Aryl hydrocarbon receptor activity of plant extracts

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Vorgelegt von DI Svjetlana Medjakovic

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Zusammenfassung

Der Arylhydrocarbon Rezeptor (AhR) ist ein wichtiger Regulator von vielen physiologischen Prozessen, darunter auch der Zellzykluskontrolle und der Reproduktion. Ist dieser Signalweg aus dem Gleichgewicht, kann es zu schweren Krankheiten und einem homoöstatischen Ungleichgewicht führen. Ein tieferes Verständnis von Substanzen, die den AhR aktivieren, sowie die Identifikation noch unbekannter AhR Aktivatoren, die einerseits umweltverschmutzende Chemikalien aber auch natürliche Pflanzen- und Lebensmittelinhaltsstoffe sein können, könnten die Ausgangssubstanzen für Therapeutika für die Krebsbehandlung, Wechseljahrsbeschwerden und hormonell-abhängiger Erkrankungen sein.

In dieser Arbeit wurden Extrakte essbarer Pflanzen, Lebensmittelinhaltsstoffe, sowie ausgewählte Pestizide, die potentiell in Lebensmitteln vorkommen können, untersucht. Rotklee wurde als potenter Aktivator des humanen AhR identifiziert. Der Wirkung konnten chemische Strukturen zugewiesen werden, nämlich den potenten AhR Liganden Formononetin und Biochanin A. Auch aus Sauerkraut konnte eine neue Verbindung, Indolylfuran (1-(2-furanyl)2-(3-indolyl)ethanone), isoliert werden, die ebenfalls ein starker AhR Ligand ist. Sauerkrautextrakt und Indolylfuran zeigten *in vivo* antiöstrogene Wirkung. In dieser Studie konnten auch grundlegende Wechselwirkungen zwischen dem AhR und dem Östrogenrezeptor (ER) aufgeklärt werden.

Eine Aktivierung des AhR kann auch durch unsachgemäße Anwendung von Pestiziden erfolgen. Nicht-persistene Pestizide, die in der Obstkultivierung und dem Weinbau verwendet werden, wurden untersucht, ob sie über nukleäre Rezeptoren, wie AhR, ER und dem Androgenrezeptor (AR), das hormonelle Gleichgewicht beeinflussen können. Einige Pestizide zeigten *in vitro* diese Wirkung. Die *in vitro* Daten zeigen, dass bei einer Anwendung entsprechend den gesetzlichen Richtlinien, nicht jene Konzentrationen erreicht werden, die so einen Effekt auslösen können. Da aber die Exposition von Landarbeitern und der Landbevölkerung durchaus höher ist, kann eine Beeinflussung des Hormonhaushaltes nicht ausgeschloßen werden.

Zusammenfassend kann man sagen, dass Lebensmittel das Potential haben den AhR und die von ihm regulierten Stoffwechselwege zu beeinflussen.

Abstract

The aryl hydrocarbon receptor (AhR) is an important regulator of several physiological processes including cell cycle regulation and reproduction. A dysregulated AhR signalling can lead to severe diseases due to homeostatic imbalance. Deeper insights of the impact of the AhR pathway, but also the knowledge of possible AhR activators that can be found in the environment as pollutants but also in plants and normal diet, facilitate the use of the AhR as drug target in cancer therapy, menopausal complaints and hormone-dependent diseases.

In this study, edible plants, food ingredients but also possible food contaminations such as pesticides were tested for their ability to transactivate the AhR. Red clover and sauerkraut were identified as potent activators of the human AhR. This was further elucidated by the identification of red clover compounds formononetin and biochanin A and the novel sauerkraut ingredient 1-(2-furanyl)2-(3-indolyl)ethanone, common name indolylfuran, as potent AhR agonists. Sauerkraut and indolylfuran showed *in vivo* anti-estrogenic effects. This study showed also basic interactions of the AhR with the estrogen receptor (ER).

Activation of the AhR can also be caused by food contaminants such as pesticide residues. Non-persistent pesticides, commonly used in fruit and wine cultivation, were tested for their ability to transactivate receptor pathways affecting hormonal balance such as the AhR, the ER and androgen receptor (AR). Several pesticides were identified that activate AhR, ER and AR. Application within legally permitted doses does not reach concentrations that can disturb hormonal balance of fruit and wine consumers. Nevertheless, this assumption bases only on *in vitro* data. The exposure of rural population and farm workers is higher, so that an impact on hormonal pathways cannot be excluded.

It can be concluded that diet has indeed the potential to affect the AhR pathway and to hereby modulate AhR regulated physiological functions.

Introduction

The aryl hydrocarbon receptor

Every cell is a complex and fine-tuned machinery that enables the organism to react to endogenous and exogenous stimuli. Important instruments for this purpose are receptors, proteins that are found in the cytoplasm, the nucleus or embedded in the cell membrane and that mediate the cellular response to various signals.

The aryl hydrocarbon receptor (AhR) is a transcription factor that belongs to the basic-helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) protein family. Members of this family are known to mediate adaptive responses to cellular stress, xenobiotic signals and hypoxia. But they also regulate basic and fundamental physiological processes such as the circadian rhythm and embryonic or organ development. According to their physiological importance, this protein family has been conserved during evolution. Flies still share the same structural motifs for the regulation of the circadian rhythm with mammals (reviewed in [1]).

The AhR is also a phylogenetically ancient protein [2]. But although the AhR pathway is inducible by light (reviewed by Rannug and Fritsche [3]), its functions are far beyond the circadian rhythm. This receptor is in charge of housekeeping functions such as the metabolism and cell cycle regulation but also reproduction. Additionally, studies with knockout mice and analysis of expression pattern during embryonic maturity indicate a great importance in development and organogenesis, as will be discussed in the following chapters. Nevertheless, this receptor was first and foremost intensively studied by toxicologists, because of toxic responses of the organism to the prototypical AhR ligand 2,3,7,8-tetrachlorodibenzodioxin (TCDD).

The AhR pathway

The pathway of the AhR resembles those of steroid hormone receptors and is dependent on ligand activation. In non-activated stage the AhR is found in the cytoplasm [4] in complex with chaperones. The unchaperoned receptor is not very stable [5]. The AhR remains in complex with the molecular chaperone hsp90 until the AhR dimerizes with its partner protein, the aryl hydrocarbon receptor nuclear translocator (ARNT), in the nucleus [5]. Despite the term nuclear translocator, ARNT is not responsible for the translocation of the AhR from cytoplasm to nucleus. ARNT is constitutively found in the nucleus [4].

In the cytoplasm, the AhR is also associated with the AhR interacting protein, AIP (also called ARA9 or XAP2) and p23 protein. But recent findings by Flaveny et al. [6] show that p23 is not necessarily required *in vivo* for ligand binding and gene expression, contradictory to *in vitro* data [7, 8]. For AIP there is no *in vivo* data available, but *in vitro* tests show that AIP seems to impact AhR-mediated signalling and is important for the transcriptionally active complex [9, 10] and not only for the stabilization of the AhR [11, 12].

The chaperone hsp90 is extremely important for the AhR pathway. It interacts with two spatial distinct AhR-domains, the PAS domain and bHLH domain and mediates therefore at several key points of the AhR cascade different functions [13]. Hsp90 is also essential for the AhR ability to bind ligands [14].

Upon binding of an agonist, a conformational change of the AhR is induced resulting in the exposition of the nuclear localization signal, which enables the translocation into the nucleus [15]. The existence of both, a nuclear localization signal as well as a nuclear export signal within the AhR structure indicates that this receptor is shuttling between cytoplasm and nucleus [16, 17]. After export from the nucleus, the AhR is degraded rapidly by the cytoplasmatic proteasome complex within the cytosolic compartment [18]. A simplified scheme of the AhR pathway is shown in Figure 1.

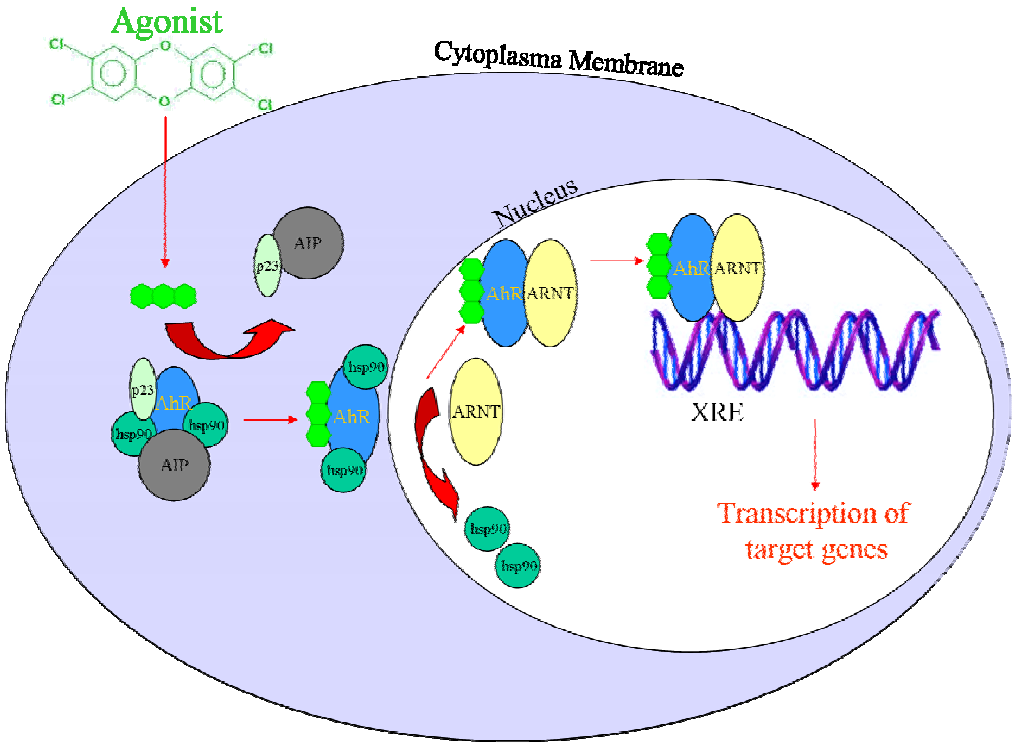


Figure 1. The AhR pathway.

In the nucleus the agonist-activated receptor meets its partner protein ARNT, which alone has no affinity for the specific DNA response elements upstream of AhR target genes, so called xenobiotic response elements (XREs). ARNT strongly promotes DNA binding of ligand-activated but not ligand-free AhR and dimerizes also exclusively with the ligand-activated receptor form [19]. The dissociation of hsp90 is required for the formation of the AhR/ARNT heterodimer [20]. This heterodimer represents the active DNA-binding form [21, 22] and the interaction with the XREs is highly specific [23]. During DNA-binding, both components of this complex directly contact the XRE [24] and mediate the transcription of target genes.

Contrary to the action of agonists, antagonists only bind to the ligand binding site of the AhR, but fail not only to initiate nuclear translocation but also to form a DNA-binding AhR/ARNT complex [25, 26].

Physiological significance of the AhR

To grasp the possibilities regarding prevention or therapy of diseases by a modulation of the AhR pathway, it is essential to understand the physiological significance of this receptor. Most toxic responses that are mediated by the AhR pathway are a consequence of a deregulated or an over-activated AhR cascade, resulting in impaired AhR functions and a homeostatic disequilibrium. Upon constitutive activation of the AhR pathway, as it happens with hardly metabolizable agonists such as TCDD, impaired liver regeneration [27], inflammatory skin lesions [28], long-lasting functional alterations in the developing immune system [29] and the development of several tumor types [30-32] have been observed. Brunnberg et al. [33] suggested the constitutively active AhR mouse as model for TCDD-mediated toxicity [33].

In the following chapter, the impact of the AhR during fetal development and postpartum as well as the involvement in various signal pathways will be enlightened.

Learning from the AhR knockout mouse

Knockout mice are important models for the investigation of physiological functions. For this purpose, a targeted gene is turned off. The resulting effects on the phenotype compared to that of the wild-type conspecifics provide indications about the physiological significance of the gene and the processes that are regulated or affected by this gene.

As the AhR is an orphan receptor and hence no endogenous ligand has been identified unambiguously, the significance in normal physiology is not fully comprehended.

Albeit it is still unknown which endogenous molecules are responsible for the activation of the AhR cascade, the AhR knockout mouse has filled gaps and has led to a better understanding of AhR regulated processes. A long time, the AhR has been defined only through its ability to modulate xenobiotic metabolism, which led to focusing on toxicological processes that are mediated by AhR-induced bioactivation of compounds to carcinogens or the constant activation of the AhR pathway. These processes can be better understood in the context of disrupted endogenous functions of the AhR. Moreover, it could be helpful for the prevention and therapy of several diseases considering the multiplicity of AhR-regulated functions.

Tijet et al. [34] described these functions with a transcriptomic analysis of liver tissue from AhR knockout mice. They investigated not only the gene expression changes after TCDD application on AhR wild type and AhR knockout mice, but also transcriptional differences between knockout mice and wild type conspecifics without ligand treatment. They reported that the AhR regulates genes related to reproduction, growth, development, protein synthesis, cell cycle, cell growth, differentiation, apoptosis, tissue maintenance, and xenobiotic metabolism. A gene expression profiling by Yoon et al. [35] in which TCDD effects on AhR knockout mice and AhR wild type animals were studied, expand AhR target genes to chemotaxis, inflammation, carcinogenesis, immune response, cell metabolism, cell proliferation, signal transduction and tumor suppression.

These findings have been strongly indicated by *in vitro* data and have been mirrored in various AhR knockout mice models. But while in the latter animal models mostly only the phenotype was characterized, the studies of Tijet et al. [34] and Yoon et al. [35] demonstrated the magnitude of AhR impact on the level of gene expression *in vivo*.

AhR and reproduction

Since the early beginning of AhR research it has been known that AhR ligands can mediate anti-estrogenic effects without direct activation of the estrogen receptor (ER) pathway. The intertwined action between the AhR and ER is also observable in knockout models, where TCDD significantly showed anti-estrogenic effects as measured via estrogen responsiveness markers in the uterus, when administered alone or in combination with estrogen [36].

But beyond ligand-induced anti-estrogenicity, the AhR affects reproduction basically although male and female AhR knockout mice still were able to reproduce [37]. Nevertheless, the maintenance of conception, survival during pregnancy and lactation as well as reproductive success at all were decreased [37]. AhR controls reproduction amongst others by regulation of ovarian aromatase (CYP19) [38], the key enzyme in estrogen synthesis, the regulation of ovary development, especially of ovary follicle growth [39] and the regulation of ovulation capacity of follicles [40]. Moreover, there is incidence that the AhR shapes reproduction at a very fundamental stage, namely by regulation of the oocyte number that remains after birth [41]. This is probably caused by AhR-induced apoptosis of germ cells that includes the activation of Bax, a pro-apoptotic protein [42, 43]. The AhR regulates the formation of primordial follicles but also in later stages postnatally folliculogenesis and controls hereby preantral and antral follicle growth [44, 45].

Most studies that investigate the correlation between AhR and reproduction deal with the female reproduction, but there is also evidence that the AhR has an essential role in male reproduction [46, 47].

AhR in development, organogenesis and organ physiology

Expression pattern during normal embryonic development show that the expression of the AhR is specific for cell type, organs and tissues as well as developmental stage. Without much doubt this can be attributed to the ability of the AhR to induce apoptosis, a feature that is essential in the formation of organs during organogenesis. Abbott et al. [48] reported developmental-stage-dependent AhR expression in neuroepithelial cells of the developing brain, the visceral arches, limb bud, liver, heart, gut epithelial cells, lung, kidney, sites of bone formation, hippocampus, epidermis and adrenal gland of mice.

Assuming an important role in organ development, it is not astonishing that AhR knockout mice showed severe organ lesions that were not found in wild type control mice. Fernandez-Salguero et al. [49] reported serious age-related abnormities of heart, liver, uterus, skin, glandular stomach, large intestine and spleen in AhR knockout mice aged from 6 to 13 months.

The main lesion of the heart manifests in cardiac hypertrophy [49, 50] and is most likely due to elevated vasoactive peptides (endothelin I and angiotensin II) and resulting increased mean arterial blood pressure [51].

Especially liver development is impaired in AhR knockout mice. Necrotic lesions and increased extramedullary hematopoiesis were observed in the developing AhR knockout embryo [52]. Schmidt et al. [53] report also slowed early liver growth and reduced liver weight, transient microvesicular fatty metamorphosis, prolonged extramedullary hematopoiesis and portal hypercellularity with fibrosis.

AhR and the vascular system

The AhR is essential for normal vascular development. From knockout mice experiments it has been known that the AhR is involved in vasculogenesis, vascular remodelling of the placenta and the control of angiogenesis. AhR knockout mice showed a rare lesion that manifests in an unclosed hepatic vascular shunt (ductus venosus) after birth, whereas it normally closes within 48h hours [54]. In the fetus this shunt is in charge of the blood flow of the umbilical vein that is directed into the inferior vena cava. This bypass of the liver delivers oxygenated blood from the placenta to the developing fetal brain. After birth, the ductus venosus normally closes within 48h. The failure of ductus venosus closure leads to a lifelong impaired blood flow through the liver and a malfunctioning in toxin clearance from the blood. This impairment is abrogatable by AhR activation [55].

In Medaka fish it was demonstrated that the AhR is important for proper development of vasculature [56] and that retinoic acid is involved in the maintenance of functional AhR signalling in this process [57].

AhR, cell cycle regulation and cancer

Every nanosecond the cell decides about its further fate. The options range from resting in G_0 phase, normal replication during mitosis, apoptosis and necrosis. What happens with the cell depends on intercellular and extracellular signals that shift the balance at several check-points in a direction that finally leads to a decision. A misregulation can cause uncontrolled growth and cancer.

The AhR is a regulator of cell cycle progression through control of several other cell cycle regulators such as Akt, p21, p27, p53, Bax, RelB, and NFκB [58-62]. The modulation of cyclins and cyclin-dependent kinases (CDKs) [63], which function as regulatory molecules of the cell cycle, as well as the transcriptional regulation of tumor promotors and tumor suppressors, is the fundament of AhRs cell cycle arresting, apoptotic and anti-tumorigenic effects.

Various AhR ligands have been reported to exert these effects by activation of the AhR signalling cascade. 3-Methylcholanthrene (3-MC) decreased in an AhR-dependent mechanism the protein level of cdk2, while it concomitantly induced p21 and p27 expression [59]. The carcinogenic AhR ligand benzo[a]pyren (BaP) has been reported to arrest the cell cycle in various cycle phases by induction and accumulation of p53 and p21 in an AhR-dependent manner [64-66]. TCDD did not alter expression of p21, p53 and cyclins [67], but induced G_1 arrest by AhR-dependent enhanced expression of p27 and hypophosphorylation of Rb protein [67, 68]. But TCDD also induced the expression of epiregulin, a mitogen and epidermal growth factor, and increased mouse keratinocyte growth by that [69]. Polycyclic aromatic hydrocarbons (PAHs) up-regulated p53, Bax and other pro-apoptotic genes [69, 70]. Mathieu et al. [71] reported that the metabolizable AhR agonists 3-MC and BaP increased p53 levels and induced *multi drug resistance gene 1* (*mdr1*), that encodes a protein that plays a role in drug elimination but can cause also drug resistance and impede cancer therapy. Interestingly, TCDD, which is more resistant to metabolic breakdown, had no effect and Mathieu and coworkers concluded that the metabolization into reactive compounds is necessary to trigger p53 activation. Patel et al. [72] reported that several AhR ligands were able to suppress NFκB-regulated gene expression, especially of the acute-phase gene *serum amyloid A* (*Saa*). The protein product of this gene is secreted during the acute phase of inflammation and has been associated with several chronic inflammatory diseases. As chronic inflammation is a progenitor of cancer development, the down-regulation of NFkB is a strategy in cancer therapy.

The AhR interacts also specifically with the breast cancer 1 factor (BRCA1), an important tumor suppressor; this stimulates transcriptional activation of BRCA1 [58]. A mutation of the *BRCA1* gene causes increased cancer risk, especially breast and ovarian cancer [73]. This is probably due to BRCA1 regulation of mitotic progression; a dysfunction leads to accumulation of multinucleated cells and cytokinesis defects [74]. Interestingly, BRCA1 is also recruited along with AhR/ARNT to promoter regions of AhR target genes upon AhR activation [75]. In all likelihood, these pathways depend on each other for properly signalling. Given that both are involved in cell cycle regulation, an impairment of one or both signalling cascades can lead to the inversion of originally intended physiological functions.

Another interaction with an important cell cycle regulator is the interplay with the transforming growth factor β (TGFβ) family. These cytokines act as tumor suppressors, but loss of TGFβ functions mediates tumor progression. The AhR down-regulates the expression of TGFβ isoforms [63, 76].

Due to the above-mentioned regulations, inductions and repressions of cell signalling factors, the AhR pathway can induce cell cycle arrest at G_0/G_1 or G_2/M check-points [65-67, 76-82]. In which cell cycle phase an arrest is induced seems to depend on affected signalling factors or inducing agonists.

Angiogenesis is deeply involved in cancer progression, as the growing tumor has to be fed via new blood vessels with nutrients and oxygen. Hence, anti-angiogenetic drugs have been implemented for cancer therapy. The AhR is an anti-angiogenetic drug target, given that this receptor controls angiogenetic processes and regulates vascularization. In the TRAMP mice model (transgenic adenocarcinoma of the mouse prostate), AhR inhibited prostate carcinogenesis [83], probably by sequestration of ARNT which results in decreased vascular endothelial growth factor (VEGF) expression [84]. VEGF is the major signal molecule that stimulates the growth of new blood vessels. Ichihara et al. [85] found also in AhR knockout mice enhanced ischemia-induced angiogenesis, while in wild type mice an activated AhR led to decreased levels of VEGF [86]. Nevertheless, it was also reported that the AhR pathway increased VEGF in the retina [87], fibroblasts [88] and endothelial cells [89]. It is possible that the AhR-regulation of angiogenesis or vascularization is tissue-specific or varies with other cofactors.

AhR and the xenobiotic metabolism

Every organism, from arthropod, to insect and mammal, is exposed all along to a plethora of compounds and stimuli. The metabolization and probably elimination of exogenous but also endogenous compounds, is an elementary task of organisms. The AhR mediates the adaptive response to a great variety of compounds. This is probably a phylogenetic new property of the AhR, since ancestral AhR orthologs do not bind xenobiotics that are potent activators of the vertebrate AhR [90, 91]. Although this has gained great importance in mammals, the activation of the AhR by exogenous compounds has to be clearly separated from the housekeeping functions of the AhR in normal physiological processes, which are phylogenetically older features.

The gene products of AhR target genes include enzymes of phase I (cytochrome P450 enzymes) and phase II (glutathion-S-transferase subunit Ya (GST-Ya), NAD(p)H:quinone oxidoreductase (Nqo1), aldehyde dehydrogenase 3 (ALDH3) and UDP-glucuronosyltransferase) of the xenobiotic metabolism [92-97]. Further drug-metabolizing enzymes are induced via the activation of the NRF2 (NF-E2 p45related factor) pathway, that regulates enzymes via antioxidant response elements and oxidative stress as inducer. NRF2 is a downstream target of the AhR [98].

The expression of *CYP1A1*, one of the most important *cytochrome P450* target genes, is also part of negative feed back mechanisms that regulate the AhR. CYP1A1 prevents by metabolization and depletion of the activating agonist a constitutively active AhR signalling. This mechanism is only impaired in the case of agonists that are hardly metabolizable, such as TCDD [99, 100]. Here, a constitutive activation of the AhR pathway leads to dysfunction of normal AhR mediated processes, manifesting in several toxic responses for the organism.

CYP1A1 is a major enzyme of phase I of the xenobiotic metabolism, in which compounds are oxidized. Resulting metabolites have a higher reactivity and are prepared for following phase II conjugation reactions (Figure 2). This finally produces more hydrophilic metabolites that are easier excretable. But phase I reactions can also bioactivate compounds and result in carcinogenic metabolites.

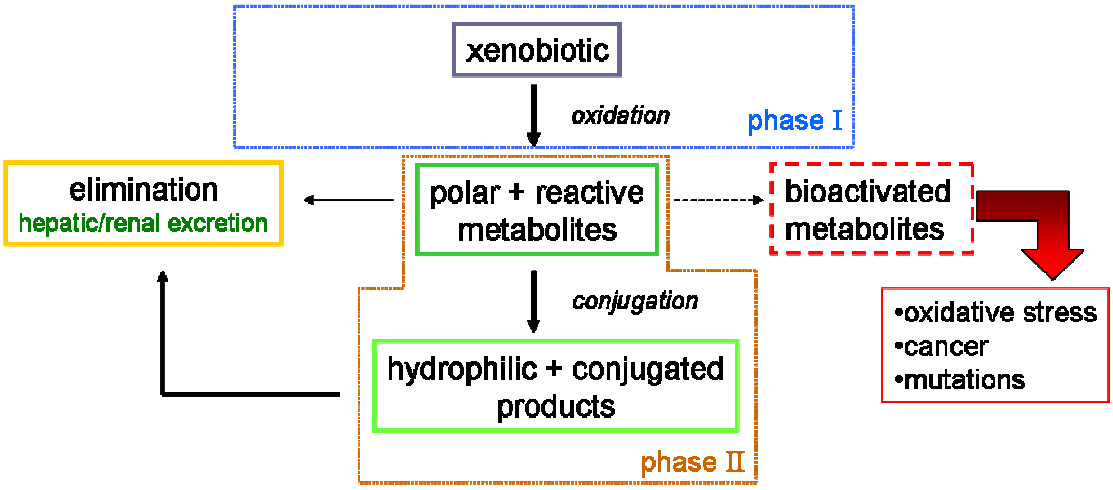


Figure 2. Simplified scheme of the xenobiotic pathway.

Nevertheless, a detoxification without this activation seems to be impeded. CYP1A1 metabolizes BaP to its carcinogenic epoxid form. But the absence of CYP1A1 leads to

a much more fatal and acute BaP toxicity, as it was shown in CYP1A1 knockout mice [101]. Hosoya et al. [102] reported also that super induction of CYP1A1 in a mice model, which had a knockout in AhR repressor gene that normally regulates the AhR pathway down, resulted in highly delayed skin tumor development. The authors concluded that overexpression of CYP1A1 shifts the balance in favor of the detoxification of carcinogens. Hence, the induction of CYP1A1 is not synonymous with toxic or pro-carcinogenic effects. Several FDA-approved and marketed therapeutics are AhR agonists and produce no toxicity in rats or humans [103].

CYP1A1 catalyzes also preferentially the 2-hydroxylation of estradiol. Estradiol itself increases 16α-hydroxylase activity and causes in long-time treatment rather a reduction in 2-hydroxylase activity. This metabolization pattern has been associated with increased breast cancer risk [104, 105]. The AhR-agonists indole-3-carbinol (I3C) and TCDD acted converse to estradiol and increased 2-hydroxylation of estradiol [106, 107]. The anti-estrogenicity of these compounds is at least partly due to this effect, mediated via the AhR pathway and CYP1A1 induction.

The constitutive and inducible expression of AhR regulated CYP enzymes, differs in various tissues. Basal CYP1A1 levels are negligible, but increase rapidly upon AhR activation in various tissues such as liver, lung, brain, digestive tract, kidney and ovaries [108, 109]. In comparison to that CYP1A2 is constitutively expressed especially in the liver, but is only inducible in a few other tissues [108, 109]. CYP1B1 is constitutively expressed at significant levels in heart, kidney, intestine, testis, thymus, uterus, ovary, and brain, but was low in liver and lung [110]. Interestingly, CYP1B1 induction by AhR agonists is much higher in organs that have a low basal CYP1B1 expression than in organs that constitutively express this enzyme [110].

CYP1A1, CYP1B1 and CYP2S1, another AhR transcription target [111], are also induced in human skin upon UV exposure [112-114]. This is probably linked to UV-induced generation of tryptophan derivates such as 6-formylindolo[3,2-*b*]carbazole (FICZ), which strongly induces the AhR pathway.

Induction of AhR-regulated CYP enzymes is also an important factor in the metabolization of drugs and therapeutics. This can be used for activation of drugs such as in the case of Phortress© [115, 116]. But it can also be an undesired effect and lead to drug-drug-interactions or altered drug clearance, which can have significant pharmacological consequences and affect therapy to a great extent. So, the

clearance of theophylline, an important asthma therapeutic agent is increased in smokers compared to non-smokers due to CYP1A induction [117]. Given that about 20-30% of asthmatics are smokers, this is of great importance for appropriate therapy [118-120].

AhR interaction with nuclear receptors

The AhR signalling cascade resembles that of other nuclear receptors in several aspects. AhR ligands are also mostly of hydrophobic and low-molecular nature, similar to ligands of other nuclear receptors. Various crosstalk mechanisms between nuclear receptors and the AhR have been observed. Inhibitory crosstalk with the androgen receptor (AR) was reported, in which AR protein stability may play a role [121]. Ohtake et al. showed that the AhR modulates the transcriptional activity of ERα and AR by direct association, but also functioning as E3 ubiquitin ligase and promoting proteasomal degradation of ERα and AR [122, 123]. AhR ligands exerted also anti-androgenic effects due to inhibition of the binding of AR to its response elements upstream of target genes such as the prostate-specific antigen (PSA) [124]. The AhR is also a negative regulator of adipose differentiation [125-128], a process that is normally regulated by the peroxisome proliferator activated receptor (PPAR) family. Transcriptional regulation of AhR by PPARα was also observed [129, 130]. Then again, PPARγ is inhibited by an activated AhR [131, 132]. An implication in the involvement in adipogenesis pathways is given, since it was shown that the AhR transcriptionally regulates *serpina 12*. The gene product serpina 12, also known as vaspin (visceral adipose tissue-derived serpin) is an insulin-sensitizing adipocytokine [133]. Vaspin may be a compensatory mechanism in response to impaired glucose metabolism, as in diabetes type 2 patients expression of vaspin was found more frequently, especially in obese subjects [134].

The most prominent interaction has been shown between the AhR and the ER pathway. That the AhR and the ER pathways are intertwined is implicated by the importance of the AhR in reproduction, as it has been discussed in a previous chapter. The AhR/ER crosstalk results in anti-estrogenicity and is mediated by AhR ligands that do not agonize or antagonize the ER directly. This applies for toxic AhR ligands such as TCDD [135], but also for naturally occurring compounds that are considered as safe and non-toxic such as indole derivates. This feature has already been discussed for the treatment of estrogen-dependent breast cancer. Nevertheless, the AhR has also

been reported to exhibit anti-proliferative effects on ER negative (ER-) breast cancer cells [136], probably by induction of transforming growth factor α (TGFα) [137] and was propagated as potential drug target for treatment of ER- breast cancer. Recently, Dusell et al. [138] have shown that the active metabolite 4-OH-tamoxifen of the selective ER modulator tamoxifen, which is approved by the FDA as breast cancer therapeutic since 1977, induces the AhR pathway in absence of ER and results in the transcription of genes involved in estradiol metabolism, cellular proliferation and metastasis.

AhR ligands alter estrogen metabolization and shift the metabolite ratio towards a pattern that has been linked to a decreased cancer risk and is opposed to the effect of estrogen [104]. This has been observed for indole compounds such as I3C [107], but also for TCDD [106]. Other interactions between the AhR and ER pathways are mediated by a decreased binding ability of ER to estrogen response elements (EREs) on the DNA in presence of AhR ligands [139]. In *in vitro* studies, an abolishment of estrogen-induced gene expression has been observed due to AhR ligands [140, 141].

The AhR influences the estrogen pathway by direct interaction with ERs [142, 143] and the EREs [144]. Sharing of a cofactor pool [145] and induction of proteasome degradation of the ER [146, 147] have also been postulated as possible crosstalk mechanisms. The ER functions also as coregulator of AhR-regulated transcription; this is induced by ER recruitment by the AhR/ARNT heterodimer [148, 149]. The human *CYP1B1* gene, which is a primary target gene of the AhR, seems also to be regulated by ERα [150]. The CYP1B1 enzyme again catalyzes estradiol hydroxylation [151].

There is evidence that TCDD inhibits ERβ stronger than ERα [152]. Results of Kietz et al. [153] suggest that this could be due to inhibition of ERα mediated induction of ERβ expression by TCDD. Nevertheless, TCDD differs in several aspects from other compounds; beginning from an extreme inertness regarding metabolization and includes also the mode of action in comparison to other AhR ligands that are metabolizable [154-159].

Ligands of the AhR

The AhR is a very heterogeneous receptor that binds a great variety of ligands that comprise naturally occurring compounds such as indoles and flavonoids as well as anthropogenic xenobiotics. As mentioned previously, the AhR is a receptor that has

been highly conserved during evolution. Bisson et al. [160] showed sequence identities greater than 85% between the AhR ligand binding domain (LBD) of the human AhR and the homologues of rats, mice, guinea pigs and rabbits. Albeit, similarity is great, the remaining differences bring also different binding affinities for ligands about. So, while TCDD docks similarly in the binding pockets of the mouse, human and zebrafish AhR-LBDs (Figure 3), the binding affinities differ and reveal a decreased binding affinity of TCDD to the human AhR-LBD. Similar results were obtained for other potent AhR ligands such as FICZ and 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE).

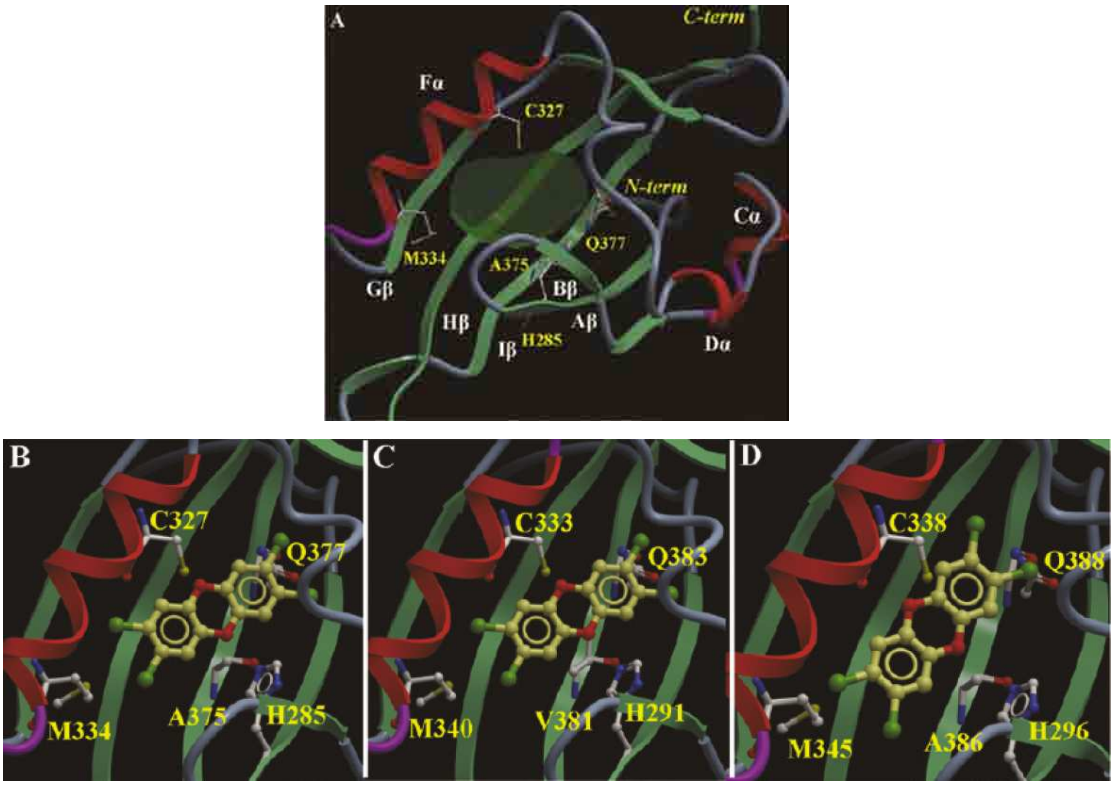


Figure 3. Model of the AhR-LBD calculated with the program ICM Pocket Finder (ICM v3.5-1n, Molsoft) as published by Bisson et al. [160]. Protein backbone displayed as ribbon and colored by secondary structure and the residues are displayed as sticks and colored by atom type, with the carbon atoms in white. The binding pocket volume is green colored. (**A**) Homology model of the mouse AhR-LBD. TCDD docking orientation into (**B**) mouse (**C**) human (**D**) zebrafish isoform 2 AhR-LBD binding pocket. The figure is kindly provided by S. K. Kolluri.

Endogenous ligands

The AhR is an orphan receptor, which means that its endogenous ligand or ligands, has/have not been identified so far. Representing the Holy Grail of AhR research, manifold attempts have been made to find the endogenous activator(s) of this

receptor. Several groups have presented compounds that could be endogenous ligands. Consistent with the widespread roles in physiology, the receptor could have more than one endogenous ligand.

An endogenous ligand has to be synthesized in the organism. Additionally it probably has a very high binding affinity to the receptor. Several compounds that have been presented as endogenous AhR ligands meet those demands, but the ultimate and sound evidence is still lacking, especially since most of these compounds are either ubiquitous present in the organism or are found specifically only in a certain tissue. Several structures of putative endogenous ligands are shown in Figure 4.

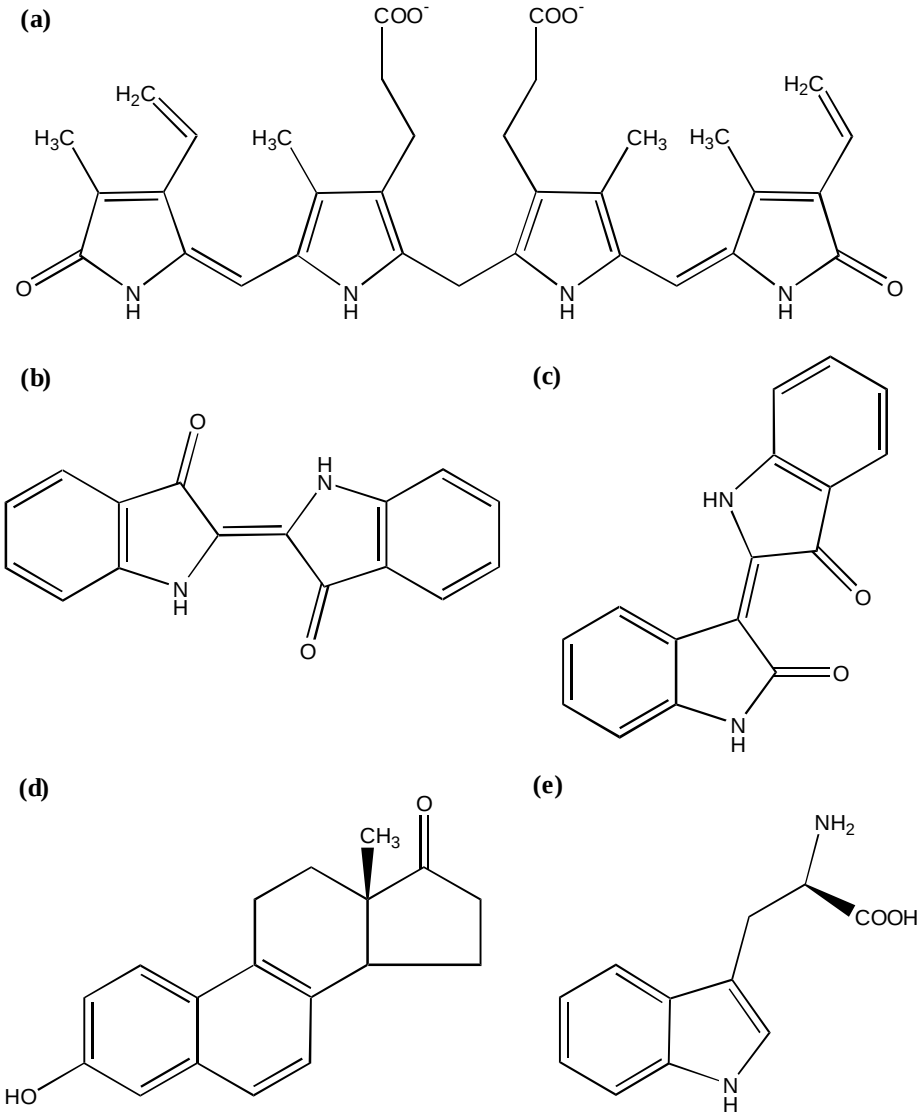


Figure 4. Putative endogenous AhR ligands: **(a)** bilirubin, **(b)** indigo, **(c)** indirubin, **(d)** equilenin and **(e)** tryptophan.

Both, the constantly ubiquitous presence, as well as the restricted distribution to compartments, is not compatible with the hypothesis of one endogenous ligand; especially as the AhR is responsible for various pathways in several tissues and that the regulation mechanism of the AhR indicates that a constant activation of this receptor is not intended.

An example for a ubiquitous present AhR ligand is tryptophan. Although tryptophan itself is a very weak ligand of the AhR, with an EC_{50} of ~300 µM [161], it is a precursor for several more potent AhR ligands. Tryptophan plasma levels are relatively high and range about 60-80 µM in healthy human adults [162]. Tryptophan is metabolized to other AhR ligands via the gastrointestinal tract [163] as well as UV irradiation [164, 165]. FICZ, a photoproduct of tryptophan, is a very potent AhR agonist and seems to be part of the circadian rhythm regulation [166]. Other tryptophan metabolites such as tryptamine and indole acetic acid are just as weak ligands as tryptophan itself [167].

Further candidates as AhR endogenous ligands are the low-density lipoprotein (LDL) [168], the heme degradation products bilirubin and biliverdin [169], the indigoids indirubin and indigo [170-172], ITE [173, 174], the equine estrogen equilenin [175], the oxysterol 7-ketocholesterol [176], cAMP [177], arachidonic acid metabolites [178, 179], eicosanoids [180] and vitamin A [181].

Some of those candidates are potent AhR activators and have an EC_{50} in the nanomolar range such as indirubin [172], indigo [172], ITE [173], while others are weak ligands and levels in the millimolar range are needed to activate the AhR. The latter one applies to equilenin [175], prostaglandin [178] and bilirubin [169]. Very potent are FICZ, ITE and ICZ with EC_{50} values in the nanomolar to nearly picomolar range [173, 182, 183].

Xenobiotic ligands

Several environmental pollutants and chemicals activate as well the AhR. Given the interaction with steroid hormone receptor pathways and the AhR, this can lead to disruption of endocrine signalling. Compounds that exhibit anti-estrogenic or anti-androgenic activities via the AhR pathway, as well as compounds that disturb directly the estrogen, androgen or thyroid hormone pathways are so-called “endocrine disrupters”.

Halogenated aromatic hydrocarbons (HAHs) including polychlorinated biphenyls (PCBs) and polychlorinated dibenzodioxins (PCDDs), but also non-halogenated polycyclic aromatic hydrocarbons (PAHs) affect the AhR signalling cascade [184-187]. The structures are shown in Figure 5.Those chemicals are ubiquitously found in the environment all around the world [188-194] due to their widespread use in industry and combustion reactions. Other examples are benzophenones, used in cosmetics and plastics as UV-blocker [195] and polybrominated diphenyl ethers (PBDEs) that are used as flame retardants [196, 197].

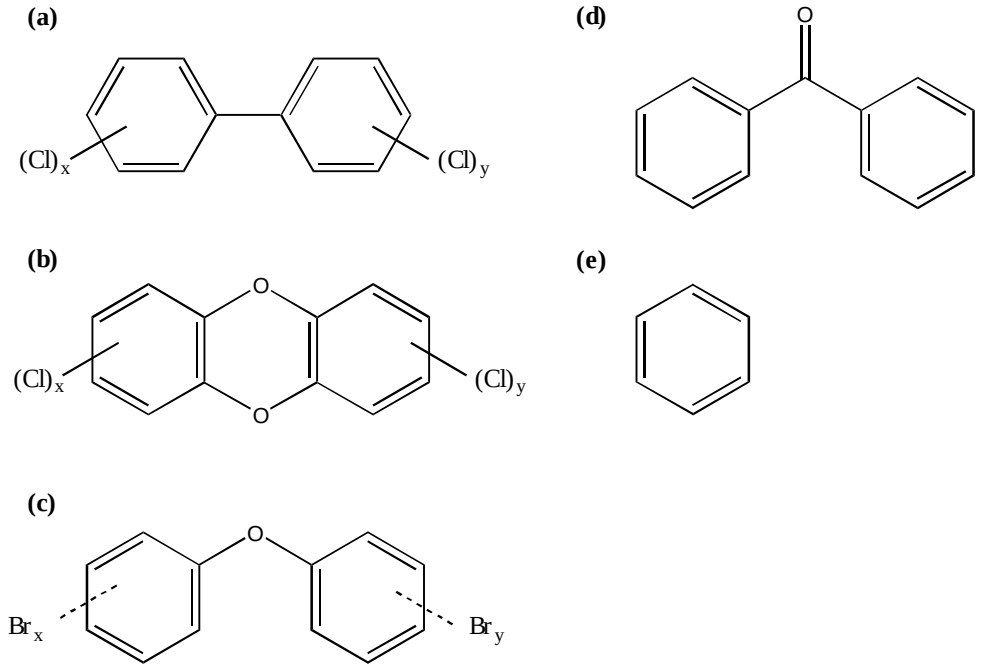


Figure 5. General structures of (a) PCBs, (b) PCDDs and (c) PBDEs and (d) benzophenone and (e) benzene as basic structures of benzophenones and PAHs.

TCDD

The most potent PCDD regarding AhR affinity is the prototypical agonist TCDD. It has a half-life of several years [99, 100] in humans, due to its poor metabolization. As it is a very hydrophobic compound it tends to accumulate in the body fat. It has been shown that nearly all toxic effects of TCDD are mediated through the AhR pathway. On closer examination is becomes evident that this is probably caused by a constant activation of the AhR cascade and hence a deregulation of this pathway. This applies not for other AhR ligands, which are metabolized after they activate the AhR pathway. Given the uniqueness of TCDD in metabolization, it is questionable if it the

common use of TCDD as reference compound in AhR research is recommendable. It is not clear if the TCDD effects that are mediated via the AhR pathway are comparable to effects of normally metabolizable AhR ligands.

Diet as AhR activator

Many naturally occurring AhR ligands are constituents of plants and food. This is especially of interest as the AhR is highly expressed in the gut where these compounds could be easily ingested. As the AhR expression is extremely high in the liver, potential ligands could activate the AhR cascade during metabolization. Furthermore it has to be kept in mind that with an indole-rich and/or flavonoid-rich diet, probably a constant level of AhR ligands could be achieved.

The *Brassicacea* plant family

The *Brassicaceae* plant family, also known as crucifers, contains well-known vegetable species with highly bioactive compounds. Amongst them are cabbage and cauliflower (*Brassica oleracea*), rapeseed (*Brassica napus*), turnip and Chinese cabbage (*Brassica rapa*), horseradish (*Armoracia rusticana*), and garden cress (*Lepidium sativum*). The agriculturally most important genus is Brassica.

Numerous epidemiological studies have been published and most of them associate a higher intake of cruciferous vegetables with an decrease in cancer risk (Table 1). But there is an inconsistency between prospective cohort studies, which show often at the best only weak associations, and retrospective case-control studies. This could be due to genetic variations that affect the metabolization of bioactive compounds as well as study design and interpretation of data [198]. Nevertheless, the chemopreventive effect has been attributed to the bioactive ingredients of cruciferous vegetables, which include glucosinolates and breakdown products thereof such as indoles and isothiocyanates.

Total glucosinolate content in cabbage ranges between 10.9 and 27g per dry weight [199]. During sauerkraut fermentation glucosinolates are hydrolyzed [200]. Indole glucosinolates, the precursers of indole compounds such as I3C, add up to 20-40% [200-202] of total glucosinolates in cabbage. In white cabbage the content of indole glucosinolates decreases also drastically during cooking [202].

Glucobrassicin is one of the main indole glucosinolates and has been reported to be the second most common glucosinolate in cabbage with ~20-40% of the main

glucosinolates [199, 202]; main glucobrassicin levels of ~2.7-404 μg/g dry weight [199, 203, 204] or ~143-344.9 μg/g fresh weight [201, 202, 205] have been reported.
The content of glucobrassicin is not constant and varies with harvest time and further food processing. In spring cabbage and fall cabbage the levels vary up to 10% [199]. Cooking alters glucobrassicin levels dramatically; after 2 minutes boiling levels drop about 50% and fall under 5% after long-term cooking of 150 minutes [202]. In fermented cabbage, about 5% of glucobrassicin are degraded to I3C and this level remains stable up to 17 weeks after fermentation [205].
In cell culture the extract of cauliflower leaves was anti-proliferative in ER positive (ER+) and ER- cells, although through different mechanisms; while in ER+ cells proliferation was inhibited by reduction of CDK6 expression, induction of p27 expression and decreased Rb phosphorylation, in ER- cells only the latter one was observed [206]. Ju et al. [207] showed in endometrial adenocarcinoma cells that cabbage extract acted as ERα selective agonist, while extract of fermented cabbage activated ERα and ERβ. The extracts were anti-proliferative in ER+ breast cancer cells at low concentrations, but increased proliferation when applicated in high concentrations [207]. In animal experiments white cabbage showed anti-tumorigenic effects [208, 209] and other Brassica vegetables such as red cabbage and Brussels sprouts were chemopreventive [210, 211]. Steinkellner et al. [212] showed a significant increase in human urine mutagenicity after fried meat consumption, which was significantly decreased when red cabbage was given prior to meat meals.

Table 1. Epidemiological studies evaluating the association of cancer risk and intake of vegetables (all) or especially cruciferous vegetables.

Ref	Consumption	Cancer site (risk;effect)	Population/Region	Study design
[213]	Vegetables Crucifers	Kidney cancer (OR=0.64 ⇓) Kidney cancer (OR=0.68 ⇓)	Eastern and Central Europe	Case-control
[214]	Crucifers	Renal cell carcinoma (OR=0.6 ⇓)	Canada	Case-control
[215]	Crucifers	Renal cell carcinoma (OR=0.53 ⇓)	L.A. (USA)/non-Asians	Case-control^{P}
[216]	Vegetables White cabbage	Renal cell carcinoma (RR=0.60 ⇓) Renal cell carcinoma (RR=0.65 ⇓)^{T}	Women/Sweden	Prospective cohort^{P}
[217]	Vegetables Crucifers	Rectal cancer (-) Rectal cancer (⇓ women only)	Canada	Case-control
[218]	Vegetables Crucifers	Colorectal cancer (-) Colon cancer (men ⇓)^{T} Colon cancer (women OR=0.51 ⇓) Rectal cancer (women ⇓)^{T}	Netherlands	Prospective cohor
[219]	Crucifers	Colorectal cancer (OR=0.51 ⇓)	Chinese/Singapore	Case-control^{H}
[220]	Crucifers	Colon cancer (RR=0.48 ⇓) Rectal cancer (RR=0.50 ⇓)	Majorca	Case-control^{P}
[221]	Vegetables Broccoli Chinese cabbage Broccoli	Colorectal cancer (OR=0.22 ⇓) Colorectal cancer (OR=0.18 ⇓) Stomach cancer (OR=0.61 ⇓) Stomach cancer (OR=0.60 ⇓)	Rural area/Japan	Case-control^{H}
[222]	Chinese cabbage	Stomach cancer (⇓)	China	Case-control
[223]	Vegetables Crucifers	Stomach cancer (RR=0.6 ⇓) Stomach cancer (weaker effects, after adjustment to smoking non-significant)	Japanese/Hawaii	Case-cohort
[224]	Vegetables	Gastric cancer (HR=0.56 ⇓)	Sweden	Prospective cohort^{P}
[225]	Vegetables	Lung cancer (⇓)^{T}	Barcelona (Spain)	Case-control

Ref	Consumption	Cancer site (risk;effect)	Population/Region	Study design
	Vegetables (yellow/orange) Crucifers	Lung cancer (OR=0.37⇓) Lung cancer (⇓)^{T}		
[226]	Vegetables Crucifers	Lung cancer (RR=0.7 ⇓) Lung cancer (RR=0.5 ⇓) Effects stronger in current than in former smokers	Netherlands	Prospective cohort
[227]	Vegetables Crucifers	non-Hodgkin lymphoma (OR=0.58 ⇓) non-Hodgkin lymphoma (OR=0.62 ⇓)	USA	Case-control
[228]	Vegetables Crucifers	non-Hodgkin lymphoma (RR=0.62 ⇓) non-Hodgkin lymphoma (RR=0.67 ⇓)	USA	Cohort
[229]	Crucifers	Multiple myeloma (OR=0.5 ⇓)	Women/Conneticut (USA)	Case-control^{P}
[230]	Crucifers	Multiple myeloma (OR=0.7 ⇓)	USA	Case-control^{P}
[231]	Crucifers	Hypopharyngeal cancer (OR=0.41 ⇓)	Smokers/India	Case-control^{H}
[232]	Crucifers	Thyroid cancer (-)	Kuwait	Case-control^{P}
[233]	Goitrogenic (primarly crucifers) vegetables	Thyroid cancer (⇓)	Hawaii (USA)	Case-control^{P}
[234]	Vegetables Crucifers	Pancreatic cancer (OR=0.45 ⇓) Pancreatic cancer (OR=0.76 ⇓)	San Francisco Bay Area (USA)	Case-control^{P}
[235]	Crucifers	Pancreatic cancer (⇓)^{T}	White men/Minneapolis-St.Paul area (USA)	Case-control
[236]	Fruits and vegetables (with high crucifer intake)	Pancreatic cancer (OR=0.51 ⇓)	Canada	Case-control^{P}
[237]	Vegetables Crucifers Cabbage	Pancreatic cancer (-) Pancreatic cancer (HR=0.7 ⇓)^{T} Pancreatic cancer (HR=0.62 ⇓)	Sweden	Prospective^{P}
[238]	Vegetables	Pancreatic cancer (-)	USA	Prospective

Ref	Consumption	Cancer site (risk;effect)	Population/Region		Study design
	Crucifers	Pancreatic cancer (-)			multiethnic cohort
[239]	Vegetables (cooked, primarily crucifers)	Pancreatic cancer (⇓)	Netherlands		Case-control^{P}
[240]	Vegetables	Prostate cancer (-)	Denmark, Germany, Italy, Spain, Netherlands, Sweden, United Kingdom		Prospective cohort
	Crucifers	Prostate cancer (-)			
[241]	Vegetables	Prostate cancer (OR=0.65 ⇓)	Seattle area (USA)		Case-control^{P}
	Crucifers	Prostate cancer (OR=0.59 ⇓)			
[242]	Vegetables	Prostate cancer overall (-)	USA		Prospective
	Vegetables	Extraprostatic prostate cancer (RR=0.41 ⇓)			
	Crucifers	Extraprostatic prostate cancer (RR=0.55 ⇓)			
	Broccoli	Extraprostatic prostate cancer (RR=0.55 ⇓)			
	Cauliflower	Extraprostatic prostate cancer (RR=0.48 ⇓)			
[243]	Crucifers	Prostate cancer (-)	Men 40-75 years	USA	Prospective cohort
	Crucifers	Organ-confined prostate cancer (⇓)^{T}			
	Crucifers	Prostate cancer (RR=0.81 ⇓)	Men<65 years		
	Crucifers	Organ-confined prostate cancer (RR=0.72 ⇓)			
[244]	Crucifers	Prostate cancer (OR=0.69 ⇓)	Canada		Case-control
[245]	Vegetables	Prostate cancer (-)	USA		Prospective multiethnic cohort^{P}
	Crucifers	Prostate cancer (-)			
[246]	Vegetables	Ovarian cancer (OR=0.77 ⇓)	Canada		Case-control^{P}
	Crucifers	Ovarian cancer (OR=0.76 ⇓)			
[247]	Vegetables	Survival; ovarian cancer (HR=0.75)	Australia		Case-control^{P}
	Crucifers	Survival; ovarian cancer (HR=0.75)			
[248]	Crucifers	Endometrial cancer (⇓)	Postmenopausal women/Sweden		Case-control
[249]	Vegetables	Breast cancer (OR=0.28 ⇓)	Guangdong (China)		Case-control^{H}
	Crucifers	Breast cancer (OR=0.58 ⇓)			

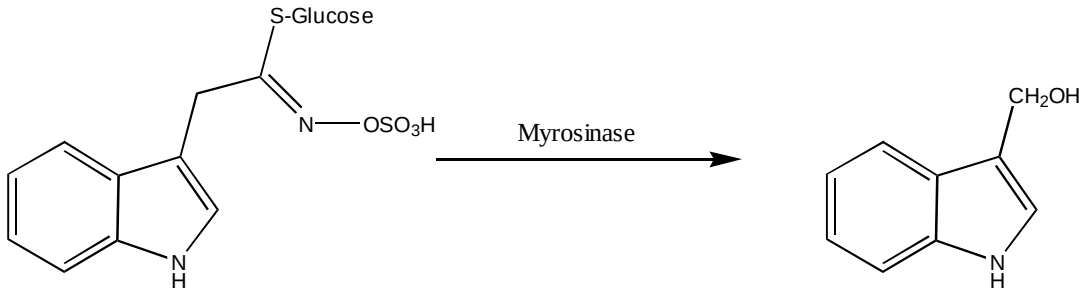
Ref	Consumption	Cancer site (risk;effect)	Population/Region	Study design
[250]	Vegetables	Breast cancer (OR=0.46 ⇓)	Premenopausal women/USA	Case-control
[251]	Fruits and vegetables Fruits and vegetables Fruits and vegetables	All breast cancer (-) ER-positive breast cancer (-) ER-negative breast cancer (IRR=0.90 ⇓)	Postmenopausal women/Denmark	Prospective cohort
[252]	Vegetables (raw) Vegetables	Breast cancer (OR=0.51 ⇓) Breast cancer (OR=0.62 ⇓)	Germany	Case-control
[253]	Crucifers	Esophageal squamous cell carcinoma (HR=0.44 ⇓)	Middle-aged men/Japan	Cohort^{P}
[254]	Crucifers (raw) Crucifers (total)	Bladder cancer (OR=0.64 ⇓) Bladder cancer (⇓)^{T} ?????	New York (USA)	Case-control^{H}
[255]	Vegetables Crucifers	Bladder cancer (⇓)^{T} Bladder cancer (RR=0.49 ⇓)	Men/USA	Prospective cohort
[256]	Crucifers	Bladder cancer (-)	Sweden	Prospective cohort^{P}

Risk: odds ratio (OR), relative risk (RR), hazard ratio (HR), incidence rate ratio (IRR), Trend but not significant (^{T}). Effect: decreased risk (⇓), no effect (-). Study design: population-based (^{P}), hospital-based (^{H}).

Indoles as bioactive compounds

Several indole compounds have been reported to be agonists of the AhR. Best investigated are I3C and 3,3'-diindolylmethane (DIM). Both, I3C and DIM are weak agonists of the AhR [161, 182, 257], but have also been described as partial antagonists of the AhR[258].

I3C is generated by enzymatic degradation (*myrosinase*) of glucobrassicin, a main glucosinolate of cruciferous vegetables (Figure 6).



Glucobrassicin Indole-3-carbinol

Figure 6. Simplified display of the enzymatic degradation of glucobrassicin to indole-3-carbinol.

DIM is the main product of I3C and is converted by acidic conditions such as found in the stomach [259]. Reed et al. [260] found DIM as primary metabolite of I3C during a phase I trial conducted with women with an elevated breast cancer risk. In cultured human breast tumor cells treated with I3C, DIM was detected in cells and seems to be accumulated in the nucleus [261], suggesting an active sequestration into the nucleus and providing possible interactions with nuclear pathways. I3C and DIM have been reported to be anti-estrogenic compounds [262, 263]. Both compounds down-regulate ERα expression, being DIM the more efficient compound and data indicated that this is due to AhR-inhibited ERα transcription [264]. Sundar et al. [265] confirmed the ERα down-regulation induced by I3C, and showed furthermore that this takes place without alteration of ERβ levels. On the contrary, I3C stimulated ERβ activation. Since a higher ERβ to ERα ratio is associated with reduced breast cancer risk, this explains partially anti-proliferative effects of I3C in breast cancer. Beyond that, I3C alters the metabolization pattern of estradiol in favor of a reduced breast cancer risk. While estradiol significantly increased 16 α-hydroxylation of estradiol, but had no effects on 2-hydroxylation in breast cancer cells [104], I3C exerted the contrary effect [107]. This was mirrored in an animal study, where mammary tumor incidence and multiplicity was lower due to I3C treatment, accompanied by cytochrome P450 induction, a well known indicator of AhR pathway activation [266]. At last, these observations were corroborated by clinical studies,

which showed also elevated 2-hydroxylation in men and women [267, 268]. TCDD, the prototypical AhR agonist has the same effect on human breast cancer cells [106], which clearly indicates that the altered estradiol metabolization depends on AhR activation.

Both, I3C [269] and DIM [269, 270] showed also anti-androgenic activities.

Another indole compound, which is a metabolite of I3C, is indolo[3,2-*b*]carbazole (ICZ). While DIM arises due to acidic conditions in the stomach, ICZ is also found in colon and cecum [271]. Kwon et al. [271] concluded also that gut bacteria are involved in the formation of ICZ, since in germ-free rats feeded with cabbage diet significantly lower ICZ levels were found compared to conventional littermates. In comparison to DIM, ICZ arises in much smaller amounts from I3C [259]. Nevertheless, ICZ is a much more potent AhR ligand than I3C or DIM, with reported binding affinity of 3.6 nM (IC_{50}) in a competitive ligand binding assay [272] and EC_{50} in a yeast transactivation assay of 1.9 nM [182]. Bjeldanes et al. [273] reported a lower EC_{50} of about 260 nM for the induction of CYP1A1 as measured by ethoxyresorufin *O*-deethylase (EROD) activity in murine hepatoma Hepa1c1c7 cells. The difference in the magnitude of the EC_{50}s is explainable by the hepatic cell line, which is much more metabolic active, as well as by the long incubation time of 2 days. Chen et al. [274] showed that the effect of ICZ on CYP1A1 expression in Hepa1c1c7 cells differed greatly depending on incubation time; EC_{50} values differed about two orders of magnitude between 4h and 24h incubation and were in the nM-range after 4h treatment, while after 24h an EC_{50} similar to the results of Bjeldanes et al. [273] was observed. Chen and coworkers [274] report further that the effect of ICZ after 24h and 48h did not change significantly. ICZ represents a potent AhR agonist that occurs naturally in frequently consumed food. Pohjanvirta et al. compared toxic responses of TCDD and ICZ in rats and found no toxic effects of ICZ, neither after single treatment nor after repeated ICZ application, whereas TCDD elicited the expected toxicity symptoms [275].

Structurally akin to ICZ is FICZ. This indole derivate arises after UV irradiation of tryptophan and is a highly potent AhR agonist [276]. It has a potency of about 3.5 nM in the yeast transactivation assay [182] and an IC_{50} of 70 pM in a competitive ligand binding assay [165]. In human keratocytes (HaCaT cell line) FICZ and ICZ induced CYP1A1 expression already at concentrations of 100 pM [277]. Wei et al. showed that a UV-induced CYP1A1 expression in mouse hepatoma cells in presence of tryptophan is AhR dependent [278] and that FICZ is rapidly metabolized by the induced CYP1A1 and involved in autoregulation of CYP1A1 transcription [279]. FICZ degradation due to AhR activation involves several phase I and phase II enzymes of the metabolism, being CYP1A1 the most important enzyme for the

initiation of metabolization [280]. For AhR activation studies, the formation of FICZ due to light exposure of tryptophan has to be taken into account. Photoproducts of tryptophan, including FICZ that reached a concentration of 8 pM, have been identified in cell culture medium after normal laboratory light exposure [183]. Nevertheless, FICZ is not the only light-induced derivate of tryptophan that elicits CYP1A1 induction [281]. As FICZ metabolites have been found in human urine samples [282] and given the high potency of AhR activation of this compound, FICZ is one of the most likely candidates as endogenous AhR ligand. However, it is very likely that more than one endogenous ligand exist, given the multitude of AhR regulated effects, which are certainly not all mediated by UV and tryptophan derivates that arise thereof.

The majority of drugs have a natural origin. Plants and microorganisms produce complex secondary metabolites as defense mechanism against vermin and predators but also for signalling within the organism or with the environment. Historically, drug discovery has been developed from folk medicine and observation of effects after the administration of plant remedies.

Drug discovery from plants

Since the development of a new drug costs at least $800 millions [283], pharma industry is aimed to position drug blockbusters on the market. For some time this led to intensified research with combinatorial chemistry as the throughput is higher and it was assumed that this would lead to more inventions. However, the expected increase in the discovery of new chemical entities (NCEs) failed to appear. As an analysis of NCEs from 1981 to 2002 by Newman et al. [284] shows (Figure 7), a great deal of NCEs is based on natural products. This is especially true for anticancer drugs where 74% have to be classified as non-synthetics and the majority of drugs were natural products, were based thereon or mimicked those [284]. It seems that the biodiversity of natural products is not easily reproducible.

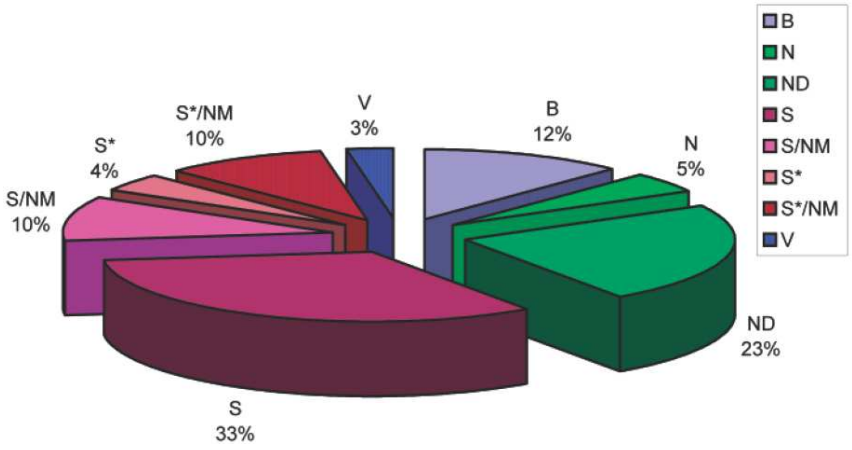


Figure 7. New chemical entities from 1981-2002, by source (N = 1031) published by Newman et al. 2003 [284]. B: biological, usually peptides or proteins. N: natural product, ND: derived from a natural product, usually a semisynthetic modification, S: totally synthetic drug, usually by random screening/modification of an existing agent, S*: totally synthetic product, but with the pharmacore of a natural product, V: vaccine, NM: natural product mimic. The figure was kindly provided by D. J. Newman.

What is the strategy for the development of a drug derived from a plant? The prerequisite is of course the specification which application is indented or which disease should be medicated. Then, preliminary to laboratory research, criteria for the selection of plants that will be analyzed have to be established. This can be the plant distribution, e.g. herbs that are domestic in a specific area, or it can be a taxonomic approach, where only plants of a certain plant family are included. Another possible approach is the ethnomedical use of plants. For example, if a drug for the amelioration of menopausal complaints should be developed, it is advisable to include plants that have been used for this application traditionally. The determination of selection criteria is of great importance as it limits the sample pool. Accompanying, a literature research regarding the selected plants should be performed to gather as much information as possible about distribution, known ingredients and biological activity. The available information can be very limited, but the scantiest information could be useful and prevent eventually false results.

After the selection and collection of plant material, the preparation and the extraction of this material have to be optimized in dependence on the properties of possible active compounds and assay requirements. Especially, the selection of the extraction solvent and the extraction method is a critical factor. Depending on the conditions the extraction can be highly selective for a certain group of compounds or very general regarding the extracted compounds. The polarity index is an important factor, when deciding which solvent to choose. The more hydrophobic the desired compound, the more unpolar should the solvent be. Another factor

could be the miscibility with aqueous systems. This could be essential for the functionality of the following bioactivity assays which are mostly performed with aqueous systems.

The bioassays should allow an easy screening of numerous extracts and detect the desired bioactivity. Pharmaceutical companies screen for NCEs with high-throughput assays that are half- or fully automated, which can include automation of sample preparation, assay preparation and data collection. The bioassays are oriented on the therapeutic areas that belong to the portfolio of the company. When the screening battery is upgraded, already existing NCEs and extracts are tested with the new assays to characterize the biological profile.

If an extract is found with a high potency in the assays, the next step would be the isolation of the active compound. For this purpose the extract is fractionized with chromatographic methods. The fractions are again tested on bioactivity and potent fractions can be separated further or they are used to identify an unknown active compound with nuclear magnetic resonance (NMR) spectroscopy. Even though the sensitivity of NMR technique is high, it is still very likely that several fractionations and the pooling of fractions will be necessary to obtain the required amount for the structure elucidation. If the compound is a metabolite or is assumed to belong to a certain compound class, it is eventually possible to identify the compound with HPLC-MS in combination with databases and mass spectra libraries.

If a hit was found and could be isolated as pure compound that also has a high potency in the bioassays, a high amount of the compound will be needed for the following toxicity tests, animal assays and clinical studies. The isolation from plant material is laborious, time-consuming and leads to a low yield. When the structure is elucidated it is better to synthesize the compound with chemical methods. The variation of chemical side groups can also help to identify the pharmacophore, which is the part of the compound that is the carrier of the desired bioactivity mostly by representing the receptor binding site. Other side groups of the molecule can modulate the binding properties of the molecule by influencing the steric alignment of the molecule. It is advantageous to synthesize derivates of the hit that vary only in side groups. These analogs could possibly have a higher bioactivity, a better solubility or can be better tolerated by the patient than their precursor. Structure-activity studies with bioassays have to be carried out.

The well known drug Aspirin (acetylsalicylic acid) is an example where the derivate of the active compound succeeded better than the precursor [285, 286]. Plants that contain salicylic acid such as willow were traditionally used to treat pain. Isolated salicylic acid from this plant had the same effects as the extract but was not well tolerated especially of the stomach. It was

not until the discovery of the acetyl derivate by the company Bayer that this problem was solved.

The isolation and identification of a novel active compound from a plant is just the beginning of a long procedure that maybe leads to the introduction of a new drug on the market. The following animal tests that should evaluate toxicity and bioactivity *in vivo* are to be followed by clinical studies in humans that will investigate further the biological effects and safety but also pharmacokinetics and bioavailability, as requested before approval by statutory agencies such as the Food and Drug Administration (FDA) or the European Commission. After each test the program can reach its end, be it that the compound turns out to be toxic, not effective or elicit unwanted side effects. The *in vivo* situation can differ to a great extent from the results *in vitro*. This can be caused by accumulation in the organism or too quick elimination processes, metabolization to more or less active compounds and metabolization to toxic compounds. From a medically active plant to an approved drug it takes years or even up to decades. For Taxol, one of the best known anticancer drugs, which has been derived from the bark of the Pacific yew tree, it took three decades up from discovery to utilizability for the treatment of breast, lung, and ovarian cancer [287].

Long before the market launch, the investor will protect the invention of the extract or the active compound by filing a patent. This is possible with a natural product when it is tied to a non-evident bioactivity. The patent is stronger when a novel compound is found and even stronger when a biological mechanism is elucidated and can be corroborated with structure-activity studies.

Drug discovery from plants is subjected to many difficulties, beginning from the availability per se of the plant up to the available amount of the plant. If the active compound has been isolated from a plant, normally a recollection and replication of the tests should be performed. But plant material is subject to great fluctuations regarding compound composition. The newly found active compound could be produced only during stress such as extreme drought or infection with microorganisms or viruses. The results may not be reproducible with newly collected plant material of the same plant. This could be also true for the same plants that were grown in a different habitat. If it was possible to identify the structure of the isolated active compound, it is possible to continue with the program by chemical synthesis of the hit. But if it was not possible to identify the active compound from the biological active plant extract it complicates the drug discovery process or even leads to a dead end.

The academic approach is similar to the above described strategy. During this study, the isolation and identification of the novel compound indolylfuran from sauerkraut was

accomplished. This compound has a high potency to activate the AhR *in vitro* and as it was demonstrated with rats also *in vivo*. The implementation of this discovery is displayed schematically in Figure 8.

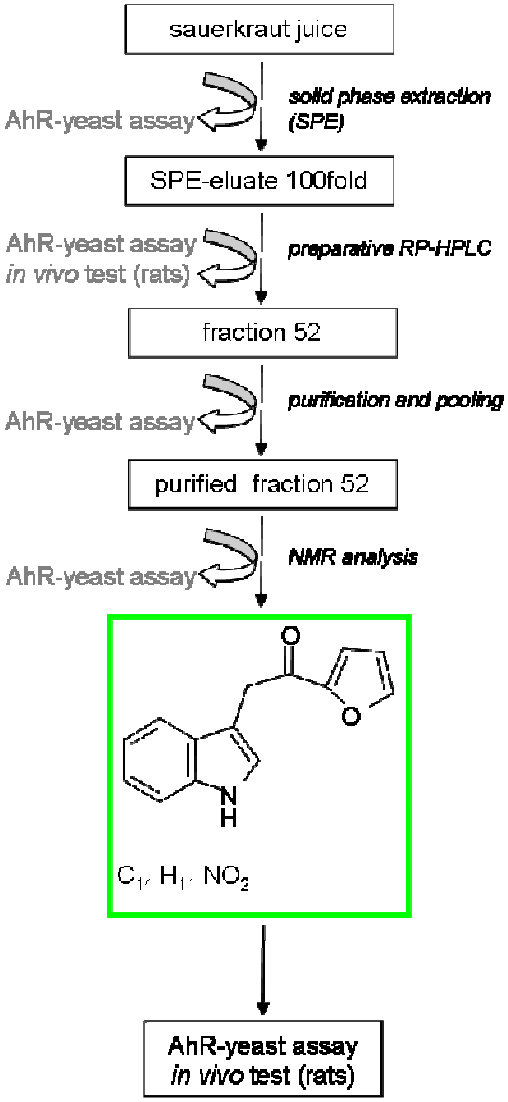


Figure 8. Scheme of the isolation and identification of indolylfuran from sauerkraut juice.

Objective

The aim of this study was to evaluate AhR activating potential of edible plants, food components and food contaminants, to get a general idea of the concerted impact of diet on AhR signalling.

As flavonoids and indoles widespread in the plant kingdom and several representatives of those compound classes are AhR ligands, manifold plants are AhR activators. These plants have been known for a long time to exert beneficial effects regarding amelioration of menopausal complaints, to be anti-estrogenic, and have been associated with decreased cancer risk. The identification of AhR active plants with *in vitro* tests provides a basis for further *in vivo* tests that will elucidate AhR action on the molecular level that is induced by normal diet. The isolation of active compounds from plants facilitates the use of AhR ligands in therapy and prevention of diseases that can be modulated by the AhR cascade and establishes the AhR as drug target.

Objectives in particular were:

- The identification of edible plants containing potent AhR activators.
- The isolation and identification of these activators.
- A further characterization of active components by e.g.: evaluation of their binding and transactivating potential on other receptors (ERα, ERβ, AR, PR, PPARγ) and/or their (anti)-estrogenicity *in vivo* (uterotrophic assay with rats).
- The evaluation of *in vitro* receptor modulating effects (ERα, AR, AhR) of pesticides.

Conclusion

The AhR mediates the adaptive response to a plethora of compounds. This receptor is expressed in most healthy tissues, but also highly in several cancers. The structures of ligands able to activate the AhR are very heterogenic. Among them are food ingredients such as retinoids, but also secondary plant compounds such as flavonoids or indoles. Contaminations with pesticides, combustion derived compounds and industry additives have a share in the activation of the AhR pathway via food. Nevertheless, the question remains: how great is the influence of normal diet on cellular processes and pathways?

A diet rich in vegetables and fruits, providing a high level of flavonoids and indoles has been associated with a decreased cancer risk in epidemiological studies (see Table 1, Introduction). In all likelihood, the prevention of several diseases is achievable by a healthy diet. The treatment of diseases via diet is not feasible, but the isolation of bioactive plant ingredients can provide a basis for drug development, as the isolated compounds can be administered more tightly focused and in higher doses.

A deliberate modulation of the AhR pathway is not an easy task, given the complexity of AhR signalling. Several AhR ligands exert toxic effects via activation of this pathway. This is predominantly explained by an insufficient metabolization of these compounds that leads to a constant activation or the metabolization to carcinogenic derivates.

The AhR plays a role in cell cycle regulation and has been associated with apoptotic processes, but has also been linked to cancer development. It has emerged that the AhR controls and regulates a plethora of other factors that modulate the cell cycle. Under normal conditions this empowers the AhR to ensure the proper progression through the cell cycle. Under pathological conditions this ability can be misguided. Peng et al. [288] suggested that the AhR functions as tumor suppressor, but is silenced during tumor progression. From the pharmacologic point of view it is of interest, if AhR properties can be utilized for the treatment of diseases. This is complicated by the fact that the interactions and modulations could be a) tissue specific and/or b) depending on the concerted action with other regulators.

Several studies report an increased expression of the AhR in some cancer tissues such as in gastric cancer [289], lung cancer [290, 291] and pancreatic cancer [292]. This phenomenon was also observed in mammary epithelial cells [293], where the results indicated that this was due to the loss of ERα functions and seems to represent the substitute for the normal function of ER signalling. Whether the AhR is overexpressed because it is part of a misled cell-surviving program in cancer cells is not quite clear. Nevertheless, it was shown that AhR agonists were able to inhibit the cancer cell growth and mediate a cell cycle arrest in these

cancer tissues [289, 292]. This strengthens the potential of the AhR to serve as drug target in cancer therapy.

