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Chapter 1

Molecular and Cell Biological Investigations of the Mode of Action of Established and Potential Phytoestrogens for the Development of Strategies in the Prevention and Treatment of Cancer

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Abstract

Phytoestrogens are naturally occurring, plant-derived, non-steroidal phytochemicals. The major structural classes of phytoestrogens are the isoflavones and lignans found at high levels in various plants such as soybeans, clover or flax. Since their chemical structures are similar to endogenous estrogens, they are able to bind to human estrogen receptors (ERα and ERβ) and act as selective estrogen receptor modulators (SERMs).

Epidemiological data support the idea that consumption of phytoestrogens could be associated with beneficial effects regarding the prevention or inhibition of carcinogenesis of hormone-dependent malignancies. Furthermore, clinical studies have demonstrated that phytoestrogens are potentially beneficial in treating osteoporosis and arthrosis, as well as mammalian and endometrial carcinoma (primary and secondary prevention). Due to an

apparent increase in the incidence of breast cancer in the Western World compared to most countries in Asia the interest in phytoestrogens has increased tremendously. However, up to now the modes of action of the different phytoestrogens at the molecular and cellular level are not well understood.

To enlighten the mechanisms underlying phytoestrogen function, we investigated the effects of synthetic isoflavones and lignans, and of phytoestrogen extracts from various plants in comparison to synthetic estrogens and antiestrogens in human mammalian, endometrial and trophoblast tumor cells as well as primary cells (cell vitality, cell proliferation, cytotoxicity and gene expression). The extracts from flax roots of *Linum usitatissimum* and from the bark of *Ulmus laevis* inhibited the cell vitality and cell proliferation in a concentration-dependent manner without showing strong cytotoxicity. Concentrations >100 μg/ml induced oncocidal effects in our tumor cells.

To analyze the substance classes of the flax root and elm bark extracts Pyrolysis Field Ionization Mass Spectrometry (Py-FIMS) was performed. Flax root extracts are composed mainly of phenols and lignans, while elm bark extracts contained primarily sterols, phenols, lignans and flavonoids. Furthermore, HPLC-MS analysis demonstrated that the flax root extracts are comprised of more representatives of lignans compared to isoflavones. Considering also that the metabolism of phytoestrogens in the human organism is little-known, further research in clinical studies needs to be conducted to develop strategies in the prevention and treatment of cancer.

1. Introduction

1.1. Medical Background and Therapeutic Goals

Of all malignant tumors in women, those of the breast (mammary carcinoma) have the highest annual incidence rate, around 25 new cases per 100,000 females. Mammary carcinomas have thus become a key theme in the fields of gynecology and oncology. In 1990, 38,000 cases were registered in Germany whereas by 2002 the number had risen to 55,100, making it clear that preventive research must be given priority. Despite the introduction of new adjuvant and palliative chemotherapeutic treatments and the establishment of primary tumor surgery, a favorable prognosis can hardly be expected in the near future [Page 1996, Kuo et al. 2006]. Up to now, there have been no consolidated findings on the prevention (chemoprevention) of mammary carcinomas, even though environmental and nutritional factors play an important role. Epidemiological studies have revealed that Asian women, compared to European or North American women have a significantly lower incidence of mammary carcinomas and lower mortality rates for hormone-dependent tumors. They also suffer less from climacteric symptoms and have high phytoestrogen levels in their urine. These effects are attributed to the high dietary intake of soy-based products, which are rich in isoflavones [Steinmetz et al. 1991]. Japanese women who migrated to Hawaii had a three-fold higher breast cancer risk. Foods and supplements containing phytoestrogens could thus constitute the basis for a chemoprevention (prophylaxis) of mammary carcinoma in the future.

It has been discussed for several years whether fruits, vegetables, or whole-grain foods have a high potential for cancer prevention and whether the increase in diet-dependent tumors

is caused by a lack of protective constituents in the diet, e.g., vitamins, minerals, trace elements, fiber, secondary plant metabolites. Secondary plant metabolites can thus be considered for their role in cancer prevention as well as in modulation of tumor growth [Mothes 1980, Nahrstedt 1990, Knight and Eden 1996]. Approximately 30,000 secondary plant metabolites are known to date, of which 5,000–10,000 are found in the diet [Ames et al. 1990].

Within the past ten years, interest in the physiological role of bioactive compounds in plants has increased sharply, especially with regard to the group of substances known as phytoestrogens in relation to human health. The phytoestrogens are compounds from several diverse classes of non-steroidal secondary plant metabolites, including isoflavones, lignans, and coumestans, exhibiting clinical efficacies similar to those of estrogens [Adlercreutz et al. 1991, 1992, 1995, Adlercreutz 1995] because of structural similarities (Figure 1). They bind to estrogen receptors (ERα, ERβ) and, in addition, exert an estrogenic and/or antiestrogenic effect on various target organs by influencing the biosynthesis and metabolism of endogenous hormones. Previous findings have shown that phytoestrogens exhibit only 0.1% the efficacy of human estrogens, but it is interesting to note that the concentration of phytoestrogens in human urine is 10 to 10^3 times that of endogenous human estrogens [Setchell und Adlercreutz 1988]. However, with an appropriate diet, it is possible to reach phytoestrogen plasma levels of 50-800 ng/ml, which is 10^3 to 10^4 times that of estradiol plasma levels. Compounds that fail to exert all of the effects of estradiol but exhibit a more or less selective activity profile in a given organ are called “Selective Estrogen Receptor Modulators” (SERMs). This term, when applied to phytoestrogens, has lead to the classification of some phytoestrogens as phytoSERMs.

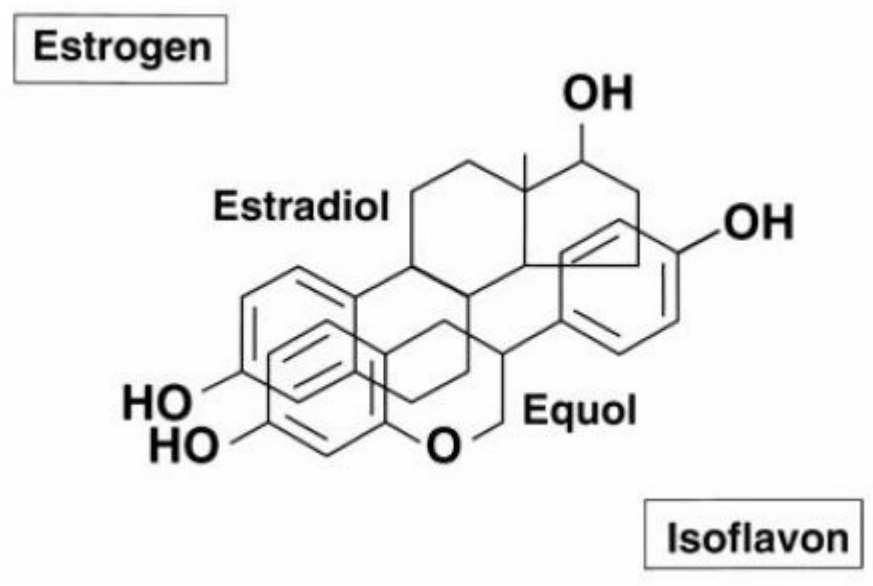

Figure 1. Structural similarities of estrogens and phytoestrogens (e.g. isoflavones).

1.2. Impacts of Phytoestrogen-Rich Diets

"Let food be thy medicine and medicine be thy food"— this advice given 2500 years ago by Hippocrates has never been more relevant than it is today [Kleine-Gunk 2008].

There are promising epidemiological studies that associate a phytoestrogen-rich diet with fewer deaths due to chronic diseases, such as breast and prostate cancer, cardiovascular disease, and osteoporosis [Messina 1999, Clarkson 2002, Setchell et al. 2003]. Studies involving immigrants have shown that a diet containing soy products reduces the breast cancer risk, especially when soy intake took place before puberty or during adolescence [Adlercreutz 2003, Stephens 1997]. According to our present state of knowledge, the premenopausal risk of mammary carcinoma can be reduced by isoflavones (incidence lowered by 50%). Opposing results were obtained in a comprehensive retrospective study on isoflavone intake by 16,165 Dutch women, which found no evidence for a protective effect of phytoestrogens with regard to cardiovascular disease [van der Schouw and Grobbee 2005]. Phytoestrogens may also play a role in brain development and in the prevention of neurodegenerative disease [Branca and Lorenzetti 2005, Kreijkamp-Kaspers et al. 2005, Patisaul 2005, Schreihofer 2005]. Setchell et al. [1997] reported on soy-based infant formula diets. Children on a soy-rich diet had levels of serum isoflavone between 10^6 and 10^7 pg/ml, whereas in adults it was between 10^4 und 10^6 pg/ml, so it is possible to achieve high isoflavone serum concentrations. In a 14-day case-control study, 35 women were given a daily dietary supplement of 60 g soy protein (45 mg isoflavone). After 2 weeks, significantly higher serum concentrations of both genistein and daidzein could be detected [Harding et al. 1997]. The biological effects of genistein have been studied most extensively. Investigations have shown that genistein, which can be detected in human urine after soybean intake [Adlercreutz et al. 2004b], suppresses the growth of new blood vessels *in vitro*. Tumor growth and metastasis thus could be blocked [Fotsis et al. 1993]. Since 1991, five case-control studies on the problem of soy-based diet and mammary carcinomas are known [Messina et al. 1997]. Three of these studies, in which soy was part of the regular diet, showed that the risk for premenopausal mammary carcinomas was substantially lowered, whereas only one of the studies confirmed this effect for postmenopausal mammary carcinomas. Mammary carcinomas in younger women (< 35 years of age) differ biologically from those of older women: the proliferation rate is higher and the differentiation stage is lower. The surface marker p53 is more often expressed [Henderson und Patek 1997]. It could be shown that a soy-rich diet increased the menstrual cycle length in proband women. Other clinical investigations, if only involving a small number of cases, showed that within the menstrual cycle progesterone, estrogen, and androgen levels can be lowered by a daily isoflavone intake of 100 mg daidzein and 100 mg genistein [Lu et al. 1996]. In the premenopausal phase, the serum concentrations of FSH and LH were lowered significantly. Ingram et al. [1997] published a case-control study on the relation between isoflavone intake and mammary carcinoma risk. This study has a definite advantage over the sole use of diet anamneses because objective criteria, i.e., measurement of isoflavone metabolites in the urine, were used for the evaluation. The measurement of isoflavones in the urine provides information on the dietary intake of these plant metabolites as well as on their metabolism by intestinal bacteria (flora) and their bioavailability. Between 1992 and 1994, 149 patients

(from the Perth area, Western Australia) who had been diagnosed with mammary carcinomas were admitted. Urine collected over a 72-hour period and a venous blood sample were used to determine FSH and estradiol levels in the laboratory. Control patients exhibited a higher median excretion of all isoflavones, and the median excretion of the mammalian lignan enterolactone was even 50% higher in the control group. High urinary equol and enterolactone excretions could thus be associated with reduced breast cancer risk. When evaluating the results, it should be taken into consideration that the study participants were subjected to exceptional stress (a mentally stressful situation in this case) and it is presently unknown if this has an effect on phytoestrogen excretion. In addition to the beneficial effects of isoflavones on health, i.e., cancer, osteoporosis, and possibly cardiovascular diseases, they also relieve premenstrual and climacteric symptoms during the premenstrual or climacteric phase. This is of clinical relevance because more than half of the women suffer from premenstrual symptoms, e.g., depressed mood, irritability, aggressiviness, breast tenderness, headaches, having to deal with weight gain as well. Besides, 30% of the women in Germany are in the postmenopausal phase of life. Due to large-scale studies in the past few years, hormone replacement therapy (HRT) is no longer generally recommended; besides, fewer than 5% of the women use HRT for more than 5 years. Prospective controlled, randomized studies have demonstrated the favorable effect of functional food (soy bread) and a dietary supplement on subjective discomfort in the climacterium. In addition, the serum levels of bone-specific alkaline phosphatase increased (osteoblast activity) and pyridinoline/deoxypyridinoline (osteoclast activity) decreased. None of the „soy-diet experiments“ had a detectable influence on thyroid gland, prolactin, FSH, LH, testosterone, insulin, or progesterone parameters. In single cases, an increase in serum DHEA-S and serum estradial concentrations was observed.

In animal experiments on the influence of the glucosinolate metabolite indole-3-carbinol in the diet, the formation of estrogens (e.g., catechol estrogen) increased to a level only slightly beneficial to tumor growth. In a clinical study, the daily administration of an estimated 500 mg indole-3-carbinol (equivalent to 400 g white cabbage) led to a 50% increase of catechol estrogen synthesis after 7 days and to its urinary excretion [Michnovicz and Bradlow 1990].

Phytoestrogens possess antiangiogenic and estrogenic as well as antiestrogenic properties due to competitive binding on estrogen receptors and activation of metabolizing enzymes such as aromatase and the estrogen-specific 17ß-hydroxysteroid oxidoreductase [Santti et al. 1998]. Phytoestrogens reduce by way of an aromatase inhibition the conversion of androstendion, so the concentration of circulating estrogens is lowered. *In vitro* studies have shown that phytoestrogens inhibit the binding of xenestrogens (DDT) to target cells [Zava et al. 1997, Zava and Duwe 1997]. Xenestrogens on the other hand could be important for carcinogenesis. Furthermore, phytoestrogens induce in humans the synthesis of the “Sex - Hormone - Binding - Globulin (SHBG)“ in the liver, so more of the circulating estrogens are bound to this transport protein, rendering them biologically inactive [Watzl et al. 1994, Watzl and Leitzmann 1995]. In women of various age groups, the plasma SHBG concentration correlated positively with the excretion of phytoestrogens in urine [Adlercreutz et al. 1987, Chie et al. 2002], whereby vegetarian women showed a higher excretion rate than non-vegetarian women.

However, within this spectrum of investigations, the results are sometimes opposing and at present difficult to interpret [Adlercreutz et al. 2004a, Sacks 2005]. Current retrospective analyses of diets with regard to fruit, vegetables, and fiber also are controversial. In a recent review summarizing prevention studies published to date, clear-cut relations have not yet been proven [Gikas et al. 2005]. This could be explained especially by the diversity of the phytoestrogens consumed and by their individual absorption behaviors [Hanf and Gonder 2005]. In a similar sense, this is also true for extracts containing phytoestrogens. Therefore, further extensive research is required.

1.3. Phytoestrogens – Isoflavones and Lignans Isolated from Plants

An important area of biomedical research is the search for new active ingredients (especially natural substances). The *in vitro* testing of supposedly active ingredients from plants is carried out if possible in stages, progressing from a multiconstituent mixture with many constituents to one with one or a few constituents (single constituent). Depending on the manufacturing process used, different active ingredients could be obtained from the same starting material and these may differ in their pharmaceutical, pharmacotoxological, and clinical properties. The various constituents of a plant extract may exhibit differing biopharmaceutical properties and pharmacological activities, which as a whole account for the therapeutic efficacy. The isoflavones and lignans are two groups of chemical compounds that are of special importance acting as phytoestrogens.

