Effects of Isoflavones on Estrogen Receptor Mediated Estrogenicity and Aromatase Activity

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Abstract

Phytoestrogens are plant derived estrogens that can have a estrogenic effect in humans. A number of women undergoing breast cancer treatment are taking phytoestrogens to relieve postmenopausal symptoms that are the result of breast cancer medication. In literature different effects of phytoestrogens on the Estrogens receptor and aromatase activity are described. These different effects could influence estrogen positive breast cancer, therefore it is important to make a risk assessment for these compounds. This thesis focuses on current literature surrounding a selection of isoflavone phytoestrogens and their relative potencies (REP) on the estrogen receptor (ant)agonism and aromatase interaction (inhibition/induction) in order to determine the most relevant mode of action with respect to estrogen-dependent breast cancer. Most information was available on the isoflavones genistein therefore, this was the main compound studied. Genistein exposure *in vitro* and *in vivo* results in ER mediated estrogenicity. Genistein is able to induce estrogen positive breast tumor proliferation in multiple *in vivo* and *in vitro* experiments. More research needs to be conducted on the effects of genistein on aromatase activity since results on the effect of genistein on aromatase are not conclusive.

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Introduction

Every year many women are diagnosed with breast cancer. In 2011 more than 230,000 women were diagnosed with breast cancer in the United States alone (Siegel et al. 2011). Approximately 75% of breast cancer cases are in postmenopausal women and 70% of the breast cancers are estrogen dependent (Harlan et al. 2002). Endogenous estrogens stimulate tumour growth of estrogen dependent breast tumours. Therefore it is important to decrease the level of endogenous estrogens or prohibit estrogenic signalling in breast tumours (Endogenous Hormones and Breast Cancer Collaborative Group et al., 2011, Yager and Davidson., 2006, Key., 2011). Contemporary treatment uses two types of medication in addition to therapies such as chemotherapy, surgery and radiation. These medications inhibit the effect of the estrogens on the tumour growth. The first are selective estrogen receptor modulators (SERMs) which compete with the estrogen receptor (ER) and thus can be used to inhibit activation by estrogens of the receptor (Wood, Riggs and Hartmann., 2003). The second are aromatase inhibitors, which can be used in postmenopausal women to inhibit the local conversion of androstenedione to estrone or estradiol. Inhibition of aromatase lowers the concentration of endogenous estrogens in tissue (Bhatnagar., 2007). Decreasing endogenous estrogen levels and enabling the effects on the ER however results in side effects. These side effects, also referred to as postmenopausal symptoms, include vasomotor symptoms (hot flushes and night sweats), increased bone loss and cardiovascular risks. To relieve some of these symptoms a number of women take phytoestrogens. Phytoestrogens are naturally occurring plant-based estrogens and can found in e.g. soy products and in dietary supplements. Although these supplements are marketed as having beneficial health effects and menopausal symptom relief, there is little scientific data supporting these arguments (Kurzer., 2003). Studies have been conducted on the effects of Phytoestrogens on estrogen dependent breast tumors. This is relevant since phytoestrogens increase in blood and breast tissue levels after consumption of soy products or supplements (Bolca et al., 2010, King and Bursill., 1998, Watanabe et al., 1998, Hargreaves et al., 1999, Xu et al., 1995, Xu et al., 1994, Morton et al., 1994). Though the affinity of phytoestrogens for the estrogen receptors is lower than endogenous estrogens, they still exert estrogenic effects *in vitro* and *in vivo* (Hsieh et al., 1998). These effects are undesirable for women undergoing breast cancer treatment or those susceptive to breast cancer. Research has also been conducted on the effects of phytoestrogens on aromatase activity. Results between studies however are inconsistent and some show decreased aromatase activity after phytoestrogen exposure, which is a beneficial effect in breast cancer tissue (Van Meeuwen et al., 2007a)(Brooks and Thompson., 2005). Two modes of action of phytoestrogens are investigated in relation to estrogen positive breast cancer; the estrogen receptor and the enzyme aromatase. Both modes must be taken into account in order to conduct a proper risk assessment for phytoestrogens.

This thesis focuses on current literature surrounding a selection of isoflavone phytoestrogens and their relative potencies (REP) on the estrogen receptor (ant)agonism and aromatase interaction (inhibition/induction) in order to determine the most relevant mode of action with respect to estrogen-dependent breast cancer.

1. Background Information

In addition to the evaluation of the most relevant mode of action for isoflavones, this chapter will firstly provide background information on breast cancer and the role of the estrogen receptor and aromatase expression in breast cancer. Secondly, the currently available breast cancer medications will be discussed. Finally, information on phytoestrogens, phytoestrogen intake and relevant *in vivo* phytoestrogen concentrations will be given.

1.1 Estrogens and Breast Cancer

In recent years, it has been accepted that exposure to estrogen leads to an increased risk of developing breast cancer (Chen, Zeng and Tse., 2008). The duration of the exposure and the concentration of the dose are the main factors influencing the risk (Endogenous Hormones and Breast Cancer Collaborative Group et al., 2011). Estrogens are produced in both males and females with the purpose of regulating growth, differentiation and the physiology of the reproduction.

In premenopausal females, the production of estrogens is mainly located in the adrenal cortex and ovaries. Ovaries produce 17β-estradiol (E2) while the adrenal glands produce androstenedione which may be converted to estrone (E1) by the enzyme aromatase in peripheral tissues. Both estrone, estradiol and estriol are endogenous estrogens. In postmenopausal women, the production of 17β-estradiol in the ovaries stops resulting in a decrease of circulating estradiol, however, as androstenedione is still produced in the adrenal glands, its production continues. In peripheral tissues such as adipose tissue, androstenedione is converted into estrone (Bulun and Simpson., 2008)(Figure 1). The circulating estrogens bind two different ER; the ERα and ERβ (Chen, Zeng & Tse 2008). These receptors have a 96% amino acid similarity in their ligand binding domain but only 53% homology (Yager and Davidson., 2006). As a result of the differences in the binding domains, ligands have different binding affinities for both receptors. In breast tumors, ERα increases while ERβ decreases. This ERα increase contributes to the tumor growth. The role of ERβ in breast tumors, however, is still under discussion (Pearce and Jordan., 2004).

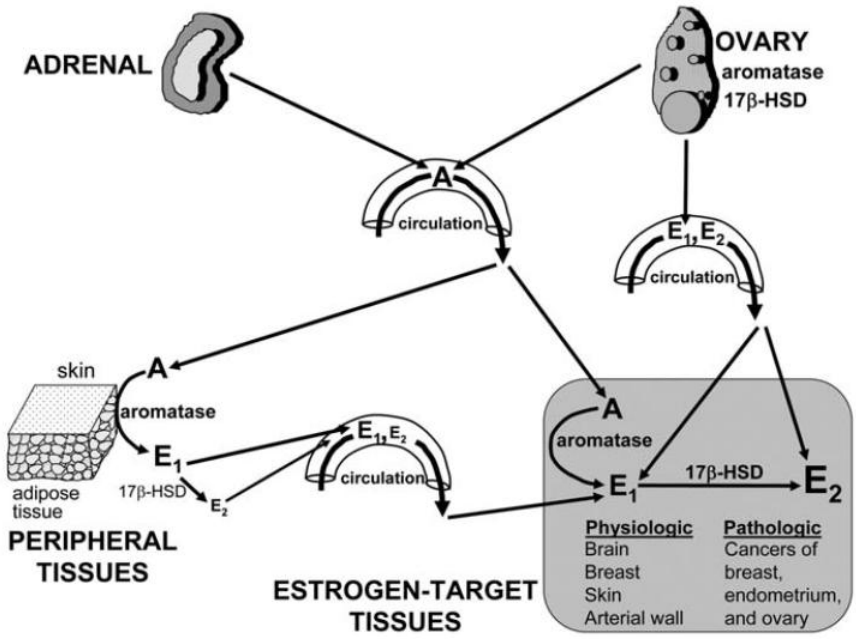


Figure 1. Production of estrogens in the female premenopausal body. 17β-estradiol (E2), estrone (E1) and androstenediones (A) (Bulun and Simpson., 2008).