Additionally, the well described AhR/ER interaction lends itself to a targeted intervention in estrogen-dependent diseases such as breast cancer, but also for the amelioration of menopausal complaints. In this study, food supplements of red clover (*Trifolium pratense*) that have been used in recent years for the amelioration of menopausal complaints, were identified as potent AhR activators. In former times this plant was used as flour extender but also as salad ingredient. It is also widely used as fodder crop. The predominant active compounds in red clover are the isoflavones formononetin and biochanin A, but daidzein and genistein are also found in traces [294, 295].
Currently, isoflavones are hotly debated. Although these compounds have been consumed for decades in Asian food, the question about their safety was raised. Evaluating *in vitro* data, isoflavones seem to be safe compounds (as reviewed in ***publication IV***).
The activation of the AhR signalling is due to the AhR transactivating abilities of formononetin and biochanin A (***publication I***). These compounds are also agonists of ERα, ERβ and PPARs [77, 294, 296] and can be regarded as highly bioactive compounds. The health-modulating effects of phytoestrogens and especially isoflavones have been discussed in ***publication V*** and ***publication VI***. Additionally to the modulation of the ER and PPAR pathways, isoflavones have been reported to be anti-proliferative, anti-inflammatory, to activate the endothelial NO synthase [297, 298], to affect cognitive functions in postmenopausal women [299], to modulate detoxification and exert hereby chemopreventive effects [300-302] and as mentioned above to ameliorate menopausal complaints [303]. Several of these properties are due to the activation of the AhR pathway. It can be assumed that isoflavones are putative candidates for the treatment of cancer and hormone-dependent diseases.

AhR agonists are found in a great diversity of plant species. Besides Leguminosae such as red clover, the AhR is also activated by Brassicacea, a commonly consumed plant family that includes cabbage, broccoli, cauliflower, horseradish and garden cress. Fermented cabbage (sauerkraut) contains high levels of indoles. Interestingly, during this work it emerged that raw sauerkraut juice is already a potent activator of the AhR cascade and was chosen as model plant for further investigations (***publication II***). Already known indole compounds such as I3C and DIM that are known to be moderate AhR agonists, are not fully responsible

for the high activation of the AhR. We isolated and identified a new indole compound, indolylfuran, which is a stronger AhR agonist from sauerkraut juice. The bioactivity of indolylfuran was completely unknown thitherto. Sauerkraut and indolylfuran were confirmed as AhR activators *in vivo* in an uterotrophic test with rats. Indolylfuran regulated *in vivo* expression of ERα and ERβ. Given that indolylfuran had no effect on uterus weight, which is a measure for ER activation in this test model, this regulation is most likely due to AhR activation. Sauerkraut did not stimulate uterus growth, but sauerkraut showed anti-estrogenic properties when administered with estradiol and reduced the expression of the proliferating cell nuclear antigen (PCNA), a proliferating marker. Indolylfuran also had no effect on uterus weight, but decreased estradiol-induced expression of the proliferation markers Ki-67 dose-dependently. The AhR/ER interaction became apparent as estradiol decreased expression of AhR and ARNT, although estradiol itself is no ligand of the AhR. Reduced expression of AhR and ARNT was also observed after indolylfuran treatment.
Data of this work strengthens not only the working hypothesis of a bidirectional AhR/ER pathway interaction (see also ***publication VII***), but also of the putative use of AhR agonists in hormone-dependent diseases. According to the results of this work, the isolated compound indolylfuran has potential as therapeutic for the treatment of estrogen-dependent cancers.

A possible modulation of AhR signalling via food contaminants was evaluated in further work (***publication III***). Non-persistent pesticides were investigated on their ability to transactivate the human AhR. As several pesticides have been described as endocrine disrupters, the transactivating potential on the human ERα and AR was also evaluated. Several pesticides were identified that activate AhR, ERα and AR. But an application within legally permitted doses should not lead to high enough concentrations to disturb the mentioned pathways. This conclusion is based only upon *in vitro* data and does not take possible metabolization into account. Hence, a modulation of these pathways cannot be excluded, especially for rural population and application workers.

The main objective of this study, to demonstrate the impact of plants and especially diet on the human AhR was fulfilled. Selected plants as well as pesticides were further characterized in detail:

- Several plants that are commonly used as food or food supplements were identified as potent activators of the AhR signaling machinery. Among them are raw sauerkraut juice and red clover.
- The isoflavones formononetin and biochanin A, main components of red clover were identified as potent AhR agonists.
- The new AhR agonist indolylfuran was isolated from sauerkraut. The structure of this compound was known, but it was unknown that it is an ingredient of sauerkraut and its bioactivity has never been investigated before.
- Sauerkraut and indolylfuran were further investigated *in vivo* in rats and *in vitro* results were confirmed. Moreover, anti-estrogenic effects as well as basic AhR/ER interactions could be demonstrated.
- Non-persistent pesticides that are currently used in fruit and wine cultivation were shown to exert transactivating potential on the human AhR and to some extent on the human AR and ERalpha.

Publication list

1st author

- **Red clover isoflavones biochanin A and formononetin are potent ligands of the human aryl hydrocarbon receptor.** (***publication I***)
  S. Medjakovic and A. Jungbauer
  Published: Journal of Steroid Biochemistry & Molecular Biology 108 (1-2), 2008, p. 171–177

- **Effect of non-persistent pesticides on estrogen receptor, androgen receptor and aryl hydrocarbon receptor.** ***(publication II)***
  S. Medjakovic, A. Zoechling, B. Schildberger, M. Gartner and A. Jungbauer
  Accepted with revisions: Environmental Health Perspectives

- **Indolylfuran a potent aryl hydrocarbon receptor agonist from sauerkraut interacts with estrogen pathway.** ***(publication III)***
  S. Medjakovic, A. Zoechling, G. Vollmer, O. Zierau, G. Kretzschmar, F. Möller, S. Kolba, A. Papke, M. Opietnik, P. Kosma, T. Rosenau and A. Jungbauer
  Submitted: The Journal of Nutritional Biochemistry

Additional publications

- **Isoflavones are safe compounds for therapeutical applications – Evaluation of in vitro data.** ***(publication IV)***
  E. Reiter, V. Beck, S. Medjakovic and A. Jungbauer
  Published Review: Gynecological Endocrinology 25(9), 2009, p. 554-580

- **Potential health-modulating effects of isoflavones and metabolites via activation of PPAR and AhR.** ***(publication V)***
  S. Medjakovic, M. Mueller and A. Jungbauer
  Accepted Review: Nutrients, Special Issue “Isoflavones and Lignans”

- **Phytoestrogens and Related Polyphenols for Prevention of Hormone-Related Diseases.** ***(publication VI)***
  S. Medjakovic, A. Zoechling and A. Jungbauer
  Published Review: Book chapter XII of “Polyphenols and Health: New and Recent Advances”, 2008, Nova Science Publishers, Inc., Editor Neville Vassallo, p. 289-366

- **Estradiol regulates aryl hydrocarbon receptor expression in the rat uterus.** ***(publication VII)***
  G. Kretzschmar, A. Papke, O. Zierau, F. J. Möller, S. Medjakovic, A. Jungbauer and G. Vollmer
  Accepted: Molecular and Cellular Endocrinology

- **Comparison of hormonal activity of isoflavone-containing supplements used to treat menopausal complaints.** ***(publication VIII)***
  E. Reiter, V. Beck, S. Medjakovic, M. Mueller and A. Jungbauer
  Published: Menopause 16 (5), 2009, p. 1049-1060

- **Effect of mycorrhization on the isoflavone content and the phytoestrogen activity of red clover.** ***(publication IX)***
  T. Khaosaad, L. Krenn, S. Medjakovic, A. Ranner, A. Lössl, M. Nell, A. Jungbauer and H.Vierheilig
  Published: Journal of Plant Physiology 165 (11), 2008, p. 1161-1167

- **Red Wine Technology: Impact of Toasting of Oak Wood on the Activation of the Arylhydrocarbon Receptor.** ***(publication X)***
  A. Zoechling, L. Falk, M. Opietnik**,** S. Medjakovic and A. Jungbauer
  For submission to: American Journal of Enology and Viticulture

Patents:

- **Medicament, cosmetic or food product comprising an indole compound, the use thereof and the method of isolation thereof from sauerkraut.** ***(patent I)***
  Publication number: WO2007110243 (A1)

- **Plant extracts, plant components and the uses thereof.** ***(patent II)***
  Publication number: WO2007110241 (A1)

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Publication I

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Red clover isoflavones biochanin A and formononetin are potent ligands of the human aryl hydrocarbon receptor

S. Medjakovic [a,b], A. Jungbauer [a,b,*]

[a] *Department of Biotechnology, University of Natural Resources and Applied Life Sciences Vienna, Muthgasse 18, 1190 Vienna,Austria*
[b] *Christian Doppler Laboratory for Receptor Biotechnology, Vienna, Austria*

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Abstract

Aryl hydrocarbon receptor (AhR) activation affects the cell cycle and drives cells to apoptosis. Thus, selective AhR modulators (SAhRMs) have previously been implicated in cancer therapy and prevention, particularly for hormone-dependent cancers. In the present study, isoflavones a remedy used to ameliorate menopausal complaints were tested for their potential in transactivating AhR in order to investigate the biological function of red clover isoflavones. The results were compared to the transactivation potentials of other flavonoids and plant-derived indole compounds. We found that the isoflavones biochanin A and formononetin were potent AhR agonists *in vitro*, with EC_{50} values of 2.5×10^{-7} and 1.3×10^{-7} mol/l, respectively. These isoflavones are 10 times more potent compared to the indole compounds indole-3-carbinol and diindolylmethane, publicised as powerful AhR agonists with EC_{50} values of 5.8×10^{-6} and 1.1×10^{-6} mol/l, respectively. Because activated AhR crosstalks with estrogen receptor α, future risk-benefit assessments of isoflavones should take into consideration their AhR transactivating potential.

Keywords: Isoflavones; Biochanin A; Formononetin; AhR; Aryl hydrocarbon receptor; Indole compounds

1. Introduction

Thirty years ago, Poland et al. [1] discovered a protein that binds with high affinity to the toxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). This protein was later designated the aryl hydrocarbon receptor (AhR). Subsequent research regarding this receptor focused on the AhR-mediated toxic response that is induced through ligand binding. TCDD and other polyhalogenated hydrocarbons (PAHs) are extremely potent activators of the AhR pathway and induce the expression of AhR-regulated genes that encode for the enzymes of phase I and II metabolic pathways. However, PAHs are not the sole exogenous ligands of the AhR. In fact, the AhR is very promiscuous and binds a wide variety of both agonists and antagonists.

Although there is substantial speculation regarding a potential endogenous ligand [2–8] for AhR, currently there is no clear evidence for any particular moiety. Nevertheless, the existence of an endogenous ligand is supported by a number of studies. Experiments with AhR knockout mice have suggested the AhR may play roles in cardiac physiology and function [9], liver development [10,11], ovary development [12], and immune system regulation [13]. Other studies have established AhR involvement in reproductive physiology [14,15], cell cycle control [16], and apoptosis [17].

Phytoestrogens are plant-derived, non-steroidal chemicals that bind the estrogen receptor. A number of phytochemicals are exogenous AhR ligands, including flavonoids [18–21], the stilben resveratrol [22,23], indole compounds [24], and furocoumarins [25]. Some AhR ligands have also antiestrogenic properties and can be characterized as "endocrine disrupters", because upon binding AhR they may crosstalk with steroid hormone receptors and disturb normal hormone pathways. Interestingly, this crosstalk was recently exploited in the creation of selective AhR modulators (SAhRMs) [26] that can inhibit the development of hormone-dependent cancers and tumours. While endocrine disrupters are mainly anthropogenic chemicals and pollutants, SAhRMs, for the

* Corresponding author. Tel.: +43 1 360066226; fax: +43 1 3697615.
E-mail address: alois.jungbauer@boku.ac.at (A. Jungbauer).

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most part, are naturally occurring plant compounds or derivates that do not exhibit toxic effects.

The AhR can interact with estrogen receptors (ER) in multiple ways. AhR ligands can also modify estradiol metabolism [27] by increasing 2-, 4-, 6α- and 15α-hydroxylation. In particular, indole compounds alter estrogen metabolism by producing a metabolite ratio of C-2 to C-16 hydroxylated estrogen that is the inverse to that observed in women with breast cancer [28,29]. In a number of *in vitro* studies, AhR ligands were associated with an abolishment of estrogen-induced gene expression [30,31]; presumably, the presence of AhR ligands hindered the ability of the ER to bind to estrogen response elements (EREs) on DNA [32]. Various other examples of AhR–ER interactions are known, and offer a wide range of potential approaches for intervening in ER-dependent diseases.

Foods rich in phytoestrogens or food supplement based on red clover, have beneficial physiological effects. The AhR transactivating potential of many of the so-called phytoestrogens has not been investigated, thus it is not clear whether AhR plays a role in their beneficial effects. Isoflavones are phytoestrogens used for the amelioration of menopausal complaints. Recent clinical studies have demonstrated the beneficial effects of 80 mg per day of red clover isoflavones [33–35]. The spectrum of isoflavones in red clover is different than those found in soy. Red clover is rich in formononetin and biochanin A, while soy has less biochanin A and no formononetin. The isoflavone coumestrol has been shown to have AhR transactivating potential, but only very low amounts are present in standardized red clover isoflavone extracts. Thus, red clover and soy can be expected to produce different biological effects.

In this study, we tested a selection of phytoestrogens and compared their AhR activation potentials with a variety of known AhR ligands. In addition, we investigated polyphenols that partly exhibit estrogenic activity. AhR activation potencies were measured with a high-throughput bioassay based on yeast as a model organism. These *in vitro* results serve as a guide for further *in vivo* investigations and for clinical studies that focus on the development of plant-based pharmaceuticals and/or herbal remedies.

2. Material and methods

2.1. Chemicals and media

Dimethylsulfoxide (DMSO) was obtained from Sigma–Aldrich (St. Louis, MO). Buffer reagents, *N*-lauroylsarcosine (sodium salt), di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$), potassium chloride (KCl), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), sodium carbonate (Na_2CO_3) and *o*-nitrophenyl-β-galactopyranoside (ONPG), were purchased from Sigma–Aldrich (St. Louis, MO), Fluka (Buch, Switzerland) and Merck (Darmstadt, Germany). *o*-Aminoazotoluene, apigenin, baicalein, 1-benzylimidazole, biochanin A, biotin, biphenyl, butyl paraben, caffeic acid, caffeine, (+)-catechin hydrate, clotrimazole, coumestrol, curcumin, daidzein, diethylstilbestrol, diindolylmethane, diosmin, dioxin, (−)-epicatechin, (±)-equol, 17-β-estradiol, *trans*-ferulic acid, fisetin hydrate, formononetin, 6-formylindolo[3,2-b]carbazole (FICZ), gallic acid, genistein, ICI 182,780, harmaline, indole-3-carbinol, indolo[3,2-b]carbazole (ICZ), ITE (2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid), indomethacine, melatonin, 3-methylcholanthren, β-naphthoflavone, naringenin, nicotine, phloretin, phtalic acid dibutyl ester, piperine, progesteron, quercetin, quinine, *trans*-resveratrol, rhein, rutaecarpine, silibinin, β-sitosterol, tangeretin, theobromin, tryptamin and tryptanthrin were obtained from Sigma–Aldrich (St. Louis, MO), Fluka (Buchs, Switzerland), Biomol International L.P. (Plymouth Meeting, PA), Indofine Chemical Company (New Jersey, USA) and Tocris Cookson (Avonmouth, United Kingdom). The solvents used were HPLC grade.

For the yeast media, yeast nitrogen base was obtained from Difco (Franklin Lakes, NJ), amino acids from Serva Feinbiochemica (Heidelberg, Germany) and dropout medium without tryptophan from Sigma–Aldrich (St. Louis, MO).

2.2. Media preparation for yeast cultivation

Media were sterilised at 121 °C and 10^5 Pa overpressure for 15 min and stored at 4 °C. The Dropout medium without tryptophan (DO-trp), was used for the cultivation of the overnight culture and the yeast stock. GOLD medium with galactose, without tryptophan (GOLD-trp), and adjusted to pH 5.0 was employed for induction. GOLD medium is made of two parts. Part 1 and 2 of the medium were aseptically mixed after autoclaving and a cool down. Part 1 consisted of 0.34 g yeast nitrogen base (without amino acids and ammonium sulphate), 0.67 g ammonium sulphate, 12 mg/l uracil, and amino acids were added as follows: 24 mg/l adenine sulfate, 12 mg/l L-arginine·HCl, 60 mg/l L-aspartic acid, 60 mg/l L-glutamic acid, 12 mg/l L-histidine·HCl, 18 mg/l L-lysine·HCl, 12 mg/l L-methionine, 30 mg/l L-phenylalanine, 225 mg/l L-serine, 120 mg/l L-threonine, 18 mg/l L-tyrosine, 90 mg/l L-valine, and 36 mg/l L-leucine. Part 2 was 1.2% galactose solution in distilled water.

2.3. Yeast AhR screen with β-galactosidase

LacZ-buffer and *Z*-sarcosyl-buffer were adjusted to pH 7.0 and filtered (0.22 μm filter). LacZ-buffer was composed of 60 mM $Na_2HPO_4{\cdot}2H_2O$, 40 mM $NaH_2PO_4{\cdot}2H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, and 1 mM DTT. For the *Z*-sarcosyl-buffer, 0.5% *N*-lauroylsarcosine was dissolved in LacZ-buffer with 2 mM DTT.

o-Nitrophenyl-β-galactopyranoside (ONPG) solution was used for the β-galactosidase assays (4 g ONPG in 1 l of LacZ-buffer). The colour reaction was stopped with 1 M Na_2CO_3.

2.4. Yeast AhR screen

The recombinant yeast strain YCM3 of *Saccharomyces cerevisiae* was a gift from Charles A. Miller III (Tulane University, Louisiana). The assay procedure was essentially as described by Miller [36]. The human AhR gene is integrated into chromosome III of the yeast together with the gene for the AhR dimer partner protein, the aryl hydrocarbon receptor nuclear translocator (ARNT). The galactose-regulated GAL 1,10 promotor is included to ensure equal levels of expression for AhR and ARNT when induced with galactose, the carbon source of the medium (GOLD medium-trp). When a ligand for the AhR was included in the assay, an AhR/ARNT heterodimer was formed and bound to the five xenobiotic response elements of the lacZ-reporter plasmid to induce the expression of the lacZ-gene. Thus the transcriptional activation of a ligand was quantified via β-galactosidase activity.

A colony of YCM3 was transferred to DO-trp medium and grown to an OD_{600} of approximately 2.0. For each experiment a new overnight culture was prepared with a 1:10 dilution of the YCM3 yeast stock in DO-trp medium. After overnight incubation the culture was diluted to an OD_{600} of 0.4 and grown again to an OD_{600} between 1.0 and 1.5. For the assay, the culture was diluted 1:50 in GOLD-trp media which contains 1.2% galactose for induction of the AhR and ARNT genes.

The test substance (1 μl in DMSO) was added to 100 μl of the yeast culture in galactose media in a 96-well sterile microtiterplate (flat bottom). Each assay was carried out in duplicate. A calibration curve was performed with β-naphthoflavone in each test run. The microtiterplate was incubated for 17 h at 30 °C with gentle shaking.

The disintegration of the yeast cells assay was performed with the addition of *N*-lauroylsarcosine, a strong detergent. A related method was described by Kippert [37], who used 1.5 ml centrifuge tubes and disintegrated the yeast cells with a 0.2% sarcosyl-solution. A modified version was used for the microtiterplate assay. The disintegration was performed with a 0.5% sarcosyl-solution.

After overnight incubation of the culture, 150 μl of *Z*-sarcosyl-buffer was added to each well and the OD was measured at 600 nm. Next the cells were completely disrupted by incubating the plate for 20 min at 30 °C. Afterwards 50 μl of ONPG solution (diluted 1:6 in LacZ-buffer) were added to each well and the microtiter plate was incubated at 37 °C until a yellow colour developed. The reaction was stopped by adding 50 μl of 1 M Na_2CO_3 and the reaction time was noted. The absorption was measured at 405 nm (reference wavelength 620 nm).

2.5. Evaluation

The expression of β-galactosidase is proportional to the transactivating activity of the test substances and the quantity of the samples. The specific β-galactosidase activity, measured at 405 nm, is expressed in AhR-units, and is normalised

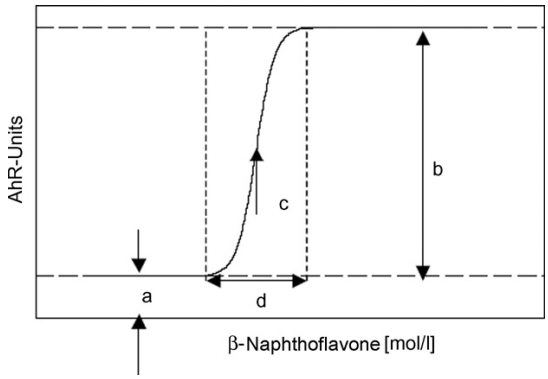

Fig. 1. Logistic dose–response function. This illustrates the parameters used for fitting the data obtained by the yeast assay; *a* corresponds to the baseline, *b* the efficiency; *c* the potency and *d* the transition width.

to the optical density of liquid in the microtiter well at a wavelength of 600 nm. The data were evaluated according to Jungbauer et al. [38].

The calculation of the AhR-units (AU) is as follows:

$$\mathrm{AU} = \left(\frac{\mathrm{OD}_{405} \times 1000}{\mathrm{OD}_{600} \times \mathrm{ml\ of\ cell\ suspension\ added}}\right)\left(\frac{1}{\Delta t}\right) \quad (1)$$

OD_{405} and OD_{600} are the optical densities at wavelengths of 405 nm and 600 nm, respectively, and Δt is the time, in minutes, that the sample was incubated at 37 °C.

The activity in AhR-units was then plotted against the concentration of the test substance (logarithmic scaling). The resulting curve was fitted using a logistic dose–response function. The calculation and fitting was performed with Table Curve 2D software (Jandel Scientific).

The logistic dose–response curve is described as

$$Y = a + \frac{b}{1 + (c/x)^d} \quad (2)$$

where a is the baseline, b is the plateau of the curve designated as the ligand efficiency, and c gives the transition center designated as the ligand potency, which is the concentration that causes 50% efficiency (Fig. 1). Parameter d is the transition width, Y is the AhR units, and x is the concentration. Parameters a and b depend on the growth of the yeast, which can vary to a large extent. To enable direct comparisons between assays the AhR-units were normalised to the maximum efficiency, i.e. the sum of a and b.

The equivalent concentrations of the extracts were calculated from the logistic dose–response curve of β-naphthoflavone.

3. Results

The list of test compounds and their chemical abstract service numbers (CAS no.) is shown in Table 1. Known

Table 1
Results of screening the pure compounds with the yeast AhR assay

Compound	Potency (mol/l)	CAS no.
o-Aminoazotoluene	5.6×10^{-8}	97-56-3
Apigenin	Active	520-36-5
Baicalein	1.1×10^{-6}	491-67-8
1-Benzylimidazol	Active	4238-71-5
Biochanin A	1.3×10^{-7}	491-80-5
Biotin	n.d.	58-85-5
Biphenyl	n.d.	92-52-4
Butyl paraben	Active	94-26-8
Caffeic acid	n.d.	331-39-5
Caffeine	n.d.	58-08-2
(+)-Catechin hydrate	n.d.	225937-10-0
Clotrimazole	Active	23593-75-1
Coumestrol	1.0×10^{-6}	479-13-0
Curcumin	n.d.	458-37-7
Daidzein	n.d.	486-66-8
Diethylstilbestrol	n.d.	56-53-1
Diindolylmethane	1.1×10^{-6}	1968-05-4
Diosmin	n.d.	520-27-4
(−)-Epicatechin	n.d.	490-46-0
(±)-Equol	Active	94105-90-5
17-β-Estradiol	n.d.	50-28-2
trans-Ferulic acid	n.d.	537-98-4
Fisetin hydrate	Active	528-48-3
Formononetin	2.5×10^{-7}	485-72-3
6-Formylindolo[3,2-b]carbazole (FICZ)	3.5×10^{-9}	172922-91-7
Gallic acid	n.d.	149-91-7
Genistein	n.d.	446-72-0
Harmaline	n.d.	304-21-2
ICI 182,780 (antiestrogen)	n.d.	129453-61-8
Indole-3-carbinol	5.8×10^{-6}	700-06-1
Indolo[3,2-b]carbazole (ICZ)	1.9×10^{-9}	241-55-4
2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE)	7.8×10^{-10}	448906-42-1
Indomethacine	n.d.	53-86-1
Melatonin	n.d.	73-31-4
3-Methylcholanthrene	8.4×10^{-8}	56-49-5
β-Naphthoflavone	1.0×10^{-8}	6051-87-2
Naringenin	Active	67604-48-2
Nicotine	n.d.	54-11-5
Phloretin	n.d.	60-82-2
Phtalic acid dibutyl ester	n.d.	84-74-2
Piperine	1.4×10^{-6}	94-62-2
Progesterone	n.d.	57-83-0
Quercetin dehydrate	n.d.	6151-25-3
Quinine	n.d.	130-95-0
trans-Resveratrol	n.d.	501-36-0
Rhein	Active	478-43-3
Rutaecarpine	4.5×10^{-9}	84-26-4
Silibinin	n.d.	22888-70-6
β-Sitosterol	n.d.	83-46-5
Tangeritin	n.d.	481-53-8
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	5.0×10^{-9}	1746-01-6
Theobromin	n.d.	83-67-0
Tryptamine	Active	61-54-1
Trypanthrin	3.4×10^{-7}	13220-57-0

The potencies (EC_{50}-values) are defined as concentrations that elicit a half-maximal response. Compounds that exhibited transactivational potentials with potencies $\geq 10^{-5}$ mol/l are described as "active", and could not be fitted with a logistic dose–response curve due to the lack of saturation of receptor activation. Not detectable (n.d.) is defined as ≤than the signal of the blank +3 times the standard deviation.

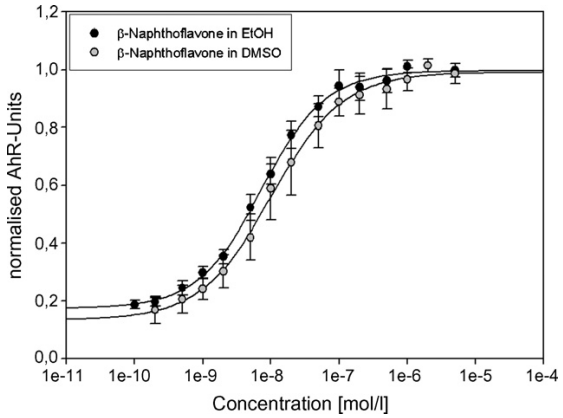

Fig. 2. The mean logistic dose–response curves of β-naphthoflavone. Comparisons are shown between activity in DMSO and in ethanol. Mean values are shown with standard deviations.

anthropogenic compounds and the so-called SAhRMs indole derivates were selected as test substances in order to get an overview of naturally occurring AhR agonists. Compounds with estrogenic activity were included in order to assess AhR crosstalk activity with ERα ([14,30,32] and reviewed by Safe and Worme [39]).

The potencies are shown for each substance that transactivated at levels that could be fitted by a logistic dose–response curve (Eq. (2)). The dose–response curve for the reference compound, β-naphthoflavone, is shown in Fig. 2.

As representatives of natural plant constituents particularly found in cruciferous vegetables, the indole compounds indole-3-carbinol and diindolylmethane were tested. They displayed similar AhR transactivating potentials with EC_{50}-values of 5.8×10^{-6} and 1.1×10^{-6} mol/l (Fig. 3). These results are consistent with those reported by Miller et al. [4] who reported an EC_{50}-value of approximately 10 μM for indole-3-carbinol in an AhR yeast assay.

Among the naturally occurring compounds, the isoflavones biochanin A and formononetin showed very high AhR-activity (Fig. 4), though they were approximately a magnitude less potent than β-naphthoflavone. Given that

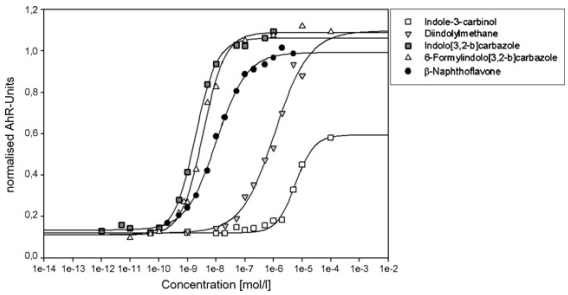

Fig. 3. The logistic dose–response curves of indole compounds. Comparisons are shown with the reference β-naphthoflavone. Mean values are shown.

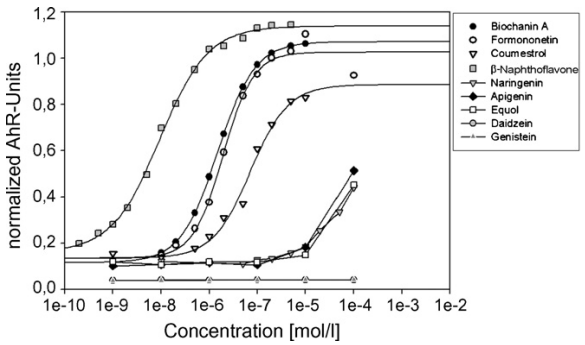

Fig. 4. The logistic dose–response curves of polyphenols. Comparisons are shown with the reference β-naphthoflavone. Mean values are shown.

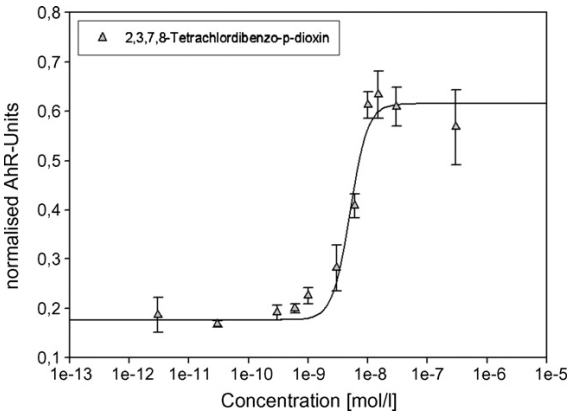

Fig. 5. Mean logistic dose–response curve of dioxin. Measurements were made in ethanol. Mean values are shown with standard deviations.

these compounds are the main constituents of red clover, extracts of this plant were also tested and were found to be extremely potent for AhR activation. We measured that 1 g of extract was equivalent to approximately 7–20 μmol of β-naphthoflavone per gram sample.

Among the other isoflavones only coumestrol displayed a moderate agonistic potential with an EC_{50}-value of 1.0×10^{-6} mol/l. Daidzein and genistein displayed no agonistic effects for AhR. Interestingly, the isoflavone metabolite equol apparently displayed transactivation at concentrations of 1.0×10^{-5} mol/l, but the saturation of receptor activation could not be achieved within the tested concentration range. The same was true for naringenin, apigenin, fisetin, rhein and tryptamine. Concentrations higher than 1.0×10^{-4} mol/l were not feasible, because they impacted yeast growth rate and were difficult to solubilise in a sufficient high concentration. Furthermore, concentrations above 1.0×10^{-4} are out of the physiological range.

Some compounds previously shown to be ligands of the AhR, including daidzein, genistein, tangeretin, resveratrol, and quercetin, did not show activity in our yeast assay. In order to assess whether anthropogenic compounds displayed biological activity in our assay, dioxin was investigated. The DMSO and toluol solvent was inappropriate for dioxin; thus, this assay was performed in ethanol, and β-naphthoflavone dissolved in ethanol was tested for control. There was no significant difference between the transactivating potential of β-naphthoflavone in DMSO and in ethanol. In both cases the potency was about 10 nM, while dioxin had a potency of 5 nM (Fig. 5). These results are similar to those of Kawanishi et al. [40], who tested dioxin and β-naphthoflavone with a similar assay based on the same yeast strain YCM3 and reported EC_{50}-values of 10 and 5 nM, respectively.

4. Discussion

A number of studies support the claims that the consumption of fruits and vegetables can influence general well being and furthermore, prevent cancer and other diseases. Many studies report effects of bioactive compounds on various physiological functions and pathways via their anti-oxidative and anti-inflammatory properties, and others report their ability to act as ligands for important cell receptors such as the steroid hormone receptors or the AhR. These bioactive compounds are mainly polyphenols such as flavonoids (with their important subclass of phytoestrogens), indole compounds, or organosulfur compounds.

In the present study, the isoflavones formononetin and biochanin A were found to be potent agonists of the AhR. To our knowledge, formononetin has not been associated previously with the AhR. However, a recent report by Han et al. [42] suggested that biochanin A might be a ligand of the AhR. These isoflavones are classified as phytoestrogens and comprise the main constituents of red clover, a plant used for easing menopausal disorders [34]. They are the precursors of two other isoflavones in red clover, daidzein and genistein. In mammals, daidzein is further metabolised to equol, which also has transactivational activity. Previously, equol was associated with a reduced incidence of breast cancer [41]. Though in the present study agonistic activity could not be detected for daidzein or genistein, equol showed transactivating potential at high concentrations (100 μM).

An interesting source of isoflavones was reported by Sakakibara et al. [43]. They detected high amounts of formononetin (50 μg/l) and equol (210 μg/l) in milk from goats fed with clover. These amounts translate to concentrations of 1.9×10^{-7} and 8.7×10^{-7} mol/l, respectively. These concentrations are in the range of the EC_{50}-values for estrogenic activity determined with *in-vitro* tests [38,44,45] and they are also in the range of the EC_{50}-values for AhR obtained in our yeast assay.

Biochanin A and formononetin are well-established activators of the ER and recently they were shown to be potent activators of the peroxisome proliferator-activated receptors (PPAR) α and γ [46]. Especially, PPARγ and its ligands were used to treat diabetes type II and the metabolic syndrome. The spectrum of properties of these isoflavones is broad. Biochanin A acts as a chemopreventive agent [46–48] by

inhibiting the metabolism of benzo(a)pyren, the initial step in its carcinogenicity. It is likely that the anticarcinogenic effects of biochanin A are related to its modulation of the AhR pathway.

The negative results presented here for daidzein and genistein are in contrast to those reported by Amakura et al. [19], who tested these compounds in a CALUX assay performed in mouse hepatoma cells. They reported EC_{TCDD25}-values (concentration producing luciferase activity equal to 25% of the maximal response to TCDD) of 3 and 2.4 μM for daidzein and genistein, respectively. We could not explain our negative results for daidzein and genistein by a lack of uptake by the yeast cells. We were able to show transactivating potentials of daidzein and genistein for ERα and ERβ in yeast-based transactivating assays [38]. A potential explanation might be that in the CALUX assay the compounds were metabolised by the enzymes of the mouse hepatoma cells, which might potentially cause the release of more potent compounds. This would not occur in the yeast assay used for the AhR because yeast is devoid of metabolising enzymes. Similar to our results, the CALUX assay demonstrated equol and apigenin inductions of luciferase activity at concentrations on the order of 10–100 μM. Moreover, in our yeast assay naringenin also showed weak transactivating potential with an EC_{50} of 10–100 μM, consistent with the EC_{TCDD25}-value of 53 μM observed in the CALUX assay. However, our results regarding resveratrol and coumestrol were again inconsistent with results reported from the CALUX assay. In our yeast assay coumestrol appeared to be a moderate agonist for AhR with a potency of 1 μM, and resveratrol showed no agonistic potential. In contrast, Amakura et al. [19] reported the inverse, where resveratrol displayed an EC_{TCDD25}-value of 7.3 μM and coumestrol induced very little luciferase activity. The reason for these inconsistencies is unclear.

Most studies on flavonoids investigate the antagonistic effects of these compounds on the AhR, with particular focus on their suppressive effects on dioxin toxicity. Ashida et al. [21] demonstrated inhibitory actions of a number of flavonoids, including apigenin, tangeretin, quercetin, and naringenin and reported IC_{50}-values of 3.2, 9.0, 1.5 and 6.7 μM, respectively. They reported that the isoflavones daidzein and genistein showed only slight inhibitory effects at concentrations above 50 μM. Nevertheless, after comparing the IC_{50}-values with physiological levels of flavonoids reported in the literature, the authors reasoned that dietary levels of flavonoids would be able to inhibit dioxin toxicity.

We found the indole compounds also exhibited notable potencies. These compounds are found in cruciferous plants, such as broccoli, cabbage and cauliflower and are proven to have antitumorigenic and antiestrogenic properties [24].

In previous studies the isoflavones of soy and red clover have been regarded as potential risk factors for cancer, due to their estrogenic activity. However, the present study indicates that isoflavones are also agonists for AhR at physiologically relevant concentrations. This suggests a reconsideration of their potential risk, because AhR ligands are potent compounds for the prevention and treatment of cancer, particularly breast cancer [49].

Acknowledgment

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Publication II

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Effect of non-persistent pesticides on estrogen receptor, androgen receptor and aryl hydrocarbon receptor

SVJETLANA **MEDJAKOVIC**[1,2*], ALFRED **ZOECHLING**[1,2*], BARBARA **SCHILDBERGER**[3], MICHAEL **GARTNER**[4] and ALOIS **JUNGBAUER**[1,2]

[1]Christian Doppler Laboratory for Receptor Biotechnology,

Muthgasse 18, A-1190 Vienna, Austria

[2]University of Natural Resources and Applied Life Sciences Vienna, Department of Biotechnology, Muthgasse 18, A-1190 Vienna, Austria

[3]Federal College and Research Institute for Viticulture and Pomolgy,

A-3400 Klosterneuburg, Austria

[4]LVA GmbH, Blaastrasse 29, A-1190 Vienna, Austria

* These authors contributed equally to this work.

Corresponding author: Alois Jungbauer, University of Natural Resources and Applied Life Sciences Vienna, Department of Biotechnology, Muthgasse 18, A-1190 Vienna, Austria

FAX: 0043 1 3697615, Tel.: 0043 1 36006 6226,

e-mail: alois.jungbauer@boku.ac.at

Non-persistent pesticides and nuclear receptors

KEY WORDS: pesticides, aryl hydrocarbon receptor, estrogen receptor, androgen receptor, maximum residue levels, wine

List of all abbreviations and definitions used in the manuscript

Acceptable daily intake (ADI)

Androgen receptor (AR)

Arylhydrocarbon receptor (AhR)

Dichlorodiphenyltrichloroethane (DDT)

Estrogen receptor (ER)

Maximum residue levels (MRLs)

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)

Yeast androgen assay (yAS)

Yeast AhR assay (yAhR)

Yeast estrogen assay α (yESα)

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Abstract

BACKGROUND: Non-persistent pesticides are attributed as less harmful for the environment, but their impact on nuclear receptors is still not fully explored.

OBJECTIVES: The objective was to evaluate the potential of pesticides that are customary in wine and fruit cultivation to transactivate the human estrogen receptor α (ERα), androgen receptor (AR) and arylhydrocarbon receptor (AhR).

METHODS: An application experiment with the pesticide Switch was performed on grape vine. Maximum residue concentration of the active ingredients was quantified with GC-MS and HPLC-MS. The transactivational potential of the pesticides Acorit, Frupica, Steward, Reldan, Switch, Cantus, Teldor and Scala and their active compounds hexythiazox, mepanipyrim, indoxacarb, chlorpyrifos-methyl, cyprodinil, fludioxonil, boscalid, fenhexamid and pyrimethanil were tested on the human ERα, AR and AhR with *in vitro* yeast assays.

RESULTS: Tested residue concentrations of Switch ingredients on grapes were below the maximum residue limits. Fludioxonil and fenhexamid were agonists of the ERα with EC_{50}-values of 3.7 and 9.0 µM respectively. Five of nine pesticides were AhR-agonists: fludioxonil, mepanipyrim, cyprodinil, pyrimethanil and chlorpyrifos-methyl (potencies of 0.42, 0.77, 1.4, 4.6 and 5.1 µM), but no AR-agonist was found. A model calculation of the maximum possible pesticide uptake within maximum residue levels range resulted in higher theoretical serum concentrations than the AhR-EC_{50}-values of some pesticides.

CONCLUSIONS: Our *in vitro* evidence suggests that pesticides can switch on pathways affecting hormonal balance, even within permitted limits. Nevertheless, we conclude that a safety margin still remains, due to the fact that the permitted maximum is never reached when the pesticides are properly applied.

Introduction

The ways of pesticide exposure are manifold and range from intake through food to exposure via air (indoor and outdoor) and soil. As it is not possible to exclude totally the contact to pesticides, the legislature protects people by legal regulations in terms of maximum residue levels (MRLs) that restrict the maximum concentration (expressed as milligrams of pesticide per kilogram of food) of a certain pesticide in a specific food product.

Nevertheless, some routes of exposure cannot be averted. This applies especially for farm workers or people living in rural areas, for whom the exposure is much higher than for the rest of the population and not truly covered by normal risk assessment arrangements. However, the main route of pesticide exposure for the majority is via food and drinking water. Regulatory requirements, an improved knowledge as well as understanding of possible after-effects have led to reduced residue levels. Unfortunately, this was not always practiced this way. Bioaccumulation and emission of persistent pesticides even in regions where these chemicals have never been implemented are evidence of a naïve usage in the first decades of pesticide application (e.g. DDT). Nowadays, persistent pesticides are mostly forbidden, but it remains the question if pesticides which are bioavailable and degradable, are truly safe compounds. This cannot be answered generally for all pesticides as the heterogeneity is high in their structure and mode of action.

An important point plays the effect of chemicals on the endocrine system, as it is known that a lot of persistent pesticides have endocrine disruptive effects (Hodges et al. 2000; Kojima et al. 2004; Lemaire et al. 2006; Soto et al. 1994). But evidence is strong that also many non-persistent pesticides have impact on the endocrine system, e.g. as observed for pyrethroid insecticides (Kim et al. 2004). This is possible via direct activation of ERs or ARs, but also through the stimulation of the aryl

hydrocarbon receptor (AhR) pathway. It is known that the AhR and the ER interact in multiple ways (Beischlag and Perdew 2005; Bemanian et al. 2004; Gierthy et al. 1988; Hayes et al. 1996; Kharat and Saatcioglu 1996; Klinge et al. 1999). The activation of the AhR pathway can result in antiestrogenic effects without direct activation of the ER. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is one of the most toxic anthropogenic compounds and the prototypical ligand of AhR. The toxicity of TCDD is mainly attributed to an inappropriate constant activation of this receptor, due to the fact that TCDD is hardly metabolized. Beside the toxic effects of an AhR activation, this receptor is known to play a significant role in normal developmental processes (Benedict et al. 2000; Gonzalez et al. 1995; Kawamura and Yamashita 2002; Walisser et al. 2004) and cell cycle procedures (Levine-Fridman et al. 2004; Ma and Whitlock Jr 1996; Zaher et al. 1998). The antiestrogenic effects that are mediated by the AhR have been newly investigated for possible therapeutic applications for the treatment of hormone-dependent diseases (Safe and McDougal 2002).

All the same, the binding of chemicals to ER, AR or AhR is an indicator for possible endocrine disruptive effects. Most studies deal with persistent pesticides such as organochlorines. The goal of our study was on the one hand the determination of residual levels, and on the other hand of the evaluation of the transactivational potential on the ER, AR and AhR of several pesticides that are nowadays commonly used in fruit and wine cultivation (see Figure 1). Knowing this, a better understanding and assessment of health risks for consumers of fruits and wine as well as for pesticide applicators, farm workers and people living nearby agricultural areas, could be obtained.

Material and Methods

Materials

Dimethylsulfoxide (DMSO) was obtained from Sigma Aldrich (St. Louis, MO). Buffer reagents, N-lauroylsarcosine (sodium salt), di-sodium hydrogen phosphate dihydrate (Na_2HPO_4*2 H_2O), sodium dihydrogen phosphate dihydrate (NaH_2PO_4*2 H_2O), potassium chloride (KCl), magnesium sulfate heptahydrate ($MgSO_4$*7 H_2O), sodium carbonate (Na_2CO_3) and o-nitrophenyl-β-galactopyranoside (ONPG), were purchased from Sigma Aldrich (St. Louis, MO), Fluka (Buch, Switzerland) and Merck (Darmstadt, Germany). Estradiol (E2), β-naphthoflavone, 5α-dihydrotestosterone (5α-DHT), hexythiazox, mepanipyrim, indoxacarb, chlorpyrifos-methyl, cyprodinil, fludioxonil, boscalid, fenhexamid and pyrimethanil were obtained from Sigma Aldrich (St. Louis, MO). The pesticide formulations Acorit, Frupica, Steward, Reldan, Switch, Cantus, Teldor and Scala were obtained from Kwizda Agro GmbH (Vienna, Austria), Bayer CropScience (Monheim, Germany), Syngenta Agro GmbH (Dielsdorf, Switzerland) and BASF SE (Ludwigshafen, Germany). For yeast media preparation, yeast nitrogen base was obtained from Difco (Franklin Lakes, NJ), amino acids from Serva Feinbiochemica (Heidelberg, Germany) and dropout medium without tryptophan from Sigma Aldrich (St. Louis, MO).

The standards used for GC-MS analysis were purchased either from Dr. Ehrenstorfer or from Sigma-Aldrich with the highest available purity. Magnesium sulfate anhydrous ($MgSO_4$), sodium chloride (NaCl) and sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2\ H_2O$) were purchased from J.T.Baker, di-sodium hydrogen citrate sesquihydrate ($C_6H_6Na_2O_7 \cdot 1.5\ H_2O$) was provided from Fluka and Bondesil-PSA 40 µm was from Varian. Ultra-residue reagent acetone, ultra-residue reagent ethylacetate, ultra-residue reagent acetonitrile, HPLC/MS grade methanol, ultra HPLC/MS grade water and HPLC/MS grade formic acid were purchased from J.T.Baker.

Pesticide application experiments and analysis of residues

The grapes from the Austrian variety Grüner Veltliner which were grown in the research vineyards of the Federal College and Institute for Viticulture and Pomology, Klosterneuburg, Austria were used. They were treated with the active ingredients cyprodinil and fludioxonil against Botrytis cinerea. The variants were split into the following groups: 1. with observance in the waiting period of at least 5 weeks, 2. without observance the waiting period (~3.5 weeks) and, 3. without the final spraying. Until the final application, the spraying followed the established programme. Cyprodinil and fludioxonil were applied to the grapes on the 23rd July 2008 for group 1 (with observance in the waiting period) and on the 19th September for group 2 (without observance in the waiting period). The grape harvest was made on the 13th October 2008.

Sample preparation for GC-MS analysis

The samples were prepared according to the QuECheRS method as explained (Lesueur et al. 2008). Triphenylphosphate (TPP) was used as internal standard and spiked at the initial step to reach a 1 µg/ml concentration in the HPLC/MS extract and a 10 µg/ml concentration in the GC/MS extract.

Sample analysis with GC-MS

The GC-MS analyses were performed on 3 Hewlett-Packard (Agilent Technologies) GC/MS Model 6890N Series gas chromatography coupled to 5973N and 5975 mass selective detectors. A HP 5 MS (30m x 0.25 mm i.d.) (Agilent Technologies, Waldbronn, Germany) fused silica capillary column with a 0.25 µm film thickness was used with helium as carrier gas at a constant pressure daily adjusted (chlorpyriphos-methyl RT relocked to 16.596 min). 1.0 µl of the sample was injected in the splitless mode at 280 °C. The GC oven was operated with the following temperature program: initial temperature 70 °C held for 2 min, ramped at 25 °C/min to

150 °C not held, followed by a ramp of 3 °C/min to 200 °C not hold, followed by another ramp of 8 °C/min to 280 °C held for 10 min and finally ramped to 320 °C at 15 °C/min held for 2.47 min. The total run time was 47 min. The interface was kept at 250 °C, the quadropole at 150 °C and the mass spectra were obtained at an electron energy of 70 eV.

The Agilent Chemstation Software G1701DA version D.02.00.237 was used for data analysis and the analyses were operated on the principle of a simultaneous full scan/SIM mode method presented elsewhere (Lesueur and Gartner 2005).

Sample analysis with HPLC-MS

The high-performance liquid chromatography system was an Agilent Technologies HP-1100 Series (Agilent Technologies, Waldbronn) controlled with the Agilent Technologies Chemstation for LC 3D System Software. Chromatographic separation was achieved using a Zorbax SB-C18 analytical column 2.1 x 150 mm, 3.5 µm particle size from Agilent Technologies at a flow rate of 300 µl/min. The mobile phases consisted of A: H_2O – MeOH, 90 % – 9.95 % (v/v) with 0.05 % HCOOH and B: H_2O – MeOH, 9.95 % – 90 % (v/v) with 0.05 % HCOOH. The gradient was 100 % A at 0 min, 100 % A at 1 min, 0 % A at 10 min, 0 % A at 17 min, 100 % A at 20 min. The post time was 2 min with 100 % A and the stop time 22 min. The HPLC system was interfaced to an Agilent Technologies mass spectrometer LC/MSD trap XCT Plus (Agilent Technologies, Waldbronn) equipped with an electrospray ionisation (ESI) interface operated in positive mode and controlled with the Agilent Technologies LC/MSD trap software 5.3. The nebulizer gaz (nitrogen) pressure was 40 psi, the drying gas flow rate was 8 ml/min and the drying gas temperature was 325 °C. The capillary voltage was – 4500 V, the endplate offset was fixed at – 500 V. The ion trap was operated in the Ion Charge Control (ICC) mode with a target ion count of 150 000 and a maximum accumulation time of 50 ms. The IT mass detector operated in full

scan and Selected Ion Monitoring (SRM) modes for confirmation with a 0.6 V fragmentation amplitude as already presented elsewhere (Lesueur et al. 2007)

Sample preparation for in vitro *assays*

Stock solutions of the pure compounds in DMSO were prepared with a concentration of 0.01 M and further diluted for testing in 1:10 dilution steps. Extracts of the pesticide preparations were performed as follows: 20 mg of the preparations were dissolved in 1 mL DMSO and further diluted in DMSO for testing.

Yeast estrogen assay α (yESα) and yeast androgen assay (yAS)

The assays have been accurately described by Reiter et al. (Reiter et al. 2009). Summarized, the assays are two-plasmid systems that contain expression plasmids with the accordant human receptor gene (ERα or AR respectively) and a LacZ-gene reporter plasmid. The expression plasmid is expressed upon induction with copper. The presence of a ligand provokes the binding of the hormone receptor as homodimer to a hormone response element (HRE) on the reporter plasmid. The gene product β-galactosidase is quantified and a measure for the agonistic effect of the ligand.

Yeast AhR assay (yAhR)

The method was described in detail by Medjakovic and Jungbauer (Medjakovic and Jungbauer 2008). Briefly, a yeast construct, which has the human AhR and aryl hydrocarbon receptor nuclear translocator (ARNT) genes integrated into chromosome III, is used. They are under control of a galactose-regulated promotor. After induction with galactose, those genes are expressed in equal shares. By binding of an AhR-ligand, an AhR-ARNT complex is built and is able to bind to xenobiotic response elements of the lacZ-reporter plasmid. The induced expression of the lacZ-gene can be quantified photometrically.

Evaluation

The expression of β-galactosidase is equivalent to the quantity of the transactivating activity. The specific enzyme activity is expressed in Miller Units (MU), which are the β-galactosidase-activity normalized to the optical density at a wavelength of 600 nm.

The calculation of the Miller Units is realized as follows:

$$MU = \left(\frac{OD_{405} * 1000}{OD_{600} * \text{ml of cell suspension added}} \right) * \left(\frac{1}{\Delta t} \right) \quad (1)$$

OD_{405} and OD_{600} are the optical densities at a wavelength of 405 nm respectively 600 nm, and Δt is the incubation time at 37°C in minutes.

For evaluation the MU are plotted against the concentration (logarithmic scaling). The resulting curve is fitted using a logistic dose response function. The calculation and fitting was performed with Table Curve 2D software (Jandel Scientific) and plotted with SigmaPlot 10.0 (Systat Software).

The logistic dose response curve is described as:

$$Y = a + \frac{b}{1 + (c/x)^d} \quad (2)$$

Parameter a equals the baseline and b the plateau of the curve designated as the ligand efficiency. Parameter c gives the transition center and equals the ligand potency which is the concentration that causes 50% efficiency (EC_{50} value). Parameter d equals the transition width. Y are the Miller Units and x the concentration.

The equivalent concentration of pesticide preparations produces the same transactivational activity as the reference compound (estradiol for yESα and β-naphthoflavone for yAhR) at a certain concentration. It is calculated with the parameters a, b, c and d of the fitted logistic dose response curve of the reference compound that is performed within the same test run and the MU of the sample:

$$equivalent\ concentration\,[mol/L] = c * \left\{ \left(\frac{b}{(\ \mathrm{MU - a}\ )} - 1 \right)^{\left(\frac{1}{d}\right)} \right\} \qquad (3)$$

In a final calculation step, the sample dilution factor D of the assay (= 0.01) and the concentration of the extract have to be taken into account:

$$equivalent\ concentration\,[mol/g] = \frac{(equivalent\ concentration\,[mol/L] * D)}{extract\ concentration\,[g/L]} \qquad (4)$$

For calculation, only the extract concentrations that produced a transactivation signal in the linear range of the logistic dose response curve were used.

Results

Transactivating potential of the pesticides in the yAhR

Three of the eight tested pesticide preparations exhibited remarkable transactivating potential in the yAhR (Figure 2). Especially Frupica and Switch showed equivalent β-naphthoflavone concentrations of 36.1 and 37.9 µM per gram sample, respectively (calculated as described). Scala showed weaker transactivating potential of the AhR with 4.7 µM equivalent β-naphthoflavone concentration per gram sample, whereas Reldan exhibited the weakest transactivation of the AhR with 0.5 µM equivalent concentration of the reference compound per gram sample. Cantus showed extremely weak transactivation of the AhR ranging slightly above the blank value + 3times the standard deviation. It was not possible to calculate an equivalent β-naphthoflavone concentration for this low activity, since it is not within the linear range of the logistic dose response curve of the reference compound. Consequently, this preparation is defined as “active”.

The transactivation of the AhR of the pure compounds is consistent with these results. The compounds chlorpyrifos-methyl, pyrimethanil, cyprodinil, mepanipyrim and fludioxonil were able to transactivate the AhR in the yeast assay (mentioned compounds in ascending order to their potency (Table 1)) as it is displayed in Figure

3. For boscalid, the active compound of Cantus, no activity could be detected up to 100 µM. Beside this exception, the agonistic activity of the pure compounds correlated very well with the transactivating potential of the pesticide preparations.

Transactivating potential of the pesticides in the yESα

Only Switch and Teldor showed transactivation of the ERα (Figure 4). The calculated equivalent estradiol concentrations were 8.0 and 30.6 nM respectively per gram sample.

Correlative to these results, only fludioxonil and fenhexamid were ERα agonists with potencies of 3.7 and 9.0 µM (Figure 5A) respectively, while the response of cyprodinil was just slightly above the blank +3times the standard deviation. Due to the fact that the change of the yeast growth medium resulted in a higher background signal, the illustration of the ERα transactivating and non-transactivating compounds were splitted in two figures (Figure 5A and 5B), whereas the mean logistic dose curves of the respective test-runs are plotted in every illustration.

Transactivating potential of the pesticides in the yAS

No EC_{50} values of the pesticides in the yAS could be obtained, as the pesticides showed not high enough agonistic activity to establish a logistic dose response curve (Figure 6). Fenhexamid and chlorpyrifos-methyl showed agonistic activities at highest applicable concentrations of 100 µM, while fludioxonil transasctivated the AhR also at 10 µM. Fludioxonil significantly inhibited the yeast growth at 100 µM, explaining why the signal is decreasing.

GC-MS analysis of Switch residues on grapes

The pesticide formulation Switch was chosen for an application experiment, because of the potent bioactive properties of its active ingredients cyprodinil and fludioxonil. Switch is a potential candidate for the contamination of wine grapes and must, because of the long waiting period and the late application of this pesticide as it is normally

practiced. Nevertheless, GC-MS analysis showed that with and without waiting period the residue levels of cyprodinil and fludioxonil were clearly below MRLs (Table 2).

Discussion

Our analysis of the pesticide residue levels in wine grapes were below the MRLs of the pesticides. This is consistent with previous reports. A literature-analysis regarding the pesticides that we tested in this study showed that these pesticides mostly cannot be detected or that their concentrations are under MRLs in various food items. This applies to peaches and nectarines (Juan-Garcia et al. 2005), strawberries (Angioni et al. 2004), fruits for baby-food production (Ticha et al. 2007) or in infant formulas (Mezcua et al. 2007), leafy vegetables (Gonzalez-Rodriguez et al. 2008) as well as fruit-based soft drinks (Garcia-Reyes et al. 2008). Also exceptions have been reported by Safi et al. (Safi et al. 2002); they found pyrimethanil levels in strawberries form Palestine that exceeded the MRL about twofold. Angioni et al. (Angioni et al. 2004) found in Italian strawberries pyrimethanil residues below the MRL, but fenhexamid concentrations were in some samples over the MRL, although the mean level was slightly below. Similar results were found for chlorpyrifos-methyl in tomatoes from Ghana; the mean level of these residues was below MRL but the maximum peak of the samples was higher (Darko and Akoto 2008). In the “Italian Ready-Meal Residue Project“ (Lorenzin 2007) the pesticides pyrimethanil, cyprodinil and chlorpyrifos-methyl were found in ready-meals, wine and fruits. The calculated daily intake was below the acceptable daily intake (ADI) value, but the maximum peaks were over 100 and 200% for adolescents and children, respectively.

These results are eased by the fact that a great deal of pesticides is further reduced by food processing procedures (Angioni et al. 2004; Balinova et al. 2006; Christensen et al. 2003) or that the levels drop during pre-harvest interval (Ticha et al. 2007). This is

also true for wine production, where pesticides are reduced due to fermentation (Ruediger et al. 2005) or as consequence of mechanic processing steps such as filtration or the use of clarifying agents (Fernandez et al. 2005; Ruediger et al. 2004).

Nevertheless, our results and the implication thereof of a possible activity of the pesticides *in vivo* sound a note to caution. Potent AhR agonists were found among the tested pesticides. A model calculation of fruit consumption with residues below the MRLs compared with the potency of the pesticides in the yAhR shows that effects on the endocrine system and the AhR pathway are possible within legally restrictions. As displayed in Figure 7, the consumption of 250 g of fruits and vegetables could result in a higher serum concentration of fludioxonil, which was the most potent AhR agonist, than the potency of this compound in the yAhR. For this model calculation we assumed a theoretical uptake of 100% and that no metabolization takes place. It was calculated with the highest permitted MRLs of fludioxonil for grapefruits, lettuce or other salad plants. The next highest MRLs are 7 mg/kg for other citrus fruits or peaches and 5 mg/kg for apples, apricots or pears. Again, under assumption of 250 g fruit consumption, the calculated maximal achievable blood concentration for these fruits amounts to 1.16 µM and 0.83 µM, respectively and are also several fold above the AhR EC_{50} value of fludioxonil.

The consumption of 250 g of blackberries or salad that containing cyprodinil reaching the permitted MRL (10mg/kg) would also result in a higher blood concentration (2.2 µM) than the EC_{50} value of this compound in the yAhR (1.4µM). For all other calculated cases of the AhR-agonists mepanipyrim, pyrimethanil and chlorpyrifos-methyl, the maximal achieved blood concentrations (calculation always based on an average portion of 250 g food) are below their AhR EC_{50} values. Nevertheless, it should be kept in mind, that concentrations ranging slightly under the EC_{50} value can also have physiological effects. This could apply to mepanipyrim (calculated

concentrations of 0.67 µM and 0.45 µM, respectively, for MRLs of 3 and 2 mg/kg of table and wine grapes and strawberries, respectively, in comparison to the EC_{50} of 0.77 µM), cyprodinil (calculated concentrations of 1.11 µM and 0.45 µM, respectively, for MRLs of 5 mg/kg of table and wine grapes and strawberries, respectively, in comparison to the EC_{50} of 1.4 µM) or pyrimethanil (calculated concentrations of 2.51 µM for MRLs of 10 mg/kg of citrus fruits and peaches in comparison to the EC_{50} value of 4.6 µM).

Fludioxonil was the only compound that showed for all tested receptors transactivating potential (see Table 1). Cyprodinil is an agonist of the AhR and the ERα, whereas fenhexamid transactivates the AR and the ERα. It is not really possible to compare those data with literature results, since there are scanty studies dealing with the same pesticides. Especially for the most active pesticides of this study, no relevant comparative date could be found. As far as we know, it is the first time that fludioxonil, mepanipyrim, cyprodinil and pyrimethanil were tested on transactivating potential on the human AhR, although a few groups have screened other pesticides with *in vitro* bioassays (Long et al. 2003; Takeuchi et al. 2008). Only chlorpyrifos-methyl was also tested by Takeuchi et al. (Takeuchi et al. 2008). They found no AhR-agonistic activity for this pesticide, while we evaluated an EC_{50}-value of 5.1 µM. This discrepancy is allegeable by the differences of the test systems; Takeuchi and coworkers used mouse hepatoma cells, while we were working with a yeast assay having the human AhR gene integrated into its genome.

Similar to that, there is surprisingly little data of estrogenic or androgenic activities of the pesticide panel that was tested during the present study. Most studies analyzed the receptor activation potentials of persistent pesticides (Lemaire et al. 2006; Soto et al. 1994), especially organochlorine pesticides (Hodges et al. 2000) or pyrethroid insecticides (Kim et al. 2004). Again, only chlorpyrifos-methyl has been mentioned

more often; consistent with our results, this compound showed no estrogenic activity *in vitro*, as tested with a yeast two-hybrid system by Nishihara and coworkers (Nishihara et al. 2000) or in a hamster ovary cell assay with a human ERα expression vector by Kojima et al. (Kojima et al. 2004). In the same study, Kojima et al. they found neither androgenic activity (with the same test principle and a human AR expression vector) nor antiandrogenic activity. Whereas *in vivo*, chlorpyrifos-methyl shows on the one hand no estrogenic or anti-estrogenic activity, but on the other hand anti-androgenic activity in the rat Hershberger assay (Kang et al. 2004). Except for chlorpyrifos-methyl, no comparative studies were found for the other pesticides that we tested.

Beside direct receptor-interaction data, animal tests and clinical studies indicate for some currently used pesticides influence on the hormonal balance and especially on reproductive health issues. 3,5,6-Trichloro-2-pyridinol (TCPY), the main metabolite of chlorpyrifos and chlorpyrifos-methyl, was associated with reduced estradiol concentration (Meeker et al. 2008) and reduced testosterone levels (Meeker et al. 2006) in men. Meeker et al. (Meeker et al. 2004) found also a suggestive association for TCPY with human sperm concentration and motility. In rats, chlorpyrifos-methyl showed weak reproductive toxicity by exposition in adulthood, as well as anti-androgenic effects after long-term exposure from in utero through sexual maturation in the offspring (Jeong et al. 2006). However, TCPY does not seem to be fetotoxic or teratogenic in animals (rats and rabbits) (Hanley et al. 2000).

TCPY was also associated with an altered thyroid hormone function in men (Meeker et al. 2006). Similarly, pyrimethanil seems to enhance the excretion of thyroid hormones and hepatic metabolism (Hurley et al. 1998).

The second most potent AhR-agonist of this study, mepanipyrim, is known to affect intracellular trafficking and thereby altering metabolism and exocytosis of

sphingolipids and low density lipoprotein (LDL) (Miura et al. 1996). In rats, mepanipyrim alters the lipoprotein profile significantly (Terada et al. 1998) and induces a fatty liver (Terada et al. 1998). Nevertheless the hepatotoxic effects may be species-dependent, since they were shown in rats, but not mice and dogs (Terada et al. 1998). On the intracellular level, mepanipyrim inhibits the transport of hepatic very low lipid protein (VLDL) from the Golgi apparatus to the cell surface (Terada et al. 1999) and induces Golgi dispersion (Nakamura et al. 2003). If these effects are intertwined with the AhR activation is unknown and needs further examination.

In contrast to persistent pesticides, chlorpyrifos-methyl is metabolized and rapidly eliminated. Tests in rats showed that 90% of chlorpyrifos-methyl is eliminated via urine, expired air and faeces after 3 days. In addition to that, a bioavailable amount of 4% were found in selected organs (Neskovic et al. 1992). Chlorpyrifos, the analogous diethyl ester of chlorpyrifos-methyl is metabolized to the same principle metabolite TCPY. Pharmacokinetic studies with human volunteers showed that only a small amount of dermally applied chlorpyrifos is absorbed (Meuling et al. 2005; Nolan et al. 1984). Nolan et al. (Nolan et al. 1984) report that about 70% of orally administered chlorpyrifos was excreted in the urine as TCPY by the human probands with an half-life of 27 hrs. But while Nolan and coworkers (Nolan et al. 1984) state that chlorpyrifos and TCPY have a low potential for bioaccumulation, Meuling et al. (Meuling et al. 2005) disagree and suggest that these compounds might be accumulated in the body.

It is necessary to emphasize that an AhR-activation is not a byword for toxic effects. The toxic effects of several compounds are clearly mediated by the AhR pathway, either by bioactivation of the AhR-triggered xenobiotic metabolism or by a constantly activated and unbalanced pathway. Notwithstanding, the physiological role of this receptor provides an implication for normal involvement in cell procedures.

The aim of this study was to gain better insight in possible bioactive effects of pesticides that are nowadays commonly used in wine and fruit cultivation. It was examined, which effects the pesticides could provoke within legally limits if the maximum of permitted exposure is applied. It can be concluded that some pesticides have influence on pathways (at least *in vitro*) that could lead to endocrine disruptive effects, referring in particular to the AhR pathway. For Switch, the most potent pesticide of this study, it was confirmed that the exposure for consumers is below the statutory MRLs. Also, there is still a safety margin, considering metabolization, the unlikelihood of 100% uptake and bioavailability and the fact that the MRLs are in most instances much higher than the de facto residue levels in food. On the basis of the results from other studies, we recommend consumers the thoroughly washing of fruits, as rinsing with tap water and food processing seem in most cases to reduce noticeably residue levels. However, further studies will help to elicit possible health hazards for high-risk groups that demand special attention, such as small children, highly exposed rural population and farm workers.

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Tables

Table 1. Summary of the transactivating potential of the tested pure compounds on the AhR, the ERα and the AR.

Compound (Cas. No.)	Active compound of	EC_{50} AhR	EC_{50} AR	EC_{50} ER
Hexythiazox (78587-05-0)	Acorit	n.d.	n.d.	n.d.
Mepanipyrim (110235-47-7)	Frupica	0.77 µM	n.d.	n.d.
(±)-Indoxacarb (144171-61-9)	Steward	n.d.	n.d.	n.d.
Chlorpyrifos-methyl (5598-13-0)	Reldan	5.1 µM	active[a]	n.d.
Pyrimethanil (53112-28-0)	Scala	4.6 µM	n.d.	n.d.
Fludioxonil (131341-86-1)	Switch Component 1	0.42 µM	active[b]	3.7 µM
Boscalid (188425-85-6)	Cantus	n.d.	n.d.	n.d.
Cyprodinil (121552-61-2)	Switch Component 2	1.4 µM	n.d.	active[a]
Fenhexamid (126833-17-8)	Teldor	n.d.	active[a]	9.0 µM

The potencies (EC_{50}-values) are defined as concentrations that elicit the halfmaximal response and are given as mean values. The EC_{50}-values of the reference compounds are 12.5 nM for β-naphthoflavone in the yAhR, 0.22 nM for estradiol in the yESα and 9.5 nM for 5α-dihydrotestosterone in the yAS.
Tested compounds that exhibited transactivational activity only at highest concentrations are termed active (active[a] :response at 10^{-4} mol/l > signal of the blank +3 times the standard deviation; active[b] :respose at 10^{-5} mol/l > signal of the blank +3 times the standard deviation). Not detectable (n.d.) is defined as ≤than the signal of the blank +3 times the standard deviation.

Table 2: Residue levels of cyprodinil and fludioxonil on grapes analyzed with GC-MS.

	Cyprodinil [mg/kg grapes]	Fludioxonil [mg/kg grapes]
Switch with waiting period	0.049	0.051
Switch without waiting time	0.074	0.065
Control	0.038	n.d.

Maximum residue levels of cyprodinil and fludioxinil according to EU regulation No 839/2008 (EU-Commision 2009) are 5 and 2 mg/kg respectively. The detection limit of the GC-MS method is 10 µg/kg for both compounds.

Figure legend

Figure 1. Classification of the active compounds of tested pesticides.

Figure 2. Equivalent β-naphthoflavone concentration of the tested pesticide preparations per gram sample.

Figure 3. Transactivating potential of the active compounds of tested pesticides on the human AhR. Logistic dose response curves were fitted for those compounds that achieved receptor binding saturation. In some cases, the yeast growth was inhibited by higher concentrations; these values were not included in the fitting. Miller Units were normalized to the maximum of the reference compound (sum of parameter a and b; set as 100%).

Figure 4. Equivalent estradiol concentration of tested pesticide preparations per gram sample.

Figure 5. Transactivating potential of the active compounds of tested pesticides on the human ERα. (A) Logistic dose response curves of fenhexamid and fludioxonil. (B) Diagram of the weak ERα-transactivating compound cyprodinil and the non-transactivating compounds. Miller Units were normalized to the maximum of the reference compound (sum of parameter a and b; set as 100%).

Figure 6. Transactivating potential of the active compounds of tested pesticides on the human AR. Miller Units were normalized to the maximum of the reference compound (sum of parameter a and b; set as 100%).

Figure 7. Model calculation of maximum achievable serum concentrations of fludioxonil within the legally permitted MRLs and comparison with the potency of this compound on the AhR.