The soybean is the main source of isoflavones, a group of phenolic compounds found in nature that belong to the flavonoids [Coward et al. 1993]. Around 100 natural isoflavones and structurally related compounds, such as isoflavones, isoflavanes, and complex isoflavanes, have been isolated from higher plants, especially leguminoses (pulses/legumes). The isoflavones mainly differ structurally on the 3rd ring at the position of the hydroxyl and methoxy groups. The complex isoflavones also contain one to several isoprenoid substituents. Carbohydrate components are in particular glucose and rhamnose. Well-known isoflavones include the widely occurring genistein from various broom species. At present, the soybean is the most important source of these nutritionally essential compounds, together with the glycosides of genistein, daidzein and glycitein, which in part are bound to proteins. There are dietary supplements on the market made from red clover, which is rich in phytoestrogens, particularly glycosides of the isoflavones formononetin und biochanin A. These compounds are converted to daidzein und glycitein by intestinal bacteria. Genistein has also been found in curry, among other sources, so apparently phytoestrogen intake is more complex than previously assumed [Clarke et al. 2004].

The biotransformation of isoflavone glycosides from soybeans is carried out by intestinal microflora. Absorption in the small intestine is followed by transport to the liver (enterohepatic circulation) [Setchell und Adlercreutz 1988, Wang 2002]. Glucosidases from intestinal bacteria split off carbohydrate, leading to the formation of the biologically active isoflavones daidzein and genistein as well as enterolactone und enterodiol (phase II des enterolactone and enterodiol metabolism). In phase I, the formation of enterolactone sulfate,

enterolactone glucuronide, and enterodiol glucuronide takes place in the colon epithelium [Jansen et al. 2005]. In adults, these compounds are converted to the metabolites equol, ortho-desmethylangolensin (o-DMA), and p-ethylphenol, whereby the conversion to equol (30–50%) and o-DMA (80–90%) is partial [Atkinson et al. 2005]. Additional compounds in urine samples could be identified by mass spectroscopy, for example, 3‘-methoxy-3‘-hydroxy-equol, 6‘-methoxy-equol, α-methyl-deoxy-benzenoid, angiolensin, und 6‘hydroxy-o-DMA [Heinonen 2004]. In contrast to other mammals, only 30–50% of humans are capable of converting isoflavones to other metabolites [Frankenfeld et al. 2004, Wiseman et al. 2004].

Lignans are present in a number of plants in the diglucosidic form and play an important role in cell-wall structure [Peeters et al. 2003]. The best-known representatives of the lignans are secoisolariciresinol (SECO) and matairesinol (MATA), whereas a number of additional lignan structures should exist, some of which have been described and some whose structure has not yet been clarified [Ho et al. 1998]. The intestinal microflora convert by demethylization SECO, MATA, and the isoflavones to the body’s own, so-called “mammalian lignans” [Adlercreutz 1995, Nesbitt et al. 1999, Bowey et al. 2003]. These compounds, as do their chemical precursors, have inhibitory effects on tumor growth. The quantitative analysis of these compounds in urine or serum is used to predict the metabolizing capability of the organism [Yamamoto et al. 2001, Kilkkinen et al. 2001]. At present, the lignans known are found in grain kernels and fruit, whereby linseeds (flax) are the best source of lignans [van Kranen 2003]. Linseeds contain approximately 53 µg lignan per 100 g linseed flour, based on the phytoestrogen content [Stark et al. 2002]. Lignans inhibit the growth of tumor cells in mammary-carcinoma cell lines [Chen et al. 2004] and trophoblast Jeg3 cell lines [Abarzua et al. 2007]. Concentrations between 1 µmol/ml and 100 µmol/ml exhibited a concentration-dependent activity [Adlercreutz et al. 1993]. By combining the use of linseed metabolites and the antiestrogenic tamoxifen, metastasis processes (cell adhesion, invasion, and migration) were arrested [Chen et al. 2003]. It thus becomes clear that phytoestrogens can develop completely different efficacies, depending on the receptor status and the individual hormone constellation. When administered together, however, both active substances inhibited tumor-cell proliferation. This indicates that both active substances are agonists in competition for estrogen receptor occupancy.

1.4. Phytoestrogens Tested in Cell Cultures

Many investigations and studies with humans have shown in some cases a correlative relation between a diet containing phytoestrogens and beneficial effects on health. Causal relations however can be better demonstrated by using *in vitro* tests. For this reason, experiments with cancer cell lines are the method of choice. By using different cell lines, the efficacies of phytoestrogens could be tested and, above all, detailed cellular and molecular effects analyzed.

An extract from *Epimedium brevicornum*, a medicinal plant in traditional Chinese medicine, proved to be effective on the proliferation of mammary carcinoma cells [Yap et al. 2005]. Low doses (1,3 µg/ml) caused a stimulation of the estrogen-receptor activity and, on the other hand, higher dosages inhibited growth. Following fractionation, a new

prenylflavone, breviflavon B, as active substance was found. High breviflavone dosages led to elimination of the α-ER protein, an occurence that should be viewed in connection with increased proteasome degradation. Similar dose-dependent results were obtained with MCF-7 cell lines and biochanin A [Hau et al. 1999]. At biochanin A concentrations less than 10 µg/ml, cell proliferation and the de novo DNA synthesis were enhanced. On the other hand, concentrations between 30 und 40 µg/ml resulted in an inhibition of cell growth and DNA synthesis. It was also demonstrated on MCF-7 cells that a low concentration of genistein stimulated the cell proliferation but a higher concentration inhibited proliferation. It is not yet known, which effect varying concentrations of phytoestrogens have on normal breast tissue and on triggering of precancers, especially of importance for long-term use [Dimitrakakis et al. 2004]. A potential anticancerogenic effect on mammary carcinoma cell lines (for example, MCF-7) turned out with the antiproliferative effect of genistein und daidzein (concentration 1 µmol/ml) [Hawrylewicz et al. 1995]. Inhibition of the tyrosine-specific protein kinase and of angiogenesis by genistein could be demonstrated [Akiyama et al. 1987, Fotsis et al. 1993]. In experiments with mammary carcinoma cell lines treated with phytoestrogens, receptor-dependent as well as receptor-independent mechanisms affected DNA synthesis, and inhibition of cell growth was dependent on the phytoestrogen concentration [Wang and Kurzer 1997]. At low concentrations (0,01 - 10 µmol/ml) of genistein and coumestrol, there was an increase in estradial-induced tyrosine kinase-dependent DNA synthesis in mammary carcinoma cell lines. At high concentrations, there was inhibition [Wang and Kurzer 1998]. Further tests demonstrated that especially the isoflavone genistein in physiologial concentrations is capable of inhibiting cell growth of mammary carcinoma cell lines and is thus a potent estrogen agonist [Zava et al. 1997, Zava and Duwe 1997]. Both genomic and non-genomic mechanisms have been made responsible for the anticarcinogenic properties of the phytoestrogens, including induction of apoptosis, inhibition of tyrosine kinases, and inhibition of DNA topoisomerases [Lechner et al. 2005].

When interpreting *in vitro* assays of active substances of plant origin, it must be kept in mind that the actual *in vivo* concentrations of relevant constituents are unknown because they are affected in their biopharmaceutical properties by other constituents (cofactors) and/or are subject to metabolic processes. The conclusions based on results from *in vitro* systems must therefore take the biopharmaceutical properties and metabolism of the substances being studied into consideration, e.g., the evaluation of the biological functions, in addition to quantitative assertions, should address factors such as food intake, metabolism, and bioavailability with regard to the mechanisms of action of phytoestrogens on mammary carcinomas. Investigations along this line are thus absolutely necessary before any soy products or products of other plants could be recommended for the prevention of mammary carcinoma.

2. Results

2.1. Anticancerogenic Effects of Phytoestrogens at the Cellular Level

2.1.1. Phytoestrogens from Plant Extracts

2.1.1.1. Flax (Linum usitatissimum) Extracts

The major classes of phytoestrogens are the isoflavones and lignans found at high levels in legumes such as soybean, chickpea, clover, flax and in various plant parts, including roots, stems, leaves, flowers, fruits and seeds [Kulling and Watzl 2003, Lee and Xiao 2003, Rickard-Bon and Thompson 2003]. The addition of flaxseed products reduced tumor incidence or cell multiplicity in tumor models of the breast, colon, prostate, liver, oesophagus and lung [Rickard-Bon and Thompson 2003, Westcott and Muir 2003]. Because of these anticancerous effects of the seeds, other organs of the flax plant might also be biologically effective. Therefore we isolated and identified potential phytoestrogens from leaves, stems and roots of the flax plant *Linum usitatissimum* and tested their effect on human trophoblast and mammalian tumor cell lines in *in vitro* cell cultures.

Effects on Human Trophoblast Tumor Cell Lines

Preparation of phytoestrogen extracts from leaves, stems and roots of L. usitatissimum:

The seeds, cultivar Barbara, were obtained from the Agricultural Research Institution Mecklenburg-Vorpommern (LUFA), Rostock, Germany, sown on soil and grown under field conditions. When the plants reached a height of about 1 m, they flowered and the leaves, stems and roots were harvested. These plant organs were frozen in liquid nitrogen and stored at –70°C till extraction. Different extraction methods [Franz and Köhler 1992, Luyengi et al. 1996, Windhövel et al. 2003] were performed to obtain either isoflavones or lignans from the various plant organs of *L. usitatissimum* [Abarzua et al. 2007]. The most effective extraction procedure was the lignan extraction method according to Luyengi et al. [1996].

It is known from other studies that isoflavones and lignans occur in glycosilated forms in planta and are therefore often biologically inactive [Muir and Westcott 2003, Rickard-Bon and Thompson 2003]. To improve the bioavailability *in vitro*, nonspecific HCl hydrolysis and specific β-glucosidase hydrolysis were used to release the aglycons [Abarzua et al. 2007].

Identification of isolated phytoestrogens with HPLC-MS:

The phytoestrogen extracts were dissolved in methanol and used for analysis. Chromatographic separation of the isolated phytoestrogen fractions was performed using reversed-phase HPLC using a gradient elution program: 0.2 ml/min, 20% methanol (A), 80% water with 0.1% formic acid (B), linearly to 80% A: 20% B in 15 min, followed by a hold for 25 min to reach initial conditions for an additional 10 min. A Discovery C18 (15 cm x 2.1 mm) column produced by Supelco (Taufkirchen, Germany) was used.

For MS analysis a LCQ-Advantage (Thermo Finnigan, San Jose, USA) mass spectrometer was used. Identification of the compounds was obtained by ion trap technology; using the ESI mode and positive ion. The source voltage was 4.5 kV and a mass range of 150 – 2000 amu was used for detection.

Table 1. Classes, representatives and forms of phytoestrogens identified in leaf, stem and root extracts of *Linum usitatissimum* using HPLC-MS analysis. Phytoestrogen extracts were

prepared according to Franz and Köhler [1992], Luyengi et al. [1996] and Windhövel et al. [2003] with and without HCl-or β-glucosidase hydrolysis [Abarzua et al. 2007].

Class of phytoestrogen	Representative	Chemical form
Isoflavones	Genistein Daidzein Biochanin A	Aglycone Glycoside Diglycoside Dimer Glycoside dimer Deoxydiglycoside
Lignans	Secoisolariciresinol Matairesinol Pinoresinol Lariciresinol Isolariciresinol Arctigenin 6-Methoxypodophyllotoxin	

The leaf, stem and root extracts from the flax species, *L. usitatissimum*, contain measurable concentrations of isoflavones such as genistein, daidzein and biochanin A, and lignans such as secoisolariciresinol, matairesinol, pinoresinol, lariciresinol, isolariciresinol and arctigenin. All extracts contain more representatives of lignans compared to isoflavones, as has been shown for other *Linum* species [Westcott and Muir 2003]. The compounds were found in the extraction procedure as aglycones or as glycosides, independently of whether additional HCl- or enzyme hydrolysis was used or not. Therefore it can be concluded that isoflavones and lignans were present in the flax extracts prior to hydrolysis as aglycons and glycoside derivates. In the case of the special lignan/toxin extraction [Windhövel et al. 2003] the aryltetralin lignan, 6-methoxypodophyllotoxin, was additionally found in the leaf extracts (Table 1). The lignan podophyllotoxin is of special interest, since its derivatives such as Etopophos are presently used in anticancer therapy [Fuss 2003]. One of the future tasks will be to determine the quantity of 6-methoxypodophyllotoxin and other lignans and isoflavones in plant extracts.

In Vitro Cell Studies:

The *in vitro* cell studies were performed with the human trophoblast tumor cell line Jeg3, obtained from the Department of "Human and Animal Cell Cultures" Braunschweig, Germany. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, BioWhittaker) with 10% inactivated fetal calf serum and antibiotics (1% penicillin/streptomycin) and antimycotic (0.5% amphotericin) at 37°C and 5% CO_2.

As a representative cell study test the cell proliferation and viability assay (MTT test), based on the activity of mitochondrial dehydrogenases, was used. Cell viability was analyzed using an MTT-kit according to the instructions of the manufacturer (Roche, Germany) [Abarzua et al. 2007]. The test conditions were optimized in preliminary experiments and the optimal cell number was found to be $1x10^6$ Jeg3 cells/ml.

The phytoestrogen extracts were dissolved in 1% DMSO to get a stock solution of 100 mg/ml. From this stock solution, aliquots were taken and added to 0.1 ml supplemented culture medium producing final concentrations of 0.05 mg/ml, 0.5 mg/ml, 1 mg/ml and 5 mg/ml (0.05% final concentration of DMSO). Jeg3 cells ($1x10^5$/0.1 ml supplemented culture medium) were grown in 96-well tissue culture plates for 48 h in the absence (controls) and presence of different concentrations of phytoestrogen extracts at 37°C and 5% CO_2. Two negative controls were prepared with (i) Jeg3 cells in DMEM and (ii) Jeg3 cells in DMEM and DMSO (0.05% final concentration of DMSO). In general, the negative controls 1 and 2 did not differ in absorbance values, indicating that 0.05% DMSO did not inhibit cell growth (data not shown). After incubation with MTT for 4 h at 37°C and 5% CO_2, solubilization solution was added and the plates were incubated in a humidified atmosphere (37°C, 5% CO_2) overnight. The spectrophotometrical absorbance of the purple formazan crystals was measured at 570 nm using a microplate ELISA reader (BioRad, Hercules, California, USA). The reference wavelength was 670 nm.

All lignan extracts obtained from leaves, stems and roots of *L. usitatissimum* with and without HCl hydrolysis revealed significant inhibition of cell viability. The strongest decrease in cell growth was induced by treatment of Jeg3 cultures with root extracts which had not undergone HCl hydrolysis (Figure 2). Incubation of Jeg3 cells with these extracts at 1 and 0.5 mg/ml reduced cell viability by about 93%. Most lignan extracts exhibit concentration-dependent effects. Since extractions performed with and without HCl hydrolysis result in extracts which are effective to different extents, it can be concluded that several different compounds in the extracts are responsible for the bioactivity.

Statistical analysis was performed using the Student´s *t*-test for comparison of the means. Data were presented as mean ± standard deviation (SD) of mean. A p value of < 0.01 was considered as being statistically significant and denoted by an asterisk.