There are two main pathways assumed to contribute to carcinogenicity of estrogens. Firstly, estrone and estradiol can stimulate proliferation and secondly their metabolites may also produce genotoxic effects (Figure 2). Estrone and estradiol metabolism occurs in various tissues by Cytochrome P-450 enzymes. These metabolites are suggested to be able to initiate, promote or progress breast cancer via damaging DNA and/or covalent binding to the DNA. Estrone and estadiol influence breast cancer by increasing cell proliferation and decreasing apoptosis. The direct effects of estrone and estradiol on breast tumors are mediated by the estrogen receptors ERα and ERβ. The binding of the estrone or estradiol to the ER eventually results in increased cell proliferation and decreased apoptosis. Currently three main estrogen receptor mediated signaling pathways have been identified. Cross-talk occurs between these pathways. Estrogen receptors have been identified in the nucleus, mitochondria and bound to the cell membrane. The binding of estrogens to these receptors results in activation of the signaling transduction pathways (Yager and Davidson., 2006). Stimulation of the three differently located receptors induces altered gene expression and can result in increased cell proliferation and decreased apoptosis.

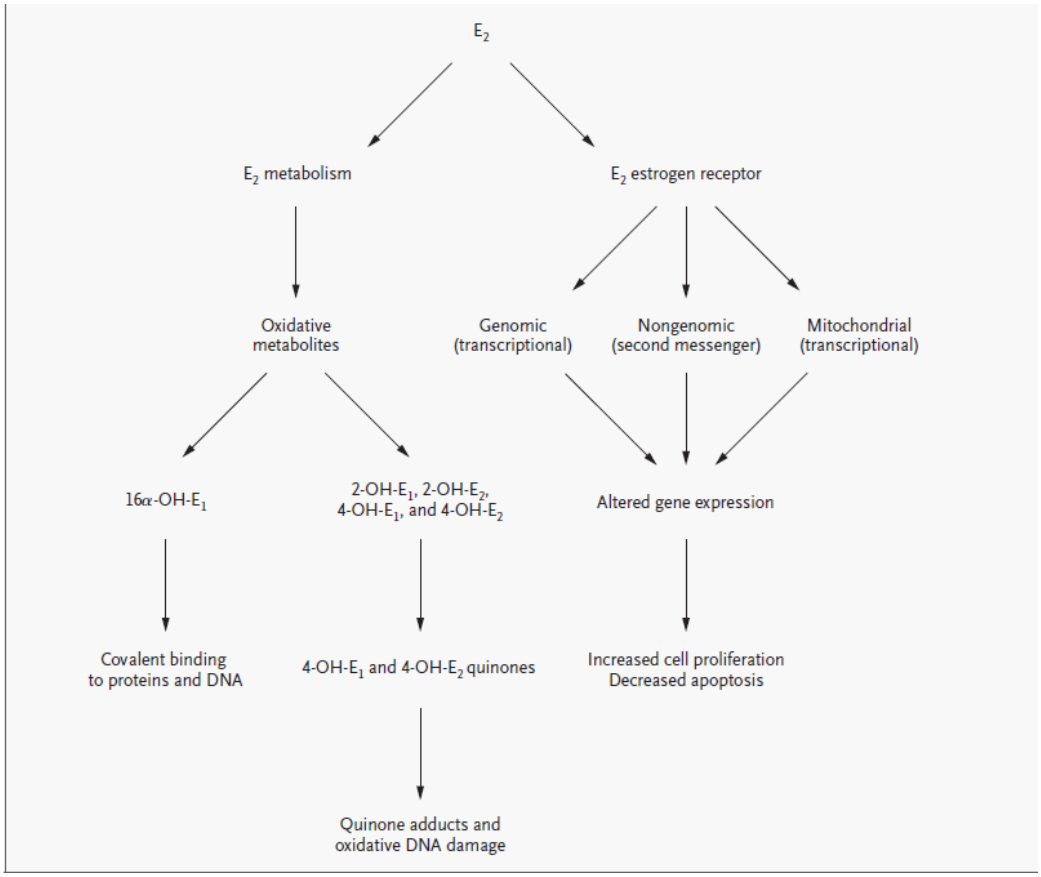


Figure 2 Two pathways that contribute to the carcinogenicity of estrogens. E1 denotes estrone, E2 estradiol, 2-OH-E1 2-hydroxyestrone, 2-OH-E2 2-hydroxyestradiol, 4-OH-E1 4-hydroxyestrone, 4-OH-E2 4-hydroxyestradiol, and 16a-OH-E1, 16a-hydroxyestrone (Yager and Davidson., 2006).

Because the endogenous estrogens can increase proliferation via the ER, the ER is a good therapeutic target to decrease the effect of estrogens on breast tumors. Selective ER modulators (SERMS) are used as adjuvant in breast cancer therapy. SERMS are competitive ER modulators and inhibit the estrogen action. They are agonists of the ER in some tissues and antagonists in others. In breast tissue they exert an antagonistic effect on the ER. Two examples of these medications are tamoxifen and raloxifene. When estrogens bind to the ER, a conformational change takes place and this leads to dimerization of the receptor. The dimer is able to directly interact with the estrogen responsive element (ERE). The binding of estrogen facilitates the recruitment of coactivators. The binding of tamoxifen leads to recruitment of corepressors and after binding of tamoxifen the conformational state of the receptor is different compared to the dimerized state (Figure 3) (Wood, Riggs and Hartmann., 2003, Bhatnagar., 2007). By inhibiting the estrogen action, tumor growth decreases. There are several groups of SERMs. Tamoxifen is a triphenylethylene and whereas Raloxifene is a tenzotiophene. Both these medications are used for treatment of estrogen positive breast cancer (Chen, Zeng and Tse., 2008).

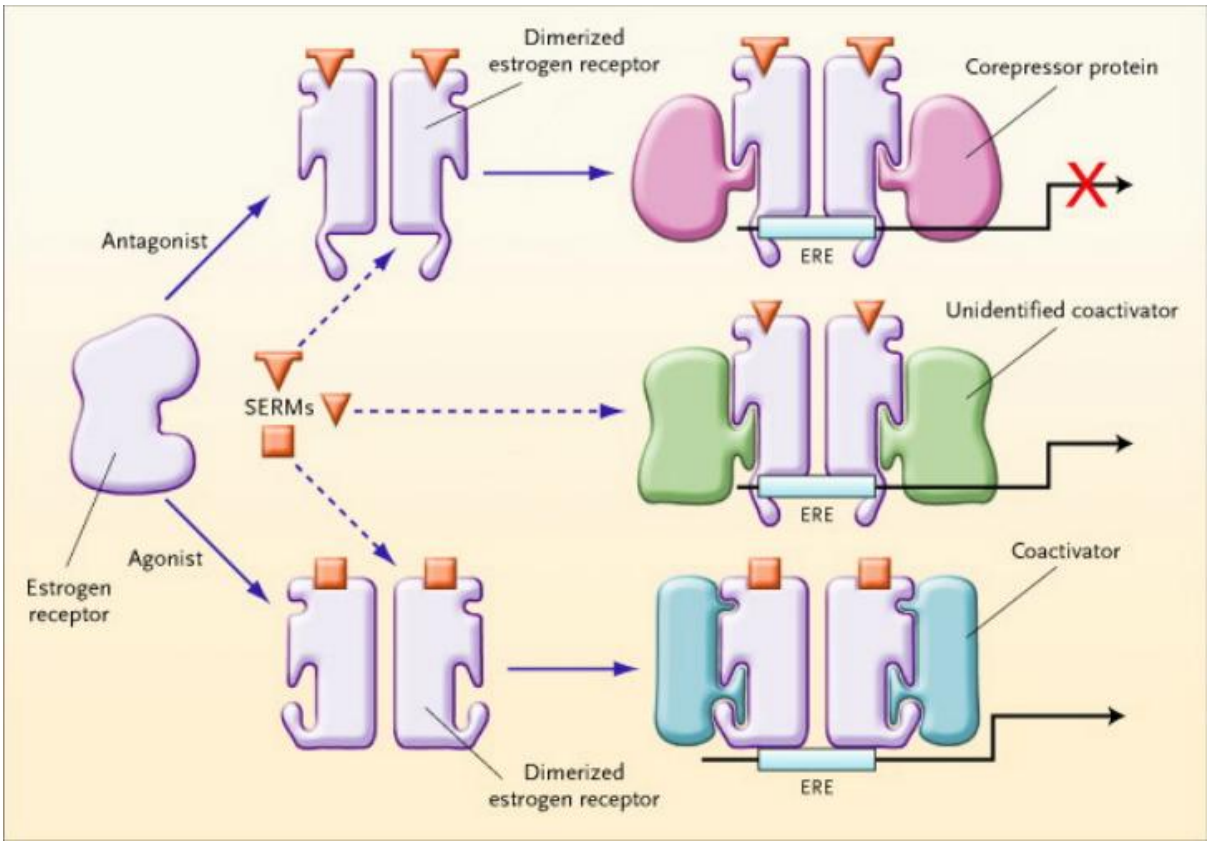


Figure 3. Working mechanism of selective ER modulators (SERMs)(Wood, Riggs and Hartmann., 2003)