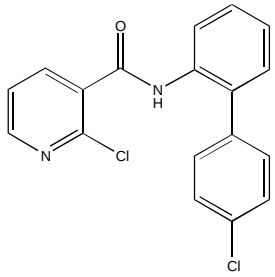
Figures

Figure 1

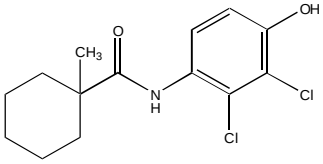
1. **Fungicides**

- Anilide Fungicide
  - Boscalid

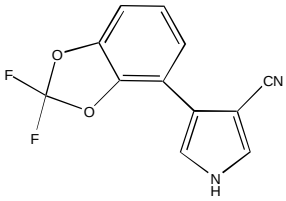
(also as pyridine fungicide classified)



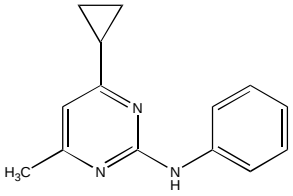
  - Fenhexamid



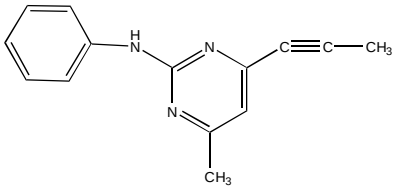
- Pyrrole Fungicide
  - Fludioxonil



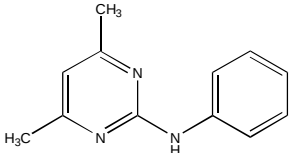
- Anilinopyrimidine Fungicide
  - Cyprodinil



  - Mepanipyrim

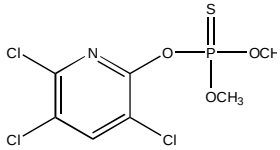


  - Pyrimethanil

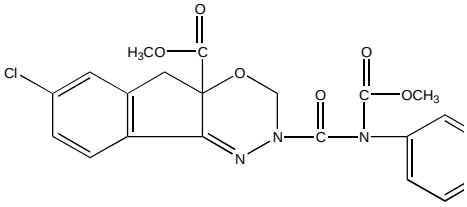


2. **Insecticides**

- Organophosphorus Insecticide
  - Chlorpyrifos-methyl



- Oxadiazine Insecticide
  - Indoxacarb



3. **Acaricides**

- Thiazolidine Acaricides
  - Hexythiazox

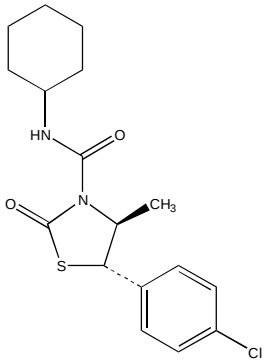


Figure 2

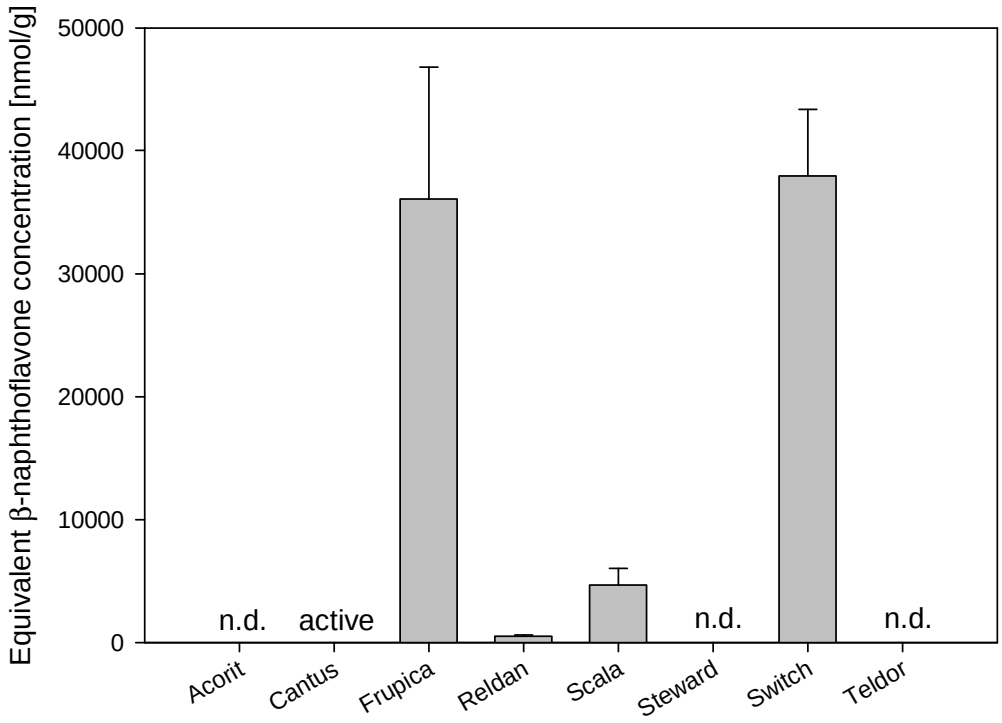


Figure 3

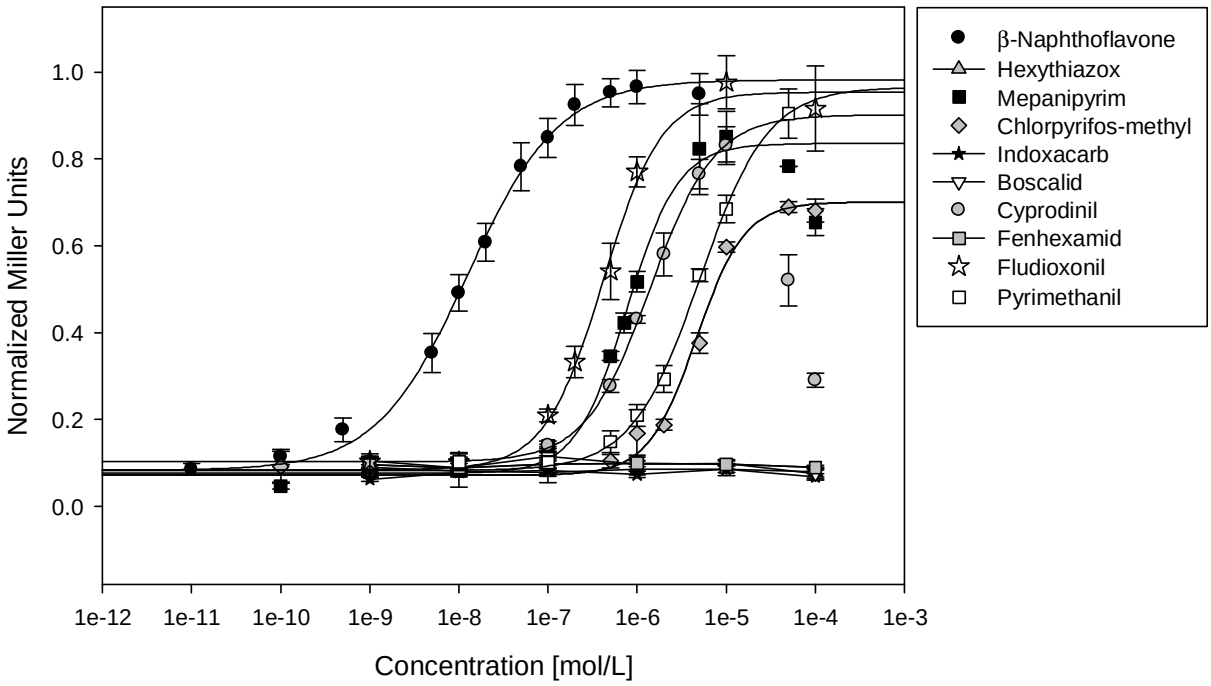


Figure 4

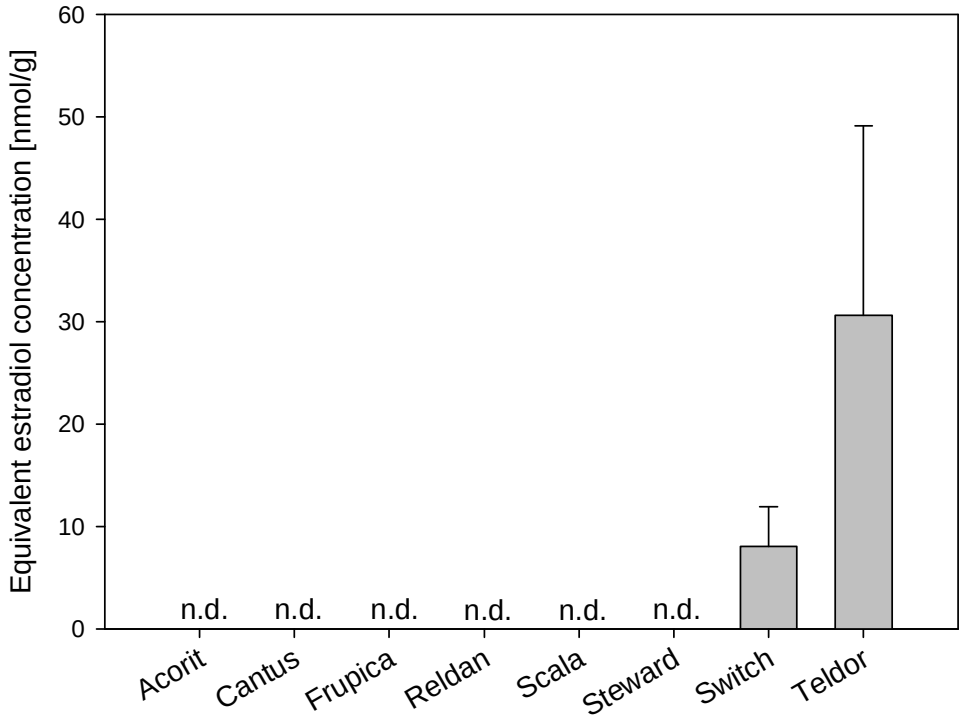
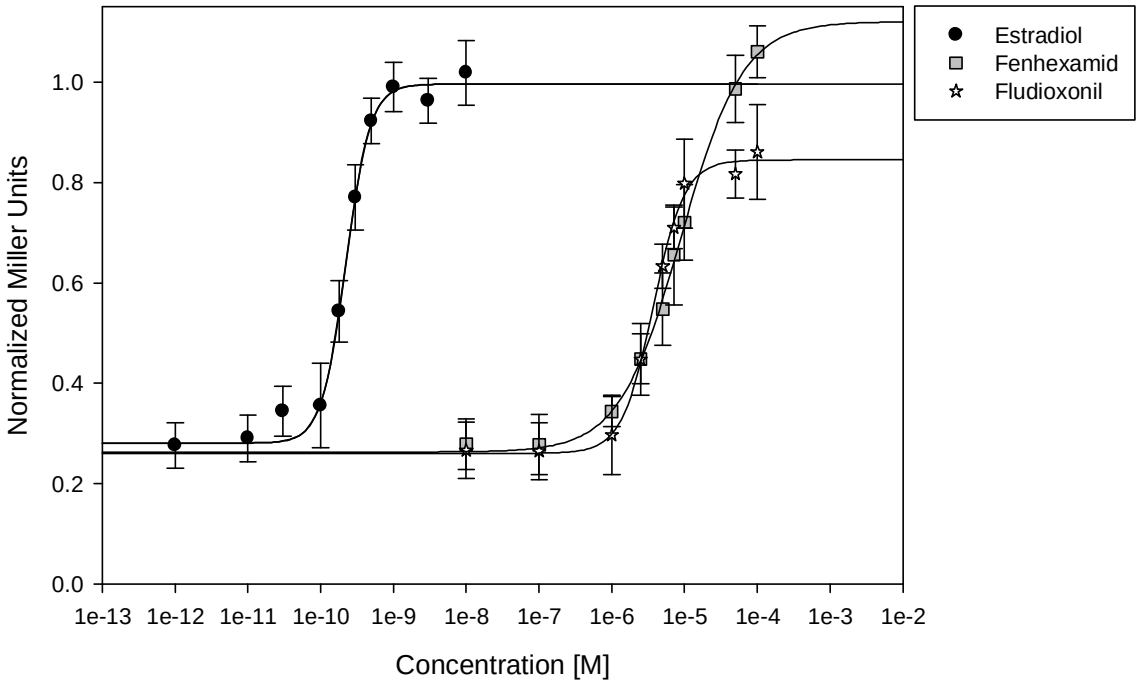

Equivalent estradiol concentration [nmol/g]
60
50
40
30
20
10
0
n.d.
n.d.
n.d.
n.d.
n.d.
n.d.
Acorit
Cantus
Frupica
Reldan
Scala
Steward
Switch
Teldor

Figure 5

(A)


Estradiol
Fenhexamid
Fludioxonil
Normalized Miller Units
0.0
0.2
0.4
0.6
0.8
1.0
1e-13
1e-12
1e-11
1e-10
1e-9
1e-8
1e-7
1e-6
1e-5
1e-4
1e-3
1e-2
Concentration [M]

(B)

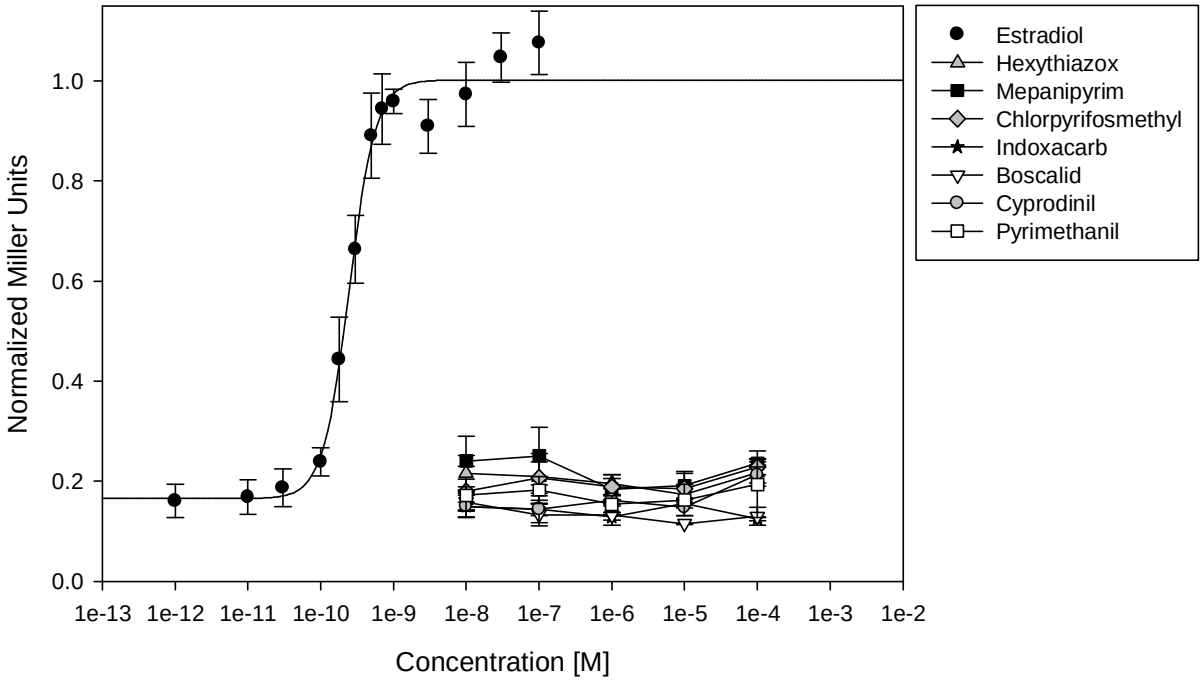

Estradiol
Hexythiazox
Mepanipyrim
Chlorpyrifosmethyl
Indoxacarb
Boscalid
Cyprodinil
Pyrimethanil
Normalized Miller Units
0.0
0.2
0.4
0.6
0.8
1.0
1e-13
1e-12
1e-11
1e-10
1e-9
1e-8
1e-7
1e-6
1e-5
1e-4
1e-3
1e-2
Concentration [M]

Figure 6

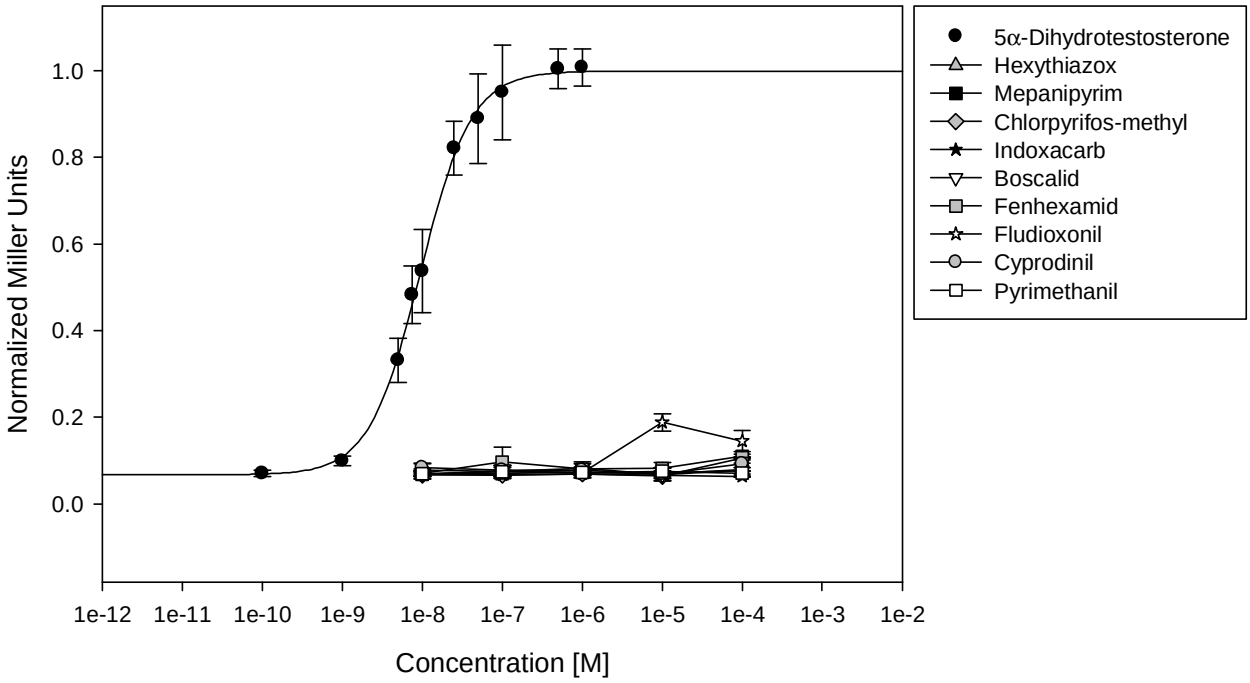
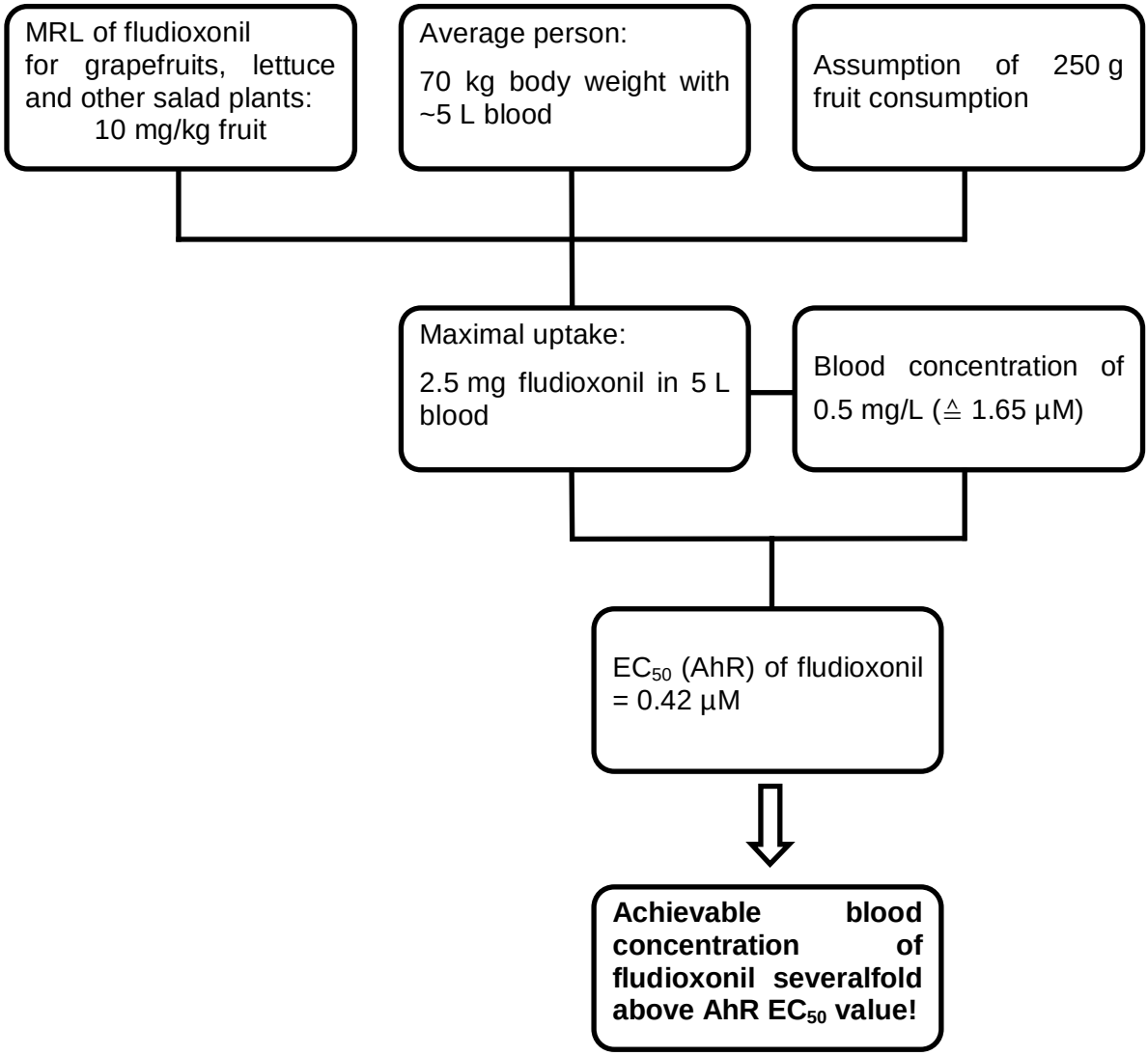

Normalized Miller Units
1.0
0.8
0.6
0.4
0.2
0.0
1e-12
1e-11
1e-10
1e-9
1e-8
1e-7
1e-6
1e-5
1e-4
1e-3
1e-2
Concentration [M]
5α-Dihydrotestosterone
Hexythiazox
Mepanipyrim
Chlorpyrifos-methyl
Indoxacarb
Boscalid
Fenhexamid
Fludioxonil
Cyprodinil
Pyrimethanil

Figure 7


MRL of fludioxonil for grapefruits, lettuce and other salad plants: 10 mg/kg fruit
Average person: 70 kg body weight with ~5 L blood
Assumption of 250 g fruit consumption
Maximal uptake: 2.5 mg fludioxonil in 5 L blood
Blood concentration of 0.5 mg/L (≙ 1.65 µM)
EC50 (AhR) of fludioxonil = 0.42 µM
Achievable blood concentration of fludioxonil severalfold above AhR EC50 value!

Publication III

For submission to the Journal of Nutritional Biochemistry

Indolylfuran a potent aryl hydrocarbon receptor agonist from sauerkraut interacts with estrogen pathway

Svjetlana Medjakovic[a,b], Alfred Zoechling[a,b], Günter Vollmer[c], Oliver Zierau[c], Georg Kretzschmar[c], Frank Möller[c], Susanne Kolba[c], Anja Papke[c], Martina Opietnik[d], Paul Kosma[d], Thomas Rosenau[d], Alois Jungbauer[a,b*]

[a]University of Natural Resources and Applied Life Sciences Vienna, Department of Biotechnology, Vienna, Austria

[b]Christian Doppler Laboratory for Receptor Biotechnology, Vienna, Austria

[c]Molecular Cell Physiology & Endocrinology, Institute of Zoology, TU Dresden, Dresden, Germany

[d]Department of Chemistry, University of Natural Resources and Applied Life Sciences Vienna, Vienna, Austria

*Corresponding author: Alois Jungbauer, Muthgasse 18, A-1190 Vienna, Austria.

e-mail: alois.jungbauer@boku.ac.at, Telephone: +43 1 47654 6226, Fax: +43 1 36 97 615.

Running title: Potent Ah receptor agonist from sauerkraut

Keywords: *Brassicaceae*, anti-estrogen, uterotrophic assay, nuclear receptors, 1-(2-furanyl)2-(3-indolyl)ethanone, cell proliferation

Abstract

Epidemiological studies suggest a correlation between the intake of cruciferous vegetables and decreased cancer risk. These vegetables contain highly bioactive compounds, including ligands of the aryl hydrocarbon receptor. We found a potent activator of the aryl hydrocarbon receptor pathway in sauerkraut juice. The active ingredient was isolated and identified to be the novel AhR ligand, (1-(2-furanyl)2-(3-indolyl)ethanone, synonymous with 2-(Indol-3-yl)acetylfuran), common name indolylfuran. The synthesized compound exerted a similar potency as the isolated indolylfuran; their EC_{50}-values in the aryl hydrocarbon receptor yeast assay were 160 nM and 123 nM, respectively. Hence, this compound transactivates the aryl hydrocarbon receptor about a factor of ~50 more potent than other known indole compounds from cabbage. Our i*n vivo* studies enlightened basic interactions between the aryl hydrocarbon receptor and the estrogen receptors. Also anti-estrogenic effects of sauerkraut extract were shown. Indolylfuran is a regulator of erstrogen receptor α and β expression, most likely via the aryl hydrocarbon receptor pathway since indolylfuran had no effect on uterus weight and did not agonise estrogen receptor α. Here we provide further elucidation for the anti-carcinogenic effects of sauerkraut. Sauerkraut and indolylfuran may have potential for the prevention or treatment of cancer through modulation of aryl hydrocarbon receptor regulated processes and, indirectly, the estrogen receptor pathway.

1 Introduction

Different types of cruciferous vegetables are consumed depending on the geographic area. In Central and Eastern Europe, cabbage and sauerkraut are traditional foods. Indole derivates of glucobrassicin, the main glucosinolate of those vegetables, are reported to be moderate AhR agonists [1-3]. Together with other plant-derived AhR-ligands they are known for anti-estrogenic, anti-proliferative, apoptotic, and cell cycle regulating properties. The AhR is a transcription factor; its induction of the expression of phase I and II enzymes of xenobiotic metabolism has been particularly studied, but it also plays a significant role in normal developmental and cell cycle procedures (as reviewed in [4-9]).

This receptor's interaction in cancer development is not clear. Although several studies show cancer promoting effects after ligand-activation of the AhR [10-13], others report tumor suppression and anti-carcinogenic properties [14-19]. Both scenarios are actually true, which makes sense if the AhR is an important check point in the cell cycle and apoptosis. Improved knowledge and understanding of AhR-dependent mechanisms may lead to cancer treatment or prevention.

The mode of action of the AhR also includes anti-estrogenic effects due to multiple interactions with the ER pathway. This feature may have possible therapeutic applications for the treatment of hormone-dependent diseases. Modified estradiol metabolization [20], that resulted in an AhR ligand concentration-dependent increase of 2-, 4-, 6α and 15α-hydroxylation has been reported. Indole compounds that are also AhR ligands alter estrogen metabolism, so that the resulting metabolite ratio is inverse to that observed in women with breast cancer [21, 22]. Anthropogenic AhR ligands act in a similar manner. The anti-estrogenic properties of TCDD are explainable by the same mechanism [23]. Further AhR/ER interactions arise from a direct decrease of the ability

of ER to bind to the estrogen response element (ERE) on the DNA in the presence of an AhR ligand [24]. The abolishment of estrogen induced gene expression was observed in *in vitro* tests. Treatment of TCDD decreased ERα and vitellogenin (precursor of egg yolk protein) expression in fish hepatocytes [25].

AhR, but not ARNT, interacts directly with ERα in a ligand-specific manner [26]. Klinge et al. [27] also reported that ERα was not able to bind to the xenobiotic response element (XRE) of AhR, whereas the AhR/ARNT heterodimer specifically binds to oligomers containing naturally occurring estrogen response elements (EREs) [27]. The results of Beischlag and Perdew [28] are contradictory to Klinge et al. [27]. They could not find an interaction of ARNT with ERα [28], but report a direct protein-protein interaction of both AhR and ARNT with ERα. Other interaction modi have been postulated that also include the competition for a shared pool of cofactors [29] and the induction of proteasome-dependent degradation of the ER [30].

The AhR has been associated with anti-apoptotic, cell cycle regulating and anti-estrogenic properties. We aimed to evaluate potential AhR activating compounds from edible plants. Here we show, *in vitro* and *in vivo*, that sauerkraut juice is a potent activator of the AhR pathway and exhibits also anti-estrogenic effects in an uterotrophic assay with rats. The novel compound indolylfuran differs in some respects in mode of action and activation of enzymes of the xenobiotic metabolism in comparison to sauerkraut, but it counteracted also estrogen-driven expression of proliferation markers and decreased the expression of the ERs. The differing mode of action between sauerkraut juice and the isolated indolylfuran suggest that although indolylfuran can be regarded as a lead compound in respect to AhR pathway modulation, other compounds could be responsible for the complete mechanism of action that is mediated by sauerkraut.

2 Materials and methods

2.1 Reagents

Dimethyl sulfoxide (DMSO), acetone, hexane, acetonitrile and trifluoroacetic acid were obtained from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). Buffer reagents, N-lauroylsarcosine (sodium salt), o-nitrophenyl-β-galactopyranoside (ONPG), were purchased from Sigma Aldrich (St. Louis, MO). All reagents used for synthesis were of the highest grade available and were commercially available from Sigma Aldrich or Fluka. For yeast media preparation, yeast nitrogen base was obtained from Difco (Franklin Lakes, NJ), amino acids from Serva Feinbiochemica (Heidelberg, Germany) and from Sigma Aldrich (St. Louis, MO). β-Naphthoflavone, was obtained from Sigma Aldrich (St. Louis, MO) and estradiol was purchased from Sigma Aldrich (Deissenhofen, Germany). Sauerkraut juice was purchased in local groceries. After preliminary screening all experiments were performed with “Ja! Natürlich” sauerkraut juice (Wr. Neudorf, Austria).

2.2 Solid phase extraction (SPE)

Juices were centrifuged and then filtered through 0.45-µm and 0.22-µm filter (Millipore). pH was adjusted to 3.5 using H_2SO_4. 5% acetone were added and samples were subjected to solid phase extraction (SPE) using Bakerbond C18 SPE columns (Baker, Phillipsburg, NJ), which were preconditioned with 5 ml acetone, 5 ml hexane, and 5 ml acidified water (pH 3.5). After percolation of the samples, the solid phase was dried using nitrogen and the adsorbed compounds were eluted with acetone. The solvent was then removed under vacuum, and the residues were dissolved in DMSO and stored at 4°C until use.

2.3 Yeast AhR assay (yAhR)

The method was described in detail previously by Medjakovic and Jungbauer [31]. Briefly, the yeast construct contains the human AhR and aryl hydrocarbon receptor nuclear translocator (ARNT) genes integrated in chromosome III. They are under the control of a galactose-regulated promoter and are expressed equally after induction with galactose. Upon AhR-ligand binding, an AhR-ARNT complex forms and is able to bind xenobiotic response elements of the lacZ-reporter plasmid. Induced expression of the lacZ gene can be quantified photometrically.

2.4 Reversed phase HPLC (RP-HPLC)

HPLC-analysis was performed with a Luna C18(2)-column (Phenomenex 100 x 4.6 mm, particle diameter of 3 µm). Eluent A was 5% acetonitrile (AcCN) + 0.1% TFA in water and eluent B was AcCN supplemented with 0.1% TFA. The flow rate was constantly 0.5 ml/min. The following step gradient was used: 5 min, 0% B; 25 min, 0-17.5% B; 25 min, 17.5-50% B; 10 min, 50% B and then up to 90% B for regeneration. The preparative RP-HPLC was performed analogue with a Luna C18(2)-column (Phenomenex 250 x 21.20 mm, particle diameter of 15 µm). The conditions were retained as described for the analytical HPLC with a flow rate of 26.5 ml/min (based on ~0.3 column volume per minute for both HPLC columns).

2.5 NMR spectroscopy

NMR spectra were recorded with Bruker Avance 300 and Avance II-400 spectrometers operating at 300.13 MHz and 400.13 MHz, respectively, for ^{1}H and at 75.47 MHz and 100 MHz, respectively, for ^{13}C NMR. Chemical shifts, relative to TMS as internal standard, are given in ppm, coupling constants in Hz. The signals were assigned by homo- and heteronuclear two-dimensional techniques.

2.6 Synthesis of indolylfuran

An authentic sample of FIE was synthesised according to a biomimetric sequence: condensation of 3-hydroxymethyl-indole with ascorbic acid (vitamin C) and thermal degradation of the resulting ascorbigen intermediate (reaction scheme is shown in Supplemental Figure 1).

2.7 Uterotrophic assay

Juvenile female Wistar/Unilever rats (150 g) (Harlan Winkelmann, Borchen, Germany) were kept under controlled surrounding conditions (20°C, 50-80% humidity, 12h day/night cycle) and had free access to water and fodder (Harlan 2019 Global Rodent, Harlan, Borchen). The Dresden regional council licensed the experiment. All animal handling and experimental conditions were in accordance with the Institutional Animal Care and Use Committee guidelines, regulated by the German federal law for animal welfare.

After an adopting phase of 3 days, animals were ovariectomized. The following 21 days were for recovery and decay of endogenous E2 levels. Six random groups of 6 animals were used for each experiment. Duration of treatment was 3 days. The application of test compounds always took place at the same time of day. Test compounds were applied subcutaneous with caster oil as the carrier substance and DMSO as the solvent vehicle (<10%). The application dosage was

administered in relation to body weight. In the interaction study, the positive control E2 was used at a suboptimal dose of 1 µg/kg BW/d and SKE as a positive control at 100 mg/kg BW/d or FIE at 15 mg/kg BW/d. For the first experiment, 3 animal groups were additionally treated, with SKE (1, 10, and 100 mg/kg BW/d) combined with E2 (1 µg/kg BW/d). For the second experiment, 3 groups were treated with FIE (0.15, 1.5, and 15 mg/kg BW/d) combined with E2 (1 µg/kg BW/d). Statistical analysis was performed with Student's T-test. Significance criteria were *p <0.05, **p<0.01, ***p<0.001 compared to the vehicle-treated control and +p <0.05, ++p<0.01, +++p<0.001 compared to the E2 positive control. Animals were sacrificed 24h after the last subcutaneous treatment by inhalation of CO_2, after light anesthesia by O_2/CO_2 inhalation. Uteri were dissected, the wet weights determined, and snap frozen in liquid nitrogen for RNA preparation.

2.8 Gene expression analysis in the uteri

Complete cytoplasmatic uterine RNA was extracted by the standard TRIzol® method (Life Technologies) and quantified photometrically. DNA-contamination was removed enzymatically with deoxyribonuclase I (Ambion). Quantitative realtime PCR (qPCR) was performed with Platinum® Taq DNA polymerase (Life Technologies) and an iCycler Thermocycler System (Biorad). PCR reactions were performed at least 3 times in triplicate. After the first denaturating cycle at 95°C for 3 min, 50 reaction cycles followed at 95°C for 10 sec, 60°C for 15 sec, and 72°C for 30 sec. Primer sequences are summarized in Supplemental Table 1.

3 Results

3.1 Sauerkraut transactivates hAhR in vitro

During a wide screening to identify plant extracts and vegetable/fruit juices able to transactivate the human AhR, we found in sauerkraut juice an activator of the AhR pathway. Different commercially available sauerkraut juices were tested in the yeast AhR assay (yAhR) to identify their transactivational potential. All raw sauerkraut juices activated the AhR in an agonistic manner without further pre-concentration. The juices exhibited similar activity as β-naphthoflavone, which has an EC_{50}-value of 10 nM (Supplemental Fig. 2). A solid phase extraction (SPE) of sauerkraut juice was conducted to enrich the hydrophobic compounds. This extract also transactivated the AhR. The SPE enrichment was linear since the 1:100 dilution of the 100-fold concentrate was in the same range as the raw juice.

3.2 Isolation and characterization of indolylfuran

Sauerkraut juice extract (concentrated 100-fold by SPE) was separated by analytical RP-HPLC (C 18 (2) column). Fractions (2 minute intervals) were collected to separate the different compounds and to determine AhR active ones. The fractions were dried in the speed-vac, dissolved in DMSO, and AhR activity was measured with the yeast assay. Fractions with longer retention times (45 to 55 minutes) showed higher AhR activity. The transactivational activity of the fractions is shown in Supplemental Fig. 3. The highest activity was measured in the 52 to 54 minute fraction, which coincides with the highest peak in the chromatogram.

The analytical separation procedure was scaled-up to a preparative method using a ~50 times bigger C18-column, the eluate of the active fraction was subjected to NMR-analysis and identified as indolylfuran (1-(2-furanyl)2-(3-indolyl)ethanone, synonymous with 2-(Indol-3-yl)acetylfuran), (see Supplemental Fig. 4). Indolylfuran (CAS No. 413599-89-0) has an indole structure, with an empirical formula of $C_{14}H_{11}NO_2$ and thus a molecular weight of 225.24 g/mol. To our knowledge, this compound was mentioned only once as a side product of a synthesis paper [32], but its bioactivity has never been investigated.

The synthesized indolylfuran occurs in equilibrium of a keto and an enol form (Fig. 2a). It had a similar potency as the isolated compound in the yAhR; the EC_{50}-values were 160 nM and 123 nM, respectively (Fig. 2b). In comparison, other indole compounds from cabbage, such as 3,3'-diindolylmethane and indole-3-carbinol, exert potencies of 1 and 6 µM and are up to a factor of 50 less potent AhR-agonists [31]. Indolylfuran can be considered the potent lead compound.

3.3 In vivo *effects of sauerkraut extract*

Sauerkraut juice extract (abbreviated SKE, concentrated 200-fold by SPE) was tested for its anti-estrogenicity in an uterotrophic assay with ovariectomized Wistar/Unilever rats. We were able to confirm SKE clearly as AhR-agonist *in vivo*, since it stimulated expression of AhR-dependent genes that encode for phase I (CYP1A1) and phase II (glutathione *S*-transferase, GST-Ya) enzymes in the uterus. The interaction of the extract and estradiol (E2) was observed on physiology level and gene expression level. SKE did not stimulate proliferation, but rather showed a strong anti-estrogenic trend, which was also significant when E2 was combined with the highest FIE dosage. Not only did proliferation of the E2-stimulated uterus decrease dose-dependently by the addition of SKE, but the extract alone caused no proliferation (Fig. 3a). In

contrast to E2-triggered enhanced expression of the proliferating marker, SKE alone slightly, but significantly, reduced expression of the proliferating cell nuclear antigen (PCNA). This effect was not seen with the combination treatment of E2 and SKE (Fig. 3b). To investigate the effect of SKE on the ER pathway, the expression of E2-regulated marker genes was observed. E2 treatment caused strong up-regulation of the complement C3 gene and down-regulation of clusterin. SKE alone also produced a significant increase of C3 mRNA, but to a much smaller extent than E2; while SKE up-regulated the mRNA level two-fold, E2 caused an up-regulation of about a factor of 1200. The effect of SKE alone was also significantly smaller in comparison to E2 as a positive control (Fig. 3c). SKE exerted a significantly contrary effect to E2 on the expression of clusterin. As expected, E2 down-regulated clusterin expression, but SKE had no significant effect when compared to the vehicle-treated control (Fig. 3d). Nevertheless, the addition of SKE did not alter the effects of E2 on expression of clusterin or C3, which indicates SKE does not necessarily impact estrogen response element (ERE)-independent functions of estrogen action.

ERα mRNA data from the uterus were not conclusive. It seemed there was no significant effect at all (Fig. 3e). This was also true for SKE alone and ERβ expression, although here, two combinations of SKE and E2 led to a reduction of ERβ expression (Fig. 3f). In the uterus, SKE clearly showed its ability to transactivate the AhR and to induce the expression of CYP1A1 and GSTYa (Fig. 3g and Fig. 3h), while E2 down-regulated the expression of these genes.

3.4 In vivo *effects of indolylfuran*

The pure compound indolylfuran was examined using the uterotrophic test described above. Treatment with FIE had no effect on the body weight of the rats (data not shown). Weights of the uterus and liver also remained unchanged, with the exception of one treatment group; the

combination of E2 and 1.5 mg/kg BW/d FIE caused a slight but significant decrease in liver weight (Supplemental Fig. 5).

To examine possible effects on proliferation, the mRNA expression of two proliferation markers, PCNA and the antigen identified by monoclonal antibody Ki-67 (Ki-67), was evaluated with quantitative real time PCR. Expression of the markers would implicate proliferating effects. A 3-day subcutaneous treatment of ovariectomized Wistar/Unilever rats with E2 caused a 7-fold increase in the expression of Ki-67 in the uterus, compared to the control. FIE alone had no statistically significant effect on Ki-67 expression and was in the same range as the vehicle-treated control. FIE was able to decrease E2-induced Ki-67 expression dose-dependently. This effect was statistically significant for the higher dosages of FIE (1.5 and 15 mg/kg BW/d) in combination with E2 (1 µg/kg BW/d) and in comparison to the E2 positive control and the vehicle-treated control (Fig. 4a). Treatment of FIE alone had no significant effect on PCNA mRNA levels. In combination with E2, a decrease was observed, although no clear correlation with dosage could be found. Two of the 3 combination treatments of FIE and E2 showed a significant decrease compared to the vehicle-treated control group, but not in comparison to the E2 positive control (Fig. 4b).

As expected, expression of ERα and ERβ was strongly and significantly down-regulated following E2-treatment. Treatment with FIE also caused a decrease in the mRNA levels of both ERs, but the regulation was not so distinctive. The combined treatment of E2 and FIE showed no additive effects (Fig. 4c and Fig 4d). The effect on the ER-pathway, in terms of expression of ER marker genes C3 and clusterin was similar to that observed for SKE. FIE had no effect on clusterin mRNA levels and when combined with E2, no alteration of the E2-regulation was

observed (Fig. 4e). Similarly, FIE alone produced only a slight increase of C3 mRNA and there was no effect when combined with E2 (Fig. 4f).

As FIE was an AhR-ligand *in vitro*, its impact on the expression of AhR and its dimerization partner ARNT was evaluated. E2 decreased mRNA levels of AhR and ARNT2 strongly. The effect on ARNT1 was milder. FIE also decreased AhR, ARNT1, and ARTN2 mRNA levels, but to a lesser extent than E2. No additive or synergistic effect was observed when E2 and FIE were combined, but both E2 and FIE modulated the AhR pathway at the gene expression level (Fig. 5a, Fig 5b and Fig. 5c). Finally, FIE's ability to trigger transactivation of the AhR pathway *in vivo* was examined. For this, the expression of AhR-dependent genes, that encode for phase I enzyme CYP1A1 and phase II enzyme GSTYa, was measured. We confirmed FIE to be an AhR-agonist *in vivo*. Yet, unlike sauerkraut juice extract, FIE was not able to induce the expression of phase I and phase II enzymes simultaneously. Only CYP1A1 was induced significantly; the mRNA levels were 5-fold higher than the control (Fig. 5d and Fig 5e).

4 Discussion

Epidemiological studies inversely link cancer risk with the intake of cruciferous vegetables. A moderate decrease in cancer risk, approximately 30-40%, has been observed; although, data are not consistent. While these reports are discordant, many research articles provide mechanistic evidence how crucifers could provide protective effects from cancer. Vegetables that are consumed as food should not have a great influence on physiological processes involved in the cell cycle and the endocrine system. Otherwise, they would be medicine by definition and not food. Nevertheless, compounds from moderately bioactive food hold great potential for cancer therapy. Manifold active ingredients of cruciferous vegetables, especially isothiocyanates and

indoles, have been studied intensively and anti-estrogenic, anti-angiogenic, anti-tumorigenic, and anti-carcinogenic effects have been reported [33-35].

We identified sauerkraut as an activator of the human AhR. Indole compounds derived from white cabbage (*Brassica oleracea* var *capitata*) are known to act as moderate AhR-ligands. Even so, the high AhR-transactivational potential of commercially available raw juice from fermented cabbage is new. Moreover, we have isolated a novel compound, named indolylfuran, from sauerkraut juice that is a potent AhR-agonist. *In vitro*, indolylfuran has an AhR EC_{50}-value of ~160 nM. It is only a factor of ~10 less potent than the reference compound β-naphthoflavone and up to 50-fold more potent than previously described indole compounds derived from sauerkraut, such as indole-3-carbinol or 3,3'-diindolylmethan. This could be due to the planar structure of indolylfuran provided by its keto-enol tautomerism. Coplanarity is the preferred structure of the AhR ligand binding domain and common in potent AhR-ligands [36].

Currently, we can just assume that this compound could elicit AhR-dependent anti-proliferative effects, as they have been reported for this activated pathway and for other non-toxic AhR-agonists. The AhR cascade regulates various cell cycle-regulators, such as Akt, p21, p27, p53, Bax, RelB, and NFκB towards apoptosis [14, 19, 37-42]. These effects were also mediated by the AhR-agonists 3,3'-diindolylmethane (DIM) and indole-3-carbinol (I3C), that are components of cruciferous vegetables [43-45]. The decreased expression of AhR, ARNT1, and ARNT2 due to indolylfuran could be utilized for cancer treatment; ARNT2 is especially of interest. It could facilitate indolylfuran anti-tumorigenicity independently of the AhR. In comparison to ARNT1, ARNT2 shows a decreased ability to induce AhR-dependent gene expression when complexed with AhR [46, 47], while both ARNT1 and ARNT2 regulate the hypoxia inducible factor 1α (HIF1α) and its regulatory signal pathway equally well [45]. The HIF1α pathway would provide

an anti-tumorigenic mechanism for indolylfuran, as decreased ARNT2 levels would also reduce functionally active HIF1α/ARNT2 dimers. HIF1α levels are elevated in many tumors and linked to therapy-resistance and poor prognosis, since it leads to angiogenesis and cancer metastasis. HIF1α has already been discussed as a cancer drug target (reviewed in [48-50]) and different approaches have been investigated to utilize HIF1α inhibitors for cancer therapy [51-53]. Indolylfuran could inhibit angiogenesis in tumors by decreasing ARNT2. *In vivo* studies with TRAMP-mice showed inhibition of prostate tumor metastasis by sAhRMs via AhR-dependent mechanisms that inhibit vascular endothelial growth factor (VEGF) transcriptionally regulated by the HIF1α pathway [15, 54].

In addition to modulation of xenobiotic metabolism, AhR is known to cross-talk with the estrogen pathway in manifold ways and exert (anti-)estrogenic effects. This has been observed at gene expression level [24, 27, 55], as well as the protein level [28, 56] and i*n vivo* [57, 58]. This interaction may allow the AhR to be utilized in the treatment of hormone-dependent cancers. An attempt has been made by Safe and co-workers [15, 17, 34, 59], who searched for selective AhR modulators (sAhRMs) that could aid therapy for breast or prostate cancer.

We confirmed the AhR-agonistic abilities of sauerkraut extract and indolylfuran *in vivo*; although differences in their modes of action were resolved, which allows us to conclude that further potent active ingredients in sauerkraut juice may exist. Sauerkraut extract showed anti-estrogenic properties in the uterotrophic assay and abrogated E2-induced uterus proliferation. Application of sauerkraut without E2 had no effects on the uterus. Indolylfuran did not directly show anti-estrogenic properties, but counteracted E2-triggered expression of the proliferating marker Ki67 and decreased ERα and ERβ expression like E2, but to a milder extent.

As safety demands, bioactive compounds from extracts that could cause adverse effects on the endocrine system are hotly debated. Often, the uterotrophic assay is utilized to determine risk potential in terms of estrogenic effects. In this regard, indolylfuran can be considered a safe compound, as it did not affect uterus proliferation.

The present findings indicate the newly found AhR-agonist, indolylfuran, could modulate AhR and ER pathways. Further investigation is needed to determine whether it can be used as a sAhRM for the treatment or prevention of cancer, particularly hormone-dependent cancers.

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Figure legends

Figure 1. RP-HPLC analysis of sauerkraut juice extract. Sauerkraut juice was concentrated 100-fold with C18 reversed phase SPE before analysis. The fraction with the highest AhR-transactivational potential is circled.

Figure 2. Structure of indolyfuran and its transactivational AhR-activity. a, Keto-enol tauterism of indolylfuran calculated by the Spartan 04' programme. **b,** *In vitro* transactivational activity of synthesised and isolated indolylfuran in the yAhR compared to the reference compound β-naphthoflavone.

Figure 3. Effect of sauerkraut extract on ovariectomised Wistar/Unilever rats after 3-day treatment. a, Effect of uterine wet weight. **b,** Relative mRNA levels of the proliferation marker PCNA. **c,** Relative mRNA levels of C3. **d,** Relative mRNA levels of clusterin. **e,** Relative mRNA levels of ERα. **f,** Relative mRNA levels of ERβ. **g,** Relative mRNA levels of CYP1A1. **h,** Relative mRNA levels of GSTYa. *p <0.05, **p<0.01, ***p<0.001 compared to the vehicle-treated control and +p <0.05, ++p<0.01, +++p<0.001 compared to the E2 positive control.

Figure 4. Effect of indolylfuran on gene expression in Wistar/Unilever rats after 3-day treatment. a, Relative mRNA level of Ki-67. **b,** Relative mRNA level of PCNA. **c,** Relative mRNA levels of ERα. **d,** Relative mRNA levels of ERβ. **e,** Relative mRNA levels of clusterin. **f,** Relative mRNA levels of C3. *p <0.05, **p<0.01, ***p<0.001 compared to the vehicle-treated control and +p <0.05, ++p<0.01, +++p<0.001 compared to the E2 positive control.

Figure 5. Modulating effects of indolylfuran on the AhR-pathway in Wistar/Unilever rats after 3-day treatment. a, Relative mRNA levels of the *AhR* gene. **b,c,** Relative mRNA levels of AhR dimerisation partner *ARNT* genes (*ARNT1* and *ARNT2* homologues). **d,e,** Relative mRNA levels of AhR-regulated target genes *CYP1A1*and *GSTYa*. *p <0.05, **p<0.01, ***p<0.001 compared to the vehicle-treated control and +p <0.05, ++p<0.01, +++p<0.001 compared to the E2 positive control.

Figure 1

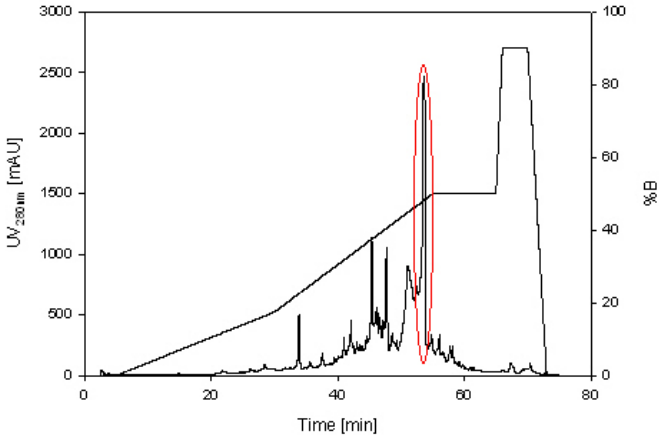
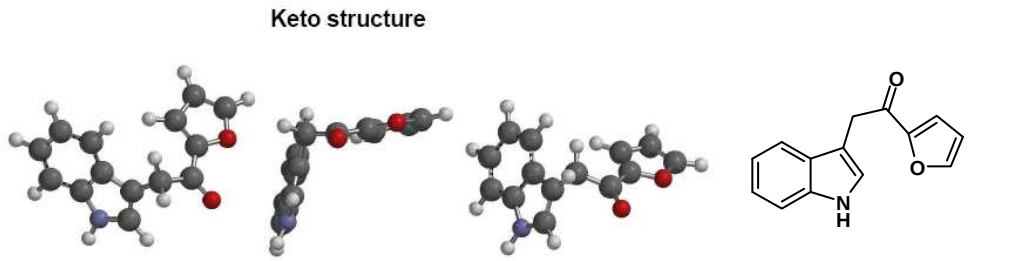

3000
2500
2000
1500
1000
500
0
UV280nm [mAU]
100
80
60
40
20
0
%B
0
20
40
60
80
Time [min]

Figure 2

a



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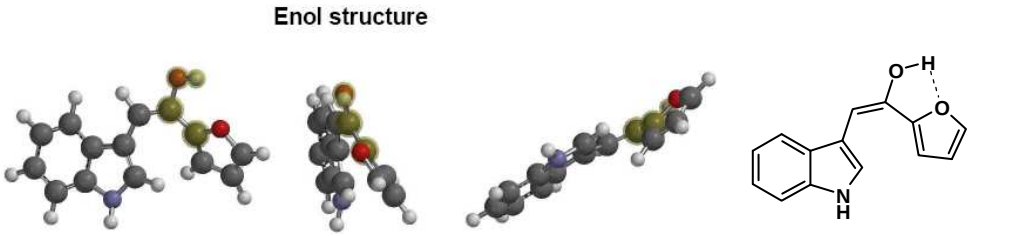

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b

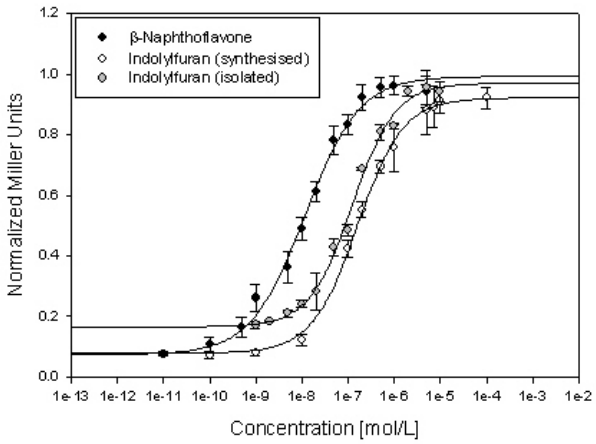


Figure 3

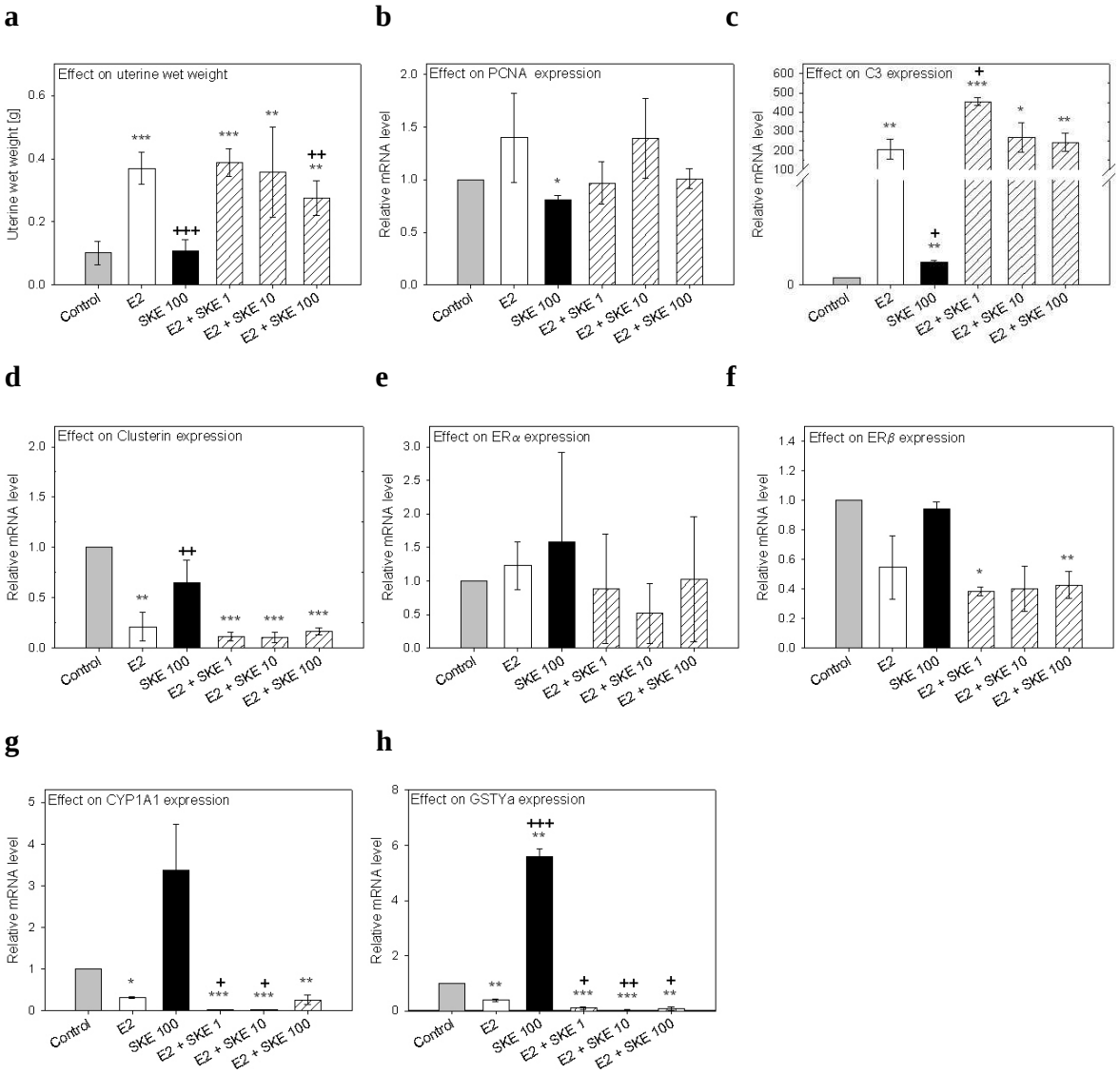

a
Effect on uterine wet weight
Uterine wet weight [g]
b
Effect on PCNA expression
Relative mRNA level
c
Effect on C3 expression
Relative mRNA level
d
Effect on Clusterin expression
Relative mRNA level
e
Effect on ERα expression
Relative mRNA level
f
Effect on ERβ expression
Relative mRNA level
g
Effect on CYP1A1 expression
Relative mRNA level
h
Effect on GSTYa expression
Relative mRNA level
Control
E2
SKE 100
E2 + SKE 1
E2 + SKE 10
E2 + SKE 100

Figure 4

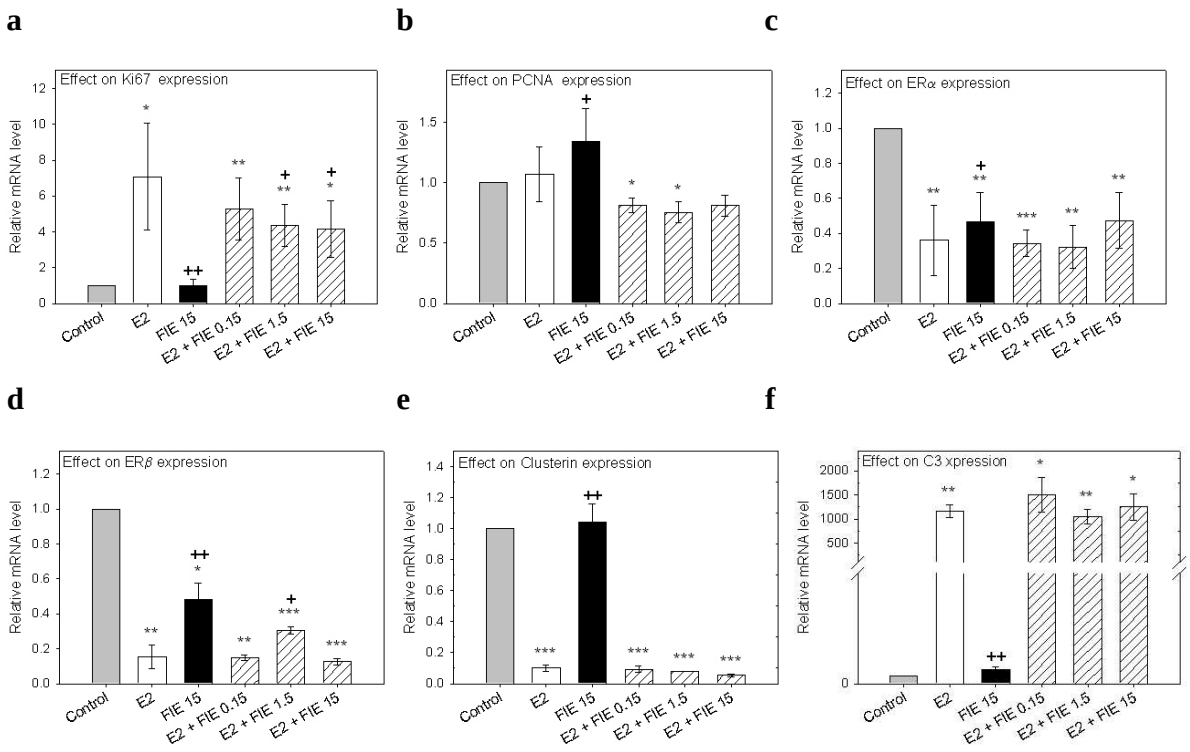
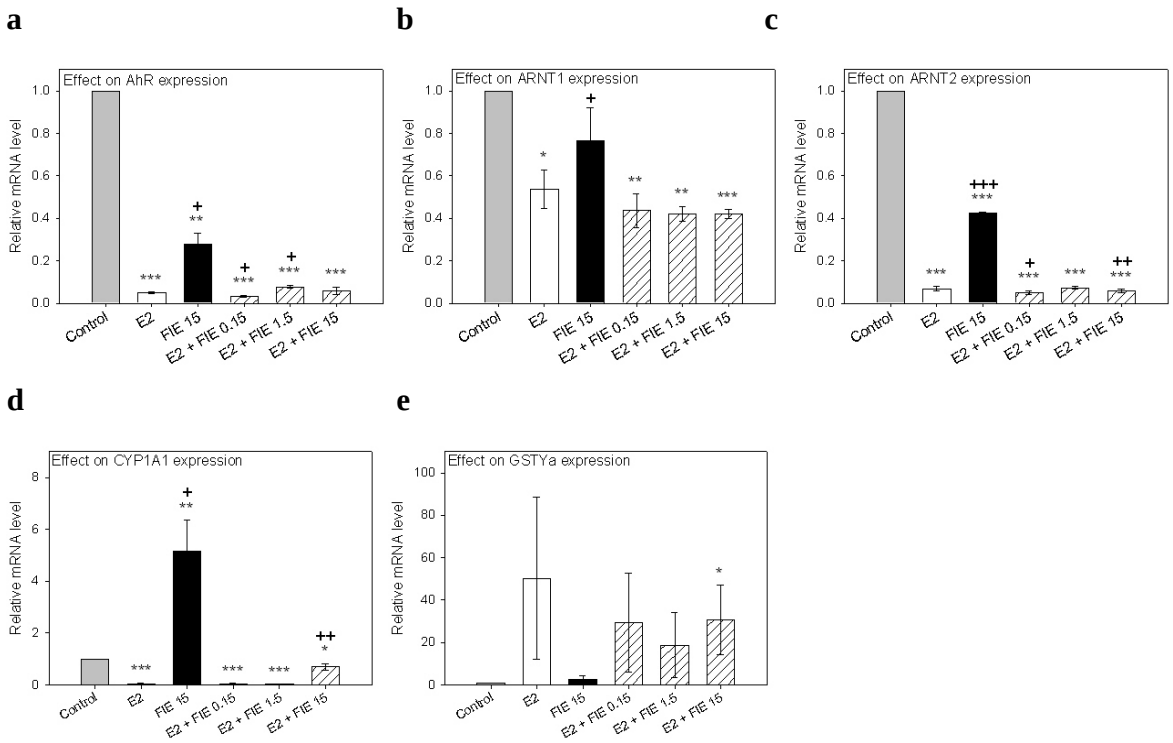
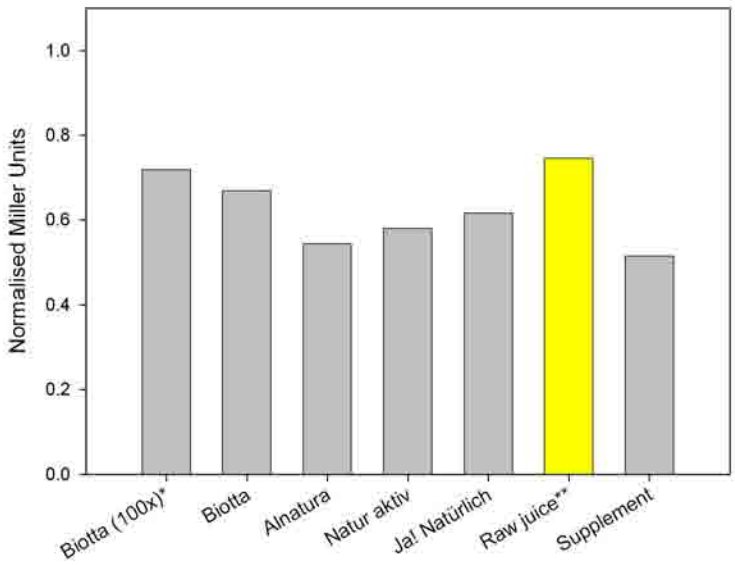

a
Effect on Ki67 expression
Relative mRNA level
b
Effect on PCNA expression
Relative mRNA level
c
Effect on ERα expression
Relative mRNA level
d
Effect on ERβ expression
Relative mRNA level
e
Effect on Clusterin expression
Relative mRNA level
f
Effect on C3 xpression
Relative mRNA level
Control
E2
FIE 15
E2 + FIE 0.15
E2 + FIE 1.5
E2 + FIE 15

Figure 5

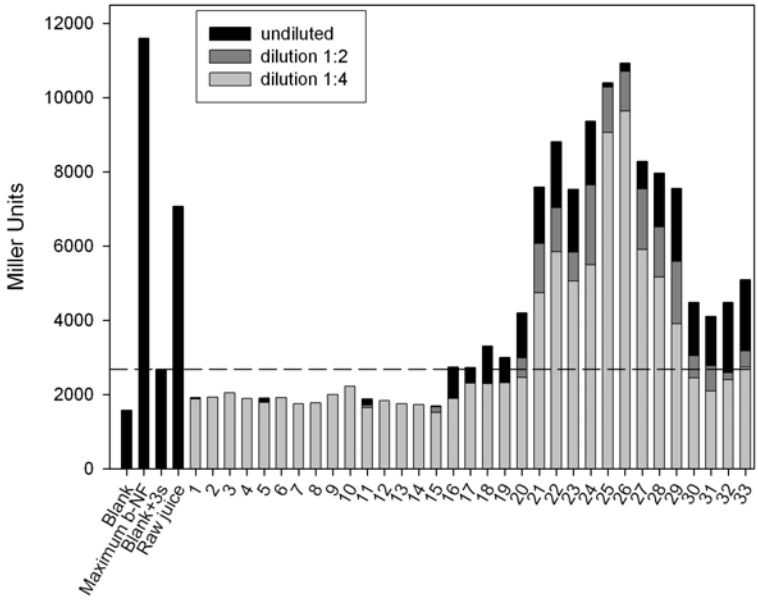

a
Effect on AhR expression
Relative mRNA level
b
Effect on ARNT1 expression
Relative mRNA level
c
Effect on ARNT2 expression
Relative mRNA level
d
Effect on CYP1A1 expression
Relative mRNA level
e
Effect on GSTYa expression
Relative mRNA level
Control
E2
FIE 15
E2 + FIE 0.15
E2 + FIE 1.5
E2 + FIE 15

Supplementary Figures and Tables

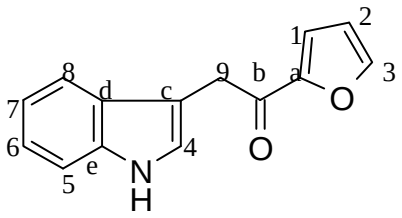
Figures



Supplementary Figure 6. Activity of various sauerkraut juices and a dietary Sauerkraut supplement. Transactivational activity of sauerkraut juices from different commercially available brands (©Biotta, Alnatura, Natur aktiv, Ja! Natürlich) are displayed. Biotta (100x)* is a 1:100 diluted SPE concentrate of a 100fold enriched extract. Its activity is in the same range as the raw juice from Biotta. Raw juice** is the basic raw material from Ja! Natürlich that was further processed and employed for the isolation of indolylfuran. Additionally a dietary supplement based on Sauerkraut juice powder was extracted with DMSO (0.25 g/mL) and tested in the yAhR. Normalised Miller Units are Miller Units related to the maximum of β-naphthoflavone response. Potency of the reference compound β-naphthoflavone (10nM) causes 50% of the maximum response, here normalised to 0.5.



Supplementary Figure 7. Transactivating potential of the HPLC fractions of sauerkraut juice on the AhR. Fractions collected every 2 minutes (fraction number multiplicated with 2 = retention time). Maximum b-NF is the maximum response of the reference compound β-naphthoflavone (5 µM) in the yAhR. The blank was conducted with DMSO.



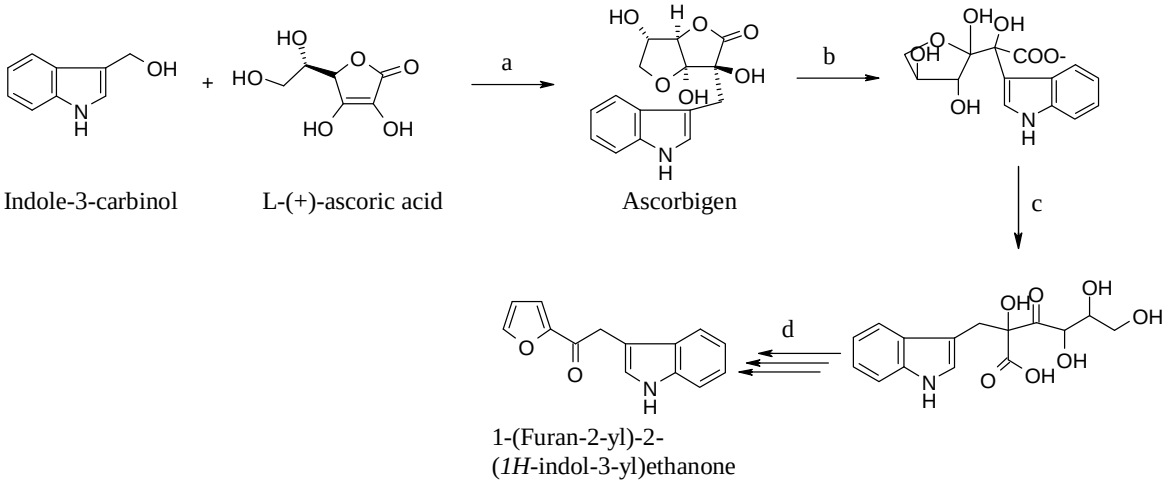
I

^{1}H NMR (DMSO, 300 MHz): δ 4.21 (9), 6.71 (2), 6.97 (7), 7.06 (6), 7.27 (4), 7.34 (5), 7.51 (8), 7.60 (1), 7.98 (3), 10.94 (NH).
^{13}C NMR (DMSO, 75.5 MHz) δ 35.07 (9), 107.20 (c), 111.37 (5), 112.44 (2), 118.48, 118.57 (8,7), 118.89 (1), 121.01 (6), 124.32 (4), 127.15 (d), 136.05 (e), 147.72 (3), 151.46 (a), 186.10 (b).

II

^{1}H NMR (CD_3OD, 400 MHz): δ 4.245 (s, 2H, 9), 6.6004 (q, J = 1.8 Hz, 1H, 2), 7.0009 (dt, J = 1-1.1, 7.5 Hz, 1H, 7), 7.0876 (dt, J = 1.2-1.3, 7.6 Hz, 1H, 6), 7.1748 (s, 1H, 4), 7.3324 (dd, J = 0.9, 8.1 Hz, 1H, 5), 7.406 (dd, J = 1, 3.6 Hz, 1), 7.5281-7.5602 (m, 1H, 8), 7.7594 (q, J = 1.6 Hz, 1H, 3).
^{13}C NMR (CD3OD, 100 MHz) δ 47.788 (9), 101.401 (c), 112.297 (5), 113.494 (2), 119.529 (8), 119.829 (1), 119.938 (6), 122.513 (7), 124.959 (4), 128.655 (d), 138.01 (e), 148.616 (3), 153.518 (a), 189.429 (b).

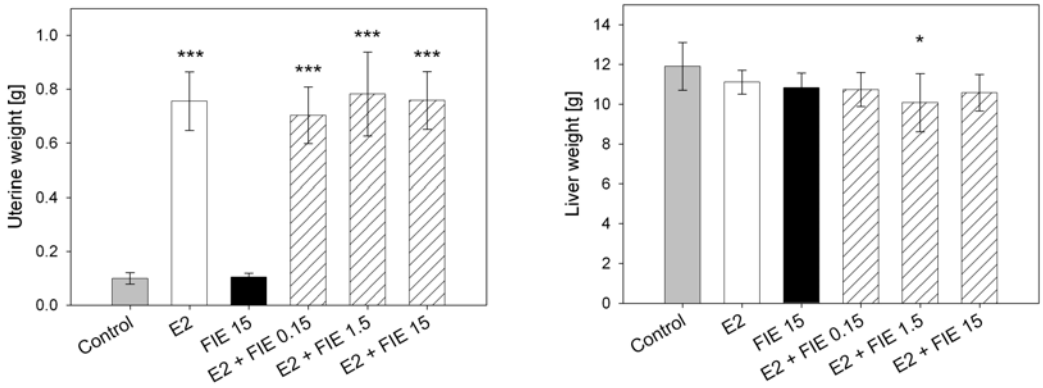
Supplementary Figure 8. NMR data assignment of 1-(2-furanyl)2-(3-indolyl)ethanone. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz on a Bruker Avance 300 spectrometer or Bruker Avance II 400 spectrometer. I: NMR data of isolated 1-(2-furanyl)2-(3-indolyl)ethanone from Sauerkraut juice extract. II: NMR data of synthesised 1-(2-furanyl)2-(3-indolyl)ethanone.



a. McIlvaine buffer (pH4), rt, over night, 51%. b. NEt_3, CH_2Cl_2 - MeOH, rt, 30 min;
c. acidification with 2N HCl until pH 2; c. heating up to 50°C, 4.2%.

Supplementary Figure 9. Scheme of indolylfuran synthesis.

a **b**



Supplementary Figure 10. Effect of indolylfuran on organ weight (mg/g body weight) after a 3-day treatment. Positive controls E2 (1 µg/kg BW/d) and indolylfuran (15 mg/kg BW/d). 3 treatment groups of indolylfuran (0.15; 1.5 and 15 mg/kg BW/d) in combination with E2 (1 µg/kg BW/d). *p<0.05, ***p<0.001 compared to vehicle-treated control. **a**, Relative uterus weight of ovariectomised Wistar rats in comparison to vehicle-treated control and positive controls. **b**, Relative liver weight of ovariectomised Wistar rats.

Tables

Supplementary Table 1. Primer sequences of the semiquantitative real time PCR experiments. Abbreviations: bp=base pair, fwd=forward, rev=revers, r=rat, 1A=subunit of cytochromoxidase.

Gene	Position cDNA (bp)	Sequence	Melting point
r1A	fwd-73	5´-tga gca gga ata gta ggg aca gc-3´	86°C
	rev-333	5´-gag tag aaa tga tgg agg aag ca-3´	
rKi-67	fwd-8673	5´-aac cag gac ttt gtg ctc tgt aa-3´	89°C
	rev-8881	5´-ctc ttt tgg ctt cca ttt ctt c-3´	
rPCNA	fwd-432	5´-gag caa ctt gga atc cca gaa cag g-3´	88°C-88.5°C
	rev-589	5´-cca agc tcc cca ctc gca gaa aac t-3´	
rERα	fwd-1019	5´-gga agc aca agc gtc aga gag at-3´	90°C
	rev-1401	5´-aga cca gac caa tca tca gga t-3´	
rERβ	fwd-1898	5´-cta cag aga gat ggt caa aag tgg a-3´	89.5°C-90°C
	rev-2113	5-ggg caa gga gac aga aag taa gt-3´	
rC3	fwd-3529	5´-aca gcc ttc ccg gga gca tca aca-3´	91.5°C-92°C
	rev-3804	5´-agc gca cca cag gag gca cag agt c-3´	
rAhR	fwd-4005	5´-caa cca aac caa aca aca gag a-3´	82°C
	rev-4201	5´-atc cat cat ctt tca acc cat c-3´	
rARNT1	fwd-2112	5´-act gct gcc tac cct act ctt c-3´	88.5°C
	rev-2303	5´-gct act tgg ttg tgc tga tgt t-3´	
rARNT2	fwd-3761	5´-tga aag aag gag aag ccc aat a-3´	88°C
	rev-3961	5´-cat cag agt tat gcc gag aca g-3´	
rGST-Ya	fwd-247	5´-gcc agc ctt ctg acc tct tt-3´	86.5°C
	rev-408	5´-gct ccg cta aaa ctt gaa aat c-3´	
rCYP1A1	fwd-702	5´-tta tga cca cga tga cca aga g-3´	84°C
	rev-912	5´-gcc ctt ctc aaa tgt cct gta g-3´	

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Isoflavones are safe compounds for therapeutical applications – Evaluation of *in vitro* data

Keywords: isoflavones, *in vitro*, metabolism, cancer, ER, AhR, PPAR

Evelyne Reiter[1,2#], Verena Beck[1#], Svjetlana Medjakovic[1,2#] and Alois Jungbauer[1,2*]

1) Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria

2) Christian-Doppler-Laboratory of Receptor Biotechnology, Muthgasse 18, 1190 Vienna, Austria

[#] These authors have contributed equally to this work.

[*]Corresponding author:
Alois Jungbauer
Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria
Telephone: 0043 1 360066226
Fax: 0043 1 3697615
E-mail: alois.jungbauer@boku.ac.at

Abstract

Isoflavone-rich food and food supplements have gained increasing popularity also in the Western world. Their weak estrogenic effect has been considered as a potential risk, although all epidemiological studies and clinical trials show a significant cancer protection and decreased risk of cardiovascular diseases. *In vitro* data suggest that the concerted action of the isoflavones and their metabolites show antiproliferative behavior, reduce angiogenesis, reduce tumor progression and exert antiinflammatory effects. For the evaluation of the biological effects, special emphasis has to be put on the concerted action between the isoflavones and their metabolites. For instance, while isolated genistein shows some growth promoting effect at low concentrations, the metabolite equol or soy extract show growth retardation as well as higher concentrations of genistein do. The isoflavones have multiple affinities to other members of the steroid hormone receptor superfamily. The beneficial effect on metabolic diseases and weight reduction by isoflavone consumption can be partly explained by its affinity for the PPAR family. In light of the *in vitro* experiments, together with the epidemiological observations and the clinical experience, isoflavones can be considered as safe compounds and their consumption as food and food supplements has to be promoted.

1. Introduction

Beneficial health effects of isoflavones are known for a long time from epidemiological studies from Japan. A lot of these effects have been attributed to the high soy consumption, in particular to the intake of isoflavones. The health benefits of an isoflavone-rich diet are out of question and have been supported by numerous clinical investigations including a recently published nested case-control study. This study shows a decreased breast cancer risk with increased plasma isoflavone level [1] and confirms a lot of previous studies.

Isoflavone-rich food supplements have been developed from soy and red clover. These extracts have shown clinical benefit for the amelioration of menopausal complaints. Clinical efficiency has become significant with an isoflavone intake of 80 mg per day which corresponds to the estimated average isoflavone intake of soy-consuming Japanese women.

The main isoflavones present in soy and red clover are daidzein, genistein, glycitein, formononetin and biochanin A. In plants they are present as conjugates or free aglycones. It seems that the aglycones are more rapidly absorbed although the conjugated form is bioavailable too.

Despite all these clinical and epidemiological experience, it has been questioned if isoflavones are safe compounds. The concern mainly arises from the fact that in certain *in vivo* experiments a growth promoting effect on ER-positive cancer cells has been observed.

Here we try to give an overview on *in vitro* data in context of evaluation of isoflavones as possible compounds promoting cancer growth. We will review at the molecular level mainly the receptor binding properties, effects on steroid synthesis and then focus on isoflavones in cancer models. Also other effects will be discussed such as preventing effects on osteoporosis, metabolic diseases and inflammation, although they are exclusively considered as beneficial health effects.

2. *In vitro* evidence for beneficial effects of phytoestrogens

Affinity of phytoestrogens to ERα and ERβ

The steroid-thyroid receptor superfamily

A lot of polyphenols have an affinity for the estrogen receptors α (ERα) and β (ERβ). Thus they are known as phytoestrogens, although only a small part of their biological action is based on ER interaction. The estrogen receptors are part of a large group of ligand activated transcription factors, the nuclear receptor superfamily, and share structural and functional similarities with other members of this group such as the progesterone receptor (PR), androgen receptor (AR) or the glucocorticoid receptor (GR).

Architecture of estrogen receptors

The estrogen receptors consist of six functional domains (Figure 1). The N-terminal A/B-region is highly variable and contains a ligand-independent activation function 1 (AF-1) which is responsible for the interaction with components of the transcriptional machinery and with coactivators. The DNA binding domain (DBD), also called C-domain, which contains two zinc fingers responsible for DNA recognition, is highly conserved among species. Downstream of the DBD the D-domain, also called hinge region is located. The variable ligand binding domain (LBD or E-region) is quite large and is responsible for various functions: ligand binding, dimerization, nuclear localization, and heat-shock protein association. Furthermore the LBD contains the ligand-dependent activation function 2 (AF-2) which is responsible for transactivation. For the C-terminal F-region no specific function could be found yet, but it is suspected to contribute to the transactivation capacity of the receptor.

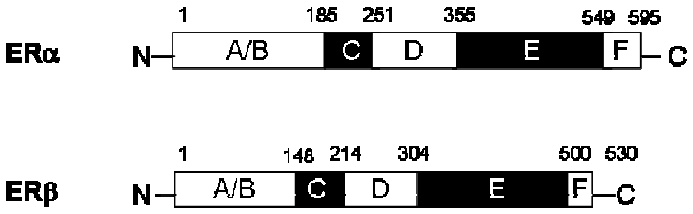

Figure 1: Schematic drawing of the domain structure of estrogen hormone receptors. A/B: N-terminal region containing AF-1, C: DNA Binding Domain (DBD), D: variable hinge region, E: Ligand Binding Domain (LBD) containing AF-2, F: C-terminal region, ERα: estrogen receptor alpha, ERβ: estrogen receptor beta.

Biological significance

The classical mode of estrogen receptor (ER) action is the genomic pathway: in their inactive state ERs are located in the cytoplasm of the cell in association to a multiprotein complex made up of heat-shock proteins and other receptor associated proteins. Upon ligand binding the ER is activated, changes its conformation and dissociates from the multiprotein complex. Receptor molecules form dimers and translocate to the nucleus where the activated ERs can bind to specific estrogen response elements (EREs) in the promoter region of their target genes on the DNA. EREs are palindromic sequences and receptor dimers can bind to those sequences via the zinc fingers in the DBD. Target gene transcription is triggered upon formation of a stable transcription initiation complex by interaction of the ER with components of the basal transcription machinery of the cell as well as with coactivator proteins. In addition to the classical genomic pathway also alternative pathways leading to transcription of genes are known for the ER: they can either regulate gene expression without directly binding to DNA but to other classes of transcription factors via protein-protein interactions or through activation by ER-phosphorylation without ligand binding.

In addition to the slow, genomic effects of estrogens mediated by the traditional ERs there are also known some rapid, non-genomic effects of estrogens such as the activation of endothelial NO-synthase (eNOS) or the activation of various pathways involving second messengers such as mitogen-activated protein kinase (MAPK) or phosphatidylinositol-3-kinase (PI3K)

pathways. It is still controversial if these non-genomic actions of estrogens are either mediated by the classical steroid receptors located at the cell membrane or by steroid binding proteins unrelated to the steroid receptors. The latter hypothesis is also confirmed by Segars and Driggers [2], who classify the non-genomic action of estrogen receptors in antiestrogen-sensitive and -resistant pathways which suggests the existence of an estrogen binding factor distinct from the known ER subtypes. Some studies also reported a smaller ER with a size of 46 kDa, called ER46, which is a splice variant of ERα and might be expressed in ERα knock-out mice. However, the role of this smaller ER in membrane associated estrogen responses is not clear [2]. Additionally, a G-protein-coupled 7-transmembrane estrogen receptor (GPR30) has been found by different groups. This receptor is bound by estrogen and estrogen antagonists such as tamoxifen and ICI 182,780 as well as by xeno- and phytoestrogens and is responsible for some non-genomic effects such as the activation of MAP kinase Erk1/2 and of adenylyl cyclase.

