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„Effects of Actaea racemosa *L.* on steroid hormone metabolism in human breast cancer cells“

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1. Abstract

Actaea racemosa *L.*, commonly known as black cohosh, is promoted as a remedy against climacteric symptoms. However, its safety is questionable, as the extract of black cohosh (BCE) may have an adverse effect regarding breast cancer growth due to its allegedly estrogenic activity. There are ongoing discussions about its links to breast cancer, as some studies reported an induction of cellular proliferation, while other studies suggested an antiproliferative effect of BCE on breast cancer cells. To study the effects of BCE and its major active chemical compound, actein, MCF-7 cells (expressing estrogen receptor alpha) and MDA-MB-231 cells (with negative estrogen alpha receptor status) were incubated and cellular proliferation was analyzed after 48 h in the presence and absence of dehydroepiandrosterone (DHEA) and estrone (E1) as hormonal precursors. An inhibition of cell proliferation with BCE as well as with actein was observed, suggesting that BCE treatment appears safe for women diagnosed with breast cancer. Subsequently, the metabolism of the ten major steroids of the estrogenic pathway were analyzed using a validated liquid chromatography-high resolution mass spectrometry assay. The experimental settings revealed no changes in estrogen metabolization but a massive rise in T and AD formation between the control group and the group treated with BCE/actein. This was caused by inhibition of SULT2A1 leaving more DHEA for 3β-HSD and 17β-HSD to be biotransformed. Overall, this study reveals an influence of black cohosh on androgen metabolism. Due to the lack of insight in this specific mechanisms of action further human studies regarding BCE are needed.

2. Introduction

2.1. Breast cancer

In the year 2020, about 19.3 million cases of cancer were reported globally. Of those cases, female breast cancer overtook lung cancer with 2.3 million cases (11.7%) to 2.2 million cases (11.4%) (Sung *et al.*, 2021). Annually 42,000 people in Austria are diagonised with cancer. In 2018, 29% of those cases were breast cancer, thereby establishing its highest incidence rate also in Austria (statistik.at, 2021). Cancer is the second most common cause of death in Austria and breast cancer in particular has the highest death rate of all cancers among Austrian women. Consequently, the research regarding breast cancer, its treatment options and possible side effect relief are of great scientific importance. The type of breast cancer is indicative for patient's treatment options. Patients suffering from breast cancer may undergo a biopsy (removal of a small part of the affected breast tissue) and using monoclonal antibodies as well as heat-induced antigen retrieval determine whether the cancer expresses hormone receptors that stimulate cell proliferation and growth or not. The two receptors that come to mind are the estrogen receptor alpha (ERα+) and the progesterone receptor (PR+). At this point of time four different kinds of breast cancer are known: ERα+/PR+, ERα+/PR-, ERα-/PR- and ERα-/PR+ though the existence of the ERα-/PR+ type breast cancer is actively discussed in the field (Beltjens *et al.* 2021). Studies carried out using tissue samples of the USA and South America showed that a majority of breast cancer cases were ERα+, underlining the importance of remedies for antihormonal therapy (Nadji *et al.* 2005). Statistically speaking, the outcome for the cancer patient seems more favorable in presence of ERα (Beltjens *et al.* 2021). Additionally, triple negative breast cancer is known, a cancer that doesn't express ER, PR or human epidermal growth factor receptor (HER2) (Cdc 2021), which in turn reduces treatment options.

ERα has been found in all kinds of tissues including but not limited to the brain, the hypothalamus in the pituary gland, the urogenital tract and for

the purpose of this work most importantly the breast, while ERβ is ubiquitously distributed across all tissues (Wallach *et al.* 1996).

ERs are found mainly in the nucleus (Dahlman-Wright *et al.* 2006). When activated by the presence of estrogen, ERs undergo conformational changes and interacts either as a transducer or a transcription factor probably by dissociation of hsp90, hsp70 and other proteins (Klinge 2000). Dimerization makes it possible to bind to an estrogen response element (ERE) on the gene, though not every gene possesses this palindromic consensus sequence (Dahlman-Wright *et al.* 2006). While direct activation (by binding to ERE) of the gene is a possibility, gene expression can also be stimulated by activating genes for transcriptions factors. To that end, a number of genes were found that contain no ERE but a SF-1 response element (SFRE) as a binding site for the orphan nuclear hormone receptor SF-1 (Björnström and Sjöberg 2005). Not every effect and action of estrogens can be explained through binding to ERE, as some outcomes are that fast making them impossible to be RNA- and protein-synthesis dependent. Therefore, also nongenomic action and activation of various protein-kinase cascades through membrane-associated ERs exists besides classical nuclear receptor mediated acticivity (Björnström and Sjöberg 2005). Non genomic signal transduction crosses the genomic expression of gens by phosphorylation and leads to indirect expression of genes via different signal ways. 17β-estradiol induces the phosphorylation of Elk-1, C/EPBβ, cAMP response element binding protein (CREB) via the mitogen-activated protein kinase (MAPK) signaling pathway (Björnström and Sjöberg 2005). Transcriptional activity by AP-1 is also mediated by the MAPK phosphorylation and leads to enhanced AP-1 DNA binding and transcriptional activity. While 17β-estradiol can activate the MAPK-pathway and with it AP-1, 17β-estradiol can also repress the activity of AP-1 by inhibition of c-jun amino-terminal kinase (Kousteni *et al.* 2003). Furthermore the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) transcription factor is phosphorylated by the Akt kinase and leads to expression of genes containing NF-kB binding sites (Kawagoe *et al.* 2003). Looking at the different pathways one can determine two main mechanisms of gene expression regulation. On the one hand there is

protein-protein interaction in the nucleus, on the other activation of signal transduction pathways. Looking at this small selection of pathways it is no wonder, that high amounts of estrogens in combination to high amounts of ERα in breast tissue raise the chance of developing breast cancer, as hyperproliferation caused by ER-activation may get out of control. To that end, cyclin D1 has been well established as a target for estrogens in breast cancer cells, as it plays a key role in the progression of the G1 phase of the cell cycle (Björnström and Sjöberg 2005). Studies suggest that elevated cyclin D1 expression is correlated with worse prognosis in the treatability of breast cancer (Itzel 2013). As of now, no ERE-like sequence has been found on the cyclin D1 gene, but studies are hinting at an interaction of ERα with specificity protein 1 (Sp1) and c-Jun/ATF-2 with a variant CRE (Castro-Rivera *et al.* 2001). There might also be an activation of CREs by the cAMP/proteinkinase A signaling pathway, again suggesting a non-genomic action. Additionally, the activation of the cyclin D1 gene can be activated through the Scr kinase and the PI3-kinase in breast cancer cells (Castoria *et al.* 2001) (Figure 1).

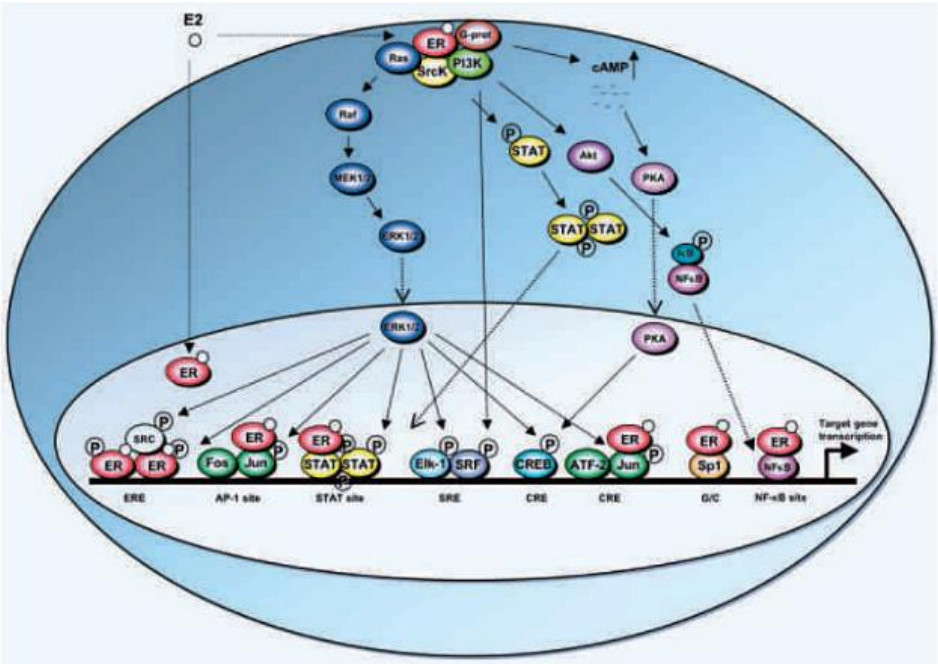

Figure 1 Illustration of estrogen-binding to the estrogen receptor and its genomic and non-genomic actions (Björnström and Sjöberg 2005)

During breast cancer initiation, estrogens seem to exert a strong effect on ERα, thereby effecting cellular proliferation, but as time goes on, cancer cells lose their dependence on estrogens and develop into an estrogen-independent growth type (Dahlman-Wright *et al.* 2006). This makes early detection of breast cancer especially important as ERα+ breast cancer seems favorable to the treatability. Classical therapy of breast cancer is executed with a combination of chemotherapy and radiotherapy, but in case of ERα expression, a lot of success is gained by endocrine disruptive hormone therapy, essentially cutting the cancer off from any estrogens by antagonistic activity through pharmaceutical drugs.

2.2. Antiestrogenic breast cancer treatment

There are multiple ways to stop the interaction of estrogen with their receptor beginning with antagonistic activity using selective estrogen receptor modulators (SERMs) (e.g. tamoxifen, toremifen), selective estrogen receptor receptor degrader (SERDs) (e.g. fulvestrant) or aromatase inhibitors (e.g. anastrozol, letrozol). In the following section a detailed insight in selected active ingredients is given to highlight the mechanisms of adverse effects of estrogen-antagonism.

Anastrozol (Figure 2) is a drug of the aromatase inhibitors. The aromatase or CYP19A1 is a member of the CYP450 family. Aromatase aromatizes testosterone into estradiol by catalyzing the demethylation of testosterone and producing aromatic estrogens. By doing so the concentration of testosterone is reduced while increasing the concentration of estradiol. In case of ERα+-breast cancer this increase of estrogens would be detrimental to the patient. Different to SERMs and SERDs the resulting effect isn’t caused by ERα inhibition but by influencing the metabolism and lowering the overall concentration of estrogens.

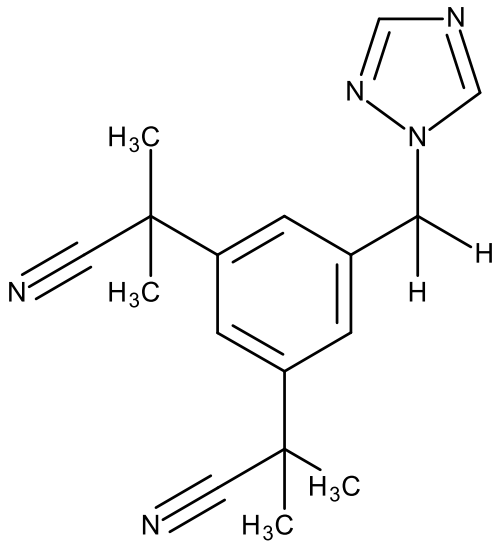

Figure 2 Chemical structure of anastrozol

Tamoxifen (Figure 3) is a SERM which inhibits the activation of ERα by competitive antagonism of estriol and estron. While SERMs have antiestrogenic effects in ERα+ cancer cells, they also have some estrogenic activity in bones and endometrial cells (Drăgănescu and Carmocan 2017), which reduces the severity of adverse effects like menopausal symptoms or osteoporosis caused by antagonistic activity of estrogens.

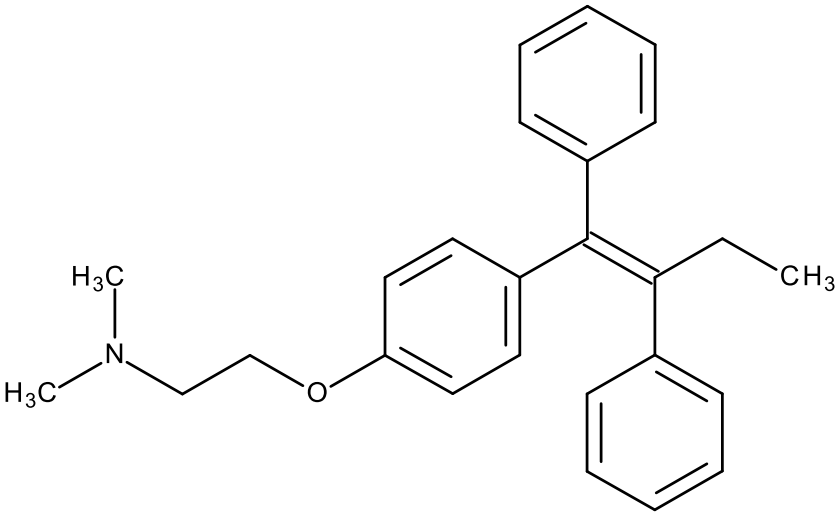

Figure 3 Chemical structure of tamoxifen

Tamoxifen is a prodrug which is metabolized in two ways: Either by CYP3A4/5 to N-Des-methyltamoxifen and in the next step to the active component N-Desmethyl-4-hydroxytamoxifen through CYP2D6 or alternatively, tamoxifen can be metabolized into the active constituent 4-Hydroxytamoxifen through CYP2D6 and following that further metabolized into endotoxifen (Figure 4).

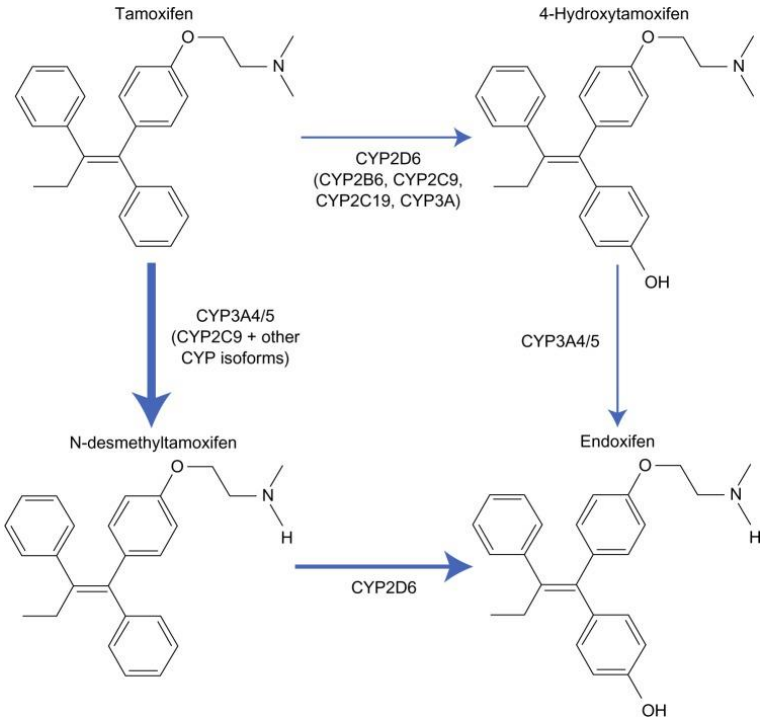

Figure 4 Tamoxifen metabolism (Nazarali and Narod 2014)

Even though endotoxifen persists in lower concentrations than tamoxifen, its affinity to ERα is about 100-fold higher, and therefore considered to be the active component in ER-antagonism (Cronin-Fenton and Damkier 2018). Since the change into its active metabolite is mediated through CYP2D6, any polymorphism would change the effectiveness of tamoxifen. As of today, there are a lot of CYP2D6-polymorphism variants the receiving patient should always be checked for, to ensure that the active endotoxifen concentrations are sufficient. Furthermore, the intake of CYP2D6 dependent medications like SSRIs, which inhibit enzyme activity (MacLeod

et al. 2017), needs to be carefully monitored or an alternative treatment option like aromatase inhibitors is recommended.