Effects on Human Mammalian Tumor Cell Lines

The aim of this study was to prepare flax leaf, stem and root extracts from *L. usitatissimum* (Figure 3) and to test their effects in cell studies *in vitro* in ER positive and ER negative human mammalian cancer cell lines to distinguish between ER dependent and independent effect mechanisms of the flax extracts tested. The different role of estrogen receptor dependent and independent effect mechanisms of phytoestrogens has so far only been poorly investigated. We therefore started a systematic investigation to test the influence of the flax extracts on the receptor positive mammalian cell line MCF 7 and the receptor negative mammalian cell line BT 20 (Marlen Szewczyk, Sibylle Abarzua, André Schlichting, Dagmar-Ulrike Richter, Barbara Nebe, Birgit Piechulla, Volker Briese, unpublished results 2009).

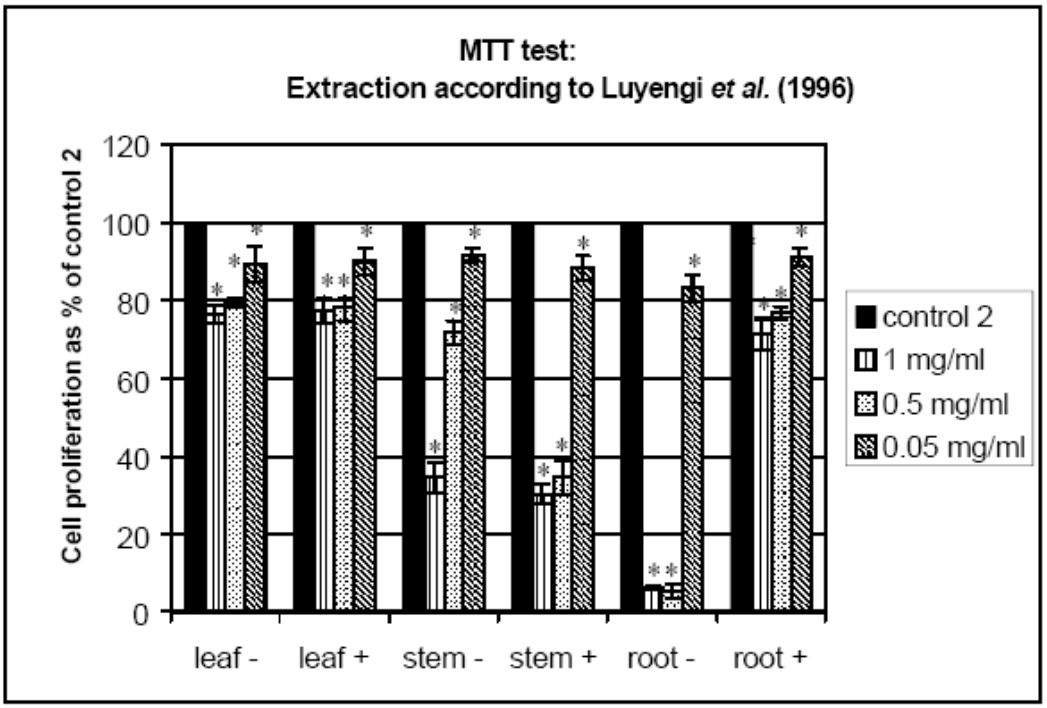

Figure 2. Effect of different concentrations of leaf, stem and root extracts from *Linum usitatissimum* on the cell proliferation and viability of Jeg3 cell lines measured by the MTT test. Extracts were prepared according to Luyengi et al. [1996] with (+) and without (-) hydrolysis with 1 M HCl. Data (mean±SD) represent relative formation of formazan from MTT in % in comparison to negative control 2 (100%) obtained in at least 3 experiments. Asterisks (*) indicate significant differences between treated Jeg3 cell lines and the negative control 2 ($p<0.01$) [Abarzua et al. 2007].

Figure 3. *Linum usitatissimum*.

In Vitro Cell Studies:

The *in vitro* cell studies were performed with the human mammalian cancer cell lines MCF 7 (ER positive) and BT 20 (ER negative), obtained from the Department of "Human and Animal Cell Cultures" Braunschweig, Germany. The cell cultivation procedure was the same as for the human trophoblast cell line Jeg3 (see above).

As representatives for the *in vitro* cell studies the cell vitality (also designated cell proliferation and cell viability test, see before), proliferation and cytotoxicity of the human mammalian cell lines MCF 7 and BT 20 (5×10^5 cells/ml) treated with different concentrations of phytoestrogen extracts from *L. usitatissimum* were analysed. For these studies the MTT, the BrdU Cell Proliferation ELISA kit (colorimetric) and the cytotoxicity detection kit (LDH kit) were used as recommended by the manufacturer (Roche, Germany). The phytoestrogen extracts were dissolved in 100% ethanol to provide a stock solution of 100 mg/ml. Aliquots from this stock solution were added to the supplemented culture medium to give final concentrations of 0.01, 0.1, 1, 10, 50, 100, 500 and 1000 µg/ml (final concentration of ethanol: 1%). Two negative controls were examined in all tests: (i) cells in DMEM (control 1) and (ii) cells in DMEM and ethanol, final concentration of ethanol: 1% (control 2).

Flow Cytometric Measurement of Apoptosis:

MCF 7 cells (5×10^5 cells/ml) were grown to confluence for 24 h in 6-well-plates. After refreshing the medium, root extracts and the negative controls 1 and 2 (see above) were added and incubated for 24 h at 37°C and 5% CO_2. Cells were washed with PBS, trypsinized, centrifuged and washed again. Cells were treated with 1 mg/ml RNase at 37°C for 20 min and incubated with propidium iodide (50 µg/ml) for 3 h on ice. Measurements were performed on BD FACSCalibur, equipped with an argon-ion laser of the wavelength 488 nm (BD Bioscience). For data acquisition, the software CellQuest Pro 4.0.1 (BD Bioscience) was used.

The Influence of Flax Extracts on Cell Viability and Proliferation of MCF 7 and BT 20:

The leaf, stem and root extracts from *L. usitatissimum* at low concentrations nearly did not affect the cell vitality and cell proliferation of MCF 7 and BT 20 cell lines. However, at higher concentrations of the root extract a significant inhibition of the cell activity and cell proliferation was found. BT 20 cells were repressed stronger in comparison to MCF 7 ones.

These differences point out that flax root extracts probably can affect the growth of MCF 7 and BT 20 carcinoma cell lines through ER mediated as well as ER independent mechanisms of action, whereby the ER independent mechanisms of action seem to play a greater role. On the other hand the differences could also indicate a higher sensitivity for flax root extracts in the ER negative BT 20 cell lines than in the ER positive MCF 7 cells.

Our results are in line with an *in vitro* study which showed that the mammalian lignans have stimulatory as well as inhibitory effects on the cell growth of breast cancer cells, depending on the concentrations used. Using DNA synthesis as a marker of cell growth, 1-10 µmol/l of the mammalian lignan enterolactone was found to be stimulatory in the breast cancer cell line MCF 7 [Wang and Kurzer 1997, 1998], but higher levels (>50 µmol/l) were inhibitory [Wang and Kurzer 1997]. In terms of cell proliferation, 0.5 to 10 µmol/l

enterolactone was found to stimulate the growth of MCF 7 cells, whereas concentrations above 10 µmol/l were inhibitory [Mousavi and Adlercreutz 1992].

Statistical analysis was performed as described before.

The Effect of Flax Extracts on Cytotoxicity of MCF 7 and BT 20 Cells:

Low concentrations of flax leaf stem and root extracts did not induce cytotoxic effects in MCF 7 and BT 20 cell lines. However, higher concentrations of the stem and leaf extracts caused a significant cytotoxicity. By contrast over the whole range of flax root extract concentrations there were no cytotoxic effects on MCF 7 cells. However, in BT 20 cell lines the addition of high flax root extract concentrations caused significant cytotoxic effects. These results correlate with the strong inhibition of cell vitality of the BT 20 cells after the addition of high flax root extract concentrations.

Induction of Apoptosis:

Flow cytometric analyses were performed for examining induction of apoptosis induced by flax root extracts. Addition of low flax root extract the percentage does not increase apoptotic cells, however high concentrations of flax root extracts resulted in a significant increase of apoptosis.

Several studies described the induction of apoptosis as a respond to phytoestrogens [Jo et al. 2005, Danbara et al. 2005]. We suggest that apoptosis of MCF 7 cells might be induced by the phytoestrogens found in the flax root extract of *L. usitatissimum* (Table 1).

Since flax root extracts of *L. usitatissimum* induce significant inhibition of cell vitality and proliferation without performing strong cytotoxicity in the human mamma carcinoma cell lines MCF 7 the potential phytoestrogens in flax roots could have beneficial effects for the prevention of hormone-dependent tumors. Forthcoming research will be directed at identifying the active molecules, testing the flax root extract effects in hormone-dependent and independent mechanisms of action and finally disclosing the relevant intracellular processes.

2.1.1.2. Elm Bark (Ulmus laevis) Extracts

Traditional Chinese medicine indicates that the bark of *Ulmus sp.* has positive effects against oedema, mastitis, inflammation and cancer [Wang et al. 2004]. Elm bark extracts also provide resources for anticancer drug recovery [Tai and Cheung, 2005, Kulp et al. 2006]. Elm tree components are present in different herbal teas, which are available as medical-tea products. An *in vitro* study found out that FlorEssence (herbal remedy tea) significantly inhibits the proliferation of human breast cancer (MCF7, MDA-MB-468) and leukaemia cells (Jurkat, K562) [Tai and Cheung 2005].

At present, the substances responsible for these effects are unknown. Naturally occurring substances such as terpenes [Wattenberg 1983), glycopeptides [Dong et al. 1997], polyphenols [Gamet-Payrastre et al. 1999, Caltagirone et al. 2000] and phytoestrogens [Adlercreutz 1995, Rickard-Bon and Thompson 2003, Abarzua et al. 2007] have been considered to have anti-cancerogenic effects. The cytostatic agent Taxol A is a diterpene-polyester from the bark of *Taxus brevifolia* and has been successfully applied against mammalian and ovarial carcinoma [Ofir et al. 2002]. Bark of the regional elm (*Ulmus laevis*) may also contain substances with anti-cancerogenic potential against hormone-dependent

gynaecological tumors. Therefore, the aim of the present study has been to identify potentially active substances of crude extracts from bark of *Ulmus laevis* (Figure 4) and to analyse their effects on cell vitality, cell proliferation and cytotoxicity in human chorion carcinoma cell lines Jeg3 and BeWo and the human endometrial cell line RL95-2. The placental cell culture model is suitable for the direct comparison of the human tumor cell lines Jeg3 and BeWo with a primary cell culture under *in vitro* conditions [Jeschke et al. 2003].

Figure 4. *Ulmus laevis*.

Effects on Human Trophoblast Tumor Cell Lines

Extract Preparation from Elm (Ulmus laevis) Bark:

Bark was collected from *Ulmus laevis* Pallas (identified by Prof. Porembski, Botany, University of Rostock) in a forest near Rostock (Mecklenburg-Western Pomerania, Germany). A voucher specimen of *U. laevis* from individuum studied was deposited at the Herbarium of the Department of Botany, University of Rostock. The extracts were prepared according to Luyengi et al. [1996] as modified by Matscheski et al. [2006]. The extracts were dissolved in 100% ethanol to provide a stock solution of 100 mg/mL. Aliquots of this stock solution were added to the supplemented culture medium to give final concentrations of 0.25, 0.5, 1, 5, 10, 50, 100, 150, 250 and 500 µg/ml (final concentration of ethanol: 1%) (Anna-Maria Hartmann, Sibylle Abarzua, André Schlichting, Marina Chwalisz, Dajana Domik, Kai-Uwe Eckhardt, Dagmar-Ulrike Richter, Peter Leinweber, Volker Briese, unpublished results, submitted for Planta Medica 2009).

Chemical analysis with pyrolysis-field ionization mass spectrometry (Py-FIMS):

For Py-FIMS, about 5 µL of the extract was transferred to a quartz crucible that was placed in the micro-oven of the direct inlet system of a double-focusing Finnigan MAT 900 mass spectrometer (Finnigan, MAT, Bremen, Germany). The analyte was evaporated to dryness in the fore-vacuum (10^{-1} hPa). The micro-oven heated the sample from 110 to 700°C at 20 K-increments in 12 min, and 91 magnetic scans were recorded for the mass range 15 to 900 Dalton (single spectra). These were combined to obtain one thermogram of total ion intensity (TII) and an averaged Py-FI mass spectrum. For each of the single scans, the absolute and relative ion intensities of 14 classes of chemical compounds were calculated by summation of the ion intensities of 8 to 39 indicator signals [Schulten and Leinweber 1999],

including the protonated molecule ion mass signals ((M+H)+) if present. All Py-FIMS data were normalised per mg sample. This procedure was carried out for each of five replicate measurements per sample and the results were averaged for statistical analyses. Py-FIMS of the crude bark extract of Ulmus laevis isolated by the Luyengi-procedure [Luyengi et al. 1996] indicated mainly triterpenes and sterols, fatty acids with lower amounts of lignans. These results are in agreement with the findings of Martin-Benito et al. [2005] and Rowe et al. [1972].

In Vitro Cell Studies:

The *in vitro* cell studies were performed with the chorion carcinoma cell lines Jeg3 and BeWo (Figure 5), obtained from the LGC Standards GmbH, Wesel, Germany. Cells were cultured in Dulbecco`s Modified Eagle`s Medium (DMEM, Bio Whittaker) with 10% inactivated fetal calf serum (FCS) and antibiotics (penicillin/streptomycin) and an anti-mycotic (amphotericin) at 37°C and 5% CO_2. The primary trophoblast cell culture was directly isolated from the placenta according to Jeschke et al. [2003]. As representatives of the *in vitro* cell study the MTT test was performed as described before. Additionally two positive controls, dissolved in ethanol were examined: 1 µg/ml 17β-estradiol (estrogen) and 10 µg/ml tamoxifen (anti-estrogen).

Statistical analysis was performed as described before.

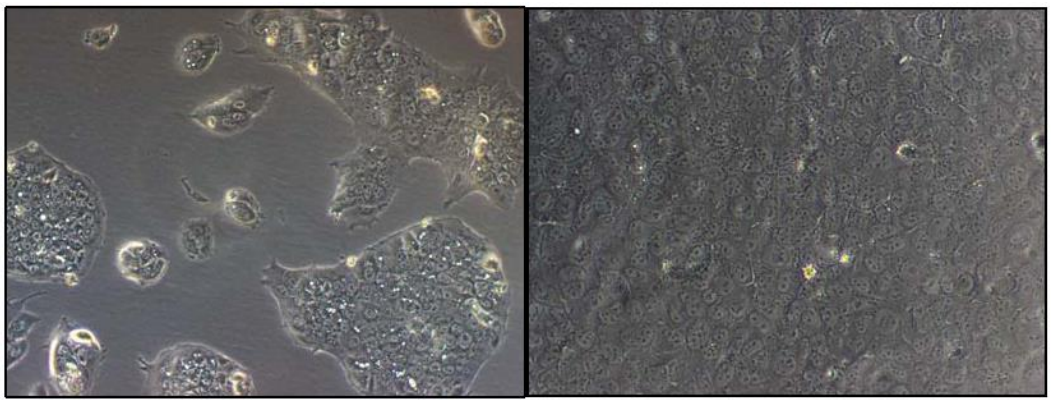

Figure 5. Cell morphology of the chorioncarcinoma cell line BEWO (left) and the endometrial cell line RL 95-2 (right). Light microscopy, magnification 10x.