In vitro test systems for the assessment of estrogenic activity

In vitro screening assays are useful tools to asses the activity of ligands toward ERs prior to subjecting them to more complex, more expensive and more time-consuming test methods. A collection of *in vitro* methods is summarized in various reviews and some of them are described in the following section.

Ligand binding assays measure the ability of a (mostly radioactively) labeled ligand to bind to a receptor. As a receptor source either recombinant proteins or extracts from cell lines are used. Affinity constants can be determined by binding of varying concentrations of labeled ligand to a constant receptor concentration. Such data can be visualized by means of a linear Scatchard plot. In competitive binding assays the inhibitory concentration IC_{50} of a ligand, which is the concentration of a compound that leads to a 50% decrease of maximal binding of labeled receptor, can be determined. Different concentrations of the test compound compete

with a constant concentration of known labeled ligand for binding to the receptor. Competition data are analyzed by means of logistic dose response curves. Ligand binding assays can not discriminate between agonistic or antagonistic binding of a ligand.

Cell proliferation assays such as the E-screen [3] measure the ability of a ligand to induce growth of the hormone responsive breast cancer cell line MCF-7. Such assays are useful to determine if a ligand is a partial or full agonist of the receptor and modifications of such assays are suitable for the determination of antagonist activity.

In transactivation assays the genomic pathway of steroid hormone receptor action is mimicked in cells. Either mammalian cell lines expressing the receptor are used or they are transfected with a plasmid for expression of the receptor. Additionally cells are transfected with a reporter plasmid expressing a reporter gene under the control of the estrogen response element (ERE). Upon binding of a hormone, the receptor forms dimers and binds to the ERE, which leads to transcription of the reporter gene. Metzger et al. [4] could show about twenty years ago that the human estrogen receptor functions in yeast and nowadays also yeast cells are frequently used as a host for transactivation assays. Yeast has the advantages of fast growth, easy cultivation, cheap media components, and robustness against toxic effects of sample compounds or solvents. Furthermore no endogenous receptors are expressed in yeast cells. The sensitivity of such screening assays in yeast cells is not as good as in mammalian cells due to the reduced permeability of the yeast cell wall, but the advantages of yeast are sufficient to make them a useful tool for screening the estrogenic activity of ligands. However, transactivation assays in yeast cells can not discriminate between agonistic or antagonistic properties of ligands.

Estrogen receptor α and β affinity and transactivation

Compounds with a structure similar to estrogen such as the isoflavones can bind to the ER. Important structural prerequisites for binding of a ligand to the ER are an aromatic ring and a

hydroxyl group. Computational methods such as Quantitative Structure-Activity Relationship (QSAR) are suitable to find a relationship between the structure and the activity of a compound and to describe it mathematically. By evaluation of binding data of a large, diverse set of natural, synthetic and environmental chemicals to ER in rat uterine cytosol Fang et al. [5] found five essential criteria for the xenoestrogen activity of a compound: (1) the H-bonding ability of the phenolic ring mimicking the 3-OH of E2, (2) an H-bond donor mimicking the 17-OH of E2, (3) precise steric hydrophobic centres mimicking steric 7α- and 11β-substituents of E2, (4) hydrophobicity and (5) a ring structure. Most of these criteria (except the steric bulk) are fulfilled by the class of isoflavones and thus they are binders of ERs. The effect of small structural differences on the estrogenicity of various flavonoids was also assessed and described by means of QSAR by Choi et al. [6]. They found in various *in vitro* methods (yeast transactivation assay, E-screen assay, and ER binding assay) that flavonoids with the exception of biochanin A and daidzein showed greater selectivity to ERβ compared to ERα and concluded that these methods including QSAR are a useful tool for the screening of natural phytoestrogens suitable for the treatment of breast cancer.

The affinity and biological activity of phytoestrogens for the two isoforms of the ER have been examined in many *in vitro* studies and literature data is summarized in Table 1. In contrast to the estrogen hormones which show similar binding affinities to ERα and ERβ [7] most of the isoflavones have greater binding affinities to ERβ compared to ERα in binding assays.

Table 1: Relative binding affinities (RBA), relative transactivation activities (RTA), or relative estrogenic activities (REA) of phytoestrogens.

Compound	CAS-No.	RBA/RTA/REA*		Test system	Reference
		ERα	ERβ		
17β-Estradiol	50-28-2	100	100		
Biochanin A	491-80-5	0.017	-	RBA	[8]
		0.019	0.059	RBA	[9]
		0.022	-	RBA	[10]
		0.34	0.18	RBA	[6]
		0.062	0.021	YES	[11]
		0.0464	-	YES	[12]

		0.00025	-	YES	[13]
		0.058	-	YES	[10]
		0.041	-	YES	[14]
		0.043	0.089	YES	[15]
		0.0005	-	HeLa	[16]
		0.045	-	HepG2	[10]
		0.011		E-Screen	[13]
		0.0027		Ishikawa	[9]
Daidzein	486-66-8	0.25	0.79	RBA	[17]
		<0.01	3.3	RBA	[18]
		0.22	1	RBA	[19]
		0.027	-	RBA	[20]
		0.082	-	RBA	[21]
		0.038	0.002	RBA	[9]
		0.01	0.04	RBA	[22]
		0.56	0.46	RBA	[6]
		0.0052	0.017	YES	[11]
		0.0045	-	YES	[12]
		0.014	-	YES	[23]
		0.00028	-	YES	[13]
		0.0041	-	YES	[14]
		n.d.	0.011	YES	[24]
		0.0041	0.017	YES	[15]
		72.29	80.95	293 hEKC	[17]
		0.01	-	HeLa	[16]
		0.0084	0.11	HEC-1	[22]
		0.011		E-Screen	[13]
		0.053		Ishikawa	[23]
		0.012		Ishikawa	[9]
		0.013		Ishikawa	[25]
		0.0002	0.003	MCF-7	[26]
		n.d.	0.00001	MCF-7	[27]
Daidzein glucuronide	38482-80-3	0.009	-	RBA	[21]
Daidzin	552-66-9	n.d.	0.00015	YES	[24]
		0.014	-	Ishikawa	[25]
Dihydrodaidzein	17238-05-0	n.d.	0.12	RBA	[17]
		55.42	53.33	293 hEKC	[17]
Dihydrogenistein	21554-71-2	0.04	0.37	RBA	[17]
		59.04	76.19	293 hEKC	[17]
Equol	94105-90-5	0.2	1.60	RBA	[22]
		0.086	2.5	RBA	[18]
		0.28	1.87	RBA	[17]
		0.29	2.85	RBA	[28]
		0.073	-	YES	[14]
		0.023	-	YES	[13]
		80.72	96.19	293 hEKC	[17]
		0.011	0.15	HEC-1	[22]
		0.013		E-Screen	[13]
		n.d.	0.0023	MCF-7	[27]
		0.003	0.03	RBA	[28]
Formononetin	485-72-3	0.0063	0.004	RBA	[9]
		0.07	0.36	RBA	[6]
		0.049	0.008	YES	[11]
		0.023	-	YES	[12]
		0.021	-	YES	[14]
		0.021	0.011	YES	[15]
		0.0033	-	HeLa	[16]
		0.0012		Ishikawa	[9]
Genistein	446-72-0	0.05	-	RBA	[8]
		5	36	RBA	[7]
		3.10	18.13	RBA	[17]
		0.2	25	RBA	[18]

		0.64	12.6	RBA	[19]
		0.49	-	RBA	[20]
		0.87	-	RBA	[21]
		0.3	-	RBA	[23]
		0.01	3.25	RBA	[29]
		2.17	13.3	RBA	[9]
		0.017	7.4	RBA	[22]
		0.8	-	RBA	[30]
		1.43	0.038	RBA	[28]
		2.07	14.78	RBA	[6]
		0.077	1.71	YES	[11]
		0.05	-	YES	[12]
		0.025	-	YES	[23]
		0.0045	-	YES	[13]
		0.046	-	YES	[14]
		0.2	-	YES	[31]
		0.05	1.1	YES	[24]
		0.046	3.80	YES	[15]
		4.9	0.53	YES	[32]
		83.13	84.76	293 hEKC	[17]
		0.013	-	MVLN	[29]
		0.08	-	HGELN	[29]
		0.01	-	HeLa	[16]
		0.026	1.67	HEC-1	[22]
		0.01	-	ELT3	[30]
		0.03	-	ERαCALUX	[33]
		0.026		E-Screen	[13]
		0.013		E-Screen	[29]
		0.4		Ishikawa	[23]
		0.028		Ishikawa	[9]
		0.066		Ishikawa	[25]
		0.0003	0.004	MCF-7	[26]
		0.14	6.6	SKBR3	[34]
		0.0001	0.011	MCF-7	[27]
		0.015	0.38	RBA	[28]
Genistin	529-59-9	>0.002	0.0039	YES	[24]
		0.081		Ishikawa	[25]
Genistein glucuronide	38482-81-4	0.018	-	RBA	[21]
Glycitein	40957-83-3	0.028	-	RBA	[20]
		0.32	0.44	RBA	[6]
O-Desmethylangolensin	21255-69-6	0.06	0.37	RBA	[17]
		65.06	52.38	293 hEKC	[17]
Tetrahydrodaidzein	304892-20-4	0.15	0.14	RBA	[17]
		48.19	56.19	293 hEKC	[17]

*RBA/RTA/REA: Relative binding affinity/relative transactivation activity/relative estrogenic activity are the IC_{50} values obtained with the standard compound 17β-estradiol divided by the IC_{50} value of the respective compound

n.d.: not detectable

293 hEKC: Transactivation assay in 293 human embryonic kidney cells transiently transfected with ERα or ERβ

ELT3: Transactivation assay in ELT3 cells transiently transfected with ERα (Leiomyoma cells from rat uterus)

ERαCALUX: Transactivation assay in stably transfected ERα CALUX cells (derived from human osteoblastic osteosarcoma cell line U2-OS)

E-Screen: Estrogen screen in MCF-7 cells

HEC-1: Transactivation assay in human endometrial carcinoma cells transiently transfected with ERα or ERβ

HeLa: Transactivation assay in HeLa cells transiently transfected with ERα

HepG2: Transactivation assay in HepG2 cells transiently transfected with ERα

HGELN: Transactivation assay in transgenic HGELN cells (derived from HeLa cells)

Ishikawa:	Induction of alkaline phosphatase activity in Ishikawa cells (ER positive endometrial adenocarcinoma cell line)
MVLN:	Transactivation assay in transgenic MVLN cells (derived from MCF-7 cells)
RBA:	Radioactive competitive binding assay
YES:	Yeast estrogen screen: transactivation assay in yeast cells heterologously expressing ERα or ERβ

The isoflavones with the highest potencies in binding assays are genistein and equol, which is a metabolite of daidzein. Only few yeast transactivation assays using ERβ have been found in literature and in most cases isoflavones have a higher transactivation potential on ERβ compared to ERα. The only exception is formononetin, for which higher potencies could be obtained in ERα- compared to ERβ-yeast transactivation assays [15]. Genistein is the isoflavone with the highest potency in yeast transactivation assays. In cell proliferation assays such as the E-screen all of the isoflavones contained in red clover and soy showed some estrogenic activity which is about four orders of magnitude lower than the estrogenicity of E2. In summary it can be stated that isoflavones show estrogenic potential in a number of *in vitro* assays which is a prerequisite for their biological activity. They exert their action predominantly on ERβ and their ability to induce growth of breast cancer cells (E-Screen) is rather small compared to E2.

Biological significance of ERβ

Ever since the discovery of ERβ by Kuiper et al. [35] in 1996, the endocrinology research gained new input and the following research articles and results kept the pot boilong. Although there are a lot of structure similarities between the ERα and ERβ, small differences have a great impact on the endocrine system. Enmark et al. [36] also demonstrated that ERα and ERβ are encoded by two independent genes.

In some tissues both receptors are equally coexpressed. In other organs and tissues either ERα or ERβ is the dominant expressed receptor. ERβ is also found in tissues that are not classically linked to estrogen modulation. Depending on the exclusive activation of one ER form or the

activation of both receptors in tissues that coexpress ERα and ERβ, the biological response varies to a great extent. Moreover, although estradiol has the same affinity for both receptors, especially phytochemicals show different affinities and transactivation potencies for the ERs. It became apparent that the newly found ERβ is not only "another" ERα, but that the receptor has a unique biological role that even counteracts sometimes the ERα response.

The estrogen receptors are found in most tissues as for example in the central nervous system, the cardiovascular system, the urogenital tract, in bone and the reproductive system. The distribution pattern can vary during development, as well as during the estrous cycle as it could be observed in breast tissue. Another important point is that the ERs may be expressed in the same tissue, but the distribution within this tissue deserves closer attention. For example, ERα and ERβ are both expressed in the prostate and are found in the stroma, but ERβ is mainly in the epithelium.

A special issue is the expression of ERs in the breast, since this tissue is a target for hormone-related cancer. In the normal human breast only a minority of the cells are ER positive. Similar, in the rodent mammary gland most of the proliferating cells do not express ERs. Nevertheless, the ER expression grade varies depending on the stage of development from prepubertal rats to pregnant or nursing rats.

In breast cancer cells the expression of ERs ungergoes a radical change. Therefore the normal healthy breast and breast cancer tissue are not responding in the same way to estrogens. Clarke et al. [37] demonstrated that only ~10-15% of luminal epithelial cells of the normal breast express ERs. Unfortunately, it was not distinguished between ERα and β in this study. On the contrary, up to 84% (median 27%) tumor cells expressed ERs. Saunders et al. [38] found in a breast cancer biopsies study that 80% of the tumors expressed ERα, 94% expressed ERβ and 74.5% coexpressed the receptors. Middleton et al. [39] showed also 75% and 70% positive for ERα and ERβ, respectively in lobular carcinoma *in situ*. Murphy et al. [40] found similar high ER expression pattern in male breast cancer (87% ERα and 87% ERβ). Saji et al.

[41] summarized reported ERα/β expression in breast cancer studies and came to the conclusion that about 50% of breast cancer patients express both receptors.

But some tumors are positive for ERβ but negative for ERα. So, Skliris and coworkers [42] found an expression of 60% of ERβ in ERα negative breast tumors. A metaanalysis showed that the most frequently occurring ER profile is ERα+/ERβ+ with 58% while ERα-/ERβ+ tumors represent only 18% [43]. To complicate the matter, during the phases of breast cancer progression, ER expression is also changing. There is strong evidence that the ERβ expression is declining during carcinogenesis. Strikingly, Behrens et al. [44] revealed that ERβ overexpression in MCF-7 cells after stable ERβ transfection led to a loss of tumorigenicity of these breast cancer cells. ERβ and its ERβ mRNA isoforms may be involved in a self-limiting mechanism of estrogenic stimulation promoted either by the natural hormone or by weaker estrogen agonists like genistein.

All those expression differences gain importance because of the differing transactivation properties of ERα and ERβ. It is well known that the estrogen receptors mediate target gene transcription by binding to EREs, but they also effect gene transcription from an AP1 enhancer element, which requires not only the ligand but also transcription factors like Fos and Jun. Paech et al. [45] reported that ERα and ERβ show a different effect of ligands regarding their AP1-element related gene expression. This might be an explanation for the ability of ERβ to enhance the treatment of breast cancer with antiestrogens and for the effect that an adjuvant treatment of ERα-negative but ERβ-positive breast cancer with tamoxifen results in an improved survival of patients, since the transcription of tamoxifen regulated genes under the control of a classic ERE is inhibited compared to AP1 regulated genes. Another explanation might be the downregulation of ERα mediated gene transcription via ERβ, which is a result of the differences in their transactivation regions. As a result of these observations, the presence of ERβ can provoke a cancer-protective effect in ERα-positive cancer cells, which was shown for genistein in MCF-7 cells by Gougelet et al. [46] and was

also observed *in vivo* [47]. The original paper from Kuiper et al. [7] showed that isoflavones transactivate ERβ with a similar efficiency than ERα, although they have a high binding affinity for ERβ. In forthcoming work it has been clearly demonstrated that isoflavones preferably transactivate ERβ with a difference of at least one order of magnitude; see also Table 1.

Tissue specificity of phytoestrogens

Phytoestrogens can be considered as natural SERMs because they influence the conformation of the ER in a way like other SERMs do. Furthermore phytoestrogens can selectively recruit coregulators to ERβ by creating an AF-2 surface that has greater affinity for coregulators than ERα. The binding of coregulators triggers the transcriptional function of the ER and thus isoflavones selectively activate the transcriptional pathways of ERβ. Similar results are found by Routledge et al. [48]: ERβ has an enhanced ability to recruit the coactivators TIF2 and SRC-1a in the presence of xenoestrogens such as genistein compared to ERα. As a consequence, the ability of ERβ to potentiate reporter gene activity in transiently transfected HeLa cells expressing SRC-1e and TIF2 was greater compared to ERα.

Another mechanism by which distinct effects of ERα and ERβ are exerted is the regulation of different downstream target genes, which results in different transcriptional activities of both ER subtypes depending on ligand, cell type and promotor context. In ERα- and ERβ-expressing U2OS osteosarcoma cells it was shown by microarray that depending on the ligand (either E2, tamoxifen or raloxifene) bound to the receptor, different target genes are expressed. Chang et al. [49] found that also the ligand dose influences the transcription of genes. Low doses of genistein regulated gene expression more effectively in cells coexpressing ERα and ERβ while at high concentrations genistein had effects on gene transcription similar to E2. In mammalian cell transient transfection assays using four

different estrogen-responsive promotors it could be shown that different promotors differentially influence the ability of ERα and ERβ to assume transcriptionally active conformations and that the transcriptional activities of both ER subtypes are dependent on the bound ligand. Transactivation by genistein-bound receptor was almost the same as by E2-bound receptor with some promotors and also differences between the two receptor subtypes could be observed.

The tissue specific effects of phytoestrogens can thus be explained by the selectivity of isoflavones for ERβ, the different tissue distribution of both receptor subtypes and the regulation of different target genes of the receptor subtypes.

Isoflavones have different estrogenic activities on both ER subtypes making them suitable for the use as natural SERMs. They have greater potential on ERβ and thus exert their action predominantly in ERβ-expressing tissues. The growth-promoting effects of isoflavones on breast cancer cells as determined in the E-screen are about four orders of magnitude lower compared to the effects of E2. It has to be kept in mind that concentrations of compounds used in *in vitro* assays are not always relevant for *in vivo* situations because such high concentrations of phytoestrogens in plasma are not always reached.

Effects of phytoestrogens on enzymes of the estrogen metabolism

E2 plays an important role in the development and progression of many hormone-dependent cancers, especially breast cancer. The intracellular formation of E2 is performed by various enzymes that convert circulating precursors to the active hormone (Figure 2). These enzymes are also present in breast cancer cells and are targets for therapeutic approaches based on the control of intracrine activity.

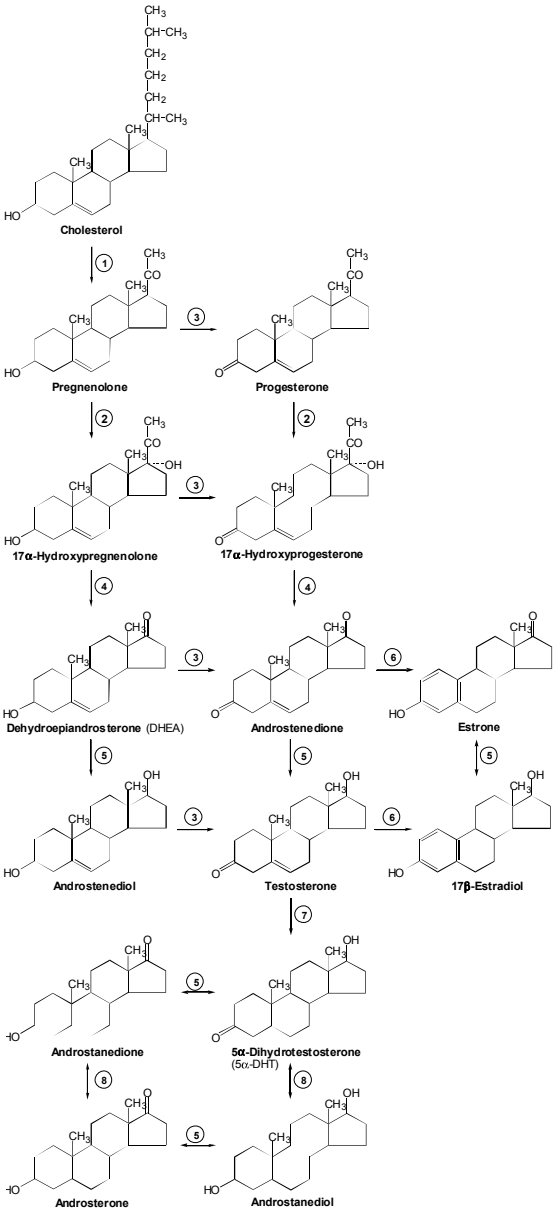

Figure 2: Enzymatic pathways involved in steroid hormone metabolism; numbers are designating the following enzymes: 1: P450ssc (P450-linked side chain cleaving enzyme), 2: P450c17 (17α-hydroxylase), 3: 3β-hydroxysteroid dehydrogenase Δ5/Δ4 isomerase (3β-HSD), 4: 17,20-desmolase, 5: 17β-hydroxysteroid dehydrogenase (17β-HSD), 6: aromatase, 7: 5α-reductase, 8: 3α-hydroxysteroid dehydrogenase (3α-HSD)

Some phytochemicals inhibit enzymes of the steroid pathway and the cancer-protective effects of phytoestrogens might be mediated by their interference with estrogen metabolism [50].

However, it has to be kept in mind that phytoestrogens additionally to their enzyme-inhibiting effects also have estrogenic properties and often those two effects occur in the same concentration range of a compound. The advantages and disadvantages of phytoestrogen consumption via food supplements have to be considered very carefully.

Effects of isoflavones on aromatase activity

Aromatase is the enzyme which is responsible for the conversion of androgens to estrogens by aromatization: androstenedione and testosterone are converted to estrone and estradiol, respectively (Figure 2). In cells that cannot produce estradiol by themselves aromatase is the enzyme responsible for the production of E2 from androgens. Many *in vitro* studies have been published about the effects of isoflavones on this enzyme in different cell types.

In MCF-7 breast cancer cells genistein was found to have an inhibitory effect on aromatase activity [50] while Almstrup et al. [51] found no inhibitory effects for this compound in the same cell type. Biochanin A and formononetin however, inhibited aromatase activity at low concentrations. These results are in accordance with the outcome of Wang et al. [52], who found no inhibitory effect of genistein, but inhibitory effects of biochanin A on aromatase activity expressed by stably transfected MCF-7 cells.

Biochanin A was the only red clover-derived isoflavone for which an inhibitory effect on aromatase could be detected in human placental microsomes. It was found that isoflavonoids with the phenolic B ring in the 3 position on the pyran ring such as genistein, daidzein or biochanin A are much better inhibitors of 3β-hydroxysteroid dehydrogenase (3β-HSD) and/or 17β-HSD than of aromatase.

The conversion of androstenedione to estradiol by aromatase/17β-HSD was inhibited in a dose-dependent manner by daidzein, while the inhibitory effects of biochanin A and genistein were not dose-related in human granulosa-luteal cells. However, inhibition did neither occur via reduction of expression of the enzymes as shown by Western blots nor by cytotoxic

effects of the phytoestrogens on the cells. In another study using human granulosa-luteal cells biochanin A did not show any significant inhibitory effect on the conversion of testosterone to estradiol by aromatase while genistein and daidzein showed dose-dependent effects on aromatase inhibition. The results of aromatase inhibition were paralleled by a reduction of aromatase mRNA expression detected by real-time PCR which suggests an inhibitory effect of phytoestrogens by reduction in aromatase expression. The reduced expression might not be detected on Western blots because of the high stability and long half-life of the aromatase protein compared to its mRNA. In the same study also inhibitory effects of low dose combinations of the three isoflavones genistein, daidzein and biochanin A were detected which suggests that dietary exposure to such compounds might have consequences for aromatase activity. The inability of biochanin A to inhibit aromatase in human granulosa-luteal cells was confirmed by Whitehead and Lacey [53].

In human endometrial stromal cells derived from women without endometriosis genistein even could enhance the activity of aromatase while daidzein hat no effect. The increased activity of aromatase is likely due to an increased enzyme expression in human endometrial stromal cells. In cells derived from women with endometriosis phytoestrogens did not inhibit aromatase activity whereas both genistein and daidzein were weak inhibitors of human recombinant aromatase.

In rainbow trout ovaries aromatase activity was inhibited strongly by equol, a metabolite of daidzein, and slightly by biochanin A and genistein whereas formononetin and daidzein did not inhibit aromatase activity. Aromatase inhibitors are used for cancer therapy. Aromatase is a key enzyme in the development of breast cancer. The weak aromatase inhibitory property of isoflavones is one of the events in the concerted actions of isoflavones in cancer prevention, which is observed by all epidemiological studies.

Effects of isoflavones on the activity of 17β-hydroxysteroid dehydrogenase (17β-HSD)

The group of 17β-HSD consists of twelve isozymes that are responsible for either 17β-reduction or oxidation of estrogens and/or androgens. 17β-HSD type 1 is responsible for the reductive conversion of estrone to estradiol whereas 17β-HSD type 2 catalyzes the oxidation of estradiol to estrone and of testosterone to androstenedione.

An inhibition of 17β-HSD type 1 in MCF-7 breast cancer cells by genistein treatment and thus a reduced production of estradiol have been found by Brooks and Thompson [50]. Additionally they detected a reduced proliferation of MCF-7 cells after treatment with genistein and concluded that the modulation of enzyme activity by isoflavones might be responsible for the cancer protective effects of such compounds. In contrast Brueggemeier et al. [54] found unchanged activity of 17β-HSD type 1 upon genistein treatment in MCF-7 cells but activity of 17β-HSD type 2 was increased leading to a higher production of estrone, which exerts less estrogenic activity than estradiol.

Experiments with human placental microsomes showed that 17β-HSD could be inhibited by the isoflavones occurring in red clover and that this effect is dependent on hydroxylation of the isoflavones in the positions 5, 7 and 4'. In another study it was found that the conversion of [^{3}H]estrone to [^{3}H]estradiol by purified 17β-HSD type 1 from human placenta is weakly inhibited by genistein [55] and only compounds with the highest inhibitory activity on the purified enzyme such as coumestrol or genistein were inhibitory to 17β-HSD type 1 in wild type T-47D breast cancer cells. From their results of the analysis of the inhibitory potential of phytoestrogens from different classes Mäkelä et al. [55] found various structural demands necessary for inhibition of 17β-HSD type 1: They agreed that a certain degree of hydroxylation of a compound is necessary and found that an isoflavonoid structure makes a compound a potential inhibitor whereas flavonoids are inactive. Additionally to their enzyme-

inhibiting properties isoflavones are also estrogenic and can not inhibit estrone-induced growth of T-47D$_{21}$ cells expressing high amounts of recombinant 17β-HSD type 1.

In human granulosa luteal cells genistein also had an inhibitory effect on 17β-HSD type 1 in the highest concentration tested. Biochanin A, however had no inhibitory effect on 17β-HSD type 1 in human granulosa luteal cells [53].

In genital skin fibroblasts 100 µM of genistein and biochanin A led to an inhibition of over 80% of 17β-HSD type 2 which catalyzes the conversion of testosterone to androstenedione. The reverse reaction is catalyzed by 17β-HSD type 5 in peripheral tissues others than testis. This reaction is inhibited by the isoflavone biochanin A with an IC_{50} value of 14 µM and by genistein and daidzein with an IC_{50} value > 20 µM.

These findings add another possible explanation for the low incidence of breast and prostate cancer in populations with a high intake of isoflavones.

Effects of isoflavones on the activity of other enzymes of the steroid metabolism

Further enzymes that are inhibited by phytoestrogens are the 3β-hydroxysteroid dehydrogenase (3β-HSD), the 5α-reductase and the 3α-hydroxysteroid dehydrogenase (3α-HSD). The first of those enzymes is on the one hand responsible for the conversion of dehydroepiandrosterone (DHEA) to androstenedione and on the other hand for the interconversion of 3β-hydroxy- and 3-keto-5α-androstane steroids. A study with purified bovine adrenal 3β-HSD showed that daidzein, genistein, biochanin A and formononetin inhibit the dehydrogenase- as well as the isomerase activity of this enzyme. Similar results were also obtained with 3β-HSD from human placental microsomes and in human adrenal H295R cells. In contrast, in cultures of human granulosa luteal cells biochanin A was the only isoflavone exerting dose-dependent inhibition of the conversion of pregnenolone to progesterone, while genistein was only active in the highest dose tested (100 µM).

5α-reductase catalyzes the conversion of testosterone to the more potent androgen dihydrotestosterone (DHT). The most potent isoflavones to inhibit 5α-reductase activity in human genital skin fibroblasts were equol, genistein and biochanin A. Biochanin A could inhibit isozyme 2 (acidic pH-optimum, high affinity for testosterone) of 5α-reductase to a greater extent than isozyme 1 (broad basic pH optimum, low affinity for testosterone). This observation was also shared when recombinantly produced human 5α-reductase was used.

3α-HSD is responsible for the conversion of DHT to androstanediol and is another target for inhibition by phytoestrogens. Blomquist et al. [56] found that 3α-HSD from microsomes of human lung could be inhibited by coumestrol, genistein and daidzein and these compounds have the ability to influence the androgen status of the human lung.

Effects of isoflavones on the activity of sulfotransferase and sulfatase

Estrone sulfotransferase (EST) is the enzyme that sulfonates estrone to biologically inactive estrone sulfate, which circulates in the plasma whereas steroid sulfatase catalyzes the formation of free estrone from estrone sulfate.

Kirk et al. [57] found that genistein was the most potent of the isoflavones to inhibit sulfotransferase from platelet cytosol fractions. Its IC_{50} value is in the range of 0.3-0.4 μM depending on the substrate used and these values are comparable to circulating concentrations of genistein and its conjugates in individuals on high soy diet. However, no effects of phytoestrogens on estrone sulfatase activity in physiologically relevant concentrations could be detected in this study. This outcome was confirmed in a study by the same authors reporting an inhibition of sulfotransferases but not sulfatases by flavonoids [58]. In contrast, an inhibition of estrone sulfatase activity by the natural flavonoids quercetin, kaempferol and naringenin in a human hepatic microsomal preparation was reported. The inhibitory activity of isoflavones derived from red clover was not investigated in this study.

A large number of enzymes involved in steroid synthesis are a possible target for inhibition by phytoestrogens. By changing the active hormone concentration through inhibition of its synthesis in a specific target tissue the development and progression of hormone-dependent diseases might be retarded by treatment with or dietary uptake of isoflavones. It has to be kept in mind that the isoflavones also exert estrogenic activity and the beneficial inhibitory effects on enzymes of the steroid metabolism have to be put into relation to the adverse growth promoting effects on breast cancer cells. This is an important issue to be considered when administering phytoestrogens via the diet or as nutritional supplements.

3. Metabolism of isoflavones *in vitro*

Isoflavones are mainly found in Fabaceae plants, predominantly in glycoside form. In plants they function as mediators and facilitate Rhizobium symbiosis for nitrogen fixation of the root nodules.

The absorption of the isoflavone glycosides requires a hydrolysis of the sugar moiety. The absorption of isoflavone aglycones from red clover and soy does not seem to differ.

In vitro studies show that glucosidases from gastrointestinal bacteria are responsible for hydrolysis and the resulting production of isoflavonoid aglycones, which are further metabolized. Moreover, also human hepatic microsomal enzymes are able to metabolize isoflavones. As experiments showed, cell-free extracts from human small intestine and liver have also β-glucosidase that has a high affinity for isoflavone glycosides. There is also evidence that the luminally exposed enzyme lactase phlorizin hydrolase (LPH) is involved in the hydrolysis of isoflavone glycosides. In Figure 3, the *in vivo* metabolism of isoflavones is illustrated schematically.

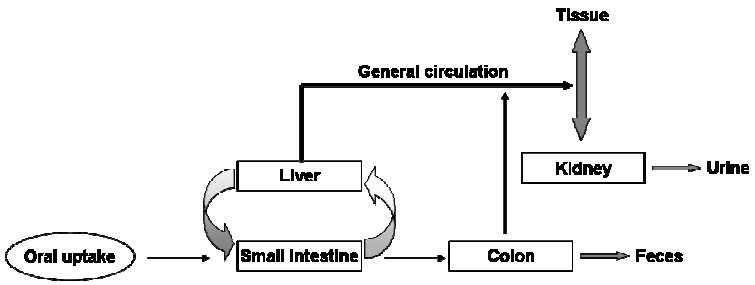

Figure 3: The metabolism of isoflavones *in vivo* illustrated schematically; from uptake to renal/hepatic elimination.

Studies obtained with rat and human liver microsomes show an oxidative metabolization of isoflavones. In human liver microsomes mainly cytochrome P450 enzymes are responsible for the demethylation and hydroxylation of genistein and daidzein. The CYP-mediated hydroxylation of daidzein affects its antiproliferative activity in MCF-7 cells (human breast cancer cell line; ER-positive). Due to a metabolization via CYP1A2 an enhancement of the

antiproliferative activity was observed. Moreover, in rat liver microsomes isoflavones were able to decrease side-effects of tamoxifen, due to an inhibition of CYP1A2 which also brought about an inhibition of α-hydroxytamoxifen formation.

Similar to liver microsomes, an isoflavone metabolization also occurs in breast cancer cell lines. However, the comparison of growth inhibiting effects of isoflavones on non- cancer breast tissue (normal mammary epithelial cells; HME cell line) and the MCF7 breast cancer cell line reveals metabolism variations. While no significant metabolism of genistein and biochanin A could be observed in non-cancer breast cells, in MCF-7 cell culture an extensive metabolization takes place. Though metabolism of genistein brings a decrease in growth inhibition of MCF-7 cells, the metabolization of biochanin A increases growth inhibition. Glycitein, also a soy isoflavone, is metabolized *in vitro* using rat liver microsomes and human liver microsomes to 8-hydroxyglycitein, 6-hydroxydaidzein, 6-hydroxy-DHD, 5´-hydroxy-ODMA, 5´-methyl-ODMA and 6-hydroxyequol.

During normal metabolism a demethylation takes place: formononetin is converted to daidzein and biochanin A to genistein. The degradation pathway of isoflavones is shown in Figure 4.

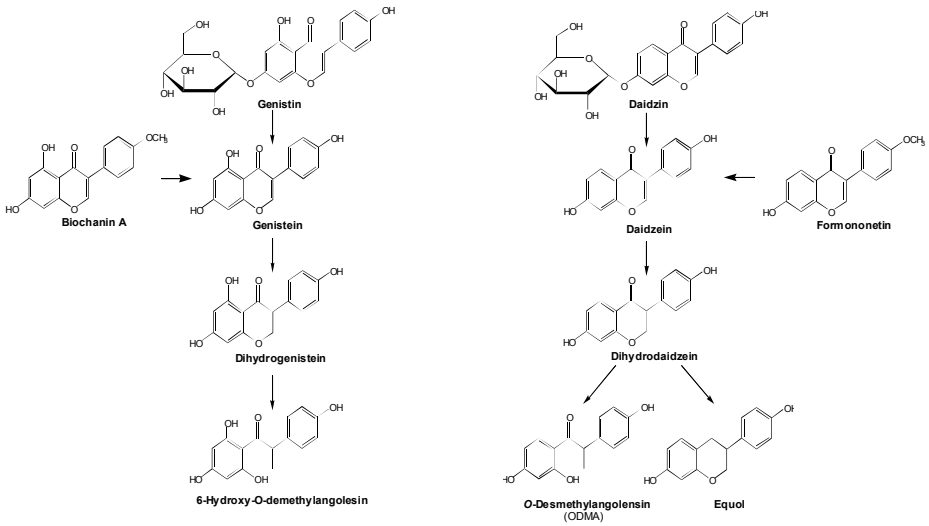

Figure 4: Degradation pathway of isoflavones.

This metabolization occurs not only in humans but also in other species, as *in vitro* experiments with bovine rumen fluid showed for example. An important metabolite of daidzein is equol; it is produced via the intestinal microflora of some individuals. Studies show that not everyone is able to produce equol. Only ⅓ to ½ of human individuals can metabolize daidzein to equol. The production of equol is attributed to a mixture of microflora bacteria and not a single pure culture. Equol excretion is associated with a reduced risk of breast cancer, probably due to hormonal differences between equol producers and non-producers. High variations in intestinal microflora of individuals account for various isoflavone metabolites, so *in vitro* fermentation with human fecal bacteria show also other metabolites of daidzein such as dihydrodaidzein, *O*-desmethylangolensin (ODMA) and benzopyran-4,7-diol,3-(4-hydroxyphenyl).

In vitro experiments with feces of equol-producers and equol-non-producers suggest that there is a negative correlation between ODMA and equol production during the metabolization of daidzein, although some individuals are able to produce both metabolites.

Not only humans are able to produce equol, but also other animals. From *in vivo* studies it is known that in some species the equol production is much higher than in humans such as observed in rhesus monkeys and rats. Nevertheless, *in vitro* incubation of daidzein with intestinal bacteria from rhesus monkeys showed similar metabolism variations to those of humans. On the contrary, rat flora metabolizes daidzein rapidly to aliphatic compounds, which could not be observed in humans. All these differences have to be taken into consideration and interpretations of animal studies in this field have to be carried out with caution.

4. Promiscuity of isoflavones

Isoflavones are multipotent bioactive compounds. Best known sphere of action is of course their estrogenic/antiestrogenic activity, which is primarily due to their binding to the estrogen

receptors. But they exert a wide range of effects through activation of several other receptor pathways such as other steroid hormone receptors, but also the aryl hydrocarbon receptor (AhR) or the peroxisome proliferator activated receptors (PPARs). The resulting effect on the organism is arising out of the multitude of those actions. Beside the interaction of the endocrine system via the steroid hormone receptors and the AhR, there are manifold properties of isoflavones due to the scope of the effected pathways of the PPARs as well as the AhR. To understand the possible benevolent effects of isoflavones it is of interest to dwell on these receptors. This subchapter will enlighten some of their properties.

Isoflavones as AhR ligands

Although the AhR belongs to the basic helix loop helix protein family, its pathway bears resemblance to those of steroid hormone receptors. It is a ligand-activated transcription factor that leads to an expression of a certain gene battery. The genes that are regulated by this receptor emphasize its ramified physiological importance. Among them there are genes involved in Ras/MAPK related signaling pathways, in calcium regulation, in cardiovascular and pulmonary function, in cell cycle regulation, in differentiation and apoptosis, in development, cell adhesion and metastasis as well as genes that are involved in drug metabolism and DNA stability. Results that based on gene expression studies were confirmed with knock-out mice experiments.

Probably best investigated is the role of the AhR in xenobiotic detoxification, since the most responsible AhR-controlled genes are those of the cytochrome P450 enzymes. The sustained activation of those enzymes redounds to the toxic effects that are exerted by the prototypical and scarcely metabolizable AhR-ligand dioxin. Likewise, the formation of very reactive metabolites is also possible during the normal detoxification of compounds that can become noxious or carcinogenic. Best example is benzo[a]pyren. Nevertheless, it seems that although the AhR pathway cascade is responsible for the formation of the toxic benzo[a]pyren

metabolites, it is the same cascade that causes the further metabolization and therewith elimination of the compounds and metabolites thereof. Most toxic effects of dioxin are mediated by the AhR or rather the sustained activation of the AhR pathway. Experiments with a constitutively active AhR show for example the promotion of hepatocarcinogenesis or stomach tumors in mice.

As aforementioned, some of the antiestrogenic action of isoflavones may be exerted via the AhR, since this receptor interferes with the estrogen receptor pathway in multiple ways; the AhR complex binds to the estrogen response elements (ERE), the receptors interact directly with each other, the ability of ER to bind to EREs is decreased as well as an abolishment of estrogen induced gene expression could be observed in presence of an AhR ligand. Moreover, the activated AhR cascade is able to modify estradiol metabolism. This fact is exceptionally remarkable since the resulting metabolite ratio is contrary to that observed in women with breast cancer. Vice versa, the ER modifies the AhR cascade too in various forms.

Recently these interactions were utilized to create drugs for the treatment of hormone-dependent cancers. The so-called selective aryl hydrocarbon receptor modulators (SAhRMs) are especially developed for the treatment of breast cancer [59-61] and circumstantiate the AhR as therapeutic target. SAhRMs are natural occurring AhR ligands (such as indole compounds) or derivates thereof that show no toxic effects. But the AhR-mediated signal pathway on its own, without influence of the ER transduction has been utilized for the creation of antitumor agents. The prodrug Phortress is a benzothiazole derivate and an AhR agonist that induces its own activation via the AhR-dependent induction of CYP1 enzymes. The activation of the AhR signalling pathway is important for the antitumor activity of the benzothiazole compounds. Phortress is currently in phase I of a clinical study.

Beside the xenobiotic detoxification and the antiestrogenic effects, the AhR is also well-known for its cell cycle controlling abilities. But there is also evidence that AhR action is cell cycle phase dependent and considerably decreased in G_2/M phase. The involvement of the

AhR in cell cycle control is intricate and ramified. AhR-defective mouse hepatoma cells (Hepa 1c1c7) show a prolonged transition through G_1 phase independently of ligand-activation, while the introduction of cDNA AhR led to a switch to normal wild type cell behavior. There are manifold underlying mechanisms that lead to cell cycle arrest. For one, the unliganded as well as the preferably liganded AhR is interacting directly with the retinoblastoma tumor suppressor protein Rb. It is possible that Rb is a coactivator of the AhR, since the AhR-Rb interaction seems to be necessary for maximal ligand-induced G_1 arrest. The AhR induces also $p27^{Kipl}$, a cyclin dependent kinase inhibitor protein, by altering *Kip1* transcription directly. The consequence is a suppression of proliferation. Mice lacking $p27^{Kipl}$ display increased body size, multiple organ and retinal hyperplasia and pituitary tumors. It is also known that reduced levels of p27 protein occur in many cancer types. So the AhR, cell cycle progression and cancer are intertwined in multiple ways. The AhR is also repressed in liver tumors and it is hypothesized that AhR silencing may be associated with cancer progression. Meanwhile, the AhR is overexpressed in other cancer types, such as in lung carcinomas or pancreatic cancer. In the latter case, this overexpression offers simultaneously therapeutic possibilities, given that AhR agonists were able to inhibit pancreatic cancer cell growth in a dose-dependent manner.

In vitro experiments showed that isoflavones are ligands of the AhR and can probably activate several of the above mentioned effects. Data on daidzein and genistein as AhR ligands are inconsistent; they are described as weak agonists, while they were not able to show activity in transactivation assays. Their antagonistic abilities were also tested by Ashida et al. [62], who reported only slight inhibitory effects at concentrations of ≥ 50 µM. Genistein also was not able not compete for AhR binding with the potent ligand methylcholanthrene. Nevertheless, Kajta et al. [63] showed for genistein anti-apoptotic effects against glutamate-induced apoptosis in neuronal cells. Interestingly, evidence showed that both ER as well as AhR were

involved in the demonstrated genistein action. This could be a novel approach for the treatment of neurodegenerative disorders.

Interestingly, biochanin A and formononetin are more potent agonists and exhibited in an *in vitro* test based on yeast EC_{50}-values of about 130-250 nM, while equol showed only at high concentrations of 100 µM a transactivating potential. Biochanin A was previously shown to induce CYP1A1 expression in MCF-7 cells and to compete with dioxin for binding to the AhR in isolated rat cytosol.

Whether isoflavones mediate some of the above mentioned AhR actions via this pathway need to be further investigated. Due to their sAhRM-like behavior an antiestrogenic effect of isoflavones can be expected. Altough this is a speculation, plant derived sAhRMs are currently tested for cancer prevention and treatment by [59-61]. These compounds have nothing in common exept that they activate the AhR.

Isoflavones as PPAR ligands

Isoflavones are also ligands of the PPAR's. Those receptors were described for the first time by Issemann and Green in 1990 [64]. The three isoforms PPAR α, β (or δ) and γ of this receptor family are all ligand-activated transcription factors and bind to peroxisome proliferator response elements (PPREs). As well as the AhR, those receptors have great impact on several crucial physiological pathways via the regulation of several target genes. The functions of the gene products are especially important for the carbohydrate and lipid metabolism as well as in inflammation processes.

Characteristics of the metabolic syndrome are dyslipidemia, obesity, hyperglycaemia and high blood pressure. This medical dysfunction leads to an exceptionally increased risk for diabetes type 2 (diabetes mellitus type 2), cardiovascular diseases and artheroclerosis and ranks among others as leading disease of civilization. Because of their physiological roles, the PPARs are

suitable as targets for the treatment and therapy of the metabolic syndrome and consequential diseases.

So, an active PPAR α alters lipid metabolism, given that it aids the increase of high density lipoprotein (HDL) due to the modulation of apolipoprotein AI and apolipoprotein AII levels. Since HDL takes cholesterol away from arteries and leads it to the liver, higher levels of HDL were associated with a decrease in cardiovascular risk. Also, the lowering of plasma triglyceride levels via induction of the apolipoprotein apoAV was observed. The PPARα agonists, the fibrates lower plasma triglycerides by the decrease of apolipoprotein apoC-III expression *in vivo* and *in vitro*. Knowing that apoC-III inhibits lipoprotein lipase (LPL) activity and leads to a decreased rate of lipolysis of human VLDL by LPL, it is not surprising that the activation of PPARα leads to a healthier lipid rate in the blood count. That apoC-III inhibits the catabolism of triglycerid-rich lipoproteins by LPL was also confirmed by Ginsberg et al. *in vivo* [65]. Interestingly, it seems that both PPARα and PPARγ are able to induce LPL expression after activation trough their selective agonists. This induction was restricted to the tissue in which the respective PPAR is predominantly expressed.

PPARα influences the degradation of fatty acids directly in at least two ways. First, the active PPARα induces transport proteins and enzymes that affect the passage of fatty acids across the plasma membrane and enable the entry of fatty acids into the mitochondria where β-oxidation takes place. In addition, the expression of enzymes of the β-oxidation is regulated. Increased plasma levels of fatty acids are a symptom of obesity and exactly that could be the link between obesity and diabetes mellitus. Diabetes II is mainly characterized by insulin resistance, which means that normal insulin levels are not enough to provoke insulin response. Higher levels of fatty acids or long term exposure of β-cells (Islets of Langerhans, type beta) to free fatty acids lead to a decrease of responsivness of those cells to glucose or even to β-cell apoptosis. Again, it was demonstrated that activation of PPARα via its

agonists, improves insulin sensitivity and glucose homeostasis and was also able to reduce adiposity. PPARα exhibits also various anti-inflammatory and anti-atherosclerotic properties that are mediated for example by the down-regulation of the monocytic tissue factor, inhibition of NF-κB and consequentially inhibition of vascular cell molecule-1 (VCAM-1). Li et al. [66] report on the inhibition of atherosclerosis *in vivo* via inhibition of macrophage foam cell formation. Experiments with apolipoprotein E deficient mice show also inhibited atherosclerosis as consequence of PPARα agonist treatment.

PPARδ also activates fat metabolism as the activated receptor induces β-oxidation of fatty acid, a better lipid profile and reduced lipid accumulation. Moreover, this receptor regulates glucose metabolism and enhances insulin sensitivity. PPARδ reduces artherosclerosis, doing so by inhibition of atherosclerotic lesion formation and anti-inflammatory effects mediated by regulation of genes involved in inflammation.

The last of the PPAR isoforms, the PPARγ, has similar physiological effects. Its ligands, the thiazolidinediones (TZDs), are for a long time in use as anti-diabetic pharmaceuticals. Their action is mediated via the PPARγ, but although the receptor is expressed in high levels in adipose tissue where it is important for differentiation, it seems that the action of TZDs is not restricted to adipocytes. The deletion of PPARγ in skeletal muscle led to insulin resistance, which was not abrogated by TZD treatment. It was hypothesized that enhanced insulin action in normal skeletal muscle is due to the evoked decrease in triglycerides and the rearrangement of fat out of the muscle. Steppan et al. [67] described also the influence of TZDs on resistin. This protein is specifically expressed and secreted by adipocytes and is induced during adipocyte differentiation. Remarkably, its level is also increased in obesity. TZD treatment decreased resistin and led to enhanced insulin-stimulated glucose uptake by adipocytes. In addition to the mentioned effects of TZDs, they can also reduce hyperglycemia, dense LDL and the expression of several factors such as: tumor necrosis factor α (TNF-α), interleukin 1

and interleukin 6, endothelin-1, interferon-γ-inducible chemokines and matrix metalloproteinases. Altogether, this redounds to the anti-inflammatory and anti-atherogenic effects of TZDs that are mediated via the activated PPARγ.

The isoflavones are well described in literature as PPAR ligands (see Table 2).

Table 2: The isoflavones biochanin A (Bio), formononetin (For), genistein (Gen), daidzein (Dai), equol (Equ) and glycitein (Gly) as PPAR α, β/δ and γ ligands, as described in literature. (* activity in Hela cells, but not in HepG2 cells)

Reference	PPARα	PPAR β/δ	PPARγ
Chako et al. [68]			Bio, Gen, Dai, Equ
Ricketts et al. [69]	Gen, Dai		Gen, Dai, Gly
Kwon et al. [70]			Gen, Dai, Gly
Dang et al. [71]			Gen
Kim et al. [72]	Gen		
Mezei et al. [73]	Gen, Dai		Gen, Dai
Shen et al. [74]	Bio, For, Gen		Bio, For, Gen, Dai*
Dang and Löwik [75]	Dai	Dai	Dai

Whether the isoflavones can trigger all of the above mentioned effects of the PPARs, has to be elucidated. Nevertheless, some of them have been demonstrated in various experiments. So, biochanin A and formononetin induced differentiation of 3T3-L1 preadipocytes at low doses [74]. Genistein down-regulated PPARγ during adipocyte differentiation and decreased also adipocyte cell number via an ER-dependent mechanism. Rayalam et al. [76] tested the effects of genistein and resveratrol (alone or in combination) on preadipocytes and mature adipocytes. They found increased apoptosis and decreased lipid accumulation in all assay setups, but all observed effects were stronger in the combinatorial testing. Also, only in combination genistein and resveratrol were able to induce lipolysis and to down-regulate PPARγ. Further it was demonstrated that soy isoflavones enhance insulin-stimulated glucose uptake in 3T3-L1 adipocytes via the PPARγ pathway [70]. Again, genistein inhibited antilipolytic action of insulin [77]. The authors concluded that this may contribute to the decreased triglyceride accumulation in adipose tissue. Daidzein is not only able to activate all PPAR isoforms, but also showed biphasic dose-dependent effects on osteogenesis and adipogenesis [75]. Genistein treatment of liver cells (HepG2) actuated the increased

expression of genes involved in lipid metabolism in an ER-independent way but through activation of PPARα [72]. Interestingly, isoflavones also inhibited TNF-α induced monocyte adhesion during flow and via activation of PPARγ in endothelial cells [68].

The PPAR pathways are also mired together with the AhR cascade. Shimba et al. [78] suggest that the AhR is a negative regulator of adipose differentiation. Worth mentioning is also that *serpina12* is part of the AhR gene battery. The protein serpina 12, also known as vaspin (visceral adipose tissue-derived serpin) is described by Hida et al. [79] as insulin-sensitizing adipocytokine. Klöting et al. [80] found more frequently expression of vaspin in patients with type 2 diabetes and especially in obese subjects and hypothesized that vaspin may be a compensatory mechanism in response to impaired glucose metabolism.

The isoflavones are interesting natural compounds for the regulation of blood glucose and may contribute in prevention of the metabolic syndrome and cardiovascular diseases, since they act as dual or even triple PPARα/β/γ agonists or antagonists. This property could improve the clinical efficacy and may have edge over agents that activate only one PPAR isoform, as it is also described for some TZDs. But their ultimately advantage is that the isoflavones are also activators of other important pathways such as the ERs or the AhR.

5. Role of isoflavones on bone health

Given that the bone structure after adolescence is influenced strongly by sex hormones, loss of mineral bone density and osteoporosis are often a consequence of menopause. Then, bone resorption and bone remodelling procedures are out of balance. Osteoblasts are responsible for bone formation, while osteoclasts mediate bone resorption. To redress the balance or to shift it in favour of bone remodelling is quite of an interest regarding millions of women who suffer from osteoporosis.

Various factors are involved in these processes. The paraythyroid hormone (PTH) induces the osteoclastogenesis by binding to osteoblasts. This leads to the expression of the receptor activator of NF-κB Ligand (RANKL). This ligand activates precursors of osteoclasts by binding to a protein that is expressed at the surface of preosteoclasts, named receptor activator of NF-κB (RANK). These processes result in mature osteoclasts (see Figure 5).

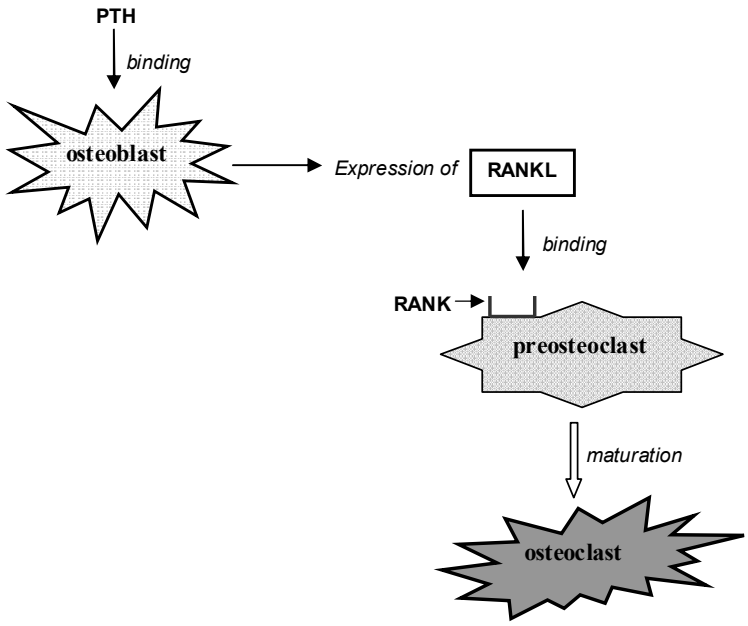

Figure 5: Initiation of osteoclastogenesis by the parathyroid hormone.

Another protein, so-called osteoprotegerin (OPG) is a negative regulator of osteoclastogenesis. It is a RANK homolog and is able to bind to RANKL, which inhibits the RANK-RANKL action.

The hindrance of the osteoclast formation could be a good approach for the prevention of osteoporosis. As we will illustrate, isoflavones may achieve this by intervening at several points in the osteoblast-osteoclast control cycle.

Also, a factor that has been associated with increased osteoporosis risk is the cytokine interleukin-6. Rachon et al. [81] demonstrated that estrogen deprivation enhances the expression of this cytokine. This is not astonishing since interleukin-6 is produced in response to PTH [82] and PTH increases around menopause due to ER depletion. Nevertheless, interleukin-6 promotes also osteoclast formation in a RANKL-independent manner [83].

Generally, isoflavones have been described as agents that have a stimulatory effect on osteoblastic bone formation or decrease the number of osteoclasts or even inhibit differentiation to osteoclasts. By now, the mechanisms that underlie these effects are better understood.

In various *in vitro* experiments genistein, daidzein or derivates have demonstrated their ability to inhibit PTH-dependent osteoclastogenesis, increase OPG levels and decreased RANKL or rather improve the OPG:RANKL ratio towards bone formation and decrease interleukin-6 levels. Also, the alkaline phosphate (ALP) activities as well as Ca2+ content in bone tissue [75] are also increased. An alkaline environment in osteoblasts provides the required conditions to lay Ca^{2+} down into bone.

Other factors that influence bone metabolism are also influenced by isoflavones such as osteocalcin. This protein is secreted by osteoblasts and holds a stake in Ca^{2+} homeostasis and bone mineralization.

A lot of those effects are ER-dependet, since they are abrogated by co-treatment with the ER-antagonist ICI 182,780. Although it seems that some effects are partly ER-independent, as observed for daidzein. Notwithstanding, other effects of daidzein on osteoblasts are indeed ER-dependent. So, daidzein inhibits osteoclast differentiation and activity via caspase-8 and caspase-3 in an ER-dependent manner. Similarily, genistein decreased osteoclasts due to a induced caspase-3 increase. Moreover, it is surmised that the topoisomerase II inhibiting effect of genistein also promote the inhibition of osteoclastogenesis.

Bone marrow stromal cells can due to their pluripotent character differentiate into osteoblasts and adipocytes among others. An inverse relationship between osteoblastogenesis and adipogenesis has been postulated as well as between osteoporosis and increased marrow adipose tissue (reviewed by Nuttall and Gimble [84]). Isoflavones shift the balance in the favor of osteoblastogenesis [85, 86].

The in vitro data are in accordance with the clinical and epidemiological findings that isoflavones prevent osteoporosis.

6. **Isoflavones and cancer**

***Growth stimulation of isoflavones* in vitro**

Isoflavones act through estrogen receptor α (ERα) and β (ERβ). In order to understand the effects of isoflavones in terms of their potential growth-promotion or inhibition in cancer cells, the receptor status and eventual mutations have to be known. Isoflavones exert multiple actions in cancer cells and the isolated view as weak estrogens hampers a sound conclusion on their real biological properties.

Natural estrogens regulate the growth, differentiation and function in many reproductive tissues like the uterus and the mammary glands through activation of ERα. Tissue specific response of estrogens is explained by differential expression of coactivator and repressor proteins. Due to their affinity for the ER, isoflavones may exert similar effects on tissues like estrogen although their potency is 10-1000 times lower. Thus they have been also categorized by toxicologists as endocrine disrupters only taking into account their actions through the narrow window of weak estrogens, whereas the other biological properties have not been considered.

Besides ER mediated growth stimulation, cell proliferation may also be mediated through other mechanisms. The G-protein coupled receptor 30 (GPR30), a seven domain transmembrane receptor has been shown to be responsible for growth stimulation of tumor cells through releasing a cascade of events which lead to cell cycle progression like upregulation of *c-fos*, phosphorylation of ERK and cyclin A and D1 expression.

In different studies, genistein stimulated proliferation in ER positive breast cancer cell lines MCF-7 and T47D in a dose-dependent manner [3, 87] (Table 3).

At low concentration, up to 20µM, genistein increases cell growth via ER-dependent pathways. Up from 20µM, the proliferation stimulating effect was diminished and genistein

exerts cytotoxic activity [88-95]. This biphasic effect also know as bell-shaped response curve (Figure 6) was also reported for daidzein and red clover extracts [96, 97].

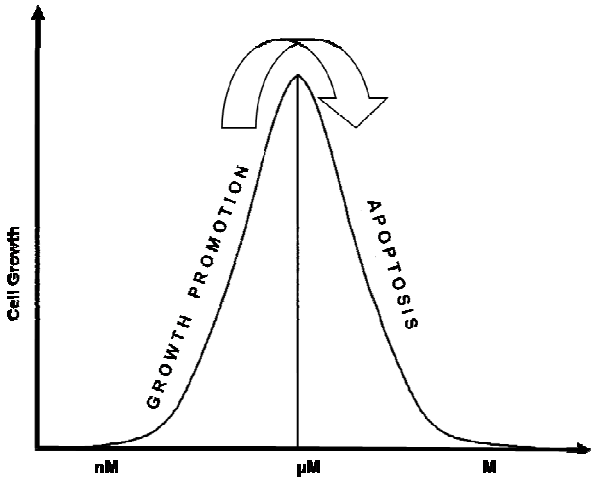

Figure 6: Biphasic, dose-dependent effect of isoflavones on growth of ER-positive cancer cells.

Often phytoestrogens and especially the isoflavones have been lumped with the xenoestrogens (endocrine disrupters), which are also weak estrogens, but do not show this growth inhibition at higher concentrations and lack all other effects on cancer cells. Aforementioned, Soto et al. developed a screening method for detecting the estrogenic action of different compounds [3]. In this so-called E-Screen, the growth promoting action of estrogenic substances is quantified by the proliferation of MCF-7 cells. Due to the apoptotic action of isoflavones at concentrations higher than 20µM, the E-Screen can only produce reliable results below the turn-around point. This narrow analytical window challenges the applicability of the E-screen at high concentrations especially for isoflavones.

Isoflavones prevent cancer growth of a large number of different cancer cells. The mechanisms involved therein are beside others apoptosis or the induction of different events leading to apoptosis. A summary of the exerted action of isoflavones regarding apoptosis and cell growth is listed in Table 3.

The process of programmed cell death can be activated through different mechanisms. It is a component of various processes like tissue development, cell turnover, proper development, functioning of the immune system et cetera.

Apoptosis differs from necrosis in essential mechanisms. During apoptosis, no cellular constituents are released into the surrounding matrix. Phagocytosis of cells undergoing apoptosis is quickly induced to prevent induction of necrotic events. The cells responsible for phagocytosis do not produce anti-inflammatory cytokines after contact with apoptotic cells. Entering apoptotic pathways, the cell shows defined symptoms. Cell shrinkage, chromatin condensation, membrane bebbling, protein cleavage or cross-linking, DNA cleavage and phagocytic recognition are examples for such distinctive events. Apoptosis can be induced through different biochemical features which result in entering either the intrinsic, the extrinsic or the perforin/granzyme pathway always leading to different execution pathways. The extrinsic pathway is induced through activation of so-called death receptors. Those receptors are members of the tumor necrose factor receptor gene family (TNF). A ligand for this receptor is the tumor necrose factor α (TNFα). The proapoptotic activity of TNFα is counteracted by the activation of nuclear factor κ B (NFκB). The products of NFκB target gene suppress apoptosis; therefore the downregulation of NFκB can lead to induction of apoptosis via TNFα. Upon binding of TNFα to its receptor, a signal cascade is induced, resulting in the activation of procaspases and caspases. Caspases are proteolytic enzymes, which can be categorized into apoptosis initiators and executioners and inflammatory caspases. As soon as execution caspases are activated, the final pathway of apoptosis is initiated. Cytoplasmic endonuclease is activated, which leads into degradation of nuclear material. Caspase-3 is one of the most important executive caspases, inducing not only endonuclease activation but also cytoskeletal reorganization and disintegration of the cell. The intrinsic pathway also leads into the executive pathway but does not involve receptor-mediated events. Intracellular signals are produced, which result in activation of pro-apoptotic

events or in suppression of anti-apoptotic events. All these events lead to a change in the inner mitochondrial membrane and release of two groups of pro-apoptotic proteins. These apoptotic mitochondrial events are under the control of a protein-family called Bcl-2, which are regulated by the tumor suppressor protein p53. Some of the members of the Bcl-2 family are pro-apoptotic like BAX, or anti-apoptotic like Bcl-2. All these events activate caspase-dependent cascades leading to the executive pathway. The effect of isoflavones on apoptosis-regulating pathways is listed in Table 3.

DNA damage is one of the elicitors of apoptosis. Tumor suppressor protein 53 (p53) recognises DNA damage and constitutes a checkpoint for cell fate. p53 leads then to a recruitment of factors for transient cell cycle arrest paralleled with the activation of factors for DNA repair. If defective repair appears and the cell survives or the p53 check-point is abrogated, the development of cancer can be promoted. Severe DNA damage, which can not be repaired leads to a p53-stimulated downregulation of apoptosis-repressor genes such as Bcl-2 and upregulation of apoptosis-effectors such as BAX. A third p53-mediated answer to DNA damage can be also cellular senescence.

All factors, which lead to an activation of p53 can therefore induce apoptosis.

The cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ is one of these factors involved in the p53 tumor suppressor pathway, acting as G1 cyclin kinase inhibitor. It plays a crucial role in cell cycle regulation. Upregulation of $p21^{waf1/cip1}$ leads the cells to growth arrest and execution of apoptosis.

Isoflavones also affect directly genes responsible for error-free DNA repair like the breast cancer-susceptibility genes BRCA1 and BRCA 2. The BRCA1 and BRCA2 proteins have multiple biological functions including proper repair of DNA double strand breaks. Mutation of these genes and therefore an increase in defective DNA repair are associated with an increased risk in developing cancer. Upregulation of these genes, which is facilitated by isoflavones, benefits the accurate repair of DNA damage [98-105] (Table 3).

Table 3:Apoptosis-inducing effects of isoflavones in different human cancer cells.

Substance	Tissue / Cell line	Effect	Reference
Genistein	Breast cancer cells MDA-MB-231 MCF-7 MCF7D40 T47D MDA-MB-435 MDA-MB-468	• Activation of Caspase-3, • Decreased NFκB activity • Cell cycle arrest • Upregulation of BAX and downregulation of Bcl-2 • Upregulation of $p21^{waf1/cip1}$ • Upregulation of BRCA1 and BRCA2	[26, 106-118]
	Prostate cancer cells DU145 LNCaP PC-3 PCa	• Activation of Caspase-3 • Decreased NFκB activity • DNA fragmentation • Cell cycle arrest • Upregulation of $p21^{waf1/cip1}$ • Upregulation of BRCA1 and BRCA2	[107, 108, 112, 114, 119, 120]
	Colon cancer cells HT29	• Upregulation of BAX and downregulation of Bcl-2	[121]
	Bladder cancer cells RT4 J82 HT1376 T24	• Cell cycle arrest	[122]
	Leukaemic cells HL60 M07e MOLT-4	• DNA degradation • Nuclear condensation • Nuclear fragmentation	[49, 123]
	Stomach cancer cells HCS-41E6 HSC-45M2 SH101-P4 AGS	• DNA fragmentation • Nuclear fragmentation • Chromatin condensation • Activation of Caspase-3	[124, 125]
	Lung cancer cells H460	• Decreased NFκB activity • Upregulation of BAX • Upregulation of $p21^{waf1/cip1}$	[108, 126, 127]
	Pancreas cancer cells BxPC-3 HPAC PANC-1	• Decreased NFκB activity • Cell cycle arrest	[108, 128]
	Liver cancer cells Bel 7402 HepG2	• Cell cycle arrest • DNA fragmentation • Upregulation of $p21^{waf1/cip1}$	[129-131]
	Kidney cancer cells SMKT R-1 SMKT R-2 SMKT R-3 SMKT R-4	• Cell cycle arrest	[132]
Daidzein	Cervical cancer cells HeLa	• Cell cycle arrest	[133]
	Colon Cancer cells LoVo	• Increased Caspase-3 activity • DNA fragmentation	[96]
	Prostate Cancer Cells LNCaP DU145 PC-3	• Cell cycle arrest • DNA fragmentation	[120]

	Breast Cancer Cells MDA-MB-231 MCF-7 MDA-468	• Upregulation of BRCA1 and BRCA2	[26, 106]
Biochanin A	Breast Cancer Cells MDA-468 MCF-7	• DNA fragmentation • Nuclear fragmentation • Chromatin condensation • Activation of Caspase-3	[124, 125]
	Stomach cancer cells HCS-41E6 HSC-45M2 SH101-P4 AGS	• DNA fragmentation • Nuclear fragmentation • Chromatin condensation • Activation of Caspase-3	[124, 125]
Soy extracts	Prostate Cancer Cells LNCaP DU145 PC-3	• Cell cycle arrest • DNA fragmentation • Upregulation of $p21^{waf1/cip1}$	[120, 134]
Red Clover Extracts	Breast Cancer Cells MCF-7	• Cell cycle arrest	[97]

In a variety of in vitro models a growth retardation and apoptotic behavior have been observed. This is consistent with the epidemiologic and clinical experience.

Influence of isoflavones on angiogenesis

Angiogenesis is a natural process of normal tissue growth. The formation of new vessels is important for adequate oxygen and nutrient supply as well as for the removal of waste products. Excessive angiogenesis is part of the pathology of different diseases like cancer. Therefore, the prevention or prohibition of neo-angiogenesis in tumor-tissue exerts a target for cancer prevention or cancer treatment.

The progress of angiogenesis can be influenced by isoflavones either by downregulation or negatively-influencing pro-angiogenic factors or by upregulation or positively-influencing anti-angiogenic factors like angiogenesis inhibitors (Table 4).

The initiating step of angiogenesis is endothelial cell activation through cytokines released in response to hypoxia or ischemia. Vascular endothelial growth factor (VEGF) production and the expression of its receptor are under the control of hypoxia inducible factor (HIF). VEGF affects vasodilation via endothelial NO production, influences vascular permeability and induces the expression of proteases and receptors for cellular invasion and tissue remodelling.

VEGF inhibition has been demonstrated with several cancer cell lines. Anti-angiogenic effects of isoflavones, such as VEGF inhibition are listed in Table 4. After endothelial cell activation, the penetration of cells into the surrounding matrix is facilitated by degradation of the body matrix through so-called matrix metalloproteinases (MMPs). MMP expression is promoted by NFκB, which gives the evidence, that NFκB could also play a role in cancer treatment by influencing angiogenesis. Genistein shows the inhibition of MMP expression tested in various cell systems (Table 4).

Besides VEGF, other pro-angiogenic factors have also been reported. Such factors are beside others, platelet derived growth factor (PDGF) or fibroblast growth factors (FGFs), which exert direct proangiogenic activity. FGF is mobilized through plasmin, which degrades fibronectin, laminin and the protein core of proteoglycans.

After endothelial cell activation and degradation of the surrounding matrix, the next step is the invasion of endothelial cells into the matrix followed by capillary tube formation (see Figure 7). This step is influenced by an interaction of many factors and ends up in vessel remodelling and matrix stabilization of the newly formed capillary [135, 136].

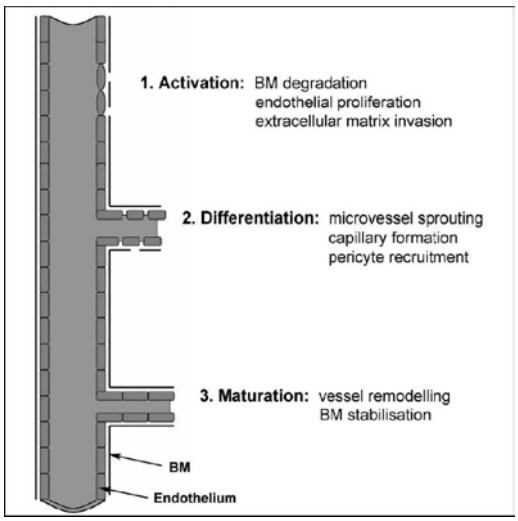

Figure 7: The stages of neovascularization (BM, basement membrane); produced by kind permission of Atkin and Chopada [135].

The balance between capillary formation necessary in normal tissue function and the overcharge of angiogenesis in tumor growth is a sensitive interplay between pro- and anti-angiogenic factors.

Such anti-angiogenic factors are beside others endostatin, tumstatin, angiostatin, thrombospondin-1 and platelet factor-4, which exert their action on various targets. Endostatin interacts with cell surface proteins like integrins and with VEGF. Furthermore it forms a stable complex with MMPs, which blocks the catalytic domain of the enzyme, causes cell cycle arrest of endothelial cells and activates apoptotic pathways. Thumstatin also causes cell cycle arrest and induction of apoptosis. So do angiostatin and thrombospondin-1. Platelet factor-4 is known to inhibit the upregulation of MMPs [137].

Isoflavones interfere in angiogenesis on several levels either by downregulation factors promoting angiogenesis or upregulating factors preventing angiogenesis (Table 4).

Table 4: Anti-angiogenic effects of isoflavones on different human cancer cells.

Substance	Tissue / Cell line	Effect	Reference
Genistein	Bladder cancer cells RT4 J82 5637 T24	• VEGF inhibition • MMP inhibition • Upregulation of Endostatin • Upregulation of Angiostatin • Upregulation of Thrombospondin-1	[138]
	Breast cancer cells MDA-MB-435 MCF-7 435.eB	• MMP inhibition	[109, 116]
	Endothelial cells HUVEC	• Cell cycle arrest • VEGF inhibition	[139, 140]
	Pancreas cancer cells Capan-1 Capan-2 AsPc-1 PANC-1 Mia PaCa-2	• VEGF inhibition • Impaired HIF-1 activation	[141]
	Prostate Cancer Cells PC3 PC3-M DU145	• MMP inhibition • VEGF inhibition	[139, 142]

Isoflavones and inflammation

Tissue injury represents an initiating step of the inflammatory response leading to cancer. The host response activated in order to re-establish proper tissue function involves a huge network of signals. Leukocytes and mast cells are directed to the site of injury through so-called chemokines. Such chemokines, which play an important role in chronic inflammatory diseases and in the development of chancer are, beside others tumor necrose factor-α (TNF-α), interleukins (ILs) and interferons (IFNs).

Many of these factors associated with chronic inflammation are found in cancer cells and may exert a new drug target for cancer prevention.

TNF-α appears in the cell either as membrane-integrated protein or as a soluble cytokine. Binding of TNF-α to its receptor leads to activation of NF-κB, which suppresses apoptosis and induces IL-6 expression. If NF-κB is not expressed, TNF-α can mediate apoptosis through caspase activation and other pathways.

NF-κB is a transcription factor, which is, beside in apoptosis, involved in many mechanisms associated with cancer development or cancer progression. The activation of NF-κB comes along with generation of inflammatory cytokines, stimulation of proliferation and angiogenesis. The production of reactive oxygen species (ROS) and thus the induction of DNA-damage is also a side-effect of NF-κB activation.

The induction of IL-6 expression can also be associated with NF-κB activation. IL-6 exerts different functions in host defence like activation of acute phase response upon viruses, bacterial endotoxins or inflammatory cytokines like TNF-α [143-147].

Isoflavones have been shown to influence the levels of TNF-α, NF-κB, IL-6 or to exert antioxidative activity by reducing ROS levels. Therefore, they might play a role in inflammatory induced cancer induction. The influence of isoflavones on these mechanisms is listed in Table 5.

Table 5: Anti-inflammatory effects of isoflavones on different cancer cells.

Substance	Tissue / Cell line	Effect	Reference
Genistein	Prostate cancer cells LAPC-4	• Antioxidative activity	[148]
	Pancreas cancer cells BxPC-3	• Downregulation of NF-κB	[149]
	Ovarian cancer cells Caov-3 NIH:OVCAR-3	• Reduction of IL-6 level	[150]
	Breast cancer cells MDA-MB-231	• Reduction of IL-6 level	[151]
	Liver cancer cells HepG2	• Reduction of IL-6 level	[152]
	Colon cancer cells Caco-2	• Reduction of IL-6 level	[153]
	Bone cancer cells G-292	• Reduction of IL-6 level	[26]
Daidzein	Ovarian cancer cells Caov-3 NIH:OVCAR-3	• Reduction of IL-6 level	[150]
	Bone cancer cells G-292	• Reduction of IL-6 level	[26]
Formononetin	Colon cancer cells HT29	• Antioxidative activity	[154]

Xenografts – the reduction to a common denominator

The effects of isoflavones regarding *in vitro* studies, suggest, that soy- or red clover- derived food supplements might exert anti-tumorigenic action *in vivo*. Supporting this hypothesis, the xenograft mouse model exerts an insight to *in vivo* action of isoflavones. Genistein was shown to inhibit angiogenesis by decreasing vessel density in MDA-MB-231 (ER negative) cells implanted in nude mice. Pretreatment of ER negative breast cancer cells (MDA-MB-435) with genistein reduced the cells' tumorigenic potential. Genistein fed to nude mice after tumor removal decreased the metastatic potential significantly [116, 155, 156]. Bel 7402 hepatocellular carcinoma xenograft model, showed an inhibition of tumor invasion and reduced tumor growth [131]. LNCaP prostate cancer xenograft model showed a decrease in tumor cell proliferation, an increase in apoptosis and a reduced vessel density upon soy phytochemical concentrate fed to the mice [120]. In a pancreatic cancer xenograft model with BxPC-3 cells, genistein supported the antitumor activity of cisplatin, which is used for treatment of this cancer. Besides enhancing the effect of cisplatin, genistein itself shows a

tumor growth inhibiting effect [157]. Biochanin A inhibits tumor growth in LNCaP xenografts [158].

A MCF-7 breast cancer (ER positive) xenograft mouse model, showed a slight dose-dependent increase in tumor growth in mice fed with genistein or daidzein [88, 159]. Mice, fed with equol, did not show any increase in tumor size [159]. By contrast, a pre-treatment of implanted MCF-7 cells with genistein diminished the tumorigenic potential of the cells and it was also reported that injection of genistein in implanted mice inhibited tumor cell growth and stimulated apoptosis [116, 155]. With a similar model daidzein, equol and genistein as well as soy extract and a food supplement have been tested [88, 90, 159, 160].

A summary of the different effects of isoflavones on xenograft mouse models is listed in Table 6.

The *in vivo* tests have to be interpreted with care since a lot of rodents are weak equol-producers. Equol does not promote tumor growth [159]. In all other cancer cell xenografts except the MCF-7 cells a cancer protective effect can be observed which clearly is in line with the *in vitro* assays.