1.2 Aromatase and Breast Cancer

Aromatase is a rate limiting enzyme in estrogen biosynthesis in women. Aromatase is encoded by a single gene –CYP19 – and is located on human chromosome 15. Aromatase converts androstenedione to estrone and testosterone to estradiol. The expression of the aromatase gene occurs in various tissues including the ovaries and in peripheral tissues such as adipose tissue. In post menopausal women, the peripheral estrogen production plays a dominant role in stimulating the proliferation of breast tumors. Since estrogen production is dependent on aromatase activity, aromatase

In breast tissue there two cell types the adipose stromal fibroblast and malignant epithelial cells, that are known to have paracrine interaction. In normal healthy breast tissue, adipose stromal fibroblasts express aromatase and mRNA is transcribed by the I.4 aromatase promoter. Activation of this promoter, results in low levels of aromatase and therefore normal levels of estrone and estradiol are maintained. In breast tumor tissue a different set of aromatase promoters is activated. These are the active promoters I.3, II and I.7 which result in increased aromatase expression (Bulun and Simpson., 2008, Bulun et al., 2005) (see Figure 4). This increase in aromatase expression results in local increased production of estradiol and estrone in breast tumor tissue. This stimulates the proliferation of the breast tumor and inhibits apoptosis (Chen et al., 2009).

In more detail, the activation of the set of aromatase promoters I.3 and II in breast tumors is induced by a paracrine interaction between malignant epithelial cells and adipose stromal cells. As a result of prostaglandin E_2 (PGE2) secretion by the malignant epithelial cells (Zhao et al., 1996). In healthy breast tissue differentiated adipose stromal cells do not express aromatase. The undifferentiated adipose stromal cells, the pre-adipocytes and/or adipose fibroblasts express aromatase by promoter I.4 activity. In breast cancer tissue, malignant epithelial cells secrete cytokines. These cytokines prevent the pre-adipocytes from differentiating to mature adipose stromal cells. In breast cancers, there are more undifferentiated adipose fibroblasts which results in more aromatase. The undifferentiated cell fibroblasts make a promoter switch from the I.4 to the I.3 and II promoter. This promoter switch is a result of prostaglandin E_2 (PGE2) secretion by the malignant epithelial cells. In addition to the increased aromatase expression in the undifferentiated fibroblasts in breast cancers, aromatase is also expressed in the endothelial cells by promoter I.7 activity. Thus, the promoter activity of aromatase differs between healthy breast tissue and malignant tissue which indicates that aromatase is a good target for breast cancer therapy. However, inhibition of aromatase activity has side effects such as bone loss. Limiting the activation of promoters I.3 and II the favorable option for future therapy due to its specificity (Chen et al., 2009).

Aromatase inhibition is a good endocrine therapy in postmenopausal women with estrogen receptor positive breast cancer. This hormonal therapy is an adjuvant to the main therapy. There are three aromatase inhibitors that are often prescribed: anastrozol (Arimidex®), exemestane (Aromasin®) and letrozole (Femara®). letrozole works directly on the aromatase enzyme as a non-steroidal drug which mimics the steroidal backbone of androstenedione. Letrozole mimics the natural substrate of aromatase and is able to bind aromatase. By binding the iron of the heme moiety of the aromatase

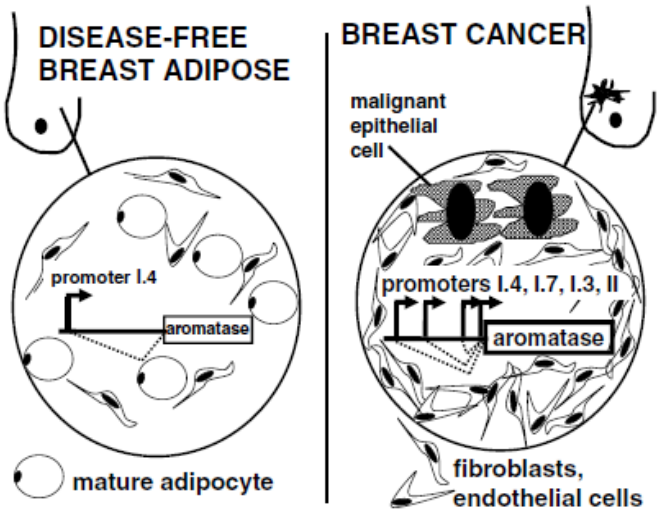


Figure 4 different aromatase expression in healthy and malignant breast tissue. Left: in the healthy breast tissue aromatase is primary expressed in the adipose stromal fibroblasts by the weak I.4 promotor. Right: in malignant breast tissue aromatase promoters I.3 and II are activated and malignant epithelial cells and adipose fibroblasts give an increased aromatase expression via these promotors. Promotor I.4 activity is enhanced by cytokines secreted by malignant epithelial cells. The excreted cytokines also inhibit adipogenic differentiation this results in more aromatase expressing adipose fibroblasts via the I.4 promotor. In the breast cancer endothelial cells aromatase is expressed via the I.7 promotor (Khan et al. 2011).

cytochrome p450 subunit letrozole inhibits the aromatase activity. When bound by letrozole,

aromatase is not able to convert androstenedione to estrone or estradiol (Figure 5) (Bhatnagar., 2007, Khan et al., 2011).

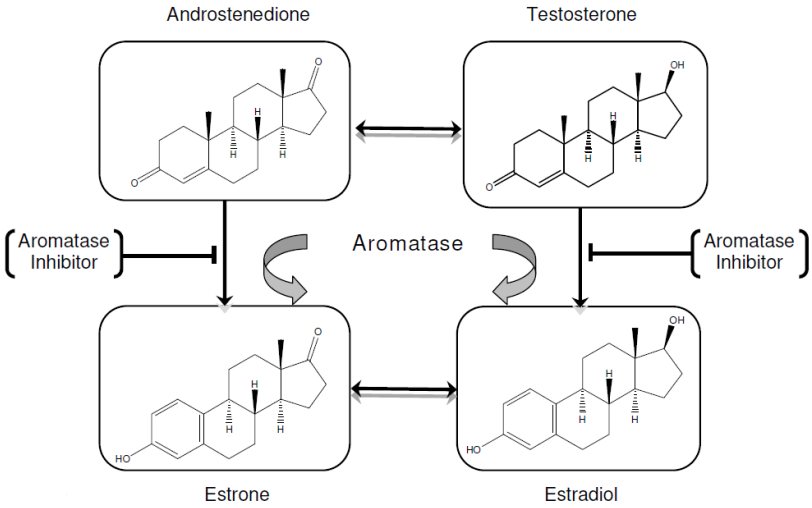


Figure 5 The reaction catalyzed by aromatase and the point of interference by the aromatase inhibitors in the reaction (Khan et al., 2011).

1.3 Phytoestrogens

Phytoestrogens are plant derived estrogens which can have either estrogenic or anti-estrogenic effects on humans. Their structural similarity to human endogenous estrogens may produce these (anti)estrogenic effects in humans by binding to ER (Figure 6 and Figure 7). Phytoestrogens are generally less potent ER agonists than endogenous estrogens (Hsieh et al., 1998).

In the case of breast cancer treatment, SERMs and aromatase inhibitors decrease estrogenic responses in women. This causes postmenopausal symptoms such as vasomotor symptoms (hot flushes and night sweats), increased bone loss and cardiovascular risks. Many women take phytoestrogens to try to alleviate these symptoms. Phytoestrogens are believed to replace the endogenous estrogens and thus potentially decreasing these symptoms. However, literature on the effectiveness of phytoestrogens to relieve postmenopausal symptoms is inconsistent and more research is required to investigate these. Despite the inconsistent results between studies, consumption of phytoestrogen supplements remains high (Kurzer., 2003, Sacks et al., 2006).

There are three major groups of phytoestrogens: isoflavones, lignans and coumestans. Isoflavones are present at high concentrations in soy and red clover. Isoflavones, including genistein, diadzein, biochanin A and formononetin, are all found in soy products. (Borrelli and Ernst., 2010).

This thesis specifically focuses on isoflavones and in particular genistein. This is because more research has been conducted on genistein compared to the other isoflavones. Because of many data on genistein and the effects it is a suitable compound to assess the most important mode of action of this compound. One such effect is that genistein binds to the ER with an affinity that is approximately 100-fold lower than estradiol producing an estrogenic effect (Hsieh et al., 1998).

