breast cancer co-culture model, which are further described in chapter 3

An in vitro co-culture breast cancer model.

It is widely accepted that (mammary) carcinomas should be considered as complex organs consisting of tumor epithelial cells interacting with surrounding cell types via intercellular communication (23-26). Therefore, an *in vitro* co-culture model combining breast tumor cells and primary BAFs creates a more realistic micro-environment to study hormonal interactions in breast cancer compared to mono-cultures of breast tumor cell lines only (26). In breast cancer co-culture system, Chen demonstrated the aromatase promotor switched from a glucocorticoid-stimulated promoter, I.4, in normal tissue to cAMP-stimulated promoters, I.3 and II, in cancerous breast tissue (27). A co-culture breast cancer model can be established for example by applying a one-compartment, layering technique, or a two-

compartment, trans-well co-culture system. While in the one-compartment co-culture model it is not possible to separate different cell types after exposure (26), the benefit of a one-compartment co-culture model is the direct cell-cell contact causing paracrine interactions. This paracrine interaction has been shown to facilitate the induction of aromatase in fibroblasts in a one- but not a two-compartment co-culture system (23, 24). This difference between both co-culture models was clearly illustrated by the absence of MCF-7 cell proliferation in the presence of breast fibroblasts and dexamethasone (aromatase inducer) in the two-compartment co-culture system (23, 24). The one-compartment co-culture breast cancer model, as used in our studies, also shows ductal formation similar to breast tissues. The ability of a one-compartment co-culture breast cancer model to convert androgens into estrogens was also used to determine the actual role of androgens toward breast cancer cell proliferation in Chapter 3. In a clinical study done by Key et al. indications were found that aromatase can play a key role in the (indirect) stimulation of androgens on cell proliferation (22, 28). In Chapter 3 we demonstrate using a co-culture breast cancer model that the differential effect of aromatizing androgens (i.e. testosterone), and non-aromatizing androgens (i.e. DHT) on breast cancer cells arises from two reasons. Firstly, the presence of the aromatase enzyme in breast adipose fibroblasts can only convert aromatizing androgens such as testosterone into estrogen. The second reason lies in the breast cancer cells' ontology: hormone receptor-positive breast cancer can be stimulated by estrogens, while hormone receptor-negative or triple negative breast cancer cells cannot be stimulated by estrogens. In this study, we also indicated that non-aromatizable androgens can inhibit the growth of breast cancer cells with have AR expression, and they can induce apoptosis regardless of their ER/PR status.

Taken together, the results from Chapter 2 and 3 suggest that androgens that cannot be aromatized, like DHT, may provide a perspective for treatment of breast cancer patients with triple negative breast cancer tumors. Moreover, our results are in line with clinical and epidemiological findings, which clearly support the relevance of using a co-culture system of breast tumor cells and BAFs for breast cancer research, for example in the search for cancer treatment and prevention options.

Natural extracts chemicals and breast cancer.

Nowadays, many naturally occurring compounds are investigated for their potential anti-cancer properties. Some have already become important anti-cancer drugs (29, 30); such as vincristine (31-33) and taxol (34, 35). Among various naturally occurring, biologically active compounds, resveratrol and melatonin have been suggested to act as aromatase inhibitors. Also, the naturally occurring depsidones from the marine fungus Aspergillus unguis are known to have anti-cancer activity, but their mechanism of action remains largely elusive. Using our *in vitro* co-culture breast cancer model, we have investigated resveratrol and melatonin (Chapter 4) and depsidones (Chapter 5 and 6) for their potential use in treatment and/or prevention of hormonal positive breast cancer.

Resveratrol is a non-flavonoid phytoestrogen found in grapes. Several epidemiological studies have indicated that high resveratrol intake could reduce breast cancer risk (36-38). Multiple mechanisms of action have been identified that could explain the anti-carcinogenic properties of resveratrol, which include its role as a SERMs, aromatase inhibitor and/or anti-oxidant (36-38). With respect to its properties as an aromatase inhibitor, several *in vivo* and *in vitro* studies have confirmed that resveratrol can modulate this key enzyme in sex steroidogenesis (39-42). As a consequence, it has been suggested that the anti-aromatase activity of resveratrol is its major property with respect to chemoprevention of breast cancer (40). Melatonin (N-acetyl-5-methoxytryptamine) is an indolic and endogenous compound that is naturally produced by the pineal gland in the human body. Melatonin plays a primary role in the circadian pattern and is regulated by the hypothalamic suprachiasmatic nucleus (SCN). In addition, melatonin is believed to have oncostatic properties against many forms of cancer such as leukemia, breast, colorectal and prostate cancer (43-46). Multiple mechanisms have been proposed to explain the chemopreventive properties of melatonin for breast cancer (47-50) of which two are especially important with respect to our present study. Firstly, melatonin can act as SERM by reducing estrogen binding to ERα receptors and secondly, it can inhibit the binding of the E2-ERα complex to the DNA. The latter mechanism of this anti-estrogenic effect of melatonin does not depend on its binding to the ER, but depends on the high affinity binding to membrane melatonin receptor(s) (MT1). This complex will interfere with the estrogen-binding activity of ERα without changing its affinity, but will reduce the transactivation of ligand-receptors (50-52). Another important mechanism of melatonin with respect to prevention of breast cancer may be its role as aromatase inhibitor. Melatonin could decrease cAMP formation and down-regulate expression of promoter regions pII, pI.3 and p1.4-regulated CYP19A1 gene in MCF-7 cells (53). In Chapter 4, we described that melatonin and resveratrol have aromatase inhibitor property.