Besides aromatase inhibitors like anastrozol and SERMs like tamoxifen a third option for breast cancer is fulvestrant (Figure 5). Fulvestrant is a selective estrogen receptor degrader (SERD) and has in contrast to tamoxifen no estrogenic activity. By binding to the ER, it inhibits its activity and builds a complex that leads to break down of overall ER. Fulvestrant is used when a cancer is recurring after therapy with SERMs or aromatase inhibitors.

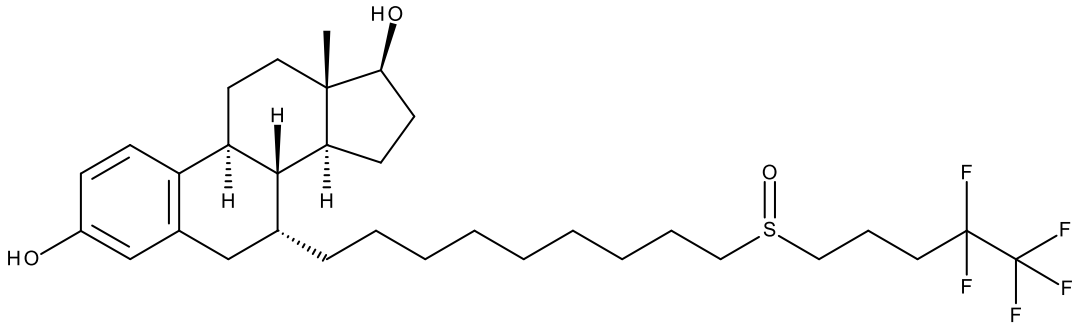

Figure 5 Chemical structure of fulvestrant

2.3. Estrogen metabolism and its role in breast cancer

Estrogens are sex hormones which are mostly produced by the ovaries in females. A small amount of estrogen is also produced by the testicles in males. The three primary estrogens are estrone (E1), estradiol (E2) and estriol (E3). As all steroid hormones, they are synthesized from cholesterol (Saha *et al.* 2019) via an array of enzymatic pathways (Sommer and Fuqua 2001). Enzymatic metabolization starts inside the mitochondria. Consequently, the rate of steroid hormone synthesis is directly correlated to the amount of cholesterol being transferred into the mitochondria as steroidogenic cells do not have the ability to store large quantities of cholesterol or steroids in general (Miller and Strauss 1999). The inflow of cholesterol is mediated by the steroidogenic acute regulatory protein (StAR) and constitutes a regulatory protein. In the next step cholesterol is catalyzed into pregnenolone by a mitochondrial cholesterol-monooxygenase for short CYP11A a P450side-chain-cleavage(scc)-

enzyme which is bound to the inner membrane of the mitochondrion (Sanderson 2006). The expression of the CYP11A-enzyme is poor in nonsteroidogenic tissue. Following metabolization into pregnenolone, 3β-hydroxysteroid dehydrogenases (3β-HSD) convert it into progesterone. Pregnenolone and progesterone are the starting steroids for all other steroid hormones. They are further converted by 17β-Hydroxysteroiddeyhdrogenases (17β-HSD) into androstenedione. Androstenedione is either directly converted into E1 by the aromatase or using 3β-Hydroxysteroiddeyhdrogenases (3β-HSD) into testosterone. As stated in section 2.2, the aromatase is responsible for the synthesis of estrogens from androstenedione and testosterone. Estrogens have a myriad of functions including the development of the female reproductive system in puberty, the regulation of the menstrual cycle, promoting and maintaining of uterus lining, triggering ovulation and influencing the sex drive. Those are just a selection of functions estrogens are responsible for. Even this small selection of functions should point out the high number of adverse effects that their absence caused by antagonistic therapy may exert. As already mentioned, the three primary estrogens are E1, E2 and E3 although the existence of Estetrol (E4) should be mentioned too, which is found only during pregnancy and is mostly produced by the fetal liver (Holinka *et al.* 2008). E2 is the most potent estrogen in general and most abundant in premenopausal women, while in postmenopausal women, E1 is playing the biggest role and is synthesized mainly in adipose tissue (Saha *et al.* 2019), whereas in pregnant women the predominant estrogen is E3. Those three estrogens are primarily metabolized using three pathways utilizing irreversible hydroxylations catalyzed by NADPH-dependent cytochrome p450 (CYP) enzymes (Samavat and Kurzer 2015). E1 and E2 are converted into catechol estrogens by hydroxylations of the positions C2, C4 and C16. The catechol estrogens are further metabolized by methylisation to methoxyestrogens by the catechol-O-methyltransferase (COMT) (Samavat and Kurzer 2015). Additionally, the original estrogens and the catechol estrogens are also conjugated with glucuronic acid and sulfate by hepatic phase II enzymes like UDP-glucuronsylstransferases (UGTs) and sulfontransferases (SULTs). Steps

like conjugation are assessed to be detoxification and inactivation pathways by raising the water solubility of the hormones to be either eliminated in urine or to be converted into soluble inactive hormone depots in the blood stream that can be reactivated at their respective target sites when needed. The main metabolic pathways are:

2.3.1. The 2-hydroxylation pathway

Compared to the 4- and 16-hydroxylation-pathway, the 2-hydroxylation pathway is the main path of estrogen hydroxylation in quantitative view, and is utilized using the CYP-450 enzymes mainly expressed in breast and liver tissue like CYP1A1 and CYP1B1 (Huang *et al.* 1996). In combination with CYP1A2 they catalyze C2 hydroxylation of their original estrogen into the catechol estrogen (2-hydroxyestrone, 2-hydroxyestradiol). A study revealed that those metabolites might actually inhibit MCF-7 cell proliferation (Schneider *et al.* 1984), suggesting a correlation of reduced metabolization activity and breast cancer. In experiments using methoxyestrogens like 2-methoxyestradiol, an inhibition of carcinogenesis and suppression of cell proliferation of MCF-7 cells has been observed (Lakhani *et al.* 2003). However, it should be noted that it has been shown that 2-hydroxyestrogens can damage DNA and generate free radicals (Liehr and Roy 1990), thereby potentially inducing carcinogenesis of other tissues.

2.3.2. The 4-hydroxylation pathway

The primary enzymes of the 4-hydroxylation of estradiol are CYP3A4/3A5 in the human liver microsomes (Kerlan *et al.* 1992). Similary to the 2-hydroxylation pathway, the metabolites of this pathway carry carcinogenic potential because 4-hydroxylated catechol estrogens have the ability to cause DNA damage by forming depurinating adducts, and generating mutations by causing oxidative damage (Cavalieri *et al.* 1997). Examination of human mammary fibroadenoma and adenocarcinoma found that formation of 4-hydroxyestradiol was higher than 2-hydroxyestradiol by four times. This should come to no surprise given that 2-hydroxyestradiol inhibit MCF-7 cell growth and proliferation. Therefore,

the 4-/2-hydroxy catechol estrogen ratio might be a biomarker for higher breast cancer risk.

2.3.3. The 16-hydroxylation pathway

The product of the 16-hydroxylation pathway is 16α-hydroxyestrone, which has been proven in mouse mammary epithelial cells to be a tumor initiator by promoting unscheduled DNA synthesis and anchorage independent growth (Bradlow *et al.* 1986). In another animal study, a correlation was drawn between urinary concentration and increased proliferation of mammary cells (Telang *et al.* 1992), Ras oncogene expression (Suto *et al.* 1992) and tumor incidence (Suto *et al.* 1993).

A study performed on human breast tissue found that 16α-hydroxyestrone was eight-fold higher in cancerous mammary terminal duct lobular units than in nearby mammary fat issue (Osborne *et al.* 1993). This in turn might suggest an important role in breast cancer induction.

Taking a look at the hydroxylation pathways, it is easy to see that polymorphism of the CYP450 family can influence the rate of which cancer occurs. As already brought up, inactivation of the partially carcinogenic catecholestrogens is executed by COMT and turning them into methoxyestrogens detoxifying them in the process (Guldberg and Marsden 1975). This also stops the biotransformation of catechol estrogens into quinone-DNA adducts, which in turn develop into reactive oxygen species (ROS), thereby reducing risk of cancer by damage to the DNA, lipids, and proteins even further. Given that information it is obvious that COMT plays an important role in breast cancer, but going into further details of its polymorphisms and subsequent consequences would escape the scope of this work.

2.3.4. Estrogen glucuronidation

Already mentioned mechanisms of detoxification and elimination are the glucuronidation and sulfation of drugs and hormones. Glucuronidation is the major mechanism of action regarding detoxification, executed by UGTs that catalyze the reaction to add the uronic acid of glucose. This in consequence raises hydrophilia, making it easier to eliminate substances

via the kidneys. Glucuronidation also alters the conformation of the steroid, hence stopping its binding to the receptor. This combination of increased clearance and changed conformation reduces the effects of estrogens or steroids in general (Harrington *et al.* 2006). The highest glucuronidation activity is found in the liver (Guéraud and Paris 1998). E2-17β-(βglucoronide) is catalyzed mainly by UGT2B7 with some contributions of UGT1A3, UGT1A4 and UGT2B15. While seen as a detoxifying process, glucuronidated steroids such as testosterone-17β-(β-glucuronide), estradiol-17β-(β-glucuronide), estriol-16α-(β-glucuronide) and estriol-17β-(β-glucuronide) are able to cause cholestasis (Mackenzie *et al.* 1996).

2.3.5. Estrogen sulfation

Sulfated steroids were seen as the inactive metabolized counterpart to active estrogens, as they cannot bind to ERs and therefore do not exert any estrogenic effects, and it was suggested for a long time that their main purpose is renal elimination as sulfation increases water solubility. But after 20 years of research a new assessment had to be made about the role that sulfated steroid hormones have in our bodies. The main task of sulfation is not necessarily detoxification and elimination; it is rather the modification of lipophilic hormones, fulfilling two tasks: For one, hydrophilic sulfated conjugates allow transportation of steroids via the bloodstream. The other task is building a circular reservoir accessible by desulfation of the needed compound (Geyer *et al.* 2017). The two main enzyme families responsible for these processes are the sulfotransferases (SULTs) and sulfatases. Within the human genome five SULTs are associated with steroid sulfation: SULT1A1, SULT1E1, SULT2A1, SULT2B1a and SULT2B1b (isoforms of SULT2B1) (Geyer *et al.* 2017), with SULT1E1 and SULT2A1 as the most relevant for this thesis, as they have implications on the biotransformation of the steroids of interest. While SULT2A1 is mainly responsible for sulfation of DHEA, the task of SULT1E1 is sulfation of E1. However, in addition to SULT2A1 DHEA does have low affinity to SULT1E1 and SULT2B and is also sulfated by these enzymes. Sulfated steroids don't possess the ability to cross cell membranes, as they are too hydrophilic. To address this issue, cells express sodium dependent organic anion

transporters (SOATs), these channels are expressed highly in steroid sensitive tissue (Foster and Mueller 2018).

Table 1 Sulfotransferases and their predominant steroid substrates (Mueller et al. 2015)

Steroid	SULT	K_m Values
DHEA	SULT2A1	0.8–3.7 μM
	SULT1E1	0.2 μM
Androsterone	SULT2A1	2.1 μM
Pregnenolone	SULT2A1	1.9–4.9 μM
	SULT2B1a	4.4 μM
E_1	SULT1E1	0.2 μM
E_2	SULT1E1	4–300 nM
	SULT1A1	240 nM
Cholesterol	SULT2B1b	1.2 μM

2.4. Estrogens and the postmenopausal syndrome

Throughout a female's life, estrogen level and synthesis are fluctuating, broadly split up in three segments: reproductive phase, transition and the postmenopausal phase (Stevenson 2011). Estrogen concentration in the body is high in the reproductive phase, but synthesis declines in subsequent phases. While females in their reproductive years run lower risk of having cardiovascular disease this doesn't hold true when estrogen levels drop. Between men and women, significant differences are seen in myocardial infarction mortality, as women in their reproductive phase have a 3-5-fold lower rate to die of heart disease. This rate goes down with age and sinking estrogen synthesis (Stevenson 2011). Health problems caused by the lack of estrogens whether through menopause or through endocrinal cancer treatment entails uncomfortable side effects like hot flashes, osteoporosis, osteoarthritis, or mood swings in combination with depression. As reduced function of the ovaries over time is inevitable, there is substantial interest in finding alleviating drugs. In the past, hormone replacement therapy (HRT) was the go-to treatment for postmenopausal women. But in 2002, a study published by the women's health initiative (WHI) found the risk-benefit to be lacking, as higher rates of breast cancer,

stroke and coronary heart disease in otherwise healthy postmenopausal women occurred (Caufriez 2007; Chen *et al.* 2019). Therefore, treatment with estrogens should only be administered under close physiological and medical care and with the lowest dose possible. This risk led to increased efforts to find alternative treatment options that are also available for persons suffering from breast cancer.