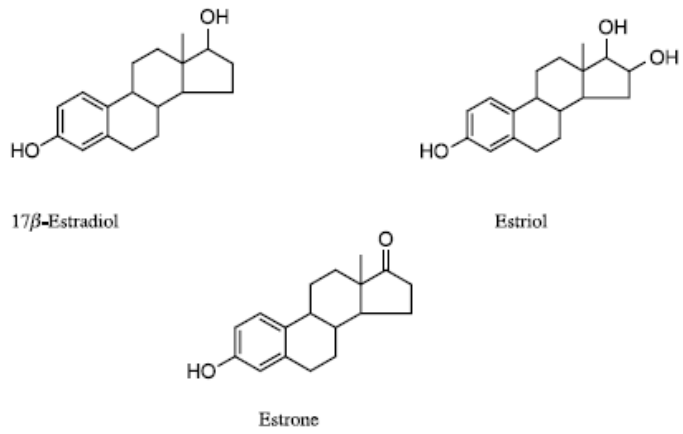
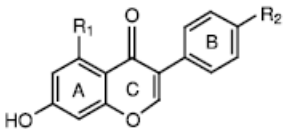


Figure 6 The structures of mammalian endogenous estrogens (Ososki, Kennelly 2003).



Isoflavone	R_1	R_2
Biochanin A	OH	OCH_3
Daidzein	H	OH
Formononetin	H	OCH_3
Genistein	OH	OH

Figure 7 The structures of isoflavones (Ososki and Kennelly., 2003).

1.4 Human Phytoestrogen Intake and Concentrations

To determine relevant concentrations of isoflavones for *in vivo* and *in vitro* research, the isoflavone concentration range present in human blood plasma and breast tissue must be established. Exposure to isoflavones comes from the consumption of soy products and/or concentrated supplements. These products contain varying concentrations of isoflavones and are not necessarily consumed at set amounts. The concentration of isoflavones in blood plasma and breast tissue is dependent on uptake, metabolism and many other factors which differ between individuals (Breinholt and Larsen., 1998, Peterson et al., 1998). Therefore *in vivo* human concentration ranges are used to investigate relevant research concentrations instead of average blood/breast tissue values or intake values. Human plasma genistein concentrations can approximately vary between 0.1 and 6uM (Bolca et al., 2010, King and Bursill., 1998, Watanabe et al., 1998, Hargreaves et al., 1999, Xu et al., 1995, Xu et al., 1994, Morton et al., 1994). Approximately 6 hours after intake the plasma levels of genistein can

increase significantly (Figure 8) (Watanabe et al., 1998, Xu et al., 1995) Genistein has also been found in breast tissue (92.33 to 493.8 pmol/g) and nipple aspirate (approximately 400 ng/ml) confirming that, isoflavones are able to reach the breast and thus, exert effects on breast tissue (Bolca et al., 2010, Hargreaves et al., 1999).

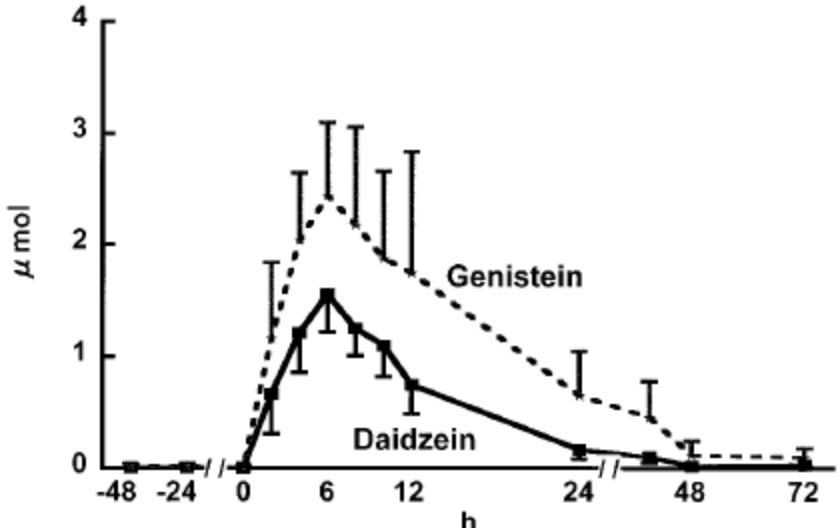


Figure 8 Plasma genistein and daidzein concentrations in men after ingestion of 60 g kinako (baked soybean powder). The plasma levels of genistein and daidzein reached their highest plasma levels after 6 hours. Each point represents the mean +/- SD, n= 7 (Watanabe et al., 1998)**.**

2. Estrogenicity of the Isoflavones and Their Effect on Aromatase Activity

Different endpoints of the estrogenic receptor mediated effects were monitored in the different studies. Some common methods to measure the estrogenic receptor mediated effects are: determining the estrogenic effect of the isoflavones in different assays by measuring pS2 mRNA. The transcriptional activity of the pS2 gene is an adequate measurement to assess the estrogenicity of the isoflavones since the transcriptional activity of the pS2 gene is a primary response in the MCF-7 cell line to estrogenic compounds (Soto et al., 1997).

Another possibility is an MCF-7 cell line with a luciferase reporter gene which is under control of the estrogen regulatory element (ERE). Proliferation was often measured by Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) incorporation in the DNA during DNA replication and monitoring tumor size (DeFazio et al., 1987). The effects of genistein on aromatase can be determined by measuring the mRNA expression or the enzyme activity with different techniques (Lephart and Simpson., 1991).

2.1 In vitro

This chapter provides an enumeration of the currently available literature on *in vitro* research. The different effects of genistein on the aromatase activity and ER *in vitro* will be discussed. First the

current literature on the effects of isoflavones on aromatase activity will be discussed and secondly the estrogenicity via the ER.

2.1.1 Aromatase

The effect of isoflavones and genistein on aromatase activity is mainly investigated *in vitro.* Research on the effects of isoflavones on aromatase activity is done in many different cell assays (Van Meeuwen et al., 2007a, Brooks and Thompson., 2005, Pelissero et al., 1996, Wang et al., 2008, Le Bail et al., 2000, Campbell and Kurzer., 1993, Ju et al., 2008, Whitehead and Lacey., 2003, Edmunds et al., 2005, Sanderson et al., 2004, Myllymaki et al., 2005, Almstrup et al., 2002). Different isoflavones were tested in a concentration range varying from 0.01 till 100 µM. Different articles find different effects of isoflavones on aromatase activity.

Some articles describe the aromatase inhibitory effect of genistein. For example, Brooks *et al,* 2005 measured estrone production *in vitro* in MCF-7 cells and found that 10 µM genistein was able to reduce estrone production by 70%. Though these MCF-7 wild type cells are not transfected with aromatase it has been determined that there is enough aromatase expression to elicit an estrogenic response in this study (Burak et al., 1997). The regulation of aromatase is tissue-specific and also changes when breast cancer develops. Therefore it was preferred by brooks et al, to examine the effects of isoflavones in wild-type MCF-7 cells. The inhibitory effect of genistein on aromatase resulted in a reduced estrogen production and this was related to the reduced cell proliferation (Brooks and Thompson., 2005).

Additionally, Pelissero *et al* (1996), found that biochanin A and genistein were able to slightly inhibit the aromatase activity in the ovarian of rainbow trout. The study was conducted *in vitro* and the IC50 values were determined by extrapolation of the data. The extrapolated IC50 values for Biochanin A and Genistein were 2200 µM and 3500 µM respectively.

Previously discussed articles find inhibition of aromatase with relatively high isoflavones concentrations (Pelissero et al., 1996, Le Bail et al., 2000). However, Almstrup *et al,* (2002) found that in MCF-7 cells, isoflavones can have an aromatase inhibitory effect at low concentrations and an estrogenic effect at higher concentrations this resulted in U-shaped dose-response curves. The isoflavones genistein, biochanin A and formononetin were tested in MCF-7 cells *in vitro*. pS2 mRNA and cell proliferation were monitored. To calculate the relative response, the pS2 mRNA response to 100 nM testosterone was set as 100%. 0.1 µM Biochanin A was able to reduce pS2 mRNA resulting from testosterone exposure and gave an induction of 64.5% pS2 mRNA. But exposure to 10 µM resulted in 109.5% induction of pS2 mRNA and thus gave an estrogenic response. Formononetin was also able to inhibit aromatase but was less potent than biochanin A. Genistein however did not inhibit aromatase at all (Figure 9)(Almstrup et al., 2002). More information on ER mediated estrogenicity will be discussed in the next chapter.