Table 6: Effects of isoflavones on xenograft mouse models

Substance	Implanted cell line	Effect	Reference
Genistein	Liver Cancer Bel 7402	• Inhibition of tumor invasion • Reduced tumor growth	[131]
	Pancreas Cancer BxPC-3	• Enhanced of chemosensitivity • Growth inhibition	[157]
	Breast Cancer (ER-) MDA-MB-231 MDA-MB-435	• Inhibition of angiogenesis • Decreased vessel density • Reduction of tumorigenic potential • Reduction of metastatic potential	[116, 155, 156]
	Breast Cancer (ER+) MCF-7	• Slight dose-dependent increase in tumor size of mice fed with genistein • Inhibition of tumor growth upon injection of genistein • Reduction of tumorigenic potential upon pre-treatment of the implanted cells with genistein	[88, 116, 155, 159]
Daidzein	Breast Cancer (ER+) MCF-7	• Slight dose-dependent increase in tumor size	[159]

Biochanin A	Prostate Cancer LNCaP	• Inhibition of tumor growth	[158]
Equol	Breast Cancer (ER+) MCF-7	• No increase in tumor size	[159]
Soy Concentrate	Prostate Cancer LNCaP	• Decrease in tumor cell proliferation • Increase in apoptosis • Reduction of vessel density	[88, 120]
	Breast Cancer (ER+) MCF-7	• Slight dose dependent increase in tumor size	

Further effects of isoflavones

Influence on cancer treatment

Besides directly affecting malignant cells, isoflavones have also been shown to influence the treatment of cancer [161-168] (see Table 7). In some cases, chemotherapy comes to its limit because of the resistance of cancer cells to different chemicals. This effect is called multidrug resistance [159, 169, 170]. Cells contain drug efflux transporters, such as P-glycoproteins (P-gps). An overexpression of these molecular pumps or other multidrug resistance-associated proteins (MRPs) is due to a decrease of intracellular drug concentration by active removing of compounds out of the cell [163, 169, 170]. In other cases, isoflavones have been detected to enhance the effects of radiotherapy and chemotherapy [161, 164-166]. The different effects of isoflavones on cancer treatment are listed in Table 7.

Table 7: Influence of isoflavones on cancer treatment.

Substance	Tissue / Cell line	Effect	Reference
Genistein	Prostate Cancer	Radiosensitizing effect	[164, 165]
	Pancreatic Cancer	Chemosensitizing effect	[161]
	Leukaemia Cells	Chemosensitizing effect	[166]
	Lung Cancer	MDR Inhibition	[167]
Daidzein	Prostate Cancer	Radiosensitizing effect	[164]
Biochanin A	Breast Cancer	MDR inhibition	[162, 167, 168, 171]

eNOS

Isoflavones derived from soy or red-clover, especially genistein, influence cardiovascular diseases via endothelial nitric oxide expression. Nitric oxide (NO) is a vasodilator produced

by the enzyme endothelial NO synthase (eNOS). NO upregulation or activation of NO has been demonstrated with pure isoflavones but also isoflavone enriched plant extracts used as food supplements. NO regulates many functions including platelet aggregation, leukocyte adhesion and smooth muscle cell mitogenesis. High-density lipoprotein, shear stress and VEGF have also been shown to induce an increase in eNOS. A decrease in endothelial-derived NO leads to an impaired reactivity of the vessel wall, which is diagnosed during the development of atheriosclerosis.

7. General conclusions

Safety of isoflavones as food supplements or in food can be approached on four levels of complexity: the molecular and cellular level, *in vivo* experiments with animals and epidemiological studies and clinical intervention studies. Clinical and epidemiological studies showed clear evidence that isoflavones are safe and that isoflavone intake is significantly correlated with lower incidence of breast cancer, cardiovascular diseases, just as there is also evidence on beneficial effects of isoflavones on metabolic diseases.

The weak estrogenic activity of isoflavones has been debated in context of increased risk of breast and prostate cancer. Isoflavones show weak affinity for ERα and higher affinity for ERβ in transactivation and binding assays. In cell culture assays using estradiol-dependent cell lines a bell-shaped dose-dependent response behavior has been observed. At low concentrations a growth promoting effect was observed, altough in the micromolar range this compounds exert significant apoptotic behavior. In *in vivo* assays a similar behavior has been shown with the isolated isoflavone genistein, while the metabolite equol or soy extracts did not show growth promoting properties. It seems that this growth promoting behavior is an artificial effect of the isolated compound genistein, whereas in food supplements and in food a mixture of isoflavones is present. In addition they are metabolized to compounds with apoptotic behavior. Also epidemiological data show that the so-called equol-producers have a lower incidence for breast cancer. Isoflavones also inhibit angiogenesis, which is a key step in tumor formation and also reduction of inflammation. This is another *in vitro* evidence for a cancer protective effect of isoflavones. Additional health benefits of isoflavone consumption concern bone health through promotion of osteoblast formation and positive effects on metabolic diseases. The weight reduction by isoflavone-rich diet or food supplements can be explained *in vitro* by its affinity to the PPARs.

In light of the in vitro data and the clinical and epidemiological data, the isoflavones are safe compounds. Due to the beneficial health effects a promotion of isoflavone consumption is mandatory at todates knowledge base.

8. SPECIAL NOTE

Further references can be obtained from the corresponding author on request.

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Review

Potential Health-modulating Effects of Isoflavones and Metabolites via Activation of PPAR and AhR

Svjetlana Medjakovic [1,2#], Monika Mueller [1,2#] and Alois Jungbauer [1,2]*

[1] Department of Biotechnology, University of Natural Resources and Applied Life Sciences Vienna, Muthgasse 18, 1190 Vienna, Austria; Emails: svjetlana.medjakovic@boku.ac.at (S.M.); monika.mueller@boku.ac.at (M.M.)

[2] Christian-Doppler-Laboratory of Receptor Biotechnology, Muthgasse 18, 1190 Vienna, Austria

[#] These authors contributed equally to this work.

* Author to whom correspondence should be addressed: E-mail: alois.jungbauer@boku.ac.at

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Abstract: Isoflavones have multiple actions on cell functions. The most prominent one is the activation of estrogen receptors. Other functions are often overlooked, but are equally important and explain the beneficial health effects of isoflavones. Isoflavones are potent dual PPARα/γ agonists and exert anti-inflammatory activity, which may contribute to the prevention of metabolic syndrome, atherosclerosis and various other inflammatory diseases. Some isoflavones are potent AhR agonists and induce by that cell cycle arrest, chemoprevention and modulate xenobiotic metabolism. This review discusses effects mediated by the activation of AhR and PPARs and casts a light on the concerted action of isoflavones.

Keywords: Isoflavones, PPARα, PPARγ, AhR, inflammation, metabolic syndrome, atherosclerosis, cell cycle control, xenobiotic metabolism

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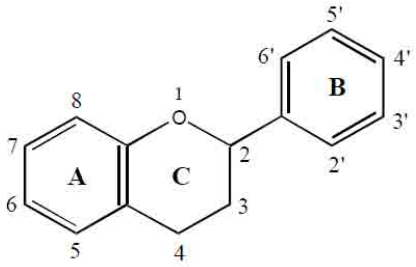
1. Introduction

1.1 Systematics of isoflavones

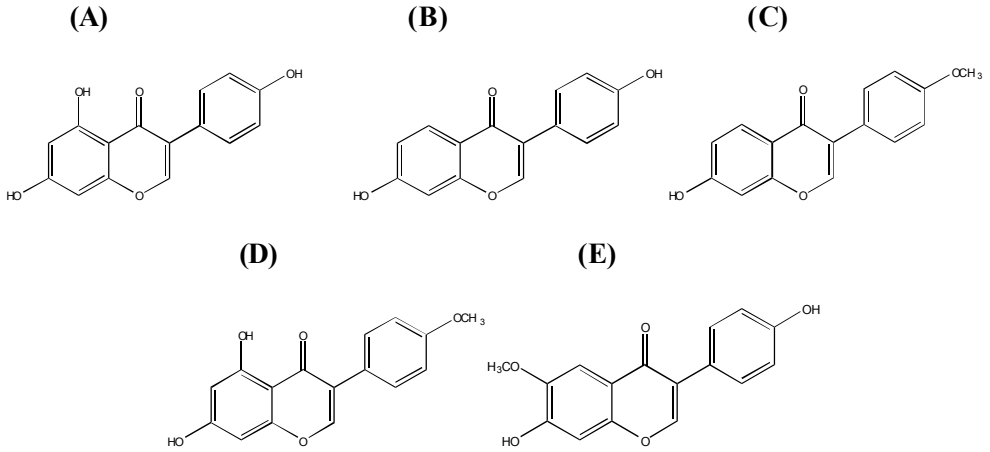
Isoflavones are a subgroup of plant phenols, which make up a group of aromatic secondary plant metabolites derived from the shikimate pathway and phenylpropanoid metabolism [1]. These compounds are widely distributed in all plant species and include simple phenol, phenolic acids, phenylacetic acids, hydroxycinnamic acids (e.g., caffeic acid, ferulic acid), coumarins, stilbens (e.g., resveratrol), flavonoids, lignans, lignins, and condensed tannins. Flavonoids are characterized by a core structure of a C6-C3-C6 flavone skeleton in which the C3 portion is commonly cyclized with oxygen (**Figure 1**). They vary in the degree and location of unsaturation and oxidation [1, 2].

Figure 1. Structure of the flavonoids [with two aromatic benzol rings (A and B rings)] and a C3 portion cyclized with oxygen (C ring).



The group of flavonoids includes anthocyanins, flavans, flavanones, flavones, flavonols, and isoflavonoids. Isoflavonoids are characterized by being substituted by various hydroxyl and/or methoxy groups. This group includes, for example, genistein, daidzein, formononetin, biochanin A, and glycitein [2, 3] (**Figure 2**).

Figure 2. Structure of isoflavones: (A) genistein, (B) daidzein, (C) formononetin, (D) biochanin A, and (E) glycitein.



1.2 Dietary sources and intake of isoflavones

Isoflavones are found in trace amounts in fruits such as apples [4] and strawberries [5] and plant seeds such as sesame [5] and sunflowers [4]. But the main sources are legumes, especially the Fabaceae family, in particular soy [4, 6, 7] and red clover [8, 9].

Soy is widely used in Asia as a staple food and consumed regularly in traditional food items such as tofu, miso, natto, edamame (whole soybeans), soybean paste, and shoyu (fermented soy sauce). Hence, the isoflavone intake among Asians is about a factor of 100 higher than that of people in the Western world. The daily isoflavone intake among Southeast Asians ranges between 15 and 47 mg [10-16], while Western people consume only between 0.15 and 1.7 mg isoflavones per day [17-21].

Red clover (*Trifolium pratense*) is widely used as a fodder crop in the Western world. In former times, it was also used in dried and milled form as a flour extender and as a salad ingredient. Today, it is mostly consumed as a food supplement for the amelioration of menopausal complaints.

The isoflavone composition of soy and red clover differs. Soy isoflavones are mainly daidzein, genistein, and glycitein, but the predominant isoflavones of red clover are formononetin and biochanin A, while daidzein and genistein are found only in trace amounts [8, 9].

1.3 Metabolism and bioavailability of isoflavones

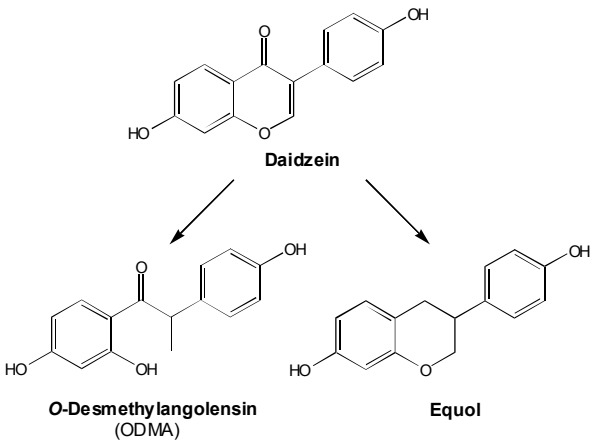
Most of the isoflavones are bound as glucosides in plants. There is evidence that hydrolysis of the sugar moiety is needed for absorption [22], but the data are inconsistent; some studies report no difference between the absorption of aglycones and glucosides [23-25], while others found that aglycones were absorbed more efficiently [26, 27]. Nevertheless, aglycone absorption seems to be unaffected by food matrix and food processing [28] or isoflavone source [29].

After oral uptake, the gastrointestinal tract is the main absorption site of isoflavones. Intestinal β-glucosidases catalyze hydrolysis of the sugar moiety [30], and the gut microflora further metabolize the agylcones. The metabolites that result depend on the individual microflora and can differ to a great extent. During metabolism, formononetin and biochanin A are demethylated to daidzein and genistein, respectively.

The most significant metabolite, however, is certainly equol. Excretion of this metabolite of daidzein has been associated with a reduced risk of breast and prostate cancers [31-34]. The incidence of breast and prostate cancers is lower among Asians in comparison to people in the Western world [35], although breast cancer incidence is rising in Asia [36-39], probably because of lifestyle and nutrition changes that increasingly are oriented towards a Western lifestyle. Not everyone can produce equol, and the prevalence of so-called equol producers ranges from 30–50% [40-49].

Another metabolite of daidzein is *O*-desmethylangolensin (ODMA). In comparison to daidzein and equol, ODMA has a weaker affinity for estrogen receptors (ERs) [50]. Daidzein is converted to ODMA because of a ring cleavage, while equol arises after the elimination of a carbonyl-group (**Figure 3**).

Figure 3. Possible metabolization products of daidzein.



Various other metabolites of isoflavones have been identified [51-53]. As mentioned, the emerging metabolite pattern is inter-individually different and depends on the intestinal microflora. For further information on bioavailability, there are several excellent reviews that have their main focus on this topic [54-58], but it should be noted that isoflavones are among the best bioavailable polyphenols.

1.4 Metabolic diseases

Cardiovascular diseases like myocardial infarct and cerebrovascular diseases are the principal cause of death worldwide, representing 30% of all global deaths in 2005. If current trends continue, by 2015, an estimated 20 million people will suffer from cardiovascular diseases [59]. A sedentary lifestyle and excessive energy intake lead to an increase in the prevalence of obesity. An excess of body fat, especially visceral fat, is a key factor for developing the metabolic syndrome [60, 61]. The International Diabetes Federation has defined the metabolic syndrome as central obesity (waist circumference ≥94 cm for male Europeans and ≥90 cm for male South Asians, Chinese, and Japanese and ≥80 cm for female Europeans, South Asians, Chinese, and Japanese) plus any two of the following four factors: raised triglycerides ≥150 mg/dL (1.7 mmol/L) or specific treatment for this lipid abnormality; reduced HDL (high density lipoprotein) cholesterol of <40 mg/dL (1.03 mmol/L) in males and <50 mg/dL (1.29 mmol/L) in females or specific treatment for this lipid abnormality; raised blood pressure, with a systolic blood pressure ≥130 or diastolic blood pressure ≥85 mm Hg or treatment of previously diagnosed hypertension; raised fasting plasma glucose ≥100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes [62]. Cardiovascular diseases are more prevalent among patients with this syndrome [63-67].

Adipose tissue is an active endocrine organ producing a great variety of hormones and cytokines that are involved in glucose metabolism, lipid metabolism, inflammation, coagulation, and blood pressure. An increase in visceral fat mass is associated with an increase in secreted bioactive molecules including tumor necrosis factor (TNF)α, interleukin (IL)-6, angiotensinogen, and plasminogen activator inhibitor type 1 [68-71]. The concentration of adiponectin, a hormone that increases insulin sensitivity, has been identified to be significantly lower in the adipose tissue or serum of obese mice or

humans than in lean control mice [72, 73]. The enhanced secretion of inflammatory factors in adipose tissue from obese animals and humans results in a low chronic inflammatory stage that is associated with enhanced development of diabetes mellitus, the metabolic syndrome, and atherosclerosis [61, 73].

1.4.1 Peroxisome proliferator-activated receptors α and γ

Isoflavones activate the ligand-dependent transcription factors known as peroxisome proliferator-activated receptors (PPARs). These are class II nuclear receptors, a class that heterodimerizes with retinoid X receptor and binds to direct repeat sequences of nucleotides, which are PPAR response elements in the case of PPARs [74]. The subtypes PPARα and γ vary concerning tissue distribution. PPARγ is found mainly in adipose tissue but also in liver, kidney, intestine, and muscle [75, 76]. PPARα is mainly expressed in liver, kidney, heart, muscle, and small intestine [76, 77]. Furthermore, PPARγ and α are found in inflammatory and immune cells such as monocytes, macrophages, B and T cells, and dendritic cells, and in vascular wall cell types such as endothelial and smooth muscle cells, linking them to a role in inflammatory responses [76-78]. Fatty acids and their derivatives are the main natural ligands of all PPAR subtypes. PPARγ ligands include the fatty acids palmitic acid, petroselinic acid, oleic acid, linolenic acid, linoleic acid, and arachidonic acid [79, 80], and fatty acid derivates like 15-deoxy-delta 12,14-prostaglandin J2 (15d-PGJ2) [81, 82]. PPARα is activated by the peroxisome proliferator WY 14,643 and by linoleic, α-linolenic, γ-linolenic, arachidonic, docosahexaenoic, and eicosapentaenoic acids, and by eicosanoids like 8(S)-hydroxyeicosatetraenoic acid, ±8-hydroxyeicosapentaenoic acid, and carbocyclin [81]. The synthetic ligands of PPARγ comprise the glitazones [82, 83], tyrosine-based agonists, and non-steroidal anti-inflammatory drugs like fenoprofen, ibuprofen, and indomethacin [80], and the synthetic ligands of PPARα include the fibrates [81].

PPARs play a role in improving several perturbations of the metabolic syndrome. The main function of PPARγ, which has been defined as a drug target for type 2 diabetes, is adipocyte differentiation and insulin sensitization [83-85]. PPARγ activation leads to a modulation of factors secreted by adipose tissue. Factors that promote insulin resistance, namely TNFα, leptin, IL-6, and resistin, are reduced, and factors that promote insulin sensitivity, like adiponectin, phosphoenolpyruvate carboxykinase, fatty acid transport protein, and insulin receptor substrate-2, are upregulated [86-90]. Activation of PPARγ further promotes adipogenesis and lipid storage in subcutaneous adipose tissue. The result is a redistribution of adipose tissue from harmful visceral fat mass to subcutaneous depots by activation of the involved genes, including fatty acid binding protein, phosphoenolpyruvate carboxykinase, acyl-CoA synthase, diacylglycerol acyltransferase 1, fatty acid transport protein, and lipoprotein lipase [87, 91, 92].

PPARα activation leads to an improved lipid profile by elevating HDL levels and reducing plasma triglyceride levels. The reduction of plasma triglyceride levels is achieved by induction of genes that decrease the availability of triglycerides for hepatic very low-density lipoprotein (VLDL) secretion [93, 94] and by an increased lipoprotein lipase (LPL)-mediated lipolysis of triglyceride-rich plasma lipoproteins like chylomicrons and VLDL particles [95]. This pathway is mediated by increased expression of LPL and the LPL activator apolipoprotein A-V and reduced expression of the LPL

inhibitor apolipoprotein C-III [96, 97]. HDL levels are elevated by increased hepatic apolipoprotein A-I and –II expression through PPARα activation [98, 99].

1.4.2 Inflammation and atherosclerosis

Atherosclerosis is a complex, chronic process involving the contribution of several factors including injury to the endothelium, proliferation of vascular smooth muscle cells, migration of monocytes or macrophages, and involvement of mediators like growth factors and cytokines [100]. In brief, endothelial dysfunction, an early marker of atherosclerosis, can be induced by elevated low-density lipoproteins (LDL), hypertension, or toxins after smoking and is associated with decreased nitric oxide (NO) synthesis [101]. An inflammatory response plays a major role in the progression of atherosclerosis. Oxidized lipoprotein, T cells, and macrophages enter into the vessel wall, which leads to enhanced oxidative stress in vascular cells and to an activation of intracellular signaling molecules. T cells recognize oxidized LDL or heat shock proteins and locally release pro-inflammatory cytokines [102]. Macrophages induce collagen breakdown in atherosclerotic plaques by secreting matrix metalloproteinases (MMPs) [103, 104]. In this way, the inflammatory response plays a major role in the initiation of atherosclerotic plaque formation and their destabilization. The rupture of a plaque underlies most of the acute coronary syndromes such as myocardial infarction, unstable angina, and coronary death [105].

PPARs are expressed in cells that are involved in several processes of atherosclerosis. In this way, PPARγ plays a role in improving cellular processes that contribute to atherosclerosis. Mechanisms are based on the correction of endothelial dysfunction, suppression of a chronic inflammatory process [86], reduction of foam cells and fatty streak formation [77, 106], attenuating plaque evolution, and promoting plaque stabilization [107, 108].

PPARα activation contributes to improvement of several atherosclerotic stages by downregulating pro-inflammatory genes [109] and inhibiting foam cell formation by enhancing expression of ATP-binding cassette A1 transport protein and thus increasing cholesterol efflux from macrophages and foam cells to HDL [110, 111]. Furthermore, a PPARα agonist was reported to inhibit MMP-12 expression in monocyte-derived macrophages, thus leading to an inhibition of atheromatous plaque rupture [112]. By decreasing tissue factor expression, the PPARα agonist fenofibrate reduces initiation of blood coagulation and thus thrombotic complications after plaque rupture. Furthermore, fenofibrate significantly enhances endothelial regrowth and plaque stability [113].

1.4.3 PPAR activation in in vitro *assays*

Activation of PPARα and γ and modulation of adipocyte differentiation in vitro are associated with putative antidiabetic or antilipidemic activity in vivo. Several studies have shown binding and/or activation of PPARα or PPARγ by the isoflavones genistein, daidzein, biochanin A, formononetin, and glycitein and the metabolites equol, ODMA, 6-hydroxydaidzein, 3´-hydroxygenistein, 6´-hydroxy-ODMA, angolensin, dihydrogenistein, dihydrobiochanin A, dihydroformononetin, dihydrodaidzein, and p-ethylphenol (Table 1). Generally, the transactivational activities were higher for biochanin A

and genistein than for daidzein or formononetin. Several metabolites showed higher PPARα or PPARγ binding and activation properties than their precursors, including equol, ODMA, 6-hydroxydaidzein, and 3´hydroxygenistein [114, 115].

Table 1. The isoflavones as PPARα and PPARγ ligands or activators.

PPARα Transactivation	**PPARγ Ligands**	**PPARγ Transactivation**	**Ref**
		biochanin A, genistein, daidzein, equol	[116]
	genistein	genistein	[117]
daidzein		daidzein	[118]
genistein			[119]
		daidzein	[120]
genistein, daidzein		genistein, daidzein	[121]
	biochanin A, genistein, daidzein, equol, ODMA, 6-hydroxydaidzein, 3´-hydroxygenistein, 6´-hydroxy-ODMA, angolensin, dihydrogenistein, dihydrobiochaninA, dihydroformononetin, dihydrodaidzein, p-ethylphenol	biochanin A, genistein, daidzein, equol, ODMA, 6-hydroxydaidzein, 3´-hydroxygenistein, 6´-hydroxy-ODMA, dihydrogenistein, dihydrodaidzein	[115]
biochanin A, genistein, daidzein, ODMA, 6-hydroxydaidzein, 3´-hydroxygenistein			[114]
genistein, daidzein		genistein, daidzein, glycitein	[122]
daidzein, equol			[123]
biochanin A, formononetin, genistein	biochanin A, genistein, daidzein	biochanin A, formononetin, genistein	[124]

Obesity and adipose tissue mass are associated with the number and volume of adipocytes, which result from adipocyte differentiation and triglyceride storage. Several studies have investigated the influence of isoflavones on adipocyte differentiation in 3T3-L1 cells. In these assays, 3T3-L1 preadipocytes are incubated with a differentiation medium and isoflavones simultaneously to test the effect on differentiation and the inhibition of lipid accumulation. In the maturation of preadipocytes, the transcription factors PPAR and CCAT/enhancer binding protein (C/EBPs) play a major role. First, the expression of C/EBPβ and C/EBPδ is induced by components of the differentiation medium (such as insulin, dexamethasone, and 3-isobutyl-1-methylxanthine) [125]. This induction leads to increased expression of PPAR2, C/EBPα, and sterol responsive element-binding protein (SREBP)-1, which in addition to a role in adipogenesis is responsible for the expression of mature adipocyte-specific genes like lipogenic enzymes, fatty acid binding proteins, and other secreted factors [85, 126, 127].

Much of the literature has focused on genistein, which inhibits adipogenesis at concentrations between 1 and 200 μM through various mechanisms: downregulation of the expression of adipocyte-

specific genes including C/EBPα and β, PPARγ [128, 129], fatty acid synthase [128-130], adipocyte fatty acid binding protein, SREBP-1, perilipin, LPL, and hormone-sensitive lipase [128]; downregulation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) activity [131] and the action of AMP-activated kinase [132]; enhancement of leptin secretion, increased expression of the adipogenesis inhibitor preadipocyte factor 1 (Pref-1) [129], and inhibition of janus-activated kinase (JAK)2-mediated adipocyte differentiation [130]. Interestingly, genistein, a PPARγ activator, inhibits adipocyte differentiation *in vitro* and thereby exerts putative anti-obesity activity. Other mechanisms for putative anti-obesity activity of genistein include the inhibition of lipid accumulation in human adipocytes [128, 130], possibly caused by inhibition of the activity of glycerol-3-phosphate dehydrogenase [128] and induction of apoptosis of mature adipocytes [132, 133].

Only a few studies have investigated adipocyte differentiation in the context of the other isoflavones. Shen et al. [124] showed that biochanin A induces lipid accumulation in preadipocytes at a low concentration (1 μM) and formononetin and genistein at higher concentrations (3 or 15 μM). Daidzein did not induce adipocyte differentiation at this concentration range. Cho et al. [123] reported that daidzein enhanced adipocyte differentiation in 3T3-L1 cells at concentrations between 10 and 100 μM and C3H10T1/2 stem cells at concentrations between 1 and 20 μM and that even its metabolite equol increased adipocyte differentiation in C3H10T1/2 cells at concentrations between 0.1 and 20 μM. These data indicate the putative role of the isoflavones genistein (only at high concentrations), daidzein, formononetin, and biochanin A and the metabolite equol in fat redistribution and thus in reducing harmful visceral fat mass and simultaneously insulin resistance.

Dang et al. [117, 118] found that in mesenchymal progenitor cells that can differentiate into osteoblasts or adipocytes, genistein and daidzein showed a biphasic effect. Adipogenesis was inhibited at low concentrations of genistein (0.1–10 μM) or daidzein (10–20 μM) and enhanced at high concentrations of genistein (>10 μM) or daidzein (>30 μM). Dang et al. [117, 118] explained the observed effects by an interaction of PPAR and ER with activation of ER, leading to an inhibition of adipogenesis at a low concentration and PPAR activation leading to enhancement of adipogenesis at a high concentration.

In addition to adipocyte mass, inflammation plays a major role in chronic diseases like diabetes and in the progression of atherosclerosis. Therefore, the anti-inflammatory activity of isoflavones and their metabolites in various cell culture systems is of great interest (Table 2). Cells are exposed to an inflammatory stimulus like lipopolysaccharide (LPS) or interferon (IFN)-γ. The subsequent inflammatory response is characterized by a sequential release of pro-inflammatory cytokines like TNFα, IL-6, IL-8, IL-1β, or IFN-γ [134] The nuclear transcription factor-κB (NFκB) controls the expression of pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, or inducible enzymes such as cyclooxygenase 2 (COX-2) and the inducible nitric oxide synthase (iNOS). Successively, iNOS and COX-2 induce the production of pro-inflammatory mediators [135]. The inflammatory state is resolved by anti-inflammatory cytokines including IL-4, IL-10, IL-13, and IFN-α [134].

In cell culture assays, isoflavones downregulate several pro-inflammatory mediators like TNFα, IL-6, IL-8, IL-1β, NO, prostaglandin E2 (PGE2), monocyte chemoattractant protein-1, IL-8, and intercellular adhesion molecule-1, or upregulate anti-inflammatory cytokines like IL-10 (Table 2). The expression of various proteins involved in the production of inflammatory mediators like iNOS, COX-

2, NFκB, and signal transducer and activator of transcription 1 (STAT-1) is downregulated or their activity is inhibited. Most data on putative anti-inflammatory activity are from studies with genistein, but daidzein, formononetin, biochanin A, glycitein, and the metabolites equol and ODMA also positively influence the profile of secreted mediators.

Furthermore, isoflavones inhibit monocyte adhesion to TNFα–activated human umbilical vein endothelial cells during flow. Because monocyte adhesion to endothelial cells is among the early steps of the inflammatory cascade and contributes to atherosclerotic development, isoflavones could help to prevent atherosclerosis by this mechanism [116].

Table 2. Influence of isoflavones on the secretion of various inflammatory markers in cell lines.

Compounds	**Cell line**	**Downregulated pro-inflammatory mediators**	**Upregulated anti-inflammatory mediators**	**Ref.**
genistein, equol	RAW 264.7	NO, PGE2		[136]
genistein, daidzein, formononetin biochanin A equol ODMA	RAW 264.7	TNFα, IL-6, iNOS, NFκB TNFα, IL-6, iNOS, NFκB iNOS TNFα, IL-6, iNOS, NFκB, Cox-2 TNFα, IL-6, COX-2 TNFα, IL-6	 IL-10 IL-10	[114]
genistein	HBMEC	TNFα, IL-1β, monocyte chemoattractant protein-1, IL-8, intercellular adhesion molecule-1		[137]
genistein, daidzein	murine J774 macrophages	iNOS, NO		[138]
genistein	Human chondrocytes	COX-2, NO		[139]
biochanin A	MC3T3-E1 cells	TNFα, IL-6, NO		[140]
genistein	PBLs	TNFα, IL-8		[141]
genistein	mesencephalic neuron-glia cultures	TNFα, NO, superoxide		[142]
daidzein, formononetin	mesencephalic neuron-glia cultures	TNFα, NO, superoxide		[143]
biochanin A	mesencephalic neuron-glia cultures	TNFα, NO, superoxide		[144]
genistein	alveolar macrophages	TNFα		[145]
daidzein genistein	PBMC	higher concentrations reduced IL-10 and IFN-γ levels IL-2, IL-4, IL-10, IFN-γ	low concentration increased IL-2, IL-4, and IFN-γ	[146]

		mRNA and protein	
genistein	RAW 264.7	NO, PGE2	[147]
genistein	RAW 264.7	PGE2, iNOS, COX-2	[148]
genistein, daidzein, glycetein	RAW 264.7	NO, iNOS	[149]
genistein, daidzein, equol	MCF-7 cells	COX-2	[150]

HBMEC (human brain microvascular endothelial cells); MC3T3-E1 (osteoblasts); MCF-7 (human breast cancer cell line); PBL (human peripheral blood mononuclear and/or polymorphonuclear leukocytes); PBMC (peripheral blood mononuclear cells); RAW 264.7 (mouse macrophage).

1.4.4 PPAR activation by isoflavones and its health effects

Given that cardiovascular diseases have reached epidemic proportions, it is of great interest that isoflavones exert *in vitro* activities that link them to putative antilipidemic, anti-obesity, antidiabetic and anti-inflammatory effects *in vivo*. The isoflavones genistein, daidzein, biochanin A, formononetin, and glycitein and several red clover metabolites like equol, ODMA, 6-hydroxydaidzein, 3´-hydroxygenistein, 6´-hydroxy-ODMA, dihydrogenistein, and dihydrodaidzein activate PPARα and γ, indicating putative antilipidemic and antidiabetic properties *in vivo*. Furthermore, adipogenesis is modulated by isoflavones. Most studies report an inhibitory effect of genistein, which may result in anti-obesity activity. Other studies report an inducing effect of genistein on adipogenesis. Biochanin A, formononetin, daidzein, and the metabolite equol enhance adipocyte differentiation and thus may promote fat redistribution from harmful visceral fat to subcutaneous fat. With a reduction in visceral fat mass, the risk for the metabolic syndrome and consequently cardiovascular diseases is reduced. Furthermore, isoflavones modulate cytokine secretion in cell culture assays, which indicates putative anti-inflammatory activities *in vivo*. Because inflammation plays a major role in atherosclerosis, anti-inflammatory activity may have a great influence on improving this disease.

Several results of *in vitro* assays are in agreement with outcomes from human or animal studies. Most animal studies were performed with genistein supplementation. An improvement of glucose levels or insulin resistance with isoflavone supplementation has been shown in obese or hypertensive rodent models [121, 151-153] and in human studies [154]. Genistein supplementation further led to lower lipid levels and increased HDL levels [151, 152, 155], to an improvement in vascular health attributable to NO- and prostaglandin-dependent pathways [151, 156], and to a stabilization of the atherosclerotic lesion, possibly because of reduced MMP-3 expression, based on results in rodent models and rabbits [157].

Supplementation with isoflavones from red clover or daidzein alone improved the lipid profile by increasing HDL and decreasing LDL, plasma total cholesterol, or triglyceride levels in rodent or rabbit models [153, 158]. Furthermore, supplementation with isoflavones led to an attenuation of atherosclerosis in studies with rabbits, possibly because of an inhibition of LDL oxidation [159] or reduction of fatty streak formation [158].

In human studies with postmenopausal women with type 2 diabetes, isoflavones from red clover reduced diastolic and systolic blood pressure [160]. With administration of only 40 mg of isoflavones, however, no effect on lipid profile was observed in postmenopausal women with hypercholesterolemia

[161]. In another study with postmenopausal hypercholesteremic participants, after a 6-week daily intake of 90 mg of isoflavones, vascular reactivity was improved, but blood cholesterol was not lowered [162]. A recent meta-analysis determined that soy isoflavones significantly reduced serum total and LDL cholesterol but had no influence on HDL cholesterol. The extent of LDL level reduction was greater in participants with hypercholesterolemia than in those without hypercholesterolemia [163].

Although several isoflavones function as PPARγ agonists, their intake does not cause weight gain as has been described for full agonists like glitazones. In fact, in various animal and human studies, isoflavone intake has led to a slight weight reduction [133, 152, 164-166].

The anti-inflammatory activity of isoflavone supplementation was also demonstrated in several human and animal studies. In animal models, soy isoflavones reduced LPS-induced inflammation by reducing IL-1β, IL-6, NO, and PGE2 production [167]. In hyperlipidemic rabbits, the level of C-reactive protein (CRP) was reduced [158]. Soy isoflavone intake has led to a significant reduction of blood CRP, IL-6, and TNFα levels in a study of patients with end-stage renal failure and systemic inflammation [168]. Conclusively, isoflavones exert simultaneous anti-inflammatory and antilipidemic activity, thus putatively leading to more effective agents for preventing or reducing atherosclerosis.

The anti-inflammatory activity of isoflavones not only improves atherosclerosis but also helps with other diseases associated with inflammation. Examples are the improvement of chronic colitis in a rodent model [169], inhibition of LPS-induced dopaminergic neurodegeneration in rats [143], amelioration of collagen-induced rheumatoid arthritis in a rodent model [170, 171], inhibition of pro-inflammatory cytokines in a neurodegenerative cell system [137], reduction of airway inflammation in an *in vitro* system due to inhibition of eosinophil leukotriene synthesis [172], amelioration of alveolitis [145], and putative prevention of osteoporosis due to anti-inflammatory activity in osteoblasts [140].

Of great importance is the physiological relevance of *in vitro* data. The serum concentration of isoflavones in humans after administration of supplements of concentrated isoflavones can reach approximately 10 μM [173]. An isoflavone-rich diet leads to plasma concentrations of 1 to 2.4 μM [174]. Those are ranges in which isoflavones already exert their PPAR activation or anti-inflammatory activities.

1.5 Xenobiotic metabolism and cell cycle control

Isoflavones are known as multitasking bioactive compounds. Their best-investigated aspect is their (anti)estrogenic activity. But as described above, they also modulate PPAR signal cascades. Beyond that, these compounds are ligands of the aryl hydrocarbon receptor (AhR). In the following section, we will describe this receptor and its implications in physiological processes, as well as possible effects of isoflavones via AhR activation.

1.5.1 The aryl hydrocarbon receptor

The AhR is a transcription factor involved in developmental processes as well as in normal physiological pathways such as cell cycle regulation or xenobiotic metabolism. It is a member of the

basic helix-loop-helix (bHLH) Per-ARNT-Sim (Pas) family and also shares elementary features of the mode of action of nuclear receptors. Reports have clearly established manifold crosstalk and interaction with nuclear receptors [175-177]. The AhR is a phylogenetically ancient protein that has been conserved during evolution [178] because of its important adaptive functions regarding extrinsic signals, such as light and exogenous compounds as well as metabolism and cell cycle control. These functions are also reflected in the diversity and heterogenicity of its ligands, which include physiologically occurring compounds like tryptophan [179], arachidonic acid metabolites [180, 181], heme metabolites [182], indigoids [183, 184], cAMP [185], equine estrogen [186], and UV products of tryptophan [187]; plant-derived compounds such as indoles [179, 188, 189] and flavonoids [190, 191]; and anthropogenic chemicals such as dioxin [192], polybrominated diephenyl ethers [193], and polychlorinated biphenyls [194]. Beyond that, it is believed that the AhR has endogenous ligands that have not been found so far, although it has been intensively studied since its discovery in 1976 by Poland et al. [195]. Furthermore, its expression patterns during embryonic stages indicate a significance of this receptor in development and ontology that is very likely not driven by exogenous ligand activation. Studies with AhR knockout mice have shown severe impairment of organ functions including liver, immune system, and reproductive organs because of deficient differentiation processes arising from lost AhR functions.

Given the role of AhR in mediating adaptation responses to environmental signals, important AhR target genes include those of the xenobiotic signal transduction pathway, such as those encoding enzymes of phase I and II of xenobiotic metabolism like *CYP1A1* and *GSTYa*. But as would be expected from its functions in cell regulation and apoptosis, this receptor also controls genes encoding regulators of growth, cell proliferation, and the cell cycle.

The entirety of AhR functions that are mediated via isoflavones through agonistic or antagonistic modulation of this pathway remains elusive. Nevertheless, isoflavones can be regarded as selective AhR modulators (sAhRMs).

1.5.2 AhR in vitro *assays*

Given the heterogenicity and variety of AhR ligands [179-186, 193, 194, 196-209], using easily executed screening assays to identify its ligands only makes sense. Several *in vitro* test systems that screen for AhR ligands have been reported. First and foremost, these screenings have been implemented as operative instruments in the search for endocrine disrupters, as it has been shown that pollutants can exert anti-estrogenic effects via AhR that include a modulation of ER pathways without direct interaction with the ERs [210-214]. Because of this background and the high affinity of anthropogenic halogenated aromatic hydrocarbons (HAHs) for the AhR, a chemical class that includes polychlorinated biphenyls (PCBs) and polychlorinated dibenzodioxins (PCDDs), but also non-halogenated polycyclic aromatic hydrocarbons (PAHs) [194, 207-209], toxicologists have intensively studied the AhR for a long time. Over the years, the research focus has shifted towards naturally occurring AhR ligands that could act as sAhRMs and could be useful in cancer prevention and therapy [215, 216]. Because a wide spectrum of flavonoids that occur abundantly in medicinal plants as well as in food function as AhR ligands [189, 191, 217-223], the elucidation of AhR activation via those compounds has become of great interest.

In vitro bioassays can be used to examine whether a compound can induce (a) AhR transformation, nuclear accumulation, and DNA binding as measured by gel retardation analysis, (b) displacement of labeled AhR ligands in competitive ligand binding assays, or (c) expression of target genes or enzyme induction. Examples of applied assays are listed in Table 3. Some of the assays allow a distinction between agonist and antagonists. The chemically activated luciferase expression assay is a transactivation assay that has been used to measure whether a compound can induce AhR-dependent gene expression in intact cells. Similar test systems based on yeasts as model organisms rather than mammalian cells as well as other reporter systems (e.g., β-galactosidase instead of luciferase) have been used. Cell lines with endogenous receptor expression can be used for the measurement of endogenous target gene expression. These tests are more complex and time-consuming but also provide more specific information.

Overall, in various *in vitro* bioassays, isoflavones exhibit agonistic or antagonistic effects on the AhR, as summarized in Table 3.

Table 3. Agonistic and antagonistic effects of isoflavones on the AhR.

Agonistic effects	Antagonistic effects	Assay	Ref.
Dai(+)*	Dai(-), Gen(-)	Gel mobility shift assay (agonistic effects) LBA (rat hepatic cytosol) (antagonistic effects)	[220]
	Dai(-), Gen(+), Gly(-), Equ(+)	LBA (mammalian liver cell cytosol)	[218]
Dai(+), Gen(+), Gly(+), Equ(-)		CALUX (mouse hepatoma cells)	[217]
	Gen(-)	LBA (rat hepatic cytosol)	[224]
	Dai(+)*,Gen (-) Dai(-),Gen (-)	SW-ELISA (Hepa-1c1c7) CALUX (HepG2 cells)	[225]
Dai(+), Gen(+) Dai(-), Gen(-) Dai(-), Gen(-)		Transactivation assay (Hepa-1 cells) Transactivation assay (HepG2 cells) Transactivation assay (MCF-7 cells)	[190]
	Dai(-), Gen(-)	LBA (rat hepatic cytosol)	[191]
Dai(+)*, Gen(+)*	Dai(+), Gen(+)	CYP1A1 expression in HepG2 cells	[226]
Bio(+)	Bio(+)	CYP1A1 expression in MCF-7 cells LBA (rat hepatic cytosol)	[227]
Bio(+)*	Bio(+)	CALUX (MCF-7 cells) CYP1A1 and CYP1B1 expression in MCF-7 cells	[228]
Bio(+)#, Dai(-), Equ(+)*, For(+)#, Gen(-)		Transactivation assay (yeast)	[189]

Biochanin A (Bio), Daidzein (Dai), Equol (Equ), Formononetin (For), Genistein (Gen), Glycitein (Gly), (+) effect, (-) no effect, * weak ligand, # potent activator, ligand binding assay (LBA), HepG2 (human hepatocellular carcinoma cell line), Hepa-1 (murine hepatoma cell line), MCF-7 (human breast cancer cell line).

Depending on test systems, small discrepancies among the results exist. Daidzein and genistein seem to be only weak agonists or partial agonists [220, 226], while biochanin A and formononetin

have exhibited potent agonistic properties in a recombinant yeast transactivation assay [189]. Chan et al. [228] found biochanin A to be only a weak AhR agonist. The reasons for the inconsistency of results are explained by different cell lineages as well as the origin of the AhR. Generally, it is recommended that assays should involve human AhR in recombinant systems because species differences in sensitivity have been observed [229]. Also, there is the consideration that most assays are performed with mammalian cell lines, which contain more metabolizing enzymes than yeast. Metabolization via hepatic cells could lead to different results because the compound that elicits the measured effect could be the metabolite and not the parent compound. On the other hand, these results are expected to be a better reflection of the real *in vivo* situation.

1.5.3 Cytochrome P450 enzyme CYP1A1

Organisms are exposed to a multitude of compounds through environment and food. Whether the exposure is volitional or not, eventually most of these compounds must be eliminated in one form or another from the body. To cope with the elimination of endogenous or exogenous compounds, the organism has a detoxification system that includes various enzymes. During phase I of xenobiotic metabolism, compounds are oxidized with the objective of achieving higher polarity and reactivity in preparation for the conjugation reaction of phase II, which leads to production of more hydrophilic compounds. Phase I reactions are accomplished mostly by cytochrome P450 enzymes that catalyze monooxygenase reactions. Among others, the enzymes CYP1A1, CYP1A2, CYP1B1, and CYP2S1 are classical target genes of the AhR [230-232]. Toxicologists have intensively studied CYP1A1 because it is responsible for the bioactivation of several carcinogenic compounds. The current general view on the impact of CYP1A1 has been undergoing a change, however. Some compounds cannot be detoxified without a preceding CYP1A1 activation and the aftermath without CYP1A1 is much more severe, which appears to contradict the fact that this same enzyme is responsible for bioactivation pathways producing noxious metabolites. Although CYP1A1 knockout mice are viable, develop normally, and show no obvious difference in phenotype compared to wild-type littermates [233], they die within 30 days after benzo[a]pyrene exposure while wild-type mice show no outward signs of toxicity [234].

Thus, a total blockade of CYP1A1 is not advisable because it is indeed part of the detoxification system. The crucial factor is a balanced action of phase I and phase II enzymes. Nevertheless, a modulation of this pathway as a whole, instead of a targeted knockdown of one enzyme, could be useful. Also potentially useful would be knowledge of exactly how the modulation occurs, considering that the composition of ingested food could interfere with administered therapeutics. An example is grapefruit juice, which alters the pharmacokinetics of several drugs via interaction with CYP3A4 (as reviewed by Nowack [235]).

Many naturally occurring plant compounds interact with the xenobiotic pathway, functioning as AhR ligands, including isoflavones. Their modulation of CYP1A1 can take place in various ways, as will be discussed in the following. Most studies report a suppression of AhR-agonist–induced CYP1A1 expression [236-241]. It is not quite clear to what extent this effect is caused by AhR-antagonistic abilities of the isoflavones or if other bioactive properties of these compounds are responsible. Backlund et al. [236] reported for genistein and daidzein an inhibition of omeprazole-

induced CYP1A1 expression but not for the CYP1A1 expression mediated by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo[a]pyrene. Moreover, genistein potentiated induction caused by TCDD. Daidzein, on the other hand, inhibited omeprazole-stimulated CYP1A1 gene transcription but not complex formation of the AhR with its xenobiotic response elements, mediated by omeprazole. Also, daidzein did not inhibit TCDD-mediated CYP1A1 induction at the enzyme, mRNA, and transcriptional levels. The different modes of action may arise from the fact that genistein is a tyrosine kinase inhibitor. Lemaire et al. [241] investigated this question experimentally and found that another tyrosine kinase inhibitor inhibited CYP1A1 induction caused by omeprazole. In that study, genistein could not inhibit omeprazole-induced CYP1A1 expression, but the authors concluded that the failure was the result of a lower genistein concentration that was used because of the sensitivity of the cell model. As noted in earlier sections, isoflavones have been described as agonists as well as antagonists of the AhR. Thus, it is not surprising that studies report a direct induction of CYP1A1 expression mediated by isoflavones [226-228, 237], while other studies did not report such results [242, 243].

Isoflavones act also at a non-transcriptional level and directly inhibit the enzyme activity of CYP1A1 [226, 228, 237, 244-247]. The resulting inhibited metabolization of various compounds could account for the chemopreventive effects of isoflavones.

Whether or not the CYP1A1-modulating effects of isoflavones are beneficial will depend not only on concerted action with other enzymes of the xenobiotic pathway but also on cell type or content. For example, CYP1A1 expression differs in human breast epithelial cells and breast tumor cells. While non–tumor-derived cells express intermediate CYP1A1 mRNA levels, ERα-positive tumor cells express high levels, and CYP1A1 mRNA expression in ER-negative tumor cells is minimal or negligible [248].

1.5.4 Cell cycle control

The control of the cell cycle is one of the principal tasks of the cell. Although the process is routine, the cell makes a decision at every nanosecond about its fate that can compromise normal replication, apoptosis, necrosis, or uncontrolled growth that can finally lead to cancer development. The AhR is known to regulate cell cycle progression through the control of several cell cycle checkpoint regulators. AhR ligands can arrest cells in various cycle phases. Examples of AhR-regulated cell regulators are Akt, p21, p27, p53, Bax, RelB, and NFκB [249-254]. Among others, these proteins cause cell growth inhibition through arrest or lead cells toward apoptosis.

Normally, Akt triggers survival signals in cells and functions as an anti-apoptotic factor. Because deregulated Akt signaling is associated with tumor promotion, the downregulation of Akt could be a target in cancer therapy. AhR-deficient cells show impairment in the Akt pathway, leading to the postulation that AhR antagonists could be useful as agents in cancer therapy [250]. A dysregulated NFκB cascade has also been associated with tumor promotion and inflammation. Patel et al. [255] reported the suppression of NFκB target gene expression arising from AhR activation by ligands, although the data indicated that no AhR target gene transcription was involved in this process. The antiproliferative effects of an agonist-activated AhR pathway are also mediated via the induction of tumor suppressors or the pro-apoptotic proteins p21, p27, p53, and Bax [249, 252-254].

Several reports have shown the cell cycle–arresting effects of isoflavones. Given that the isoflavones act not only through the AhR pathway, it is not quite clear to what extent these effects are mediated via the AhR. Nevertheless, the effects obviously can be attributed at least partly to the AhR cascade. The ER pathway seems unlikely to be a mediator of the cell cycle–arresting effects of isoflavones, given that estrogens instead are assoicated with cell cycle promotion according to their physiological role in normal tissue proliferation. This association is true not only for tissues that are known to depend on the ER pathway for proliferation such as the breast, but also for others such as the urinary system [256].

Because isoflavones are also known PPAR ligands, this route would also be a possibility for their cell cycle–interfering abilities. The natural PPARγ ligand, 15d-PGJ_2, a prostaglandin, represses cyclin D1 and inhibits cells in G1/S transition in a PPARγ-pathway–dependent manner [257].

As Table 4 shows, most studies have focused on genistein, and only a few reports have involved daidzein or other isoflavones. Also, it is evident that genistein causes an arrest in the G2/M phase of the cell cycle, while it seems that daidzein arrests cells in G0/G1. Concomitant with this arrest, several tumor suppressors are induced and key proteins modulated. Some studies have also reported tumor growth reduction in xenograft models or induction of apoptosis.

Table 4. Effect of isoflavones on the cell cycle in human cells.

Effect on cell cycle (cell type)	Further effects	Tested isoflavone (concentration)	Ref.
G2/M arrest (colon cancer)[a]		Genistein (111 μM)	[258]
G2/M arrest (prostate cancer)[b]	Concomitant decrease of cyclin B	Isoflavones from soybean cake; genistein most efficient (30–50 μM)	[259]
G2/M arrest (bladder cancer)[c]	Inhibition of cdc2 kinase activity	Genistein (37 or 185 μM)	[260]
	Direct induction of apoptosis without alteration of cell cycle distribution	Daidzein (39.3 or 196.7 μM) and biochanin A (35.2 or 175.9 μM)	
	Suppression of tumor growth *in vivo* (xenograft model; mice)	Genistein and combined isoflavones	
G2/M arrest (prostate cancer)[d]		Genistein (18.5–74 μM)	[261]
G2/M arrest (breast cancer cells overexpressing Bcl-2)[e1]		Genistein (50 μM)	[262]
G0/G1 arrest (control breast cancer cells)[e2]		Genistein (50 μM)	
G2/M arrest (bladder cancer)[f]	Reduction of tumor volume *in vivo* (xenograft model; mice)	Genistein (50 μM)	[263]
G2/M arrest (androgen-insensitive prostate cancer)[g1]	Induction of tumor suppressor gene expression (p21, p16)	Genistein (10 or 25 μM)	[264]

G0/G1 arrest (androgen-sensitive prostate cancer)[g2]	Induction of apoptosis (only in androgen-insensitive cells)	Genistein (10 or 25 μM)	
G2/M arrest (liver cancer)[h]	Induction of tumor suppressor genes expression (p21), Accumulation of p53 protein	Genistein (37–111 μM)	[265]
G2/M arrest (leukemia cells)[i]	Stimulates Raf-1 activation, Decreases Akt activation, Induction of p21 and cyclin B expression, Induction of apoptosis	Genistein (10 or 25 μM)	[266]
G2/M arrest (prostate cancer)[j]	Increased p21 expression, Decreased cyclin B expression, Decreased NFκB activity	Genistein (15 or 30 μM)	[267]
G1 cell arrest (androgen-sensitive prostate cancer)[k]	Increased p27 and p21 expression	Genistein (≤20 μM)	[268]
	Induction of apoptosis	Genistein (40–80 μM)	
G2/M arrest (non-tumorigenic breast cells)[l]	Enhanced expression of p21 and p53, but not p27	Genistein (30 μM)	[269]
G2/M arrest (prostate cancer)[m]		Genistein (20–100 μM)	[270]
G2/M arrest (B cell leukemia)[n]	Decreased IL-10 secretion, Upregulation of IFNγ	Genistein (7.5–60 μM)	[271]
G2/M arrest (breast cancer)[o]	Increased cyclin B	Genistein (15 or 30 μM)	[272]
G2/M arrest (eye cancer; choroidal melanoma)[p]	Induction of p21, but not required for cell cycle arrest	Genistein (30 or 60 μM)	[273]
G2/M arrest (eye cancer; choroidal melanoma)[q]	Upregulation of CDK1 and p21, but no effect of CDK2 and p27	Genistein (30 μM)	[274]
G1 cell arrest (eye cancer; choroidal melanoma)[q]	Upregulation of CDK2 and weakly p21 and p27	Daidzein (150 μM)	
G2/M arrest (eye cancer; choroidal melanoma)[r]	Impairment of CDK1 dephosphorylation, Weak accumulation of p53 protein	Genistein (60 μM)	[275]
G2/M arrest (metastatic melanoma)[s]		Genistein (60 μM)	[276]
G2/M arrest (gastric cancer)[t]		Genistein (25 or 60 μM)	[277]
G1 cell arrest (gastric cancer)[t]		Daidzein (25 or 60 μM)	
G2/M arrest (metastatic melanoma)[u]		Genistein (60 μM)	[278]
S phase arrest		Daidzein (60 μM)	

(metastatic melanoma)[u]			
G0/G1 arrest (colon cancer)[v]	Biphasic effect on cell growth	Daidzein (5–100 μM)	[279]

Listing of cell lines: **a**: Caco2-BBe, **b**: LNCap and PC-3, **c**: RT-4, J82, HT-1376, T24, TSGH8301, BFTC905 and E6,**d**: PC-3, **e1**: MCF-7/PV, **e2**: MCF-7/Bcl-2, **f**: HT-1376, UM-UC-3, RT-4, J82, and TCCSUP, **g1**: DuPro, **g2**: LNCap, **h**: HepG2, **i**: HL60 and NB4, **j**: PC-3, **k**: LNCap, **l**: MCF-10F, **m**: DU-145, **n**: Raji, 2F7 and JVM-13, **o**: T47D, ZR75.1, MDAMB-231 and BT20, **p**: OCM-1, **q**: OCM-1, **r**: OCM-1, **s**: UISO-MEL-6, UISO-MEL-4, UISO MEL-7 and UISO-MEL-8, **t**: HGC-27, **u**: WM451, **v**: LoVo.

1.5.5 AhR activation by isoflavones and health effects

In addition to a role in prenatal development and organogenesis, the AhR is in charge of several housekeeping functions. In normal physiology, this transcription factor regulates the cell cycle, metabolism, and reproduction. Transcriptomic analysis of tissue from AhR knockout mice has revealed that the AhR also regulates genes involved in protein synthesis, tissue maintenance, cell growth, differentiation, and apoptosis [280]. Gene expression profiling by Yoon et al. [281] extended the AhR sphere of influence to chemotaxis, immune response, signal transduction, inflammation, and tumor suppression. An activated AhR mediates all these functions. Because isoflavones act as selective AhR modulators, they are putative activators of the abovementioned AhR functions.

The AhR has been intensively studied by toxicologists, because of TCDD-induced toxic responses. In the meantime, it emerged that those effects are mediated by a deregulated or over-activated AhR pathway resulting in a homeostatic imbalance (reviewed by Bock et al. [282]). TCDD has a half-life of several years in humans [283, 284]. Due to its poor metabolization, TCDD activates the AhR cascade constitutively and elicits toxic responses such as impaired liver regeneration [285], the development of several tumor types [286-288] and inflammatory skin lesions [289] have been reported. Several studies evaluated the antagonistic properties of naturally occurring plant compounds on the AhR and the possibility to antagonize TCDD effects [191, 203, 218, 220-222, 225].

But beside a constitutive activation of the AhR signalling cascade, the activated AhR can lead to the bioactivation of compounds during the xenobiotic metabolism. But as we have discussed in a previous chapter, a detoxification without a preceding CYP1A1 activation is even more problematic. It is noteworthy to mention that an activation of the AhR and the induction of CYP1A1 is not synonymous with toxic effects. Several AhR agonists are FDA-approved marketed therapeutics and are not toxic to rodents or humans [290].

Nevertheless, possible negative aspects mediated by AhR activation can not be excluded. This could be also true for isoflavones, especially when the intake is extremely high due to excessive recommendations in package inserts of some dietary supplement products. Recommendations that are based on the intake of isoflavones by Asians, will probably not exert harmful effects.

The AhR functions as a master regulator of several other cell cycle regulators. Among others, the AhR leads cells towards apoptosis by regulation or interaction with Akt, NFκB, RelB, p21, p27, p53, and Bax. As described above, all of these proteins have influence on cell fate and can shift the balance to apoptosis when they are upregulated or downregulated, respectively.

Studies have reported the same effects for the isoflavones (see also Table 4). Because they are bioactive compounds that stimulate more than the AhR cascade, it is not quite clear which of these

effects can be attributed solely to AhR activation. It is only of theoretical interest, however, to separate the AhR-mediated isoflavone actions because *in vivo*, the sum of all effects will always be displayed.

The anticarcinogenic properties that have been attributed to isoflavones arise in all likelihood from the concerted action that is partly the result of AhR modulation and manifests in a) cell cycle regulation, b) chemoprevention due to CYP enzyme activation, c) antiproliferative and apoptotic effects mediated by up- or downregulation of tumor suppressors or promotors, d) anti-estrogenicity that is a result of the AhR/ER interaction, and e) anti-inflammatory responses.

2 General conclusion

Certain effects of isoflavones are mediated by either the PPARs or the AhR. With the analysis of *in vitro* effects it is possible to assign them to a mode of action and the associated receptor that mediates those effects. This is a methodical approach to dissect isoflavone action for a better understanding. Methodological shortcoming of in vitro studies is often the use of high isoflavone concentrations, which limits interpretation of the results and makes a comparison with in vivo data difficult.

From the receptor interaction it is clear that isoflavones have an effect on the blood lipid profile, which is explained by the activation of PPAR pathways. This may also counteract certain symptoms of the metabolic syndrome. Isoflavones have also been suggested for prevention of the polycystic ovary syndrome.

Its action on cancer may be partially due to an activation of the AhR pathway and the interaction of the AhR with the ER. Both effects have also been seen *in vivo* in clinical trials. Effects *in vivo* are modulated by bioavailability, which can limit the uptake of bioactive compounds to a great extent, but also metabolization to probably more or less active compounds. This also explains the inter-individually response to isoflavones.

Isoflavones are one of the best studied class compounds, but the focus was primarily on estrogenicity and other effects were mostly overlooked.

3 References

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Publication VI

Phytoestrogens and Related Polyphenols for Prevention of Hormone-Related Diseases

Authors: Medjakovic S., Zoechling A., Jungbauer A.

Department of Biotechnology, University of Natural Resources and Applied Life Sciences Vienna, Austria

Christian Doppler Laboratory for Receptor Biotechnology, Vienna Austria

Corresponding Author: Jungbauer A.

Department of Biotechnology, University of Natural Resources and Applied Life Sciences Vienna
Muthgasse 18, 1190 Vienna, Austria
Telephone: +43- 1- 360066226
Fax: +43-1-3697615

E-mail address: alois.jungbauer@boku.ac.at

Abstract

Phytoestrogens and related polyphenols are omnipresent in vegetarian food. They have been associated with beneficial health effects. Several epidemiological studies demonstrate these effects. An overview is given on the presence of phytoestrogens and related phenols in vegetables, fruits, berries and wine, including examples of different ethnic food. Examples of chemical nature and synthesis in plants will be overviewed. Then the endocrinological activities of phytoestrogens and related polyphenols will be reviewed. Receptor binding activities and *in vivo* and *in vitro* data will be compared. Focus will be put on prevention of breast and prostate cancer. Phytoestrogens are also commonly used to ameliorate menopausal complains. Resent studies will be discussed in the context of literature. For selected phytoestrogens bioavailability and metabolism will be shown.

1. Introduction

1.1 Discovery of estrogenic nature of certain plants

First reports on the estrogenic nature of plant-derived compounds are reported during World War I. In Germany governmental institutions were considering red clover as a substitute for vegetable especially lettuce due to extreme shortage of food production. Already at time certain concerns were made on the estrogenic nature of red clover although estrogens as such have not been discovered. A clear milestone of estrogenicity of red clover is a report back in 1946 in The Australian Veterinary Journal. Bennetts et al. [1] reported on fertility problems of sheeps grazing on clover pastures. They concluded that an estrogenic compound must be present in this clover species.

1.2 The estrogen action on estrogen receptors

1968 Jensen discovered the estrogen receptor α (ER α), at that time reported as cytosolic estrogen binding protein [2]. The DNA sequence of estrogen receptor α has been published in 1986 [3-5]. The receptor possesses a ligand (hormone) binding domain, a DNA binding domain, and several other domains interacting with the general transcription factor complex [6]. The N-terminus has a certain role to enhance transcription [7, 8]. ER α is considered as a ligand dependent transcription factor. ER α belongs to the so-called steroid thyroid receptor superfamily [6]. Common to all these receptors belonging to this superfamily is the architecture of the receptor, with the five different domains, although varying in length and sequence (Figure 1).

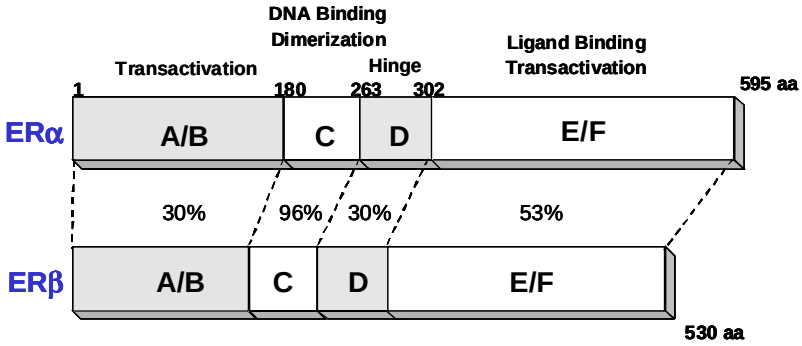

Figure 1: The architecture of estrogen receptor α and β.

Upon binding of the ligand to the receptor the conformation changes, receptor sheds of its accompanying proteins, dimerises and binds to a specific DNA-sequence, the hormone response

element (HRE) [9]. Upon binding to the HRE transcription of certain genes are initiated [10, 11] (Figure 2).

The transcription is cell and tissue specific. Also the nature of the ligands has a certain effect on the transcription. Certain ligands act as agonist others as partial agonist, or antagonist. In order to describe such behaviour, the term SERM, selective estrogen receptor modulators, has been proposed [12]. A lot of natural compounds binding to ER are considered as SERMs [13, 14].

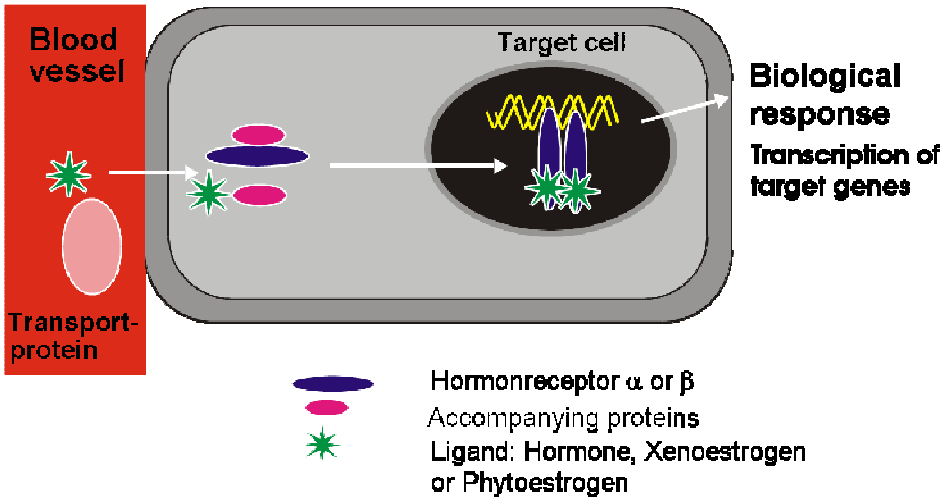

Figure 2: Principle of activation of estrogen receptor; details of inactive receptor protein complex are not shown.

1988 Metzger and Chambon [15] showed that ER can also exert its transactivational activity in yeast. So simple transactivation assays are available [16] as displayed in Figure 3. It has been shown that such a system may also detect the transactivational activity of natural plant derived compounds, although there is a slightly different response to mammalian cell culture systems [17]. For rapid screening such a system is well suited, although false negative may be more frequently observed than in cell culture systems [18].

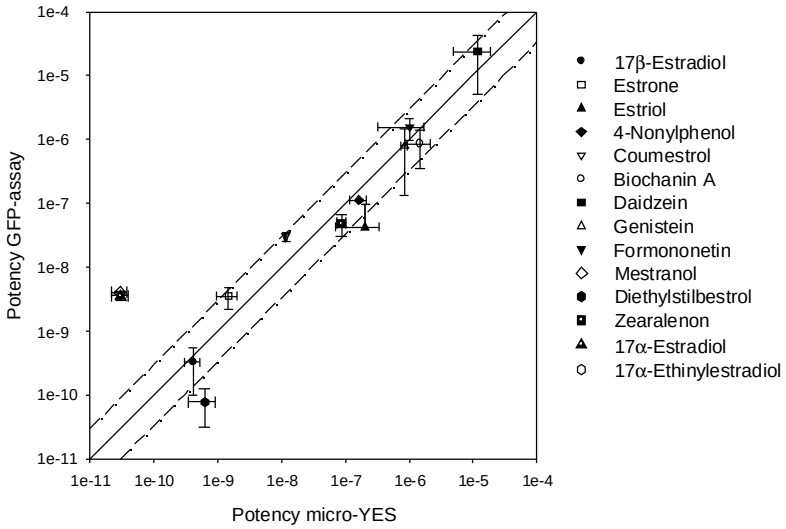

Figure 3: Comparison of potencies measured with micro plate system and large scale system using GFP as reporter. Potencies obtained in microplate assay are plotted on the abscissa and potencies obtained in large scale assay were plotted on the ordinate. The dashed lines x = 3y and 3x = y are also shown. Symbols between the lines designate similar estrogenic activity in both test systems. Values outside the lines show potencies that are at least three times higher or lower in one assay than in the other. The data have been obtained from Beck et al. [19].

1996 Kuiper et al. [20] discovered the second estrogen receptor, the estrogen receptor β (ER β). This receptor has a similar architecture as ER α (Figure 1), but is expressed in different tissues and cells [21-23] as shown in Figure 4.

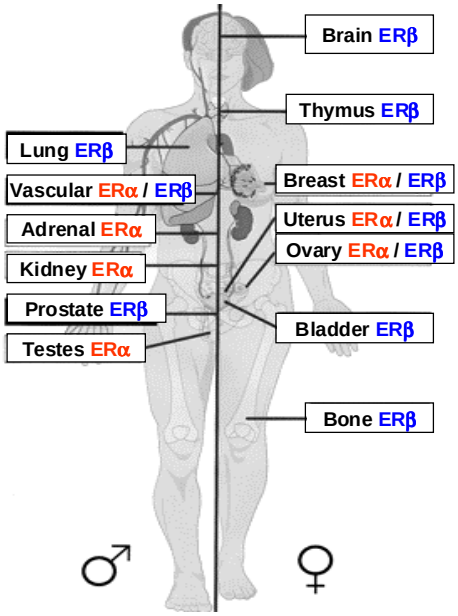

Figure 4: Tissue distribution of ER α and ER β.

With ER β a lot of estrogen actions could be explained, which was not possible with ER α alone. Examples are the action of estrogens on bone [24], brain development and heart. For example ER β is found in bone while ER α is not. A short time later Ogawa et al. [25] discovered a larger form of the receptor, but the function remained the same. It became soon clear that a lot of the so called "phytoestrogens" have a higher affinity to ER β compared to ER α [26, 27]. This also helped to explain why phytoestrogens exert different biological activity compared to the natural or synthetic estrogens.

With ER α and ER β the slow and long lasting effects can be explained. It is also quite clear how coactivators function [28]. They enhance the ER-dependent transcription complex. Mediate between receptor and basal transcription machinery, the so-called bridging factors. Coactivators may also exert histone acetyltransferase activity, by acetylation of histone and therefore loosening the DNA structure, which eases transcription [29-31]. Some natural plant derived compounds may act on coactivators or transcription factor although they do not have an affinity to ER α or ER β. In the presence of estrogen they may enhance the estrogen action and thus they may be accounted as estrogenic compounds, applying the narrow definition of estrogens. An example of such a compound is naringenin [32]. This compound acts on the general transcription factor NFκB, but the affinity for ER is very weak.

It has been also proposed that ER α and ER β may form heterodimers. How relevant the heterodimer formation is remains an open question [8, 25]. Interesting is that ER α and the arylhydrocarbon receptor (AhR) may form a heterodimer (Figure 5). This has been described for the response elements controlling the alkaline phosphatase [33]. If this heterodimer formation is physiologically highly relevant remains open. A compound without affinity to ER may exert anti-estrogenic action through the AhR. Activation/binding of AhR by polyphenols has been reported in several papers [34-38]. Again it depends on the specific context of a test system, if a compound can produce an estrogenic/anti-estrogenic response.

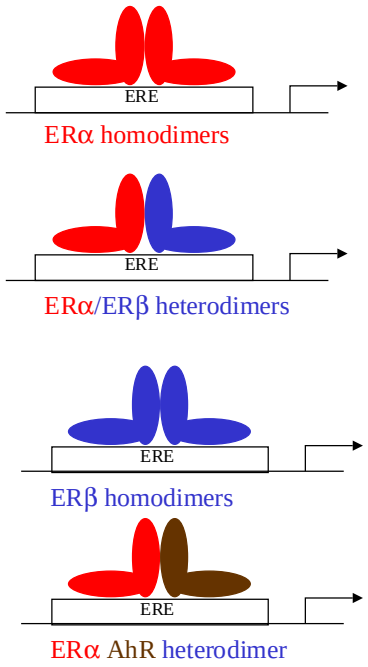

Figure 5: Alternative estrogen signaling pathway.

Assays on binding of the compound on ER do not suffice to draw a conclusion, if a compound has estrogenic properties. In-vitro assays only provide a preliminary assessment of the hormonal properties. An international recognized test for estrogenicity is the uterotropic assay [39]. Ovarectomized rats are supplemented with estrogens and the uterus weight and differentiation is measured. Compounds can be tested in presence and absence of estrogen and indirect effects can be assessed. This in-vivo assay has been also used for assessment of plant derived compounds. A typical example is *Cimicifuga racemosa* extract used for treatment of menopausal complaints. These extracts do not have binding affinity to ER [26], but show weak affinity in the uterotrophic assay [40].

The slow but long lasting effects of estrogens, the genomic effects can be explained by ER α and ER β. It has been known for a long time that estrogens have effects on the cardiovascular system, especially the vasodilatory effects. In 1977 Pietras and Szega [41] described binding sites for estrogen at the outer surfaces of isolated endometrial cells. Parik et al. [42] identified high affinity binding sites in calf uterine membranes. These binding sites seem to be different from ER α and ER β present in the cytosol. Finally a 46 kD form of ER α has been discovered [43, 44]. Upon palmioylation at Cys447 the receptor gets anchored in the inner side of the cell membrane. These forms have been initially reported in MCF7 cell, osteoblasts, and vascular endothelial cells

[43-45]. This receptor can use the same signally cascade as G-protein coupled receptors and signal through ERK/MAPK and tyrosine kinase pathway [46-48]. Estrogenic effects depending on this pathway are also described as non-genomic effects. A lot of phytoestrogens exert non-genomic estrogenic effects [49, 50]. The most prominent non-genomic effect is the activation of endothelial nitric oxide synthetase (eNOS) [51-53]. This enzyme has been associated with vasodilatation and in a broader sense with the effects of phytoestrogens on coronary heart disease. Ho and Liao [54] from the Department of Medicine at Harvard Medical School reviewed the non-nuclear actions of estrogen as now targets for prevention and treatment of cardiovascular disease.

The mER is present in cancer cells, such as MCF7 or T47D cells [46]. Estrogen can rapidly activate protein kinase c. The rapid response is definitely membrane mediated. mER is involved in cell specific signaling effects. In particular the estrogen mediated inhibition of cytokine related signal then solution to cell differentiation, proliferation, migration or cell death. Ho and Liao have reviewed these tissue specific non-nuclear activities of ER [54].

1.3 The estrogenicity of polyphenols

As early as 1946 reports on weak estrogenicity of *Trifolium subterranean* L.G. has been speculated [1]. Farnsworth et al.[55, 56] reported on antifertilty agents in plant without further discussion, why these compounds may act in this way. 1963 phloretin has been described as weak estrogen [57]. 1978 Martin et al. described the interaction of plant estrogens with the estrogen receptor from the human breast cancer cells [58]. This is the first evidence that polyphenols may interact with estrogen receptors. 1985 genistein and daidzin were found to be estrogenic in mice [59].

In three consecutive papers Miksicek [60-62] has shown that a variety of flavanoids has an affinity for ER α. After that time a lot of reports have been published showing polyphenols with affinity for the estrogen receptor. After discovery of ER β, it has been shown that these compounds also interact with this receptor variant. A lot of polyphenols have even higher affinity to ER β than to ER α [63]. Moreover, a lot of papers have been published in the past decade on the biological effects of phytoestrogens which go beyond ER-binding and transactivation [64].

In the late 90thies the chemical nature of the phytoestrogens present in hops has been identified [65]. Although it has been known for long time that hops have estrogenic activity [66, 67] the activity has been attributed to Xantohumol without evidence. Indeed, 8-prenylnaringenin is the

active compound [65]. This compound is barely found in beer, since it is decomposed during beer processing such as worth cooking and fermentation. The estrogenicity of beer may also stem from isoflavones and not from prenylated compounds [68].

The estrogenicity of polyphenols has been explained by the similarity between these compounds and the natural steroid hormone 17β-estradiol [69] (Figure 6). These classes of compounds have a similar structure and fit in the binding pocket of the ligand binding domain of both receptors; ERα and ER β.

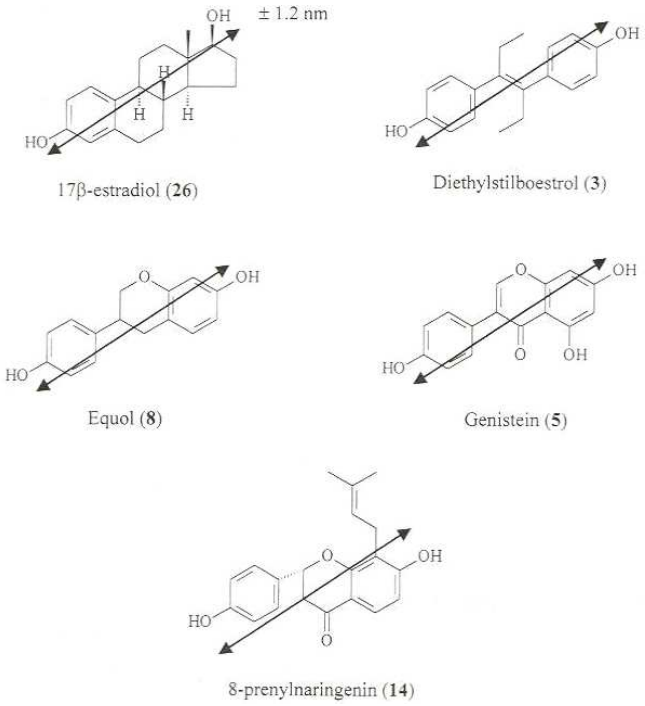

Figure 6: Structural requirements for ER-binding according to Cos et al. 2003[69]

2. Chemical classification of polyphenols

Plant phenols have been defined by Haslam et al. as aromatic secondary plant metabolites derived from the shikimate pathway and phenylpropanoid metabolism [70]. These compounds are ubiquitous among all plant species and have been investigated by numerous scientists. The chemical classification with some examples is listed in Table 1.

Table 1: Chemical classification of plant phenols

Basic skeleton	Class	Examples	Diet source
C_6	Simple Phenols	Catechol	
C_6-C_1	Phenolic acid	Salicylic acid	Sea buckthorn
C_6-C_2	Phenylacetic acid		
C_6-C_3	Hydroxycinnamic acids	Caffeic acid	Apple, Tea
		Ferulic acid	Coffee
	Coumarins	Umbelliferone	Citrus
C_6-C_2-C_6	Stilbenes	Resveratrol	Red wine
C_6-C_3-C_6	Flavonoids	Quercetin	Onion
		Cyanidin	Cherry
$(C_6$-$C_3)_2$	Lignans	Secoisolariciresinol	Rye, Flax seed
$(C_6$-$C_3)_n$	Lignins		Stone fruits
$(C_6$-C_3-$C_6)_n$	Condensed Tannins		Red wine

The most important compounds belong to the group of flavonoids with a core-structure of a C_6-C_3-C_6 flavone skeletone in which the C_3 part is commonly cyclised with oxygen (see Figure 7).

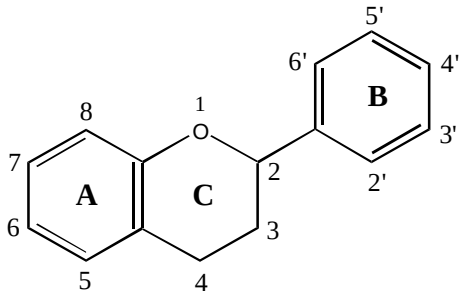

Figure 7: Core-structure of the flavonoids (with two aromatic benzol rings (A and B ring) and a C3 part cyclised with oxygen (C ring).

They differ in the degree of unsaturation and the degree of oxidation. The various flavonoid classes with some examples and sources are shown in Figure 8 and Table 2.