2.5. Herbal remedies as therapeutic alternative to HRT

When studies found that Asian women had less severe cases of post-menopausal symptoms and less cases of breast cancer, scientists didn't take long to find that isoflavones found in soy had agonistic capabilities for ERα in postmenopausal women (Messina 2014). As of today, there are three main groups of phytoestrogens: Isoflavones, lignanes and coumestans:

2.5.1. Isoflavones

Isoflavones are found almost exclusively in vegetables of the legume family. They are part of the phenyl-propanoid pathway, which are common in both legume and non-legume plants (Parr and Bolwell 2000). Though for the synthesis of isoflavones isoflavone-synthase is necessary, an enzyme limited to legumes (e.g. soy or red clover) and only a few other plants (Yue *et al.* 2016). Soy components include mostly genistein (Figure 6) and daidzein (Figure 7), and red clovers active isoflavones consist mostly of biochanin A and formononetin. They possess SERM properties caused by their structure, which is similar to 17β-estradiol. Furthermore, they possess strong antioxidant capacities (Larkin *et al.* 2008). Isoflavones bind to ERα and block estrogen binding, and also modulate ERβ with higher affinity (Anderson *et al.* 1999). Their capacity for estrogenic or anti-estrogenic effects depends on the endogenous estrogen levels (Wang and Kurzer 1998). These properties might contribute to their effect to alleviate postmenopausal symptoms and cancer prevention. The effects of isoflavones are still actively discussed: On one hand, looking at data showing the incidence of breast cancer in Asian women, one would have

to assume the soy consumption and its components to be cancer cell inhibiting, but on the other hand, genistein showed concerning results in *in vitro* studies with MCF-7 (ERα+) breast cancer cells. In concentrations below 10 µM, the effect was cancer cell proliferating in the MCF-7 cell line, with little to no effect on MDA-MB-231 and MDA-MB-468 (ERα-) cells, indicating an effect on ER. In spite of its proliferating effect in lower concentrations, raising the concentration to >10 µM showed an inhibiting effect on cell growth in both ERα+ and ERα- cell lines (Le Bail *et al.* 2000). Soy also shows indications of beneficial effects on lipid levels. However, those might rather be attributed to the combination of soy proteins and soy isoflavones than to either component alone (Steinberg *et al.* 2003).

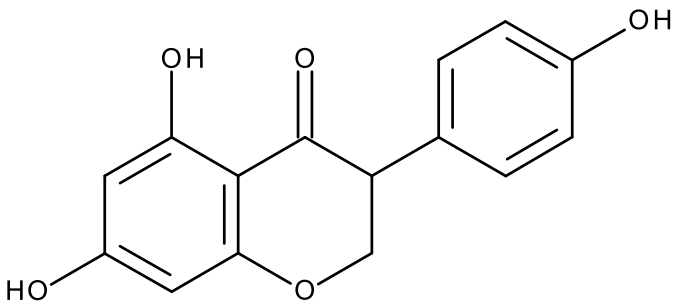

Figure 6 Structure of the isoflavone genistein

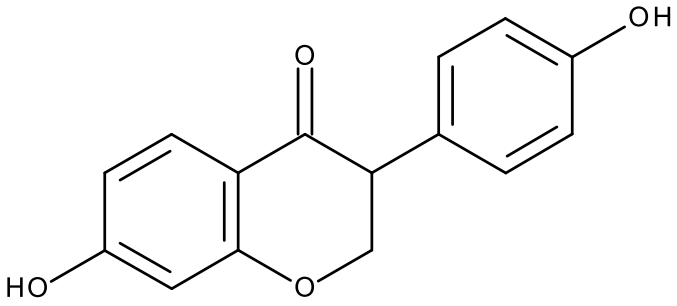

Figure 7 Structure of the isoflavone daidzein

2.5.2. Lignans

Another group of phytoestrogens are lignans. Those are components of e.g. cereals, fruits or flaxseed, mainly matairesinol (Figure 8) and seco-

isolariciresinol. After oral intake, they are processed in the human gut into enterolignanes. There are some contradicting studies regarding lignans: While some studies found no changes in sex hormone levels (Patisaul and Jefferson 2010; Nicholls *et al.* 2002), one study found a reduction in serum concentrations of E2 and E1-sulfate (Hutchins *et al.* 2001; Sturgeon *et al.* 2008). Another study revealed beneficial effects only in overweight women (Sturgeon *et al.* 2008), suggesting an interaction with aromatase, an enzyme mainly expressed in fatty tissue. An additional aspect observed was a positive effect on blood pressure in individuals with a high lignan diet (Nicholls *et al.* 2002).

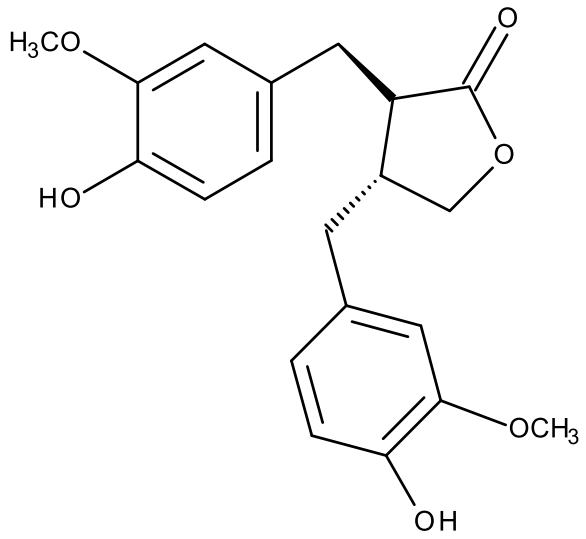

Figure 8 Chemical structure of the lignan matairesinol

2.5.3. Coumestans

Coumestans are phytoestrogens derived through oxidation of pterocarpan, similar to coumarin (Tuskaev 2013). One of the most studied coumestans is coumestrol (Figure 9), which can be found mainly in soy and clover. Similar to isoflavones, coumestrol binds to ERβ with higher affinity than to ERα, but at an even higher affinity than the isoflavones (Bingham *et al.* 1998). Additionally, incubation of MCF-7 cells with coumestrol (50 µM) showed an upregulated expression of CDKI p21 and p53, causing cell cycle arrest (Zafar *et al.* 2017).

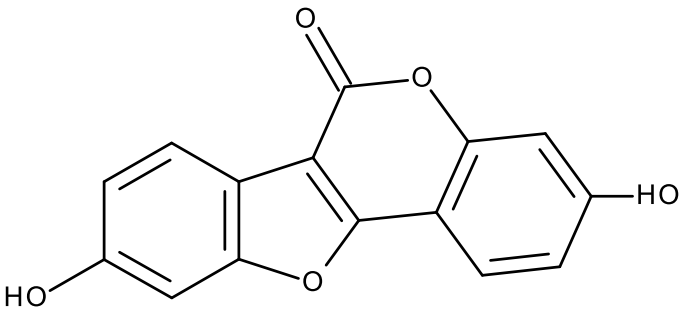

Figure 9 Chemical structure of coumestrol

Regarding the effectiveness of phytoestrogens, a lot of questions remain, as the results of the studies are not entirely conclusive. While statistics, *in vitro (Wang and Kurzer 1998)* and animal studies (Barnes 1997) seem promising, human studies resulted in contradicting data (Patisaul and Jefferson 2010). This discrepancy might be in part contributed to the fact that most phytoestrogens are glucosidated in native plant material (Larkin *et al.* 2008), whereas glucoside composition varies depending on the processing conditions of plant-based products (Coward *et al.* 1993). Glucose-conjugated compounds can't pass through the membranes of the intestine and are therefore inactive (Miksicek 1995), so that deglucosidation is required to form the active aglyka. This task is taken on by the microflora in the intestines (Bowey *et al.* 2003), which is variable between human individuals. This in turn might be a hint why there are different results between *in vitro* and *in vivo* studies.

2.5.4. Actaea racemosa

Figure 10 Actaea racemosa L.in an open field (Zell., 2021)

Actaea racemosa *L.*, formerly known as Cimicifuga racemosa, is a perennial dicot plant of the Ranunculaceae family which is commonly called black cohosh, black bugbane, black snakeroot or fairy candle (Figure 10). It is mostly found in the eastern part of Canada and the USA (Predny *et al.* 2006), where it prefers shadowy and moist places to grow. It is a large bushy plant, stands between 0,75 to 2,5 meters tall with compound toothed leaves and thin and lengthy flowers (Figure 11).

Figure 11 Illustration of Actaea racemosa plant parts (https://www.biodiversitylibrary.org/item/84253#page/71/mode/1up)

Black cohosh was already used by American and Canadian natives as a remedy against a myriad of ailments like colds, coughs, rheumatism, or menstrual disorders (Predny *et al.* 2006). Today, it is frequently used as a dietary supplement against menopausal symptoms such as hot flashes, premenstrual syndrome (PMS) or as a relief against menstrual cramps (Guo *et al.* 2017). What started as a native traditional remedy developed into a go-to treatment option of menopausal symptoms (Predny *et al.* 2006). While black cohosh in general is ruled as safe, there might be some side effects associated with its use, most in common is transient gastric upset (Dog T. *et al.* 2003). As is the case with a lot of phytotherapeutics, the exact chemical constituents and its mechanism of its relieving effects are not entirely understood. Its chemical and most likely active components consist of triterpene glycosides like actein (Figure 12), cimicifugoside (Figure 13), and 23-epi-26-deoxyactein (Figure 14), but also aromatic acids such as ferulic acid, salicylic acid and caffeic acid as well as tannins, resins, fatty acids and sugars are found, especially in the roots (Predny *et al.* 2006). The aforementioned triterpene glycosides are used as markers for the content of active components.

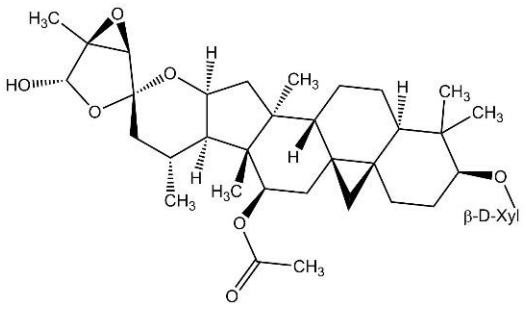

Figure 12 Actein, a triterpene glycoside characterized by its 9,19-cycloartane group, is considered to be the main active component of black cohosh (https://phyproof.phytolab.com/de/referenzsubstanzen/details/actein-89154).

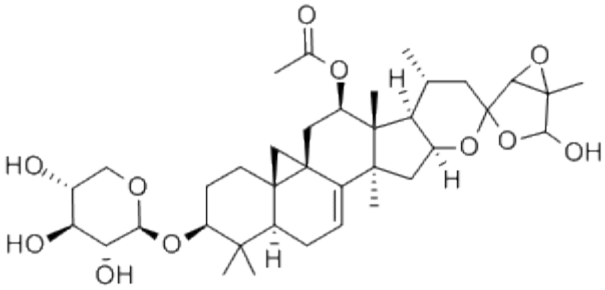

Figure 13 Cimicifugosid, one of the markers for the content of active components (https://www.chemicalbook.com/ChemicalProductProperty_DE_CB4202590.htm).

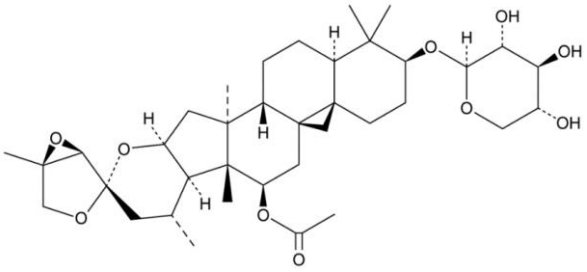

Figure 14 23-epi-26-deoxyactein, formerly classified as 27-deoxyactein (https://www.biomol.com/products/chemicals/biochemicals/23-epi-26-deoxyactein-cay25126-500).

Many phytoestrogens utilize some of their physiological reaction by binding to ER, which reduces the effects of postmenopausal symptoms. However, studies performed on ovariectomized rats using BCE did not result in physiological changes such as increase in uterine weight or changes in the vaginal cytology, which would be typical for ER-binding effects (Burdette *et al.* 2003). Furthermore, *in vitro* studies using cloned ERα and ERβ showed no ER-binding by black cohosh constituents (Wuttke and Seidlová-Wuttke 2015). This seems to be further confirmed by the fact that

BCE does not stimulate cell growth of MCF-7 cells (Szmyd *et al.* 2018; Marsh 2009), which on the other hand, is induced by low-dose isoflavones. For those reasons other mechanisms of action seem to be responsible for the alleviating properties of BCE regarding postmenopausal symptoms. Some studies found a direct modulation of $GABA_A$ by BCE (Cicek *et al.* 2010; Strommer *et al.* 2014). Additionally, stimulation of osteoblast functions and inhibition of bone-resorbing mediators has been observed in an *in vitro* study, suggesting a counteracting effect on osteoporosis in postmenopausal women (Choi 2013). BCE is able to bind to 8 serotonin receptor subtypes, with the highest affinity to 5-HT_7 and 5-HT_{1A} (Burdette *et al.* 2003). 5-HT_7 as well as 5-HT_{1A} are found to be involved in thermoregulation in the body (Guscott *et al.* 2003). This in turn might explain the positive effects of BCE as treatment against hot flashes.

2.6. Cancer cell culture as *in vitro* model for estrogen activity and metabolism

Established and highly researched cancer cell lines have been used for decades to investigate the effects of natural compounds on cancer cell activity, resulting in reproducible results that can be easily compared even between different research groups. The probably best established cell lines as breast cancer models are MCF-7 cells (ERα+) and MDA-MB-231 cells (ERα-).

2.6.1. MCF-7 cells

The MCF-7 cell line was established 1973 by isolating the cells of a pleural effusion of a 69 year-old Caucasian woman with metastatic cancer and represents an *in vitro* model for ERα+ breast cancer. The name abbreviation MCF stands for “Michigan Cancer Foundation”. To the year 2015, nearly 25,000 publications were published for MCF-7, only peaked by publications of the HeLa cell line (Lee *et al.* 2015). It is especially noteworthy that maintaining ERα in cancer cell lines is difficult, making the MCF-7 cell line even more valuable in breast cancer research. Interestingly enough, the ability to maintain ERα seems to be a fortunate coincidence as Dr. Soule didn’t seem to make special preparations to this one cell line

further confirmed by the fact that originally, the ERα functionality wasn't in the description. The scientific impact of having a stable ERα positive cell line was first reported in the studies performed by Lippman and Horwitz two years later (Lippman and Bolan 1975; Horwitz *et al.* 1975). While the inhibition of ERα using antimetabolites and with that, the inhibition of growth was easy enough to prove, the demonstration of growth using estrogens deemed a challenge and had no reproductive results in over a decade (Osborne *et al.* 1983). Only when Katzenellenbogen discovered the weak estrogenic activity of phenol red, reliable scientific advances using the MCF-7 cell line could be made (Berthois *et al.* 1986). With the help of MCF-7 cells, it was discovered that ERα is predominantly localized in the nucleus (King and Greene 1984). Even with the tremendous contributions that MCF-7 cells did for the breast cancer research, it is to note that other than the ER, MCF-7 cells also express androgen, progesterone and glucocorticoid receptors (Horwitz *et al.* 1975). All ways of how estrogens induce cell proliferation are still not entirely explained as shown in the small but complex selection of genomic and non-genomic interactions. Even though the MCF-7 cell line is still a prime subject for ERα+ breast cancer research advances were also made in the field estrogen and anti-estrogen resistance. While prolonged exposure to either estrogen antimetabolites or lack of estrogen initially slowed the cell growth, variants emerged that were independent of ERα and regained their ability to grow even without estrogens. In long-term estrogen deprivation (LTED) of MCF-7 cells, a downregulation of ERα and heightened sensitivity to growth factors other than estrogens should be expected, but paradoxically, in a study of 1998 the opposite effect was observed. ERα expression in LTED was compared to the wild-type upregulated in turn getting hypersensitive to E2 (Jeng *et al.* 1998). Though different results in different MCF-7 cell experiments were achieved and a downregulation of ERα was also observed (Oesterreich *et al.* 2001). This in turn highlights the adaptability of MCF-7 cells and thus the adaptability of breast cancer cells in general. Even worse, some studies showed that after sufficient exposure to tamoxifen, the small agonistic activity of tamoxifen was enough to even induce cell proliferation (Couillard *et al.* 2000). Since 1973, the MCF-7 cell

line is helping understanding ERα+ breast cancer and with its adaptability under therapeutic pressure, MCF-7 cells are a great representation of breast cancer in real life conditions.