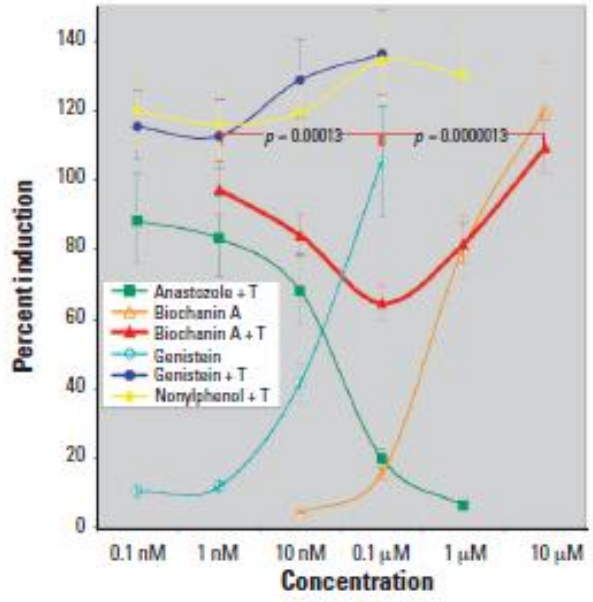


Figure 9 Expression levels of the estrogeninduced pS2 mRNA in MCF-7 cells after incubation with the indicated concentrations of the different compounds in the culture media. All values are shown as percentage relative to the response to 100 nM testosterone (T), which we set to 100%. Each point represents the mean of four independent samples; error bars indicate SEM. The *p*-values indicate the statistical significance of the decline between the 1 nM and 0.1 µM data points of the biochanin A plus testosterone curve and the increase between 0.1 µM and 10 µM data points, thus representing the U shape (Almstrup et al., 2002)**.**

Although Brooks et al (2005), Pelissero (1996) and Almstrup et al (2002), describe inhibitory effects of genistein on the aromatase activity, multiple assay could not find an inhibitory effect of genistein on aromatase activity. A first example is a study conducted by Wang *et al* (2008). Genistein was found not to influence aromatase activity however, Biochanin A from red clover was found to inhibit aromatase activity and expression *in vitro* in MCF-7 cells. These MCF-7 cells were stably transfected with *CYP19*. Biochanin A displayed an inhibitory effect on aromatase in the MCF-7 cells with an IC50 value of 8 µM. The testosterone-induced proliferation in the MCF-7 cells was also inhibited as a result of aromatase inhibition by biochanin A. 12.5 µM biochanin A was enough to significantly reduce proliferation. Exposure to 25 µM biochanin A brought the proliferation back to levels comparable with the cell growth in absence of testosterone. The promoter activity was also investigated in MCF-7 cells and SK-BR-3 cells. Though SK-BR-3 cells are a breast cancer ER negative cell type they use the promoters I.3 and II for aromatase transcription. A concentration of 50 µM biochanin A was able to suppress the promoter activity in the SK-BR-3 cells. Besides biochanin A, also the isoflavones genistein and diadzein were tested. However, these isoflavones were not able to inhibit aromatase activity but it might suppress the I.3 and II promoter activity (Wang et al., 2008)

Additionally, Le Bail et al (2000), measured aromatase activity in human placental microsomes. None of the isoflavones were able to inhibit aromatase activity except biochanin A, which had an IC50 value of 49 µM. Hypothesized is that the methoxylation in position 4'of the biochanin A molecular structure increases the anti-aromatase activity over the other isoflavones. Campbell and Kurzer (1993), used a human preadipocyte cell culture to investigate the effect of isoflavones on aromatase. This cell culture was thought to be a relevant model since adipose tissue is a significant source of estrogen production in postmenopausal women. Diadzein and genistein were not able to inhibit

aromatase activity in an *in vitro* human preadipocyte cell culture system. However, Biochanin A was able to inhibit aromatase and had an IC50 of 113 µM (Campbell and Kurzer, 1993).

The aromatase inhibitory property of biochanin A was also investigated in human granulose-luteal cells. The cells were exposed for 24 hours to 10 umol/L biochanin A. However, in this research there was no significant inhibition on aromatase activity found (Whitehead and Lacey., 2003)

Besides no effects, or inhibiting effects of genistein on aromatase, there are also examples of genistein stimulating aromatase activity. Genistein can stimulate the aromatase activity in human endometrial stromal cells (ESC) with the concentration range of 1nM to 1mM. Resulting in a aromatase activity of approximately 100-125% compared to the untreated cells (Edmunds et al., 2005). Genistein was also tested *in vitro* in H295R human adrenocortical carcinoma cells expressing aromatase by Sanderson *et al,* 2004. The H295R cell line contains the I.3 and II aromatase promoter as do breast cancer cells. This makes this cell line a useful assay to investigated aromatase activity. Genistein was able to induce increased CYP 19 mRNA expression. The aromatase activity induction was 2.5 fold at a concentration of 10 µM. Besides the increased aromatase activity the mRNA levels for the specific PII and to a lesser extent the 1.3 promoter were increased. (Sanderson et al., 2004).

Another example of increased aromatase activity was found *In vitro* in immature rat ovarian follicles. Genistein is also able to concentration–dependently increase aromatase activity in this assay. At concentrations of 10^{-7} and 10^{-6}M the aromatase activity was concentration-dependently significantly increased. Aromatase activity was increased with approximately 1.5 and 2.5 fold at 10E-7M and 10E-6M, respectively compared to the vehicle control (Myllymaki et al., 2005).

Furthermore, genistein was able to induce aromatase activity in hepatic cells HepG2. The cells were exposed to 0, 0.1, 1.0 and 10 µM genistein. The concentrations of 1 and 10 µM genistein were able to significantly increase the aromatase activity in the HepG2 cells approximately 1.5 to 2 fold, respectively. As a result of exposure to 10 µM genistein, the mRNA of aromatase and elevated the usage of promoters II and I.3. (Ye, Chan and Leung., 2009).

2.1.2 ER

Though there are many different effects found of isoflavones on the aromatase activity, the effects found on the ER are more consistent. Also there is less variety in the used assays to conduct experiments with the ER and isoflavones compared to the amount of different assays used to investigate the effect on aromatase activity. The MCF-7 assay was used in most experiments although sometimes a co-culture was used. More information on the co-culture will be discussed in the next chapter. Many articles find a ER mediated estrogenic effect as a result of genistein exposure. The concentrations differ but already at low concentrations estrogenicity is found.

Concentrations as low as 0.01 – 0.1 µM were able to induce proliferation with approximately 1.5 and 2.4 fold over the vehicle control in MCF-7 cells. This was comparable to the effect of 1 nM estradiol (Figure 10). Dose dependent increase in tumor proliferation and pS2 mRNA expression were found as a result of exposure to 0.01 – 10 µM genistein. However higher concentrations 25-100 µM genistein resulted in a dose-dependent decrease of proliferation compared to the controls (Hsieh et al., 1998).

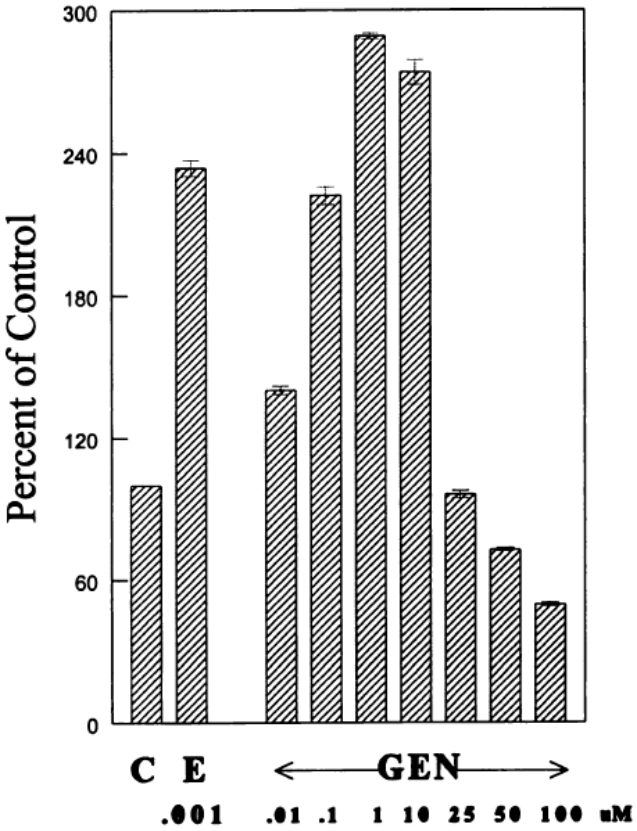


Figure 10. Dose dependent increase of proliferation in MCF-7 cells after 0.01 – 10µM genistein exposure. Genistein concentrations above 10µM gave a dose dependent decrease of proliferation compared to the control. C: vehicle control and E: 1nM estradiol (Hsieh et al., 1998).