The Effect of Elm Bark Extracts in Vitro:

It was shown that the vitality of Jeg3 and BeWo cells (MTT test) decreased significantly in a concentration-dependent manner, after application of elm bark extracts, relative to the negative control 2. The strongest inhibition of cell vitality was measured in the Jeg3 culture. The addition of 17β-estradiol did not affect the vitality of Jeg3 and BeWo cells, but the application of tamoxifen significantly inhibited the uptake of MTT by the Jeg3 and BeWo culture. The vitality of the primary trophoblast cells was increased by all concentrations of elm bark extracts and 17β-estradiol. By contrast, tamoxifen significantly reduced their vitality. Cell vitality can be enhanced by estrogens (17β-estradiol) and reduced by anti-estrogens (tamoxifen) *via* the activity of the estrogen–receptor (ER). Primary trophoblast cells

and the Jeg3 and BeWo carcinoma cell lines have been found to be positive for ERα and ERβ [Szewczyk 2007, Ho et al. 1998, Jiang et al. 1997]. The positive ER mediation becomes obvious by the significantly contrasting estradiol and tamoxifen effect on the vitality of primary trophoblast cells.

Effects on Human Endometrial Tumor Cells

In Vitro Cell Studies:

The *in vitro* cell experiments were performed with the non-polar human uterine epithelial cell line RL95-2, purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). This cell line was maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (Gibco-Life Technology, Eggenstein, Germany) and Ham's F12 medium (Sigma, Taufkirchen, Germany), supplemented with 10% fetal calf serum (FCS) (Gibco), 10 mmol/l HEPES pH 7.4 (Sigma), 5 µg/ml insulin (Sigma), 2.0 g/l $NaHCO_3$ (90%), 1% penicillin/streptomycin (Sigma) and 0.5% amphotericin B (Sigma). The cells were cultured in a humidified atmosphere at 37°C with 5% CO_2. As representatives of the *in vitro* cell studies the MTT-, BrdU- and LDH tests with statistical analysis were performed as described before (Daniel Paschke, Sibylle Abarzua, Andre Schlichting, Dagmar-Ulrike Richter, Peter Leinweber, Volker Briese, unpublished results, submitted for European Journal of Cancer Prevention 2008).

Effects of Elm Bark Extracts on Cell Vitality and Proliferation of RL 95-2 Cells:

Our experiments demonstrated a significant inhibition of cell viability and cell proliferation after application of elm bark extracts in a dose-dependent manner measured by the MTT and BrdU assay. These results suggest that elm bark extracts have tumor growth inhibiting properties as indicated by an inhibition of mitochondrial activity (MTT test) as well as decreased DNA synthesis (BrdU test).

To test the possibility that the inhibition of cell viability and cell proliferation in the presence of elm bark extract is due to cell lethality of the human endometrial carcinoma RL 95-2 cell lines the cytotoxicity of the extracts was measured by the LDH activity. It was shown that elm bark extracts did not induce cytotoxic effects. These results lead to presumption that elm bark extracts did not have lethal properties on endometrial carcinoma cells.

Different phytoestrogens have been found in the elm tree root and bark in the form of lignan xylosides and neolignan glycosides [Lee et al. 2001]. It has been suggested that plant cell walls containing significant amounts of phenolic components may be the most likely to protect against cancer (dietary fiber hypothesis) [Ferguson et al. 2001, Dembitsky and Maoka 2007]. The other analysed substance classes from elm bark could be also potent agents possessing high anticancer activities. Anticancer effects of free fatty acids were estimated using a rabbit liver cancer model [Hayashi et al. 1992]. Palmitic acid and octadecenoic acid as well as oleic acid resulted in apoptosis – inducing activity in colon tumor cells [Waterman and Lockwood 2007, Yoo et al. 2007]. Recently Juan et al. [2008] detected antiproliferative and apoptosis-inducing effects of maslinic and oleanolic acids, two pentacyclic triterpenes from olives on HT-29, on colon cancer cells. In summary, many substance classes we had found in elm bark by Py-FIMS are described as potential anti-cancer products on different

tumor cell lines. According to the incidence of the main fractions, we suggest a main responsibility for observed effects by sterols, triterpenes, free fatty acids and phytoestrogens. Single substances as well as a combined action of the analysed substance classes could be responsible for the decreased cell vitality and cell proliferation.

Conclusion

In this study we demonstrated inhibitory effects of flax root and elm bark extracts obtained from *Linum usitatissimum* and *Ulmus laevis* on trophoblast, mammalian and endometrial tumor cell lines. The observations displayed considerable significance from an oncological and botanical standpoint for future investigations into the usefulness of flax root and elm bark extracts for cancer prevention and treatment as well as drug candidates.

2.1.2. Synthetic Phytoestrogens

There is accumulating evidence that phytoestrogens, which are naturally occuring, plant-derived phytochemicals, could inhibit tumorigenesis during the development of breast cancer. Tumor metastasis and the proliferation of cells resulting in tumor cell growth in breast cells is directly connected with cell adhesion receptors, such as integrin and hyaluronan receptor expression. In maintaining tissue architecture, e.g. of the mammary gland, the integrin receptor- and steroid hormone-signaling pathways play an important role. Disruption of the delicate balance of signaling can result in dramatic changes in the cellular interactions, which might lead to breast cancer [Hansen and Bissell 2000]. The adhesion receptors of the integrin family are transmembrane receptors consisting of an α- and a β-subunit and exert important functions in signal transduction via the actin cytoskeleton [Wiesner et al. 2005, Dedhar and Hannigan 1996, Nebe et al. 1995]. With their extracellular domain, integrins bind to extracellular matrix proteins (ECM) like fibronectin (FN) as a prime target of α5β1 [Hynes 1999]. Integrins transduce extracellular signals via the cytoplasmic domain and facilitate downstream signalling cascades by organizing the cytoskeletal 'scaffold' for intracellular signaling components [Aplin et al. 1999, Nebe et al. 1996]. Thus, integrin-mediated cell adhesion and resulting cytoskeletal dynamics lead to an early cell response like intracellular calcium mobilization [Sjastaad and Nelson 1997, Pommerenke 1996] and to the control of focal adhesion kinase, which activity is sufficient for cell growth [Hansen et al. 1994], and gene expression [Roskelley et al. 1994].

The integrin function can also be influenced by other receptors, like the adhesion receptor CD44 [Wang et al. 2005]. CD44, a transmembrane glycoprotein, binds hyaluronan and plays a major role in cell-cell adhesion and cell-substrate adhesion. CD44 is also expressed in differentiated epithelial cells [Speranza et al. 2005]. This receptor is associated with tumor metastasis as demonstrated in experiments of CD44 cross-linking-induced upregulation of integrins resulting in increased adhesion of breast cancer cells (MDA-MB-435S) [Wang et al. 2005]. The direct linkage between integrins and cell growth has also been clearly indicated in recent experiments on breast cancer cells in which the inhibition of the β3-integrin function by antagonists was correlated with a decrease of proliferative subpopulations [Vellon et al. 2005]. Tumor metastasis and the enhanced motility of mammary carcinoma cells are

associated with integrin-mediated adhesion and hyaluronan receptor expression. It is important to get deeper insights into the behavior of cells and their cellular structure-cell function-dependencies under estrogen influence. The reason is that synthetic estrogens are able to modulate *in vitro* the β1-integrins, alter the cell-matrix-interaction, increase the adhesion contact numbers and are responsible for more organized F-actin in the lamellipodia of motile cells [DePasquale et al. 1999, Iype et al. 2001]. Migrating mammary epithelial cells stimulated *in vitro* with estrogens (17β-estradiol) demonstrate more so called footprints of the integrin residues (see scheme Figure 6). Less is known how phytoestrogens act in these cellular adhesion receptor dependent processes. The aim of our studies is to unravel the mode of action of phytoestrogens and the regulation of adhesion receptors like integrins and the hyaluronan receptor.

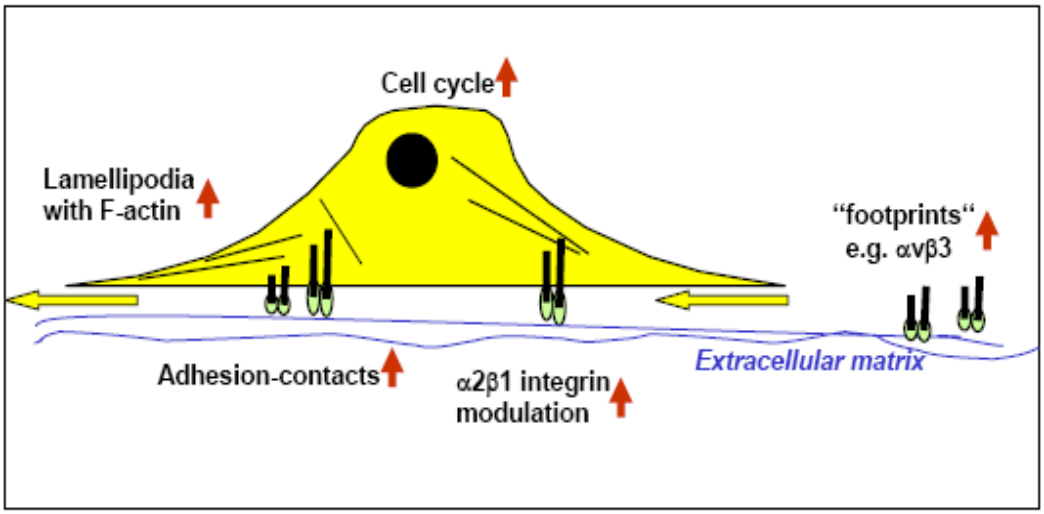

Figure 6. Schematic presentation how synthetic estrogens influence mammary epithelial cells possibly resulting in enhanced motility of the tumor cells: 17β-estradiol modulates the β1-integrins, alters the cell-matrix-interaction, increases the adhesion contact numbers and is responsible for more organized F-actin in the lamellipodia of motile cells.

In Vitro Cell Studies:

First experiments using estrogen-sensitive breast cancer cells MCF-7 (ATCC no. HTB-22) indicated that the integrin adhesion receptors were significantly up-regulated with 17β-estradiol but in contrast, genistein and daidzein did not affect the expression, which was concentration dependent [Nebe et al. 2006]. The MCF-7 cells express the integrin receptors α2, α3, β1 in the same intensity as observed in primary mammary epithelial cells [Nebe et al. 2006]. Therefore, this cell line is well suited for the phytoestrogen studies. The hyaluronan receptor CD44 was significantly increased with 17β-estradiol (1 µM) compared to untreated control cells. In contrast, the synthetic phytoestrogen genistein increased CD44 expression only at lower concentrations, whereas CD44 remained unaffected at 100 µM. The phytoestrogen daidzein did not affect the CD44 expression level at any of the concentrations tested [Nebe et al. 2006].

We also determined the influence of phytoestrogens on cell growth. In all proliferation experiments with a significant stimulation of the primary mammary epithelial cells due to 17β-estradiol, genistein and daidzein did not influence S- and G2/M-phase cells. Additionally, the stimulative effect of 17β-estradiol could be inhibited.

Our contemporary, preliminary investigations using matairesinol (MATA) and secoisolariciresinol (SECO) seem to confirm that estrogens upregulate the adhesion receptor CD44 of mammary epithelial cells, whereas phytoestrogens do not (Figure 7). However, for statistical achievements further experiments are necessary. MCF-7 cells were cultured in DMEM (Invitrogen, Karlsruhe, No. 31966) at 37°C and in a 5% CO_2 atmosphere. The cells were cultured in serum free DMEM for 24 h before incubation with the phytoestrogens Mata and Seco (further 48 h) to avoid unspecific stimulation. Integrin preparation was according Nebe et al. [2006]. Briefly, MCF-7 cells were trypsinized, washed and sedimented cells were incubated with the monoclonal anti-CD44 (Immunotech), or for control with mouse IgG_1 (BD Biosciences). For fluorescence labelling a FITC-conjugated anti-mouse IgG (Fab_2 fragment, Sigma) was used and cells were measured by flow cytometry.

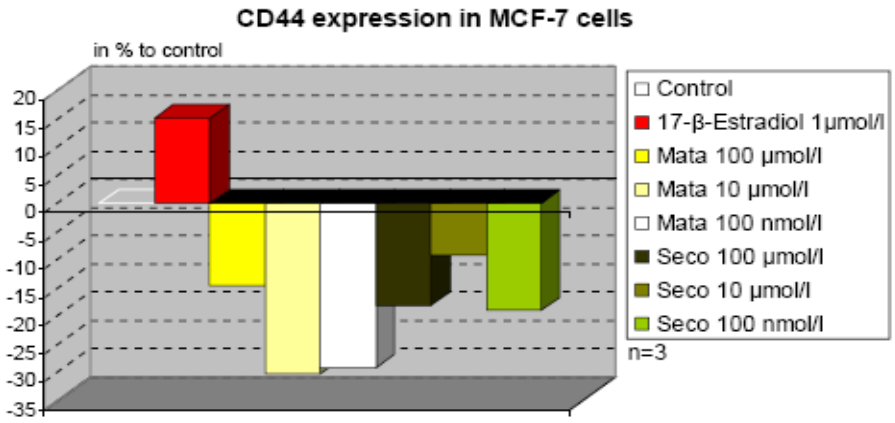

Figure 7. The CD44 hyaluronan receptor is upregulated after 17β-estradiol application and downregulated due to the influence of the phytoestrogens matairesinol and secoisolariciresinol.

It is possible that the action of estradiol is due to the expression of the estrogen receptor α and β (ER) in these cells (Figure 8). Immunocytochemical characterization of estrogen receptors were proven with cytospins [Nebe et al. 2006]. Briefly, 300 µl of suspended cells ($3x10^5$) per slide were centrifuged for 1 min at 1000 rpm. The slides were air dried, fixed with 3.7% formalin and permeabilized with cold methanol, followed by incubation with 0.1 % hydrogen peroxide (H_2O_2). After rinsing with PBS the slides were incubated with normal serum from the Vectastain ABC-Kit (Vector Laboratories, Dako, Hamburg) followed by incubation with the primary antibodies mouse anti-human estrogen receptor α (1:10, Dako) and anti-human estrogen receptor β (1:10, Serotec).