2.6.2. MDA-MB-231 cells

The MDA-MB-231 cell line was established using a pleural effusion of a 51year-old Caucasian female with metastatic mammary adenocarcinoma. They are commonly used as a triple negative breast cancer cell line. MDA-MB-231 cells are poorly differentiated and stellate shaped with an elongated cell body. Initially classed as a basal breast cancer line, it is now recognized as a claudin-4 molecular subtype (European Collection of Authentificated Cell Cultures). While MDA-MB-231 cells are generally accepted as free of ERα and PR, these cells represent an ideal model for unresponsiveness to estrogens and their antimetabolites.

2.7. Quantification of steroids in *in vitro* and *in vivo* studies

It is not always feasible to use radioimmunoassays or enzyme immunoassays to quantify steroid hormones, as their specificity is poor and cross-reactions between different steroids aren't that uncommon, making it very difficult to assess the results (Hauser *et al.* 2008). In addition to that, using antibody dependent quantification of hormones yielded inhomogenic results between different laboratories depending on the used antibodies. Therefore, using liquid chromatography – mass spectrometry (LC-MS) to quantify steroid hormones became gold standard, as these assays are highly specific. Mass spectrometry uses three main components: an ionization source, a mass analyzer and an ion detection system. The ionization source in combination with a heating source ionizes molecules by converting them into gas-phase ions that can be manipulated by an electromagnetic field. The electromagnetic field accelerates the charged ions which then fly a distance being influenced by the charge they hold and the mass they have pictured by the relation of mass over charge (m/z). There are different methods to realize the ionization, but usually, electron spray ionization (ESI) is used for these compunds. Using a capillary

charged with a high electric field, liquid samples are charged and atomized in tiny droplets. The solvent evaporates, creating even smaller droplets and raising their charge density on the surface, until they finally split into charged ions when reaching the so-called Rayleigh limit. At that point, repulsive forces between the charges are so high that they overcome the surface tension of the droplet and enter a gaseous charged state. Using a quadrupole mass analyzer, essentially four metal rods parallel to each other, the charged fragments are manipulated in their flight behavior by an oscillating electric field applied to said rods. Measuring the Time-of-flight (TOF), the ion's m/z can be determined (Figure 15). Combining the MS with liquid chromatography adds another dimension to the measurement. Being able to determine the time at which a given metabolite or steroid is fed into the MS narrows the possibility which compound at any given time is flying.

Figure 15 Maxis HD orthogonal acceleration time-of-flight mass spectrometer

3. Materials and Methods

3.1. Materials

Materials used were as followed:

Adenosine-3'-phosphate-5'-phosphosulfate lithium salt hydrate (PAPS), acetic acid, acetonitrile, actein of United States Pharmacopeia (USP) reference standard quality ([(1S,1′R,2S,3′R,4R,4′R,5R,5′R,6′R,10′S, 12′S,13′S,16′R,18′S,21′R)-2-hydroxy-1,4′,6′,12′,17′,17′-hexamethyl-18′-[(2S,3R,4S,5R)-3,4,5trihydroxyoxan-2-yl]oxyspiro[3,6-dioxabicyclo[3.1.0] hexane-4,8′-9-oxahexacyclo[11.9.0.01,21.04,12.05,10.016,21]docosane]-3′yl] acetate; CAS no. 18642-44-9; LOT, F0H264; purity, 100.0%;), ammonium acetate, androstenedione (AD), dehydroepiandrosterone (DHEA), dehydroepiandrosterone-2,2,3,4,4,6-d6 (DHEA-d6), dehydroepiandrosterone-3-O-sulfate sodium salt (DHEA-S), dehydroepiandrosterone-3-O-sulfate-2,2,3,4,4,6-d6 sodium salt (DHEA-S-d6), dimethylsulfoxide (DMSO), dithiothreitol (DTT), E2, 17β-estradiol-3-O-(β-D-glucuronide) sodium salt (E2-G), E3 (16α-hydroxy-17β-estradiol), E1, formic acid, powdered black cohosh extract (BCE;USP reference standard, CAS no. 84776-26-1; LOT, F0D086) and testosterone (T) were obtained from Merck KGaA (Darmstadt, Germany). Estrone-3-O-sulfate sodium salt (E1-S) and 17β-estradiol-3-O-sulfate sodium salt (E2-S) were purchased from Steraloids Inc. (Newport, RI, USA); sulfotransferase 2A1 (SULT2A1) was procured from tebu-bio GmbH (Offenbach, Germany); 4-androstene-3,17-dione-2,2,4,6,6,16,16-d7 (AD-d7), 16α-hydroxy-17β-estradiol-2,4,17-d3 (E3-d3), 17β-estradiol-2,4,16,16-d4 (E2-d4), 17βestradiol-16,16,17-d3-3-O-(β-D-glucuronide) sodium salt (E2-Gd3), 17β-estradiol-2,4,16,16-d4-3-O-sulfate sodium salt (E2-S-d4), estrone-2,4,16,16-d4 (E1-d4), estrone-2,4,16,16-d4-3-O-sulfate sodium salt (E1-S-d4) and testosterone-2,2,4,6,6-d5 (T-d5) were purchased from C/D/N-Isotopes Inc. (Pointe-Claire, Quebec, Canada). Purified water was produced using an arium® pro ultrapure water system (Sartorius AG, Göttingen, Germany). All solvents and reagents purchased were of HPLC quality grade. All standard compounds were separately dissolved in DMSO and stored at -80 ˚C until further use. All deuterated standards were diluted

with DMSO to their final concentration and mixed to obtain a final internal standard master mix composition, which was also stored at -80 ˚C. MCF-7 and MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle Medium F-12 (DMEM), Dulbecco's phosphate buffered saline (DPBS), fetal bovine serum (FBS), PenStrep®- and TrypLE®-solution were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). HyClone® heat-inactivated charcoal-stripped FBS (U.S. origin) was obtained from GE Healthcare Life Sciences (Logan, UT, USA).

3.2. Quantification of actein in BCE

To determine the quantity of actein in the standardized BCE (USP certified), a LaChrom L-7000 high-performance liquid chromatography system (Merck Hitachi, Darmstadt, Germany) was used. The LC-system was equipped with an ELSD 90 evaporative light scattering detector (VWR international, LLC, Radno, PA, USA), adhering to the monography of the United States Pharmacopeia 42. Separation was achieved using a Phenomenex Luna® 5 µm C18(2) LC column (250 x 4.6 nm I.D.; Phenomenex, Inc., Torrance, CA, USA) at 35°C. Solvent A consisted of 0.1% formic acid in water, while solvent B was acetonitrile; at a flow rate of 1.6 ml/min. The gradient was eluted using following settings:

Table 2 HPLC gradient for actein quantification

Time (min)	Solvent B
0	20%
8	20%
15	32%
55	64%
65	95%
70	95%
85	20%

BCE was dissolved in methanol, vortexed, sonicated and filtered with a poresize of 0.22 µm. 20 µl of the brownish filtrate were injected onto the

column. External linear calibration curves derived from samples containing purified actein in methanol were used to quantify the concentration of actein in BCE. Furthermore, cell culture media fortified with BCE were incubated at 37°C for up to 48 h and analyzed for their actein content to verify its stability under experimental conditions.

3.3. Cell culture and proliferation experiments

After seeding into cell culture flasks (Nunc 75 cm^2; BD-Falcon®, Thermo Fisher Scientific, Inc., Waltham, MA, USA), the cells were incubated with phenol red-free DMEM containing 1% PenStrep® to reduce the chance of microbiological contamination and 10% FBS at a temperature of 37°C and an atmosphere of 95% humidity with 5% CO_2. Using phenol-red in the medium was avoided, as it possesses weak estrogenic properties which may possibly falsify the results (Berthois *et al.* 1986). MCF-7 and MDA-MB-231 cells attached as monolayers within 24 h. After attachment, medium was removed and the cell layers were washed with DPBS. To detach the cells for seeding into 6-well-plates, TrypLE®-solution was applied and incubated for 15 minutes before deactivating the proteolytic process with DMEM containing 10% FBS to stop TrypLE® from destroying the cancer cells. After detachment, the suspension of cancer cells was seeded into 6-well-plates at a density of 1.0×10^6 cells/well and cultured again with DMEM containing 10% FBS over a time-span of 24 hours for attachment. After visual validation of the successful attachment, the medium was removed, and the cell layers were washed twice with DPBS. The compounds of interest, namely the estrogen precursors estrone (E1) and dehydroepiandrosterone (DHEA), acting as hormone precursors as well as BCE and actein, were dissolved in dimethyl sulfoxide (DMSO), a potent solvent capable of dissolving polar and apolar substances at a final concentration of 0.1% in DMEM fortified with 10% charcoal-dextran treated FBS and added to the cells. As DMSO may have an impact on cellular growth, DMSO-only (0.1%) control samples were incubated for each experiment.

The steroid precursor concentrations were increased starting at 25 and ending at 100 nM in increments of 25, while BCE concentrations ranged

from 1 to 25 µg/ml and purified actein from 1 to 50 µM. After adding the dissolved components to the 6-well plates, cells were incubated at the aforementioned conditions for 48 hours. After this incubation period, the supernatants were removed, the cells washed again using DPBS and detached using 400 µl TrypLE® solution for 15 minutes. Subsequently, cells were re-suspended as mono cell suspensions using an 10-1000 µl Eppendorff pipette and the TrypLE® solution was stopped using DMEM containing 10% FBS. The number of viable cells was then determined using a Casy® TT cell counter. All samples were incubated as triplicates for all concentrations in the presence and absence of BCE and actein. Furthermore, different incubation periods were examined: While 24 hours were not enough to observe a significant effect of the investigated compounds on cellar proliferation, and 72 hours resulted in cell death of both cell lines due to the limited space in each well, 48 h were determined to be most suitable for all experiments, as already observed in earlier studies (Poschner *et al.* 2018; Poschner *et al.* 2017a).

3.4. Steroid metabolism in the presence and absence of BCE or actein

To prepare the analysis of the metabolization of the steroid precursors E1 and DHEA, Eppendorf vials were washed with acetonitrile as to remove any possible remnant of lipophile contaminants and let it dry at room temperature. Oasis HLB 1 cc SPE cartridges were preconditioned twice with 1.0 ml of acetonitrile and three times with 1.0 ml ammonium acetate buffer (10 mM, pH = 5.0) using a vacuum manifold and connecting it to a vacuum. The medium, incubated at the aforementioned conditions, containing the steroid precursors E1 or DHEA (25-100 nM) in the presence or absence of BCE (1-25 µg/ml) or actein (0.5-10 µM) was collected from the incubation experiments. To investigate the proposed transformation of testosterone (T) to estradiol (E2) via the aromatase-pathway, also testosterone (100 nM) was added to the medium. Additionally, a control well only containing DMSO (0.1%) was prepared. The incubation span amounted 48 hours as that is the duration the metabolite formation takes to complete (Poschner *et al.* 2018).

3.5. Sample preparation

After removal of the medium from the 6-well plates, 2 ml were mixed with 2 ml of ammonium acetate buffer and 20 µl of the internal standard mix to ensure stable pH-conditions, and vortexed before being loaded onto the SPE cartridges. By connecting to a vacuum, the medium is drawn through the cartridges and the lipophilic hormone-metabolites were locked in the solid phase of the cartridge (Figure 16).

Figure 16 Oasis HLB 1 cc cartridge (https://www.waters.com/nextgen/th/en/shop/sample-preparation--filtration/wat094225-oasis-hlb-1-cc-vac-cartridge-30-mg-sorbent-per-cartridge-30--m-1.html)

A mixture of acetonitrile and ammonium acetate buffer 10:90 (v/) proved to be the ideal ratio to wash the SPE cartridges afterwards to remove all cell medium remnants but to keep the hormones in the solid phase (Poschner *et al.* 2017b). To elute the hormones into the prepared Eppendorf vials, the cartridges were then washed twice with a mixture of 95% acetonitrile and 5% ammonium acetate-buffer. After extraction of the metabolites, dryness was achieved by using a Concentrator plus vacuum centrifuge (Eppendorf, Hamburg, Germany) at 45°C for 1.5 h. After the initial dry process, the Eppendorf vials were shock frozen at -80°C for 15 minutes. After freezing, the caps of the Eppendorf vials were punctured using a syringe cannula and an additional dryfreeze cycle was performed using a Telstar LyoQuest® freezedryer (VWR, Vienna, Austria) for an hour. The dried remnants were reconstituted using 270 µl of a mixture of acetonitrile and ammonium acetate (10m mM, pH=5.0), in a proportion of 25:75 (v/v) for quantitative dissolution of all components and centrifuged

at 14,000 rpm for 5 minutes, the clear supernatant was transferred into two separate HPLC vials (one for positive and one for negative ion mode measurement) and stored at -80°C until LC-HRMS analysis.

3.6. Liquid chromatography conditions

Using an UltiMate 3000 RSLC-series system (Dionex/Thermo Scientific, Gemering, Germany, Figure 17) coupled to a maXis HD ESI-Qq-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) a liquid chromatography was performed. The biggest challenge was the separation of E1, DHEA and AD as they have similar elution properties. To that end, only the Phenomenex Luna® 3 µm C18(2) 100 Å LC column (250 × 4.6 mm I.D., Phenomenex Inc., Torrance, CA, USA) with a Hypersil BDS-C18 guard column (5 µm, 10 × 4.6 mm I.D., Thermo Scientific)) had the properties to successfully separate the problematic steroids from each other. Furthermore, using this column made injections of larger sample volumes possible, achieving better sensitivity. Solvent A was an aqueous ammonium acetate buffer (10 mM pH=5.0) and solvent B acetonitrile. The temperature of the column oven amounted to 43 °C and the flow rate was set to 1 ml/min. Of each sample, a volume of 100 µl was injected. Gradient settings were as followed: At 0 min 25% solvent B, 56.3% solvent B at 19 min, a washing step at 90% solvent B from 19.5 to 24.0 min and column re-equilibration with 25% solvent B from 24.5 to 30.5 min. To prevent overload of the ESI source, a T-piece splitter was positioned between the liquid chromatography system and the mass spectrometer, reducing the flow to about 12,5% for further analysis. This solvent profile has been proven ideal for the analysis of the steroids (Poschner *et al.* 2017b).