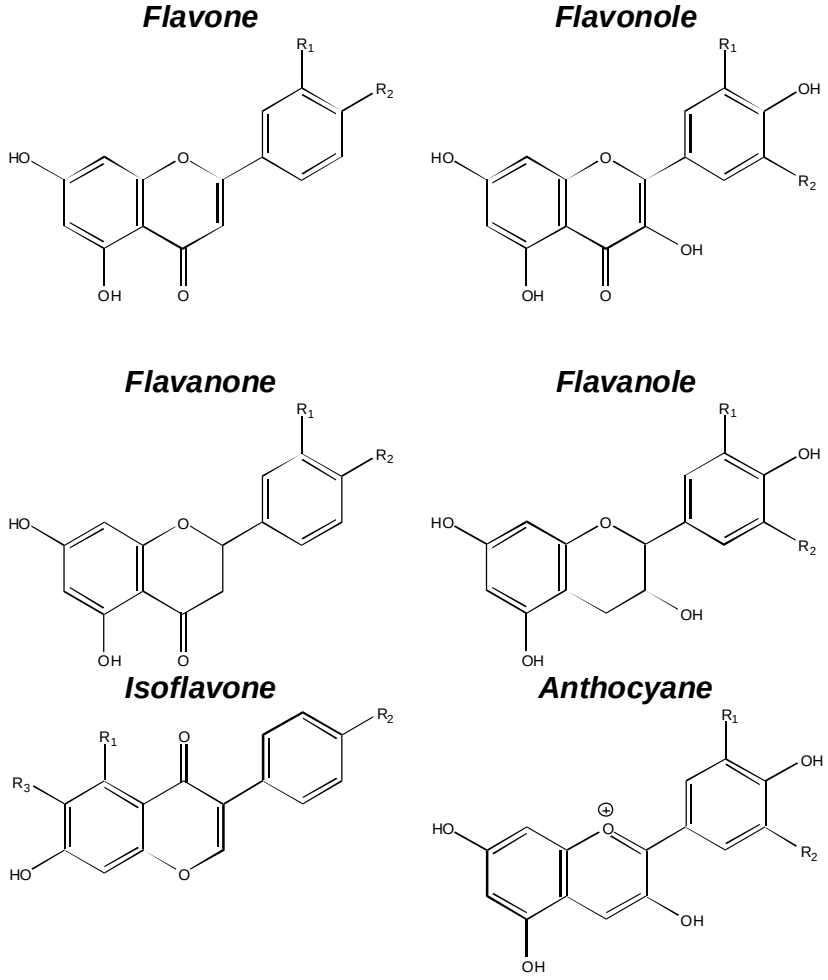

Figure 8: Schematic draw of the six flavonoid classes.

Table 2: The six flavonoid classes with some examples (adapted from [71] and [72]).

Class	Examples	Sources	Comments
Anthocyanins	Cyanidin	Cherry	Especially in coloured fruits, restricted to the skin, in
	Delphinidin	Plums	some berries they are also present in the flesh, red
	Malvidin	Red grapes	cultivars of vegetables.
	Pelargonidin	Blackberry	They are often used as colouring agents in other
	Peonidin	Red cabbage	foods/beverages.
		Red onion	
Flavans	Catechin	Tea	As mono-, bi- and triflavans, seldom glycosylated
	Epicatechin	Apples	but esterified
	Procyanidin	Peaches	
	Theaflavin	Strawberries	
		Cereals	
Flavanones	Naringenin	Citrus fruits	Major sources are citrus fruits and juices
	Hesperidin	Peppermint	They contribute to the flavour of citrus fruits,
	Neohesperidin	Licorice	naringenin and neohesperidin to the bitterness.
		Cumin	
		Rowanberry	
Flavones	Apigenin		Mostly found in herbs, but also in fruits, vegetables

	Luteolin Tangeretin Diosmetin		and cereals. They are yellow coloured, some have bitter taste (tangeritin) neodiosmin reduces the bitterness.
Flavonols	Quercetin Myricetin Kaempferol	Onion Pears Tea Berries Root vegetables Leafy vegetables	Ubiquitary in plant food In fruits flavonols and their glycosides are mainly found in the skin. Quercetin is predominant in vegetables.
Isoflavonoids	Genistein Daidzein Formononetin Biochanin A Glycitein	Soybeans Red Clover Alfaalfa Black beans Sunflower seeds	Predominantly found in the legume family. They differ from the other flavonoids in the B ring orientation. They are known for their estrogenic activity.

Additional complexity results from hydroxylation, methylation, acetylation and sulfation. The common occurrence of flavonoids is glycosylated as O-glucosides in plants, but also other sugar like galactose, fructose, xylose and arabinose occur. The range of known flavonoids is therefore huge; more than 4000 different structures have been reported. [73]. In humans mainly aglycones are adsorbed and therefore responsible for the different biological actions [74].

2.1 Biosynthesis of Different Flavonoid Classes

The main flavonoids are divided into different classes according to the oxidation level on the heterocyclic ring. They are formed by different pathways; 4-coumaroyl-Coenzyme A (the shikimate pathway and the general phenylpropanoid) is condensed with malonyl-Coenzyme A by the key enzyme of flavonoid biosynthesis, chalcone synthase (CHS). This gives the fist C_{15} condensation product (4, 2´, 4´, 6´-tetrahydroxychalcone) from which all flavonoids are derived. The different enzymes which are responsible for the formation and structural modification of flavonoids are mostly known and well characterised [75, 76].

An overview of the flavonoid biosynthesis is shown in Figure 9. This is only a sketch, since the pathway and metabolites depend on the individual plant and even they vary from clone to clone and are influenced by the habitat. This is the reason why plant extracts are difficult to standardize.

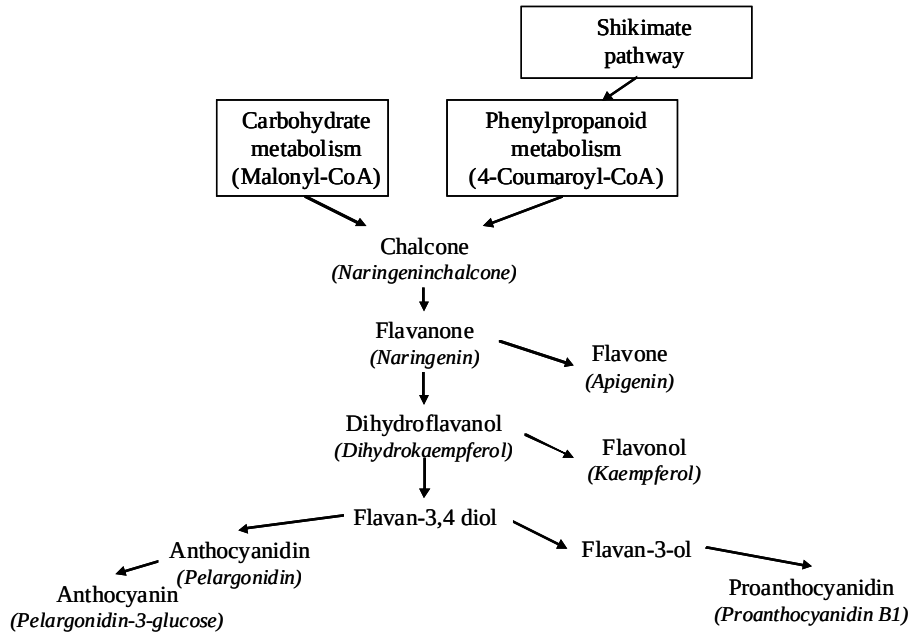

Figure 9: Overview of the flavonoid biosynthesis (adapted from [73, 77, 78])

2.2 Biosynthesis of Isoflavonoids

Isoflavonoids are mainly synthesized in the *Fabaceae* family in plants; they play an important role in plant-microbe interaction for the establishment of the Rhizobium symbioses for nitrogen fixation of the root nodules. Isoflavonoids are the most important plant derived compounds with selective estrogen modulating activity in mammals.

Isoflavonoids differ from flavonoids in the position of the aromatic B-ring which is attached in position 3 of the heterocycle. Genistein and daidzein are formed based on tetrahydroxychalcone by two isoflavone-specific enzymes: isoflavone synthase and isoflavone dehydratase (**Fehler! Verweisquelle konnte nicht gefunden werden.**).

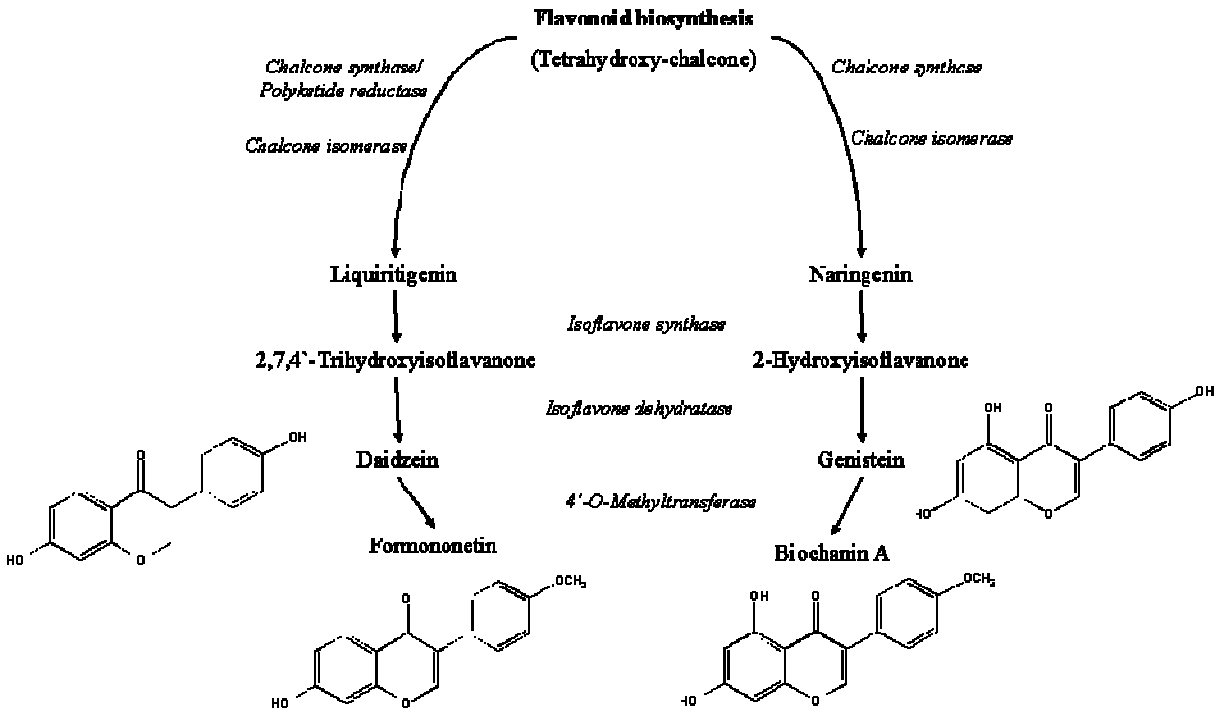

Figure 10: Overview of isoflavonoid biosynthesis.

Methylation leads to the formation of formononetin and biochanin A, respectively. A lot of polyphenols have estrogenic activity and thus they have been named plant estrogens or phytoestrogens. After ingestion a part of the isoflavonoids are metabolised by the gut micro flora into other hormonal active compounds like equol. The binding affinities of different isoflavones, apigenin, quercetin and resveratrol are listed in Table 3

Table 3: Binding affinities of different isoflavones in different test system. EC50 equals the concentration that is needed to exert the half maximal response or to bind to 50 % of the receptors. (RBA: relative binding affinity, YES: yeast estrogen screen, MCF7, HGELN, MVLN, Hela and Ishikawa cells: mammalian cell culture assay, n.d.: not detectable)

Compound	Class		EC_{50} of 17-β-Estradiol/ EC_{50} of the compound	Test system	Reference
17-β-Estradiol	Estrogen		1		
Apigenin	Flavonon		0.00300	RBA	[27]
			0.0000024	YES	[79]
			0.00015	MCF7-cells	[79]
Biochanin A	Isoflavone		0.00045	YES	[16]
		<	0.00010	RBA	[27]
			0.0000025	YES	[79]
			0.000110	MCF7-cells	[79]
			0.000005	HeLa cells	[61]
Daidzein	Isoflavone		0.00005	YES	[16]
		<	0.00010	RBA	[80]
			0.00100	RBA	[27]
			0.00027	RBA	[81]

			0.00082	RBA	[82]
			0.00053	Ishikawa	[65]
			0.00014	YES	[65]
			0.0000028	YES	[79]
			0.000110	MC7-cells	[79]
			0.000111	HeLa cells	[61]
Formononetin	Isoflavone		0.00023	YES	[16]
		<	0.00010	RBA	[27]
			0.000033	HeLa cells	[61]
Genistein	Isoflavone		0.00050	YES	[16]
			0.00200	RBA	[80]
			0.04000	RBA	[27]
			0.00490	RBA	[81]
			0.00870	RBA	[82]
			0.00400	Ishikawa	[65]
			0.00025	YES	[65]
			0.00300	RBA	[65]
			0.000045	YES	[79]
			0.000260	MCF7-cells	[79]
			0.000132	MVLN cells	[83]
			0.000800	HGELN cells	[83]
			0.000013	MCF7 cells	[83]
			0.000100	RBA	[83]
			0.000111	HeLa cells	[61]
Naringenin	Flavonone		0.00005	YES	[16]
			0.00010	RBA	[27]
			0.000078	YES	[79]
			0.000077	MCF7-cells	[79]
Quercetin	Flavonol		n.d.	YES	[16]
			0.00010	RBA	[27]
Resveratrol	Stilbene		0.00005	YES	[16]

The relative binding affinity of the compounds is rather low compared to the natural estrogen 17-β-estradiol. Usually they are 2 to 5 orders of magnitudes lower. In order to reach physiological relevant doses the bioavailability must be high or the intake substantial and constant. Endocrine activity has to be low in order not to disrupt the normal endocrine function. Baker hypothesises why some of the plant derived compounds may have endocrine activity, either through direct hormone receptor binding or interaction with enzymes for the hormone metabolism [84].

3. Presence of Polyphenols in the Diet

Due to the various biological effects, polyphenols were also referred as vitamin P (P like permeability or phenol) in the past. But no deficiency symptoms are known, so they were not recognized as vitamins. The health effects of polyphenols depend on the amount consumed and their bioavailability. Diet has changed dramatically due to economic wealth from almost plant to animal product origin. The amount of polyphenols in the diet also differs from very high intake in the eastern diet (mainly due to soy products) to low amount in the western diet with high intake

of products animal origin. In Table 4 are the contents of the isoflavones daidzein and genistein and the flavone quercetin of diverse foods listed.

Table 4: Content of daidzein, genistein and quercetin in various selected foods. (*wet weight/fresh weight, #dry weight, i.tr.: in traces)

	Phytoestrogen content (mg/kg)			
	Isoflavone		Flavone	Reference
	Daidzein	Genistein	Quercetin	
Apple	#0.1240	#i.tr.		[85]
			*4-101	[86]
			*20.0	[87]
Broccoli			#60.0	[88]
			*37.0	[87]
Strawberry	*0.0045	*0.0461		[89]
			*7.0	[90]
			*6.0	[87]
Peanuts	*0.0770	*0.1580		[89]
Blueberry			*73.0	[87]
Cauliflower			#219.0	[88]
Carrot			#55.0	[88]
Cherry			*10.0	[87]
Garlic			#47.0	[88]
Cranberry			*74-146	[90]
			*160.0	[87]
Sesame	*0.0370	*0.0170		[89]
Soybean	#105-850	#268-1.025		[85]
Soy bread	#78.53	#121.12		[91]
Soy drink	*7.0	*21.0		[92]
Sunflower seeds	#0.080	#0.139		[85]
Tea, black			#1070.0	[88]
Tofu	*73.0-97.5	*187.4-215.9		[92]
Grape. red			*37.0	[87]
Grape. white			*2.0	[87]
Onion. white			*383	[86]

3.1 Polyphenols in Fruits and Berries

Fruits, berries and juices thereof are the best sources for polyphenols due to the large serving size and the high content in fruits. The quantity and the phenol composition of the fruits vary in respect to growing conditions and area, variety, storage time and conditions and harvest time within a wide range [93-95]. Variation in the phenol content is also due to different analysis techniques [96]. The phenol content of some selected fruits and juices is listed in Table 5.

Table 5: Phenol content of some selected fruits (adapted from [97]) ([a] gallic acid equivalents / 100g fresh weight or 100 ml respectively, [b] catechin equivalents / 100 g fresh weight)

Fruit	Total phenol content	Reference
Apple	296.3 [a]	[98]
Banana	11.8-90.4 [a]	[98, 99]
Blackberry	26.7-555 [a]	[100, 101]

Blueberry	171-961 [a]	[101, 102]
Cherry	105.4 [b]	[103]
Pineapple	94.3 [a]	[98]
Plums	174-375 [a]	[104]
Red Grape	201.0 [a]	[98]
Strawberry	160-535 [a]	[98, 105]
Grape fruit juice	53.5 [a]	[106]
Orange juice	75.5 [a]	[106]

3.2 Polyphenols in Vegetables

The polyphenol content of different vegetables are listed in Table 6. The influence of storage and processing is well documented and depends on the type of polyphenol [107-109]. The highest content was observed in fresh unprocessed vegetables.

Table 6: Phenol content of some selected fruits adapted from [97] ([a] gallic acid equivalents / 100g fresh weight, [b] catechin equivalents / 100 g fresh weight, [c] ferulic acid equivalents / 100 g fresh weight)

Vegetables	Total phenol content	Reference
Broccoli	87.6 [b]	[110, 111]
	101.6 [a]	
Brussels sprouts	68.8 [b]	[111]
Cabbage	54.6 [a]	[110, 111]
	92.5 [b]	
Carrot	55.0 [a]	[110, 111]
	56.4 [b]	
Cucumber	19.5 [a]	[110, 111]
	48.0 [b]	
Spinach	91.0 [a]	[110]
Tomato	25.9 [c]	[111, 112]
	68.0 [b]	
Onion	73.3 [a]	[110, 113]
	180.8 [a]	
	Total isoflavone content (mg/100g)	
Soy	37.3-187.5	[85]
Kudzu	197.6	[85]
Mung sprouts	1.647	[114]
Sunflower seed	0.02	
	Total lignan content (mg/100g)	
Linseeds	370.1	[114]
Rye bran	0.3	[114]
Pumkin seeds	21.370	[114]

The highest intake of isoflavone is observed in Japan, where soybeans are a staff of life. The concentration of isoflavones in soybeans ranges from 0.2 to 2 mg/g. The presence of active isoflavone in the processed foods depends on the type of processing and extraction method for the soy protein [114]. Lignans are the main sources of phytoestrogens in the European diet. The precursors are present in the outer part of different grain, especially rye. Beside linseeds and rye products vegetables such as spinach, carrots, broccoli and spinach are the main sources of

lignans. The ingestion of lignans leads to the production of enterolactone and enterdiol in the gut, which were made responsible for the estrogenic activity [115].

3.3 Polyphenols in Wine and Polyphenol-rich Beverages

Wine contains a range of different polyphenols with desirable biological properties. The most abundant polyphenols in grapes and wine belong to the flavonoids, phenolic acids, proanthocyanidins. The polyphenol composition of grapes and wine has been intensively studied since epidemiological studies have shown the positive effects of red wine consumption, the so called "French Paradox" [115-117]). Despite the Mediterranean diet moderate red wine consumption has been made responsible for the lower mortality from coronary heart disease. Beside wine growing conditions like soil, climate, grape variety and sun exposure of the grapes the wine making technology is the most influencing factor on quantity and quality of polyphenols in the wine.

Table 7: Distribution of the main phenols in the grape (adapted from [118]) in mg/g.

Compound	Skin	Seed	Stem	Reference
Phenolic acids				[119-122]
Gallic acid	0.03	0.10-0.11	-	
Coutaric acid	0.03-1.23	-	-	
Caftaric acid	0.11-6.97	-	0.04	
Flavan-3-ols				[122-127]
Catechin	0-0.16	2.14-2.15	0.06	
Epicatechin	0-0.13	0.88-0.91	0.28	
Epigallocatechin	in traces	0.05	0.01	
Epigallocatechin 3-gallate	-	0.06-0.07	-	
Epicatechin 3-gallate	0.04	0.25-0.31	0.07	
Procyanidin B1	0.11-0.6	0.14-0.16	-	
Procyanidin B2	0.01-0.84	0.04-0.18	-	
Tannins	1.61	2.32	0.22-0.39	
Anthocyanins	11.47-29.82	-	-	[120, 121]
Flavonols				[120, 127]
Quercetin 3-glucoside	0.15-0.2	0.01-0.02	0.02	
Myricetin 3-glucoside	-	-	In traces	
Quercetin 3-glucoronide	0.22-0.29	0.01-0.02	0.2	
Kaempferol 3-glucoside	0.11-0.14	0.01	In traces	
Myricetin 3-glucuronide	-	-	In traces	

The general distribution of the main phenols occurring in different fractions of the grape is shown in Table 7. Most of the phenols were extracted from the grape skin during fermentation at higher temperature and ethanol content. Other sources of polyphenols in the wine are oak tannins from the barrels, which were extracted during wine maturation in small casks. The phenol content of

different wines varies in a broad range within the grape varieties and wine growing region. The total phenol content of a selection of wines is shown in Table 8.

Table 8: Total phenol content of wines from different wine growing regions (adapted from [97]) in mg gallic acid equivalents / L

Wine growing region	Total phenol content	Reference
Red wines		
Argentina	1593-1637	[128]
Brazil	1947-1984	[129]
California	1800-4059	[130]
Chile	2133	[129]
France	1847-2600	[131]
France	1018-3545	[132]
Italy	3314-4177	[129]
Portugal	1615	[129]
Spain	1869	[128]
White wines		
Argentina	216	[129]
Brazil	256-353	[129]
California	165-331	[130]
California	220-306	[128]
France	245	[131]
France	262-1425	[132]
Italy	439-854	[129]
Italy	191-296	[128]
Spain	292	[128]

During wine aging the polyphenol content decreases due to oxidation, polymerisation and reaction with other wine compounds like proteins. Additional filtration and fining agents can reduce polyphenol content dramatically [133, 134]. The best documented compound in red wine in the view of a positive health benefit is resveratrol [135, 136]. High levels of this compound were detected in red grape juice and wine up to a concentration of 15 mg/l [135, 137]. The health benefits in respect of hormone related diseases are the activation of e-NOS and the antagonistic estrogenic activity. Adlercreutz et al. also detected remarkable lignan content in red wines [138].
Tea and coffee contribute considerably to the daily polyphenol intake in humans due to high consumption of these beverages. The mean total phenolic content of some teas and coffees are listed in Table 9. Most of the phenols in tea belong to the flavans and are fermentation and condensations products thereof [139-141]. Brewing of green tea leads to the extraction of high quantities of lignans, tea can therefore also be beneficial for the phytoestrogen uptake in humans [142].

Table 9: Total phenol content of tea and coffee (adapted from [97]) in mg gallic equivalents / g dry matter.

	Total phenol content	Reference
Black tea	80.5-134.9	[139]
Black tea	154.9-162.9	[140]
Black tea	62-107	[143]
Green tea	65.8-106.2	[139]
Green tea	117.3	[141]
Green tea	61-200	[144]
Ground coffee	52.5-57	[140]
Instant coffee	146-151	[140]

3.4 Polyphenols in Food Supplements: Soy and Red Clover

Soy has been used as food fortifier in commercial foods e.g. in bread to increase the isoflavone content and also for meat substitution. Umphress et al. [145] measured a total isoflavone content of soybean products up to 109.3 mg/100g and 149.9 mg/100g in meat substitutes.

As alternative for treatment of postmenopausal complains food supplements from soy and red clover can contribute to phytoestrogen consumption. They are standardized in the isoflavone content with an average isoflavone content of 40mg/day (capsule). This is comparable with the mean isoflavone consumption in Japan (see following subchapters). Red clover (*Trifolium pratense*) belongs to the *Leguminosae* family with the isoflavones genistein, daidzein, biochanin A and formononetin. Soy only contains genistein, daidzein and additional glycitein [146]. The equivalent 17 β estradiol concentration has been investigated by Beck et al [13] determined an equivalent concentration up to 332 nmol/g for the estrogen receptor β and 167.2 nmol/g for the estrogen receptor α in different food supplements (Table 10). Other alternatives to plant estrogens are studied for their beneficial effects for menopausal complains like black cohosh (*Cimicifuga racemosa*), wild yam (*D.villosa*), ginkgo (*G. biloba*), evening primrose (*O. biennis*), Mother wort (*L. cardiaca*), St. John´s wort (*H.perforatum*) and others with not sufficient data on safety and efficacy [147].

Table 10: Equivalent 17 β estradiol (E2) concentration of different food supplements on the ER α and ER β.

Food supplement	Source	Recommended daily dose (mg isoflavone)	Equivalent E2 concentration (nmol/g) ER α / ER β
Promensil	Red clover	40	80.6 / 168.3
Rimostil	Red clover	57	167.2 / 94.2
Trinovin	Red clover	40	58.5 / 160.9
Rotklee Active tablets	Red clover	Not stated	67.0 / 165.2
Rotklee tablets	Red clover	Not stated	71.5 / 167.2
Red clover	Red clover	40	53.5 / 101.4
Isoflavones	Red clover	40	63.7 / 114.8
Menoflavon	Red clover	40-80	99.6 / 192.7
Soy Plus capsules	Soybean	80	1.7 / 43.7

Orthomol Femin	Soybean	40	0.8 / 23.9
Isoflavones from Soybean extract	Soybean	40	8.5 / 332.0
Aria	Soybean	50	1.8 / 33.7
Remifemin	Black cohosh	40	-
Life Extension	Soybean, Black cohosh	110	3.7 / 36.8
Tibolon Liviella	Synthetic	Not applicable	49.5 / 6.1

The total isoflavone content of different soy and red clover based food supplements were determined by different groups. The measured isoflavone content is up to 201 mg/g [148] food supplement. They differ from the labelled isoflavone concentration remarkable (Table 11) [149, 150].

Table 11: Isoflavone content of different food supplements: combined from Setchell et al. and Howes and Howes [149, 150].

Food supplement	Source	Recommended daily dose (mg isoflavone)	Total isoflavone content per daily dose (mg)
Carlson Easy Soy	Soybean	12.5	10.2
Carlson Easy Soy Gold	Soybean	50	36.2
Erdic (Busting Out)	Grain, hops	Not stated	-
Stroven	Soybean, black cohosh	50	7.8
Solgar	Soybean	15	9.4
Kudzu Root Extract	Arrow root	3	11.5
Healthy Woman	Soybean	55	48.8
One a Day	Soybean	42	12.8
Phyto Estrin	Soybean	14	10.3
Phyto Soy	Soybean	17.5	12.5
Soy Extract	Soybean	13	11.3
Phyto Estrogen-Power	Soybean	5.3	7.3
Promensil	Red clover	40	41.7-40.12
PhytoEstrogen Solaray	Red clover, soybean	10	10.6
H&B Soya Isoflavones	Soybean	16.7	16.2
Soyamax	Soybean	60	58.0
Soy Care	Soybean	25	23.2
N Resources Soy Isoflavones	Soybean	50	43.4
Soy Plus	Soybean	20	18.1
Naturally Preferred Soy Germ	Soybean	10	12.3
Trinovin	Red clover	40	36.9
Basic Soy Isoflavones	Soybean	25	16.6
Nature´s Bounty Flash Fighters	Soybean	21.7	16.8
Herbal Blends Menopause Balance	Soybean	8	2.3
Nova Soy	Soybean	50	40.8
New Phase-Sunsource	Soybean	80	8.6
Spring Valley	Soybean	7	12.7
Sundown	Soybean	40	39.2
Phytosoy	Soybean	4	3.4
Soy Choice Vitanica	Soybean	56	25.8
Revival	Soybean	13.8	8.9
Nutri Soy	Soybean	Not stated	2.8
Soy Life 25	Soybean	25	20.2
Phytolife one a day	Soybean	40	41.02

Soy powder plus	Soybean	68	48.75
Earth Own soy + calcium	Soybean	68	42.52
Femme phase	Soybean	235 mg soy protein	0.29
Phyto source	Soybean	22.5	16.27
Menopause	Soybean	60	0.56
Phytobalance	Soybean	90	58.12
Pretorius	Soybean, red clover, wild yam	68	50.36
Femme soy plus with red clover	Soybean, red clover	27	30.76

Meanwhile a lot of different food supplements are available on the market. It is important to standardize these products as seen in Table 11. The isoflavone content varies to a large extent.

4. Dietary Intake and Metabolism of Phytoestrogens

People in Southeast Asia have a lower risk for some cancer types and Asian women suffer to a lesser extent from menopausal symptoms. All these circumstances were linked to Asian lifestyle and especially diet. One of the main components of traditional Asian diet is soy. This plant contains several bioactive compounds, including the isoflavones daidzein, genistein and glycitein (for chemical structure see Figure 4). They are also known to bind to the estrogen receptor and modulate thereby several signalling pathways in mammals. Cancer prevention and the amelioration of climacteric symptoms are mainly attributed to soy and its constituents. These circumstances will be discussed in detail in subchapters afterwards.

Epidemiological evidence suggests that a reason why Asian people have lower breast and prostate cancer risks is that they are used to eat soy food from an early age. Strictly speaking they start to consume isoflavone-rich food before they are born. There is no indication that a whole-life soy intake as well as intake over generations and centuries has adverse effects on single individuals or populations.

The principal isoflavonoids in soy and red clover are formononetin, biochanin A, daidzein, genistein and glycitein and are found as glycosides and as aglycones. The distribution of those compounds has been described in a subchapter before. All of them exhibit estrogenic activity as aglycone [16, 17, 68, 79-81, 151-154] and are classified as phytoestrogens. For the absorption of the isoflavone glycosides a hydrolysis of the sugar moiety is needed [155]. However, there is a controversy about the bioavailability of aglycones and glucosides; while some studies emphasise no difference in the bioavailability of them [156-158] other studies indeed report a better absorption of isoflavone aglycones [159, 160]. However, there seem to be no alteration of aglycone absorption due to food matrix and processing [161]. The absorption of isoflavone aglycones from red clover and soy also seems to be very similar [162].

During metabolism, formononetin is demethylated to daidzein and biochanin A to genistein. A crucial metabolite is equol; it arises from conversion of daidzein via the intestinal microflora of humans. Equol excretion is associated with a reduced risk of breast cancer, probably due to hormonal differences between equol producers and non-producers [163]. Studies show that not everyone is able to produce equol. The prevalence of equol producing phenotypes ranges between 24 and 51% [164-173], see also Table 12.

Table 12: Equol producer prevalence.

Study subjects	Equol producers	Comments	Reference
n= 12, US	30%	Development to produce equol during chronic soy ingestion	[164]
n= 10, US	30%	women	[170]
n= 91, US (Korean)	51%	Study with girls	[169]
n= 222, US (Caucasian)	36%		
n= 6, US (ethnicity unknown)	50%		[171]
n= 19, Japanese	47%	Positive correlation of meat intake and equol production	[168]
n= 25, Australian	24%	Young women	[167]
n= 36, Irish	51%	Irish Caucasian women	[165]
n= 20, US low soy consumers	10%	78% Seventh Day Adventist	[166]
n= 25, US high soy consumers	28%	men Positive correlation of meat and soy intake and equol production	
n= 194, Japanese	40.5%	Pregnant women at delivery	[173]
n= 51, Japanese	37%	Pregnant women at delivery	[172]

Lu and Anderson [164] reported an attainment of the ability to produce equol of 3 out of 6 female study participants during one month of soy ingestion. On the contrary Védrine et al. [174] found no induction of an equol-producing ability in postmenopausal non-equol producing women after exposure to soy food over a month. A study compared the equol producing capability of Korean American girls who consumed 3 times more soy food with that of Caucasian American girls and found more equol producers among the Korean girls (51% vs. 36%) [169]. The authors emphasised higher equol-producer prevalence among Asians. Hedlund et al. [166] found in high soy food consumers also a higher prevalence compared to those of low soy consumers (28% vs. 10%). In the same study a positive correlation between consumption of meat and equol production was observed. This was also reported by Adlercreutz et al. [168] and Lampe and co-workers [175]. Interestingly the last mentioned study could not confirm the correlation with soy intake and equol-producing abilities. Whether a soybased diet is conducive to higher equol-producing prevalence is not quite clear. So, Hall et al. [165] found in a study with native Irish Caucasians 51% equol-producers, too.

Another possible metabolite of daidzein is *O*-desmethylangolensin (ODMA). A probable metabolic pathway of those compounds is displayed in Figure 11.

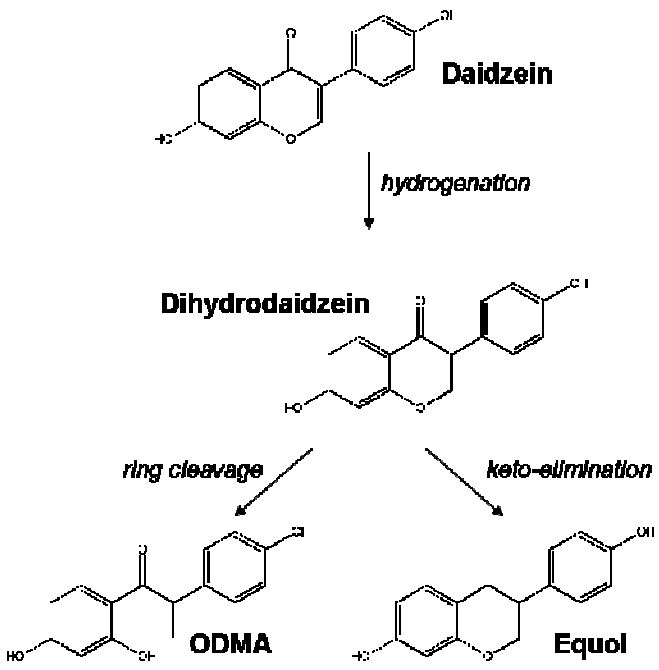

Figure 11: Metabolic conversion of daidzein adapted from Decroos et al. [176]. This is a simplified sketch of the conversion. Rate of conversion as well as pathway may vary in each individual.

Noteworthy is that it is possible to be coevally an equol-producer as well as an ODMA-producer [171]. The metabolism of phytoestrogens is widely ramified and intrinsic. A lot of metabolites have been identified until now [177-179]. It is very likely that variations in intestinal microflora account for a varying metabolisation. So far, it is not known which strains of bacteria are responsible for the production of specific metabolites. ODMA shows a weaker binding affinity for the estrogen receptors α and β compared to equol, daidzein and genistein [180].

The assessment of soy and isoflavone intake is carried out with food frequency questionnaires. Such a procedure has been validated for suitability in numerous studies [181-184], even with young girls [185]. The validation comprises the questionnaire, the evaluation of isoflavone intake according to those information and databases of food constituent contents, as well as the analysis of plasma concentrations of isoflavones. The intake of isoflavones varies around the world and is highest in Southeast Asia and Japan and lowest in Western countries, as displayed in Figure 12.

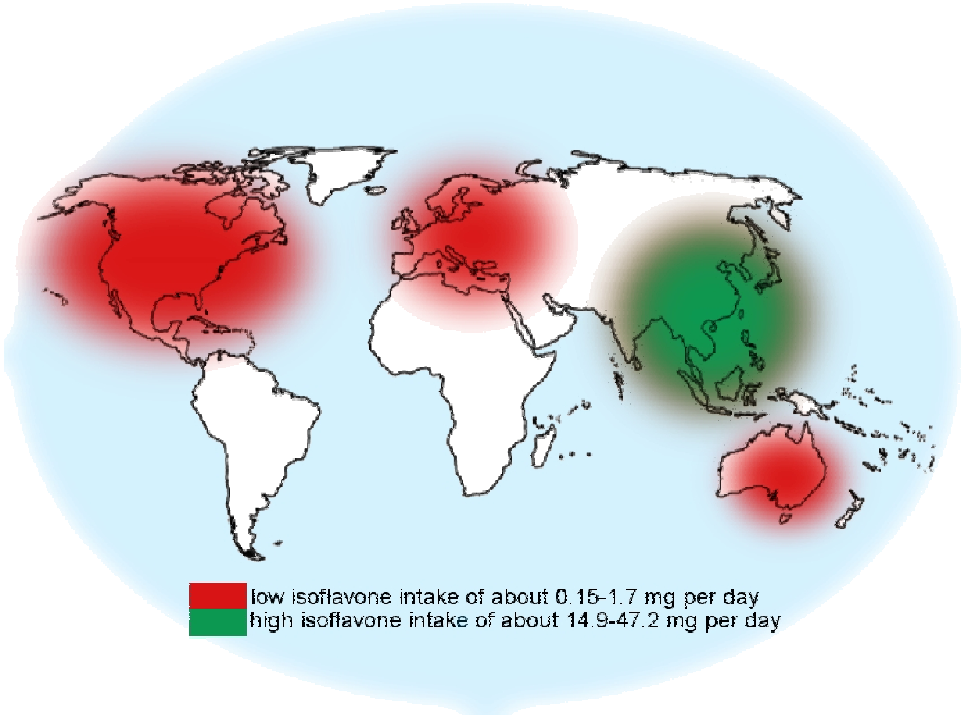

Figure 12: Isoflavone intake in Southeast Asia and Western countries.

In Western countries such as the USA, the United Kingdom or the Netherlands, the estimated daily intake of isoflavones is extremely low and ranges between 0.15 and 1.7 mg [186-190]. This is not surprising, since isoflavone-rich food is not usual in Western diet. The main sources of isoflavones named in these studies, beside soy food were beans, peas, nuts, grain products, coffee and tea. Nevertheless, Mulligan et al. [190] point out in the EPIC-Norfolk study that the average isoflavone intake of soy-consumers is a tenfold higher than of non-consumers in the UK. Heald et al. [184] reported also a median intake of genistein and daidzein of about 1.0 mg/day, but noticed also a great variation between 0.01 and 8.3 mg/day in Scottish older men.

Whereas the daily intake of isoflavones in Southeast Asia, as described in studies for Korea, China and Japan, ranges between 14.9 and 47.2 mg [191-197]. The main sources in these cases were traditionally used food items such as tofu, miso, natto, fried tofu, soybeans (whole, sprouts and as paste) and arrowroot.

Asian migrants in the USA are often objects of various studies, especially regarding intake of isoflavones and soy and cancer incidence differences. Their diet is altered to Western style but some items of the traditionally diet from the homeland are often maintained. This is also seen in the intake of isoflavones among Asian migrants and offspring that ranges between 10.2 and 18.9

mg per day [196, 198-200] and is intermediate between intake data of Caucasians and Asians. Studies conducted in Hawaii suggest that Caucasians living in this area have a daily isoflavone intake of 5.2 to 6.7 mg per day [196, 200]. These values are higher than usually reported, but have not been discussed by the authors.

The plasma isoflavone concentrations of Caucasians are naturally also lower, according to the lower intake. Uehara et al. [201] compared plasma phytoestrogen levels of Japanese and Finnish women and found in Japanese women a higher level of about a factor of 50. They report mean plasma levels of genistein, daidzein and enterolactone for Japanese and Finnish women of 109.9, 30.1, 4.0 and 1.7, 0.9, 7.5 ng/ml, respectively. Morton et al. [202] observed similar daidzein plasma concentrations such as seen among Japanese women in the last named study in Hong Kong men and found 31.3 ng/ml. In the same study they found in Portuguese and British men plasma concentrations of 1.3 and 8.2 ng/ml for daidzein. In another study with Scottish older men serum median concentrations were 9.1, 4.6 and 0.16 ng/ml for genistein, daidzein and equol respectively (for those who were able to produce equol).

It is noteworthy that isoflavones are accumulated in prostatic fluid. Whether this fact is linked to the lower prostate cancer risk of Asian men, will be discussed in a following subchapter. Anyway, exemplarily equol and daidzein are found in concentrations of 0.5-29.2 and 4.6-70 ng/ml in men from UK, Portugal, Hong Kong and China. The Asian study subjects showed higher levels of isoflavonoids and had 5 to nearly 60 times higher concentrations. Comparable values (11.8 and 13.7 for equol and 49.5 and 42.4 for daidzein) were found in Korean men, both healthy and suffering from benign prostatic hyperplasia [203]. The only significant difference between the healthy and the ill volunteers was a lower genistein concentration in the prostatic fluid of the participants diseased with benign prostatic hyperplasia.

Asians are exposed to isoflavones from an early age on. Already as foetus they consume isoflavones via diet of the mother and as infants they obtain isoflavones from breast milk. Actually, *in vivo* studies with rats show that phytoestrogens can pass the placental barrier [204], probably due to their lipophilicity. Once in the bloodstream of the rat foetus, isoflavones can reach also foetal brain and are found in concentrations like in the maternal brain [205]. Weber et al. [206] analysed in a study with rats the effects of phytoestrogens on brain aromatase and found no alterations of aromatase enzyme levels during perinatal development. Aromatase is a key enzyme of the steroid hormone metabolism (see Figure 13).

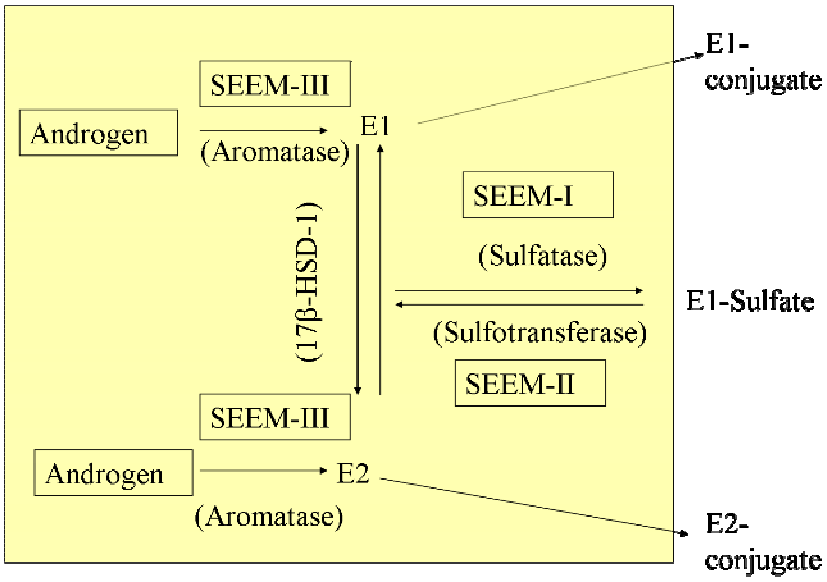

Figure 13: Aromatase action in the steroid metabolism and the formation of estrogen (E2) and estrone (E1) metabolites. The involvement of selective estrogen enzyme modulators (SEEMs) is displayed.

Whether other health implications can be expected is not quite clear, but the normal development of thousands and thousands of Asian infants suggest harmlessness.

The placental transfer is also true for humans, as studies with maternal and umbilical cord blood demonstrate [172, 173, 207]. In Japanese studies cord blood concentrations of about 19.4 to 34.3 ng/ml, 4.3 to 9.8 ng/ml and 0.9 to 1.0 ng/ml for genistein, daidzein and equol were found [172, 173]. A Malaysian study found mean concentrations of 1.4 ng/ml for daidzein and 3.7 ng/ml for genistein in cord blood. Adlercreutz et al. [208] studied Japanese women at delivery and measured mean total isoflavonoid concentrations of about 76 ng/ml in cord plasma, 59 ng/ml in maternal plasma and 57 ng/ml in amniotic fluid.

After birth Asian infants are exposed to bioactive compounds by means of breast milk, while especially infants in Western countries experience mostly for the first time an isoflavonoid exposition via soy-based infant food. Franke and Custer [209] measured a concentration range of 20-28 ng/ml and 8-13.5 ng/ml for daidzein and genistein in the breast milk of a Chinese woman, who ate daily tofu soup. They observed similar isoflavone concentrations in the breast milk of a Caucasian woman after challenge with roasted soybeans. Comparable data were found years later in mother milk (mean values of about 18 ng/ml total isoflavones) after a soy beverage intervention of several days duration. The breastfed infants showed mean plasma concentrations of total isoflavones of ~5 ng/ml. Much higher concentrations were found in older infants (9-25 months), who ate 15-90 g tofu; their plasma isoflavone concentration was 50 times higher.

Nevertheless, the daily intake and the resulting isoflavone plasma concentrations are much higher when the babies were fed with soy-based infant formulas. This kind of baby food is often used in Western countries, particularly since more and more paediatrists recommend the beginning of consumption of dairy products based on cow milk at a later date. Infants fed with those formulas have an intake of 3 to 11 mg isoflavones per kg body weight [210-212]. This is about a factor of 5 to 10 times higher than the usual daily intake of Asians eating a traditional diet (see data above) and much higher than the isoflavone intake of breastfed babies even with high-isoflavone consuming mothers. With the soy-based formula diet, infants accomplish plasma concentrations of 684 ng/ml for genistein and 295 ng/ml for daidzein [213]; amounts that translate to concentrations of 2.5 µM and 1.2 µM for genistein and daidzein respectively. Considering EC_{50}-values (for definition, see Table 3) of 10 µM (daidzein) and 0.1 µM (genistein) for estrogen receptor (ER) α [16], the obtained isoflavone concentrations could have an effect on infants.

Nevertheless, experts advise the feeding of infants with soy-based milk formulas, especially if recommended of paediatrists, since clinical trials show clearly no evidence of disruption of sexual development in infants [214-217]. So, Irvine et al. reported in the New Zealand Medical Journal that the New Zealand Ministry of Health recommended soy based formulas for replacement of cow milk, if required. They considered these products also as safe for infant feeding purposes.

5. Cancer Prevention and Diet

5.1 Cancer Risk and Incidence

Statistics show an increasing trend in cancer incidence worldwide [218]. One of the simple explanations is the increasing life expectancy. Cancer is an age-related disease, but cancer incidence has been correlated with smoking or exposition to second-hand smoke and pollution, socioeconomic status, genetic predisposition, age at first birth, age at menarche or menopause, hygiene factors and diet. Generally the cancer incidence for males is higher than for women. This is statistically confirmed for cancer of lung, pancreas, liver (except for Japanese in Los Angeles) colorectum (except for Chinese in Shanghai), stomach, bladder, oesophagus and leukaemia [219-226]. An exceptional case is skin cancer: no higher incidence for males could be found. However, white people in USA and Europeans had much higher risk than Asians, Asian Americans and Afro-Americans regarding skin cancer [227].

Worldwide incidence, prevalence and mortality was presented in the GLOBOCAN series of the International Agency for Research on Cancer (IARC) and summarised by Parkin et al. [228]. In the year 2002 about 24.6 million people suffered from cancer, in addition to 10.9 million new cancer patients. 6.7 million mortalities due to cancer were reported. Most frequently diagnosed cancer is lung cancer, followed by breast and colorectal cancer. Lung cancer is also leading cause of cancer death. Interestingly, it is not followed by breast and colorectal cancer but stomach and liver cancer as causer of cancer death.

But statistical evaluation revealed also a decline for some cancers worldwide or for some geographical areas, e.g. stomach cancer [228], oesophageal cancer [229] or ovarian cancer [230]. This is due to improved living conditions, hygiene factors (especially for *Heliobacter pylori*-induced gastric cancers) and diet. In case of ovarian cancer it is probably the consequence of the increased use of oral contraceptives during the last fifty years, which is linked to reduced ovarian cancer risk [231]. These observations of time trends suggest that indeed prevention of some cancer types is achievable, although some diseases will not be affected due to unalterable facts such as genetic factors.

In an international comparison of cumulative risk of all-site cancer [232], evaluated from the data from “Cancer Incidence in Five Continents Vol. VIII”, Afro-American men showed the highest cumulative risk. Within women, Denmark had the highest risk in females. Meanwhile Chinese immigrants living in Los Angeles showed the lowest risk in both genders. See also Figure 14.

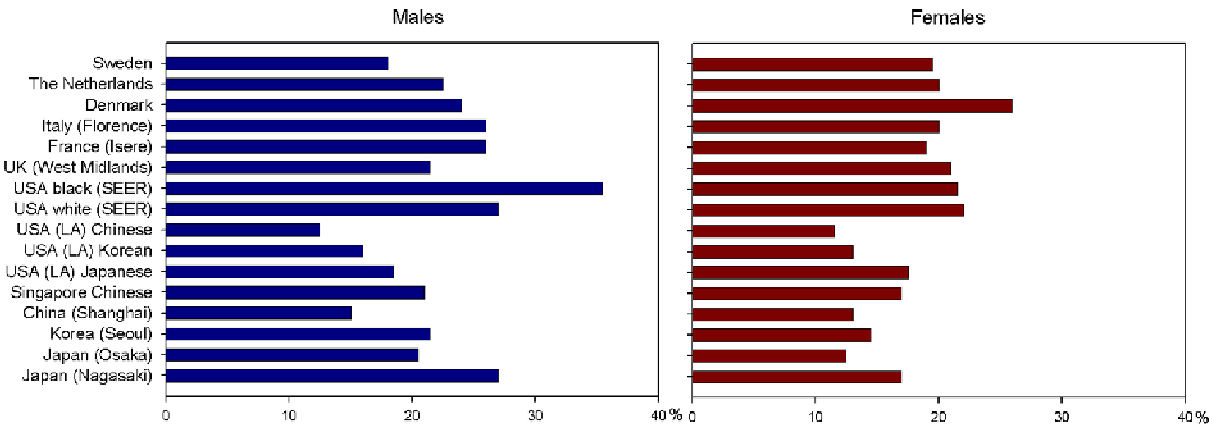

Figure 14: Cumulative incidence risk of all site cancer for males and females to age 69 years, adapted from Katanoda et al. [232]), who evaluated data from "Cancer Incidence in Five Continents Vol. VIII". SEER: Surveillance Epidemiology and End Results, LA: Los Angeles.

Cancer prevalence is differing around the world and ethnicies. Although East-Asians are known to have lower incidence rates for some cancer types such as breast and prostate cancer, they show higher prevalence for other cancer varieties. This is observed for liver cancer [220], oesophagus cancer [225] and stomach cancer [221]. Explanations are of course infections with hepatitis B and C as well as with *Helicobacter pylori*, higher intake of nitrosamines and nitrite pickling salt in preserved meat and aflatoxin-contaminated food, just as a lower intake of fresh fruits and vegetables.

Studies on minorities, emigration and cancer incidence shed light on cancer-causing factors, preventable cancer diseases and inevitable cancers in consequence of genetic determinants. Emigration can lead to higher incidence rates of cancer varieties that are prevalent in the country of emigration and lower incidence rates of cancer types that are prevailing in the country of origin, due to a new adapted diet and lifestyle. To examine those influence factors and distinguish those from genetic causes, it is of avail to compare incidence rates of migrants, their offspring and of local residents. Asian emigrants in the USA are suited for those purposes because of a great Asian population in the USA, a long immigration history with chronological different emigration phases and the well-known distinctions of cancer incidence between Asians and Caucasians. But interpretation of statistic data is not always easy and obvious.

Flood et al. [233] examined the incidence of colorectal cancer among Asian-Americans according to birth in the land of origin or in the USA (data was obtained from the Surveillance, Epidemiology and End Results (SEER) program over a period of 1973 to 1986). Japanese men

born in the USA had a twofold higher incidence for colorectal cancer than Japanese migrants and a 60% higher rate compared to US-born white men. Japanese women born in the USA had a 40% higher incidence rate in comparison to women born in Japan and US-born white women. An interpretation could be that Japanese exhibit a priori a genetic hypersensitivity for colorectal cancer. Traditional lifestyle and diet in Japan could be protective, while western lifestyle could lead to a higher cancer rate boosted through the genetic sensibility. In the meantime, there was no difference between the incidence rates of Chinese men born in China and US-white men. Chinese born in the USA showed mildly reduced rates. Chinese women, independent of place of birth, exhibited 30-40% lower incidence of colorectal cancer.

Overall, a shift of cancer pattern towards higher incidences for cancer types that are more common in the land of emigration can be observed [234-236]. The protective effect of Asian lifestyle and diet is lost more and more, depending on the duration of lifetime spent in the emigration country and the resulting greater or lesser extent of westernisation [237, 238]. Wu et al. [239] reported that the intake of tofu among Asian-Americans born in Asia is twice as high as among their offspring born in the USA, and that the intake decreases with years of residence in the US. Nevertheless some kinds of cancer seem to be unaffected by such factors. Herrington et al. [240] analysed SEER data for the period from 1973 to 1986 and compared the incidence rates of ovarian cancer among Chinese, Japanese and Filipino emigrants to the USA and their offspring with rates among US-born white women. Interestingly the incidence rates of Asian-born women and Asian-Americans were nearly the same and about 10 to 50% lower than the rates of US-white women.

5.2 Steroid Hormone Related Cancers

Among cancer types the hormone-related breast and prostate cancers are one of the most common cancers in Western world. The incidence for those cancer types is known to be much lower among Asians in comparison to Caucasians or in general people in western populations [241]. Global trends show increasing incidence rates for breast cancer, simultaneously with a stable or slightly decreasing mortality [242]. While North America had the highest incidence levels, Western Europe, Oceania, Scandinavia and Israel were in the intermediate sector and Eastern Europe, South and Latin America and Asia exhibited the lowest rates. The decreasing mortality is clearly due to improved diagnostic and breast cancer treatment and large-scale mammographic screening programmes in several countries. Unexpectedly, countries with high

and intermediate incidence rates had also the highest mortality rates, although the cancer therapy in those countries is state-of-the-art. This phenomenon is explained by genetic determinants as well as time of childbearing and breast feeding and dietary caused differences in endogenous hormone status. Although Japan had one of the lowest incidence rates in international comparison, extremely high increases during the last decades were noticed [242, 243]. Over the last decades a similar approach to western statistics regarding breast cancer incidence is documented for Taiwanese [244] and Singapore [245] women. Migrational studies show analogue to that on the one hand an intermediate incidence rates for US-born Asians in comparison to the lower rates of Asian residents and the higher rates of US-born whites [246], but then also rapidly rising incidence rates for Asian-Americans [247].

Althuis et al. [242] spotted similar age-incidence trends among Asians and estrogen receptor (ER) negative tumour types in Caucasians [248, 249]. Generally, only a minority of normal breast cells, varying in studies up to 15%, are ER positive cells (ER+) [250-252]. Most of those cells are distributed as single cells and surrounded by ER negative (ER-) cells. A smaller fraction showed a tendency to build cell clusters. This phenomenon increases with age. While those contiguous cells are uncommon in premenopausal breast tissue, it is found more often in perimenopausal tissue and climaxes in postmenopausal breast tissue but does not increase after that further [251]. Interestingly, only a small amount of normal premenopausal breast tissue expresses the estrogen receptor and the proliferation-associated marker Ki-67 simultaneously. Yet again, there is a strong increase in co-expressive cells in ER+ mammary tumours [252, 253]. To get back to the breast cancer differences of Asian and Caucasian women, not only that Japanese women have much lower ER expression in normal breast tissue [254, 255], but also they have fewer ER+ cells in breast tumours [256, 257].

The endogenous steroid hormone level is often associated with risk of breast cancer, although the studies are discordant [258-261]. In the Guernsey cohort premenopausal breast cancer patients had an increase in estradiol concentrations but not as high as postmenopausal women with breast cancer (12% vs. 29%) [262, 263]. But not only the estrogens may play an important role in breast cancer development and progression, the androgens, sex hormone-binding globulin (SHBG) and progestins, have to be taken into consideration.

Data of the European Prospective Investigation into Cancer and Nutrition (EPIC) study with a cohort of 370 000 women and 150 000 men from over ten European countries, confirmed that among postmenopausal women breast cancer risk is positively associated with higher levels of

androgens and estrogens and inversely related to SHBG levels. The results from a case-control study (677 cases that developed breast cancer after recruitment in the EPIC study and 1309 matched control subjects) nested within the EPIC study were presented by Kaaks et al. [264]. For premenopausal women the risk of breast cancer increased with elevated levels of testosterone and androstenedione and low levels of progesterone, while no clear association with estrogens and SHBG was found [265].

Zeleniuch-Jacquotte et al. [266] presented very similar results for postmenopausal women to those of the Guernsey cohort and reported further that free estradiol was higher and conversely SHBG-bound estradiol lower in breast cancer cases than in controls. A very similar distribution pattern of free and SHBG-bound estradiol was obtained for postmenopausal Japanese breast cancer patients [267]. This is illuminating since free steroid hormones represent the bioactive form that interacts directly with cells via receptors. Again, an ethnical difference between the serum estrogen levels in postmenopausal Japanese and American white women is reported [268]. Although the much lower levels of estradiol of Japanese women could not be confirmed in a smaller study of Dowsett et al. [269], the authors concluded that this could be due to the choice of study subjects. Dowsett et al. [269], who found no difference in estradiol serum levels, had subjects mainly from an urban background, while Shimizu et al. [268] deliberately chose women from a rural agricultural area in order to represent a traditional Japanese lifestyle. At first sight these data could suggest a genetic coherence, considering the much lower level of estrogen receptor, estrogens in the sera of Asian women and the epidemiological lower breast cancer incidence. Nevertheless, a great deal of the differences can be explained by lifestyle including reproductive behaviour and especially the diet. So for example, no difference in the distribution of estradiol in sera of Caucasian, Chinese, Filipina, Hawaiian and Japanese women living in Hawaii could be found [270]. Moreover, the Multiethnic Cohort Study [271] and a study of Pinheiro et al. [272] found higher levels of estradiol and free estradiol in Asian Americans and African American women compared to Caucasian Americans. Although Probst-Hensch et al. [273] found in a smaller study only little difference in estradiol levels, Japanese Americans had in their study the highest levels of estrone. This discrepancy between hormone levels of migrants and people from the homeland can be explained by dietary factors. Woods et al. [274] demonstrated that the change from a high fat – low fibre diet to a low fat – high fibre diet provoked a significant decrease of serum estrogens in African-American women. Similar results were obtained in the DIANA study of Berrino et al. [275]. The change of diet resulted in changes

in serum levels of testosterone (↓ significantly), estradiol (↓ but not significant) and SHBG (↑ significantly). Adlercreutz et al. [276] compared the estrogen metabolism and excretion in Oriental and Caucasian women and came to the conclusion that the evaluated higher estrogen level of Finnish women result from a higher fat diet. A few years earlier Goldin et al. [277] compared also the estrogen level and excretion of Oriental (Vietnamese, Laotian, Japanese, Koreans immigrants in Hawaii) women with Caucasians (Americans). They found 30-75% higher plasma estrone and estradiol levels in premenopausal Caucasian women than in the age-matching Oriental cohort. The postmenopausal Caucasians had also a 3-fold higher estradiol level. Oriental pre- and postmenopausal women had also lower levels of androgens, which could be the explanation for the lower estrogen concentration. The fat-intake of the Oriental cohort was half the intake of Caucasian women. A direct correlation between total dietary fat consumption and serum estrogen level was found. A comparison of the influence of a vegetarian vs. omnivorous diet [278] showed that levels of androstenedione, testosterone, free testosterone and SHBG were higher in omnivores than in vegetarians. In the same study breast cancer cases showed similar hormone level pattern to omnivores, but with even higher levels of hormones, although the breast cancer patients and healthy omnivores consumed a similar diet. This diet was typical "Western style", rich in proteins and fat and poor in complex carbohydrates and fibre. It is associated with low SHBG and high androgens (total and free), a hormone pattern found in breast cancer patients. A population-based case-control study among Indian migrants in England showed a strong inverse trend in the odds of breast cancer with increasing intake of vegetables and fibre [279].

Steroid hormone levels play also an important role in prostate cancer risk. In context of a possible hormone level modification by diet and lifestyle, it is of great importance to take that into account regarding cancer prevention arrangements and recommendations. East Asian men have much lower cumulative prostate cancer incidence than people living in Europe and especially in America, irrespective they are white, black or have Asian ancestries. But US-black men showed by far the highest incidence rates, followed by US-white men [241]. Whether this is due to genetic predisposition or lifestyle factors is not easily explained. No significant differences in testosterone (free or SHBG-bound) and SHBG levels by race or ethnicity is found among black, white and Hispanic men in the Boston Area Community Health (Bach) Survey study. A higher dihydrotestosterone (DHT) level and DHT to testosterone ratios where found in black men after covariate adjustment [280]. In a cross-sectional study, the results concerning testosterone were

confirmed, while Rohrmann et al. [281] simultaneously reported higher estradiol serum levels for black men. Although Wu et al. [282] report contrary results, only higher levels in SHBG and DHT for African-Americans and Japanese-Americans compared to US-whites were statistically significant. They found also significantly higher levels of total testosterone in Japanese-Americans and Chinese-Americans compared to whites.

Studies that deal with the association between hormone levels and prostate cancer incidence are inconsistent. Gann et al. [283] found no clear association between hormone levels or SHBG and prostate cancer risk. But after a simultaneously adjustment of hormone and SHBG levels, they observed a risk increase with high levels of circulating testosterone and low levels of SHBG and estradiol. In the meanwhile, other data seem to negate a relation between serum testosterone and prostate cancer incidence [284].

5.3 Cancer Prevention by Isoflavones

Nonetheless the consumption of soy products and soy constituents is associated with a reduced risk for prostate cancer. Studies in China and Japan circumstantiate this hypothesis [285-289] and provide once again an indication for the prostate cancer protective properties of traditional Asian diet. The serum phytoestrogen concentration is much higher in comparison to Western regions where the soy consumption is negligible. Morton et al. [290] found 10 to 20fold higher concentrations of isoflavones in Japanese men and women than in British men and women. The difference in equol concentration was even higher. This is probably a reason why studies conducted in Europe, found a decreased risk in prostate cancer associated with serum concentration of enterolactone, but no association with for isoflavone intake or serum level [291, 292]. Nonetheless, the American Adventist Health Study [293] revealed a 70% reduction in prostate cancer risk due to the frequent (more than once per day) consumption of soy milk. In a multiethnic case-control study Kolonel et al. [294] report an inversely relation between legumes intake (soyfood and others) and prostate cancer. A lot of epidemiological results can be buttressed by *in vitro* and *in vivo* experiments.

Beginning from cell culture [295] to in vivo animal studies [296, 297], isoflavones inhibit prostate cancer. A revealing study of Rannikko et al. [298] demonstrated that the prostate tissue is concentrating isoflavones. Patients that were supplemented with 240 mg of clover phytoestrogens over two weeks, exhibited a statistically significant 23- and 7fold increase of genistein and daidzein and the prostate tissue concentration of those components were over 2fold higher than in

their plasma. But as described in a previous subchapter, not all people are able to metabolise daidzein to equol. Akaza et al. [299] compared the ability to produce equol between prostate cancer cases and controls and found out that the rate of equol producers was significantly lower (39.7% vs. 50.0%) in prostate cancer patients. In a subsequent study [300] they evaluated producers and non-producers of equol among Japanese, Korean and American prostate cancer patients and healthy men and found similar results for Japanese and Korean men (29% vs. 46% in Japan and 30% vs. 59% in Korea). The American men showed markedly lower isoflavone levels and the percentage of equol producers amounted up to 17% for patients and 14% for controls.

There is a lot of evidence that Western diet increases the risk for hormone-related cancer types, while Asian diet seems to act contrary. One of the best known dietary factors that seem to protect against breast and prostate cancer and are highly consumed in Asian cuisine are the phytoestrogens. A case-control study in Australia demonstrated a breast cancer risk reduction with high intake of phytoestrogens. Especially the excretion of equol and enterolactone is correlated with a significant decrease of risk [301]. A German case-control study asserted a protective role of a diet high in daidzein, genistein, maitaresinol, enterodiol and enterolactone against premenopausal breast cancer, although the intake was low in comparison to Asian women [302]. Often special soyfood items were investigated on their ability to reduce breast cancer. So, Yamamoto et al. [303] found an inverse association between intake of miso soup and isoflavones but no significant correlation of other soyfood items such as soybeans, tofu, deep-fried tofu and natto with breast cancer. Fujimaki and Hayashi [304] came to similar results and reported a negative association between breast cancer and miso soup, but no association with natto and even a positive association with tofu consume. More critical studies exist: Grace et al. [305] reported even an increase in breast cancer risk with phytoestrogen consumption. A weakness of the study was the relative small number of cases and the low daily intake of isoflavones of 437 µg (on average). Nevertheless, Nishio et al. [306] conducted a large cohort study with Japanese women that definitely have a high soyfood consume and found no protective effects against breast cancer. It is not easy to find a clear and definite leitmotif in the magnitude of studies, but meta-analysis papers that compared the available studies on this area of work (including papers published in Chinese and Japanese) support the hypothesis that soyfood is associated with a decreased risk of breast cancer [307].

There are abundant studies that report on cancer risk reduction due to phytoestrogen or especially soy consumption. In addition to breast and prostate cancer, also ovarian cancer risk seems to be reduced by soyfoods and isoflavones [308].

Generally, epidemiological studies correlate intake of vegetables and polyphenol-rich food to reduced cancer risk. Literature is full of examples of the correlation of vegetables and a reduced risk of various cancer types. A few are listed now: prostate cancer [294, 309], lung cancer [310], gastric cancer [311], oral cancer (meta-analysis in [312]), thyroid cancer [313] and colorectal cancer [314]. The ORDET cohort study [315] compared four dietary patterns and identified the "salad vegetable" diet, with a high consumption of raw vegetables and olive oil as added fat, as breast cancer protective diet type with an incidence decrease of about 35%. Cui et al. [316] found in the shanghai breast cancer study an association of western diet (described as "meat-sweet" diet pattern) with increased breast cancer risk among Shanghai postmenopausal women in comparison to the "vegetable-soy" diet pattern. Equally, high tofu intake was correlated with a protective effect regarding breast cancer in Asian-Americans [239].

Another dietary factor that is often correlated to reduced cancer risk is the consumption of green tea. This beverage is traditionally drunk in Asia and gained certain popularity in Western civilisation, although the everyday intake is low compared to Asia. Actually, there is not only a significantly lower risk of breast cancer for green tea drinkers, but there is also a significant trend of decreasing risk with increasing amount of green tea intake [317]. Additionally, the work of Miyanaga et al. [318] contains a reference to the ability of green tea to enhance equol production. In their study they found a significantly higher consumption of green tea in equol producers than in non-producers. Polyphenols in green tea are mainly a subclass of flavanols called catechins. The most abundant catechins in green tea are epigallocatechin gallate, epicatechin gallate, epigallocatechin and epicatechin [141, 319]. Tea polyphenols exert similar to isoflavones a wide range of beneficial health effects and exhibit exemplarily a protective effect against breast [317, 320] and prostate cancer [321-324]. So, tea polyphenols are also found in prostate tissue of humans after tea consumption [325].

An analysis of lung cancer risk and the intake of over 240 food items showed a statistically significant inverse association between lung cancer risk and two flavonoids: quercetin, found in higher quantities in onions and apples and naringin, found in grapefruits [326]. Schabath et al. [327] found a statistically significant decrease of about 44% in men and 34% in women in lung cancer risk accompanied by increasing intake of phytoestrogens. Moreover, high intake of

enterolactone and enterodiol and use of hormone therapy was associated with a risk reduction of 50%.

Evidence suggests that a prepubertal and adolescent exposure to estrogens as well as phytoestrogens have great impact on the organism and may be an important determinate regarding cancer development in adult life. *In vivo* studies in rats indicate that a prepubertal genistein exposure acts not only chemopreventive [328] but has also no adverse effects to uterine weight, mammary gland size and circulating hormone levels [329]. Cabanes et al. [330] showed an up-regulation of the BRCA1-gene due to prepubertal exposure of rats with genistein or estradiol. BRCA1 gene (breast cancer susceptibility gene 1) is a tumour suppressor gene. A mutation at this gene is responsible for most genetic caused breast cancer diseases. The administration of genistein *in utero* and lactational at least at levels that were comparable to a human dietary exposure obtained by a soy-rich diet caused no adversely effect on the mammary gland [331]. Similar to the described influence of diet on human hormone levels, Hilakivi-Clarke et al. [332] reported a significantly higher estradiol plasma level of pregnant rats that were fed with a high fat diet. The high-fat offspring featured also an earlier puberty onset.

Three population-based case-control studies [333-335] (USA, Shanghai and Canada) that deal with the correlation of soyfood intake during adolescence and/or adult life and breast cancer risk, came to the conclusion that indeed the risk is inversely associated with higher soy consumption. Moreover Wu et al. [333] ascertained that the risk of low-soy consumers in adult life was still only intermediate, if they have been high soy-consumer during adolescence.

The exercise of influence of the diet that is consumed by the mother and acts upon the unborn foetus *in utero* has also to be taken into consideration. A phytoestrogen-rich diet is traditionally eaten in Southeast Asia and does not seem to have obviously bad influence on Asian people. Strictly speaking the known epidemiologic data suggest lower incidence for several cancer types. This is in all likelihood caused by the diet and probably other lifestyle factors such as reproductive behaviour. That the latter one can not be the exclusive reason is obvious, since the reproductive factors in other regions are similar, but the lower cancer risk is missing.

Our conclusion is that epidemiological studies, *in vitro* and *in vivo* experiments and controlled clinical trials suggest that polyphenol-rich diet has a beneficial effect for cancer prevention.

6. Ameloriation of Menopausal Complaints

6.1 Clinical Aspects and Epidemiology

The reproductive phase of females begins with the menarche and ends with the menopause. Strictly speaking is the menopause defined as the permanent stopping of the menstrual cycle. In general it is referred to as longer time period in which the slowly fading of estrogen production and the therewith resulting implications are included. These events are due to a diminishing follicular ovarian activity. This period is also called menopause transition or perimenopause and is in fact the time when the woman is being in menopause and undergoes menopausal symptoms.

The international median age of natural menopause ranges between 50 to 51 years [336]. Although this differs across the world: in Western countries such as the USA [337] and Australia [336] as well as European countries such as Spain [338], Finland [339] and Poland [340] an onset of menopause of about 51.0 to 51.8 years is reported. Reynolds et al. [338] estimate the median age of menopause in Massachusetts (USA) with 52.6 years. The mean value of years at menopause for Japanese women comes to 49.6 [341]. In comparison to that countries in Northern Africa [342, 343], Northern India [344] and Southern Korea [345] report an earlier menopause onset with 48 years.

In the middle of a normal menstrual cycle (see Figure 15) the ovulation takes place. The phases prior and after that are called follicular and luteal phase respectively. During the follicular phase, the estradiol level is low at the beginning and rises to a peak 24-48h before the ovulation. Afterwards it falls abruptly to the beginning of luteal phase and rises again to a lower and broader peak with its climax in the middle of the luteal phase. The follicle-stimulating hormone (FSH) and luteinising hormone (LH) have their peak on the verge of ovulation. The concentration of progesterone is constantly low during the follicular phase and ovulation, but rises to a high and broad peak during luteal phase with its maximum around the middle of this phase.

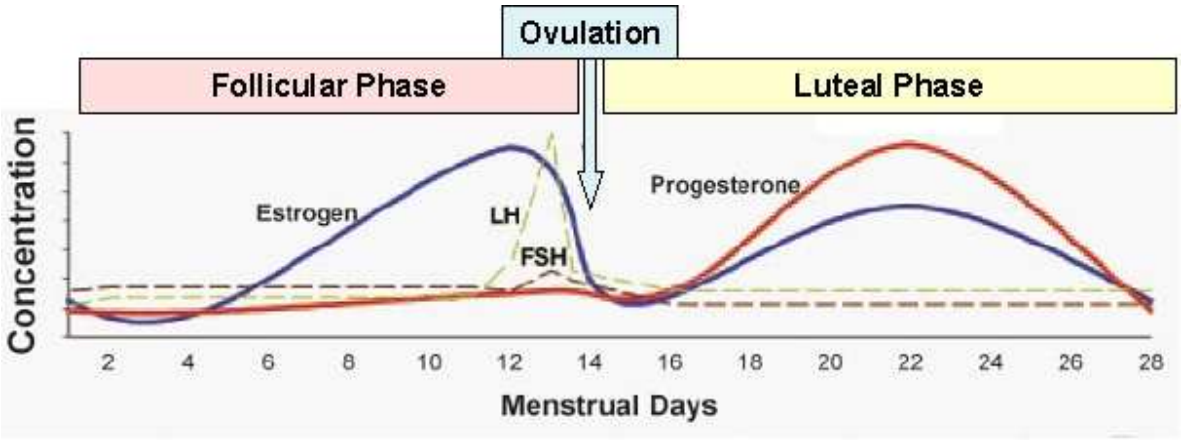

Figure 15: Hormone changes during the normal human menstrual cycle (adapted from Simmen and Simmen [346]).

Approaching menopause, the hormone pattern changes dramatically and leads to several physiological alterations that become manifest in disorders and complaints, also referred to as menopausal syndrome. Prior to the beginning of menopause, there is a time-related change of the menstrual cycles approaching the menopause. Before the final menses, a change from the normal ovulatory cycles to prolonged length cycles is observed [347]. This is characterised by a prolonged follicular phase and the missing of a luteal phase and is explained by the changing hormone levels.

During the normal ovarian aging, the number of follicles is declining. Since the oocytes are produced prenatal and the oogonial stem cells disappear thereafter, there is no possibility of post-production and the ability of reproduction is lost. Inhibin A and B are peptide hormones that are produced by follicles in different stages of the menstrual cycle [348-351] and inhibit FSH secretion and synthesis. Parallel to a declining number of follicles, the inhibin levels are also sinking approaching menopause [352]. Inhibin B level declines in early perimenopausal phase and remains constantly low in the late perimenopausal state, while inhibin A level does not seem to fall in early stages, but declines in late perimenopausal state rapidly [353]. The declining inhibin levels, especially those of inhibin B bring an increase of FSH levels about in older ovulatory women [353-356], while the estrogen level is not changing [352, 353, 355, 357]. Beginning from the late perimenopausal stage, estradiol level begins to fall and decreases most rapidly around the final menstrual period (FMP) and levels off in postmenopausal phase [353, 354, 358]. The hormone concentrations found in different ethnicities may differ, but the general pattern of declining estradiol and rising FSH levels during menopausal transition seem to be

independent of ethnicity (see Figure 16) [359, 360]. Nevertheless, some ethnic differences in hormone ratios such as the combination of lower estradiol and concomitant similar FSH levels of Chinese and Japanese women as demonstrated in Figure 16 could be an explanation for differing menopausal complaints that were reported.

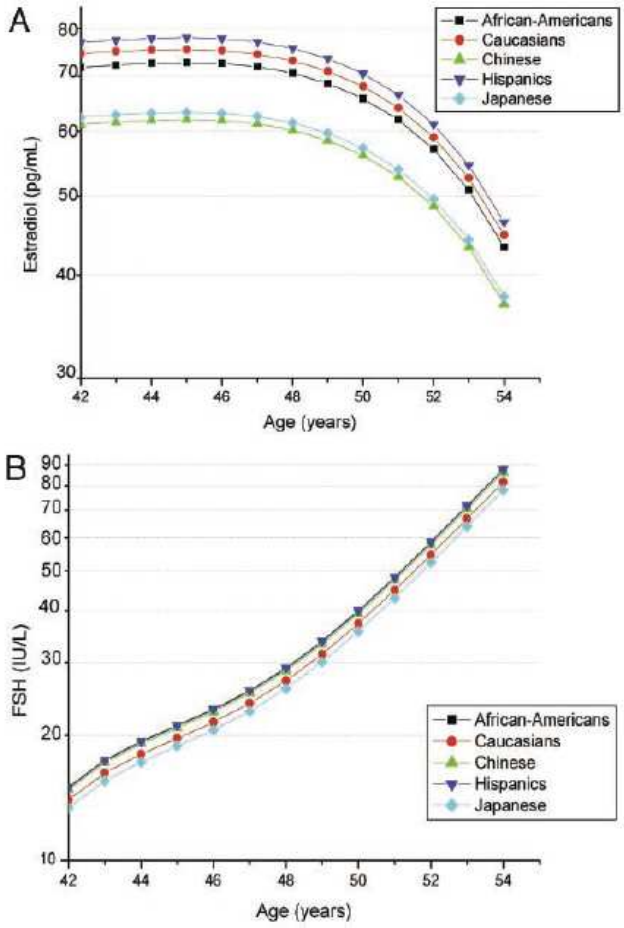

Figure 16: Average profiles of estradiol and FSH by age of different ethnicities (adapted from Randolph et al. [359]).

According to the bottom line of numerous studies, the prevalence for example of hot flushes seems to vary in different cultures and areas (also reviewed by Melby et al. [361] and meta-analised by Sievert et al. [362]). The incidence range of hot flushes experienced as menopausal symptom by women around the world, ranges from an intermediate to a high observed prevalence in Ecuador [363, 364], Brazil [365], USA [366] or Turkey [367], to a low frequency in Singapore [368] and Taiwan (China) [369] and none reported symptoms of hot flushes among Mayan women in Mexico [370].

The menopausal symptoms are sometimes not easily distinguished from general indications of aging such as sleep disturbance, mood symptoms and cognitive disturbances. Nonetheless some symptoms are definitely associated with menopausal syndrome including vasomotor symptoms (hot flushes and night sweats), vaginal dryness in combination with painful intercourse and sexual dysfunctions. Long-tail claims due to declining ovarian endocrine activity and the modified hormone levels are osteoporosis and cardiovascular diseases.

Vasomotor complaints are one of the most bothersome problems during menopause and affect life quality to a great extent. The etiology of hot flushes is still not fully understood. It seems that the thermoneutral zone, which is the temperature range in which one is neither shivering nor sweating, is extremely narrowed in postmenopausal women with hot flushes. Small elevations of the core body temperature are sufficient to trigger heat loss mechanisms. The circadian rhythm of the core body temperature is also different from that in asymptomatic women and varies especially in the time period of 00.00 to 04.00 and 15.00 to 22.00 h [371]. Sievert and Flanagan [362] hypothesised after data correlation of 54 studies that women living in warmer climates experience less frequently hot flushes because of a greater adaptation to elevated temperatures regarding their thermoneutral zone. Nevertheless, this hypothesis would not explain differences of hot flush frequency among women of different races who live in the same area [366, 372].