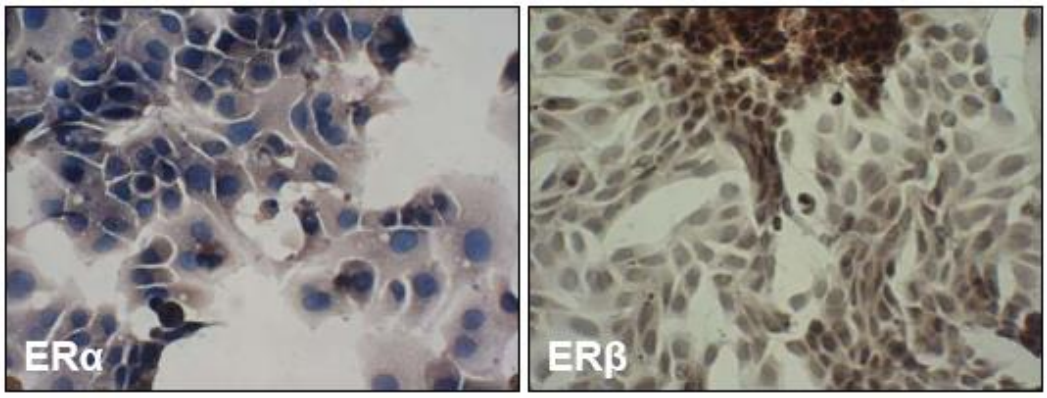

Figure 8. Estrogen receptor (ER) expression in mammary epithelial cells MCF-7. The ERα and the ERβ both are expressed (brownish color).

2.2. Anticancerogenic Effects of Phytoestrogens at the Molecular Level

Lignans are primarily found in flax, whole rye flour, pumpkin seeds, cereals and fruits. These are products that, in contrast to soy, are part of a healthy diet in Western countries. Lignans also mainly act through the estrogen receptor β (ERβ). The gene for the ERβ is located on the human chromosome 14, and is expressed in the testicles, the ovaries, the lung, the kidney, the prostate and the thymus. Five isoforms of ERβ have been found. Only isoform 1 (ERβ1) is able to produce homodimers and thus has an own function. Isoforms 2, 4 and 5, however, are able to produce heterodimers with isoform 1 and thus increase the ligand-dependant activation of transcription. Only homodimers can attach to the corresponding ERE promoter sequence. The ERα/ERβ ratio is additionally important for cellular proliferation or inhibition. Furthermore, gene activation through estrogen receptors has also been observed with genes without ERE responsive elements. It can be concluded that other ways of activation exist, e.g. the binding of complexes to AP-1 (activator protein) binding sites or of other transcription factors (e.g. NFκB, SP1).

For the present work, we investigated the activation of estrogen-sensitive genes through lignan-containing extracts of flax root and pumpkin seeds in *in vitro* settings. Previous studies have shown that extracts of these raw extracts significantly inhibit the proliferation of tumor cells (MCF7, Jeg3) in a cytotoxicity test (MTT). The trefoil factor 1 (TFF1), the estrogen receptors α and β, the progesterone receptor (PR) and the insulin receptor were selected as estrogen-sensitive genes. Increased expressions of TFF1, ER and PR would constitute favorable prognosis factors. Trefoil factor 1 is a small secretory protein consisting of 60 amino acids. It belongs to the trefoil family of which three proteins are known (TFF1, TFF2, and TFF3) and is expressed mainly in healthy tissue in the gastrointestinal tract. TFF1 is known to stimulate intestinal repair. If the expression of TFF1 in the mamma carcinoma is increased, a favorable effect on the further course of the disease and a response to the hormone therapy can be expected.

Material and Methods

Extracts from the root material of flax, *Linum usitatissimum*, variety: Barbara, and shelled pumpkin seeds of the variety Gele Centenaar were produced. The cell cultures used included the chorioncarcinoma cell line Jeg3 and the human mamma carcinoma cell line MCF7. 100 ml serum and 2 g activated carbon are incubated for 24 h at 4°C with mild rotation in order to filter the majority of the steroid hormones out of commercial fetal calf serum (FCS). For the examination of gene expression, the cells are prepared in a concentration of 300,000 cells per well.

Estradiol is used in the concentration of 1 µg/ml, secoisolariciresinol in the concentration of 10 µg/ml. The flax root extract is used in two different concentrations - 500 µg/ml and 100 µg/ml-; the pumpkin seed extract is used in the concentration of 5000 µg/ml.

Further steps comprise RNA extraction and cDNA synthesis. An oligo-d$(T)_{12}$ primer is used to this effect. A survey of the primers for real-time PCR, in part derived using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) or taken from other publications, is shown in the table 2.

Table 2. *Primers.* Number of base pairs, annealing temperature and sequence, HPRT: Hypoxanthine - phosphoribosyltransferase, TFF1: trefoil factor 1; INSR: insulin receptor, PGR: progesterone receptor, ESR1: estrogen receptor α, ESR2: estrogen receptor β

Gene	Primer designation	Length (bp)	Annealing temp.	Sequence
HPRT	HPRTup	21	61°C	TGTAATGACCAGTCAACAGGG
	HPRTdw	21		TGGCTTATATCCAACACTTCG
TFF1	TFF1up	22	65°C	GTGAGCCGAGGCACAGCTGCAG
	TFF1dw	22		TGACTCGGGGTCGCCTTTGGAG
INSR	INSRup	18	61°C	TCGTCCCCAGAAAAACCT
	INSRdw	19		GATAGCCCGTGAAGTGTCG
PGR	PRGup	24	61°C	CACAAAACCTGACACCTCCAGTTC
	PRGdw	22		GCAAAATACAGCATCTGCCCAC
ESR1	ESR1up	20	61°C	AGCCCGCTCATGATCAAACG
	ESR1dw	23		GGATCATACTCGGAATAGAGAAT
ESR2	ESR2up	20	61°C	AACCTCCTGATGCTCCTGTC
	ESR2dw	21		GCCCTCTTTGCTTTTACTGTC

For relative quantification, the concentrations of the unknown samples to be measured are analyzed in comparison to a reference gene (housekeeping gene). The ΔΔCT method is used to this effect.

We used the absolute quantification for evaluating the data collected here since the standard gene did not show any stable expression. The concentrations were related to the ethanol control which is set to one. The significances were determined using the Tukey-Kramer test (statistics program: SAS).

Results

In the hormone receptor-positive mamma carcinoma cell line MCF7, a dose-related increase in the expression of the trefoil factor 1, the insulin receptor, the progesterone receptor and the estrogen receptor α was observed after addition of the flax root extract. The gene expression was less pronounced in the hormone receptor-positive Jeg3 cell line than in the MCF7 cell line. The flax root extract resulted in an increased gene expression. At present it cannot be distinguished which compounds of the extract are responsible for the results achieved. But it can be assumed that in particular the flax root extract as a multicomponent mixture is able to support an expression of therapeutically important receptors and thus can be considered in concomitant therapies.

2.3 Clinical Studies

2.3.1. The Treatment of Climacteric Symptoms by Isoflavones Using a Prospective, Randomized, Placebo-Controlled Double-Blind Study

We performed a prospective, randomized double-blind study on a product made of soybean extract, vitamins and other nutrients.

66 peri- and postmenopausal women were randomized into the study. The treatment group included 29 participants, the placebo group 37 participants.

The treatment group received the micronutrient combination with 50 mg soy isoflavones daily for 6 months; the control group received a corresponding placebo. The patients were called in at the beginning of the study, after 6 and after 12 weeks as well as after 6 months.

The climacteric symptoms were objectivized using Hauser's Menopause Rating Scale / MRS II. The bone metabolism was examined through cross-links and ostase. Pyridinoline and deoxypyridinoline, markers for the absorption of bone, i.e. for osteoclast activity, were analyzed in urine samples. Ostase, a marker of bone formation, i.e. for osteoblast activity, was determined in serum samples.

The intensity of the following complaints was determined using the menopause rating scale as clinical standard of valuation: hot flushes, breaking out into a sweat, cardiac complaints, sleep disorders, depressive moods, irritability, timidness, physical and mental exhaustion, sexual problems, urinary tract problems, dryness of the vagina as well as joint and muscular complaints.

Results

The entirety of these subjective complaints showed a significant reduction in both groups after 6 months compared with the baseline value with statistically relevant benefit for the treatment group compared with the placebo group ($p < 0.001$).

The laboratory chemical analyses showed an improvement of the bone metabolism in the treatment group. Ostase showed a significant increase in the treatment group ($p = 0.003$), deoxypyridinoline showed a significant drop in the treatment group ($P = 0.007$).

Conclusion

The study results show that the intake of the used product containing soybean extract, vitamins and other nutrients relieves the entirety of the subjective climacteric complaints. Furthermore, the study demonstrated that the product has a positive influence on the bone metabolism. Thus, soy isoflavones constitute a useful dietetic therapy option for menopausal complaints [Anderson et al. 1999, Barnes 2003].

2.3.2. Quantitative Detection of the Phytoestrogens Daidzein and Genistein in the Urine in a Placebo-Controlled Double-Blind Study on Osteoporosis Prevention

Phytoestrogens, in particular the isoflavones daidzein and genistein present in soy, are currently in the centre of interest of scientists doing research on plant agents for the prevention and therapy of the climacteric syndrome. Up to now, isoflavone ingestion has been assessed mainly on account of diet protocols, intestinal absorption and metabolism not being taken into consideration [Yan et al. 2007]. Phenotypical differences are not identified [Pineda et al. 2001, van der Heide et al. 2003].

In the context of the present work, we have developed a method for the determination of free daidzein and genistein in the urine by means of HPLC (high performance liquid chromatography with UV detection) as well as of conjugated daidzein and genistein after splitting of the conjugates by means of acid hydrolysis. 492 urine samples of 80 participants in the "randomized placebo-controlled double-blind study on the realization of osteoporosis prophylaxis and relief of climacteric complaints using nutrient-enriched food" of the Department of Obstetrics and Gynecology of the University Rostock were examined for their content of isoflavones.

In all, 89 women were included in the study, including 23 women who have already been receiving an HRT and continued it during the study. Six women discontinued the study prematurely. The study participants were aged between 43 and 66 years (∅ 53.1 years), the women were postmenopausal and had at least one climacteric symptom. The body mass index (BMI) was between 18 and 40 (∅ 26.3).

In the randomized, placebo-controlled double-blind study, a bread enriched with 750 mg calcium, 2,5µg vitamin D, 80 mg isoflavones, 200 mg lignans, 250 mg magnesium and 1 mg fluoride for the corresponding daily quantity (250 g) was regularly sent to the women over a period of six months. The symptoms were recorded monthly by means of a standardized questionnaire and serum and urine samples were collected.

We used specific bone markers for recording the dynamics of bone metabolism: pyridinoline as well as deoxypyridinoline (cross-links) for bone resorption and ostase for bone formation [Poulsen and Kruger 2008]. In addition, triglyceride, HDL, LDL and cholesterol were determined in the serum for lipid metabolism and FSH, LH and estradiol for hormonal balance.

Urine measurements of the isoflavones daidzein and genistein:

The used HPLC (high performance liquid chromatography) method is briefly described:

- Separation procedure according to substance-specific retention times and measurement of UV detection.
- Qualitative and quantitative analysis using peak height and retention time compared with standardized chromatograms.
- 2 ml safe-lock reaction vessels + 1000 µl urine + 20 µl 4-hydroxybenzophenone (0.2 µmol/ml) + 400 µl sulfuric acid, shaking for 1 min in the gyrator.
- Extraction at room temperature by adding 500 µl diethyl ether.
- Processing of the upper organic phase after centrifugation 14,000 rpm, injection volume 10 µl.
- An isocratic ammonium formate / formic acid buffer in acetonitrile that is pumped through the column system at a pressure of 170 bars and a flow rate of 0.7 ml/min is used as mobile phase, wave length at the UV detector 260 nm.
- Collection of urine samples according to clinical study: pre-phase, six-month study phase, three-month post-phase.
- Collection of 24 h-urine in the pre-phase as "blank sample" (U1), during the study phase, one urine sample per month (U2 – U7). After another 3 months, "control sample" U8.

Results of the Clinical Study

Regarding the subjective symptoms, we found that the climacteric symptoms decreased in all three groups. Nevertheless, a significant reduction of several symptoms was detected in the treatment group compared to the placebo group. For example, the complaints of hot flushes ($p = 0.02$), heart hurry ($p = 0.03$) and attacks of vertigo ($p = 0.04$) clearly decreased both regarding number and frequency. Sleep disorders ($p = 0.01$) and irritability ($p = 0.02$) improved in the treatment group as well. Complaints in the genital area such as pruritus ($p < 0.01$) and dry vagina ($p = 0.01$) occurred less commonly, which certainly also explains the higher desire for sexuality ($p < 0.01$) in the treatment group.

Table 3. Determination of genistein and daidzein in urine samples - frequencies of sample evaluation

Isoflavones measured	Treatment group	Control group
Positive	240	30
Questionably positive	22	8
Negative	47	145
Total	309	183

Figure 9 shows the time course of daidzein concentrations. Clear differences compared with the placebo group are identified. Please note that the group of patients with hormone related therapy (HRT) received both the classical hormone and the phytoestrogens.

The laboratory chemical analyses showed a clear improvement of the bone metabolism in the treatment group. The bone resorption markers decreased significantly more strongly in this group than in the placebo group (PYD $p = 0.02$; DPD $p = 0.04$). The ostase level did not

show any significant change. Regarding the lipid metabolism, it becomes clear that a drop in the triglyceride and cholesterol levels occurs in the treatment group. This, however, does not become significant on account of the high variance and the small number of cases. No changes were found in the hormone analyses for the two groups.

The women under HRT have to be considered as a separate group. Laboratory parameters change only marginally under the additional therapy. However, a clear improvement of the complaints takes place in this group as well.

Results of the determination of daidzein and genistein concentrations in the urine samples of the nutrition study revealed isoflavone concentration (daidzein, genistein) in the treatment group (Table 3).

Summary of the Clinical Study on a Soy Supplementation:

Up to present, mainly diet protocols have been used in clinical studies on the effect of isoflavones on climacteric complaints for assessing the exposure to isoflavones. No general standard for the determination of isoflavones has been found yet. As a reasonable objective alternative, we determined the concentration of free daidzein, total daidzein, free genistein and total genistein in the urine without and with acid hydrolysis by means of high performance liquid chromatography (HPLC). Regular soy consumption was identified for 45 test persons of the treatment group. Significant quantities of isoflavones were detected irregularly in the urine of 3 test persons. It is unclear whether these results are explained by irregular soy consumption or special features of the metabolism of the test persons. For 25 of the 28 test persons in the control group, isoflavones were detected rarely or never in the urine samples during the study phase. This also means that we have to assume a small current intake of isoflavones in the population. Isoflavones were detected irregularly for 3 test persons so that food intake has to be assumed in these cases. This emphasizes that special phenotypic characteristics have to be taken into account with regard to the phytoestrogen metabolism. An administration of a certain quantity of isoflavones alone does not mean that this quantity is of biological relevance.