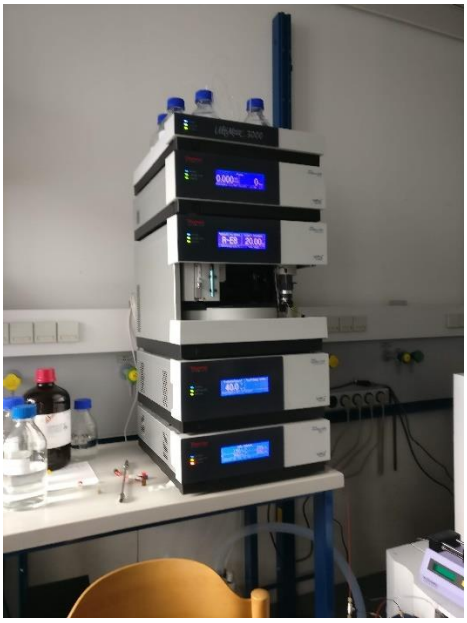

Figure 17 UltiMate 3000 RSLC-series HPLC system

3.7. Mass spectrometer conditions

According to the publication of Poschner *et al.* 2017b), detection of phenolic estrogens, sulfated and glucuronidated steroids as well as their metabolites was the most sensitive as deprotonated molecules using negative ionization mode, whereas DHEA, AD and T had better sensitivity using positive ionization, creating protonated molecule ions. As fast polarity switching is not possible with an ESI-Qq-TOF, two separate vials were necessary for separate analyses of the samples in the positive and negative ion mode. Full scan mass spectra were recorded in the range of *m/z* 150-500 in both modes, and the mass spectrometer settings were identical for both modes aside from the polarity the of ESI ion source:

- Capillary voltage: ±4.5 kV
- Nebulizer: 1.0 bar N_2
- Dry gas flow: 8.0 l/min N_2
- Dry Temperature: 200 °C
- Ion transfer parameters: 400 Vpp tunnel RF and 300 Vpp multipole RF
- Quadropole ion energy: 8.0 eV
- Collision RF: 1100 Vpp
- Collision energy: 10.0 eV

- Transfer time: 38 ms
- Prepulse storage: 18 ms

3.8. LC-HRMS assay validation

To ensure accurate quantification, assay performance characteristics had to be validated. Firstly, steroid background levels in charcoal stripped FBS had to be checked. Analyzing untreated cell culture medium resulted in detection of a low amount of DHEA-S that obviously escaped the charcoal strip process. Though carry-over-effects could be excluded, as methanol blanks between analyses didn't show any DHEA-S left over between samples. This circumstance resulted in a higher lower limit of quantification (LLOQ) for DHEA-S. The limit of detection (LOD) is calculated as the concentration where the signal to noise ratio (S/N) is higher or the same as 3, whereas the LLOQ is defined as S/N ≥9. The concentrations of the internal standard were set to 10 times LLOQ for accurate quantification results. For quantification, quality control samples were analyzed containing 6-, 60-, 600-fold the concentrations of the LLOQs for all steroids, respectively. Further details concerning the validation process can be found in Poschner *et al.* 2017b.

3.9. SULT2A1 inhibition

Metabolite examination showed that the formation of DHEA-S was inhibited by actein, but not that of E1-S or E2-S. This led to the conclusion that BCE and actein are responsible for SULT2A1 inhibition, as that is the primary enzyme responsible for DHEA sulfation. Therefore, recombinant human SULT2A1 (2.4 µg/ml) was incubated in KH_2PO_4/ KH_2PO_4 buffer (50 mM, pH = 6.5) in the presence of DTT (1mM), EDTA (0.1 mM) and PAPS (50 µM), as well as increasing concentrations of DHEA as substrate in concentrations ranging from 25 to 100 nM, in combination of BCE (1-25 µg/ml) or actein (1-25 µM). To compare the effect of BCE and actein, control samples containing the dissolution solvent DMSO, 0.1% only were analyzed as well. To ensure statistical stability, all reactions were performed in triplicate. SULT2A1 was pre-incubated with DTT/PAPS and BCE/actein for 5 minutes at 37 °C. Afterwards, DHEA was added for

additional 15 min. To interrupt the reaction, methanol at -20 °C was added, followed by centrifugation at 14,000g, for 5 minutes. The supernatants were then analyzed for DHEA-S using the same LC-HRMS assay as described before.

3.10. Kinetic Analysis and statistics

Kinetic parameters of the steroid metabolite formations were calculated for the presence and absence of BCE and actein using the GraphPad Prism 6.0 software package (Graphpad Software Inc. LA Jolla, CA, USA). The kinetics best fitted the Michaelis-Menten model $V = \frac{Vmax\ [S]}{Km+[S]}$, where V is the rate of the reaction, V_{max} is the maximum reaction velocity, [S] is the initial substrate concentration and K_m the Michaelis constant. The K_m is the value where the reaction speed has reached ½ V_{max}. The higher the K_m value is, the lower the affinity to the enzyme and the lower the value, the higher the affinity. To determine whether the mode of inhibition is competitive or non-competitive, Lineweaver Burk plots were used. This model is described by the formular:

$\frac{1}{V} = \frac{Km}{Vmax}\frac{1}{[S]} + \frac{1}{Vmax}$, where 1/[S] is plotted onto the x-axis and 1/V onto the y-axis. Using Dixon plots, also the inhibition constants (K_i) were calculated. The K_i is a constant that indicates how potent an inhibitor is. All values are reported as the mean value of three separate experiments with indication of the standard deviation. One-way analysis of variance (ANOVA) and Tukey's post hoc-test were applied to measure the significance of the findings, between the control groups and the treatment samples. When $P < 0.05$, the resulting value was considered to be statistically significant compared to its counterpart.

4. Results and discussion

4.1. Actein content of BCE and stability of actein in cell culture medium

The concentration of actein in BCE was determined to be 0.51%. Stability analyses of actein in DMEM/F-12 cell culture medium when incubated for 48 hours confirmed no changes in the actein concentration.

4.2. Impact of BCE on ERα+ and ERα- breast cancer cell proliferation

The first metric to look at was cellular growth of both MCF-7 (ERα+) and MDA-MB-231 (ERα-) cells without hormones in the presence and absence of BCE or actein (Figure 18Figure 19).

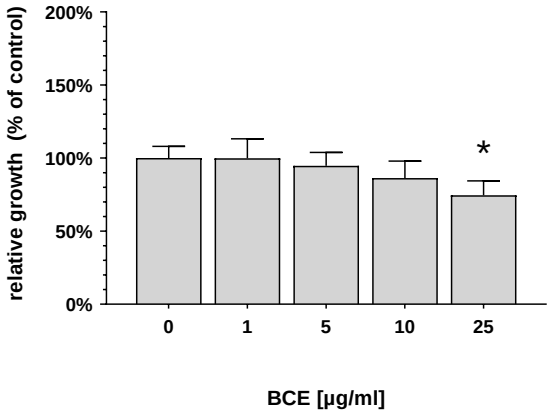

*Figure 18 Impact of increasing BCE concentrations (0-25 µg/ml) on the relative growth of MCF-7 cells (ERα+) in the absence of steroid hormones. * p < 0.05 vs. untreated controls*

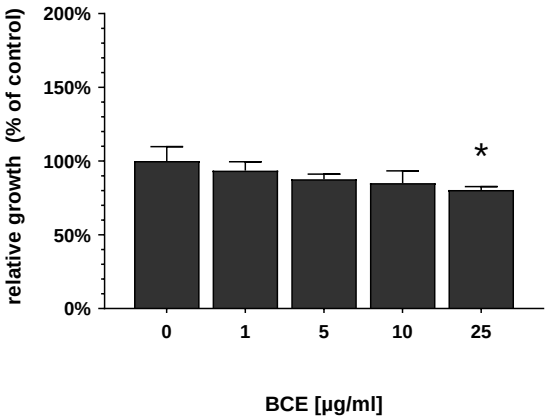

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*Figure 19 Impact of increasing BCE concentrations (0-25 µg/ml) on the relative growth of MDA-MB-231 cells (ERα-) in the absence of steroid hormones. * p < 0.05 vs. untreated controls*

Compared to the control group, a concentration dependent decline in cell growth was observable. In the MCF-7 cell line, the growth rate was

reduced by 25.5 ± 9.9% at the highest concentration (25 µg/ml) of BCE, whereas MDA-MB-231 cell growth was reduced by 19.6 ± 2.2% attributed to the highest concentration (25 µg/ml)] of BCE. To exclude the possibility of DMSO interfering with cell growth, samples containing DMSO (0.1%) were compared to blank samples without any solvent; and no difference was found regarding cellular growth. As with a lot of plant extracts the exact mechanism is not entirely understood and the active components are not completely identified. In a study of 2019 (Crone *et al.* 2019)., a possible explanation for BCE induced inhibition of cell growth was made by investigating the effect on BCE on ER. The study found that ERα expression was down by 49% in MCF-7 cells, potentially affecting cellular proliferation rates. However, as MDA-MB-231 cells don't express ERα but also exhibited an inhibition of cell growth, this can't be the only mechanism of action of BCE, highlighting the need for further investigations. Addition of 100 nM E1 to the media increased the number of viable MCF-7 cells by 67.7 ± 3.0% compared to the hormone deprived control sample, whereas MDA-MB-231 cells remained practically unchanged with a small drop of 1.8 ± 5.6%. Those values are in line with the picture of hormone dependency of the MCF-7 and independency of the MDA-MB-231 cell line. In MCF-7 cells, a concentration dependent inhibitory effect on cell growth was observed (Figure 20) with a maximum inhibition rate of 20.9 ± 2.0% at the maximal BCE concentration (50 µg/ml), as well as an inhibition of 15.5 ± 1.8% in MDA-MB-231 cells (Figure 21). However, these inhibition rates are not significantly different compared to the setting without E1. Another mechanism might be the induction of apoptosis in cancer cells. By monitoring apoptosis markers like phosphatidylserine using Annexin V adherence or CK18 degradation caused by caspase activation (Hostanska *et al.* 2004), apoptosis induced by ethanolic and isopropanolic BCE was observed, thereby inhibiting cellular proliferation rates. While our findings are in harmony with most other studies stating that BCE has no proliferating effect on breast cancer cells (Hostanska *et al.* 2004; Lupu *et al.* 2003), at least one study found that an isopropanolic BCE extract supplied in food does increase metastatic mammary cancer expressing c-erbB2 in mice (Davis *et al.* 2008). Nevertheless, BCE is generally regarded

as safe and as an effective drug against postmenopausal ailments (Friederichsen *et al.* 2020), with actein as the main constituent.

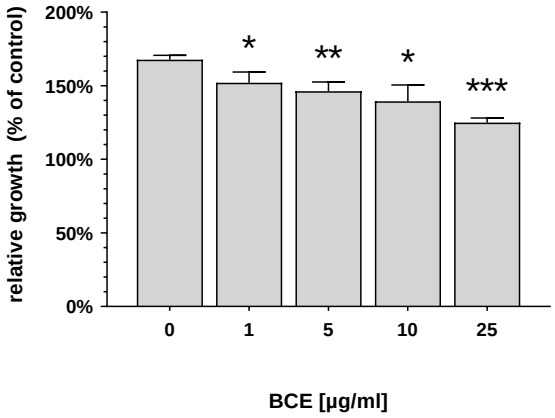

*Figure 20 Impact of increasing BCE concentrations (0-25 µg/ml) on the relative growth of MCF-7 cells in and the presence of the precursor estrogen E1 (100 nM). * p < 0.05, ** p < 0.01, *** p < 0.005 vs untreated controls*

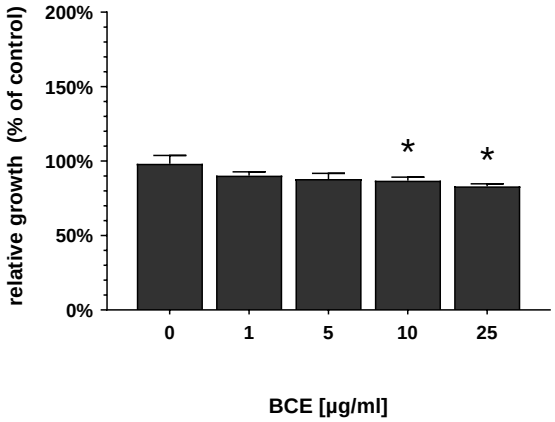

*Figure 21 Impact of increasing BCE concentrations (0-25 µg/ml) on the relative growth of MDA-MB-231 cells in the presence of the precursor estrogen E1 (100 nM). * p < 0.05 vs. untreated controls*

4.3. Impact of actein on ERα+ and ERα- breast cancer cell proliferation

Actein is assessed to be the main constituent of BCE (Einbond *et al.* 2004). Compared to BCE, actein showed a stronger inhibitory effect on cell growth both in MCF-7 (34.2 ± 2.4%) and MDA-MB-231 (30.1 ± 6.8%) cells in a hormone-deprived setting (Figure 22Figure 23). However, MCF-7 cell growth inhibition has no statistically significance compared to BCE.

Surprisingly, the MDA-MB-231 cell line showed a significantly higher inhibition by actein compared to BCE (19.6 ± 2.2%).

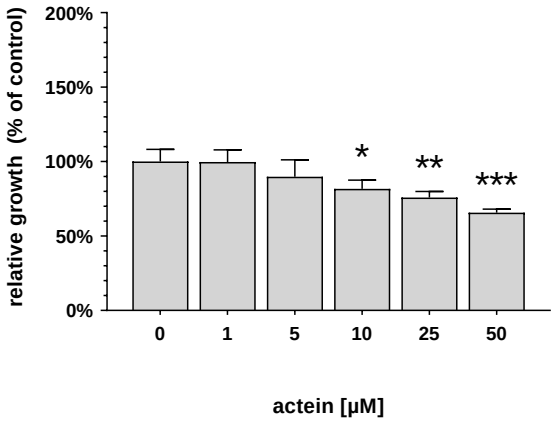

*Figure 22 Impact of increasing actein concentrations (0-50 µM) on the relative growth of MCF-7 cells (ERα+) in the absence of steroid hormones. * p < 0.05, ** p < 0.01, *** p < 0.005 vs. untreated controls*

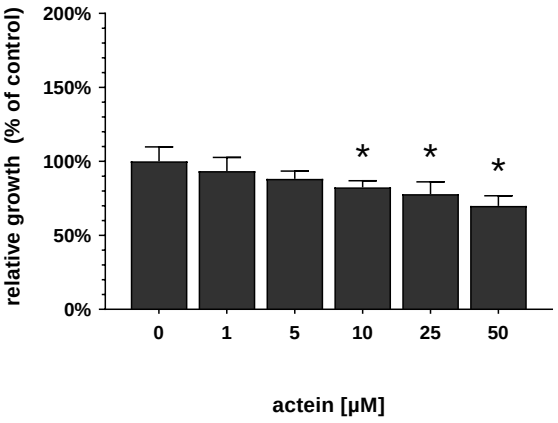

*Figure 23 Impact of increasing actein concentrations (0-50 µM) on the relative growth of MDA-MB-231 cells (ERα-) in the absence of steroid hormones. * p < 0.05 vs. untreated controls*

This result follows other studies proposing an inhibitory effect of actein on MCF-7 and MDA-MB-453 cell growth and cell cycle arrest in G1 (Einbond *et al.* 2004; Wu *et al.* 2018). *In vitro* studies employing the properties of breast cancer cells and human colon cells in combination of luciferase promoter, Western blot and histology assays revealed that actein alters genes relating to calcium homeostasis (Einbond *et al.* 2013). Additionally, actein has an inhibitory effect on the Na^+K^+-ATPase in MDA-MB-453 cells that was exponentially stronger in combination of digitoxin (Einbond *et al.*

2008) as well as a downregulatory effect on cyclin D1, CDK4, pEGFR, in combination with an upregulation of the CDK inhibitory protein $p21^{cip1}$ (Einbond *et al.* 2013). As earlier suggested, BCE influences ERα expression, which has a major influence on ERα+, but less on ERα- cancer cell lines. As actein is assumed to be the main active constituent of BCE, this effect should apply to actein too, but as of now, no studies were conducted on its effect on ER-expression. The many mechanisms of actein though should also apply to BCE albeit on a lower scale, as the actein concentration in BCE is below 1% only.