Depsidones are secondary metabolites mostly found in lichens, but also in some higher plants, where they play a role in the protection against insects and microbes or sun light. Depsidones are esters which compose of polyphenolic depsides and cyclic ethers, but the chemical structure of the depsidones formed is highly dependent on the type of fungus and its environment (54). Generally, the chemical structure of depsidones resembles prostaglandins and leukotrienes in humans, which makes these compounds may have beneficial health effects in humans. Indeed, several studies have already reported biological activities of these naturally occurring depsidones like anti-proliferative actions or bactericidal activity against infectious diseases (55-59). Recently, *in vitro* studies from our laboratory have shown that some depsidones are potent *in vitro* inhibitors of the aromatase enzyme (60). In Chapter 5 we show that two common depsidones, unguinol and aspergillusidone A, inhibit the growth of T47D tumor cells most likely via inhibition of aromatase activity in our co-culture system of human primary breast adipose fibroblasts and hormonal responsive T47D breast tumor cells. The IC_{50} values of these depsidones were 2 orders of magnitude higher than those of the therapeutic aromatase inhibitors letrozole and exemestane.

Thus, our studies with melatonin, resveratrol and depsidones show that these compounds can be aromatase inhibitors in co-cultures of breast fibroblasts and tumor cells, albeit at significantly different concentrations. The melatonin concentration of 20 nM, resveratrol concentration of 20 μM and depsidones concentration of 2 μM have an efficacy of aromatase inhibition similar to 20 nM of letrozole, which is a commonly used as an anti-aromatase drug in breast cancer treatment. Our co-culture model did not provide any indications that melatonin is also a selective estrogen receptor modulator. Such a combination of both SERM and SEEM mechanisms, especially of melatonin, may have offered more advantages for breast cancer treatment.

However, it must be noted that although cell lines are classified as ER+ or ER-, this concerns expression of ERα and not ERβ, while MDA-MB-231 is categorized as ER-, it does express low levels of ERβ (61). Therefore, it cannot be excluded that some of the observed effects in the present study might be partly attributed to interaction of an androgen metabolite or compounds with ERβ, as described above (62).

Main conclusions from this thesis.

The majority of Thai breast cancer patients had tumors that expresses the AR (80%). These tumors are mostly hormonal and HER-2 positive in nature. Expression of the AR in triple negative breast tumors are less common (33%). When compared with data from Western studies, the Thai patients show higher in AR expression and the ratio between hormonal/HER-2 responsive and triple negative breast tumors than Western patients. According to high expression of AR, we studied the role of androgens in breast cancer model. Our results demonstrate the use of a co-culture system of breast tumor cells and BAFs for anti-aromatase assays. Two main findings in our studies are first, that androgens can induce or reduce breast cancer cell growth, depending on the ability of conversion to estradiol by aromatase in the fibroblasts of breast tissue. On the other hand, non-aromatizable androgens (DHT) may inhibited proliferation of both hormone receptors, positive or triple negative breast cancers. Second, it shows that resveratrol, melatonin, and depsidones can inhibit hormonal positive breast cancer cell growth via the anti-aromatase activity. The results show the possibility of resveratrol, melatonin, and depsidones as new candidates for anti-aromatase agents to fight against hormonal positive breast cancer. Further research is needed for possible adverse effects of these compounds at dose levels that have been found to show AI properties. Three main conclusions for this thesis are as followed;

- A co-cultures of breast cancer cells and primary fibroblasts is a good *in vitro* model to study breast cancer biology for mechanisms involving the aromatase enzyme and estrogen positive breast cancer cells.
- Androgens can both induce or reduce the growth of human breast cancer cells, depending on the conversion by aromatase into estrogens.
- Natural chemicals like some depsidones and melatonin can act like aromatase inhibitors and may have the potential for future clinical treatments in the case estrogen positive breast cancer.

References.