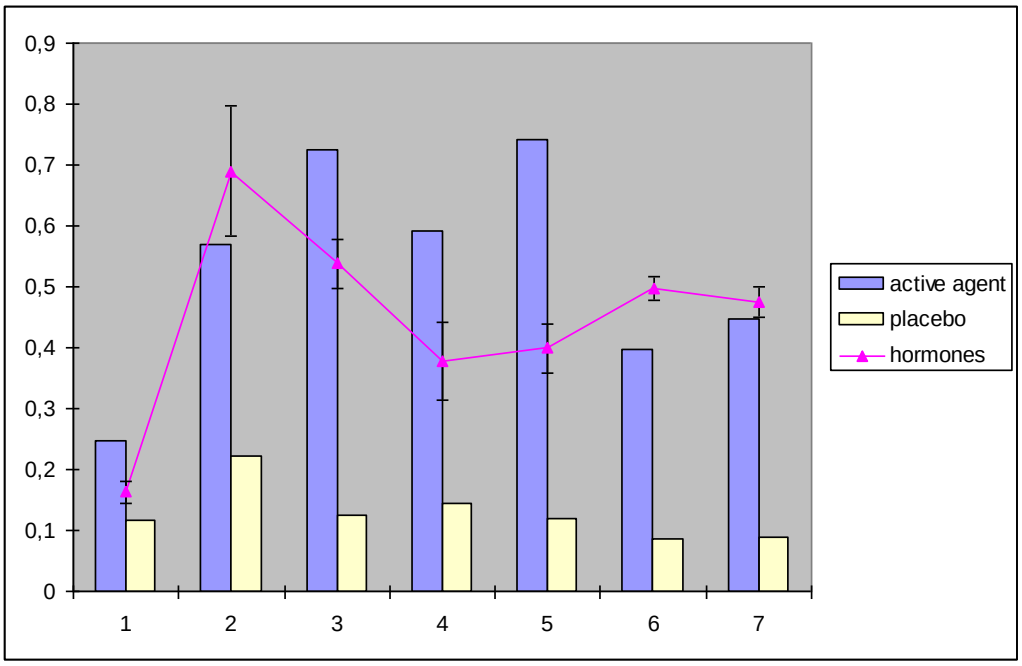

Figure 9. Presentation of daidzein concentrations (nmol/ml) in the course of the study.

Figure 10 shows the time courses of genistein concentrations. The differences between treatment and placebo group are smaller. Please note again that the hormone group additionally received the phytoestrogens.

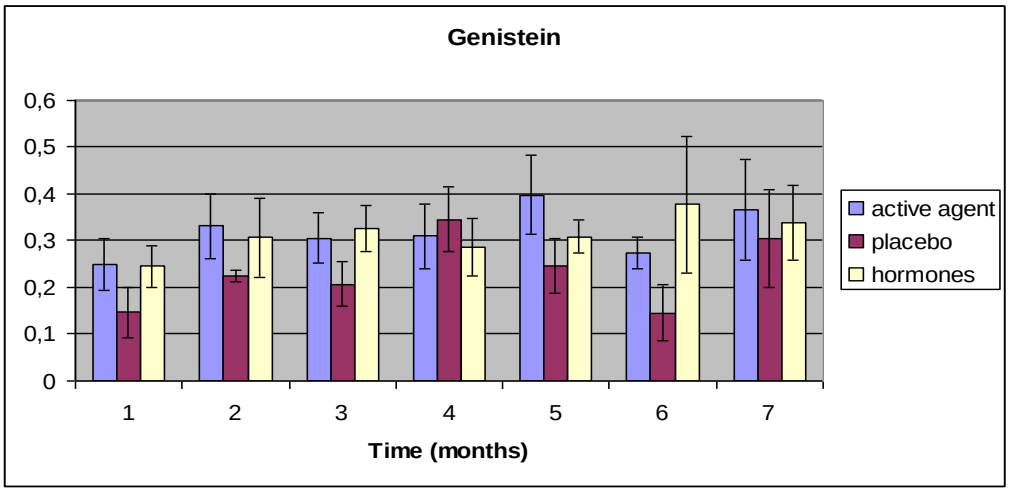

Figure 10. Presentation of genistein concentrations (nmol/ml) in the course of the study.

The evaluation of our results makes clear that the determination of isoflavones in the urine without and with acid hydrolysis by means of HPLC is well suitable for studies including a large number of samples since the presence of significant quantities of free unconjugated daidzein and genistein in the urine can be quantified relatively simply and

quickly. This is how the exposure to isoflavones can be assessed in an objective way. These results constitute decisive preparations for further studies.

A significant reduction of the climacteric complaints compared to the placebo groups was proven in the present treatment groups. For example, the complaints of hot flushes, heart hurry and attacks of vertigo clearly decreased both regarding number and frequency. Sleep disorders and irritability improved in the treatment group as well. The laboratory chemical analyses showed a clear improvement of the bone metabolism in the treatment group. There was a trend towards an increase in ostase in the serum and towards a decrease in pyridinoline (cross-links). In particular the concentration of triglycerides, but also of total cholesterol, was reduced, whereas no changes were found in the placebo group.

Thus, we can establish that prevention and therapy of the climacteric syndrome are possible both using food supplements and functional food enriched with phytoestrogens.

2.3.3. Remifemin – Prospective Cohort Study

Medicinal products that contain extracts of black cohosh (*Cimicifuga racemosa*) enjoy more and more popularity for the therapy of climacteric complaints. The two established products Remifemin® and Remifemin® plus are the subject matter of a worldwide clinical research program.

Remifemin® contains the isopropanolic extract of back cohosh. Remifemin® additionally contains an ethanolic extract of St. John's wort (*Hypericum perforatum*). Randomized, placebo-controlled clinical trials have proven the efficacy of both products.

Our study is a prospective, controlled post-authorization study [Briese et al. 2007]. More than 1,000 gynecological surgeries throughout Germany have participated in this study. Considering this great participation and the study period of two years, the random test has to be considered to be representative.

Patients with climacteric complaints who have not been treated with the study drugs during the last 6 months or with hormones during the last 4 weeks were included in the study.

The findings were established at the beginning of the study, after 3 and after 6 months. The climacteric symptoms were measured using the Menopause Rating Scale I (MRS I).

As expected, the two treatment groups showed minor differences regarding the individual demographic and anamnestic data. On average, Remifemin® patients were a bit younger slightly more frequently premenopausal. Nevertheless: Both drugs are used both for pre- and postmenopausal women.

The intensity of the dominant symptoms of hot flushes and sleeping disorders was approximately identical in both groups. On the contrary, Remifemin® plus was used more frequently in patients suffering from more intense depressive moods or nervousness / irritability than the drug containing only a single active agent.

The comparison of the therapy groups is in the center of interest. In this comparison, Remifemin® plus proves to be highly significantly superior to Remifemin® regarding the influence on the MRS subscore of psychological symptoms.

2.4. Prevention and Therapy with Phytoestrogens (Basic Research and Clinical Aspects)

Orthodox medicine as well is increasingly discovering the potential for health offered by plant hormones. Long-term hormone replacement therapy (HRT) has come under criticism [Kleine-Gunk 2008, Rohr 2004]. Therefore, a more careful and differentiated assessment of the necessity of such a therapy is required in future. Alternatives to classical hormone replacement therapy (HRT) are called for. At present, phytoestrogens constitute the most promising option [Branca and Lorenzetti 2005, Gebhardt 2008]. Phytoestrogens have many of the positive effects – albeit in a weaker form – that classical hormone preparations show as well; but without their undesired risks and side effects. Orthomolecular medicine has discovered in which way vitamins and trace elements can have a selective influence on our immune system. At present we are not sure if phytoestrogens are generally safe food additives or dangerous drugs [Wuttke 2007]. In this context, dose-effect relationships, period of administration and interindividual differences of the metabolic characteristics have to be taken into account. Phytoestrogens reveal new possibilities: plants have an influence on our hormone system. They produce hormones similar to human hormones - hormone-related substances - and, as a result, have hormone-like effects in the human body [Adlercreutz 1997, 2000, 2002]. According to human estrogens, we have to assume genomic and non-genomic mechanisms of action mainly through ERß, but also through ERα [Cabanes et al. 2004]. It has not been discovered yet to which extent the hypothalamo-hypophyseal-ovarial axis is influenced.

From the clinical point of view the phytoestrogens are weak estrogens. They act by estrogen receptors predominantly. We don't know which the crucial conditions are for their estrogenic or antiestrogenic activity, respectively. However, there are conflicting results related to differences in study design, estrogen status of the body, metabolism of isoflavones among individuals, and other dietary factors. Thus must be understandably for the clinically active physician that basic research results are needed before standards for prevention could be recommended.

The positive influence of phytoestrogens on the cardiovascular system is caused mainly by the action via the endothelial membrane sex steroid receptors. This receptor is likely to the estrogen receptor α (ERα). The activated palmityolated membrane sex steroid receptor induces the endothelial nitric oxide synthesis (eNOS) via an intracellular signal cascade [Nakaya et al. 2007].

Experiments in adult female animals have shown that estrogen induces endothelium-dependent vascular relaxation via the nitric oxide (NO), prostacyclin, and hyperpolarization pathways. Also, surface membrane estrogen receptors (ERs) decrease intracellular free Ca^{2+} concentration and perhaps protein kinase C-dependent vascular smooth muscle contraction [Oia et al. 2008]. On the other side a phytoestrogen antagonism on homocysteine-induced endothelin-1 gene expression and on reactive oxygen species accumulation could be responsible for vasodilatation. Anti-inflammatory effects of phytoestrogens are also of interest. Phytoestrogens are hypothesized to act through inflammation pathways [Pan et al. 2008]. It could be demonstrated that a consumption for 6 week of a 500 mg/d of secoisolariciresinol diglucoside may reduce CRP (c-reactive protein) serum concentrations

but had no effect on plasma lipid concentrations, serum lipoprotein oxidation resistance, or plasma antioxidant capacity [Hallund et al. 2006, Hallund et al. 2008]. No significant effects of phytoestrogens on other plasma inflammatory markers were observed [Hall et al. 2005].

Present is to be assumed that phytoestrogens are acting at the molecular level regarding to the protection of degenerative and cancer diseases. Recently, Park et al. [2008] determined the effect of genistein on adipogenesis and estrogen receptor (ER) alpha and beta expression during differentiation in primary human preadipocytes. Their study adds to the elucidation of the molecular pathways involved in the inhibition of adipogenesis by phytoestrogens. The inhibition of lipid accumulation was associated with inhibition of glycerol-3-phosphate dehydrogenase activity and down-regulation of expression of adipocyte-specific genes, including peroxisome proliferator-activated receptor gamma, glycerol-3-phosphate dehydrogenase, adipocyte fatty acid binding protein, fatty acid synthase, sterol regulatory element-binding protein 1, perilipin, leptin, lipoprotein lipase and hormone-sensitive lipase. These effects of genistein during the differentiation period were associated with down-regulation of ERα and ERβ expression.

Review of the existing literature suggests that consumption of soy foods or an exposure to a soy isoflavone genistein during childhood and adolescence in women, and before puberty onset in animals, reduces later mammary cancer risk. A meta-analysis of human studies indicates a modest reduction in pre- and postmenopausal risk when dietary intakes are assessed during adult life. These findings concur with emerging evidence indicating that timing may be vitally important in determining the effects of various dietary exposures on the susceptibility to develop breast cancer. The biochemical pathway for cancer prevention are based the ability of phytoestrogens to bind preferentially to estrogen receptor ß (ERß), inhibit enzymes that convert circulating steroid precursors into estradiol and inhibit cell signalling pathways of growth factors [Rica and Whitehead 2008]. Chen et al. [2007] indicated that genistein is involved in mechanisms in activation of insulin-like growth factor 1 receptor expression in human breast cancer cells. The studies have shown that genistein can enhance the insulin-like growth factor (IGF)-1 receptor signalling pathway via an estrogen receptor (ER) in human breast cancer MCF-7 cells. The results indicated that the induction of IGF-1 receptor promoter activity by genistein required the action of ER while the stimulatory actions of genistein on IGF-1 receptor expression required the activity of the IGF-1 receptor and de novo protein synthesis (cross-talk between IGF-1 receptor and the ER-dependent pathways). The new focus is on changes in gene expression, such as those involving BRCA1 and PTEN. Warri et al [2008] debated whether mammary stem cells are the targets of genistein-induced alterations and also whether the alterations are epigenetic. We propose that the effects on mammary gland morphology and signalling pathways induced by pubertal exposure to genistein mimic those induced by the oestrogenic environment of early first pregnancy.

Metabolism of dietary soy isoflavones to equol by human intestinal microflora [Yan et al. 2007]:

In vivo studies have shown variations in health benefits of isoflavones among individuals, which have been attributed to dissimilarities in the population of colonic bacteria responsible for isoflavone conversion [Rafii et al. 2003, Setchell et al. 2002].

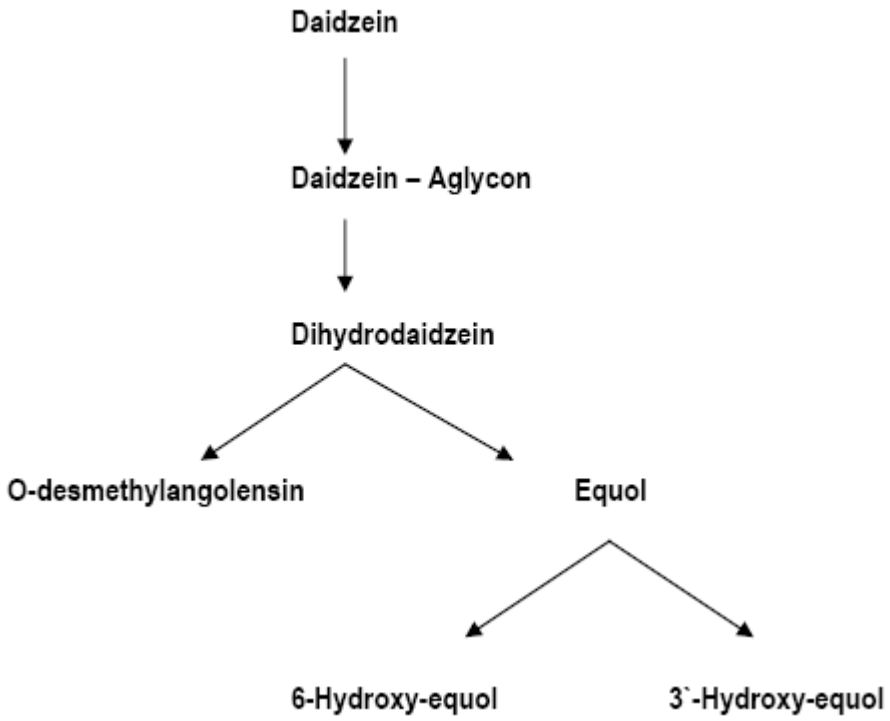

Figure 11. Bioavailability – daidzein is converted to O-desmethylangolensin and equol via the action of intestinal ß-glycosidase from bacteria.