However, the involvement of hormones on hot flush incidence is very likely. A high FSH level and a lower estradiol serum concentration are associated with a higher hot flush frequency [373-377]. Randolph Jr. et al. [378] found also an association of increased FSH concentration with hot flushes but then no association with estradiol.

As consequence of changes in the hormone pattern, which is climaxing in perimenopausal stage and settles down in postmenopausal times, the prevalence and the intensity of climacteric symptoms are increasing from premenopausal to perimenopausal and postmenopausal phase [379, 380]. It seems that the prevalence of menopausal complaints increases during the first two years after menopause and decreases afterwards [377, 381].

6.2 Intervention for the Reduction of Hot Flushes

It is still a controversial issue among scientists, whether isoflavones are able to ease vasomotor complaints due to menopause. Some researchers were not able to find any amelioration in hot flush severity and incidence, neither with soy food [382, 383] nor with phytoestrogen supplementation [384-386]. Exceptionally interesting are conflicting results obtained from

studies that use the same supplementation and came to completely different conclusions such as Tice et al. [387] and van de Weijer et al. [388]. Tice and co-workers [387] report no difference in hot flush reduction between the group that got supplements administered and placebo group. Yet, the participants that got isoflavone supplements derived from red clover (82 mg isoflavone content) registered a faster reduction of hot flush incidence in comparison to the placebo group. The study of van de Weijer et al. [388] administered the same red clover supplementation and they report a reduction of hot flush frequency by 16% during the first 4 weeks of placebo. But the subsequent double blind phase resulted in a further significant decrease of 44% in isoflavone group, but not in placebo group. Other research groups report also an improvement regarding hot flush complaints after administration of soy food [389, 390] or isoflavone treatment [391-394].

In some cases mixed supplements of red clover, black cohosh, kudzu and other herbs are used in studies and it is difficult to attribute the demonstrated amelioration of vasomotor symptoms clearly to isoflavones [395, 396]. Important is the harmlessness of supplements with a dosage of about 50 mg isoflavones. Barnes [397] reviewed 2003 current literature and concluded that doses <2 mg isoflavones per kg body weight and per day are safe for most population groups. Several studies checked parallel to the effect on hot flush occurrence also vaginal cytology and endometrial thickness and found no modification of those parameters [393, 394]. In the same way, targeted application of a single isoflavone such as genistein caused significant effects on hot flush incidence but lacked negative impact on endometrial thickness [398, 399]. Atkinson et al. [400] noticed no effect on breast density due to red clover isoflavone supplementation. However, no impact on menopausal symptoms was observed, but the baseline levels of menopausal symptoms were low among study participants.

Epidemiological studies and controlled clinical studies show that isoflavones do not increase the risk of breast cancer and thus frequently isoflavone supplementation is recommended for amelioration of menopausal complaints. It seems that a dose of at least 50 mg per day is necessary.

6.3 Effect of Soy on Plasma Lipoprotein Levels

The data of studies that deal with the effects of soy protein and isoflavones on plasma lipid concentrations and the adherent incidence of coronary heart disease are inconsistent. The opinions in the scientific scene differ greatly. Some research groups found no significant difference at all in blood lipid levels due to isoflavone-rich soy protein [401-404] or isoflavone

supplement consumption [405-407]. Critical reviews challenge the hypothesis of an improving effect of isoflavones and soy on serum lipid ratios [408] and result in recommendations of professionals against isoflavone supplements due to a missing of clear evidence for the improvement of blood lipids as it is seen 2006 of the American Heart Association Nutrition Committee [409]. A call for more significant studies and reconsideration of the if possible overhasty classification of isoflavones as wonder nutrients is heard.

Contradictory to that, meta-analysis report on a significant decrease in serum low density lipoprotein (LDL) levels with high isoflavone intake [410]. In the same way scientists argue for the beneficial effects of soy on cardiovascular health, for example on the "Symposium on the Role of Soy in Preventing and Treating Chronic Disease" [411].

Emanating from animal models indeed an effect of soy protein and isoflavones on the plasma lipid composition can be observed. An i*n vivo* study of Peluso et al. [412] with rats shows a reduction of total cholesterol concentration due to soy feeding. They found also a decrease in liver weight and liver triglyceride and cholesteryl ester concentrations. This effect was associated with the isoflavone content. The feeding of a soy isoflavone extract lowered the liver triglyceride concentration by 33% in comparison to an atherogenic diet. Blair et al. [413] report gender specific differences in hamsters. Male hamsters that were fed a soy-containing diet had lower LDL and VLDL (very low density lipoprotein) cholesterol concentrations, although the effect does not seem to be caused by the isoflavone content. Female hamsters showed no alterations in LDL and VLDL concentrations, but had higher HDL levels due to soy feeding. Again, this was not associated with the isoflavones. A positive association of total isoflavones and LDL and VLDL concentration was observed in female hamsters. Another study [414] with hamsters demonstrate a decrease in plasma total, LDL and VLDL cholesterol concentrations after soy protein feeding independent of isoflavone content. But only the soy protein without isoflavones attained a reduction in aortic cholesterol accumulation. In rabbits isoflavone extracts without soy protein lowered significantly cholesteryl ester hydroperoxide as well as the atherosclerotic lesion area of the aortic arch in comparison to cholesterol-fed rabbits [415]. It is suggested that an inhibition of LDL oxidation brings an anti-atherosclerotic effect about.

Monkeys reacted on a soy plus isoflavone diet with significant improvements in LDL and HDL cholesterol levels. Meanwhile, soy feeding without isoflavones resulted only in higher HDL levels. The isoflavone fed group had the least atherosclerosis in comparison to the casein and soy without isoflavones feeding [416]. Two years earlier Anthony and co-workers [417] reported of

positive effects of phytoestrogen-intact soy protein (in comparison to isoflavone-depleted soy protein) on plasma lipid composition, especially a significant reduction in LDL and VLDL, an increase in HDL and a lowering in total plasma cholesterol concentration in monkeys. Nevertheless, Greaves et al. [418] found contradictory the same effect in monkeys only for intact soy protein, but not for the casein diet enriched with isoflavones (with similar isoflavone content as found in the soy protein). Whether the difference in results is explained by the fact that Anthony et al. [417] studied young male and female monkeys, while Greaves et al. [418] studied adult female ovariectomised monkeys remains open.

Data of studies in humans is similar to those of animal studies and in places inconsistent in the same manner. Soy diet, based on protein alone or including isoflavones resulted in decreased levels of LDL [197, 419-427], total cholesterol [197, 419, 420, 422, 423, 425, 428, 429] and triglycerides [428, 430-432], improved LDL/HDL ratios [421-423, 429-431, 433, 434] and a longer LDL oxidation lag phase [428] or reduction of oxidation [429, 435]. An increase in HDL concentrations was observed in a few studies [426, 427, 434, 436], whereas some other studies explicit arrive at the conclusion that the HDL cholesterol level remains unaffected [419-421, 423]. The above mentioned results occurred alone or in combinations and were not consistent in all studies, as exemplified by the effect on HDL cholesterol. Some studies prove those effects solely for soy protein and some attribute it to isoflavones. A point of critique was also the relatively high intake of soy protein in some studies of about 50 g per day that is up to 50% of a normal daily protein intake. Nonetheless, most of the mentioned studies provided less soy protein within the study diet and administered 15 to 36 g per day [419, 420, 429, 433, 436] or in two studies 40 g per day [430, 431]. Sagara et al. [425] investigated the effects of soy protein with isoflavones on cardiovascular disease in middle-aged men in Scotland and concluded that 20 g soy protein per day and 80 mg isoflavones reduce the coronary heart disease risk effective. That a much lower intake of soyfood is effective is demonstrated by the study of Rosell et al. [423]. In this study instead of a soy administration, the dietary intake of the 1033 study participants was assessed by a food-frequency questionnaire and categorized in groups of <0.5 g in the lowest group to >6 g of soy protein in the highest. A significant inverse association with total cholesterol and LDL levels was found. LDL cholesterol concentrations of participants with an intake of >6 g per day was 12.4% lower than of the lowest intake group. The same effect was described by a study that was conducted among Hong Kong Chinese population and a total of 500 men and 510 women [437].

6.4 Isoflavone supplementation and Cardiovascular Disease Risk

Given that soy is not a common food item in Western world and a rearrangement of the diet towards high soy intake is unlikely for many people, a supplementation of isoflavones is often opted especially of menopausal women. Therefore several studies investigate the impact of commonly used supplements. Beside soy, red clover represents a source of isoflavones and is used as basis for nutritional supplement formulations. A 12-weeks-lasting study [438] found a significant decrease in triglyceride levels among 246 menopausal women taking red clover supplements, although the decrease was primary observed among women with elevated baseline triglyceride levels. Clifton-Bligh et al. [439] studied the effects of the same red clover preparation on postmenopausal women over a time-period of 6 months and found a significant increase of HDL cholesterol of about 15.7-28.6% and a significant decrease of serum apolipoprotein B by 11.5-17.0% with different isoflavone doses. An increase of HDL cholesterol was also reported by Campbell and co-workers [440] in postmenopausal but not premenopausal women and no alteration in total cholesterol and triglyceride concentration. Biochanin A lowered LDL cholesterol significantly about 9.5% in a randomised placebo-controlled double-blind trial in men, but had no significant effect on women [441]. In menopausal women, on the other hand, Nestel et al. [442] found plasma lipids not affected at all by a 5-week treatment with 40 and 80 mg red clover isoflavones, but a rising in arterial compliance by 23%. An improvement in aortic stiffness after isoflavone intake was also reported by Teede et al. [443] and van der Schouw and co-workers [444]. This is of great importance for the reduction of cardiovascular disease risk, since arterial compliance (arterial elasticity) is deteriorating with advancing age and linked to cardiovascular events. A benevolent effect of isoflavones on vascular function was also proved by Steinberg et al. [445] by measurement of the brachial artery reactivity. Soy with isoflavones, but not isoflavone-depleted soy treatment of postmenopausal women lowered peak flow velocity of the brachial artery, which is consistent with a vasodilatory effect.

6.5 Influence of Isoflavones on eNOS and Arteries

It is not clearly clarified if the improvement in blood lipid pattern is confined to soy protein, isoflavones or the combinatory effect of both. However, isoflavones alone exhibit several properties that associate them with reduced cardiovascular disease risk. *In vitro*, isoflavones [446, 447] and equol [448] activate endothelial nitric-oxide synthase (eNOS), an enzyme that is accountable for the release of nitric oxide (NO) in endothelial cells that line blood vessels. This leads to a relaxation of smooth muscles of arteries. The obtained dilation of the blood vessel

lowers blood pressure. Actually, an inverse association of soy food intake and systolic and diastolic blood pressure was found in a large longitudinal cohort study among 45 694 participants of the Shanghais Women`s Health Study [449]. But NO inhibits also thrombocytes aggregation and prevents so blood clots. Chin-Dusting et al. [450] found not only a vasodilatory activity of isoflavone metabolites but also a protective effect against endothelium damage caused by oxidised LDL. Biochanin A, was also able to improve vascular relaxation in an *in vivo* test with rats [451].

Studies with postmenopausal women suggest favourable effect of isoflavones on cardiovascular risk markers and hypoglycaemic effects [452-455]. In a study of Clerici et al. [456], 62 adults ate isoflavone aglycone enriched pasta (33 mg isoflavones/day and negligible soy protein) for 8 weeks. As consequence the arterial stiffness decreased and a beneficial effect on several risk markers of cardiovascular risk was observed. The effect was greatest in equol producers. Afterwards switching to conventional pasta restored diagnostic findings.

6.6 Prevention of Osteoporosis

Loss of bone mineral density and content is another consequence of menopause. Normally, bone resorption and bone remodelling are balanced. Postmenopausal women experience increased bone resorption due to low estradiol levels, since a lack of estradiol leads to an exaggerated expression of interleukin-6 [457]. This cytokine is associated with lower bone mass and increased osteoporosis risk in postmenopausal women [458, 459]. Chapurlat et al. [460] demonstrated in a longitudinal study a rapid and diffuse bone loss in perimenopausal women, while they observed no bone loss in premenopausal women. A decrease in bone mineral density was observed after the menopause, as well as a positive correlation of estradiol and bone mineral density from about three years postmenopause [358]. Epidemiological studies suggest a lower fracture incidence of elderly Asians in comparison to Caucasians [461-464]. Whether this circumstance is explainable with higher isoflavone consumption, is not entirely clear.

Studies with animals corroborate the hypothesis that isoflavones from red clover or soy and equol benefit bone mineral density and prevent bone loss. Isoflavones reduce bone turnover [465-469], although the mechanism of action could be partly different from those of estradiol [470, 471].

Nevertheless, Nagata et al. [472] found no association of bone mineral density and isoflavones in a cross-sectional study with 87 postmenopausal Japanese women. A Dutch study with 67 postmenopausal women was also unable to find preventing effects of isoflavones on bone loss

[473]. On the contrary, they report higher rates of bone loss for equol and enterolactone. Though, the authors concluded that this finding is more likely a result of differences in diet and lifestyle, because of the general extremely low isoflavone intake among study participants. Whereas, data of other epidemiological studies fits the results of animal studies and demonstrates a benevolent effect of soy and isoflavones on bone mineral density in postmenopausal women [439, 474-478]. Chiechi and co-workers [479] observed among postmenopausal women also a reduction of osteoporosis risk after 6 months of increased soy food intake, although the participants group that received hormone replacement therapy was more effective. The positive impact on bone mineral density was indicated by a significant increase in osteocalcin concentrations, a marker of bone formation. Before menopause or during menopausal transition, the effect of isoflavones on bone density is by far more unclear. Some researchers found reduced excretion of bone resorption markers in perimenopausal women after isoflavone extract supplementation [480], while others observed an association between genistein intake and bone mineral density only for premenopausal but not for perimenopausal Asian Americans with high genistein intake through diet [481]. On the contrary, Mei et al. [482] report among postmenopausal women with habitually high intake of dietary isoflavones also higher bone mineral density values at spine and hip region, but found no association in premenopausal women. A longitudinal study in Hong Kong Chinese women aged 30-40 years showed also a significant effect on the maintenance of the spinal bone mineral density [483].

7. Recommendations

In vitro data, *in vivo* experiments, controlled clinical trials and dietary intervention studies clearly indicate that polyphenol-rich diet has a beneficial effect. We have only considered the phytoestrogens and here mainly the class of isoflavones. Other classes of polyphenols the catechins, condensed tannins, lignans and flavonoids also exert a positive effect. Not all of these compounds bind to ER α and ER β, but exhibit nevertheless a variety of other beneficial effects including up or down regulation of certain genes involved in the cell cycle, prevention of cardiovascular diseases via eNOS activation and vasodilation, anticarcinogenic, antioxidant and anti-inflammatory properties as well as positive impact on hormone metabolite pathways and on lipoprotein levels.

The most popular examples for the beneficial effects of polyphenol-rich diet are the Mediterranean, Cretan and the Japanese diet. These diets are based on high consumption of vegetables, fish and vegetable oils. But the beneficial effects are not only gained by the composition of the diet. Moreover, calory restriction, physical activity and sufficient exposition to sun light are contributing the health effects. A polyphenol-rich diet is mostly associated with a healthier lifestyle. This includes not only the intake of more fruits and vegetables but also less red meat consumption. Usually, the calory intake is also lower. Thus, lower calory intake has also been associated with lower risk of breast and colon cancer and coronary heart disease.

Thus polyphenol-rich diet results into lowering blood pressure, prevention of plaque formation, reduction of bone resorption (after menopause), lowering LDL level and increasing HDL level. Consequently a reduction of coronary heart diseases (CHD) has often been observed. A cancer preventive effect has been associated with polyphenol-rich diet. This can be explained by a specific regulation of certain genes involved in cancer initiation and progression. Also an interaction with certain enzymes involved in steroid synthesis has been reported.

Polyphenol-rich food has almost the status of a functional food. Several food companies have started to merchandise polyphenol fortified food and beverages. It can be clearly stated that popularity of such food will also result in lower incidence of some of the lifestyle related diseases such as CHD and the metabolic syndrome. Eventually, fortified food may also impact on reduction of incidence of certain cancers. It is important to keep an eye on the lifestyle and not only the nutrition habits. Balanced calory intake and sufficient physical exercise cannot be replaced by functional food. Moreover this problem arises with food supplementation

Isoflavone-rich food supplements seem to be safe, but the problem of an intake of food supplements as compensation for reduced exercise and higher calory intake has to be transported to the public. There is a certain need for standards of isoflavone-rich food supplements.

The current public debate that they may be unsafe is not really scientifically justified in the light of the overwhelming studies and reports on beneficial effects of isoflavones. Moreover there is a demand for standardized labeling of the effective compound, so that the consumer can select on the basis of quality and can trust in the commercially available products.

Our general recommendation is to follow the guidelines of the various nutritional societies. The food pyramid (see Figure 17) as a guideline for the consumers has been revised toward recommendations for high intake of vegetables, fruits and berries.

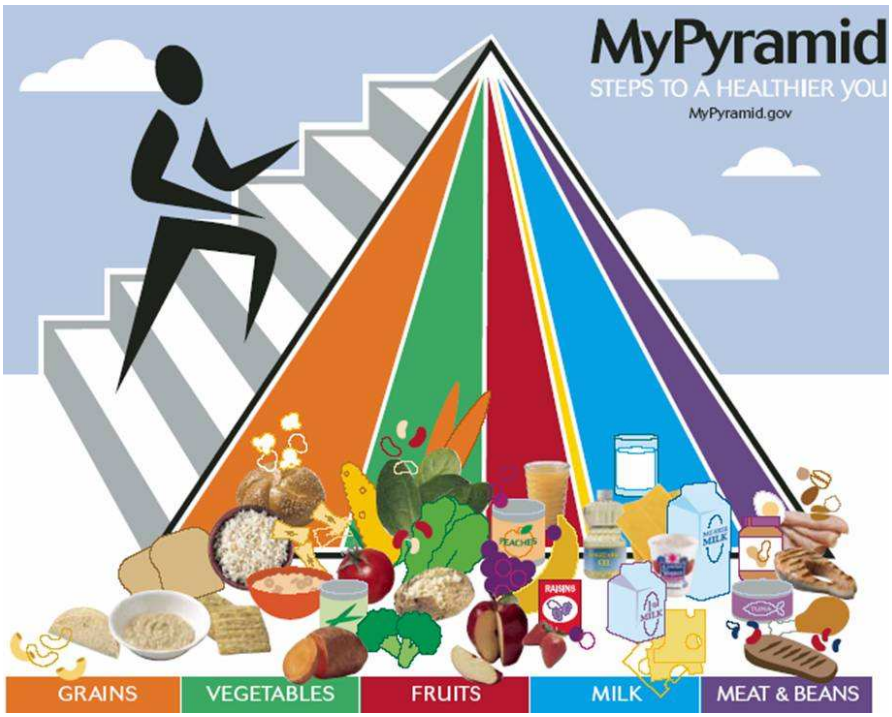

Figure 17: New food pyramid "MyPyramid" created 2005 by the USDA (United States Department of Agriculture) as nutritional guide.

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Publication VII

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Estradiol regulates aryl hydrocarbon receptor expression in the rat uterus

Georg Kretzschmar[1], Anja Papke[1], Oliver Zierau[1], Frank Josef Möller[1], Svjetlana Medjakovic[2,3], Alois Jungbauer[2,3], Günter Vollmer[1]

[1]Molecular Cell Physiology & Endocrinology, Institute for Zoology, Technische Universität Dresden, 01062 Dresden, Germany

[2]Christian Doppler Laboratory for Receptor Biotechnology, Muthgasse 18, 1190 Vienna,

Austria

[3]Department of Biotechnology, University of Natural Resources and Applied Life Sciences

Vienna, Muthgasse 18, 1190 Vienna, Austria

Abstract

Several authors have investigated the role of aryl hydrocarbon receptor (AHR) in the reproductive tract, but there are no data available whether 17β-estradiol (E2) regulates expression of members of the AHR pathway in the uterus. We therefore examined the mRNA expression of *Ahr* as well as the genes of the AHR dimerization partners ARNT1 and ARNT2 and the AHR regulated genes *Cyp1a1* and *Gsta2* in the uterus of ovariectomized rats after administration of E2 at two different doses. The data show that *Ahr* mRNA expression is downregulated while AHR protein amounts increased in all uterine tissue compartments. In addition we observed a downregulation of *Arnt1*, *Arnt2* and *Cyp1a1* while *Gsta2* mRNA expression is upregulated by E2 in a dose dependent manner. These results show that members of the AHR pathway are regulated by E2 in the uterus. AHR may therefore play an important role in the mediation of uterine estrogenic effects.

Keywords

aryl hydrocarbon receptor, ARNT, estradiol, uterotrophic assay, gene expression

1 Introduction

The aryl hydrocarbon receptor plays a key role in the activation of phase I and phase II metabolic pathways after binding of halogenated aromatic hydrocarbones like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and many other xenobiotic and endogenous ligands. This activation leads to the formation of a heterodimer with one of the AHR nuclear translocator proteins (ARNT1 or ARNT2) and the subsequent induction of gene expression of *Cyp1a1*, *Cyp1a2*, glutathione s transferase a1 and a2 (*Gsta1*, *Gsta2*), NAD(P)H menadione oxidoreductase and the genes of other enzymes of the so called AHR battery [Nebert et al., 2000; Lindros et al., 1998].

In addition the AHR is a crucial factor in development. Evidence for this stems from *Ahr* null mice which show a number of developmental defects especially in the vascular system [Lahvis et al., 2000; Lahvis et al., 2005]. Moreover, even in the fully developed animal, AHR seems to play an important role beyond the induction of the metabolization of xenobiotics.

A good example for this is the female reproductive tract. It has been demonstrated that absence of *Ahr* gene expression in AHRKO mice as well as the activation of the AHR pathway by an AHR ligand lead to abnormal phenotypes in all organs of the female reproductive system and impaired reproductive function [Hernández-Ochoa et al., 2009]. AHR has been shown to be involved in the secretory processes and epithelial proliferation of the uterus possibly by the regulation of estrogen receptor (ER) expression or activation [Buchanan et al., 2000; Kitajima et al., 2004]. In the human uterus *Ahr* expression corresponds with menstrual cyclicity peaking at ovulation [Küchenhoff et al., 1999]. In addition AHR seems to play a functional role in the establishment and maintenance of pregnancy as has been shown in the rabbit uterus where the cellular localization of AHR is altered and *Ahr* expression is

upregulated during preimplantation [Hasan and Fischer, 2001] and early gestation [Tscheudschilsuren et al., 1999]. So far the precise mechanism that regulates AhR expression in the uterus is unclear but the described findings suggest that sex steroids may be involved. To elucidate the role of 17β-estradiol (E2) in the regulation of the uterine AHR signalling cascade we measured the mRNA expression of *Ahr*, *Arnt1*, *Arnt2*, *Cyp1a1* and *Gsta2* in the uteri of ovariectomized rats following E2 treatment in tissue samples originating from a classical three day uterotrophic assay. The animals were treated with E2 at two different doses. A third group received the carrier substance castor oil as a negative control. In addition immunohistochemical staining of AHR was performed to demonstrate tissue specific distribution and regulation of a key member of this pathway.

2 Materials and Methods

2.1 Substances

17β-Estradiol (E2) was purchased from Sigma-Aldrich (Hamburg, Germany).

2.2 Animals

Young adult female Wistar Unilever rats (150 g) were obtained from Harlan-Winkelmann (Harlan-Winkelmann, Borchen, Germany) and were maintained under controlled conditions of temperature (20 ± 1℃, relative humidity 50-80 %) and illumination (12-h light, 12-h dark). All animals had free access to standard rodent diet (Harlan 2019 Rodent Breeding, Harlan-Winkelmann, Borchen, Germany) and water. All animal handling and experimental conditions were in accordance with the EC Directive 86/609/EEC for animal experiments and the Institutional Animal care and Use Committee guidelines, regulated by the German federal law for animal welfare.

2.3 Treatment of the animals and uterotrophic assay

Animals were ovariectomized and after 14 days of endogenous hormonal decline they were treated subcutaneously once daily for three consecutive days. Animals were treated with E2 (either 0.5µg/kg body weight (bw)/day (d) or 4 µg/kg bw/d). Treatment groups were composed of six to eight animals. The animals were randomly selected for treatment and vehicle groups. Substances were dissolved in the carrier castor oil, which was also applied to a third treatment group as a negative control. After 72 h of treatment, animals were sacrificed by CO_2-inhalation after light anesthesia by O_2/CO_2-inhalation. The uterine wet weight was determined and the uteri were snap frozen in liquid nitrogen for later RNA preparation.

2.4 Immunohistochemical analysis

For immunohistochemical analysis of AHR expression in the uterus the tissues were fixed and embedded in paraffin and cut into sections of 7 µm. Staining was performed using the NOVADetect HRPO/DAB Standard StreptAB kit (Dianova, Hamburg, Germany) and specific antibodies for AHR ab84833 (Abcam, Cambridge, UK).

Samples from 3 animals treated with the carrier substance DMSO and 3 animals treated with 4µg/kg bw/d E2 chosen at random were analyzed. Staining intensity was quantified using ImageJ (http://rsbweb.nih.gov/ij/) by marking the areas of interest (endometrial epithelium, endometrial stroma and myometrium) and using its histogram function. Results have been normalized against the background next to the slices to adjust for possible exposure differences.

2.5 RNA extraction and quantification

The total cytoplasmic RNA was extracted from the rat uteri by the standard TRIzol® method (Life Technologies) and RNA of all animals in one group was pooled. DNA residues were enzymatically eliminated by digestion (Desoxyribonuclease I, Ambion) and completeness of its removal was checked by PCR. MMLV Reverse Transcriptase (Life Technologies) and Oligo(dT) with a length of 15 nucleotides were used for the first-strand cDNA synthesis. Real time PCR analysis was performed as described elsewhere using cytochrome c oxidase subunit 1 (1A) as an endogenous reference gene and SybrGreen1 to detect PCR products [Kretzschmar et al., 2005]. The primers that have been used are listed in table 1. Results are expressed as amounts of mRNA relative to the expression observed in the vehicle control treated animals using the $2^{-\Delta\Delta CT}$ formula [Winer et al., 1999] after at least three measurements.

2.5 Statistical analysis

Statistical analysis of the data in this work was performed using two way analysis of variance followed by pair wise comparison of means using the Student's t-test. Significance was assumed at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) as compared to the negative control.

3 Results

In a first series of measurements we demonstrated that the uterotrophic assay performed was functional. As can be seen in Fig. 1 E2 treatment led to a dose dependend increase in uterine wet weight of about 600% following treatment with 4µg/kg bw E2. Complement component 3 (*C3*) expression was also strongly and dose dependently increased to about 1300fold following treatment with the higher

E2 dose of 4µg/kg bw. This finding is clearly indicative for a strong uterotrophic response in this experiment.

As a potentially important functional read out we measured uterine *Ahr* mRNA expression. The expression of this gene was decreased by E2 treatment in a dose dependent manner to about 10% of the level in the control animals following treatment with 4µg/kg bw E2. Similarly *Arnt1* and *Arnt2* mRNA expression decreased dose-dependently to 32% and 1% of the baseline expression rate under the same conditions. *Cyp1a1* expression decreased to about 14% following treatment with 0.5µg/kg bw E2 and was barely detectable in the uteri of animals treated with 4µg/kg bw E2.

In contrast, the expression of *Gsta2* was 2.3fold upregulated in the uteri of animals treated with 0.5µg/kg bw E2 and almost 10fold in the animals treated with 4µg/kg bw E2 (Fig. 2).

Uterine protein levels were observed for AHR following DMSO and E2 treatment with 4µg/kg/day using immunohistochemistry. Microscopic observation (Fig. 3) and quantitative evaluation of the immunohistological staining intensity (Fig. 4) revealed that AHR is present in all uterine tissue compartments but it is most strongly expressed in the endometrial epithelium followed by the endometrial stroma and the myometrium. Contrary to our expectations based on the mRNA expression data AHR protein levels seem to increase following E2 treatment. This finding could be confirmed for the endometrial epithelium as well as endometrial stroma by determining the staining intensity of samples from three different animals from each treatment group. The numbers for the myometrium also suggest an increase of AHR, but the observed changes are statistically not significant (Fig. 4).

4 Discussion

In the present study we investigated uterine tissue samples from a three day rat uterotrophic assay towards a potential impact of E2 treatment on the uterine expression of genes of the AHR pathway. We show for the first time that the expression of several members of this signalling pathway is regulated following E2 treatment in the rat uterus.

There has been only one study briefly looking at uterine *Ahr* expression in ovariectomized rats following high dose oral E2 administration of 0.6 mg/kg BW over 5 days. However, no effects were observed [Schlecht et al., 2004]. Unlike this approach we used tissue samples from a rat three day uterotrophic assay where animals received a much lower dose by subcutaneous injections of E2 to test our hypothesis that E2 is one of the key hormones regulating AHR expression in the uterus.

The ovariectomized rats had a strongly reduced uterus wet weight due to the tissue regression following subsequent estrogen ablation. The increase in uterine wet weight following treatment with E2 indicates that E2 treatment was effective. The strong increase in the mRNA amount of *C3*, a gene which is known to be expressed estrogen-dependently [Sundstrom et al., 1989], demonstrates that the ER signalling cascade was functional.

Earlier studies demonstrated that AHR protein expression in the uterus of humans is linked to the menstrual cycle with high expression rates during the proliferative phase and a peak around ovulation [Küchenhoff et al., 1999]. Furthermore high uterine levels of AHR have been observed during preimplantation and early gestation in rabbits [Hasan and Fischer, 2001; Tscheudschilsuren et al., 1999]. These data suggest a positive correlation of AHR protein levels with the level of the hormones involved in these processes and thus we also expected an E2 dependent

upregulation of *Ahr* mRNA expression. Contrary to these expectations we measured a strong downregulation of uterine *Ahr* as well as *Arnt1* and *Arnt2* expression following E2 treatment in ovariectomized rats and hence a negative correlation of *Ahr* expression and E2 levels.

Unlike mRNA expression AHR protein levels are increased in all observed uterine tissue compartmens following treatment with E2 (Fig. 3 and Fig. 4) supporting our hypothesis that E2 is a key player in the changes in uterine AHR levels observed during the human menstrual cycle and rabbit implantation and gestation. AHR may therefore play a crucial role in the mediation of the effects of E2 on estrogen dependent processes in the uterus like endometrial proliferation, implantation of the fetus and maintenance of gestation.

The reason for the inversed and seemingly contradicotry expression pattern of mRNA and protein levels are not clear, but it is well known that correlation between mRNA expression and protein amounts is often weak as has been shown for the mouse liver following treatment with three PPARα and -γ agonists [Tian et al., 2004]. In this study as well as in another one by Yang and coworkers [Yang et al., 2001] it has also been shown that opposite regulation of mRNA and protein levels frequently occurs for example with carboxylesterases which are also part of the AHR battery. Our study therefore supports their conclusion that for the regulation of protein levels regulation of translation and protein stability are equally as important as transcriptional regulation.

The upregulation of AHR should consequently lead to an upregulation of AHR target gene expression if the AHR is activated by an endogenous ligand as has been proposed by a number of authors [Backlund et al., 1997; Rannug et al., 1987, Chiaro et al. 2008]. Our immunohistological images also suggest that AHR might be partially endogenously activated in the uterus in a constitutive manner as the nuclei

of many cells seem to be stained more intensely than the cytoplasm in all observed samples (Fig. 3). Even though mRNA expression data indicate a mild downregulation of the AHR dimerization partner ARNT1 and a very strong downregulation of ARNT2 (Fig. 2) their amounts seem to be sufficient to maintain the activation of AHR. *Gs*ta1 a*nd Gs*ta2 have long been known to be such target genes of AHR as they are upregulated by activation of AHR in the rat liver [Lindros et al., 1998]. Our observation shows that uterine *Gsta2* expression is indeed also upregulated following an E2 induced increase in AHR protein amount.

The AHR dependent upregulation of hepatic *Cyp1a1* has been known even longer [Poland and Knutson, 1982]. It was therefore very surprising to find that in the uterus *Cyp1a1* mRNA expression was downregulated while the amount of AHR was increased following treatment with E2. This indicates that either AHR regulates *Cyp1a1* expression in a tissue specific manner or that E2 directly regulates the expression of this gene by activating one or both of the ERs leading to subsequent binding to a regulatory element of *Cyp1a1* without the involvement of AHR.

On the other hand it has been shown that ERα and AHR share a number of ligands although to our knowledge this has not been demonstrated for E2 itself [Abdelrahim et al., 2006; Medjakovic and Jungbauer, 2008]. Nevertheless this leaves the third possibility that the uterine expression of *Gsta2* or *Cyp1a1* could be mediated by E2 binding and activating AHR. To resolve the involvement of both receptors in the process of the regulation of *Gsta2* as well as *Cyp1a1* expression by E2 experiments involving additional treatment with an ER antagonist like fulvestrant and an AHR antagonist like α-naphtoflavone will be necessary.

The regulation of genes of the AHR pathway like *Cyp1a1* and *Gsta2* indicates that in addition to functional aspects the E2 mediated increase of AHR in the uterus may also have profound consequences on the AHR mediated detoxification. Although

the detoxification and metabolization of foreign substances is mainly performed in the liver the observed downregulation of Cyp1a1 may indicate a decrease in the ability of the uterus to perform monooxygenase reactions of phase I of the drug metabolism. The upregulation of *Gsta2* expression on the other hand is indicative of an increased ability to perform phase II reactions leading to detoxification of these substances. These results also have important implications for the evaluation of toxicity of endocrine disruptors. Since many of these substances are estrogenic they may also change the expression of genes of the AHR battery. Therefore the involvement of locally altered metabolism of the endocrine disruptors themselves and of other substances should be considered when elucidating their uterine effects. In conclusion we could show that the mRNA expression of *Ahr* as well as of its dimerization partners *Arnt1* and *Arnt2* are downregulated while AHR protein levels are increased in the uterus of ovariectomized rats. Uterine mRNA expression of *Cyp1a1* is downregulated by estrogens and *Gsta2* mRNA expression is strongly increased by the same treatment but the precise mechanism could not be elucidated within this experiment. These results support the hypothesis that AHR represents a mediator of E2 dependent processes in the uterus including proliferation, implantation of the fetus and maintenance of gestation.

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Tables

Table 1 Primers used for quantitative real time PCR

Figure captions

Figure 1 Effectiveness of E2 treatment: Dose dependent change in relative uterus wet weight and uterine expression of *complement C3* in ovariectomized rats. Measurements have been performed three times from independently synthesized cDNA of the same pooled RNA samples. Gene expression in the control group has been set to 1. Asterisks indicate values significantly different from the negative control. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 2 Uterine expression of genes of the AHR pathway: E2 dose dependent changes in uterine mRNA expression of *Ahr* and its dimerization partners *Arnt1* and *Arnt2* as well as the AHR regulated genes *Cyp1a1* and G*sta2*. Measurements have been performed three times from independently synthesized cDNA of the same pooled RNA samples. Gene expression in the control group has been set to 1. Asterisks indicate values significantly different from the negative control. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 3 Tissue sections of uterine samples stained with anti-AHR antibodies. **A** uterus of a DMSO treated rat. **B** uterus of a rat treated with E2 [4µg/kg/d]. Abbreviations: E endometrial epithelium, S endometrial stroma, M myometrium

Figure 4 Quantification of the AHR antibody staining intensity of the different uterine tissues in animals treated with the carrier substance DMSO and E2 [4µg/kg/d]. Asterisks indicate values significantly different from the negative control (DMSO). *p < 0.05, **p < 0.01.

Figure 1

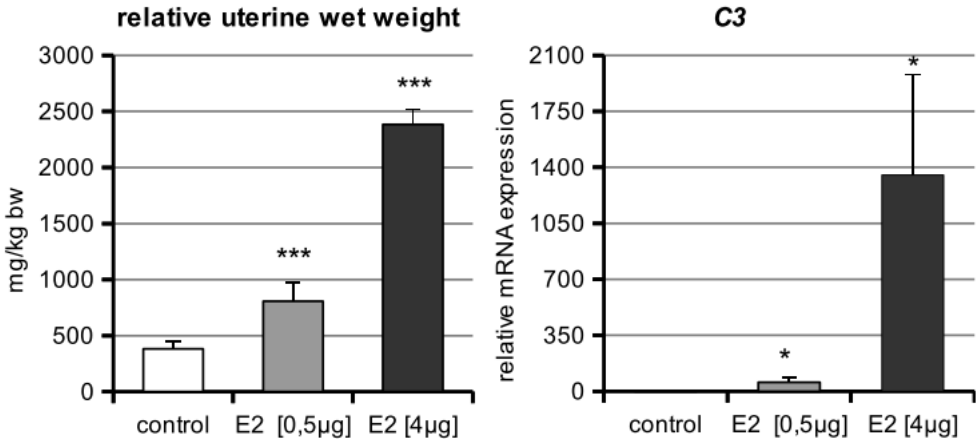


Figure 2

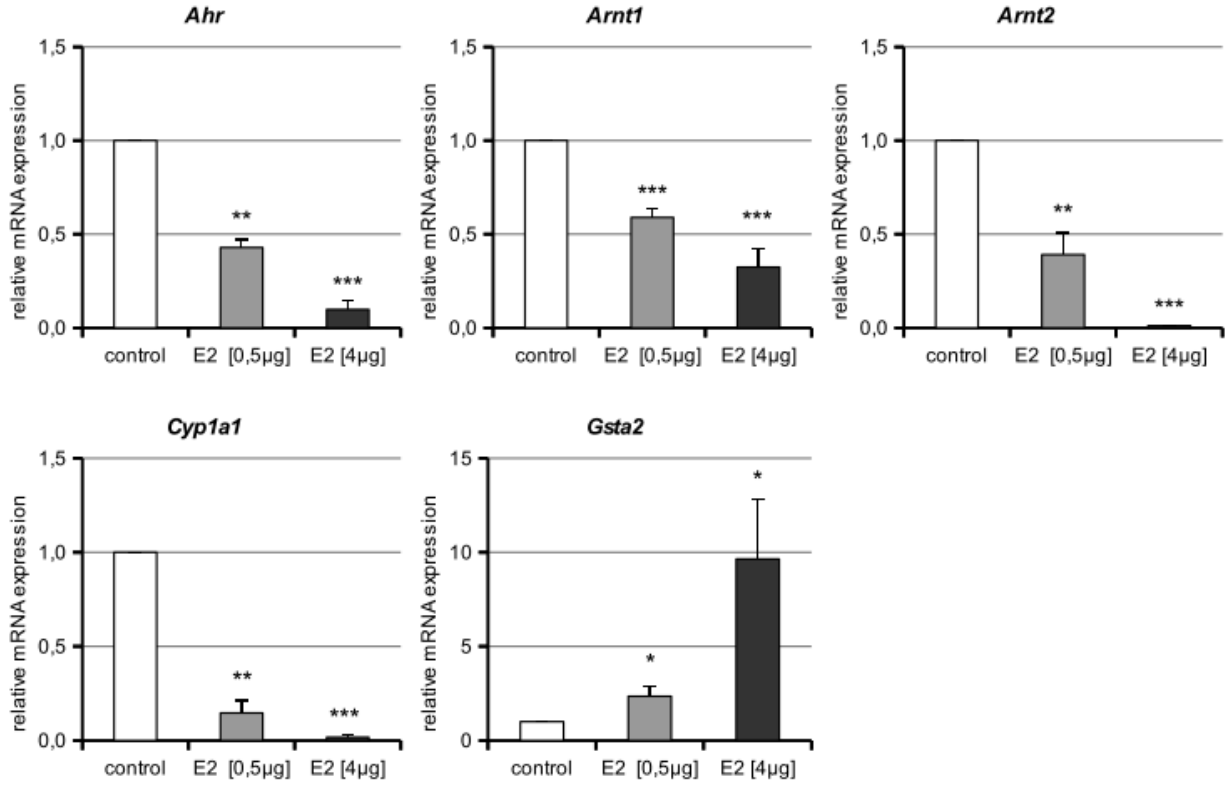


Figure 3

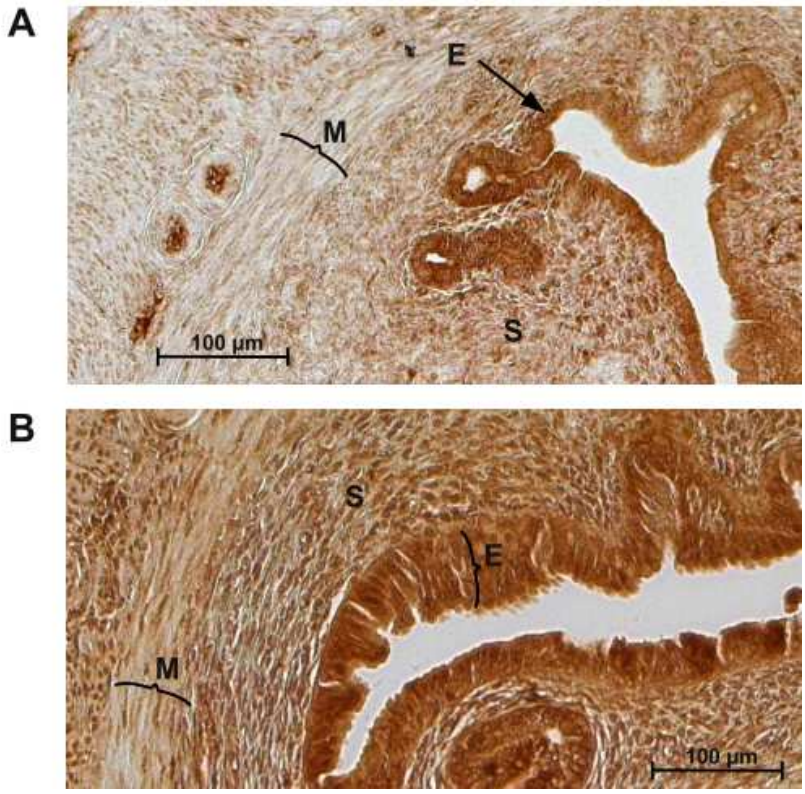
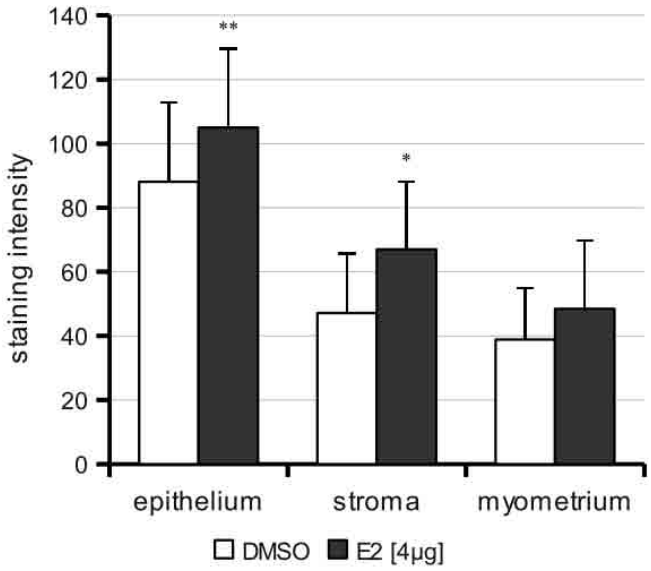


Figure 4



Publication VIII

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Comparison of hormonal activity of isoflavone-containing supplements used to treat menopausal complaints

Evelyne Reiter, MSc,[1,2] Verena Beck, PhD,[1] Svjetlana Medjakovic, MSc,[1,2] Monika Mueller, PhD,[1,2] and Alois Jungbauer, PhD[1,2]

Abstract

Objectives: The isoflavones present in red clover and soy are used as an alternative treatment for menopausal complaints and are commercially available as high-dose food supplements. These preparations contain varying amounts of active ingredients, often without detailed specifications. Thus, it is difficult to derive a recommended daily dose, and the reliability of these products is rather low.

Methods: We quantified the isoflavone content of 19 different isoflavone-containing preparations and compared their binding and transactivational activities with regard to estrogen receptor α, estrogen receptor β, androgen receptor, progesterone receptor, peroxisome–proliferator–activated receptor, and aryl hydrocarbon receptor.

Results: The food supplements that we tested bound to and transactivated both the estrogen receptors and the other receptors. After comparing the isoflavone content quantified by us with the isoflavone content specified on the package labels, we found that at least the specified isoflavone content or more could be detected in only 5 of the 19 food supplements that we tested.

Conclusions: Preparations containing isoflavones should be standardized for the isoflavone aglycone content to facilitate the prediction of theoretical hormonal activity, facilitate the intake of a controlled amount of isoflavones, and ensure greater product reliability.

Key Words: Red clover – Soy – Isoflavones – Binding affinity – Transactivation.

Isoflavone-containing food supplements derived from soy or red clover have become a popular treatment for minor menopausal complaints.[1] A daily dose of 40 to 80 mg of isoflavones has been recommended, and several clinical studies have indicated that the extracts may have a beneficial effect.[2-4] The general outcome of these studies was that a daily dose of at least 80 mg is required to exert statistically significant positive effects. We have previously compared the hormonal activity of different isoflavone-containing food supplements, and others have also quantified the isoflavone content of such preparations.[5,6] Concerns have been raised about the discrepancies between the isoflavone content specified on the package insert and the actual quantified isoflavone content in these preparations. Because the pharmaceutically active isoflavones in these preparations are derived from the natural resources red clover and soy, the isoflavone content is difficult to standardize. In addition, the active compounds consist of a large range of molecules. Thus, the concentration of active ingredients and their relative amounts vary according to climate, cultivar, agricultural factors, and extraction procedure. Furthermore, companies mix extracts from different origins and sources. Red clover and soy are the plants from which isoflavone-containing supplements are most often derived. The relative concentrations of the isoflavones genistein, daidzein, biochanin A, and formononetin differ between these plants. Whereas soy contains mainly genistein, daidzein, and glycitein, red clover contains mainly biochanin A and formononetin, as well as daidzein and genistein (in much smaller amounts).[7] These compounds occur in the plants either in a free form as aglycones or as sugar conjugates, which do not show hormonal activity in in vitro assays.[8-10] However, in vivo, the sugar residue is cleaved off in the gut, and the bioavailability of the remainder is similar to that of the aglycone, although in some experiments, the excretion time compared with that of the aglycone is delayed.[6,11] Once ingested, the isoflavones are metabolized by gut microflora, which can either produce metabolites with similar or higher hormonal activity or eradicate hormonal action entirely.[12] The methylated precursors biochanin A and formononetin are demethylated to genistein and daidzein, respectively. Genistein and daidzein are then degraded to 6-hydroxy-*O*-desmethylangolensin, *O*-desmethylangolensin, or equol. The ability to produce equol is contingent on the presence of specific gut microorganisms and does not always occur in vivo.[9,13-16] Isoflavones bind and transactivate not only estrogen receptors but also other members of the steroid-receptor superfamily, such as androgen receptor (AR),

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From the [1]Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Vienna, Austria; and [2]Christian-Doppler-Laboratory of Receptor Biotechnology, Vienna, Austria.

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Address correspondence to: Alois Jungbauer, PhD, Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria. E-mail: alois.jungbauer@boku.ac.at

progesterone receptor (PR),[17-20] peroxisome proliferator–activated receptors (PPARs),[21-23] and aryl hydrocarbon receptor (AhR),[24] which belongs to the basic helix-loop-helix family of receptors. This broad specificity and receptor-binding promiscuity is often not taken into account in studies, but it may explain some of the other effects of isoflavone intake on postmenopausal women, such as unexpected weight loss that could occur as a result of PPAR activation,[25,26] which is involved in lipid metabolism. Even the antiestrogenic activity that is frequently observed with certain isoflavones could be due to AhR activation.[27,28]

The hormonal activity of isoflavones can be reliably determined by an in vitro assay that mimics the transactivation or binding properties of the ligands with regard to their receptors. Detecting the hormonal activity of plant extracts in vitro comprises some difficulties. Isoflavone glucosides do not bind to or exert any transactivation activity on estrogen receptors; therefore, the in vitro detected activity is only a theoretical value because it may be higher in vivo after hydrolysis of the sugar residue in the gut. Another issue is the daily recommended dose, which ranges, according to the manufacturer's data, from 40 to 150 mg/day.

Recent discussions regarding the safety and efficacy of treatments with isoflavones have highlighted the importance of standardizing plant-derived isoflavone-containing supplements so that consumers can be confident that they are receiving the indicated quantity of pharmaceutically active isoflavones. In the present study, we estimated the binding affinity and transactivational potential of 19 commercially available preparations from central Europe with regard to the receptors estrogen receptor α (ER-α), estrogen receptor β (ER-β), AR, PR, PPARγ, and AhR. We also quantified the isoflavone content using reversed-phase high-pressure liquid chromatography (RP-HPLC) and compared it with the isoflavone content specified on the preparation labels.

METHODS

Chemicals and media

Buffering reagents, dimethyl sulfoxide (DMSO), and *o*-nitrophenyl-β-galactopyranoside were purchased from Fluka (Buchs, Switzerland), Sigma-Aldrich (St. Louis, MO), or Merck (Darmstadt, Germany). Estradiol (E_2), 5α-dihydrotestosterone (DHT), progesterone, glycitein, genistein, biochanin A, formononetin, daidzein, genistin, daidzin, ononin, and sissotrin were obtained from Sigma-Aldrich. Radiochemicals [2,3,6,7,16,17-^{3}H (N)]E_2, [17α-methyl-^{3}H]trienolone, and [1,2,6,7-^{3}H (N)]progesterone were obtained from PerkinElmer (Waltham, MA). Ultima Flo AP scintillation cocktail was purchased from Canberra-Packard (Frankfurt, Germany). Recombinant human ER-α and ER-β and recombinant human PR ligand-binding domain (LBD; thioredoxin fusion protein) were obtained from Invitrogen (Lofer, Austria). For yeast media preparation, yeast nitrogen base was obtained from Difco (Franklin Lakes, NJ), and amino acids were purchased from Serva Feinbiochemica (Heidelberg, Germany) and Sigma-Aldrich. The solvents used were HPLC grade. Rosiglitazone was purchased from Cayman Chemicals (Ann Arbor, MI). The polar screen PPAR competitive assay was obtained from Invitrogen Corporation (Carlsbad, CA). Dulbecco's modified Eagle's medium, used for cell culture, was purchased from Biochrom (Berlin, Germany), fetal calf serum was obtained from HyClone (Logan, UT); SuperFect was purchased from Qiagen (Germantown, MD); and the Dual-Glo luciferase assay system was purchased from Promega (Madison, WI).

Extracts in DMSO

For extracting the preparations with DMSO, the samples were milled in a mortar and, after pulverizing, stirred in a concentration of 0.1 g/mL DMSO for 24 hours. The extracted material was separated by centrifugation at 13,000 rpm for 15 minutes. The clear supernatant was stored at 4°C and used as stock solution. The extract was diluted in semilogarithmic steps down to 1:1,000 and applied to the different test systems.

Pure compounds were solved and diluted in DMSO and applied to the different test systems.

Quantification of isoflavones

Isoflavones, resveratrol, and the glucosides genistin, daidzin, ononin, and sissotrin were quantified using an HPLC 110 system from Agilent (Santa Clara, CA) with a Luna green 100 × 4.6 mm, 3 μm, C 18 column (Phenomenex, Aschaffenburg, Germany).

The binary mobile phase consisted of 5% acetonitrile and 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B) with a flow rate of 0.5 mL/minute. The linear gradient began with 100% solvent A for 5 minutes; then solvent B was increased to 17.5% for 25 minutes, further increased to 50% for 25 minutes, held for 10 minutes, increased to 90% for 5 minutes, and finally returned to starting conditions in 3 minutes and held at starting conditions for 7 minutes. The sample volume was 3 μL. The quantification was performed in duplicates. Quantification of isoflavones could only be performed with extracts showing baseline separation. With Femarelle, this was not the case, so the isoflavone and resveratrol content could not be measured (Fig. 1).

The calibration curves for the isoflavones, resveratrol, and the glucosides were obtained by applying the same methods as for the extracts for different dilutions of the pure compounds. The applied concentration was related to the obtained area under the curve. The measured limits of detection, limits of quantification, and the retention times are shown in Table 1.

Binding assays

Polar screen PPAR competitive assay

The PPAR ligand-binding competitive assay was performed using the PolarScreen PPAR competitor assay green, according to the manufacturer's protocol (Invitrogen Corporation). In brief, the PPARγ LBD and the fluorescent PPARγ ligand (Fluoromone PPAR Green) build a PPARγ-LBD/fluoromone complex that exerts a high polarization value.

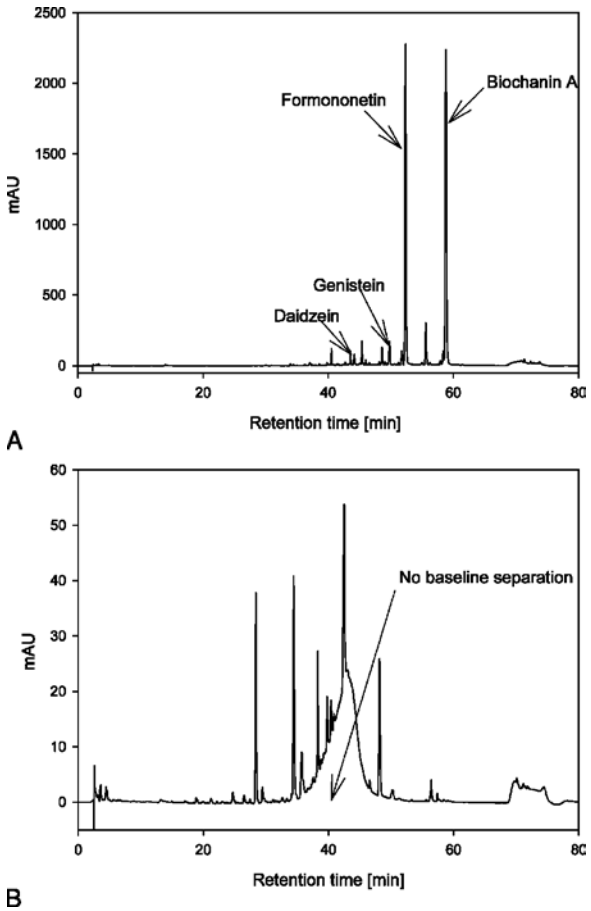

FIG. 1. Reversed-phase high-pressure liquid chromatography of two isoflavone-rich food supplements using a C-18 column (Luna green 100 × 4.6 mm, 3 μm) of Menoflavon (**A**), a highly concentrated red clover–containing food supplement, and Femarelle (**B**), containing a fermented soy preparation. AU, absorbance units.

The PPARγ ligand displaces the fluorescent ligand, which results in a low polarization value. Compounds not binding PPARγ do not displace the fluorescent ligand, which result in a polarization value that remains at a high level. The relative affinity of the test compound to PPARγ is measured as a change in the polarization value. Fluorescence polarization was measured using a GENios Pro plate reader (Tecan, Crailsheim, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The high-affinity PPARγ ligand rosiglitazone was used as a standard, and the maximal polarization value of rosiglitazone was defined as 100%.

Radiolabeled ligand binding assays: ER-α, ER-β, AR, and PR

Competitive radiolabeled ligand-binding assays (LBAs) for ER-α, ER-β, AR, and PR were performed according to Freyberger and Ahr.[29] Whereas ER assays use the full-length recombinant human ERs, AR assays use a rat recombinant fusion protein containing the hinge and LBD of the AR, and PR assays use the LBD of a human recombinant PR.

The receptor was incubated in assay buffer (10 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 10% glycerol, 1.5 mM EDTA, and 3 mg/mL bovine serum albumin for the ER LBA, or 50 mM Tris, pH 7.5, 800 mM NaCl, 2 mM dithiothreitol, 10% glycerol, and 10 mg/mL γ-globulin for the AR and PR LBA) overnight at 4°C in microplates together with the radiolabeled ligand ([^{3}H]17β-estradiol for ER-LBA, [17α-methyl-^{3}H]trienolone for AR-LBA, and [1,2,6,7-^{3}H (N)]progesterone for PR) and the compounds being tested. After incubation, a dextran-coated charcoal suspension was added, the samples were mixed for 10 minutes, and the plates were centrifuged. Aliquots of the clear supernatant were mixed with scintillation cocktail, and radioactivity was measured on a MicroBeta TriLux scintillation counter (PerkinElmer). Measurements were performed in triplicates. Data obtained (counts per minute) for each test compound were normalized with respect to the data obtained for the control (DMSO as sample). For nonspecific binding, an excess of an unlabeled standard substance (E_2, DHT, or progesterone for the assays with ER-α and ER-β, AR, and PR, respectively) was applied. This value was subtracted from the data for each test compound. The data were plotted as percentage of control on the ordinate versus the concentration of the compound being tested on the abscissa. The logistic dose-response curve of the data was fitted with Table Curve 2D software (Jandel Scientific, Erkrath, Germany). IC_{50} values (the concentration at half-maximal binding or transactivation) were determined from the transition center of each curve. All experiments were performed at least in duplicate.

Transactivation assays

Yeast transactivation screens

The yeast strain used for all of the transactivation assays was *Saccharomyces cerevisiae.* For the PR transactivation assay, we used the protease-deficient yeast strain BJ3505. For the ER and AR transactivation assays, the yeast strain 188R1, which is a hyperpermeable derivative of RS188, was used. The system is based on a two-plasmid system. The expression plasmid contains a gene for the human hormone receptor, which is expressed upon the addition of copper. In the presence of a transactivating ligand, the hormone receptor binds a hormone response element, which switches on the *LacZ* gene; β-galactosidase as a reporter is then expressed and

TABLE 1. *Limit of detection, limit of quantification, and retention time of the quantified compounds obtained with reversed-phase high-pressure liquid chromatography with a C-18 column (Luna green 100 × 4.6 mm, 3 μm)*

Compound	Limit of detection, μM	Limit of quantification, μM	Retention time, min
Genistein	625.0	1,625.0	48.6
Daidzein	3.6	18.5	42.8
Biochanin A	107.1	312.1	58.8
Formononetin	12.8	48.4	52.4
Glycitein	230.4	578.7	43.3
Resveratrol	140.5	382.5	42.3
Genistin	2.5	6.5	35.2
Daidzin	6.1	14.8	28.0
Ononin	16.0	35.0	41.0
Sissotrin	2.3	12.6	45.9

can be quantified. The plasmids for the ER-α transactivation assay (yeast estrogen screen) were a gift from C. Richard Lyttle.[30] The original setup was downscaled to microtiter format so that it would consume less time and materials. Briefly, 1 μL of sample was incubated for 5 hours at 30°C with 100 μL of yeast culture, with an optical density at 600 nm (OD_{600}) set to 0.4, and 10 μM $CuSO_4$. Yeast cells were then lysed with *N*-lauroylsarcosine disintegration buffer. After the 5-hour incubation, 150 μL of disintegration buffer was added to each well, and the OD_{600} was measured with a GENios Pro plate reader. The plates were incubated at 30°C for 20 minutes to ensure complete disruption of the cells. Afterward, 50 μL of *o*-nitrophenyl-β-galactopyranoside solution (diluted 1:6 in LacZ buffer) was added to each well, and the microtiter plate was incubated at 37°C until a yellow color developed. Fifty microliters of 1 M Na_2CO_3 solution was used to stop the reaction, and the total incubation time was noted. Absorption was measured at 405 nm, with a reference wavelength of 620 nm.

For the AhR transactivation assay, the recombinant yeast strain YCM3 of *S. cerevisiae* (a gift from Charles A. Miller III, Tulane University, New Orleans, LA), was used. The human AhR gene is integrated into yeast chromosome III, as well as the gene for the AhR dimerization partner aryl hydrocarbon receptor nuclear translocator. Upon ligand addition, an AhR–aryl hydrocarbon receptor nuclear translocator heterodimer is formed and binds to the five xenobiotic response elements of the lacZ reporter plasmid to induce the expression of the *LacZ* gene. Thus, the transcriptional activation of the ligand was quantified via β-galactosidase activity. The assay was performed as described above for ER-α, AR, and PR, with galactose as the carbon source in the media and an overnight incubation time instead of 5 hours. All experiments were performed at least in duplicate.

PPARγ transactivation assay

For PPARγ transactivation, NIH-3T3 cells (German Collection of Microorganisms and Cell Cultures, accession number 59) were seeded at a density of 1×10^4 cells per well. The cells were incubated for 24 hours, giving them time to settle. After that, the cells were cotransfected with 30 ng of pGAL4-hPPARγ-LBD, 300 ng of pFR-Luc reporter plasmid, and 15 ng of pRL-TK using SuperFect transfection reagent (Qiagen), according to the manufacturer's protocol. The transfected cells were incubated with a standard, and the test substance was dissolved in DMSO in Dulbecco's modified Eagle's medium for 24 hours, with a maximal DMSO concentration of 0.1%. The Dual-Glo luciferase assay system (Promega) was used, according to the manufacturer's protocol, to perform the luciferase assays, using *Renilla* and firefly luciferase. Luminescence was measured with the GENios Pro plate reader. The firefly-to-*Renilla* ratio was calculated by dividing the firefly luciferase activity by the *Renilla* luciferase activity, which normalizes for transfection efficiency. The firefly-to-*Renilla* ratio was normalized to the

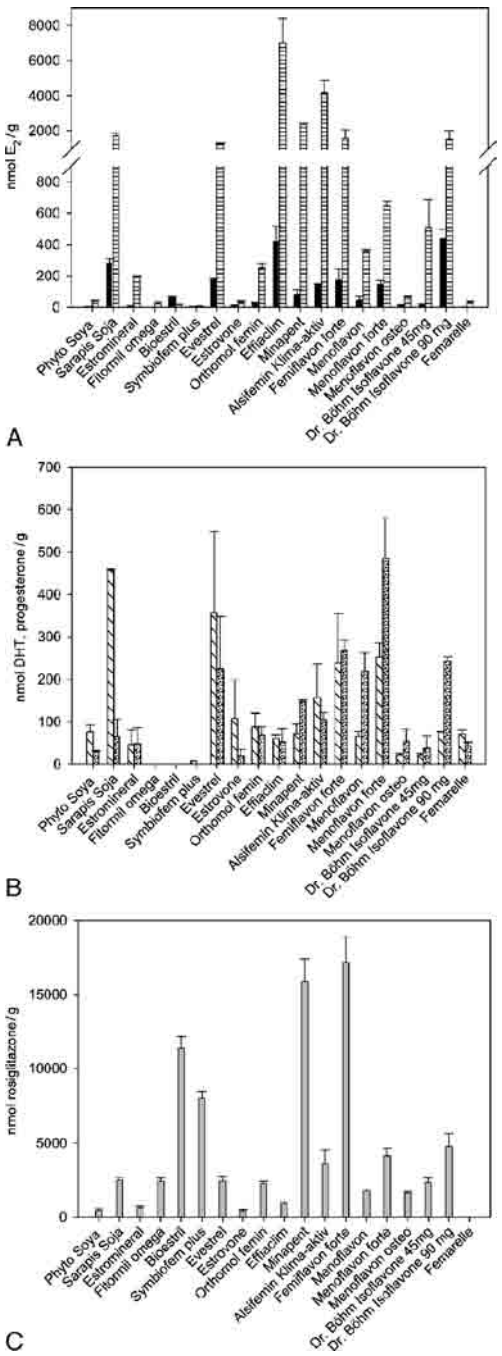

FIG. 2. Ligand-binding assays of 19 isoflavone-rich food supplements using a radiolabeled competitive assay for (**A**) ER-α (human recombinant full-length receptor) and ER-β (human recombinant full-length receptor) with [^{3}H]17β-estradiol; androgen receptor (AR) (**B**) AR (rat recombinant fusion protein containing ligand-binding domain and hinge region) and PR (human recombinant ligand-binding domain) with [17α-methyl-^{3}H]trienolone for AR and [1,2,6,7-3H (N)]progesterone; and for peroxisome proliferator–activated receptor (**C**) PPARγ (human recombinant ligand-binding domain) with fluorescence polarization. Data are given as mean value of equivalent hormone concentration calculated as described in equation 2. E_2, estradiol; DHT, dihydrotestosterone; ER-α, estrogen receptor α; ER-β, estrogen receptor β; AR, androgen receptor; PPARγ, peroxisome-proliferator–activated receptor γ.

DMSO control. Rosiglitazone, a strong PPARγ activator, was used as a positive control, and the efficiency of rosiglitazone was defined as 100%. The pGAL4-hPPARγ-LBD expression plasmid, which expresses the chimeric GAL4 DNA-binding domain fused to the human PPAR LBD, was provided by Bart Staels (Institut Pasteur, University of Lille, France).[31] The luciferase reporter plasmid pFR-Luc, containing a firefly luciferase gene controlled by five repeats of GAL4-binding elements, was purchased from Stratagene (La Jolla, CA). The *Renilla* control plasmid (pRL-tk) consisting of the *Renilla* luciferase gene and the herpes simplex virus thymidine kinase promoter for moderate levels of *Renilla* luciferase expression was obtained from Promega.

Evaluation

For all receptors, the transition center of the four-parameter logistic dose-response curve (equation 1), which equals the concentration of the half-maximal effect, is defined as IC_{50} or EC_{50} values. The logistic dose-response curve of the radiolabeled competitive assay, used for the determination of the binding affinity of the compounds on ER-α, ER-β, AR, and PR, is obtained through plotting the applied concentration on the abscissa against a relative value (% control) on the ordinate. Within the yeast assays, the logistic dose-response curve is obtained through plotting the applied concentration on the abscissa against the obtained Miller units (MUs) over AhR units (AUs) on the ordinate. For PPARγ, the logistic dose-response curve was obtained through plotting the applied concentration of the test compound on the abscissa against the polarization value for binding experiments or the transactivational efficiency for transactivation experiments on the ordinate. IC_{50} or EC_{50} values was determined using Table Curve 2D software.

$$y = a + \frac{b}{1 + (c/x)^d} \quad (1)$$

Parameter a equals the baseline. Parameter b equals the plateau of the curve designated as the ligand efficiency. Parameter c gives the transition center and equals the ligand potency, which stands for the concentration that shows 50% of efficiency. Parameter d stands for the transition width.

Binding assays

For binding experiments, the DMSO extracts of the food supplements were diluted in DMSO and applied to the assays to obtain a logistic dose-response curve and an IC_{50} value.

The equivalent hormone concentration present in the extract was calculated by dividing the IC_{50} value of the standard substance by the IC_{50} value of the plant extracts or isoflavones (equation 2):

$$\text{Equivalent hormone concentration [nmol/g]} = \frac{IC_{50(\text{substance})}[\text{nmol/L}]}{IC_{50(\text{extract})}[\text{g/L}]} \quad (2)$$

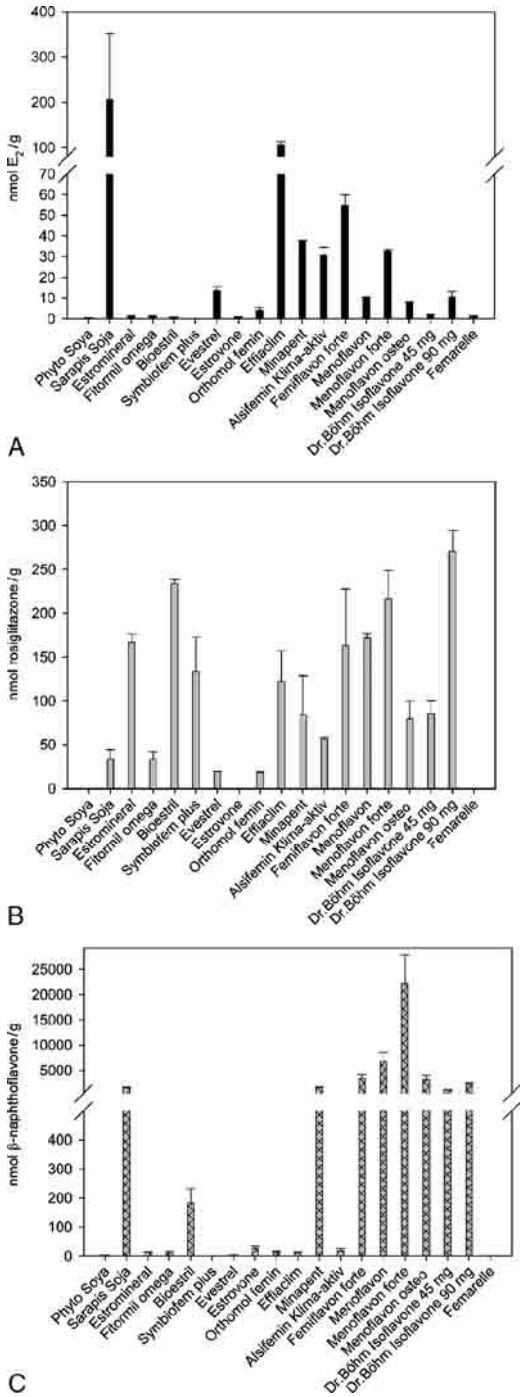

FIG. 3. Transactivation for **(A)** ER-α using a two-plasmid yeast system with a β-galactosidase reporter (yeast strain 188R1) with 17β-estradiol as standard substance; for **(B)** PPARγ using a mouse fibroblast luciferase reporter system, with rosiglitazone as standard substance; and for **(C)** AhR using a yeast system with genome-integrated receptor sequence and β-galactosidase as reporter (yeast strain YCM3), with β-naphthoflavone as standard substance. Data are given as mean value of equivalent hormone concentration, calculated as described in equation 5, including the standard deviations. E_2, estradiol; ER-α, estrogen receptor α; PPARγ, peroxisome-proliferator–activated receptor γ; AhR, aryl hydrocarbon receptor.

The experiments were performed at least in duplicate, and the data were described as mean values of the equivalent hormone concentration including the SDs in Figure 2.

Transactivation assays

The specific enzyme activity of the reporter product β-galactosidase was expressed in MUs for ER-α, AR, and PR or AU for AhR, which take into account yeast growth measured as OD_{600}. MUs or AUs are defined as follows:

$$\text{MU or AU} = \left(\frac{OD_{405}}{OD_{600}}\right) * \left(\frac{1}{\Delta t}\right) * \left(\frac{\text{sample volume protein assay } [\mu L]}{\text{sample volume } \beta - \text{gal assay } [\mu L]}\right) * 1,000 \quad (3)$$

Obtained MUs or AUs were plotted on the ordinate against the concentration of the ligand tested on the abscissa. The data were fitted with Table Curve 2D software using a logistic dose-response function (equation 1).

A standard curve with the reference compound (E_2, DHT, β-naphthoflavone, or progesterone) was performed within each test run, and determinations were carried out at least in duplicate. The transition center of the logistic dose-response curve gives the EC_{50} value. The four parameters of the logistic dose-response curve obtained were used to calculate the equivalent concentration of standard substance (mol/L) in the applied sample as follows:

$$\text{mol/L} = \left[\left\{\frac{b}{(\text{MU or AU} - a)} - 1\right\}^{\left(\frac{1}{d}\right)}\right] * c \quad (4)$$

The equivalent hormone concentration corresponds to the theoretical concentration of the hormone in the extract producing the same transactivational activity (E_2 for ER-α and ER-β, DHT for AR, progesterone for PR, rosiglitazone for PPARγ, and β-naphthoflavone for AhR). The value obtained in equation 4 is now used to calculate the equivalent concentration of standard substance in the plant extract (equation 5).

$$\text{Equivalent hormone concentration [nmol/g]} = \frac{(\text{mol/L} * 1,000)}{\text{g/mol}_{(\text{extract})}} * 10^9 \quad (5)$$

For PPARγ, the four parameters of the logistic dose-response curve obtained are applied in equations 4 and 5 as described above for the yeast assays.

The experiments were performed at least in duplicate, and the data are presented as mean values of the equivalent hormone concentration including the standard deviations in Figure 3.

RESULTS

Quantification of isoflavones

The isoflavone content of 19 different preparations was quantified using RP-HPLC. The amounts of genistein, daidzein, biochanin A, formononetin, glycitein, the stilbene resveratrol, and the glucosides genistin, daidzin, sissotrin, and ononin are shown in Table 2. The isoflavone content of Femarelle could not be detected. The chromatogram of the extract did not show a baseline separation, which is necessary for isoflavone quantification. Figure 1 shows the chromatogram of Menoflavon with baseline separation and Femarelle without baseline separation.

The highest specific genistein content was detected in Effiaclim (41.3 mg/g), followed by Dr Böhm Isoflavon forte (11.0 mg/g) and Minapent (10.4 mg/g). The highest specific daidzein content was detected in Dr Böhm Isoflavon forte (26.1 mg/g), followed by Sarapis soja (23.3 mg/g) and

TABLE 2. *Amount of the quantified compounds present in the tested food supplements*

Extract	Genistein	Daidzein	Biochanin A	Formononetin	Resveratrol	Glycitein	Genistin	Daidzin	Ononin	Sissotrin
Phyto soya	<LOQ	0.9	<LOQ	<LOQ	<LOQ	<LOQ	5.1	0.4	0.2	<LOQ
Sarapis soja	7.5	23.3	15.0	14.7	<LOQ	<LOQ	<LOQ	0.2	0.8	3.6
Estromineral	<LOQ	1.9	<LOQ	<LOQ	<LOQ	1.5	27.2	0.6	0.3	<LOQ
Fitormil omega	<LOQ	1.7	<LOQ	<LOQ	<LOQ	<LOQ	35.3	1.0	0.3	<LOQ
Biocstril	<LOQ	4.0	4.1	14.3	<LOQ	<LOQ	32.6	0.3	<LOQ	72.9
Symbiofem plus	<LOQ	0.1	<LOQ	<LOQ	<LOQ	<LOQ	0.5	0.1	<LOQ	<LOQ
Menoflavon forte	4.3	0.7	105.0	93.5	<LOQ	<LOQ	<LOQ	<LOQ	0.4	1.7
Evestrel	<LOQ	0.2	<LOQ	<LOQ	<LOQ	<LOQ	29.2	0.4	0.2	<LOQ
Estrovone	<LOQ	1.0	<LOQ	<LOQ	<LOQ	<LOQ	17.3	1.3	0.1	<LOQ
Orthomol femin	<LOQ	1.6	<LOQ	<LOQ	<LOQ	<LOQ	25.0	0.3	0.5	0.1
Effiaclim	41.3	11.7	<LOQ	<LOQ	<LOQ	<LOQ	24.3	0.2	0.2	0.1
Minapent	10.4	18.3	10.4	24.7	<LOQ	2.6	0.5	0.7	0.2	0.1
Alsifemin klima-aktiv	9.2	17.9	<LOQ	<LOQ	<LOQ	<LOQ	9.9	0.6	0.2	0.5
Femiflavon forte	9.0	19.2	23.3	36.2	<LOQ	<LOQ	2.3	1.2	0.8	2.8
Menoflavon	<LOQ	0.4	46.2	41.0	<LOQ	<LOQ	<LOQ	<LOQ	0.2	0.6
Menoflavon osteo	<LOQ	0.2	21.4	20.3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.6
Dr Böhm Isoflavon	<LOQ	4.4	1.6	6.4	<LOQ	<LOQ	0.8	0.1	0.1	<LOQ
Dr Böhm Isoflavon forte	11.0	26.1	6.6	46.9	<LOQ	<LOQ	8.1	0.2	0.4	0.4
Femarelle	nq	nq	nq	nq	nq	nq	nq	nq	nq	nq

Values are in milligrams per gram of product. Nq, not quantifiable (the extract's chromatogram shows no baseline separation); <LOQ, the detected isoflavone content is below the limit of quantification.

Femiflavon forte (19.2 mg/g). The highest specific biochanin A content was detected in Menoflavon forte (105.0 mg/g), followed by Menoflavon (46.2 mg/g) and Menoflavon osteo (21.4 mg/g). The highest specific formononetin content was detected in Menoflavon forte (93.5 mg/g), followed by Dr Böhm Isoflavon forte (46.9 mg/g) and Menoflavon (41.0 mg/g). Resveratrol was detected only in Femiflavon forte, but the detected amount was below the limit of quantification. Glycitein was detected in Minapent (2.6 mg/g) and Estromineral (1.5 mg/g), although in very small amounts compared with genistein, daidzein, formononetin, and biochanin A.

The highest specific genistin content was detected in Fitormil omega (35.3 mg/g), Bioestril (32.6 mg/g), and Evestrel (29.2 mg/g). The highest specific daidzin content was detected in Estrovone (1.3 mg/g), followed by Femiflavon forte (1.2 mg/g) and Fitormil omega (1.0 mg/g). The highest specific ononin content was detected in Sarapis soja (0.8 mg/g), Femiflavon forte (0.8 mg/g), and Orthomol femin (0.5), whereas the highest specific sissotrin content was detected in Bioestril (72.9 mg/g), followed by, although in much smaller amounts, Sarapis soja (3.6 mg/g) and Femiflavon forte (2.8 mg/g).