1. Elledge RM, Osborne CK. Oestrogen receptors and breast cancer. BMJ. 1997;314(7098):1843-4.
2. Ogawa Y, Hai E, Matsumoto K, Ikeda K, Tokunaga S, Nagahara H, et al. Androgen receptor expression in breast cancer: relationship with clinicopathological factors and biomarkers. Int J Clin Oncol. 2008;13(5):431-5.
3. Bosch A, Eroles P, Zaragoza R, Vina JR, Lluch A. Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research. Cancer Treat Rev. 2010;36(3):206-15.
4. Akiyama F, Iwase H. Triple negative breast cancer: clinicopathological characteristics and treatment strategies. Breast Cancer. 2009;16(4):252-3.
5. Rodler E, Korde L, Gralow J. Current treatment options in triple negative breast cancer. Breast Dis.32(1-2):99-122.
6. Gail MH, Costantino JP, Bryant J, Croyle R, Freedman L, Helzlsouer K, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. J Natl Cancer Inst. 1999;91(21):1829-46.
7. Fabian CJ. The what, why and how of aromatase inhibitors: hormonal agents for treatment and prevention of breast cancer. Int J Clin Pract. 2007;61(12):2051-63.
8. Thurlimann B, Keshaviah A, Coates AS, Mouridsen H, Mauriac L, Forbes JF, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med. 2005;353(26):2747-57.
9. Rao RD, Cobleigh MA. Adjuvant endocrine therapy for breast cancer. Oncology (Williston Park). 2012;26(6):541-7, 50, 52 passim.
10. Clemons M, Goss P. Estrogen and the risk of breast cancer. N Engl J Med. 2001;344(4):276-85.
11. Chang M, Peng KW, Kastrati I, Overk CR, Qin ZH, Yao P, et al. Activation of estrogen receptor-mediated gene transcription by the equine estrogen metabolite, 4-methoxyequilenin, in human breast cancer cells. Endocrinology. 2007;148(10):4793-802.
12. Siebert AE, Sanchez AL, Dinda S, Moudgil VK. Effects of estrogen metabolite 2-methoxyestradiol on tumor suppressor protein p53 and proliferation of breast cancer cells. Syst Biol Reprod Med. 2011;57(6):279-87.
13. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol. 2007;25(36):5815-24.
14. Hao Y, Montiel R, Li B, Huang E, Zeng L, Huang Y. Association between androgen receptor gene CAG repeat polymorphism and breast cancer risk: a meta-analysis. Breast Cancer Res Treat. 2010.
15. Nahleh Z. Androgen receptor as a target for the treatment of hormone receptor-negative breast cancer: an unchartered territory. Future Oncol. 2008;4(1):15-21.
16. Hao Y, Montiel R, Li B, Huang E, Zeng L, Huang Y. Association between androgen receptor gene CAG repeat polymorphism and breast cancer risk: a meta-analysis. Breast Cancer Res Treat. 2010;124(3):815-20.
17. Dreyer G, Vandorpe T, Smeets A, Forceville K, Brouwers B, Neven P, et al. Triple negative breast cancer: clinical characteristics in the different histological subtypes. Breast. 2013;22(5):761-6.
18. Gucalp A, Traina TA. Triple-negative breast cancer: role of the androgen receptor. Cancer J.16(1):62-5.
19. Narayanan R, Ahn S, Cheney MD, Yepuru M, Miller DD, Steiner MS, et al. Selective androgen receptor modulators (SARMs) negatively regulate triple-negative breast cancer growth and epithelial:-mesenchymal stem cell signaling. PLoS One. 2014;9(7):e103202.
20. Dimitrakakis C, Bondy C. Androgens and the breast. Breast Cancer Res. 2009;11(5):212.
21. Cochrane DR, Bernales S, Jacobsen BM, Cittelly DM, Howe EN, D'Amato NC, et al. Role of the androgen receptor in breast cancer and preclinical analysis of enzalutamide. Breast Cancer Res. 2014;16(1):R7.
22. Chottanapund S, Van Duursen MB, Navasumrit P, Hunsonti P, Timtavorn S, Ruchirawat M, et al. Effect of androgens on different breast cancer cells co-cultured with or without breast adipose fibroblasts. J Steroid Biochem Mol Biol. 2013;138C:54-62.
23. Heneweer M, Muusse M, Dingemans M, de Jong PC, van den Berg M, Sanderson JT. Co-culture of primary human mammary fibroblasts and MCF-7 cells as an in vitro breast cancer model. Toxicol Sci. 2005;83(2):257-63.
24. Heneweer M, Muusse M, Dingemans M, de Jong PC, van den Berg M, Sanderson JT. Direct and indirect endocrine disruption: Co-culture of primary human mammary fibroblasts and MCF-7 cells as an *in vitro* breast cancer model. Institute for Risk Assessment Sciences (IRAS). Ph.D. Utrecht Utrecht University; 2005. p. 175.
25. van Meeuwen JA, Korthagen N, de Jong PC, Piersma AH, van den Berg M. (Anti)estrogenic effects of phytochemicals on human primary mammary fibroblasts, MCF-7 cells and their co-culture. Toxicol Appl Pharmacol. 2007;221(3):372-83.
26. Miki Y, Ono K, Hata S, Suzuki T, Kumamoto H, Sasano H. The advantages of co-culture over mono cell culture in simulating in vivo environment. J Steroid Biochem Mol Biol. 2012;131(3-5):68-75.
27. Chen S, Zhou D, Okubo T, Kao YC, Yang C. Breast tumor aromatase: functional role and transcriptional regulation. Endocrine-related cancer. 1999;6(2):149-56.
28. Key T, Appleby P, Barnes I, Reeves G. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst. 2002;94(8):606-16.
29. von Schwarzenberg K, Vollmar AM. Targeting apoptosis pathways by natural compounds in cancer: marine compounds as lead structures and chemical tools for cancer therapy. Cancer Lett. 2010;332(2):295-303.
30. Chomcheon P, Wiyakrutta S, Sriubolmas N, Ngamrojanavanich N, Kengtong S, Mahidol C, et al. Aromatase inhibitory, radical scavenging, and antioxidant activities of depsidones and diaryl ethers from the endophytic fungus Corynespora cassiicola L36. Phytochemistry. 2009;70(3):407-13.
31. Mattos DM, Gomes ML, Freitas RS, Rodrigues PC, Paula EF, Bernardo-Filho M. A model to evaluate the biological effect of natural products: vincristine action on the biodistribution of radiopharmaceuticals in BALB/c female mice. J Appl Toxicol. 1999;19(4):251-4.
32. DuBois SG, Allen S, Bent M, Hilton JF, Hollinger F, Hawkins R, et al. Phase I/II study of (131)I-MIBG with vincristine and 5 days of irinotecan for advanced neuroblastoma. Br J Cancer. 2015;112(4):644-9.
33. Ceppi F, Langlois-Pelletier C, Gagne V, Rousseau J, Ciolino C, De Lorenzo S, et al. Polymorphisms of the vincristine pathway and response to treatment in children with childhood acute lymphoblastic leukemia. Pharmacogenomics. 2014;15(8):1105-16.
34. Raj KG, Chidambaram R, Varunkumar K, Ravikumar V, Pandi M. Chemopreventive potential of fungal taxol against 7, 12-dimethylbenz[a]anthracene induced mammary gland carcinogenesis in Sprague Dawley rats. Eur J Pharmacol. 2015.
35. Weaver BA. How Taxol/paclitaxel kills cancer cells. Mol Biol Cell. 2014;25(18):2677-81.
36. Alkhalaf M, El-Mowafy A, Renno W, Rachid O, Ali A, Al-Attyiah R. Resveratrol-induced apoptosis in human breast cancer cells is mediated primarily through the caspase-3-dependent pathway. Arch Med Res. 2008;39(2):162-8.
37. Lee HS, Ha AW, Kim WK. Effect of resveratrol on the metastasis of 4T1 mouse breast cancer cells in vitro and in vivo. Nutr Res Pract. 2012;6(4):294-300.
38. Leon-Galicia I, Diaz-Chavez J, Garcia-Villa E, Uribe-Figueroa L, Hidalgo-Miranda A, Herrera LA, et al. Resveratrol induces downregulation of DNA repair genes in MCF-7 human breast cancer cells. Eur J Cancer Prev. 2012;22(1):11-20.
39. Lee JE, Safe S. Involvement of a post-transcriptional mechanism in the inhibition of CYP1A1 expression by resveratrol in breast cancer cells. Biochem Pharmacol. 2001;62(8):1113-24.
40. Le Corre L, Chalabi N, Delort L, Bignon YJ, Bernard-Gallon DJ. Resveratrol and breast cancer chemoprevention: molecular mechanisms. Mol Nutr Food Res. 2005;49(5):462-71.
41. Zhang S, Cao HJ, Davis FB, Tang HY, Davis PJ, Lin HY. Oestrogen inhibits resveratrol-induced post-translational modification of p53 and apoptosis in breast cancer cells. Br J Cancer. 2004;91(1):178-85.
42. Wang Y, Lee KW, Chan FL, Chen S, Leung LK. The red wine polyphenol resveratrol displays bilevel inhibition on aromatase in breast cancer cells. Toxicol Sci. 2006;92(1):71-7.