Incubation of MCF-7 cells in the presence of E1 (100 nM) and actein (Figure 24) yielded a significantly stronger inhibition of cell growth compared to BCE (30.1 ± 6.8% vs. 20.9 ± 2.0%). Stronger inhibition by actein was also observed in MDA-MB-231 cells compared to BCE (27.9 ± 7.0 vs. 15.5 ± 1.8%, Figure 25). The hormone independency of the MDA-MB-231 cell line has been discussed in this work several times. Hence, it should come to little surprise that E1 had no positive impact on cell proliferation of MDA-MB-231 cells, therefore, the inhibitory effect of actein in the presence of E1 was comparable to the experiment with a hormone-deprived setting. As MCF-7 cells are highly dependent on E1, the initial cell growth in the control group was, as expected, elevated compared to the hormone-deprived controls.

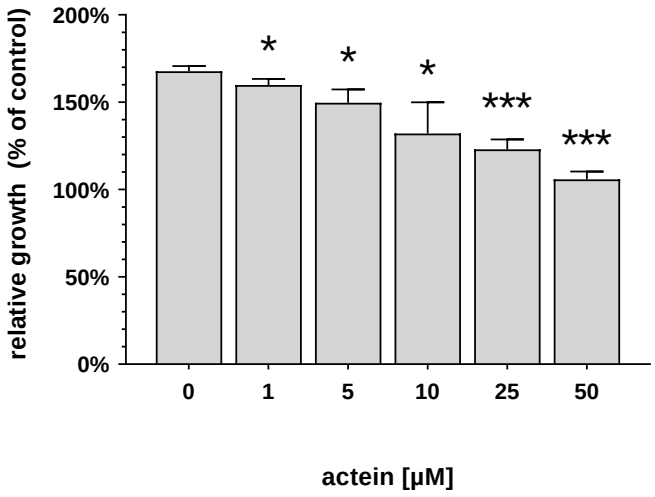

*Figure 24 Impact of increasing actein concentrations (0-50 µM) on the relative growth of MCF-7 cells in the presence of the precursor estrogen E1 (100 nM). * p < 0.05, *** p < 0.005 vs. untreated controls*

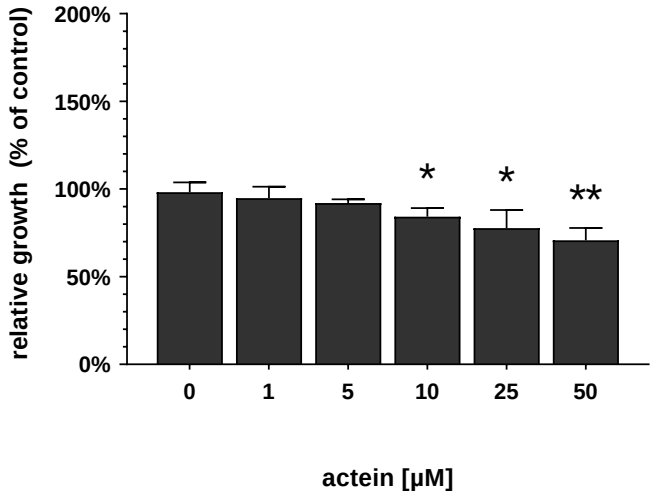

*Figure 25 Impact of increasing actein concentrations (0-50 µM) on the relative growth of MDA-MB-231 cells in the presence of the precursor estrogen E1 (100 nM). * p < 0.05, ** p < 0.01 vs. untreated controls*

4.4. Effects of BCE on steroid metabolism in MCF-7 cells

The use of BCE as a treatment option for climacteric symptoms suggests a potential interplay of BCE with endogenous steroid hormone levels, thereby questioning its administration to cancer patients. To study the impact of BCE on the steroid metabolism of estrogens, MCF-7 cells were

incubated with E1 as hormonal precursor and treated with BCE (Figure 26).

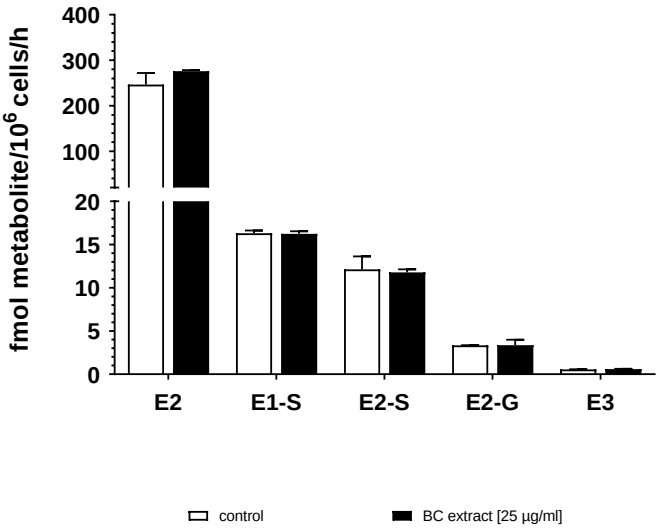

Figure 26 Formation- and metabolization rates of estrogens (fmol/10^6 cells/h) in MCF-7 cells treated with the precursor steroid E1 (100 nM) in the presence and absence of BCE (25 µg/ml).

The quantity of all biotransformation products amounted to 99.5% of the observed reduction of the precursor steroid E1, indicating that the investigated metabolites represent almost all formed biotransformation products. E1 was heavily metabolized into E2 by MCF-7 cells (275.8 ± 2.5 fmol/10^6 cells/h) in presence of 25 µg/ml BCE. Following E2 in quantity were E1-S (16.2 ± 0.3 fmol/10^6 cells/h), E2-S (11.8 ± 0.3 fmol/10^6 cells/h) and E2-G (3.36 ± 0.64 fmol/10^6 cells/h). E3 was also a product of metabolization, albeit in miniscule amounts (0.59 ± 0.04 fmol/10^6 cells/h). However, no significant differences could be observed between the BCE and the control group. This could be attributed to the fact that BCE does not have estrogenic activity (Wuttke and Seidlová-Wuttke 2015). Wuttke used recombinant ERα and Erβ and investigated binding of BCE and its constituents *in vitro*. The results showed neither binding to ERα or ERβ, which would solidify the findings in this work that no strong interference of BCE with the estrogen metabolization pathway occurs in MCF-7 cells. This also adheres to the finding that BCE doesn't increase uterine weight in mice, which would be an indicator of estrogenic activity (Burdette *et al.* 2003). Similarly, no increase of E2 in women taking 5 mg of BCE for 12

weeks was detected (Wuttke *et al.* 2006). Regarding the anticlimactic properties of BCE, different mechanisms seem to be at play. One study for example found that constituents of BCE (75% ethanolic extract) has affinity to the μ opiate receptor expressed in CHO-cells (*in vitro) (Rhyu et al. 2006)*. Other studies attributed some effects to binding to GABA (Henneicke-von Zepelin 2017) or serotonin receptors (Wobser R. W. and Takov V. 2020).These findings in turn positively support the use of BCE for the treatment of side-effects caused by endocrinal therapy in breast cancer (through lack of estrogens), as it seems to be effective against the symptoms while not interfering with the therapeutic outcome.

Subsequently, 100 nM DHEA were administered to the cells to investigate the androgen metabolism (Figure 27). Checking the quantity of the three metabolites DHEA-S, AD and T, the total proportion amounted to 99.8% of the reduction of the precursor concentration, again confirming that the investigated metabolites represent the main biotransformation products of DHEA in MCF-7 cells.

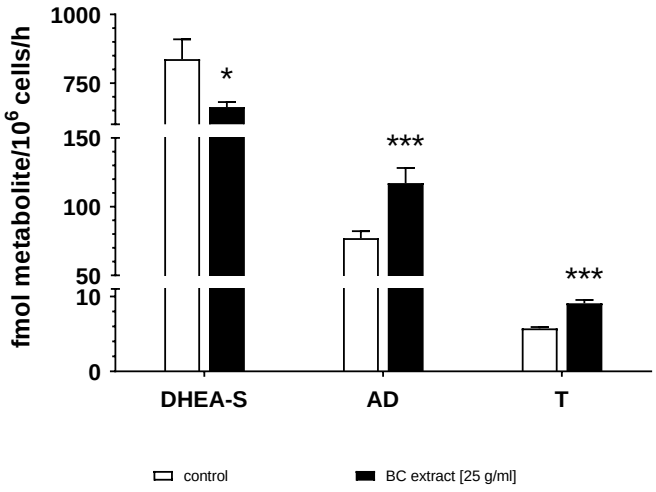

*Figure 27 Formation- and metabolization rates of androgens (fmol/10^6 cells/h) in MCF-7 cells treated with the precursor DHEA (100 nM) in the presence and absence of BCE (25 μg/ml). * $p < 0.05$, *** $p < 0.005$ vs. untreated controls*

A high synthesis of DHEA-S was exhibited by MCF-7 cells (837.7 ± 71.9 fmol/10^6 cells/h), while 3β-HSD (DHEA → AD) and 17β-HSD (AD → T)

activities were significantly less pronounced (AD: 77.1 ± 5.1 fmol/10^6 cells/h and T: 5.75 ± 0.44 fmol/10^6 cells/h). In contrast to the experiments regarding the estrogen metabolism, addition of BCE (25 µg/ml) to the media significantly reduced the synthesis of DHEA-S (to 663.0 ± 18.3 fmol/10^6 cells/h) compared to the control group, whereas AD and T synthesis rose considerably (AD: to 117.2 ± 11.0 fmol/10^6 cells/h and T: to 9.08 ± 0.44 fmol/10^6 cells/h). This is most likely contributed to inhibition of SULT2A1, the main enzyme responsible for the sulfation of DHEA. By inhibition of this enzyme, the unmetabolized precursor DHEA represents a more abundant supply for AD and T synthesis, in turn raising both metabolite concentrations. As aromatase activity in MCF-7 cells is very low, it is obvious that these accumulate in the media. These interesting findings concerning a raise in androgen levels might actually contribute to the climacteric relief women experience when supplementing with BCE, since studies showed that T alleviates postmenopausal symptoms like hot flashes (Øverlie *et al.* 2002). However, a potential biotransformation to estrogens *in vivo* via the aromatase pathway raises concerns regarding the safety for cancer patients, highlighting the need of further human studies.

4.5. Effects of BCE on steroid metabolism in MDA-MB-231 cells

When MDA-MB-231 cells were incubated in the presence of E1 (Figure 28), E2 synthesis was also observed (250.8 ± 8.5 fmol/10^6 cells/h), but further metabolization to conjugated steroids such as E1-S (0.48 ± 0.02 fmol/10^6 cells/h), E2-S (0.10 ± 0.01 fmol/10^6 cells/h) and E2-G (0.20 ± 0.02 fmol/10^6 cells/h) was executed to a much lower extent. In line with the findings in MCF-7 cells, the quantity of all metabolization products amounted 97.1% of the reduction of the precursor E1; indicating that no other metabolites than the investigated ones are formed by the cell line.

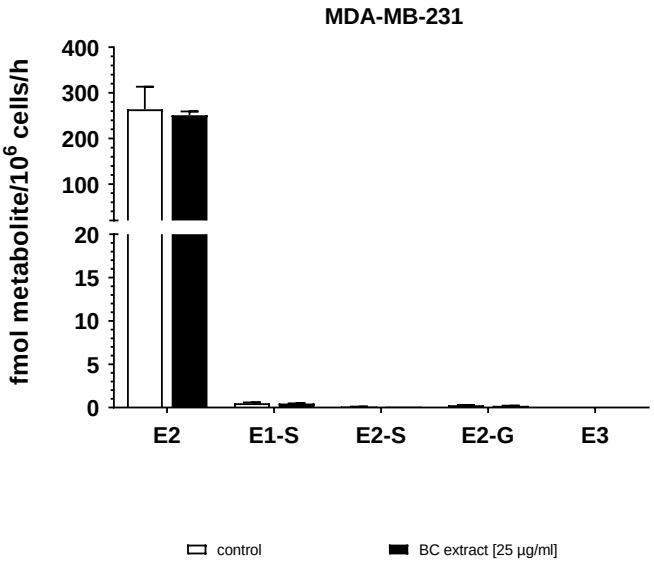

Figure 28 Formation- and metabolization rates of estrogens (fmol/10^6 cells/h) in MDA-MB-231 cells treated with the precursor steroid E1 (100 nM) in the presence and absence of BCE (25 µg/ml).

Similar to the effects in MCF-7 cells, no significant changes in the estrogen pathway were detected upon addition of BCE to the cellular media. E3 was not detectable in the supernatant of MDA-MB-231 cells at all. These data show that E2 synthesis and biotransformation is virtually unchanged by BCE in MDA-MB-231 cells. The E1-S, E2-S, E2-G and E3 values point to very low activities of SULT1A1, SULT1E1 and CYP3A4 in MDA-MB-231 cells.

Moreover, DHEA (100 nM) in combination with BCE was added to the MDA-MB-231 cell line (Figure 29). Again, the quantity of the biotransformation products in MDA-MB-231 cells amounted to the reduction of the precursor steroid DHEA (97.9%).

Metabolization of DHEA to DHEA-S was comparably low in both presence and absence of BCE (Control: DHEA 0.86 ± 0.12 fmol/10^6 cells/h vs. BCE 25 µg/ml: DHEA 0.83 ± 0.09 fmol/10^6 cells/h), indicating that SULT2A1 activity is almost nonexistent in these cells. This in turn led to high DHEA concentrations that can be further converted to AD and T. Therefore, one can assume that BCE doesn't inhibit 3β-HSD and 17β-HSD activity. Looking at both the E1 and DHEA metabolism, there were almost no noticeable changes in biotransformation upon addition of BCE. Therefore,

no further experiments in context of steroid metabolism were conducted with MDA-MB-231 cells.

