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Relevantie van estrogene en aromatase inhiberende effecten van mengsels van xenoestrogenen voor humane blootstelling

(met een samenvatting in het Nederlands)

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Relevance of estrogenic and aromatase inhibiting effects of mixtures of xenoestrogens for human exposure
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Introduction

Naturally occurring estrogens are steroid hormones that play essential roles in maintaining physiological homeostasis. On the other hand, both natural and synthetic estrogens have been linked to hormone-sensitive diseases such as breast cancer and prostate cancer. Therefore, there is a continuous concern about risks from exposure to estrogens or compounds mimicking their action. In addition to the presence of individual compounds and their levels in the environment or humans, the question is compelling what effects occur from exposure to mixtures of these chemicals. Parts of this thesis focus on mixtures of exogenous estrogens and their estrogen receptor mediated effects. Exposure to a compound may induce several effects, for instance plant derived chemicals may at the same time act on the estrogen receptor and modulate endocrine synthesis. In another part of this thesis effects were investigated on aromatase, an important enzyme in estrogen synthesis. The last part of this thesis focuses on the resultant of the ambivalent properties of some compounds. The general introduction provides background information on steroidgenesis, estrogenicity, endocrine active compounds, breast cancer and mixture-concepts.

Endocrine system and Steroidgenesis

The endocrine system comprises all hormone-secreting cells in an organism. There are four distinguished hormonal systems, based on the type of the signalling molecule. All four act in concert to regulate processes such as development, growth, reproduction, behaviour and, more general, maintain homeostasis in an organism throughout life. These four systems use catecholamines, prostaglandins, polypeptides or derived amino acids, or steroids, which are secreted from endocrine glands into the bloodstream. At their target sites they fulfil a regulatory function. These target sites are able to respond to their corresponding hormones when an appropriate receptor is available. Generally four receptor types (superfamilies) are distinguished, based on their molecular structure and nature of transduction mechanism that are type 1 (channel-linked receptors (or ionotrophic receptors)), type 2 (G-protein-coupled receptors (GPCRs)), type 3 (kinase-linked receptors) and type 4 (nuclear receptors (NR)). Briefly, ion channels are involved in signalling between electrically excitable cells, where the signalling is regulated by neurotransmitters. G-protein-coupled receptors are membrane bound proteins that serve as extracellular receptor that activate intercellular signalling transduction pathways upon binding of a ligand. Kinase-linked receptors also have an extracellular domain for ligand binding that activates the intracellular domain which exerts enzymatic activity itself. NRs are cytosolic (type I) or nucleic (type II) proteins that bind DNA or transcription factors upon activation by the ligand. These receptors directly increase gene transcription (transactivation) or indirectly decrease gene transcription (transrepression). NR are further divided in several subfamilies amongst which are the thyroid hormone receptors (subfamily 1), retinoid X receptors (subfamily 2) and estrogen receptors (subfamily 3).

This thesis regards the interaction of steroids with NRs (subfamily 3) as the most relevant and will therefore be discussed in more detail. One group of steroids that rely on NR signalling are the endogenous estrogens. Cholesterol is the precursor molecule in the biosynthesis of all estrogens, as well as for all other steroid hormones (fig 1.1). In steroidgenesis several enzymes convert cholesterol eventually into androstenedione and testosterone (T), which are interconvertible by two isoforms of the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD). Another enzyme, aromatase, will further convert androgens to estrone (E_1) and 17β-estradiol (E_2), which are again interconvertible by 17β-HSD. Partly, the androgens and estrogens formed will then be available for binding to respectively the androgen receptor (AR) and the estrogen receptor (ER), while another part will be temporarily unavailable because of formation of T, E_1 or E_2 sulfates by sulfotransferase and binding to sex hormone binding globulins (SHBG). Testosterone is further metabolized to 5α-dehydrotestosterone (DHT), which shows the highest affinity to the AR, and then to the less potent androsterone. Estrogens are metabolized by several cytochrome P450 enzymes into 2-hydoxycatechol estrogens (by

CYP1A1 and CYP1A2), 4-hydroxycatechol estrogens (by CYP1B1) or 16α-hydoxyestrogens (by CYP3A4 and CYP3A5). The catechol estrogens may be further metabolized by catechol-*O*- methyltransferase (COMT) [8].