Isoflavones (genistein, daidzein) can be metabolized by intestinal microflora and converted to dihydrodaidzein, O-desmethylangolensin, equol or 4-hydroxyequol, significantly altering its biological properties [Hedlund et al. 2003]. The bioavailability of soy isoflavones strongly depends on the activity of intestinal bacteria. The intestinal microflora plays a crucial role in the metabolism of isoflavones. The underlying interactions remain poorly understood [Clavel et al. 2005]. Equol suggests a major role as a biomarker for the effectiveness of soy isoflavones. Despite this known biological and clinical importance of equol, there have been limited studies of equol effects in vivo because of the high cost of equol and its limited availability [Selvaraj et al. 2004]. The bioavailability of isoflavone glycosides requires the conversion of glycosides to aglycones via the action of intestinal ß-glycosidase from bacteria that colonize the small intestine for uptake into the peripheral circulation. Genistein is converted to p-ethyl phenol and 4-hydroxyphenyl-2-propionic acid, while daidzein is reduced to O-desmethylangolensin and equol (Figure 11) [L`homme et al. 2002, Bowey et al. 2003].

The distinction between equol producers and equol nonproducers can be derived from urine. An equol producer is defined as someone excreting > 1000 nmol/L [Setchell 2002]. The physiologic differences between equol producers and nonproducers have not been fully elucidated [Blair et al. 2003]. However, it is clear that nutritional diets influence the metabolism of isoflavones. The role of prebiotics comes also into the question.

Phytoestrogens as Anti-Menopausal Agents

Female ovaries are designed as “temporary organs”. The endocrine function significantly reduces approximately in the late forties, at the latest however in the mid-fifties, individual very indifferently. The feed back mechanisms of the hypothalamo-hyophyseal-ovarial axis are detoriated irreversibly. This concerns autocrine, paracrine cerebral and endocrine central and peripher mechanisms. This results in the great variety of climacteric symptoms, e.g. due to the impairment of cerebral centers such as the centre for temperature, the limbic system, autonomic nervous system. About 30% of all women experience a significant impairment of their quality of life due to daily and undulant hot flushes. Only one third of women can live without any treatment. Recently symptoms of depression move into the foreground [Briese et al. 2007]. In addition to hot flushes, other psycho-autonomic complaints include sleep disorders, heart hurry or latent and obvious depressions. From numerous experiences and reports we can conclude that by means of phytoestrogens easy symptoms can be covered well.

The cancer preventive effect of phytoestrogens allows women suffering from a mamma carcinoma to take this substance for menopausal complaints. In contrast to hormone replacement therapy performed up to now, phytoestrogens constitute a therapy option during the critical stage at the beginning of the menopause as well since they do not cause hormone substitution, but hormone modulation (SERMS = selective estrogen receptor modulators). Phytoestrogens have a weak estrogen effect. Their hormone receptors are identical to the body's own hormones. They unfold their estrogen effect in case of endogenous hormone deficiency [Anderson et al. 1999, Jungbauer and Pfitscher 2005, Kleine-Gunk 2008]. On the contrary, in case of excessively high estrogen levels, phytoestrogens act as antiestrogens so that the essentially stronger endogenous hormones cannot unfold their proliferative effect. Thus, their use for the premenstrual syndrome seems to be possible as well [Kleine-Gunk 2008]. However new studies are to be considered, which warn of uncontrolled application of phytoestrogens. In our region (Germany) we dedicate ourselves at present excellently lignans.

What to Do for Women Suffering from Persistent Climacteric Complaints that Cannot Be Managed Through Phytodrugs?

We recommend first a phytoestrogen application over 6 - 12 weeks. We vary the dose of isoflavons pray if 50 and 100 mg and change then on another preparation, for example black cohosh. In some cases, the classical hormone replacement therapy (HRT) should be applied - at least for some time. The application of HRT for treating acute complaints is very effective. Then, a switchover of therapy to phytoestrogens should be tried by slowly reducing the dose, e.g. reduction from 2 mg estradiol to 1 mg estradiol with simultaneous administration of phytoestrogen [Kleine-Gunk 2008]. Under in vitro conditions genistein and daidzein could decrease proliferation rates of mammary epithelial cells stimulated by estradiol [Nebe et al. 2006].

Protection Against Osteoporosis

According to epidemiologic studies from the Asian region, we can conclude that a long-term diet rich in soy can prevent osteoporosis [Barnes 2003, Branca and Lorenzetti 2005]. In Asia the osteoporosis occurs very rarely. Just like the actual estrogens, phytoestrogens inhibit osteoclast activity. In addition, osteoblast activity is stimulated so that real new bone formation can be promoted. In the meantime, the positive effects of phytoestrogens on the

bones have been proven in animal experiments. Laboratory rats in which estrogen deficiency was established developed osteoporosis. The now started administration of genistein, the most important soy phytoestrogen, prevented the development of an osteoporosis. Human studies showed that biomarkers such as pyridinoline and osteocalcin can verify this effect of phytoestrogens after approx. 6 weeks. Women who keep to a diet rich in phytoestrogens had less products of bone absorption (pyridinoline) in their urine and more bone-stimulating substances (osteocalcin) in their blood. In accordance with our in vitro cell culture investigations we assume, which also the arthritis are favorably affected through phytoestrogens (genistein, daidzein) [Claassen et al. 2008]. Proteoglycans consisting of low and high sulfated glycosaminoglycans are the main components of articular cartilage matrix, and their synthesis is increased by insulin in growth plate cartilage. We have investigated whether glycosaminoglycan synthesis and sodium [(35)S]sulfate incorporation in female bovine articular in chondrocytes are affected by daidzein, genistein, and/or insulin. However, the stimulating effect of insulin on sulfate incorporation was enhanced significantly after preincubation of cells with 10(-11) M-10(-5) M daidzein or 10(-9) M-10(-5) M genistein but not by 17β-estradiol was estimated. In view of the risks of long-term estrogen replacement therapy, further experiments should clarify the potential benefit of phytoestrogens in articular cartilage metabolism.

In the meantime, a synthetic phytoestrogen, ipriflavone, has become available and has already been used for osteoporosis treatment in 20 countries [Kleine-Gunk 2008]. A daily dose of 600 mg ipriflavone for a period of 2 years is recommended. In addition to phytoestrogens, soy protein is associated with a positive effect on the preservation of the bone structure. The absorption of soy protein needs 30% less calcium than the absorption of animal proteins. However, we have to add that a sufficient resorption of soy proteins and phytoestrogens is attached to a well functioning intestinal flora.

Lipid Metabolism and Cardiovascular Protection

Animal experimental and human studies indicate that a reduction of high cholesterol levels and the improvement of the LDL-HDL-ratio are widely demonstrated effects of phytoestrogens. Phytoestrogens are highly effective radical scavengers (antioxidants) and can reduce the oxidation of LDL cholesterol. Oxidation of LDL cholesterol mainly is a result of aggressive molecules, the so-called free radicals. Like heparins, phytoestrogens contribute to the improvement of the thinning of the blood. They have a mild anticoagulant effect. This is why phytoestrogen, unlike the classical estrogens, are not associated with an increase in the risk of thrombosis, but with a reduction of the risk of thrombosis. With regard to the protection of the cardiovascular system, soy protein contains less homocysteine than animal protein. Soy protein is rich in B vitamins and folic acid. It is generally known that folic acid strongly reduces high homocysteine levels. The positive influence of phytoestrogens on the cardiovascular system can be explained by means of the membrane estrogen receptor. In its parmitoylated form, the membrane estrogen receptor is associated with the cell membrane and activates the endothelial nitrogen oxide synthetase (eNOS). On account of the similarity with the estrogen receptor, phytoestrogens also act through the membrane estrogen receptor or through non nuclear pathways and can thus effect a dilatation of the vessels and also contribute to a reduction in blood pressure [Ho and Liao 2002, Klinge et al. 2003].

Anticancerogenic Properties of Phytoestrogens

Cancer initiation and cancer promotion constitute the two most important steps in carcinogenesis. So-called carcinogenic as well as cancerogenic substances in the form of pollutants and so-called free radicals play a role in cancer initiation. This results in an initial damage to the hereditary material of a cell so that growth-promoting substances (promoters) can advance the further cancerogenic degeneration. As radical scavengers, phytoestrogens can prevent the malignant degeneration in both stages - both during cancer initiation and cancer promotion - in particular of hormone-dependent tumors. Furthermore, phytoestrogens act as aromatase and angiogenesis inhibitors [Chen et al. 2003]. The antiangiogenic properties of phytoestrogens have been proven by means of experiments [Fotsis et al. 1995].

Breast cancer prevention through phytoestrogens should already start before puberty [Controneo et al. 2002]. This statement is corroborated by the known Asian migration studies [Luo et al. 2004]. Asian women usually eat food rich in phytoestrogens. After migration to Western countries, the "protection against carcinoma" was preserved [Pineda et al. 2001]. Their daughters (next generations) who have grown up with food adapted to Western culture and thus poor in phytoestrogen had lost this protection.

Maskarinec and Noh [2004] compared cancer incidence trends among Japanese in Japan, and Japanese and Caucasians in Hawaii, between 1960 and 1997, and estimated the impact of migration on the incidence of different cancers. Among the 5 more common cancers, the migrant effect was strongest for colon and stomach cancers, prostate and breast cancers were affected to a lesser degree, and lung cancer risk differed little between Japanese in Japan and Hawaii. Migration led to lower risk of stomach, esophageal, pancreatic, liver, and cervical cancers, but to higher rates for all other cancers. Although the migration effect can be partially explained by known etiologic factors, a large proportion of the changing risk remains unexplained. Recently, cancer rates for Korean-American immigrants have increased for prostate, breast, colon, and rectal cancers [Lee et al. 2007].

Experiments with prepubertal rats showed that a complete differentiation of the mammary glands takes place in case of exposure to isoflavones. This "complete" differentiation is considered to be an important factor for the prevention of breast cancer. Phytoestrogens contribute to the upregulation of the expression of the mRNA for the marker protein BRCA1.

According to the Asian studies more than 15 (20) mg isoflavones per day are considered to be the ideal quantity for the prevention of mamma carcinoma. A daily intake of isoflavones of under 1 mg is assumed in the industrial nations of the US, Canada and Australia. It is essential that the intake of isoflavones is started in childhood and continued throughout life [Wu et al. 2008].

Catechins are said to have anticancerogenic properties as well. Catechins belong to the secondary plant substances and, according to their structure, to the polyphenols. An ointment for local application, the polyphenon ointment, has been available since 2007. The polyphenon ointment has indications in gynecology and dermatology for HPV viral infections (genital warts, *Condylomata acuminata*). Only few experiences are available at present. Studies indicate that catechins have a primary and/or secondary preventive effect for cardiac diseases and tumor diseases. Studies of cell cultures showed that the proliferation of tumor cells is inhibited [Cooper et al. 2005]. On the other hand, apoptosis is induced. Especially

green tea has demonstrated promise in the prevention of several cancers. Green tea contains several components including catechins, a category of polyphenols that have chemopreventive properties [Lee et al. 2006]. Besides, in vitro studies indicate that tumor suppressor genes are expressed to an increased extent. The gene expression of EGF – and of the tumor necrosis factor α (TNFα) is reduced significantly [Adachi et al. 2007]. With regard to mamma carcinoma, we currently have to assume that the prognosis can be improved by secondary preventive application of catechins in particular in stages 1 and 2. Furthermore, it is assumed for catechins that anti-inflammatory and anti-stress properties might be responsible for the prevention of degenerative diseases.

Protection of the Skin Through Phytoestrogens

The experience with hormone replacement therapy (HRT) has shown that estrogens can have a large number of positive cosmetic effects. Exactly the critical studies have shown that estrogens cannot be applied without hesitation, they remain a medicinal measure. Local creams and ointments, but also phytoestrogens offer an alternative to this systemic hormone replacement therapy. The first cosmetic containing phytoestrogen − a skin care cream produced by Vichy Company − is available on the marked under the name of Novadiol®. Further experiences in dermatology remain to be seen.

What Clinicians Need to Know According to the Newest Literature? What's the Meaning of Protective Features of Phytoestrogens against Prostate Cancer?

Accumulating epidemiological data suggest that Asian men have lower incidences of prostate cancer and benign prostate hyperplasia compared with American and European populations and may have benefited from their higher intake of phytoestrogens in their diet. However, how these phytochemicals affect prostatic diseases is still unclear [Gaynor 2003]. To determine the clinical effects of soy isoflavones on prostate cancer Hussain et al. [2003] conducted a pilot study in patients with prostate cancer who had rising serum prostate-specific antigen (PSA) levels. Patients with prostate cancer were enrolled in the study if they had either newly diagnosed and untreated disease under watchful waiting with rising PSA (group I) or had increasing serum PSA following local therapy (group II) or while receiving hormone therapy (group III). The study intervention consisted of 100 mg of soy isoflavone (Novasoy) taken orally twice daily for a minimum of 3 or maximum of 6 months. Serum genistein and daidzein levels increased during supplementation from 0.11 to 0.65 μM. The follow up of the PSA levels suggests that soy isoflavones may benefit some patients with prostate cancer. There was a decrease in the rate of the rise of serum PSA in the whole group with rates of rise decreasing from 14 to 6% in group II and from 31 to 9% in group III following the soy isoflavone intervention. It can be postulated, dietary intervention with isoflavone supplementation may have biologic activity in men with biochemical active prostate cancer as shown by a decline in the slope of PSA in pilot studies.

Pendleton et al. [2008] evaluated the efficacy of isoflavones in patients with PSA recurrent prostate cancer after prior therapy. They postulated that isoflavone therapy would slow the rate of rise of serum PSA. Twenty patients with rising PSA after prior local therapy were enrolled in this open-labeled, Phase II, nonrandomized trial. Patients were treated with soy milk containing 47 mg of isoflavonoid three times per day for 12 months. Nearly two

thirds of the patients were noted to have significant levels of free equol in their serum while on therapy. The slope of PSA after study entry was significantly lower than that before study entry in 6 patients and the slope of PSA after study entry was significantly higher than before study entry in 2 patients. For the remaining 12 patients, the change in slope was statistically insignificant.

Kumar et al. [2007] evaluated the safety of 80 mg of purified isoflavones regarding to men with early stage prostate cancer. A total of 53 men with clinically localized prostate cancer, Gleason score of 6 or below, were supplemented with 80 mg purified isoflavones or placebo for 12 wk administered in 2 divided doses of 40 mg. Changes in plasma isoflavones, and clinical toxicity were analyzed at baseline, 4, and 12 wk. A continuous, divided-dose administration of 80 mg/day of purified isoflavones at amounts that exceeded normal American dietary intakes significantly increased ($P < 0.001$) plasma isoflavones in the isoflavone-treated group compared to placebo and produced no clinical toxicity.

Which Actual Statements about Phytoestrogens and Breast Cancer Prevention Are Meaningful at Present?