The results of Le Bail, 2000 also support the ER mediated estrogenicity of genistein. Biochanin A, Genistein, daidzein and formononetin were tested for ER interaction with a luciferase reporter gene assay (MVLN) and their effect on MCF-7 cell proliferation. The phytoestrogens caused a concentration-dependent ER mediated estrogenicity. Additionally, proliferation was stimulated by the isoflavone exposure with maximum levels at 1 or 10 µM. Genistein gave an ER mediated luciferase induction of 8.7-106.8% over the estradiol control as a result of exposure to 0.1-50 µM genistein respectively. Proliferation in the MCF-7 cells was in a range of 62.8-109.2% over the estradiol control as a result of exposure to 1-10 µM genistein respectively. A concentration of 50 µM genistein resulted in a decrease in proliferation compared to the control (Le Bail et al., 2000).

2.1.3 Co-culture

A new promising model to study the effect of phytoestrogens is a co-culture. A co-culture mimics the *in vivo* situation were breast cancer cells and fibroblast are present and communicate with cytokines, prostaglandins and estradiol. In short, in human breast cancer the MCF-7 cells produce PG2 resulting in increased aromatase transcription in the breast adipose fibroblasts (BAFs) because of a promoter switch from the I.4 to the I.3 and II promoter. The estrogens produced by the BAFs as a result of increased aromatase influence the proliferation of the MCF-7 cells by binding the ER. Though, still experimental, with the co-culture it might be possible to include this paracrine positive feedback

loop. In these co-cultures both the effect of genistein on aromatase activity in BAFs and ER mediated estrogenicity in MCF-7 can be measured.

Van Meeuwen (2007), developed a co-culture of MCF-7 cells and fibroblast and tested the estrogenic effect of isoflavones. First, MCF-7 cells and fibroblast were tested in mono-culture. The effect of genistein on cell proliferation (pS2 mRNA) but also aromatase activity and mRNA was investigated. The proliferation potency on the MCF-7 cells had an EC50 of 32 nM and inhibition of aromatase in fibroblasts had an IC50 of 3.6 µM. Genistein was not investigated in the co-culture but other phytochemicals were included. Results of the mono and co-culture showed an overlap of the concentration range of phytochemicals for the inhibition of aromatase activity and ER mediated estrogenicity.

Additionally, van Duursen et al (2011), used a H295R cell line, MCF-7 cells and MCF-7 co-culture. This MCF-7 co-culture consists of the MCF-7 cells with the ER and breast adipose fibroblasts (BAF) that express aromatase via the PI.4 promoter. Genistein was able to induce estrogen dependent MCF-7 cell growth. The proliferation was concentration-dependently increased with an EC50 value of 11.2 nM compared to the exposure to estradiol. pS2 mRNA was determined after exposure to testosterone (20nM) and genistein (3µM) and was significantly increased with 2 fold compared to testosterone exposure. Genistein concentration-dependently increased the aromatase activity in the H295R cell line resulting in a EC50 value of 26 µM. The effect of genistein on the MCF-7 co-culture with BAFs resulted in activation of the ER in the MCF-7 cells and increased aromatase activity in the BAFs. The increased aromatase activity resulted in increased formation of estrogens which could also increase the ER mediated tumor growth in the MCF-7 cells. The induction of pS2 in this co-culture after testosterone (20nM) and genistein (3 µM) exposure was 3.4-4.5 fold which is more increased than the pS2 of the monoculture.

Summary

In summary, many different *in vitro* assays were used to determine the effect of isoflavones on the aromatase activity. Most frequently used were a MCF-7 cell line and the H295R human adrenocortical carcinoma cell line (Hsieh et al., 1998, Van Meeuwen et al., 2007a, Brooks and Thompson., 2005, Wang et al., 2008, Le Bail et al., 2000, Sanderson et al., 2004, Van Duursen et al., 2011). Besides mono-cultures, promising co-cultures with MCF-7 cells and BAFs are developed that are more comparable to the human breast cancer tissue because of paracrine signaling between the different cells (Van Meeuwen et al., 2007a, Van Duursen et al., 2011). The results found by different studies on the effect of genistein on aromatase activity are variable. Three different results were found for the effect of genistein on the aromatase activity. Genistein has been found to have no effect, an inhibitory effect or a stimulating effect on aromatase activity. Measured or extrapolated IC50 values for aromatase inhibition by genistein were approximately 3.6-3500 µM (Van Meeuwen et al., 2007a, Brooks and Thompson., 2005, Pelissero et al., 1996). Increased aromatase activity was found as a result of 0.01-10 µM genistein exposure. The increased activity varied between 1.5-4.5 fold compared with aromatase activity in vehicle control treated cells (Edmunds et al., 2005, Sanderson et al., 2004, Myllymaki et al., 2005, Ye, Chan and Leung., 2009). Furthermore, *in vitro* experiments were used to research the ER mediated estrogenicity of genistein. In MCF-7 cells and co-culture concentration of 0.01-10 µM genistein resulted increased proliferation. Although, previously discussed articles all find estrogenic effects as a result of genistein exposure, higher concentrations

starting at 25-50 µM can give a decrease in proliferation (Hsieh et al., 1998, Le Bail et al., 2000). An overview of the different *in vitro* experiments is presented in Table 2.

2.2 In vivo

The estrogenicity of isoflavones was also investigated in several *in vivo* mice studies. MCF-7 cells were implanted in athymic mice and tumor proliferation was monitored. Genistein blood plasma levels were measured in several studies and indicates the approximate exposure concentration of the tumor cells. Plasma concentrations in mice varied from 0.39-5.92 µM (Hsieh et al., 1998, Ju et al., 2008, Ju et al., 2001).

Exposure to 125-1000 µg/g dietary genistein resulted in plasma levels of 0.39 to 3.36 µmol/L in mice. Dietary exposure to 250 µg/g and higher concentration induced significant growth, proliferation and pS2 expression of the tumor in a dose dependent manner (Figure 11). Above a concentration of 500 µg/g the pS2 expression significantly increased 11.25 times the control (normal diet without genistein). Also the exposure to 1000 µg/g genistein resulted 15.84 times more pS2 mRNA compared to the control (Ju et al., 2001).

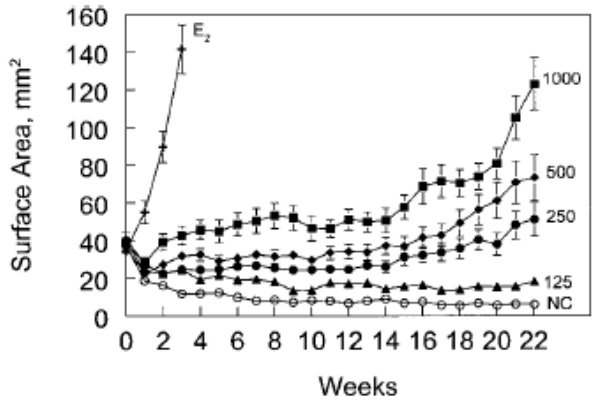


Figure 11 Increased tumor growth in athymic mice implanted with MCF-7 cells. At the time when estradiol pellets were removed from all of the mice except the PC group, mice were assigned into six treatment groups: PC (estradiol) (10 mice; *n* 5 34 tumors), NC that were fed AIN-93G diet alone (13 mice; *n* 5 50 tumors), AIN-93G 1 125 mg/g genistein (11 mice; *n* 5 42 tumors), AIN-93G 1 250 mg/g genistein (10 mice; *n* 5 39 tumors), AIN-93G 1 500 mg/g genistein (10 mice; *n* 5 39 tumors) and AIN-93G 1 1,000 mg/g genistein (9 mice; *n* 5 35 tumors) Day 0 was the first day that mice began consuming the experimental diets. (Ju et al., 2001).

Allred *et al* (2001), conducted a similar experiment. Allred *et al* investigated the effect of genistein on breast cancer growth. The experiments were conducted *in vivo* in ovariectomized athymic mice. MCF-7 cells were implanted in the mice and exposed to genistein by addition to the diet. The genistein content of the diet was 15, 150 or 300 µg/g. Cell proliferation and pS2 mRNA were evaluated. The varying amounts of genistein increased tumor growth in a dose dependent manner. The proliferation of the tumors was greatest when the mice were exposed to diets containing 150-300 µg/g genistein and also the pS2 expression was increased in these tumors. Besides the genistein exposure mice were also exposed to diets with soy isolates with a genistein content of 150-300 µg/g genistein. This exposure also resulted in a dose dependent increase in tumor growth. This shows that not only genistein but also soy isolates containing genistein can increase MCF-7 tumor growth.