43. Mediavilla MD, Sanchez-Barcelo EJ, Tan DX, Manchester L, Reiter RJ. Basic mechanisms involved in the anti-cancer effects of melatonin. Curr Med Chem.17(36):4462-81.
44. Srinivasan V, Spence DW, Pandi-Perumal SR, Trakht I, Cardinali DP. Therapeutic actions of melatonin in cancer: possible mechanisms. Integr Cancer Ther. 2008;7(3):189-203.
45. Dai M, Cui P, Yu M, Han J, Li H, Xiu R. Melatonin modulates the expression of VEGF and HIF-1 alpha induced by CoCl2 in cultured cancer cells. J Pineal Res. 2008;44(2):121-6.
46. Mills E, Wu P, Seely D, Guyatt G. Melatonin in the treatment of cancer: a systematic review of randomized controlled trials and meta-analysis. J Pineal Res. 2005;39(4):360-6.
47. Grant SG, Melan MA, Latimer JJ, Witt-Enderby PA. Melatonin and breast cancer: cellular mechanisms, clinical studies and future perspectives. Expert Rev Mol Med. 2009;11:e5.
48. Korkmaz A, Sanchez-Barcelo EJ, Tan DX, Reiter RJ. Role of melatonin in the epigenetic regulation of breast cancer. Breast Cancer Res Treat. 2009;115(1):13-27.
49. Blask DE, Brainard GC, Dauchy RT, Hanifin JP, Davidson LK, Krause JA, et al. Melatonin-depleted blood from premenopausal women exposed to light at night stimulates growth of human breast cancer xenografts in nude rats. Cancer Res. 2005;65(23):11174-84.
50. Sanchez-Barcelo EJ, Cos S, Mediavilla D, Martinez-Campa C, Gonzalez A, Alonso-Gonzalez C. Melatonin-estrogen interactions in breast cancer. J Pineal Res. 2005;38(4):217-22.
51. Treeck O, Haldar C, Ortmann O. Antiestrogens modulate MT1 melatonin receptor expression in breast and ovarian cancer cell lines. Oncol Rep. 2006;15(1):231-5.
52. Yuan L, Collins AR, Dai J, Dubocovich ML, Hill SM. MT(1) melatonin receptor overexpression enhances the growth suppressive effect of melatonin in human breast cancer cells. Mol Cell Endocrinol. 2002;192(1-2):147-56.
53. Martinez-Campa C, Gonzalez A, Mediavilla MD, Alonso-Gonzalez C, Alvarez-Garcia V, Sanchez-Barcelo EJ, et al. Melatonin inhibits aromatase promoter expression by regulating cyclooxygenases expression and activity in breast cancer cells. Br J Cancer. 2009;101(9):1613-9.
54. Armaleo D, Sun X, Culberson C. Insights from the first putative biosynthetic gene cluster for a lichen depside and depsidone. Mycologia. 2011;103(4):741-54.
55. Sultana N, Afolayan AJ. A new depsidone and antibacterial activities of compounds from Usnea undulata Stirton. J Asian Nat Prod Res. 2011;13(12):1158-64.
56. Varughese T, Riosa N, Higginbotham S, Arnold AE, Coley PD, Kursar TA, et al. Antifungal Depsidone Metabolites from Cordyceps dipterigena, an Endophytic Fungus Antagonistic to the Phytopathogen Gibberella fujikuroi. Tetrahedron Lett. 2012;53(13):1624-6.
57. Sureram S, Wiyakrutta S, Ngamrojanavanich N, Mahidol C, Ruchirawat S, Kittakoop P. Depsidones, aromatase inhibitors and radical scavenging agents from the marine-derived fungus Aspergillus unguis CRI282-03. Planta Med. 2012;78(6):582-8.
58. Khumkomkhet P, Kanokmedhakul S, Kanokmedhakul K, Hahnvajanawong C, Soytong K. Antimalarial and cytotoxic depsidones from the fungus Chaetomium brasiliense. Journal of natural products. 2009;72(8):1487-91.
59. Kokubun T, Shiu WK, Gibbons S. Inhibitory activities of lichen-derived compounds against methicillin- and multidrug-resistant Staphylococcus aureus. Planta Med. 2007;73(2):176-9.
60. Chottanapund S, Van Duursen MBM, Zwartsen A, Timtavorn S, Navasumrit P, Kittakoop P, et al. Depsidones inhibit aromatase activity and tumor cell proliferation in a co-culture of human primary breast adipose fibroblasts and T47D breast tumor cells. Toxicology Reports. 2017;4:165-71.
61. Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lakins J, Lupu R. Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. Oncol Rep. 2000;7(1):157-67.
62. Sotoca AM, Ratman D, van der Saag P, Strom A, Gustafsson JA, Vervoort J, et al. Phytoestrogen-mediated inhibition of proliferation of the human T47D breast cancer cells depends on the ERalpha/ERbeta ratio. J Steroid Biochem Mol Biol. 2008;112(4-5):171-8.