The measured isoflavone content of one recommended daily intake of each food supplement was calculated and compared with the daily serving information described on the package insert (Table 3). The difference between the calculated and measured isoflavone intake ranged from 5.7% to 186.9%. Only five food supplements contained approximately at least the specified amount of isoflavones or more as written on the package insert. These were Menoflavon

TABLE 3. *Comparison of the quantified content of isoflavones, glucosides, and resveratrol with the specified content of isoflavones and resveratrol*

Extract	Ingredients (active compounds)	Recommended daily intake	Measured isoflavone content, mg isoflavones/ packing unit	Daily isoflavone intake, mg isoflavones	% of theoretical content
Phyto soya	35 mg isoflavones per capsule	2 capsules/d (70 mg isoflavones)	2.0	4.0	5.7
Sarapis soja	37.5 mg soy extract and 25 mg red clover extract per capsule	2 capsules/d (125 mg extract)	26.0	52.0	—[a]
Estromineral	60 mg soy isoflavones per tablet	1 tablet/d (60 mg isoflavones)	28.4	28.4	47.3
Fitormil omega	50 mg soy isoflavones per dragée	1 dragée/d (50 mg isoflavones)	19.4	19.4	38.8
Bioestril	72 mg isoflavones; 60 mg derived from soy per tablet	1 tablet/d (72 mg isoflavones)	134.6	134.6	186.9
Symbiofem plus	40 mg soy isoflavones per package	1 double-chamber bag/d (40 mg isoflavones)	8.4	8.4	10.5
Menoflavon forte	80 mg red clover isoflavones per capsule	1 capsule/d (80 mg isoflavones)	82.2	82.2	102.8
Evestrel	Soy extract with 37.5 mg isoflavones per tablet	1 tablet/d (37.5 mg isoflavones)	18.0	18.0	48.0
Estrovone	50 mg soy extract per tablet—standardized to 40% isoflavones	1-3 tablets/d (20-60 mg isoflavones)	9.9	10-30	50
Orthomol femin	50 mg soy isoflavones, 10 mg lignans from flaxseed per dragée	2 dragées/d (100 mg isoflavones)	12.4	24.8	24.8
Effiaclim	80 mg soy isoflavones per tablet	1 tablet/d (80 mg isoflavones)	70.0	70.0	87.5
Minapent	300 mg red clover extract per capsule	1-2 capsules/d (300-600 mg extract)	27.2	27.2-54.4	—[a]
Alsifemin klima-aktiv	125 mg soy extract with 50 mg soy isoflavones per dragée	3 dragées/d (150 mg isoflavones)	11.5	34.5	23.0
Femiflavon forte	50 mg holy thistle extract standardized to 20% silybinin, 50 mg grape extract standardized to 0.1% resveratrol, algae, 4 mg carotinoids, 40 mg red clover isoflavones per tablet	1 tablet/d (10 mg silybinin, 0.05 mg resveratrol, 40 mg isoflavones)	52.1	52.1	130.3
Menoflavon	40 mg red clover isoflavones per capsule	1 capsule/d (40 mg isoflavones)	35.4	35.4	88.5
Menoflavon osteo	50 mg red clover isoflavones, 350 mg calcium, 30 mg magnesium, 2.5 μg vitamin D_3, 0.5 mg copper, 2.5 mg zinc per tablet	1 tablet/d (50 mg isoflavones)	51.0	51.0	102.0
Dr Böhm Isoflavon	20 mg red clover isoflavones, 25 mg soy isoflavones, 90 mg evening primrose oil per tablet	1 tablet/d (45 mg isoflavones)	13.4	13.4	29.8
Dr Böhm Isoflavone forte	40 mg red clover isoflavones, 50 mg soy isoflavones, 90 mg evening primrose oil per tablet	1 tablet/d (90 mg isoflavones)	99.7	99.7	110.8
Femarelle	322 mg of fermented soy extract, 108 mg of flaxseed per capsule	1-2 capsules/d	nq	—[b]	—[b]

nq, not quantifiable (the chromatogram shows no baseline separation).
[a] The specification contains details about the extract content only; no calculation of the % of theoretical isoflavone content possible.
[b] No calculation possible because of not quantifiable isoflavone content.

TABLE 4. *Binding potencies (IC_{50}) of pure compounds*

Substance	ER-α	ER-β	AR	PR	PPARγ
Genistein	$2.2 \times 10^{-7} \pm 2.7$	$6.2 \times 10^{-8} \pm 0.9$	nd	nd	$3.0 \times 10^{-5} \pm 0.3$
Daidzein	$1.4 \times 10^{-7} \pm 2.2$	$1.6 \times 10^{-7} \pm 2.1$	nd	nd	Active
Biochanin A	$1.4 \times 10^{-5} \pm 0.5$	$1.7 \times 10^{-6} \pm 0.8$	$1.0 \times 10^{-5} \pm 0.2$	$1.9 \times 10^{-5} \pm 0.3$	$2.6 \times 10^{-5} \pm 0.4$
Formononetin	$4.6 \times 10^{-6} \pm 2.2$	$1.0 \times 10^{-5} \pm 0.1$	nd	nd	nd
Glycitein	nd	$4.6 \times 10^{-5} \pm 1.4$	nd	nd	nd
Resveratrol	nd	$3.5 \times 10^{-5} \pm 0.2$	$3.2 \times 10^{-5} \pm 1.6$	$1.8 \times 10^{-5} \pm 0.1$	$9.3 \times 10^{-5} \pm 1.9$

Values are in moles per liter. IC_{50}, half-maximal inhibitory concentration; ER-α, estrogen receptor α ER-β, estrogen receptor β AR, androgen receptor; PR, progesterone receptor; PPARγ; peroxisome proliferator–activated receptor γ; nd, not detectable; Active, detectable binding at highest applied concentration (no determination of an IC_{50} value possible).

forte (102.8%), Menoflavon osteo (102.0%), Dr Böhm Isoflavon forte (110.8%), Femiflavon forte (130.3%), and Bioestril (186.9%). The food supplements containing less than 25% of the specified isoflavone content were Phyto soya (5.7%), Symbiofem plus (10.5%), Alsifemin klima-aktiv (23.0%), and Orthomol femin (24.8%).

Binding data

Binding experiments were performed using radiolabeled competitive assays for ER-α, ER-β, AR, and PR. For PPARγ, we used a competitive assay that uses fluorescence polarization. A summary of the binding data is shown in Figure 2. For ER-α, the highest binding activity was detected in Dr Böhm Isoflavon forte (439.5 nmol E_2/g), followed by Effiaclim (421.0 nmol E_2/g) and Sarapis soja (281.5 nmol E_2/g). For Fitormil omega, Phyto soya, Femarelle, and Symbiofem plus, almost no binding activity was detected, which can be explained by the results of the isoflavone quantification, which showed that these products contained very low amounts of isoflavones. For ER-β, the highest binding activity was detected in Effiaclim (7,000.0 nmol E_2/g), followed by Alsifemin klima-aktiv (4,194.0 nmol E_2/g) and Minapent (2,359.0 nmol E_2/g). The lowest binding activity was detected in Symbiofem plus (7.5 nmol E_2/g), Bioestril (15.0 nmol E_2/g), and Fitormil omega (22.5 nmol E_2/g). The difference between the binding data of ER-α and ER-β can be explained by the different IC_{50} values of the pure compounds for these receptors (Table 4). The detected IC_{50} values for the isoflavones were 1 order of magnitude lower for ER-β than for ER-α. For AR, the highest binding activity was detected with Sarapis soja (458.0 nmol DHT/g) and Evestrel (356.5 nmol DHT/g). For PR, the highest binding activity was detected with Menoflavon forte (485.0 nmol progesterone/g) and Femiflavon forte (267.0 nmol progesterone/g). For PPARγ, the highest binding affinities were detected for Femiflavon forte (17,145.7 nmol rosiglitazone/g), followed by Minapent (15,880.9 nmol rosiglitazone/g) and Bioestril (11,367.9 nmol rosiglitazone/g), whereas for Estrovone (373.9 nmol rosiglitazone/g) and Phyto soya (438.2 nmol rosiglitazone/g), rather low equivalent rosiglitazone concentrations were detected. For Femarelle, binding could not be detected at all.

Transactivation data

Transactivation experiments were performed using yeast and β-galactosidase as a reporter for ER-α, AR, PR, and AhR. Transactivation data for PPARγ were obtained using animal cells and luciferase as a reporter. A summary of the transactivation data is shown in Figure 3. For ER-α, the highest transactivation values were obtained for Sarapis soja (205.8 nmol E_2/g), followed by Effiaclim (105.1 nmol E_2/g) and Femiflavon forte (54.6 nmol E_2/g). For Symbiofem plus (not detectable), Phyto soya (0.5 nmol E_2/g), and Bioestril (0.9 nmol E_2/g), no or very low transactivation potentials were detected. We did not detect transactivation of AR and PR within any of the extracts.

For PPARγ, the highest transactivation was detected with Dr Böhm Isoflavon forte (270.8 nmol rosiglitazone/g), followed by Bioestril (233.7 nmol rosiglitazone/g) and Menoflavon forte (216.4 nmol rosiglitazone/g). For Phyto soya, Estrovone, and Femarelle, no transactivation was detected.

For AhR, the highest transactivation values were detected with Menoflavon forte (22,216.4 nmol β-naphthoflavone/g), Menoflavon (6,801.0 nmol β-naphthoflavone/g), and Femiflavon forte (3,560.0 nmol β-naphthoflavone/g). We did not detect any transactivation with Symbiofem plus or Femarelle. Extracts with low biochanin A and formononetin content caused either no or very low transactivation, which is a result of the low EC_{50} value of biochanin A and formononetin for AhR (see Table 5 for EC_{50} values of pure compounds).

TABLE 5. *Transactivation potencies (EC_{50}) of pure compounds*

Substance	ER-α	AR	PR	PPARγ	AhR
Genistein	$2.6 \times 10^{-7} \pm 0.7$	nd	nd	$1.9 \times 10^{-5} \pm 0.3$	nd
Daidzein	$1.9 \times 10^{-5} \pm 1.1$	nd	nd	$7.7 \times 10^{-5} \pm 1.5$	nd
Biochanin A	$4.8 \times 10^{-7} \pm 0.7$	nd	nd	$1.9 \times 10^{-5} \pm 0.3$	1.3×10^{-7}
Formononetin	$1.1 \times 10^{-6} \pm 0.3$	nd	nd	nd	2.5×10^{-7}
Glycitein	nd	nd	nd	nd	Active
Resveratrol	nd	nd	nd	Active	nd

Values are in moles per liter. EC_{50}, half-maximal effective concentration; ER-α, estrogen receptor α AR, androgen receptor; PR, progesterone receptor; PPARγ; peroxisome-proliferator–activated receptor γ; AhR, aryl hydrocarbon receptor; nd, not detectable; Active, detectable transactivation at highest applied concentration (no determination of an IC_{50} value possible).

Comparison of theoretical and detected activities

The theoretical and measured binding activities of the tested preparations were compared, which can give evidence of whether there are other active compounds present in the preparations or whether the measured activities are only due to the quantified substances. The theoretical binding affinity was calculated using the IC_{50} or EC_{50} values of the pure compounds, which were related to the quantified amount of isoflavones and resveratrol in the preparations. Glucosides were not included into the calculation because they do not exert any detectable activity in vitro.

Table 6 shows that there are indeed preparations with a significant higher measured activity than theoretical activity. These preparations are Bioestril (134%) only for ER-α, and for Symbiofem plus and Estrovone, the isoflavone content is low, so that the difference between theoretical and measured binding activity might not be significant. For ER-β, the preparations potentially containing other active substances are Sarapis soja (172%), Evestrel (137%), Orthomol femin (129%), Effiaclim (137%), and Alsifemin klima-aktiv (218%). For AR, PR, and PPARγ, nearly all preparations show a higher measured than theoretical binding activity.

Table 7 shows that the transactivation assays do not detect 100% of the theoretical activity, which might be explained through the interference and interaction of the compounds in the extract, which is a highly complex mixture. However, there is again evidence that some preparations contain substances that do transactivate the receptors additionally (Phyto soya, Sarapis soja, Estromineral, Fitormil, Estrovone, Orthomol femin, and Alsifemin klima-aktiv for ER-α; Estromineral, Fitormil omega, and Symbiofem plus for PPARγ).

DISCUSSION

All of the food supplements that we analyzed are commercially available in central Europe. They are advertised as easing menopausal complaints through their isoflavone content. The recommended daily isoflavone intake according to the package insert ranged from 40 to 150 mg of isoflavones. In addition to red clover or soy isoflavones, some of the food supplements also contained lignans from flaxseed, holy thistle extract, grape extract, or evening primrose oil. These extracts contain substances that exert hormonal activity and could contribute to the detected effects.[32] After quantification of genistein, daidzein, biochanin A, formononetin, glycitein, genistin, daidzin, ononin, and sissotrin in the extracts using RP-HPLC, the differences between the quantified isoflavone content and the isoflavone content written on the package insert allowed us to divide the food supplements into two groups. One group was well standardized and contained the specified isoflavone content or even more. In the second group, the extracts contained less than 25% of the labeled isoflavone content. Although it is not an isoflavone, we also quantified the stilbene resveratrol, which was present in one preparation along with red clover and holy thistle extract, but the detected amount was below

TABLE 6. *Comparison of theoretical and measured binding activities of the tested preparations*

	ER-α			ER-β			AR			PR			PPARγ		
	Theoretical binding	Measured binding	%	Theoretical binding	Measured binding	%	Theoretical binding	Measured binding	%	Theoretical binding	Measured binding	%	Theoretical binding	Measured binding	%
Phyto soya	5	3	56	49	41	84	—[a]	76	—	—[a]	30	—	—[a]	438	—
Sarapis soja	379	282	74	2,110	1,684	80	15	458	2,980	44	66	148	370	2,479	670
Estromineral	16	12	74	114	197	172	—[a]	47	—	—[a]	49	—	2	607	26,390
Fitormil omega	10	nd	—	88	23	26	—[a]	nd	—	—[a]	nd	—	—[a]	2,389	2,235,149
Bioestril	45	61	134	274	15	5.5	2	nd	—	5	nd	—	31	11,368	36,727
Symbiofem plus	1	4	518	6	8	123	—[a]	7	—	—[a]	nd	—	—[a]	7,982	—
Evestrel	206	176	85	940	1,286	137	—[a]	357	—	—[a]	225	—	81	2,417	2,982
Estrovone	9	14	154	63	30	47	—[a]	108	—	—[a]	21	—	1	374	25,843
Orthomol femin	39	27	69	195	252	129	—[a]	89	—	—[a]	69	—	13	2,251	16,771
Effiaclim	1,238	421	34	5,123	7,000	137	—[a]	61	—	—[a]	53	—	533	893	168
Mnapent	448	81	18	2,225	2,359	106	11	72	678	31	147	480	322	15,881	4,937
Alsifemin klima-aktiv	362	148	41	1,925	4,194	218	—[a]	158	—	—[a]	106	—	118	3,607	3,058
Femiflavon forte	427	172	40	2,068	1,567	76	24	238	985	70	268	385	544	17,146	3,154
Menoflavon	160	47	29	503	357	71	46	66	144	131	219	167	837	1,748	209
Menoflavon forte	322	148	46	971	644	67	108	253	234	312	485	156	1,967	4,119	209
Menoflavon osteo	64	12	18	191	65	34	22	23	102	63	54	85	398	1,613	406
Dr Böhm Isoflavon	97	13	13	492	509	104	2	23	1,306	5	38	782	58	2,334	4,061
Dr Böhm Isoflavon forte	523	440	84	2,706	1,530	57	6	76	1,209	18	243	1,337	244	4,756	1,952
Femarelle	—[a]	3	—	—[a]	32	—	—[a]	71	—	—[a]	43	—	—[a]	nd	—

The theoretical binding activity was calculated using the IC_{50} values of the pure compounds, which were related to the quantified amount of isoflavones and resveratrole in the preparations. Glucosides were not included into the calculation because they do not exert any detectable activity in vitro. Values are in equivalent nanomoles of reference substance per gram of sample and rounded to integer numbers. IC_{50}, half-maximal inhibitory concentration; ER-α, estrogen receptor α ER-β, estrogen receptor β AR, androgen receptor; PR, progesterone receptor; PPARγ; peroxisome proliferator–activated receptor γ; nd, no detectable activity.

[a] No significant theoretical in vitro activity because of the low aglycone content or the low activity of the compounds present in the preparations on the respective receptor.

TABLE 7. *Comparison of theoretical and measured transactivational activities of the tested preparations*

	ER-α			PPARγ			AhR		
	Theoretical transactivation	Measured transactivation	%	Theoretical transactivation	Measured transactivation	%	Theoretical transactivation	Measured transactivation	%
Phyto soya	—[a]	1	1,046	10	nd	—	—[a]	2	—
Sarapis soja	63	206	327	1,176	34	3	6,866	1,589	23
Estromineral	1	1	201	26	167	639	—[a]	13	—
Fitormil omega	—[a]	1	1,252	18	34	183	—[a]	11	—
Bioestril	14	1	7	119	234	197	2,451	183	8
Symbiofcm plus	—[a]	nd	—	1	134	10,527	—[a]	nd	—
Evestrel	18	14	75	278	19	7	12	5	42
Estrovonc	—[a]	1	246	15	nd	—	—[a]	27	—
Orthomol femin	3	4	138	54	18	33	—[a]	15	—
Effiaclim	118	105	89	1,605	123	8	—[a]	11	—
Minapent	71	37	53	1,023	84	8	7,147	1,639	23
Alsifemin klima-aktiv	27	31	114	520	56	11	—[a]	19	—
Femiflavon forte	100	55	54	1,528	163	11	12,812	3,560	28
Menoflavon	124	10	8	2,027	172	9	19,942	6,801	34
Menoflavon forte	287	33	11	4,751	216	5	46,745	22,216	48
Menoflavon osteo	59	8	14	961	80	8	9,697	3,270	34
Dr Böhm Isoflavon	15	2	15	195	85	44	1,508	1,196	79
Dr Böhm Isoflavon forte	81	11	13	917	271	30	9,258	2,386	26
Femarelle	—[a]	1	—	—[a]	nd	—	—[a]	nd	—

The theoretical transactivational activity was calculated using the EC_{50} values of the pure compounds, which were related to the quantified amount of isoflavones and resveratrol in the preparations. Glucosides were not included into the calculation because they do not exert any detectable activity in vitro. Values are in equivalent nanomoles of reference substance per gram of sample and rounded to integer numbers. Androgen receptor and progesterone receptor do not show any detectable transactivation and are therefore not included in this table. IC_{50}, half-maximal inhibitory concentration; ER-α, estrogen receptor α PPARγ; peroxisome-proliferator–activated receptor γ; AhR, aryl hydrocarbon receptor; nd, no detectable activity.

[a] No significant theoretical in vitro activity, because of the low aglycone content or the low activity of the compounds present in the preparations on the respective receptor.

the limit of quantification. We quantified also the glucosides genistin, daidzin, ononin, and sissotrin, although they do not contribute to the results detected in the in vitro assays but are metabolized to active aglycones in vivo. To standardize the isoflavone content of food supplements for the treatment of menopausal complaints, standardization for the aglycone content should be taken into account because in in vitro assays, only these are detected and a theoretical hormonal activity can be predicted.

The binding experiments on ER-α, ER-β, AR, PR, and PPARγ clearly revealed the receptor promiscuity of isoflavones. Those receptors thought to be mostly involved in menopausal complaints, ER-α and ER-β, showed detectable binding activity with all of the extracts, although the binding experiments of ER-β showed a difference in equivalent hormone concentration of 1 order of magnitude higher. It was previously shown that isoflavones have higher binding and transactivation activities with ER-β than with ER-α[5,33-35]

TABLE 8. *Equivalent daily dose of rosiglitazone in 1 g of product*

	Transactivation assay		Binding assay	
	Equivalent concentration, nmol rosiglitazonc/g	% of daily rosiglitazonc dosc[a]	Equivalent concentration, nmol rosiglitazonc/g	% of daily rosiglitazonc dosc[a]
Phyto soya	0.0	0.0	438.2	3.9
Sarapis soja	34.0	0.3	2,479.0	22.2
Estromineral	166.7	1.5	606.9	5.4
Fitormil omega	33.7	0.3	2,388.8	21.4
Bioestril	233.7	2.1	11,367.9	101.6
Symbiofem plus	133.8	1.2	7,982.3	71.3
Evestrel	18.8	0.2	2,416.5	21.6
Estrovone	0.0	0.0	373.9	3.3
Orthomol femin	17.9	0.2	2,250.5	20.1
Effiaclim	122.5	1.1	893.2	8.0
Minapent	84.0	0.8	15,880.9	141.9
Alsifemin klima-aktiv	56.4	0.5	3,607.0	32.2
Femiflavon forte	163.4	1.5	17,145.7	153.2
Menoflavon	172.4	1.5	1,747.6	15.6
Menoflavon forte	216.4	1.9	4,119.2	36.8
Menoflavon osteo	79.9	0.7	1,613.3	14.4
Dr Böhm Isoflavon	85.2	0.8	2,333.5	20.9
Dr Böhm Isoflavon forte	270.8	2.4	4,755.6	42.5
Femarelle	0.0	0.0	0.0	0.0

[a] Calculated with a daily rosiglitazone dose of 4 mg.

It has been postulated that isoflavones have beneficial effects in treatment of mild menopausal complaints eventually due to a high affinity for ER-β, although the effects and mechanisms are still debated. Recent results with T47D and T47D-ER-β cells clearly show that proliferation of breast cancer cells is dependent on the ER-α/ER-β ratio. ER-β seems to suppress ER-α-induced cancer cell proliferation. Therefore, selective estrogen receptor modulators (like isoflavones), which have a higher affinity to ER-β, may have a beneficial effect on breast cancer development, shifting the ER-α/ER-β ratio to the ER-β side.[36] This might represent an important beneficial effect for postmenopausal women.

We also detected binding to AR and PR, which is to be expected, because pure isoflavones also bind to these receptors (Table 4). For PPARγ, we detected high binding values.

Transactivation experiments showed that the food supplements caused relatively low transactivation of ER-α. This can be explained by the EC_{50} values of the pure compounds (Table 5). We could not detect transactivation of AR or PR, suggesting that isoflavones may have an antagonistic effect on these receptors, because, as mentioned previously, we did detect binding of isoflavones to AR and PR. Partial antagonistic activity on ER-α is also possible because the transactivation values are substantially lower than would be expected from the binding results. The same conclusion can be made for PPARγ. Rosiglitazone is used to treat type 2 diabetes.[37,38] The average intake during treatment is 4 mg/day. The quantity of rosiglitazone in food supplements is compared with the average daily rosiglitazone dose (Table 8). With regard to transactivation, the food supplements maximally exerted 2% of the hormonal action of pure rosiglitazone, although our binding experiments showed that some of the food supplements contained more than the equivalent daily dose of 4 mg rosiglitazone per g (Table 8). This may be an indication for isoflavones or other compounds present in the food supplements acting as PPARγ antagonists. The high transactivation values of AhR can be explained with biochanin A and formononetin being strong AhR transactivators (Table 5). The preparations exerting high transactivational potential on AhR contain a lot of biochanin A and formononetin.

Tables 6 and 7 show that isoflavone-rich preparations do contain compounds with significant additional hormonal effects, especially for PPARγ and ER-β binding, and in a lower amount for AR and PR, as well as for ER-α and PPARγ transactivation. These results indicate that isoflavone-rich food supplements are highly complex mixtures and do not only contain estrogenic compounds. They can also contain substances affecting other hormone receptors, such as AhR and PPARγ. They are drug targets for activation of the detoxification system and for altering lipid metabolism, respectively.

CONCLUSIONS

We conclude that for the prediction of a theoretical activity using in vitro assays to control isoflavone intake and for higher product reliability, food supplements containing isoflavones should be standardized for the aglycone content. Furthermore, the binding to and the transactivation of different receptors by red clover and soy extracts should not be ignored. This promiscuous receptor activity could explain the additional effects observed when isoflavones are used to treat menopausal complaints, perhaps by altering lipid metabolism or by antiestrogenic actions. With respect to standardization, mixtures of undefined extracts and fortifications should be avoided to improve the reliability of the preparations.

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Effect of mycorrhization on the isoflavone content and the phytoestrogen activity of red clover

Thanasan Khaosaad[a], Liselotte Krenn[b], Svjetlana Medjakovic[c,e], Alexander Ranner[b], Andreas Lössl[d], Monika Nell[a], Alois Jungbauer[c,e], Horst Vierheilig[a,*]

[a]*Department für Angewandte Pflanzenwissenschaften, und Pflanzenbiotechnologie, Institut für Pflanzenschutz, Universität für Bodenkultur Wien, Peter-Jordan-Street 82, A-1190 Wien, Austria*
[b]*Department of Pharmacognosy, University of Vienna, Althanstraße 14, 1090 Vienna, Austria*
[c]*Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria*
[d]*Institut für Pflanzenbau und Pflanzenzüchtung, Universität für Bodenkultur Wien; Gregor Mendel-Straße 33, A-1180 Wien, Austria*
[e]*Christian Doppler Laboratory of Receptor Biotechnology, Muthgasse 18, 1190 Vienna, Austria*

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Summary

Red clover, known for its estrogenic activity due to its isoflavones content (biochanin A, genistein, daidzein and formononetin), was inoculated with the arbuscular mycorrhizal fungus *Glomus mosseae*. Once the symbiotic fungus was well established, plants were harvested and we determined the root and shoot dry weight as well as the P-content. In roots and leaves the levels of biochanin A, genistein, daidzein and formononetin were quantified by reversed-phase HPLC and the estrogenic activity of the leaves was measured by a transactivation assay using a yeast two-plasmid system.

Mycorrhization increased the levels of biochanin A in the root and the shoot and reduced the levels of genistein in the shoot of red clover. The levels of the other isoflavones were not affected. The shoot biomass of mycorrhizal plants more than doubled compared with non-mycorrhizal control plants, and this growth-stimulating effect of arbuscular mycorrhiza did not affect the estrogenic activity of red clover. In a control P treatment, the biomass of red clover was greatly enhanced. However, the estrogenic activity was reduced.

*Corresponding author. Tel.: +43 1 47654 3352; fax: +43 1 47654 3359.
E-mail address: nonhorst@boku.ac.at (H. Vierheilig).

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These results suggest that, in contrast to an enhanced shoot biomass production after P application with a reduced estrogenic activity, with arbuscular mycorrhiza the shoot biomass of red clover can be enhanced without a negative effect on estrogenic activity.

Introduction

Plant extracts containing polyphenolic compounds with estrogenic activity, the so-called phytoestrogens, have become increasingly popular for treatment of menopausal disorders (Beck et al., 2005). One source of phytoestrogens is red clover (*Trifolium pratense*) (Saloniemi et al., 1995). In red clover, the isoflavones biochanin A, genistein, daidzein and formononetin, which exert estrogenic activity in mammals (Miksicek, 1993; Breithofer et al., 1998), are present in high concentrations (Setchell et al., 2001). Thus, red clover has become popular as a food supplement for the amelioration of menopausal disorders (Dornstauder et al., 2001; Beck et al., 2003). This positive effect on menopausal disorders has been confirmed by several clinical trials (Hidalgo et al., 2005; Imhof et al., 2006).

Legumes such as red clover are hosts for rhizobia. Signals released by rhizobia alter the pattern of flavonoid (including isoflavone) accumulation in legumes (Phillips and Tsai, 1992), including red clover (Edwards et al., 1997) roots. Moreover, rhizobial nodulation of red clover not only affects isoflavonoid accumulation in the root but also systemically reduces the levels of certain isoflavones in the leaves (Edwards et al., 1997).

Red clover is a host not only for rhizobia, but also for symbiotic arbuscular mycorrhizal fungi (AMF). Arbuscular mycorrhiza (AM) is a symbiosis between root colonizing soil-borne fungi and most land plants. Root colonization by AMF results in a positive effect on plant growth, primarily through an improved nutritional status of the host plant by transferring phosphate (P) through the AM hyphae from the soil to the plant (Smith and Read, 1997). The formation of the AM association is a result of a complex exchange of signals between the plant and the AMF. Abundant data show an effect of flavonoids with phytoestrogenic activity on spore germination, hyphal growth and root colonization by AMF (reviewed by Morandi, 1996; Vierheilig et al., 1998a). Moreover, AMF not only respond to flavonoids with a known estrogenic activity, but also to estrogens and antiestrogens (Poulin et al., 1997). During the establishment of AM symbiosis, the levels of isoflavones with estrogenic activity (e.g. of biochanin A, formononetin, genistein or daidzein) are altered in roots of mycorrhizal plants (Vierheilig et al., 1998a), such as alfalfa (*Medicago sativa*) (Volpin et al., 1994; Larose et al., 2002; Catford et al., 2006), *Medicago truncatula* (Harrison and Dixon, 1993) and soybean (*Glycine max*) (Morandi, 1996). Little is currently known, however, about systemic alterations of isoflavone content in the shoot of mycorrhizal plants.

The aim of the present work was to examine whether mycorrhization can alter the isoflavone pattern in the root and shoot of red clover, and whether these alterations can be linked to phytoestrogen activity. To exclude a simple P-mediated effect through mycorrhization, we also tested the effect of P application on the isoflavone accumulation and the phytoestrogen activity.

Materials and methods

Plant material and growth conditions

Seeds of red clover *T. pratense* L. were surface sterilized in 50% commercial bleach for 5 min, rinsed several times in sterile distilled water and germinated in autoclaved (20 min; 121 °C) perlite. After 2 weeks plantlets were transferred to 300 mL pots (1 plant/pot) into a mixture (autoclaved 20 min 121 °C; 1:1:1; by vol.) of sand:expanded clay:soil. Plants were planted into a hole in the substrate, where the inoculum (5 g per plant) had been previously added. The inoculum consisted of colonized root pieces of bean (*Phaseolus vulgaris* L. cv. Sun Gold), sporocarps, spores and hyphae of *Glomus mosseae* (Vierheilig et al., 1993) (BEG 12; La Banque Européenne des Glomales; International Institute of Biotechnology; Kent; GB; http://www.kent.ac.uk/bio/beg/).

Plants were randomly grown in the greenhouse (day/night cycle: 16h; 22 °C/8h; 19 °C; rel. humidity 50–70%; light intensity 400 $\mu E/s\,m^2$ by Radium HRI-T4W/DH lamps) for 16 weeks (8 plants per treatment) and watered with a nutrient solution with or without P. The nutrient solution without P consisted of $Ca(NO_3)_2$ 0.472 g/L, K_2SO_4 0.256 g/L; $MgSO_4$ 0.136 g/L; MoO_3 0.07 g; NH_4NO_3 8 mg/L; $Fe_6H_5O_7 \cdot 3\,H_2O$ 50 mg/L; $Na_2Bo_4O_7 \cdot 4\,H_2O$ 1.3 mg/L; $MnSO_4 \cdot 4\,H_2O$ 1.5 mg/L; $ZnSO_4 \cdot 7 \cdot H_2O$ 0.6 mg/L; $CuSO_4 \cdot 5\,H_2O$ 0.54 mg/L; $Al_2(So_4)_3$ 0.028 mg/L; $NiSO_4 \cdot 7\,H_2O$

0.028 mg/L; $Co(NO_3)_2 \cdot 6 \cdot H_2O$ 0.028 mg/L; TiO_2 0.028 mg/L; $LiCl_2$ 0.014 mg/L; $SnCl_2$ 0.014 mg/L; KJ 0.014 mg/L; KBr 0.014 mg/L. KH_2PO_4 (0.136 g/L) was also added to the nutrient solution with P.

The 'no M, no P' and the 'plus M, no P' treatment received the nutrient solution without P, whereas the 'no M, plus P' treatment received the nutrient solution with P. At the end of the experiment roots were carefully washed with tap water and root and shoot tissue samples were freeze dried. The root and leaf biomass (dry weight (DW)) was determined.

Eight plants per treatment were tested. The experiment was repeated three times.

P-content

The P content in freeze-dried leaves was determined with the ammonium–vanadat–molybdat method (Gericke and Kurmies, 1952).

Determination of root colonization

To visualize the AMF colonization, fresh roots were cleared by boiling 4 min in 10% KOH, rinsed three times with tap water and stained by boiling for 4 min in a 5% ink (Shaeffer; jet-black)/household vinegar (= 5% acetic acid) solution (Vierheilig et al., 1998b, 2005). After staining, the percentage of root colonization was determined according to the method of Newman (1966).

Extraction of samples for isoflavone analysis and determination of hormonal activity

Freeze-dried leaves were pulverized in a mortar. The powder was suspended in DMSO (0.1 g/mL DMSO) and stirred at 450 U/min for 23 h. The suspension was centrifuged at 13,200 rpm for 15 min. The supernatant was collected for further analysis and stored until use at 4 °C.

HPLC—analysis of isoflavones

Hundred milligrams of pulverized leaves or roots were mazerated for 24 h in 2 mL water for enzymatic cleavage of the glycosides. Four milliliters of methanol was then added. The mixture was refluxed for 1 h, filtered and the filtrate evaporated. The root extracts were dissolved in 800 μL DMSO and 40 μL of standard (5 mg 6-methoxyflavanon/mL) were added to 500 μL of the solution. The leaf extracts were dissolved in 1.0 mL DMSO. Forty microliters of standard (10 mg 6-methoxyflavanon/mL) was added to 500 μL of these solutions. Ten microliters of each solution was analyzed by HPLC according to Krenn et al. (2002) for the quantification of daidzein, genistein, formononetin and biochanin A.

Yeast cultivation

Media was sterilized at 121 °C and 1 bar overpressure for 15 min and stored at 4 °C. The GOLD medium without tryptophan and without uracil (Gold–Trp–Ura) was used for overnight culture, yeast stock and induction.

Yeast estrogen screen (YES)

The yeast estrogen screen is a bioassay based on yeast and is an established test system for detecting estrogenic activity. It is a two-plasmid system: the expression plasmid YEpE12 consists of a copper-inducible human estrogen receptor α gene, an ampicillin resistance gene and a tryptophan-auxotrophy marker, whereas the reporter plasmid carries the reporter lacZ, a uracil-auxotrophy marker, two copies of the estrogen response element (ERE) and the iso-1-cytochrome *c* promoter. Both plasmids are transformed into the *Saccharomyces cerevisiae* strain 188R1.

Upon induction with copper and activation of the estrogen receptor (ER) by an estrogenic active ligand, β-galactosidase is expressed. The amount of β-galactosidase is correlated to the estrogenic activity of the unknown compound and can be quantified by means of a logistic dose–response curve generated with the reference substance 17-β-estradiol, which is tested within each test run. The evaluation has been described previously by Jungbauer and Beck (2002).

For the assay, an overnight culture was prepared and diluted with medium to an OD_{600} of 0.4. Five microliters of 10 mM $CuSO_4$, 45 μL of DMSO and 5 μL of test substance were added to 5 mL of the diluted yeast culture. For the blank value, only 50 μL DMSO and 5 μL $CuSO_4$ were added to the yeast culture. A calibration curve with 17-β-estradiol was performed within each assay.

After an incubation of 4 h at 30 °C, the cell suspension was centrifuged at 2500 rpm for 5 min. The pellet was washed with 1 mL lac-Z buffer (60 mM $Na_2HPO_4 \cdot 2\ H_2O$, 40 mM $NaH_2PO_4 \cdot 2 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7 \cdot H_2O$ and 1 mM DTT, adjusted to pH 7.0 and filtrated (0.22 μm filter)) and after centrifugation at 10,000 rpm for 5 min the pellet was resuspended in 100 μL of lac-Z buffer. The cells were disintegrated mechanically with glass beads (vortexing three times for 30 s within 15 s of rest on ice between the vortexes). The disintegrated cells were centrifuged at 10,000 rpm for 10 min and the clear supernatant was used for the β-galactosidase and protein assays.

β-galactosidase assay

For the β-galactosidase assay, 5 μL of each test tube were transferred to a 96-well microtiter plate. 250 μL of an ONPG solution (*O*-nitrophenyl-β-galactopyranoside; 0.77 g dissolved in 1 L Lac-Z-buffer) were added to each well. The microtiter plate was incubated for 15 min at 37 °C until a yellow color had developed. The reaction was stopped by adding 100 μL of 1 M Na_2CO_3. The absorption was measured at 405 nm with a reference wavelength at 620 nm. Each determination was performed in duplicate.

Protein assay

The protein content of each test tube was measured according to Bradford (1976) with a modified method. Five microliters of clear supernatant were transferred to a 96-well microtiter plate. Diluted Bio-Rad protein assay reagent (250 μL) (1:10 dilution with water) was added. The absorption was measured at a wavelength of 570 nm with a reference wavelength of 690 nm. As reference substance BSA was used in each assay. Each determination was performed in duplicate.

Statistical analysis

Analysis of variance was performed after a variance check by Levene's test. Mean values were compared using Fisher's least significant difference. These analyses were performed using Statgraphics Plus 5.0 software.

Results

In inoculated plants (plus M, no P) a high level of root AM was reached (nearly 80%). Non-inoculated plants showed no signs of root colonization (Table 1). In plants supplied with P (no M, plus P) and in mycorrhizal plants (plus M, no P), a clear increase of the shoot DW and enhanced P levels in the leaves were observed (Table 1). The shoot DW and the P levels in the P treatment were always higher compared to the mycorrhizal treatment (plus M, no P). The root DW was enhanced only in the P treatment (no M, plus P).

Mycorrhization (plus M, no P) did not affect the levels of daidzein and formononetin in the root and the shoot compared to the control treatment (no M, no P). However, in mycorrhizal plants, biochanin A levels were increased in the root and the shoot and genistein levels were decreased in the shoot (Figures 1 and 2). Application of P (no M, plus P) never exhibited an effect on the isoflavone content in red clover plants, with the exception of daidzein, which was reduced in roots compared to levels in mycorrhizal plants, and genistein, which was reduced in the shoot compared to levels in mycorrhizal and control plants (Figures 1 and 2). The total isoflavone content in the root was not affected in any of the treatments. However, in the shoot the total isoflavone content was increased in the mycorrhizal (plus M, no P) and the P treatment (no M, plus P) (Figure 3).

The estrogenic activity of the plant material was similar in the mycorrhizal (plus M, no P) and the

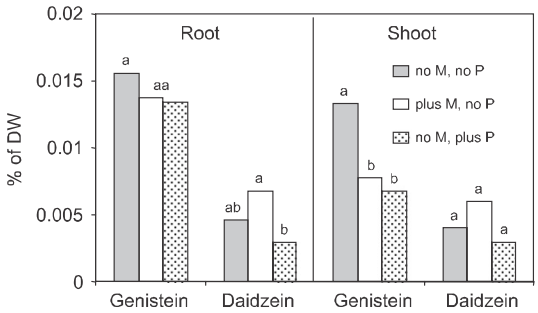

Figure 1. Genistein and daidzein content in roots and leaves of mycorrhizal (plus M) and non-mycorrhizal (no M) red clover plants supplied (plus P) or not supplied (no P) with a P solution. Within each group (e.g. root/genistein) columns with the same letter are not significantly different according to Fisher's LSD test ($P<0.05$).

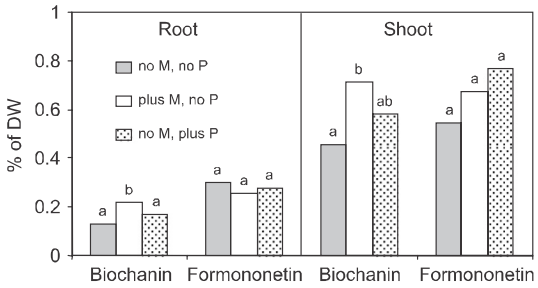

Figure 2. Biochanin A and formononetin content in roots and leaves of mycorrhizal (plus M) and non-mycorrhizal (no M) red clover plants supplied (plus P) or not supplied (no P) with a P solution. Within each group (e.g. root/biochanin A) columns with the same letter are not significantly different according to Fisher's LSD test ($P<0.05$).

Table 1. Percentage AM root colonization, root and leaf biomass (dry weight (DW)) and P status of mycorrhizal (plus M, no P) and non-mycorrhizal (no M, no P) red clover plants and of non-mycorrhizal red clover plants supplied with a P solution (no M, plus P)

	% Root colonization	Root DW (g)	Shoot DW (g)	mg P/g DW shoot	mg P/shoot
No M, no P	0a	1.76a	0.67a	1.16a	0.58a
Plus M, no P	78b	2.19a	1.51b	1.36b	2.05b
No M, plus P	0a	4.02b	3.06c	3.23c	9.88c

Within each column values with the same letter are not significantly different.

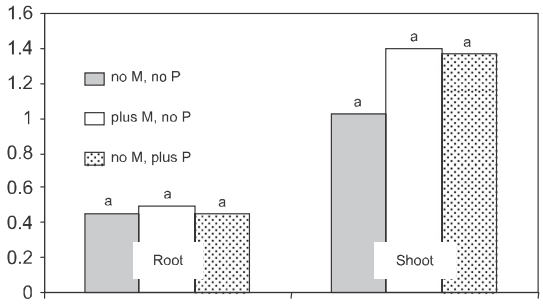

Figure 3. Total isoflavone (genistein, daidzein, Biochanin A and formononetin) content in roots and leaves of mycorrhizal (plus M) and non-mycorrhizal (no M) red clover plants supplied (plus P) or not supplied (no P) with a P solution. Within each group (roots or shoots) columns with the same letter are not significantly different according to Fisher's LSD test ($P<0.05$).

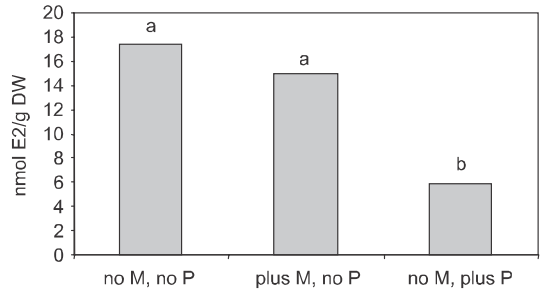

Figure 4. Estrogenic activity of leaves of mycorrhizal (plus M) and non-mycorrhizal (no M) red clover plants supplied (plus P) or not supplied (no P) with a P solution. Columns with the same letter are not significantly different according to Fisher's LSD test ($P<0.05$).

control (no M, no P) treatment but was highly reduced in the P-treated plants (no M, plus P) (Figure 4).

Discussion

In general, root colonization by AMF results in a positive effect on plant growth, mainly through an improved P status of the host plant (Smith and Read, 1997). Recently, Stürmer (2004) reported an increased shoot DW of red clover when inoculated with different AMF; he did not, however, test *G. mosseae*. In our experiment with *G. mosseae*, we were able to confirm this growth-stimulating effect of mycorrhization on red clover in examination of the shoot DW, although the root DW was not affected. Moreover, similar to the report of Stürmer (2004), in our experiment mycorrhization increased the P content.

It has been previously reported that isoflavone levels can be altered in roots of legumes when colonized by AMF (Harrison and Dixon, 1993; Volpin et al., 1994; Morandi, 1996; Larose et al., 2002; Catford et al., 2006). In our experiment the level of biochanin A was enhanced in roots of mycorrhizal red clover, but levels of genistein, daidzein and formononetin were not.

Mycorrhization can affect isoflavone levels in roots not only locally but also systemically, meaning that isoflavone levels are altered not only in mycorrhizal roots but also in non-mycorrhizal roots of mycorrhizal plants (Catford et al., 2006). A systemic effect of mycorrhization on flavonoids has been reported not only for the root system, but also for the shoot. In the shoot of white clover (*Trifolium repens*), the flavonoids levels were increased when roots were colonized by AMF (Ponce et al., 2004). In the shoot of red clover, we also found alterations of the flavonoid pattern when plants were mycorrhizal. The level of biochanin A was increased not only in the root but also in the shoot of red clover. Our data confirm that systemic alterations of the flavonoid pattern are a general pattern in mycorrhizal plants.

The main active compounds in red clover plant extracts exerting estrogenic activity are isoflavones such as biochanin A, genistein, daidzein and formononetin (Miksicek, 1993; Breithofer et al., 1998). We found that the total levels of these isoflavones were not affected in the different treatments, indicating the reduced estrogenic activity of the P treatment was not due to the total isoflavone content. Genistein is formed from biochanin A (Knight and Eden, 1996); thus, the enhanced levels of biochanin A in the mycorrhizal red clover plants compared to non-mycorrhizal plants could result in enhanced estrogenic activity. However, the estrogenic activity of mycorrhizal and non-mycorrhizal control plants was similar. This could be due to the relatively small increase of biochanin A in the mycorrhizal plant, which in its effect is possibly compensated by the decrease of genistein or a decrease of other compounds with estrogenic activity such as flavonols and coumestans. We did not determine these (Hänsel et al., 1994, Breithofer et al., 1998) by the HPLC analysis, but they should be detected by the bioactivity assay, if present.

There are some data available that a high P status can reduce the levels of flavonoids in roots. In soybean, P application resulted in reduced coumestrol levels (Morandi and Gianinazzi-Pearson, 1986) and in reduced daidzein levels in bean

(Malusa et al., 2006). To exclude a simple P-mediated effect through mycorrhization, we also tested the effect of P application on the isoflavone accumulation and the phytoestrogen activity. In contrast to other studies (Morandi and Gianinazzi-Pearson, 1986; Akiyama et al., 2002; Malusa et al., 2006), we did not find a reduction of flavonoid levels in the roots of P-treated plants, but genistein levels were reduced in the shoot.

The estrogenic activity in P-treated plants was highly reduced compared to the control and the mycorrhizal red clover plants. Application of P to red clover plants showed no effect on the shoot levels of daidzein, biochanin A and formononetin. However, genistein was reduced to a similar level as in the mycorrhizal plants. This reduction could be an explanation for the reduced estrogenic activity of P-treated plants. Moreover, other compounds with estrogenic activity such as flavonols and coumestans are possibly reduced in P-treated plants, thus explaining the lower activity.

In summary, we found that mycorrhization does alter the flavonoid pattern in the root and shoot of red clover. Moreover, the shoot biomass of mycorrhizal plants was doubled compared to non-mycorrhizal control plants without compromising estrogenic activity. P application resulted in drastic increase of biomass; the estrogenic activity, however, was highly reduced.

Acknowledgments

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Publication X

Red Wine Technology: Impact of Toasting of Oak Wood on the Activation of the Arylhydrocarbon Receptor

ALFRED ZOECHLING[1,2], FALK LIEBNER[3], MARTINA OPIETNIK[1,3], SVJETLANA MEDJAKOVIC[1,2] and ALOIS JUNGBAUER[1,2,*]

[1]Christian Doppler Laboratory for Receptor Biotechnology, Muthgasse 18, A-1190 Vienna, Austria
[2]University of Natural Resources and Applied Life Sciences, Department of Biotechnology, Muthgasse 18, A-1190 Vienna, Austria
[3]University of Natural Resources and Applied Life Sciences, Department of Chemistry, Muthgasse 18, A-1190 Vienna, Austria

*Corresponding author: Alois Jungbauer, Department of Biotechnology, University of Applied Life Science and Natural Resources Vienna, Muthgasse 18, 1190 Vienna, Phone: +43 1 36006 6226, Fax: +43 1 3697615, Email: alois.jungbauer@boku.ac.at

ABSTRACT: Oak wood is used for maturation of red wines and is consequently responsible for the formation of the typical flavor compounds. Beside the high content of red wine polyphenols the oak derived compounds are a sign for high quality red wine. Due to the traditional procedure of oak barrel making by banding the staves over a fire, there are some possible health risks concerning the formation of toxic polycyclic aromatic hydrocarbons (PAHs). Alternatively a new technology using heated oak particles is now a common practice for "flavoring" red wine. Additionally it is of great interest to elucidate the formation of PAHs during the production of oak chips and barrels. We want to estimate possible health hazards using an in vitro test system for the detection of the activation of the human detoxification system. 9 different toasting variants of oak chips or from the 4 different toasting degrees of the oak barrels were investigated. No significant activation of the arylhydrocarbon receptor could be detected. GC/MS analysis confirmed the results from the yeast test system, showing no significant amounts of the toxic PAHs. The concentrations of all toxic PAHs were below the detection limit. Hence it can be concluded that no health risk of using toasted oak material (both oak chips and oak barrels) can be expected. The quality difference of these two vinification procedure should be the aim of further investigations in respect to the formation of the typical oak aroma profile.

KEYWORDS: Arylhydrocarbon receptor, toasting, oak chips, PAH

Introduction

Maturation of red wine in oak barrels is a well established oenological practice. Oak casks have been used in wine technology for storage and transportation for a long time. The great advantage is the development of a more complex flavor profile during maturation due to extraction of wood compounds from the oak and reaction with wine compounds. The influence of oak compounds on the flavor of the wine has been intensively studied (1). Positive effects on wine aroma are described (2) as well as some negative influences like the formation of "off flavors" (3, 4). Nevertheless, oak is the favored material for the maturation of high quality wines. A great disadvantage is that an oak barrel can only be used up to three times with notable extraction of oak compounds. Thus it is very expensive for vinification, which sometimes can double the price of the wine. For that reason another technology was established and is now a common practice. Small oak particles (oak chips) with different degree of toasting (heating of the oak particles) are added to the mash or fermented wine, while the wine is stored in inert casks or tanks (most common is stainless steel). This gives a similar taste to the wines (5).

Heating of the oak barrels over a fire is used to bend the staves. During this process the typical aromatic oak compounds are formed. To imitate this process oak chips are also heated to different degrees. This heating process was made responsible for the formation of toxic compounds like polycyclic aromatic hydrocarbons (PAHs) (6). They are produced under certain conditions of organic materials. Beyond the compounds that are produced during the combustion of oak wood on air or indirect heating, the PAHs are the most likely ones, which are subsequently extracted in the wine during wine maturation. PAHs consist of at least two condensed aromatic rings (Figure 1) and can be subsequently divided into relatively soluble low molecular weight compounds and condensed reaction products which are relatively insoluble. The mechanism of PAHs formation during the toasting process is highly complex and variable. More than 500 PAHs have been identified. Among them benzo[a]pyrene is considered as one of the most toxic with genotoxic and mutagenic activity. Nisbet and La Goy (7) have established a benzo[a]pyrene (BaP) carcinogenic equivalency factor system, which indicates BaP and Dibenzo(a,h)anthracene (DBahA) as the most carcinogenic ones. Naphthalene for example is by a factor of 1000 less carcinogenic as BaP in this classification system.

The aryl hydrocarbon receptor, also known as dioxin receptor, was reported as target receptor for activation of the detoxification system. Cytochrome P450 enzymes are the gene product of the transcriptional activation of the AhR which can lead to the formation of benzo[a]pyrene-7,8-diol-9,10-epoxide, a mutagenic reaction product of benzo[a]pyrene. This is one example of a compound which becomes toxic during detoxification in humans. Therefore in 1987 the FAO and WHO expert committee on food additives regulated that the amount of benzo[a]pyrene in foods should not exceed 10 ppb. The carcinogenicity of the PAHs is summarized in the IARC Monographs on the evaluation of carcinogenic risk of chemical to humans (8). Many reports have shown, that exposure of humans to PAHs may induce the formation of different cancers, such as lung and skin cancer (9-11). Air borne PAHs were shown to activate the arylhydrocarbon receptor (12). Other compounds, which bind to the arylhydrocarbon receptor lead to an up regulation of detoxification enzymes and stimulation of the detoxification pathways (13). Some flavonoids are described as AhR antagonist; by suppression the toxic effects of dioxin and benzo[a]pyrene (14).

Consequently, it is of great interest to estimate the sum of all potential compounds with health hazards risk. A sum parameter for risk assessment could be delivered by a detection system, which quantifies the activation of the human detoxification system. We want to elucidate the maximum impact of toasted oak wood on the activation of the human aryl hydrocarbon receptor. By means of GC/MS the corresponding compounds should be identified and the amount of PAHs quantified. This allows us to summarize the potential toxic impact of the development of PAH during the toasting procedure of oak chips and oak barrels. Additionally the stimulation of the detoxification system by other oak derived compounds can be investigated.

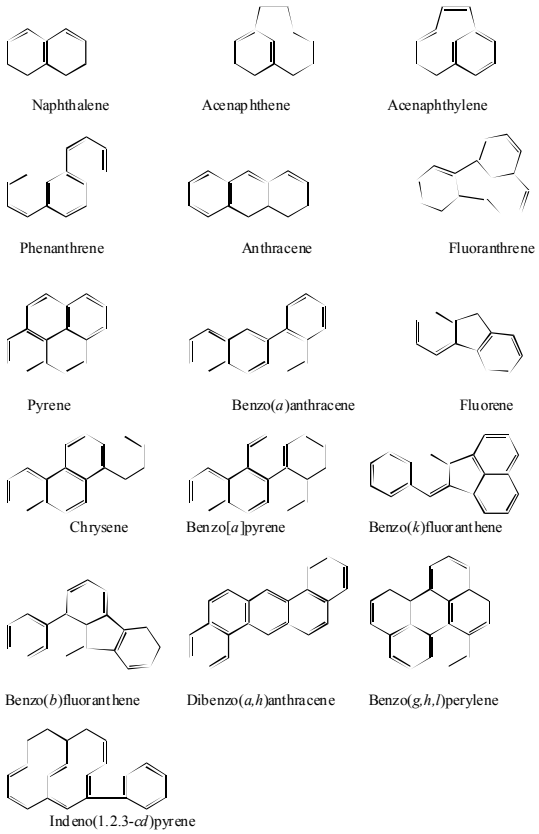

Figure 1: The chemical structures of the 16 PAHs.

Materials and Methods

Materials

Standard compounds were purchased from Sigma (Germany) and Apin Chemicals (Abingdon, U.K.). The oak chips were a kind gift from Erbslöh Geisenheim (Geisenheim, Germany) and the oak staves were provided from Tonnelerie Rousseau (Couchey, France).
Soxhlet extraction
Oak chips were controlled crushed with an impact mill (Retsch Technology, Haan, Germany) to a size < 2mm. The inner surface of the staves was removed with a planing machine up to 5mm depth. These particles were also crushed with the impact mill to a size < 2mm. Shortly, 5 g of the conditioned oak particles were placed in an extraction thimble (Whatman, Maidstone, U.K.) extracted with a soxhlet aperture for 4 hours with 150 ml toluene. After that the solvent was evaporated and the residues dissolved in 4 ml dichloromethane. An aliquot of 10 % was used for the yeast screening assay; therefore a solvent exchange with dimethylsulfoxide was performed. The dichloromethane extracts were used for GC/MS analysis

GC/MS-Analysis

GC/MS was performed using a GC 6890N/MSD 5973B instrument (Agilent Technologies). Column: HP-5MS (30 m column length, 0.25 mm diameter, 25 µm film thickness), 0.9 ml min-1 Helium. Inlet: 280 °C, split (25:1). Oven program: 50 °C (5 min), then 10 °C min-1 to 280 °C (20 min). Auxiliary temperature program: 240 °C (18 min), then 10 °C min-1 to 280 °C (14 min). Ionization: EI mode, 70 eV, 230 °C, 8.5 × 10–6 Torr. Data acquisition and processing: MSDChem software package (Agilent Technologies), NIST 2002 mass spectral library (National Institute of Standards and Technology, USA).

Yeast aryl hydrocarbon transactivation system

The yeast AhR screen is an in-vitro test to detect transactivating potential of substances on the aryl hydrocarbon receptor. The yeast strain YCM3 was a kind gift from Ch. Miller (15). The human AhR and ARNT genes are integrated into chromosome III of the yeast strain YCM3. A galactose-regulated promotor is accountable for the expression of AhR and ARNT in equal shares by induction with galactose. If a ligand of the AhR is present, an AhR/ARNT heterodimer is built and can bind to xenobiotic response elements of the reporter plasmid and induce the expression of a lacZ-gene. Therefore the transcriptional activation of a ligand is quantified via β-galactosidase activity. For that purpose a logistic dose response curve of β-naphthoflavone is done within every test trial. Each experiment was done at least in triplicate. A blank with dimethylsulfoxide was included within each test set up.
The yeast assay was performed in 96 well microtiterplates as described by Medjakovic and Jungbauer (16). The YCM3 yeast strain was grown over night in a synthetic DO-Trp medium, diluted on the following day to an OD600 of 0.4 and cultivated for further 8 hours. 300 µl of this saturated culture was then added to 15 ml of a synthetic medium containing 2 % galactose. The assay was performed in microtiter plates using 100 µl of this culture and adding 1 µl of test compound or extract. The microtiter plates were incubated for 16 hours, then 150 µl of lac-Z-buffer (containing 0.2 % sarcosyl) were added and absorbance was measured at 600 nm. After 20 min the reaction was started adding 50 µl of o-nitrophenyl-β-D-galactopyranoside. The samples were incubated for 5 min at 37°C. The reaction was terminated by adding 50 µl of a 1 M sodium chloride solution. Absorbance of the samples was read at 405 nm (reference wave length 620 nm). The AhR units were calculated as follows:

$$AhR\text{-}units = \left(\frac{OD_{405} * 1000}{OD_{600} * ml\ cell\ suspension} \right) * \left(\frac{1}{\Delta t} \right)$$

(Equation 1)

The potencies were calculated using a logistic dose response curve (Table curve 2D (Jandel Scientific, CA, USA)).

Results and Discussion

The first step in the estimation of the possible health hazards originated from oak wood was the extraction of 9 oak chips variants with different degrees of toasting and origin of the oak wood. Commercial available oak chips were used in this study. Additionally the traditional method of oak use (barrique) was

compared with the new technology of oak chips extraction. The staves from French oak barrels were planed for comparison of the wine extractable surface of the oak barrel with the surface of the oak chips (Picture 1). Therefore 5 mm of the inner surface of the staves were planed, because this is the maximum extraction depth for wines. One sample was taken only from the other part of approximately 100 cm2 of the heaviest toasted stave (sample Sch). These 4 different toasting variants of barrique from French oak were also extracted in a Soxhelt extractor. A short description of all studied oak chips and oak barrels are given in Table 1.

Figure 2: Oak staves: four different toasting variants (Heavy, medium plus, medium long and light)

Table 1: Oak chips and oak barrels.

No.		Toasting	Origin (nomenclature)	Temperature/time
1	Oaky Vin DM	medium	German oak (*Quercus robur/petraea)*	Not reported
2	Oaky Vin DH	heavy	German oak *(Quercus robur/petraea)*	Not reported
3	Oaky Vin AM	medium	American oak *(Quercus alba)*	Not reported
4	Oaky Vin AH	heavy	American oak *(Quercus alba)*	Not reported
5	Oaky Vin ASH	heavy	American oak *(Quercus alba)*	Not reported
6	Oaky Vin FM	medium	French oak *(Quercus robur)*	Not reported
7	Oaky Vin FH	heavy	French oak *(Quercus robur)*	Not reported
8	Oaky Vin FSH	heavy	French oak *(Quercus robur)*	Not reported
9	M 3066	no toasting	French oak *(Quercus robur)*	Not reported
10	G 3845	no toasting	French oak *(Quercus robur)*	Not reported
11	Rousseau Leger	light	French oak *(Quercus robur)*	<150°C/<30 min
12	Rousseau ML	medium long	French oak *(Quercus robur)*	150-200°C/ >60min
13	Rousseau M+	medium plus	French oak *(Quercus robur)*	150-200°C/~60 min
14	Rousseau F	heavy	French oak *(Quercus robur)*	>200°C /30-60 min
15	Rousseau F (sample Sch)	Heavy Only ~1 mm depth	French oak *(Quercus robur)*	>200°C /30-60 min

Prior to extraction the oak material was conditioned with a mill to a size ≤ 2 mm for equal extraction conditions. After an extraction time of four hours, the solvent toluene was evaporated and the residues dissolved in 4 ml dichloromethane. An aliquot of 10 % was used in the yeast transactivation assay; the rest was analyzed by means of GC/MS to quantify the PAHs. The 16 studied PAHs and their toxicity according to the IARC Monographs (8) are listed in Table 2.

The extracts were tested in the aryl hydrocarbon receptor yeast assay to determine the sum of the detoxification stimulating compounds. Therefore 1 µl of the extracts were applied to the assay, a standard curve with β-naphthoflavone was done within each test setup as reference (Figure 2).

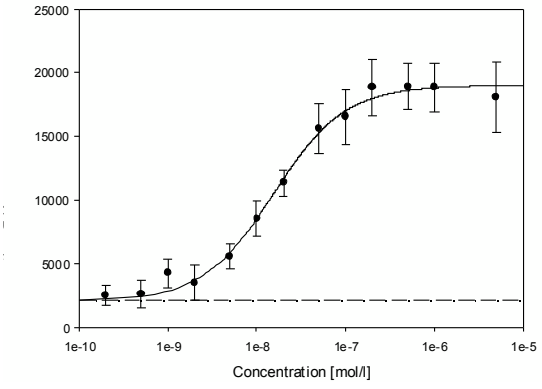

Figure 3: Logistic dose response curve for β-naphthoflavone (n=15, β-naphthoflavone was used within each test setup as reference compound)

The assay was also performed without yeast to estimate the influence of the colored samples. These blank runs were used to calculate the background of the assay. The most concentrated extract and the 1:5 diluted extracts of all samples could not be used for calculation, because of the high growth inhibitory effects on the yeast. For most extracts no significant transactivation could be observed. Only the oak extract AH and the completely burned residues from 100 cm2 of heavy toasted oak stave (sample Sch) showed a significant increase in AhR transactivation (Figure 3).

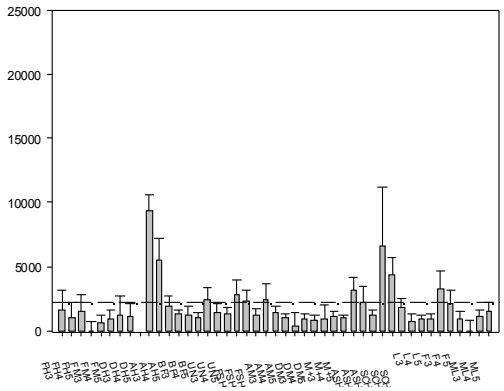

Figure 4: Yeast transactivation assay of the 14 oak extracts (n=3) dashed line represents the blank (1 µl dimethylsulfoxide) signal plus 3□; The samples were diluted in 1:5 steps, step 3 was the first evaluable one, which represents a concentration of 0.2 g oak material/mL solvent; The abbreviations are listed in Table 1.

The standard compound mixture was used in the yeast transactivation assay to assess the functionality of this assay for estimation of the health hazards of PAHs (Figure 4). The calculated potency of the 16 PAHs mixture is 5 ng/ml, assuming that only one compound in the standard mixture with a concentration of 10 µg/ml exhibit AhR transactivation. According to Saeki et al. (17) chrysene and benzo(a)pyrene are the most potent ones in the standard mixture with an EC_{50} of 1.25 and 1.37 µM, respectively They measured the potency of the neat compounds in the same yeast test system. They showed that only N-substituted and halogenated PAHs exhibit a more toxic effect.

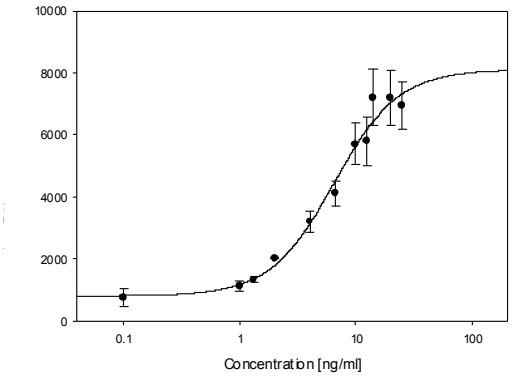

Figure 5: Logistic dose response curve for the standard solution mixture (n=2); The standard compound mixture with a concentration of 10 µg/ml was diluted with dimethylsulfoxide. The mixture of all 16 PAHs was tested in the yeast transactivation assay.

Assuming that all of the transactivation potential is due to these two compounds (with approximately the same potency and molecular weight) 1 ng/mL of each single compound can be detected with this yeast test system (Figure 5). For the yeast transactivation assay we used the extracts with a concentration of 5 g oak chips or stave/mL. The first two concentrations of the extracts could not be used because of some inhibitory effects on yeast growth. The 25 fold diluted samples give evaluable results. Only the extract AH (American oak, heavy toasted) and Sch (which are the burning residues of 100 cm2 of the heaviest toasted stave) exhibit some transactivation potential and are therefore possible candidates for higher concentrations of PAHs. No significant concentrations of these compounds can be expected from the preliminary screening assay with the yeast transactivation system for the other extracts.

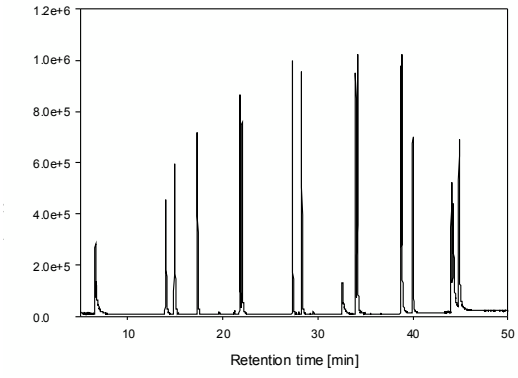

Figure 6: GC/MS analysis of the standard compound mixture. The standard solution mixture was diluted with ethlyacetate 1:10 and 1 µl was injected to the GC column.

For quantification the standard solution mix was injected in different concentrations to calculate standard curves. The retention time of each compound is listed in Table 2 and Figure 5 shows the chromatogram of the standard solution mixture performed with GC/MS. All 16 compounds could be detected with GC/MS from the standard mixture solution. Next the 14 oak extracts were injected and we tried to identify each PAH with help of retention time and mass spectrum.
The results from the GC/MS analysis are given in Table 3. Only naphthalene was found in the extracts of heavier toasted oak wood in higher concentrations. The other 15 PAHs could not be quantified in the samples with GC/MS, with a detection limit of 10-50 ng/ml. Using 1g oak chips for one liter wine and assuming 100% extraction, a maximum concentration of about 10-50 ng/L wine of each compound can be reached. One order of magnitude lower concentrations were obtained by Chinnici et al. (18) and Chatonnet and Escobessa showing only 5.5 (traditional toasting) and 3.0 ng/g (hot air convection toasting) oak wood for the molecules with toxicological risk (19).
Very high amounts of propenylsyringol and sinapylaldehyde were detected. Therefore these high concentrated compounds and naphthalene were tested in the yeast test system. The results are given in Figure 6, indicating no transactivation for naphthalene which is in agreement with other findings (17). Only a low transactivation at high concentration could be measured for propenylsyringol and sinapylaldehyde. Such high concentrations can only be reached in wood extracts and are not physiological relevant. Our two extracts with detectable transactivation potential are rich in such compounds and this can explain one part of the transactivation potential. The presence of different PAHs in minor concentration results in a detectable AhR transactivation signal, thus explaining another part of the transactivation potential.

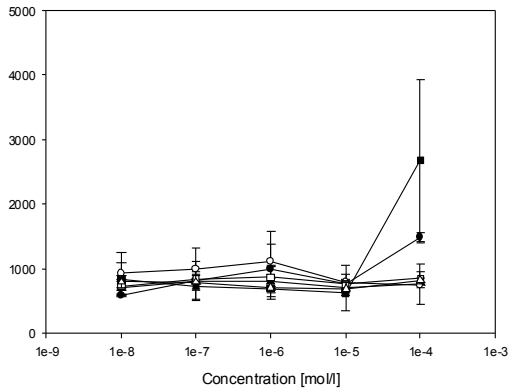

Figure 7: Yeast transactivation assay for some neat compounds (propenylsyringol, sinapylaldehyde, naphthalene, syringaldehyde, coniferylaldehyde and vinylguaiacol)

Table 2: The 16 studied PAHs (molecular weight, retention time and carcinogenicity) (1: carcinogenic to humans, 2 A: probably carcinogenic to humans, 2 B: possibly carcinogenic to humans, 3: unclassifiable as carcinogenic to humans, 4: probably not carcinogenic to humans; n.e. not evaluated).

Compound	Abbreviation	MW	Retention time	Carcinogenicity
Acenaphtene	ACN	154	14.94	n.e.
Acenaphtylene	ACL	152	14.04	n.e.
Anthracene	ANT	178	20.03	3
Benz[*a*]anthracene	B[a]A	228	34.18	2 A
Benzo[*a*]pyrene	B[a]P	252	40.00	2 A
Benzo[*b*]flouranthene	B[b]F	252	38.87	2 B
Benzo[*ghi*]perlyene	B[g,hi]P	276	44.91	3
Benzo[*k*]flouranthene	B[k]F	252	38.77	2 B
Chrysene	CHR	228	34.05	3
Dibenz[*a,h*]anthracene	D[a,h]A	278	44.28	2 A
Flouranthene	FLA	202	27.39	3
Fluorene	FLU	166	17.38	n.e.
Indeno([1,2,3]-cd)pyrene	I[1,2,3]P	276	44.11	2 B
Naphtalene	NAP	128	6.578	n.e.
Phenantrene	PHE	178	21.82	3
Pyrene	PYR	202	28.35	3

Table 3: Table 3 Quantification of the 14 different oak extracts for their PAH content (detection limit: 50 ng/g sample) n.d. not detectable, n.q. not quantifiable)

Oak chips/extract	Dosage (g/l)	ACN	ACL	ANT	B[a]A	B[a]P	B[b]F	B[g,hi]P	B[k]F	CHR	D[a,h]A	FLA	FLU	I[1,2,3]P	NAP	PHE	PYR
DM	0.5-3g/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.
DH	0.5-3g/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.
AM	0.5-3g/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.
AH	0.5-3g/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.
ASH	0.5-3g/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.
FM	0.5-3g/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.
FH	0.5-3g/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.
FSH	0.5-3g/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.
M	0.5-3g/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.q.	n.d.	n.d.
G	0.5-3g/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.q.	n.d.	n.d.
L (~30g/dm^2)	1 dm^2/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.
ML (~30g/dm^2)	1 dm^2/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.
M+ (~30g/dm^2)	1 dm^2/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.
F (~30g/dm^2)	1 dm^2/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	>50ng/g	n.d.	n.d.

Garcia-Falcon and Simal-Gandara have shown that spirits aged in traditionally made oak barrels have a higher PAH content up to a concentration of 200 ng/L. Therefore it is necessary to investigate extraction conditions at higher alcohol levels (20). We only studied the oak stave from one cooperage. These results can not be generalized for all cooperages, or all kind of wood used for barrel as well as fire making methods for stave bending. Barbosa et al. (21) have shown that the highest amount of PAH was found in wood from Eucalyptus sp., for example. One described mechanism of the origin of PAHs in oak barrels is the transfer of particular matter from the fire to the surface of the barrel. The fire temperature there can reach >400°C.This promotes the formation of PAHs. At the surface of the oak barrels such temperatures will never be reached.

Nevertheless the calculated doses of potential carcinogenic PAHs from food ranged from 1 to 12 µg / day. Charcoal boiled or smoked meat and fish are the major sources of PAHs estimated in the Total Human Environmental Exposure Study (THEES) (22). The quantity of wine derived PAHs is therefore negligible.

We investigated the maximum of toxic compounds which are present in the toasted oak, knowing that only a small part of these compounds can be extracted by the wine due to the low solubility (high hydrophobicity). We could not detect significant amounts of PAHs and no significant transactivation of the aryl hydrocarbon receptor. Our results indicate that no toxic concentration of the PAHs can be leached from oak wood neither from oak chips nor from oak barrels.

Conclusion

The yeast aryl hydrocarbon receptor assay is a helpful tool to detect possible health hazards in food and beverages after sample concentration. The concentration of PAHs in oak chips was below the detection limit for the yeast assay as well as for the GC/MS. In summary it can be said that wood extracts both from oak chips and oak staves do not contain significant amounts of toxic hydrocarbons. No impact of toasting on the transactivation of the aryl hydrocarbon receptor could be detected and thus no health hazards for the use of oak chips and barrels can be expected.

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(71) Applicant(s):
G.I.M.-GESELLSCHAFT FÜR INNOVATIVE MEDIZIN G.M.B.H. NFG OHG [AT/AT]; Alserbachstrasse 16 1090 Vienna (AT) *(for all designated states except US)*
JUNGBAUER, Alois [AT/AT]; Ichagasse 31-1 A-1210 Vienna (AT) *(for US only)*
MEDJAKOVIC, Svjetlana [AT/AT]; Pettenkofengasse 2-35 A-1030 Vienna (AT) *(for US only)*
ZÖCHLING, Alfred [AT/AT]; Teesdorferstrasse 21 A-2523 Tattendorf (AT) *(for US only)*

(72) Inventor(s):
JUNGBAUER, Alois; Ichagasse 31-1 A-1210 Vienna (AT)
MEDJAKOVIC, Svjetlana; Pettenkofengasse 2-35 A-1030 Vienna (AT)
ZÖCHLING, Alfred; Teesdorferstrasse 21 A-2523 Tattendorf (AT)

(74) Agent(s):
SONN & PARTNER; Riemergasse 14 A-1010 Wien (AT)

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(57) Abstract:

(EN): The present invention provides a medicament comprising a chemical compound according to formula A: wherein Rl is hydrogen, fluorine, chlorine, bromine, hydroxy, mercapto, methoxy, ethoxy, acetoxy, methyl, ethyl, propyl, isop- ropyl, t-butyl, nitro, amin, N,N-dimethylaminoyl, N,N-diethyl- aminoyl; R2 is hydrogen, a C1-8-alkyl e.g. methyl, ethyl, propyl, isopropyl, butyl, t-butyl; an C1-8-acyl e.g. acetyl, propionyl; R3 is a C5-12-heterocyclic ring, e. g. furanyl, pyranyl, pyrrolyl, pyridinyl, pyrazolyl, thienyl, thiazolyl, indolyl or -0R4; R4 is a C5-C12-aromatic ring e.g. phenyl; unsubstituted or substituted in any position with fluorine, chlorine, bromine, methoxy, eth oxy, C1-8-carboxy; Z is OH or 0; and n is 0, 1, 2 or 3, and m is 0, 1, 2 or 3, and its use for the activation of the aryl hydro carbon receptor.

(FR): La présente invention concerne un médicament comprenant un composé chimique répondant à la formule A : dans laquelle R1 est un hydrogène, un fluor, un chlore, un brome, un hydroxy, un mercapto, un méthoxy, un éthoxy, un acétoxy, un méthyle, un éthyle, un propyle, un isopropyle, un t-butyle, un nitro, un amino, un N,N-diméthylaminoyle, un N,N-diéthylaminoyle ; R2 est un hydrogène, un alkyle en C1-8 par exemple un méthyle, un éthyle, un propyle, un isopropyle, un butyle, un t-butyle, un acyle en C1-8 par exemple un acétyle, un propionyle ; R3 est un hétérocycle en C5-12 par exemple un furanyle, un pyranyle, un pyrrolyle, un pyridinyle, un pyrazolyle, un thiényle, un thiazolyle, un indolyle ou -OR4 ; R4 est un cycle aromatique en C5-12 par exemple un phényle non substitué ou substitué en n'importe quelle position par un fluor, un chlore, un brome, un méthoxy, un éthoxy, un

carboxy en C1-8 ; Z est OH ou O ; et n est 0, 1, 2 ou 3 et m est 0, 1, 2 ou 3. La présente invention concerne également l'utilisation de celui-ci pour l'activation du récepteur des hydrocarbures aryle.

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(71) Applicant(s):
G.I.M.-GESELLSCHAFT FÜR INNOVATIVE MEDIZIN G.M.B.H. NFG OHG [AT/AT]; Alserbachstrasse 16 A-1090 Vienna (AT) *(for all designated states except US)*
JUNGBAUER, Alois [AT/AT]; Ichagasse 31-1 A-1210 Vienna (AT) *(for US only)*
MEDJAKOVIC, Svjetlana [AT/AT]; Pettenkofengasse 2-35 A-1030 Vienna (AT) *(for US only)*
ZÖCHLING, Alfred [AT/AT]; Teesdorferstrasse 21 A-2523 Tattendorf (AT) *(for US only)*

(72) Inventor(s):
JUNGBAUER, Alois; Ichagasse 31-1 A-1210 Vienna (AT)
MEDJAKOVIC, Svjetlana; Pettenkofengasse 2-35 A-1030 Vienna (AT)
ZÖCHLING, Alfred; Teesdorferstrasse 21 A-2523 Tattendorf (AT)

(74) Agent(s):
SONN & PARTNER; Riemergasse 14 A-1010 Vienne (AT)

(54) Title (EN): PLANT EXTRACTS, PLANT COMPONENTS AND THE USES THEREOF

(54) Title (FR): EXTRAITS DE PLANTES, COMPOSANTS DE PLANTES ET UTILISATIONS DE CEUX-CI

(57) Abstract:

(EN): The present invention provides a use of a concentrated plant extract or plant juice for the production of a pharmaceutical, cosmetic or nutritional preparation for the activation of the aryl hydrocarbon receptor, wherein the plant extract or juice comprises aryl hydrocarbon receptor activity concentrated by a factor of at least 5, preferably at least 10, more preferred at least 15, most preferred at least 50, relative to the original plant or plant juice dry matter.

(FR): L'invention concerne l'utilisation d'un extrait ou d'un jus de plante concentré pour la production d'un produit pharmaceutique, d'un produit cosmétique ou d'une préparation nutritionnelle pour l'activation du récepteur des hydrocarbures aryle, ledit extrait ou jus de plante présentant une activité vis-à-vis du récepteur des hydrocarbures aryle concentrée d'un facteur d'au moins 5, de préférence d'au moins 10, encore mieux d'au moins 15, idéalement d'au moins 50, par rapport à la matière sèche de la plante ou du jus de la plante d'origine.

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Declaration of inventorship (Rules 4.17(iv) and 51bis.1(a)(iv)) for the purposes of the designation of the United States of America