Besides the discussed *in vivo* experiments many similar experiments were conducted. Al these experiments confirmed a tumor growth and increased proliferation as a result of *in vivo* genistein exposure after a dietary intake of 250-750 µg/g genistein (Hsieh et al., 1998, Ju et al., 2008).

Contradicting, are the effects of soy phytochemical concentrate with Green tea exposure containing genistein. Soy and bioactive tea components were tested on breast tumor progression *in vivo* in female SCID mice. The combination of soy phytochemical concentrate with green tea resulted in dose dependent inhibition of tumor growth via inhibition of cancer cell proliferation. The disadvantage of this study is that it gives an inconclusive answer regarding to which compound or combination of compounds is responsible for the inhibiting effect (Zhou et al., 2004).

Summary

In summary, *in vivo* conducted experiments with genistein exposure all confirmed dose dependent estrogenic effects of genistein on tumor growth and proliferation (Hsieh et al., 1998, Ju et al., 2008, Ju et al., 2001, Allred et al., 2001). However, exposure to soy phytochemical concentrate and green tea showed a dose dependent inhibition on tumor growth. An overview of the different in vivo results is presented in Table 1.

2.3 Genistein Interaction with Medication

Phytoestrogens are used by women as complementary alternative medicine during breast cancer treatment. Therefore not only the effect of Phytoestrogens on breast cancer should be investigated but also the effect on breast cancer in combination with medication. Some research has been conducted on genistein in combination with SERMs or aromatase inhibitors.

Fadrozole is an aromatase inhibiting drug and was tested in a co-culture in combination with genistein. Genistein was able to negate the inhibitory effect of fadrozole on aromatase. (Van Duursen et al., 2011). Furthermore genistein counteracts the effect of letrozole on MCF-7Ca cells. The MCF-7Ca cells were implanted in ovariectomized mice. The effect of dietary genistein of 250-1000 p.p.m. on tumor growth was investigated in combination with the aromatase inhibitor letrozole. The dietary intake resulted in a total plasma concentration range of 0.99-5.92 µM. In Figure 12 it is shown that the dietary genistein increased tumor growth even tough letrozole is present. In the MCF-7Ca cells exposed to androstenedione and letrozole genistein was able to increase tumor growth, cellular proliferation, plasma E2 levels, expression of pS2 mRNA. The tumor growth in the experiments correlates with the circulating genistein levels. For this reason it is expected that ER agonist activity might be involved in genistein stimulating tumor growth (Ju et al., 2008).

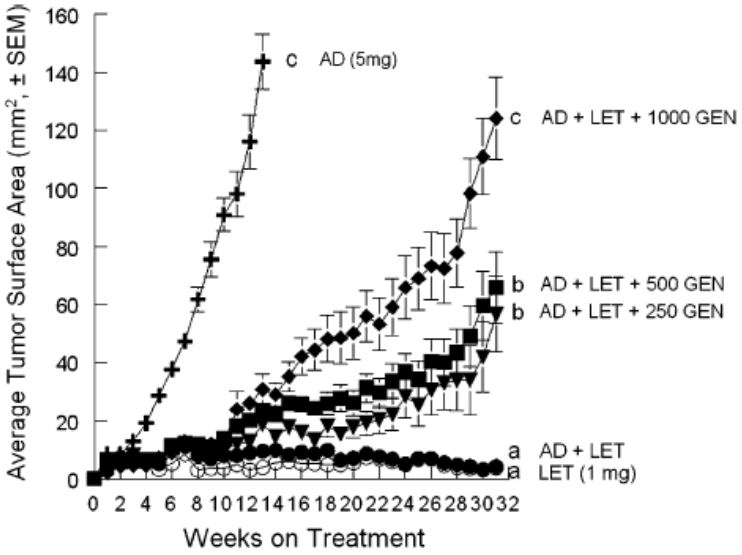


Figure 12 Effect of dietary GEN in the presence of AD and LET on the growth of MCF-7Ca cells implanted in ovariectomized athymic mice. Animals were randomly assigned into five treatment groups: LET (12 mice; n 5 15 tumors), AD (12 mice; n 5 24 tumors), AD þ LET (12 mice; n 5 17 tumors), AD þ LET þ 250 GEN (15 mice; n 5 20 tumors), AD þ LET þ 500 GEN (15 mice; n 5 24 tumors) and AD þ LET þ 1000 GEN (14 mice; n 5 25 tumors). Bars with different letters are significantly different, P , 0.05 (Ju et al., 2008).

Thus genistein can counteract the effect of letrozole and fadrozole. However genistein in combination with tamoxifen (TAM)(SERM) results in synergistically or additively delayed breast tumor growth in MCF-7 cells *in vitro* and *in vivo* in immunodeficient mice. The combination of genistein and tamoxifen decreased estrogen levels and activity. First, TAM and genistein were tested separately. 6 µM of TAM inhibited the growth of the MCF-7 cells by 61%. Genistein was able to inhibit the growth of the MCF-7 cells as well. 50 µM genistein inhibited the growth with 86%. Another isoflavones, daidzein was tested but was not a strong inhibitor as TAM or genistein. A concentration of 50 µM daidzein inhibited the growth in MCF-7 cells with 36%. When the cells were exposed to the combination of TAM and genistein (3 and 25 µM respectively, and 6 and 50 µM, respectively) there was an increased inhibition found compared to the single compounds. The exposure to the combination of TAM and genistein results in a more potent growth inhibition than the exposure to the single compounds. The results show that phytochemicals might have beneficial effects in breast cancer when taken with tamoxifen (Mai, Blackburn and Zhou., 2007).

Table 1 Overview of genistein effects *in vivo*

reference	assay	plasma concentration mice	compounds	effect	dietary intake
in vivo					
Ju *et al*, 2001	MCF-7 cells	0.39-3.36 µM	genistein	dose dependent tumor growth and increased pS2 mRNA and proliferation	250-1000 µg/g
Allred *et al*, 2001	MCF-7 cells	-	genistein	dose dependent tumor growth in creased pS2 mRNA and proliferation	150-300 µg/g
Ju *et al*, 2008	MCF-7 cells	0.99-5.92 µM	genistein	stimulation tumor growth and increased pS2 mRNA	250-500 µg/g
		0.99-5.92 µM	genistein	no significant increase in tumor growth or pS2 mRNA	1000 µg/g
		0.99-5.92 µM	genistein	aromatase mRNA inhibition no aromatase protein inhibition	1000 µg/g
		0.99-5.92 µM	genistein	aromatase protein inhibition	500 µg/g
Hsieh et al, 1998	MCF-7 cells	2 µM	genistein	stimulation tumor growth	750 µg/g
Zhou *et al*, 2004	MCF-7 cells		soy extracts + tea bioactive compounds	inhibition of tumor growth	51.9% soy isoflavones of diet

Table 2 Overview effects genistein *in vitro*. Results of articles on aromatase, ER and aromatase & ER are shown respectively. Results showing no change in aromatase activity were not included only increase or decrease of aromatase was included.

reference	assay	concentration/EC50/IC50	compounds	effect
in vitro				
aromatase				
Pelissero *et al*,1996	ovarian of rainbow trout	IC50= 3500µM	genistein	inhibition aromatase activity
Brooks *et al*, 2005	MCF-7 cells *in vitro*	10µM	genistein	reduction estrone production by 70% as result of reduced aromatase activity
Sanderson *et al*,2004	H295R human adrenocortical carcinoma cells	10µM	genistein	2.5 fold induction of aromatase activity
Edmunds *et al*, 2005	cell free microsome assay	1nM-1mM	genistein	increased aromatase activity ± 1.5 100-125% compared to control
Myllymaki *et al*, 2005	immature rat ovarian follicles *in vitro*	10^{-7} and 10^{-6}M	genistein	increased aromatase activity ± 1.5 and 2.5 fold
Ye *et al*, 2009	hepatic cells HepG2	1 and 10µM	genistein	increased aromatase activity ± 1.5-2 fold
ER (Estrogen Receptor)				
Le Bail *et al*, 2000	MCF-7 cells	0.01-10µM	genistein	significant proliferation compared to control 62.8-109.2%
		50µM	genistein	decrease in proliferation
	MCF-7 MVLN	0.1-50µM	genistein	Estrogenic effect via ER. Significant luciferase expression 8.7-106.8%