Dutch summary (Nederlandse samenvatting)

Hormonen en borstkanker.

Borstkanker kan op verschillende manieren worden behandeld. Het type borsttumor bepaalt grotendeels welke adjuvante behandeling van borstkanker in de praktijk wordt toegepast. De hormoon-gevoelige groep (oestrogeenreceptor (ER) / progesteronreceptor (PR) / HER-2-positief) vormt 50-80% van de borstkankerpatiënten (1). Deze groep heeft een goede overlevingskans, omdat er verschillende behandelingen zijn die gericht zijn op deze receptoren. De andere groep borsttumoren, vaak drievoudig negatieve of hormoon-ongevoelige tumoren genoemd, hebben geen duidelijke expressie van deze receptoren. Om deze reden zijn de behandelingsstrategieën beperkt (2-5).

Oestrogeen-gerelateerde werkingsmechanismen en therapieën.

Hormoon-gevoelige borsttumoren worden bij voorkeur met tamoxifen behandeld. Tamoxifen is een selectieve modulator van de oestrogeenreceptor (SERM). Tamoxifen is een antagonist van de ER waardoor estradiol (E2) niet meer kan binden aan de ER. Hierdoor kan E2 de ER niet meer kan activeren voor o.a. binding aan het DNA en celdeling. Tamoxifen wordt gebruikt voor de behandeling van uitgezaaide borstkanker, na operatie en bestraling. Daarnaast wordt het gebruikt voor een langere periode (5-10 jaar) om het risico op terugkeer van borstkanker na de operatie te verkleinen. Tamoxifen kan echter ook ernstige bijwerkingen veroorzaken, zoals trombo-embolische effecten, endometrium hyperplasie of tumoren (6). Een andere behandelingsmethode hormoon-gevoelige tumoren is gericht op de groeiremming van borstkankercellen door onderdrukking van de ER met een SERD: selectieve oestrogeenreceptor downregulator (SERD). Een voorbeeld van deze behandeling is het medicijn fulvestrant (7).

Meer recent is er een verschuiving geweest naar het gebruik van selectieve oestrogeen-enzymmodulators (SEEM), zoals aromataseremmers. SEEMs remmen de lokale productie van oestrogenen in het borstweefsel, waardoor de groei-stimulus van hormoon-gevoelige borsttumorcellen wordt verminderd. Er zijn aanwijzingen dat, in het algemeen, patiënten die met aromataseremmers behandeld worden langer overleven en ziektevrij blijven (8, 9). Wanneer de effectiviteit van deze aromataseremmers vergeleken wordt met de effectiviteit van een behandeling met SERD's , dan zijn de resultaten zeker niet minder gunstig voor de patiënt. Fulvestrant is echter duurder dan aromataseremmers en het is alleen toe te dienen via een injectie. Aromataseremmers kunnen ook als pil worden ingenomen. Verder dient opgemerkt te worden dat, volgens Clemens en Goss (2001), de oestrogeenmetabolieten eveneens borstkanker kunnen veroorzaken door schade aan het DNA (10). Aromataseremmers hebben dus een tweezijdig, positief effect bij de behandeling van oestrogeen positieve borsttumoren: deze SEEM's remmen de lokale aanmaak van oestrogenen in het borstweefsel en hierdoor wordt ook de vorming van mogelijk kankerverwekkende metabolieten geblokkeerd (10-12). Het is echter wel zo dat, bij patiënten met een vergevorderd stadium van borstkanker, tumorcellen kunnen

ontwikkelen die resistent zijn tegen aromataseremmers. Fabian *et al*. (7) verklaren deze resistentie doordat de tumorcellen een overgevoeligheid voor activatie van de ER ontwikkelen. Hierdoor kunnen al zeer lage concentraties van oestrogenen een toename van groeifactorreceptoren en/of bijbehorende signaal-transductieroutes (bijv. HER-2, EGFR en de receptor van de insulinegroeifactor) in tumorcel veroorzaken (7, 13). Bij aromataseremmers van de derde generatie worden twee groepen onderscheiden, waarbij het al dan niet een hormoonachtige structuur hebben een onderscheidende rol speelt. De medicijnen zonder hormoonachtige structuur kunnen reversibel binden aan het aromatase-enzym (bijv. anastrozole en letrozole). De aromatase-remmers die een hormoon-achtige structuur hebben binden irreversibel aan het aromatase-enzym (bijv. exemestaan) (7). Klinisch onderzoek heeft aangetoond dat tussen beide klassen van SEEM's geen kruisresistentie optreedt. Dit betekent dat vrouwen die een resistentie ontwikkelen tegen een van deze twee groepen nog steeds een kans hebben om met een SEEM uit een andere groep te worden behandeld, voordat overgestapt moet worden naar bijv. een nieuwe chemotherapie (7). Het blijft echter een feit dat ondanks de vele behandelingsopties voor borstkanker, dit nog steeds een van de belangrijkste doodsoorzaken bij vrouwen is, waardoor de noodzaak voor nieuwe behandelingsopties van groot belang is.