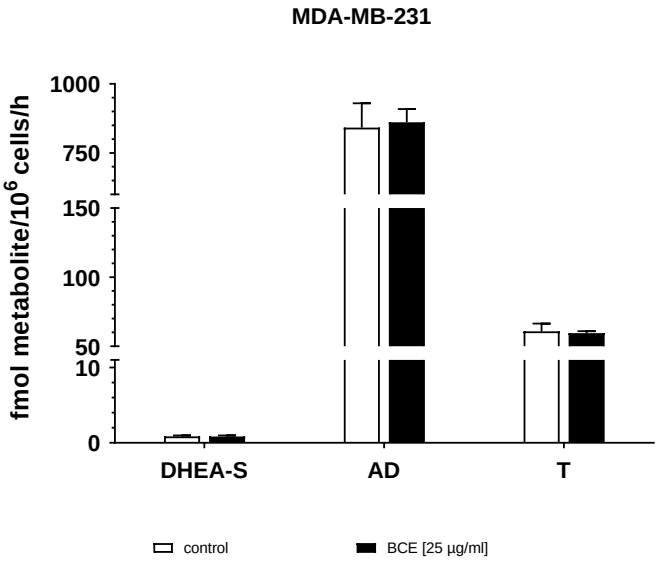

Figure 29 Formation- and metabolization rates of androgens (fmol/10^6 cells/h) in MDA-MB-231 cells treated with the precursor steroid DHEA (100 nM) in the presence and absence of BCE (25 µg/ml).

4.6. Impact of actein on steroid metabolism in MCF-7 cells

As the addition of BCE to the media of MCF-7 cells revealed significant changes in the activity of DHEA sulfation to DHEA-S, in turn raising AD and T concentrations, the main constituent of BCE, actein, was added to the cells to check for changes of the E1 metabolization caused by actein. Firstly, 100 nM of E1 were added in the presence of increasing concentrations of actein (0.5-10 µM). Monitoring the E2, E1-S, E2-S, E2-G and E3 levels, no observable changes compared to the control group could be observed, comparably to the incubations with BCE.

By contrast, strong changes were seen in the androgen pathway upon addition of actein (0.5-10 µM, Figure 30). The lowest concentration of actein (0.5 µM) was enough to inhibit the transformation of DHEA to DHEA-S by 45.2% (827.4 ± 75.5 to 453.8 ± 52.2 fmol/10^6 cells/h), while the subsequent increase of the actein concentration resulted in an almost

quantitative inhibition of DHEA-S formation (10 µM: 44.9 ± 6.1 fmol/10^6 cells/h).

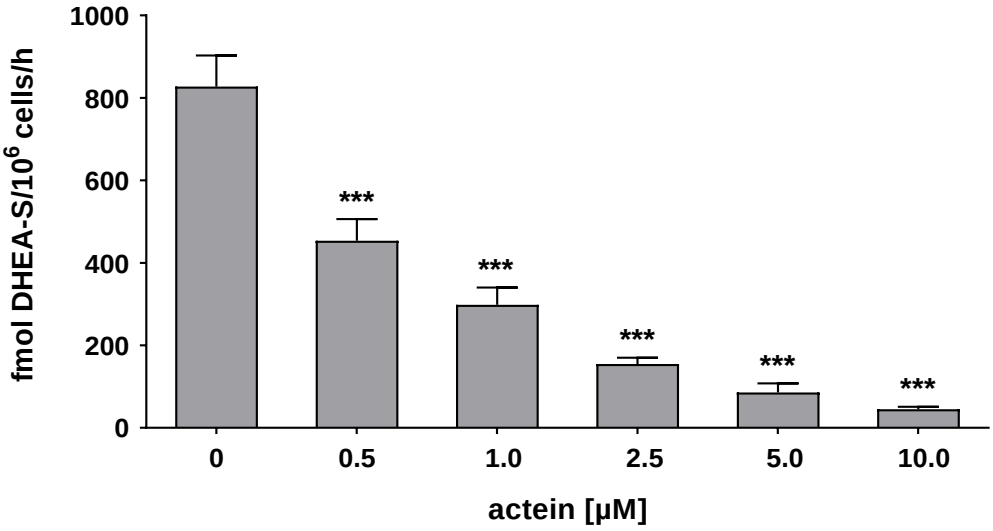

*Figure 30 Impact of increasing concentrations of actein (0-10 µM) on the DHEA-S formation in MCF-7 cells treated with the precursor DHEA (100 nM). *** p < 0.005 vs. untreated controls*

As aforementioned in the methods section, the Michaelis-Menten-kinetics model was the best fitted enzymatic model for this reaction. While K_m values remained largely unchanged upon addition of actein, V_{max} decreased considerably dependent of the actein concentration (Figure 31).

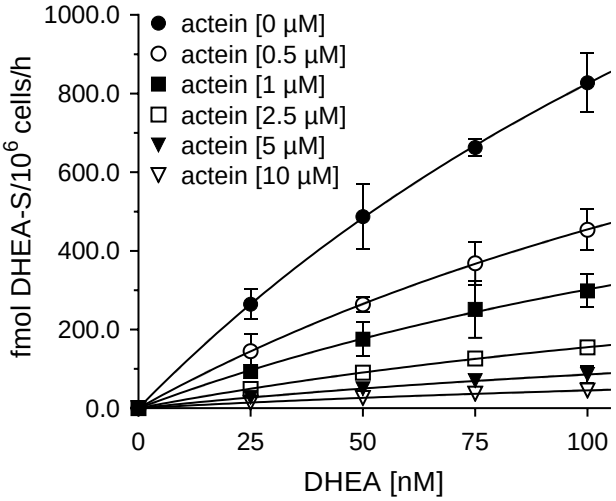

Figure 31 Michaelis-Menten kinetics for the formation of DHEA-S in MCF-7 cells upon addition of the precursor DHEA (0-100 nM) and increasing concentrations of actein (0-10 µM).

Investigating the Lineweaver-Burk plot (Figure 32), a non-competitive inhibition mode was confirmed. The Dixon plot revealed a K_i value of 0.58 ± 0.02 µM (Figure 33).

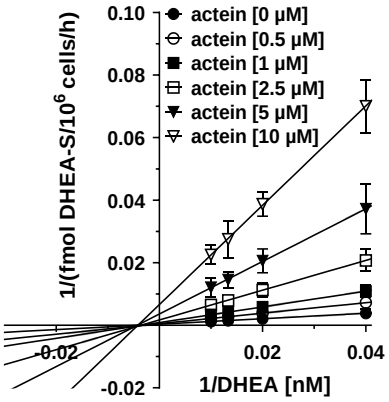

Figure 32 Lineweaver-Burk plot revealing non-competitive inhibition of DHEA-S formation by increasing concentrations of actein (0-10 µM) in MCF-7 cells.

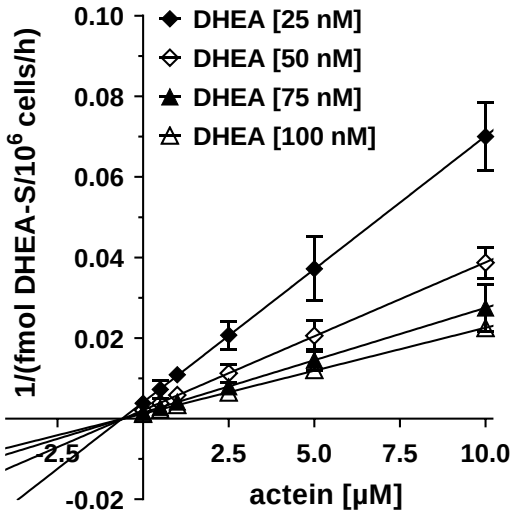

Figure 33 Dixon plot for the inhibition of DHEA-S formation in MCF-7 cells upon addition of the precursor DHEA (0-100 nM) and increasing concentrations of actein (0-10 µM).

As it was the case with BCE, actein also increased AD and T formation in cells treated with 100 nM DHEA by a huge margin (Figure 34 and Figure 35). The lowest concentration of actein (0.5 µM) was enough to raise AD formation from 66.5 ± 4.0 to 133.8 ± 15.7 fmol/10^6 cells/h; an increase by

101.2%. Subsequent biotransformation of AD to T was also elevated by 50.2% (4.60 ± 0.44 to 6.91 ± 1.08 fmol/10^6 cells/h). As the smallest concentration was enough to influence the androgen pathway to such an extent, it comes to no surprise that at maximum concentration (10 µM) a big increase in AD (550.0 ± 22.6 fmol/10^6 cells/h) and T (34.6 ± 1.0 fmol 10^6 cells/h) formation was detected.

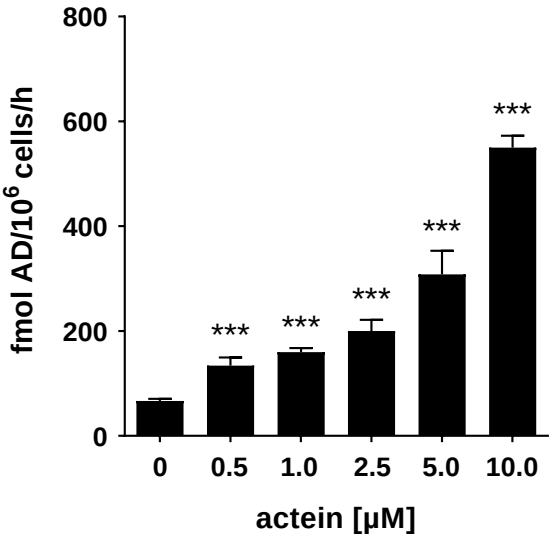

*Figure 34 Impact of increasing concentrations of actein (0-10 µM) on the formation of AD in MCF-7 cells treated with the precursor DHEA (100 nM). *** p < 0.005 vs. untreated controls*

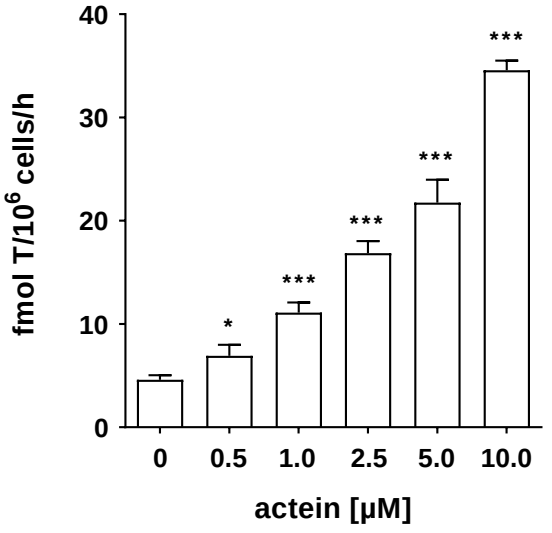

*Figure 35 Impact of increasing concentrations of actein (0-10 µM) on the formation of T in MCF-7 cells treated with the precursor DHEA (100 nM). * p < 0.05, *** p < 0.005 vs. untreated controls*

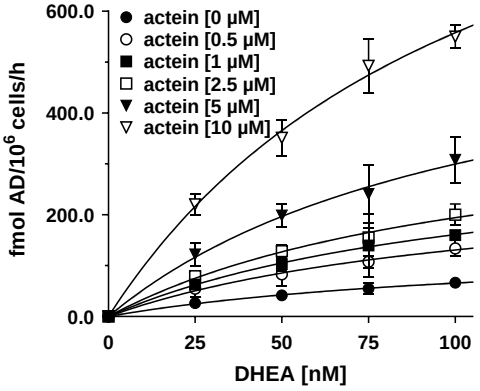

Figure 36 Michaelis-Menten kinetics for the formation of AD in MCF-7 cells upon addition of the precursor DHEA (0-100 nM) and increasing concentrations of actein (0-10 µM).

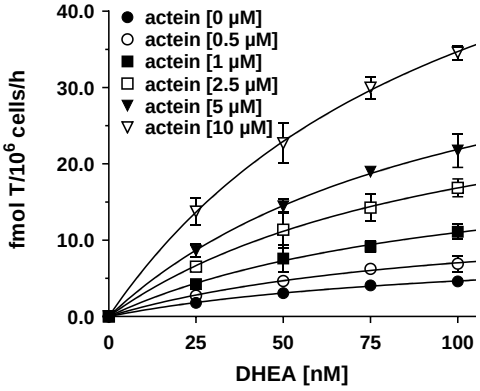

Figure 37 Michaelis-Menten kinetics for the formation of T in MCF-7 cells upon addition of the precursor DHEA (0-100 nM) and increasing concentrations of actein (0-10 µM).

These results suggest an inhibition of SULT2A1, as the concentration of DHEA-S was significantly reduced in favor of an increased DHEA to AD and T metabolization. Aromatase activity in MCF-7 cells is sparse (Rahideh *et al.* 2017); consequently, no detectable levels of E2 formation were found. Kinetics of AD (Figure 36) and T (Figure 37) formation were assessed using increasing concentrations of DHEA (0-100 nM) as a hormone precursor. Even though a lot of studies focused on estrogen-pathways, there aren't a lot of studies regarding androgens. Looking at the task that aromatase fulfills, one could assume that an increased AD and T formation might be a cause of concern, as they can be further metabolized into E1 and E2, which are both regarded as growth stimulating hormones for ERα+ breast cancer. However, as of yet, no studies could find a correlation of estrogen levels rising upon intake of BCE (Szmyd *et al.* 2018; Rostock *et al.* 2011). By contrast, the increased T formation might actually contribute to the reduction of symptoms following lack of estrogens (Øverlie *et al.* 2002). Interestingly, the observed inhibition of steroid sulfation seems to be highly specific for SULT2A1, as E1-S and E2-S levels remained largely unaffected (Figure 38 andFigure 39). Therefore,

this hypothesis should be further verified by incubating recombinant SULT2A1 *in vitro*.

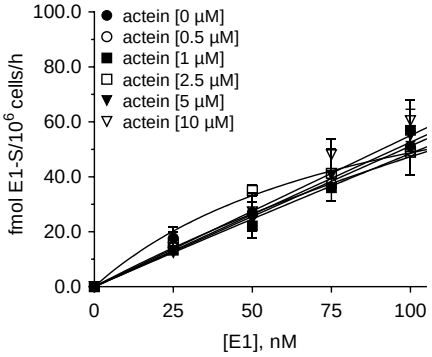

Figure 38 Kinetics for the formation of E1-S in MCF-7 cells upon addition of the precursor E1 (0-100 nM) and increasing concentrations of actein (0-10 µM).