Hsieh et al, 1998	MCF-7 cells	0.01-10 µM	genistein	dose dependent increase tumor growth and pS2 mRNA increase ± 1.5-2.4 fold
		25-100 µM	genistein	dose dependent decrease tumor growth
Mai *et al,* 2007	MCF-7 cells	50µM	genistein	reduced tumor growth 86%
ER and aromatase				
van Meeuwen *et al, 2007a*	MCF-7 cells	EC 50: 32nM	genistein	increased proliferation and increased pS2 mRNA
	MCF-7 cells	IC50: 3.6 µM	genistein	aromatase activity inhibition
van Duursen *et al* 2011	MCF-7 cells	EC50: 11.2 nM	genistein	increased proliferation and increased pS2 mRNA
	MCF-7 cells	20nM + 3µM	testosterone + genistein	pS2 mRNA increased with 2 fold
	H295R cells	EC50: 26 µM	genistein	dose dependent increase aromatase activity
	co-culture MCF-7 and BAFs	20nM + 3µM	testosterone + genistein	increased aromatase expression
	co-culture MCF-7 and BAFs	20nM + 3µM	testosterone + genistein	pS2 mRNA increased with 3.4-4.5 fold

3 Discussion

The aim of this thesis was to determine the most relevant mode of action of isoflavones by assessing their potency on the ER and aromatase with the current available literature. Comparing this literature proved challenging as research methods used were inconsistent between studies. Furthermore, the results found in literature were contradicting and not conclusive. However, it did provide information for an overview of some current insights.

More research was done on the effects of the soy-derived isoflavone genistein compared to other phytoestrogens. Because of the larger amount of available information, genistein is used for a comparison between the effect of genistein on the ER and aromatase. Research conducted on the effects on the ER mediated estrogenicity was done *in vitro* as well as *in vivo.* Experiments investigating the effects on aromatase activity were mainly done *in vitro.*

First, it is important to use relevant concentrations. Human genistein plasma concentrations vary approximately between 0.1-6 µM after consumption of soy products or supplements. Although some supplements might be more concentrated than others and increase the genistein plasma levels further. Experiments conducted with *in vivo* mouse models with human MCF-7 cells measured mouse plasma levels of approximately 0.39-5.92 µM. Thus the exposure levels in these *in* vivo experiments are in a range that is relevant for the human situation. However many different *in vitro* studies used a very wide range including high concentrations. These concentrations vary between 0.01 and 100 µM and the higher range of these concentrations might be less relevant when compared with human serum concentrations. Additionally, it is important to include the breast tissue and aspirate levels of isoflavones since these can differ from the plasma concentrations. However this is not easily measured or extrapolated from serum concentration data. First, nipple aspirate levels can reach levels that are higher than serum concentrations (Hargreaves et al. 1999). Secondly, breast tissue concentrations do not correlate with serum concentrations and do not always reach serum concentrations. Finally, the distribution of isoflavones in different cell types in breast tissues is not equal (Bolca et al., 2010).

Secondly, it is difficult to determine the relevancy of the different available assays. To test the effects of isoflavones on the ER, human MCF-7 cells *in vitro* and *in vivo* are often being used. Besides these MCF-7 cells, a promising co-culture of MCF-7 cells and fibroblasts is developed which is more comparable to human *in vivo* breast cancer (Van Meeuwen et al., 2007a). By using a co-culture the paracrine interaction of cells in breast cancer is partly mimicked. Though there are relatively good models to measure the effects of isoflavones on the ER it is more difficult to develop a model for the effects on aromatase activity. The reason for this is the difference in promoter activity between healthy and malignant tissue. In healthy breast tissue, aromatase mRNA is transcribed by the I.4 promotor while in malignant tissues the strong promoters I.3, II and I.7 activated by a paracrine interaction between the different cells in the breast. This results in the increased aromatase activity in the malignant breast epithelial cells. MCF-7 cells do not (highly) express aromatase. Sometimes, MCF-7 cells are transfected to express aromatase, but this is often not done with the appropriate promoters (Ju et al., 2008, Chen., 1998). This makes these assays less appropriate to test the effect of isoflavones on the aromatase activity. Thus a MCF-7 assay with increased aromatase expression like

the MCF-7ca assay would be a better alternative, if the right promoters regions are being used. However a mono-culture does not include the paracrine interactions between the fibroblasts and MCF-7 cells. Ideally a co-culture with aromatase expression by the I.3 and II promoters in the fibroblasts would be developed because this would be even more comparable to human breast cancer. Many other assays besides the MCF-7 assay and co-culture were used to investigate the effect of isoflavones on aromatase activity. The used cell lines for these assays are not breast cancer cells. Therefore they lack the specific properties of breast cancer cells and the specific aromatase expression. Thus, it is difficult to determine the relevance of the results of these assays when specifically searching for an effect in breast cancer.

Thirdly, there are many other phytoestrogens besides genistein that are able to induce MCF-7 proliferation or influence aromatase activity though, with different potencies (Van Meeuwen et al. 2007a). To be able to make a risk assessment of isoflavones and their effect in breast cancer patients it is also important to test these different phytoestrogens in mixture since these compounds do not occur in soy products and supplements as single compounds (Taxvig et al., 2010). Combinations of different phytoestrogens might contribute to the total estrogenicity in human blood plasma in an additive way (Van Meeuwen et al., 2007b). Furthermore the different effects of processed and unprocessed soy should be further investigated. While the Asian diet contains a lot of natural products with unprocessed soy the USA diet contains processed soy products. As a result of processing the soy the products might have lost some of the claimed, beneficial biologically active components. The least processed diet, soy flour was found to not induce tumor growth while more processed diets were able to induce tumor growth (Allred et al. 2004).

Finally, effects of isoflavones in combination with different breast cancer treatments should be further investigated. Limited research has been done on the effect of isoflavones in combination with SERMs or aromatase inhibitors. The available information shows different results for genistein combined with letrozole, tamoxifen and fadrozole. Genistein in a mixture with letrozole was found to counteract the inhibitory effect of letrozole (Ju et al., 2008). The inhibitory effect of fadrozole on aromatase was also negated combined with genistein. However, genistein was found to additively delay breast tumor growth in combination with tamoxifen (Mai, Blackburn and Zhou., 2007).

Because the effect of isoflavones on the ER and aromatase activity, is an assessment of two different endpoints it is not so straightforward. Most *in vivo* and *in vitro* results show that genistein exerts a dose-dependent estrogenic effect by binding the ER in a concentration range of 0.01-10 µM (Hsieh et al., 1998, Van Meeuwen et al., 2007a, Ju et al., 2008, Ju et al., 2001, Allred et al., 2001). Genistein is able to induce this effect already at low concentration *in vitro* and *in vivo*. These also include lower relevant concentrations that µM can be reached in human blood plasma after consuming soy products or supplements and show that the estrogenicity of genistein is relevant. The higher concentrations 25-50 µM however can induce a dose-dependent decrease in tumor growth (Hsieh et al. 1998, Le Bail et al. 2000). While most results on estrogenicity of genistein are comparable, the available data on the effect of genistein on aromatase activity are contradicting. Some results show an inhibition of aromatase after exposure to genistein while most results show no effect or increased aromatase activity. Thus the effects of isoflavones on the aromatase activity are contradicting but the inhibition of aromatase is often found at a higher concentration range and IC50 values vary from 3.6-49 µM (Van Meeuwen et al., 2007a, Brooks and Thompson., 2005). The genistein concentrations 0.01 to 10 µM resulted in an increased aromatase activity (Edmunds et al., 2005, Sanderson et al.,

2004, Myllymaki et al., 2005, Ye, Chan and Leung., 2009). Estrogenicity via the ER and increased aromatase activity are found in the same exposure concentration range 0.01-10 µM. Aromatase inhibition was found as a result of higher concentrations though with some overlap IC3.6-49 µM with increased activity and estrogenicity via the ER. More research on the effects of genistein on aromatase is needed.

4 Conclusion

There is insufficient information to determine the relevance of the effect of genistein on aromatase activity. With the current knowledge it is clear that genistein can have an estrogenic effect on breast tumors via the ER. This interaction can potentially affect breast cancer treatment with SERMs. Data indicate that the effects of genistein on the ER are more potent than on the aromatase inhibition but more research on the potential interaction with the aromatase enzyme is needed.

Abbreviations

BAF=	Breast Adipose Fibroblast
EC50=	the concentration needed to exert 50% effect of a biological response.
ER=	Estrogen Receptor
ERE=	Estrogen Responsive Element
IC50=	the concentration inhibitor needed to inhibit 50% of the biological response.
PGE2=	Prostaglandin E_2
SERMS=	Selective ER Modulators
TAM=	Tamoxifen

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