Androgeen-gerelateerde werkingsmechanismen

Er zijn steeds meer aanwijzingen dat de androgeenreceptor (AR) een extra doelwit kan zijn voor borstkankerbehandeling (14, 15). De rol van androgenen en de AR bij de ontwikkeling van borstkanker is echter nog steeds controversieel. Deze androgenen kunnen de deling van borsttumorcellen zowel stimuleren als remmen. Sommige klinische onderzoeken en *in vitro* experimenten ondersteunen de opvatting dat een lage dosis androgenen het risico op de vorming en groei van borsttumoren kan verhogen (15-17). Daarentegen geven enkele andere onderzoeken aan, dat androgenen de groei van hormoon-ongevoelige borsttumorcellen zowel *in vitro* als *in vivo* kunnen afremmen wanneer in de cel een hoge expressie van de AR is (15, 18-20). Deze androgenen kunnen hun effecten op de tumorcel via andere twee manieren uitoefenen; directe stimulatie door binding aan de AR (bij AR-positieve / ER-negatieve tumoren) of via verhoogde vorming van groeifactoren, zoals bijv. de EGF (bij ER-negatieve / AR-negatieve tumoren). Een recente studie heeft aangetoond dat de AR / ER-verhouding de respons op hormonale behandeling van de tumorcel beïnvloedt (21). Eveneens werd geconcludeerd dat de AR-antagonist enzalutamide gebruikt kan worden gebruikt voor de behandeling van AR positieve borsttumoren ongeacht de ER-status. De oorzaak hiervoor wordt gezocht in het feit, dat zowel de door AR als ER gestimileerde tumorgroei in preklinische modellen worden geblokkeerd (21). Op basis van de uitkomsten van deze studies is het duidelijk geworden, dat zowel de AR- als ER-status van een borsttumor het effect van een antiandrogeen op borsttumorceldeling kunnen bepalen. In dit proefschrift wordt in hoofdstuk 2 aangetoond dat de meerderheid (80%) van de Thaise borstkankerpatiënten een borsttumor waarin de AR tot expressie komt. Deze tumoren waren meestal ook hormoon-gevoelig en HER-2-positief van aard. Expressie van de AR in hormoon-

ongevoelige borsttumoren komt echter veel minder vaak voor: bij zo'n 33% van de onderzochte tumoren (zie hoofdstuk 2). Een andere recente studie bij Westerse patiënten heeft aangetoond dat tot zeventig procent van alle kwaadaardige borsttumoren, inclusief hormoon-ongevoelige, de AR tot expressie brengen (15). Het aantal studies is echter nog steeds beperkt en biedt nog onvoldoende duidelijkheid met betrekking tot de rol van androgenen of de AR in de etiologie van borstkanker. Voor de invloed van androgenen op de groei van borsttumoren kan echter wel een mechanistische verklaring gegeven worden, omdat sommige androgenen door het aromatase-enzym worden omgezet in oestrogenen (22). Om dit werkingsmechanisme verder te onderzoeken hebben wij *in vitro* studies uitgevoerd met borst (tumor) cellen. Dit wordt in detail beschreven in hoofdstuk 3.

Een *in vitro* borstkankermodel met een co-cultuur van borst(tumor)cellen.

Het is algemeen aanvaard dat borsttumoren beschouwd kunnen worden als complexe organen, bestaande uit epitheeltumorcellen die intercellulaire communicatie onderhouden met omringende celtypen zoals bijv. fibroblasten in het borstweefsel (BAFs) (23-26). Om die reden is een *in vitro* co-cultuurmodel, dat primaire BAFs combineert met borsttumorcellen, een meer realistische micro-omgeving om bijv. de hormonale interacties bij borstkanker te bestuderen t.o.v. monoculturen van borsttumorcellijnen (26). In een co-cultuursysteem voor borstkanker heeft Chen *et al.* aangetoond dat de door glucocorticoïde-gestimuleerde aromatase-promotor I.4.in gezond weefsel overschakelt naar de cAMP-gereguleerde promoters I.3 en II in borsttumorweefsel (27). Een experimenteel model voor borstkanker met co-culturen kan in het laboratorium worden opgezet door bijvoorbeeld een techniek van een één cellaag toe te passen, of gebruik te maken van een tweecompartimenten systeem waarbij de verschillende cellagen fysiek zijn gescheiden. In het co-cultuurmodel met één cellaag is het niet mogelijk om de verschillende celtypen na blootstelling te scheiden (26). Het voordeel van dit co-cultuurmodel is echter dat het directe cel-cel contact de paracriene interacties kan versterken. Eerdere studies hebben aangetoond dat deze paracriene interactie(s) de inductie van aromatase in BAFs kan bevorderen als het co-cultuurmodel uit één cellaag bestaat (23, 24). Dit verschil tussen beide co-cultuurmodellen werd duidelijk geïllustreerd door het ontbreken van gestimuleerde celdeling van MCF-7 borsttumorcellen, wanneer deze in verschillende cellagen met BAFs en dexamethason werden gekweekt (23, 24). Daarnaast vertoont het co-cultuurmodel van één cellaag met borstumorcellen en BAFs ook ductale vorming wat van nature voorkomt in borstweefsel.

Het co-cultuurmodel met één cellaag is in onze studies gebruikt om de omzetting van androgenen in oestrogenen te stimuleren en de daaropvolgende celdeling van borsttumorcellen te bestuderen. De resultaten hiervan zijn beschreven in hoofdstuk 3 van dit proefschrift. In een klinische studie, uitgevoerd door Key *et al.,* er werden eveneens aanwijzingen gevonden dat het aromatase-enzym een sleutelrol kan spelen bij de (indirecte) stimulatie van androgenen op de celdeling (22, 28). In Hoofdstuk 3

hebben wij aangetoond dat de effecten op de tumorcelproliferatie van aromatiserende androgenen (testosteron) en niet-aromatiserende androgenen (dihydrotestosteron) verschillend zijn. Vanuit mechanistisch oogpunt kunnen deze observaties verklaard worden door de aanwezigheid van aromatase in de BAFs, waarbij de uit androgenen gevormde oestrogenen de tumorgroei stimuleren. Dit geldt echter alleen voor ER positieve, hormoon-gevoelige borsttumorcellen en niet voor hormoon-ongevoelige borsttumorcellen. In deze studie hebben wij ook aangetoond, dat het niet de androgeen dihydrotestosteron (DHT), wat niet door aromatase kan worden omgezet in oestrogenen, de groei van borstkankercellen met AR-expressie kan remmen en celdood (apoptose) kan induceren, ongeacht de ER / PR-status van de tumorcel.