Scientific achievements in the last two decades have revolutionized the treatment and prevention of breast cancer. This is mainly because of targeted therapies and a better understanding of the relationship between estrogen, its receptor, and breast cancer. One of these discoveries is the use of synthetic selective estrogen modulators (SERMs) such as tamoxifen or raloxifen in the treatment strategy for estrogen receptor (ER)-positive breast cancer. The potential effects of phytoestrogens may alter the risk of breast cancer, but only a limited range of phytoestrogens has been examined in prospective cohort studies. Serum and urine samples from 237 incident breast cancer cases and 952 control individuals (aged 45 to 75 years) in the European Prospective into Cancer-Norfolk cohort were analysed for seven phytoestrogens (daidzein, enterodiol, enterolactone, genistein, glycitein, o-desmethylangolensin, and equol) using liquid chromatography/mass spectrometry [Ward et al. 2008]. In summary, urinary or serum phytoestrogens were not associated with protection from breast cancer. Breast cancer risk was marginally increased with higher levels of total urinary isoflavones (odds ratio = 1.08 (95% confidence interval = 1.00 to 1.16), $P = 0.055$); among those with estrogen receptor-positive tumors, the risk of breast cancer was increased with higher levels of urinary equol (odds ratio = 1.07 (95% confidence interval = 1.01 to 1.12), $P = 0.013$). There was limited evidence of an association between phytoestrogen biomarkers and breast cancer .risk in the present study.

The observation that some phytoestrogen biomarkers may be associated with slightly greater risk of breast cancer warrants further studies. At present, it remains uncertain whether the different phytoestrogens are chemo protective or whether they may produce adverse outcomes related to breast carcinogenesis.

Recently Helferich et al. [2008] reported results of animal breast cancer model focused on the effects of dietary genistein on the growth of estrogen dependent mammary tumors both in vitro and in vivo. Genistein enhances the proliferation of estrogen dependent human breast cancer tumor growth. In a similar manner, dietary genistein stimulates tumor growth in the chemically-induced mammary cancer rodent model. Genistein, the glycoside of genistein, simulates growth similar to that of genistein and withdrawal of either genistein or genistein

results in tumor regression. The extent of soy processing modulates the effects of dietary genistein *in vivo* as soy protein isolate, a highly purified and widely used source of protein that is processed to contain low, medium, and high amounts of isoflavones, stimulate the growth of the estrogen dependent mammary tumors in a dose dependent manner. In contrast to the more purified diets, studies with soy flour of equivalent genistein levels did not stimulate the growth of estrogen dependent breast cancer tumors in vivo.

There is conflicting evidence from epidemiological, intervention and experimental animal studies regarding the chemo preventing effects of soy isoflavones in breast cancer. Isoflavones are weak estrogens and their effect depends upon the dose, time of exposure and species involved. It would, therefore, not be safe to indisputably accept soy or red-clover as a source of isoflavone resource to prevent breast cancer [Tomar and Shian 2008].

Should We Recommend Adult Women in Western Countries Take a Daily Phytoestrogen Application for Breast Cancer Prevention?

Studies conducted in Asian populations have suggested that high consumption of soy-based foods, at the beginning of childhood, that are rich in isoflavone phytoestrogens is associated with a reduced risk of breast cancer. At present it must be pointed out that no context exists regarding an association between phytoestrogen rich diets and successful breast cancer prevention in Western countries. Because one of the biological effects of phytoestrogens is probably estrogenic, it is possible that the preventive effect on breast cancer differs by estrogen receptor (ER) or progesterone receptor (PR) status of the tumor. High dietary intakes of plant lignans and high exposure to enterolignans were associated with reduced risks of ER- and PR-positive postmenopausal breast cancer in a Western population that does not consume a diet rich in soy. Touillaud et al. [2007] prospectively examined associations between the risk of postmenopausal invasive breast cancer and dietary intakes of four plant lignans (pinoresinol, lariciresinol, secoisolariciresinol, and matairesinol) and estimated exposure to two enterolignans (enterodiol and enterolactone), as measured with a self-administered diet history questionnaire, among 58,049 postmenopausal French women who were not taking soy isoflavone supplements. During 383,425 person-years of follow-up (median follow-up, 7.7 years), 1469 cases of breast cancer were diagnosed. Compared with women in the lowest intake quartiles, those in the highest quartile of total lignan intake (>1395 μg/day) had a reduced risk of breast cancer (RR = 0.83, 95% CI = 0.71 to 0.95, p (trend) = 0.02, 376 versus 411 cases per 100,000 person-years), as did those in the highest quartile of lariciresinol intake (RR = 0.82, 95% CI = 0.71 to 0.95, P(trend) = 0.01). The inverse associations between phytoestrogen intakes and postmenopausal breast cancer risk were limited to ER- and PR-positive disease.

Hedelin et al. [2008] evaluated the associations between dietary phytoestrogen (isoflavonoids, lignans, and coumestrol) intake and risk of breast cancer and whether the ER/PR statuses of the tumor influence this relationship. In 1991-1992 a prospective population-based cohort study among Swedish pre- and postmenopausal women was performed, making questionnaire data available for 45,448 women. A total of 1014 invasive breast cancers were diagnosed until December 2004. However, intake of coumestrol was associated with decreased risk of receptor negative tumors (ER-PR-) but not positive tumors. The risk of ER-PR- tumors was significantly lower (50%) in women with intermediate

coumestrol intake compared with those who did not consume any. In addition, the authors found no association between intake of isoflavonoids or lignans and breast cancer risk.

Are Synthetic SERMS (Tamoxifen) and Phytoestrogens Applicable in Combined Form in the Secondary Prevention of Breast Cancer?

Not enough clinical trials exist. Label use applications are present, but no data base of follow-up observations. Dietary genistein can negate the inhibitory effects of tamoxifen on estradiol stimulated growth of MCF-7 cell tumors implanted into ovariectomized athymic mice [Helferich 2008]. An increasing number of breast cancer patients seek to take supplements together with their standard treatment in the hope that these will either prevent recurrence or treat their menopausal symptoms. Observational studies suggest a protective effect of isoflavones on breast cancer risk and the case may be similar for increasing lignan consumption although evidence so far is inconsistent. In contrast, short-term intervention studies suggest a possible stimulatory effect on breast tissue raising concerns of possible adverse effects in breast cancer patients. However, owing to the dearth of human studies investigating effects on breast cancer recurrence and survival the role of phytoestrogens remains unclear. So far, not enough clear evidence exists on which to base guidelines for clinical use, although raising patient awareness of the uncertain effect of phytoestrogens is recommended [Valentzis 2008]. Hormonal replacement therapy (HRT) is contra indicated in breast cancer survivors [Holmberg et al. 2008].

Should We Recommend Adult Women in Western Countries Take a Daily Phytoestrogen Application Regarding the Prevention of Osteoporosis?

In vitro, phytoestrogens promote osteoblastogenesis and inhibit osteoclastogenesis [Poulsen and Kruger 2008]. Human studies support a long-term substitution with phytoestrogens against osteoporotic progression [Rohr 2004]. The recommended daily dosages of isoflavone applications amounted to 40–100 mg in most studies. On the other side a relatively large number of intervention studies have been undertaken in animals and humans, the efficacy of phytoestrogens as bone-protective agents *in vivo* remains unclear. Differences in the bioactivities of individual phytoestrogens, differences in phytoestrogen metabolism and bioavailability within different study populations, and imprecise reporting of the dose of phytoestrogens administered in intervention studies may have contributed to the disparity in study findings.

What Is the Usefulness of Phytoestrogens in Reduction of Blood Pressure?

To determine whether treatment with phytoestrogens or soy proteins succeeds in lowering blood pressure, Rosero et al. [2008] evaluated all the observation studies and clinical trials in a systematic review. No significant variations in blood pressure were found, whether systolic (-1.20 mm Hg; 95% CI, -2.80 to 0.41 mm Hg) or diastolic (-1.31 mm Hg; 95% CI, -2.73 to 0.11). If there were any variations, they are clinical of little importance. There are no statistically significant or clinically important differences in blood pressure between patients treated with phytoestrogens and those not treated.

Are Isoflavones Able to Decrease Serum Total and LDL Cholesterol Concentrations in Humans?

Clinical trials have reported the cholesterol-lowering effects of soy protein intake, but the components responsible are not known. Taku et al. [2007] performed a meta-analysis to evaluate the precise effects of soy isoflavones and soy proteins on lipid profiles. Eleven studies were selected for the meta-analysis. Soy isoflavones significantly decreased serum total cholesterol by 0.10 mmol/L (3.9 mg/dL or 1.77%; P = 0.02) and LDL cholesterol by 0.13 mmol/L (5.0 mg/dL or 3.58%; $p < 0.0001$); no significant changes in HDL cholesterol and triacylglycerol were found. Isoflavone-depleted soy protein significantly decreased LDL cholesterol by 0.10 mmol/L (3.9 mg/dL or 2.77%; $p = 0.03$). Soy protein that contained enriched isoflavones significantly decreased LDL cholesterol by 0.18 mmol/L (7.0 mg/dL or 4.98%; $p < 0.0001$) and significantly increased HDL cholesterol by 0.04 mmol/L (1.6 mg/dL or 3.00%; $p = 0.05$). The reductions in LDL cholesterol were larger in the hypercholesterolemic subcategory than in the normocholesterolemic subcategory.

Weggemans and Trautwein [2003] identified literature to the relation between soy associated isoflavones and LDL and HDL cholesterol concentrations in humans. A total of ten studies were adapted in a meta-analysis. Studies were included if they had a control group or treatment, experimental diets only differed in the amounts of soy protein and isoflavones and were each fed for at least 14 days. Studies comprised 959 subjects (336 men and 623 women), average age ranged from 41 to 67 years and baseline cholesterol concentration from 5.42 to 6.60 mmol/l. The intake of soy-associated isoflavones increased by 1–95 mg/day and the intake of soy protein increased by 19–60 g/day. Feeding daily 36 g soy protein with 52 mg soy-associated isoflavones on average decreased low-density lipoprotein (LDL) cholesterol by -0.17+/-0.04 mmol/l and increased high-density lipoprotein (HDL) cholesterol by 0.03+/-0.01 mmol/l. There was no dose-response relation between soy-associated isoflavones and changes in LDL cholesterol or HDL cholesterol. Consumption of soy-associated isoflavones is not related significantly to changes in LDL or HDL cholesterol.

Thorp et al. [2008] examined the contributions of soy protein, isoflavones and equol to the hypocholesterolemic effects of soy foods in a prospective study. Nonsoy consumers (33 men, 58 women) with a plasma total cholesterol concentration > 5.5 mmol/L participated in a double-blind, placebo-controlled, crossover intervention trial. The subjects consumed 3 diets for 6 wk each in random order, which consisted of foods providing a daily dose of 1) 24 g soy protein and 70-80 mg isoflavones (diet S); 2) 12 g soy protein, 12 g dairy protein , and 70-80 mg isoflavones (diet SD); and 3) 24 g dairy protein without isoflavones (diet D). Total cholesterol was 3% lower with the S diet (-0.17 +/- 0.06 mmol/L; $p < 0.05$) than with the D diet, and triglycerides were 4% lower with both the S (-0.14 +/- 0.05 mmol/L; $p < 0.05$) and SD (-0.12 +/- 0.05 mmol/L; $p < 0.05$) diets. There were no significant effects on LDL cholesterol, HDL cholesterol, or the total cholesterol:HDL cholesterol ratio. On the basis of urinary isoflavones, 30 subjects were equol producers. Lipids were not affected significantly by equol production. Equol, a gut bacterial metabolite of isoflavone daidzein, may improve health through changes in vascular function and in estrogen metabolism. The individual function is unclear. The authors concluded that regular consumption of foods providing soy protein and isoflavones had no significant effect on plasma LDL cholesterol in mildly hypercholesterolemic subjects, regardless of equol-producing status.

Are Phytoestrogen Supplements Standardized and Comparable Among Themselves Respectively?

For guarantee of quality of isoflavone-rich supplements, raw material standards are needed. The main isoflavones must be identified and marked.

For example, the investigations of Thompson et al. [2007] clearly demonstrated that supplements regarding their phytoestrogen content are incomparable. Twenty one nonvitamin, nonmineral dietary supplements commonly consumed by women in Canada were analyzed for isoflavones (formononetin, daidzein, genistein, glycitein), lignans (pinoresinol, lariciresinol, secoisolariciresinol, matairesinol), and coumestrol. Supplements containing soy or red clover had the highest concentrations of total isoflavones (728.2-35,417.0 µg/g) and total phytoestrogens (1030.1-35,517.7 µg/g) followed by licorice and licorice-containing supplements (41.3-363.3 µg/g isoflavones; 56.5-370.0 µg/g total phytoestrogens). Other supplements had considerably less isoflavones (</= 19.0 µg/g) and total phytoestrogens (</= 44.2 µg/g). Lignans were present in all (</= 298.9 µg/g), whereas coumestrol was either not present or present in only small amounts (</= 3.0 µg/g). Supplements differed in phytoestrogen profiles.

Another problem is "functional foods". In the United States, about 25% of infant formula sold is based on soy protein, which is an important source of estrogenic isoflavones in the human food supply [Cao et al. 2008]. Urinary concentrations of genistein and daidzein were about 500 times higher in the soy formula-fed infants than in the cow milk formula-fed infants.

It Is Possible to Guarantee a Significant Lignan Substitution by Rye Bread?

Lignins are basic substances of plant scaffolds derived from lignans. They are part of high-fibre substances. Main sources of lignans are rye and flaxseed. In processed flours lignans are hardly available.

Is the Knowledge Regarding the Content of Lignans in Foods of Western Countries Extensive Enough?

Accurate information about dietary phytoestrogens is important, but there are very limited data concerning food content. Lignans were the main type of phytoestrogens detected for instance in different regions in Germany, but exact information is often absent. Tea and coffee contained up to 20 µg/100 g phytoestrogens and beer (except bitter) contained up to 71 µg/100 g, mainly lignans [Kuhnle et al. 2008].

3. Conclusion

In clinical endocrinology, phytoestrogens (isoflavones, lignans) — estrogen-like substances — are considered to be a "gentle alternative" to classical estrogen therapies. Areas of indication include: protection against hormone-dependent tumors, protection against osteoporosis and cardiovascular protection, relief of climacteric complaints, protection of the skin. Phytoestrogens prove to be an interesting group of substances in particular in oncology since they can act as anti-estrogens, aromatase inhibitors and angiogenesis inhibitors. At

present, it still has to be taken into account that the study data from experimental and human biological trials in part is inconsistent. Dose-effect relationships are particularly considered to be not clear.

According to the data available up to now, the absorption of phytoestrogen before puberty is not associated with damage to the reproductive system. A diet rich in phytoestrogen is recommended for prepubertal girls. The situation for prepubertal boys still is not clear. The question of what consequences would result from an exposure to phytoestrogen in this case cannot be answered definitely yet. The exposure to isoflavones during pregnancy and lactation in rats showed a demasculinization of the reproduction system of the offspring. Other groups did not show any influence.

Final Statements on Flax and Elm Bark Extracts as Anticancerogenic Substances

Flax root and elm bark are potential candidates for anticancerogenic active agents for hormone-dependent gynecological tumors. Two strategies could be pursued based on the *in vitro* studies realized by us together with the corresponding results:

1) Check food chemistry and food technology for processing flax root and elm bark (tea etc.).
2) Identification of subfractions of extracts and single substances and elucidation of action mechanisms.

Although we have to assume that phytoestrogens display a favorable anticancerogenic effect in combination with other active agents, no sufficient statements on the dose dependency in human biology are possible at present.

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