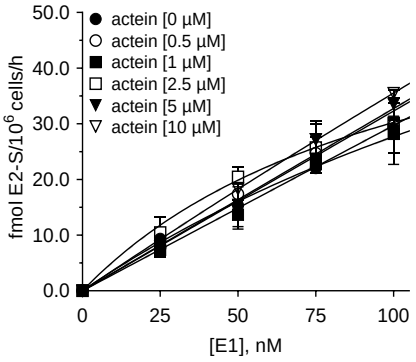

Figure 39 Kinetics for the formation of E2-S in MCF-7 cells upon addition of the precursor E1 (0-100 nM) and increasing concentrations of actein (0-10 µM).

4.7. Inhibition of SULT2A1 by BCE or actein

To confirm the proposed inhibition of SULT2A1 by BCE or actein, recombinant SULT2A1 was incubated with 25 nM DHEA and multiple concentrations of BCE (1-25 µg/ml) or actein (1-25 µM) for 15 min at a temperature of 37 °C (Figure 40). BCE at the highest concentration (25 µg/ml) caused a reduction of DHEA-S formation from 8.1 ± 1.2 pmol/min/mg protein (DMSO only control) to 6.0 ± 0.9 pmol/min/mg protein, representing an inhibition by 26.5%. Staying in line with the results from the MCF-7 cell incubation experiments, actein showed an even stronger inhibition. At maximum concentration (25 µM), inhibition reached 64.3% compared to the untreated control (from 8.1 ± 0.9 to 2.9 ± 0.1 pmol/min/mg protein).

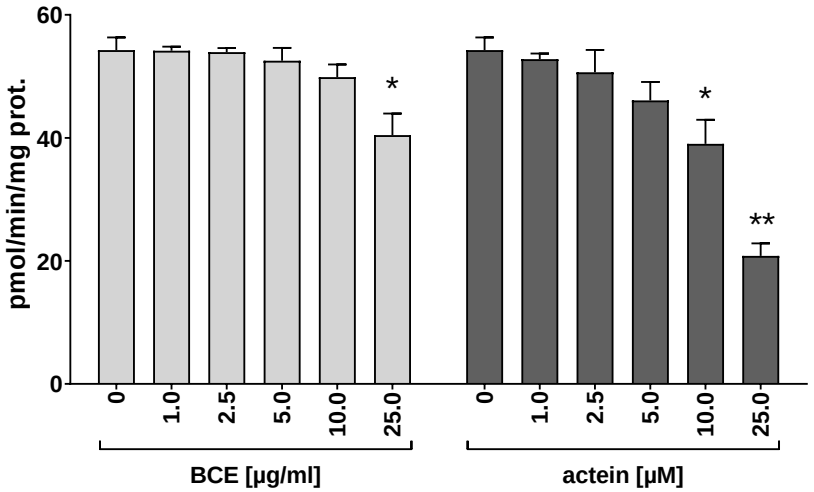

Figure 40 Inhibition of DHEA-S formation by recombinant human SULT2A1 under the influence of BCE (0-25 µg/ml) or actein (0-25 µM).

Inhibition of SULT2A1 and DHEA-S formation is visualized in Figure 43 Figure 45. Using the Dixon plots (Figure 41 and Figure 42), the K_i values were calculated to be 67.7 ± 1.5 µg/ml for BCE and 14.0 ± 0.2 µM for actein, respectively. As it was the case in the cell line experiments, the Lineweaver-Burk plots confirmed the inhibition mode to be non-competitive (Figure 44 and Figure 46).

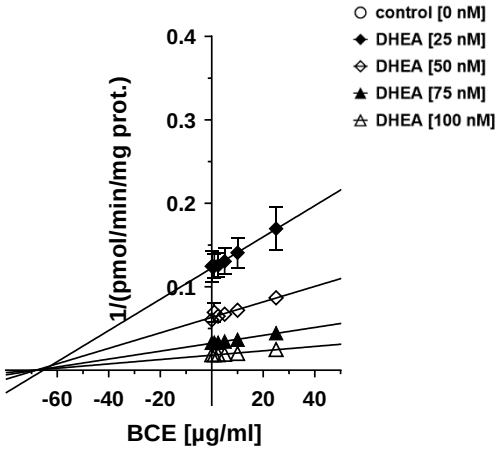

Figure 41 Dixon plot for the inhibition of DHEA-S formation by recombinant SULT2A1 upon addition of the precursor DHEA (0-100 nM) and increasing concentrations of BCE (0-25 µg/ml).

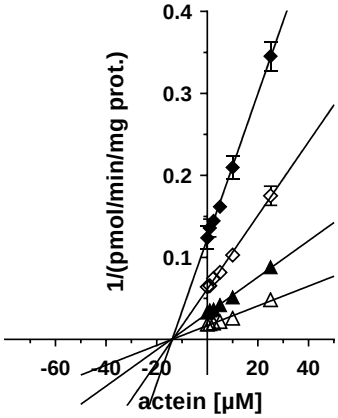

Figure 42 Dixon plot for the inhibition of DHEA-S formation by recombinant SULT2A1 upon addition of the precursor DHEA (0-100 nM) and increasing concentrations of actein (0-25 µM).

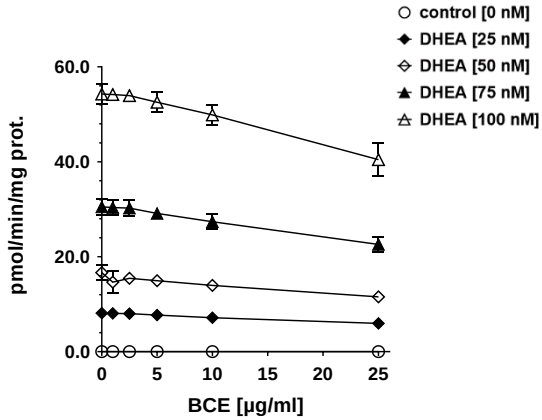

Figure 43 Inhibition of SULT2A1 activity measured by its DHEA-S formation capability upon exposure to BCE (0-25 µg/ml).

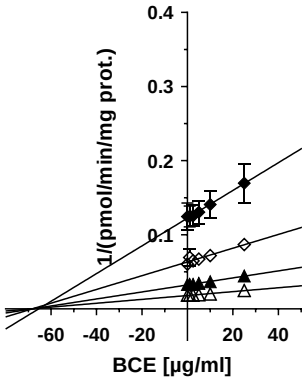

Figure 44 Lineweaver-Burk plot for the inhibition of recombinant SULT2A1 by BCE (0-25 µg/ml).

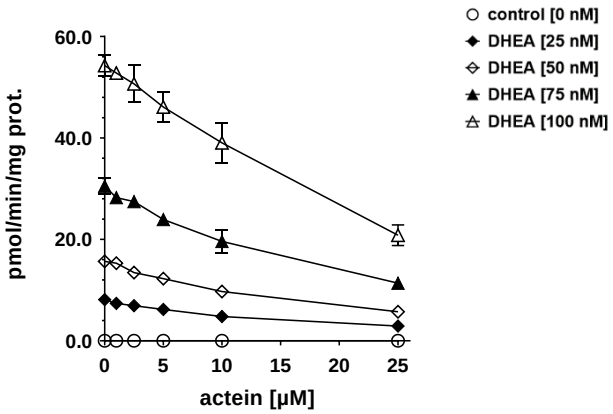

Figure 45 Inhibition of SULT2A1 activity measured by its DHEA-S formation capability upon exposure to actein (0-25 µM).

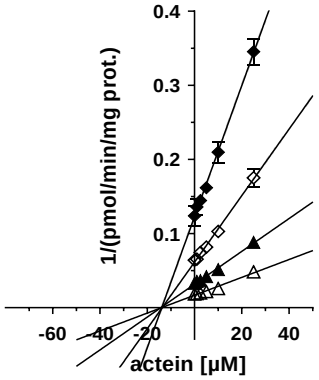

Figure 46 Lineweaver-Burk plot for the inhibition of recombinant SULT2A1 by actein (0-25 µM).

These findings support the hypothesis that actein is a selective and strong inhibitor of SULT2A1, therefore causing elevated levels of DHEA, which subsequently causes increased androgen concentrations (Figure 47). This may be – among other mechanisms – a possible explanation for the beneficial effect of BCE against disorders caused by estrogen-deprivation, such as hot flashes.

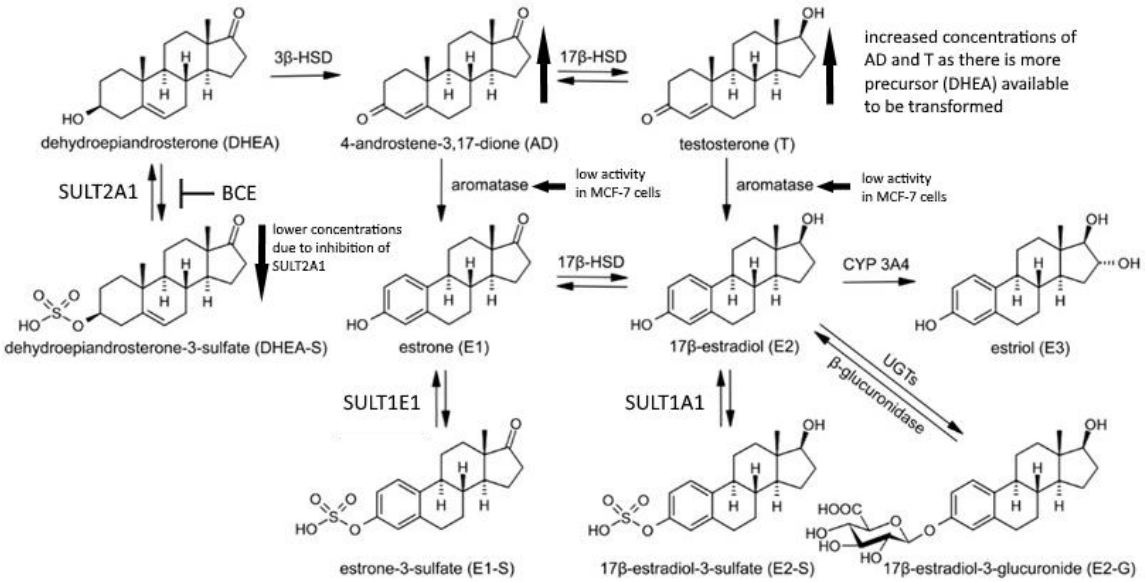

Figure 47 Pathways of steroid hormone biotransformation in human breast cancer. BCE inhibits SULT2A1 activity, thereby increasing the levels of unconjugated DHEA, which in turn is further metabolized to increased AD and T levels.

A potential risk of elevated estrogen levels due to aromatase-catalyzed conversion *in vivo* seems hypothetical, as multiple human studies (Ruan *et al.* 2019; Hernández M. and Pluchino 2003; Dog T. *et al.* 2003) regarding safety and efficacy of BCE could not confirm this increase, highlighting the benefits of BCE intake.

5. Conclusion

Using BCE as an herbal remedy to alleviate symptoms caused by lack of estrogens (postmenopausal syndrome or antiestrogenic therapy) shows a lot of promise. Cell growth and proliferation of MCF-7 (ERα+) and MDA-MB-231 (ERα-) breast cancer cells were suppressed, showing that BCE and its main compound actein don't stimulate breast cancer growth, thereby suggesting that the use of BCE appears safe for cancer patients. Using an established LC-MS assay, an influence on the biotransformation of steroid hormones was proven. While it confirmed the proposal that BCE does not have a major impact on the estrogen synthesis, androgen generation was raised considerably. This effect was more profound when cells were treated with purified actein, reinforcing the assumption that actein is the main active constituent of BCE. This increase of androgen concentrations is contributed to the inhibition of SULT2A1, an enzyme that catalyzes mainly the sulfation of DHEA and androgens. By inhibiting the sulfation of DHEA, more substrate is available for 3β-HSD and 17β-HSD mediated conversion to AD and T. However, in-depth observation and investigation are advised to rule out different mechanisms of action influencing the androgen metabolism. While theoretically higher concentrations of androgens might be a cause of concern, as E1 and E2 generation via the aromatase pathway could fuel cancer development, progression and cell proliferation *in vivo*, no studies found evidence of heightened estrogen-generation substantiating safety of taking BCE even for patients suffering from breast cancer. The increased androgen concentrations may contribute to the positive effects of BCE regarding the relief of climacteric symptoms. This study also comes to show that using immunoassays for steroid quantification might be a thing of the past, as immunoassays are prone to crossreactions and lower specificity, while an MS driven approach is capable to distinguish unconjugated steroids and their respective conjugated metabolites in a highly specific manner, allowing the observation of interactions between exogenous compounds and the endogenous hormone biotransformation pathways on the metabolomic level. Until now, little interest was shown regarding the

influence of BCE on the androgen pathway when investigating its effect in females. This thesis should highlight how little we know about BCE and that more clinical studies regarding hormone metabolism are highly warranted.

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7. List of Figures and Tables

8. Zusammenfassung

Actaea racemosa *L.*, allgemein bekannt unter dem Namen Traubensilberkerze, wird als Phytotherapeutikum gegen klimakterische Beschwerden verwendet. Die Unbedenklichkeit steht allerdings noch in Frage, da das Extrakt der Traubensilberkerze potenziell Brustkrebszellwachstum hervorrufen könnte. Derzeit existieren laufende Diskussionen bezüglich eines eventuellen östrogenen Effekts: Während manche Studien eine Krebszellstimulierung beschreiben, stellten andere eine Hemmung des Zellwachstums fest. Um die Effekte des Extraktes der Traubensilberkerze und ihres Hauptinhaltsstoffes Actein zu untersuchen, wurden MCF-7 (Östrogenrezeptor positiv) und MDA-MB-231 (Östrogenrezeptor negativ) Brustkrebszellen für 48 h mit und ohne den Östrogenvorstufen Dehydroepiandrosteron (DHEA) und Estron (E1) in An- und Abwesenheit des Extraktes sowie von Actein inkubiert. Eine Suppression des Zellwachstums wurde sowohl mit dem Extrakt als auch mit der Reinsubstanz Actein beobachtet. Dies weist daraufhin, dass eine Behandlung mit dem Traubensilberkerzen-Extrakt auch bei Brustkrebspatientinnen sicher erscheint. Zusätzlich dazu wurden die Produkte des hormonellen Kreislaufes durch eine validierte LC-MS-Methode gemessen. Die Ergebnisse zeigen, dass der Östrogenmetabolismus unter Einwirkung der Traubensilberkerze unverändert bleibt, während die Androgene Testosteron und Androstendion vermehrt produziert werden. Dies ist auf die Inhibierung von SULT2A1 zurückzuführen, wodurch mehr DHEA für 3β-HSD- und 17β-HSD-medierte Metabolisierungsschritte überbleibt. Diese Arbeit zeigt, dass Traubensilberkerzen-Extrakt einen Einfluss auf die Androgen-Synthese nimmt. Aufgrund der geringen Datenlage zu diesen Erkenntnissen, sollten diese Mechanismen in zukünftigen humanen Studien näher betrachtet werden.