Samengevat suggereren de onderzoeksresultaten in hoofdstuk 2 en 3, dat niet-aromatiseerbare androgenen, zoals DHT, mogelijk ingezet kunnen worden bij preventieve behandeling van borstkankerpatiënten met hormoon-ongevoelige tumoren. Onze resultaten komen overeen met klinische en epidemiologische bevindingen. Daarmee ondersteunen onze resultaten de mechanistische en fysiologische relevantie van een co-cultuursysteem van borsttumorcellen en BAF's in onderzoek naar nieuwe behandelings- en preventiemogelijkheden voor borstkanker.

Extracten van natuurlijke chemicaliën en borstkanker.

Tegenwoordig worden veel natuurlijke verbinding onderzocht op potentiële anti-kanker eigenschappen. Sommigen zijn al belangrijke geneesmiddelen tegen kanker geworden (29, 30); zoals vincristine (31-33) en taxol (34, 35). Van natuurlijk en biologisch actieve verbindingen, zoals resveratrol en melatonine, is reeds eerder gesuggereerd dat deze als aromataseremmers kunnen werken. Ook van depsidones is bekend dat deze een anti-tumoractiviteit kunnen hebben, maar het onderliggende werkingsmechanisme is nog grotendeels onbekend. Met behulp van ons co-cultuurmodel voor borstkanker hebben we resveratrol en melatonine (hoofdstuk 4) en depsidones (hoofdstuk 5 en 6) nader onderzocht op eigenschappen die van nut kunnen zijn bij de toekomstige behandeling en/of preventie van hormoon-gevoelige borstkanker.

Resveratrol is een niet-flavonoïde fyto-oestrogeen dat onder andere wordt aangetroffen in druiven. Verschillende epidemiologische studies hebben aangetoond dat een hoge resveratrol inname het risico op borstkanker zou kunnen verminderen (36-38). Hierbij zijn meerdere werkingsmechanismen geïdentificeerd, die de anti-carcinogene eigenschappen van resveratrol kunnen verklaren, waaronder de rol als aromataseremmer en antioxidant (36-38). Verschillende *in vivo* en *in vitro* studies hebben bevestigd dat resveratrol het aromatase enzym kan beïnvloeden (39-42). Hierbij is gesuggereerd dat de anti-aromatase-activiteit van resveratrol de belangrijkste eigenschap zou kunnen zijn met betrekking tot chemopreventie van borstkanker (40). Melatonine (N-acetyl-5-methoxytryptamine) is een indolachtige verbinding, die van nature in het lichaam wordt geproduceerd door de pijnappelklier. Melatonine speelt een primaire rol in het dag-nacht

ritme wat wordt gereguleerd vanuit de hypothalamus. Bovendien wordt aangenomen dat melatonine ook tumorremmende eigenschappen heeft bij vele vormen van kanker zoals leukemie, borstkanker, colorectale en prostaatkanker (43-46). Er zijn meerdere mechanismen voorgesteld om de chemopreventieve eigenschappen van melatonine voor borstkanker te verklaren (47-50), waarvan er twee met name belangrijk zijn met betrekking tot onze huidige studies. Ten eerste kan melatonine werken als SERM door oestrogeenbinding aan ER-receptoren te verminderen. Ten tweede kan het de binding van het E2-ER-complex aan het DNA remmen. Dit laatste antioestrogene mechanisme van melatonine hangt niet af van de binding aan de ER, maar hangt af van de binding aan de membraan-melatoninereceptor(en) (MT1). Dit complex interfereert met de oestrogeen-bindende activiteit van de ER zonder de affiniteit hiervan te veranderen, maar zal de transactivatie van ligand-receptoren (50-52) wel verminderen. Een ander belangrijk werkingsmechanisme van melatonine met betrekking tot de preventie van borstkanker kan de rol als aromataseremmer zijn. Melatonine zou de cAMP-vorming in de cel kunnen verminderen, waardoor de expressie van aromatase via specifieke promoteren pII, pI.3 en pl.4 in MCF-7-cellen kan worden verlaagd (53). In hoofdstuk 4 hebben we beschreven dat zowel melatonine als resveratrol aromatase remmende eigenschappen hebben in het co-cultuur borstkankermodel.

Depsidones zijn secundaire metabolieten die meestal worden aangetroffen in korstmossen, maar ook in sommige hogere planten, waar ze een rol spelen bij de bescherming tegen insecten, microorganismen of zonlicht. Depsidones zijn esters die bestaan uit polyfenolische depsiden en cyclische ethers, maar de chemische structuur van de gevormde depsidonen is sterk afhankelijk van het type schimmel en zijn omgeving (54). Over het algemeen lijkt de chemische structuur van depsidones op prostaglandines en leukotriënen die bij de mens, wat de mogelijk gezonde eigenschappen van depsidones zou kunnen verklaren. Verschillende studies hebben antiproliferatieve werkingen of bactericide activiteit tegen infectieziekten van depsidones gerapporteerd (55-59). Recent *in vitro* onderzoek in ons laboratorium heeft aangetoond dat sommige depsidones sterke remmers van het aromatase-enzym kunnen zijn (60). In Hoofdstuk 5 laten we zien dat twee algemeen voorkomende depsidones, unguinol en aspergillusidon A, de groei van hormoon-gevoelige T47D-borsttumorcellen kunnen remmen in een co-cultuursysteem met BAFs. Dit effect onstaat hoogstwaarschijnlijk door remming van aromatase-activiteit door deze stoffen.

Hierbij dient wel opgemerkt te worden dat de depsidones tot honderd keer minder goed zijn in het remmen van aromatase dan de therapeutische aromataseremmers letrozole en exemestaan.

Samenvattend tonen onze studies aan dat melatonine, resveratrol en depsidones aromataseremmers kunnen zijn in co-culturen van borstfibroblasten en borsttumorcellen, maar wel bij aanzienlijk hogere concentraties dan gangbare therapeutische SEEM's.

Met ons co-cultuurmodel voor borstkanker konden wij geen aanwijzingen vinden dat melatonine ook een selectieve oestrogeenreceptormodulator is. Een dergelijke combinatie van zowel SERM- als SEEM-mechanismen, in het bijzonder van melatonine, kan meerdere voordelen hebben opgeleverd voor de behandeling van borstkanker. Er moet echter worden opgemerkt dat, de door ons gebruikte borsttumorcellijnen in het verleden formeel geclassificeerd zijn als ERα positief of negatief, de expressie van ERβ niet voldoende is gedefinieerd. Het blijkt bijvoorbeeld dat de veronderstelde ER negatieve MDA-MB-231 cellijn wel lage ERβ-niveaus tot expressie brengt (61). Daarom kan in onze studies niet worden uitgesloten, dat enkele van de waargenomen effecten geheel of gedeeltelijk kunnen worden toegeschreven aan interacties van de geteste stoffen met ERβ (62).

Belangrijkste conclusies uit dit proefschrift.

- Thaise borstkankerpatiënten hebben, in vergelijking met vrouwen uit Westerse landen, borsttumoren met een hogere AR-expressie en meer hormonale / HER-2-gevoelige dan hormoon-ongevoelige of drievoudig negatieve ("triple negative") borsttumoren.
- Een co-cultuursyteem van borstkankercellen en primaire fibroblasten is een goed *in vitro* model om werkingsmechanismen te bestuderen waarbij het aromatase-enzym en hormoon-gevoeilige borstkankercellen betrokken zijn.
- Androgenen kunnen de groei van borstkankercellen zowel stimuleren als remmen, afhankelijk van de mogelijke omzetting door aromatase in oestrogenen.
- Natuurlijke chemicaliën, zoals sommige depsidones en melatonine, zijn aromatase-remmers en kunnen mogelijk in de toekomst gebruikt worden als therapeutische behandelingen bieden bij hormoon-gevoelige borstkanker.

Curriculum Vitae

Suthat Chottanapund was born in Bangkok, Thailand, on January 9, 1973. He graduated high school in 1990 at Samsen Withayalai in Bangkok. In 1991 he started his medicine study at Mahidol University, Bangkok, Thailand and in 2004 he obtained his Master of Public Health, Chulalongkorn University. During his M.P.H education, he works as physician in Bamrasnaradura Infectious Diseases Institute, Nontaburi, Thailand. After completing his M.P.H, he started his Ph.D.-research in 2008 on the possible effects of Androgens and selected natural chemicals on human breast cancer cells and the co-culture system, under supervision of Prof. dr. Martin van den Berg and Prof. dr. Majorie B.M. Van Duursen at the Institute for Risk Assessment Sciences (IRAS) at Utrecht University, and Prof. dr. Mathuros Ruchirawat at Chulabhorn Research Institute. This Ph.D.-research has provided important and novel insights in the possible effects of Androgens and selected natural chemicals on human breast cancer cells and the co-culture system, which is described in this thesis.

List of publications

Chottanapund S, Van Duursen MBM, Zwartsen A, Timtavorn S, Navasumrit P, Kittakoop P, et al. Depsidones inhibit aromatase activity and tumor cell proliferation in a co-culture of human primary breast adipose fibroblasts and T47D breast tumor cells. Toxicology Reports. 2017;4:165-71.

Chottanapund S, Van Duursen M, Ratchaworapong K, Navasumrit P, Ruchirawat M, Van den Berg M. Androgen Receptor Expression in Thai Breast Cancer Patients. Medical Sciences. 2016;4(3):15.

Chottanapund S, Van Duursen MB, Navasumrit P, Hunsonti P, Timtavorn S, Ruchirawat M, et al. Anti-aromatase effect of resveratrol and melatonin on hormonal positive breast cancer cells co-cultured with breast adipose fibroblasts. Toxicol In Vitro. 2014 Oct;28(7):1215-21.

Chottanapund S, Van Duursen MB, Navasumrit P, Hunsonti P, Timtavorn S, Ruchirawat M, et al. Effect of androgens on different breast cancer cells co-cultured with or without breast adipose fibroblasts. J Steroid Biochem Mol Biol. 2013 Nov;138:54-62.

Acknowledgements

I would like to thank her royal highness princess Chulaborn Mahidol for gave me opportunity to study in this sandwich program between Chulaborn Graduate Institute and Utrecht University. I would like to thank the my following advisors and co-advisors who supported for this thesis; Prof. dr. Martin Van den Berg, Prof. dr. Mathuros Ruchirawat, Prof. dr. M.B.M Van Duursen, and Dr. Panida Navasumrit.

I could not have started my first co-culture without the help of Sandra Nijmeijer (IRAS, UU) who taught me to isolate the fibroblasts from breast tissue. I am hugely indebted to all members of the Laboratory of Environmental Toxicology at the Chulaborn Research Institute. They helped me out since I first came into the Lab without any experience on cell cultures or PCR work. Many thank to my 2nd batch friends in CGI, we had a lot of fun during the lectures and even more fun during the research. Unbelievable, I spent almost 8 years at CGI, CRI and IRAS, these memories will be with me forever!

Finally, I would like to thank my family; Bum (My wife), Tung, Bai toing and Tata, for their love and support during my study. This in spite of me coming home late from the lab or working in the weekends. Finally, I would like to thank my mom and dad who still wait to see the completion of this Ph.D. thesis.