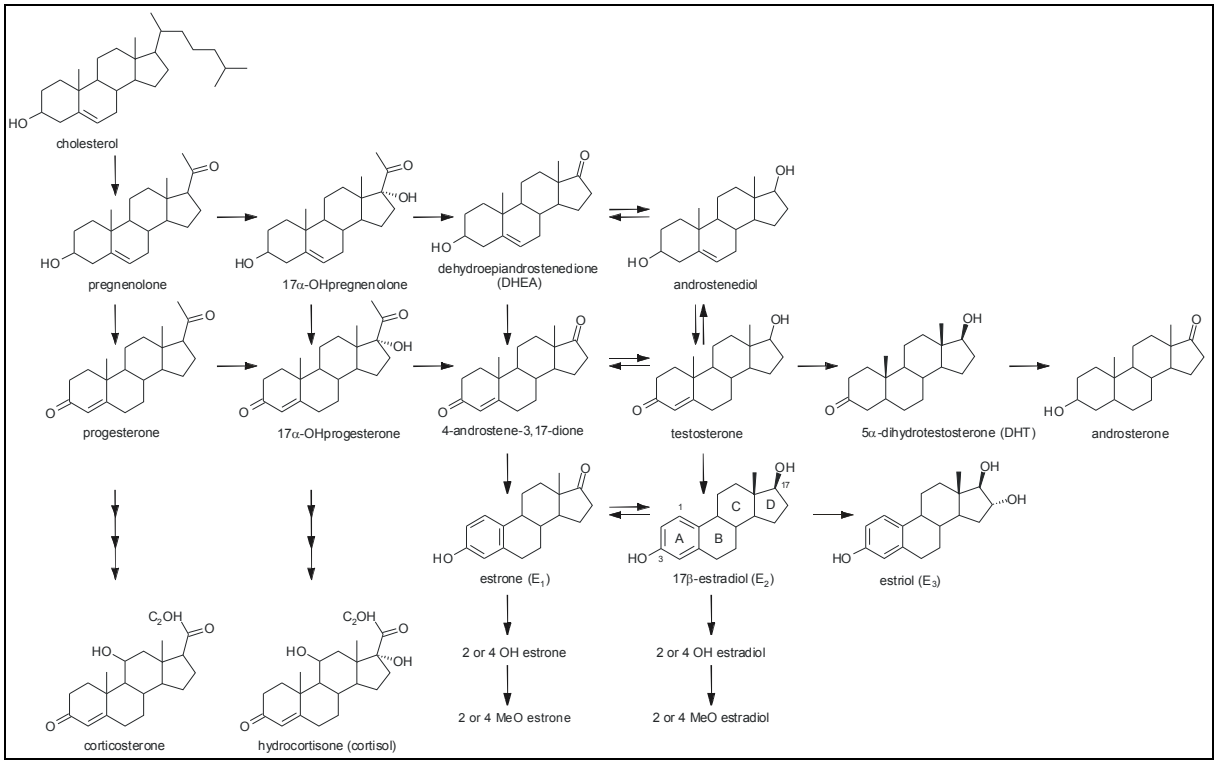


Fig 1.1. Biosynthetic pathways of steroids.

The key enzyme in this biosynthesis is aromatase, as it converts androgens to estrogen steroids. Aromatase is a member of the cytochrome P450 enzymes and consists of a $P450_{arom}$ enzyme, a haem group and an NADPH-reductase flavoprotein. This enzyme complex is bound to the membrane of the endoplasmatic reticulum, restricted to estrogen producing cells, primarily granulosa cells in the ovaries and Leydig cells in the testis, and to a lesser extent also in the adrenals. Here it catalyzes the conversion of C19-steroids such as androgens to C18-steroids such as estrogens. In this process two hydrogen atoms (1α and 1β) are released as water, but the crucial steps are demethylation of the C19 and the irreversible aromatization of the A-ring in C19-steroids (fig 1.2).

Estrogens play important and diverse physiological roles that are universal in vertebrates. They are involved in bone anabolism, cardiovascular processes, functions of the central nervous system and unmistakably in the physical changes during life (e.g. puberty, menopause) and reproduction (e.g. menstrual cycle, uterus and mammary gland). Circulating estradiol reaches the highest levels in premenopausal women (20-350 pg/ml = 73-1285 pM and even 18000 pg/ml = 66000 pM in pregnant women [136]). Nonetheless, estradiol is also produced in men and circulating levels are considerable (20-50 pg/ml = 73-184 pM). In addition, peripherally produced estrogens (e.g. in adipose tissue) are recognized as a powerful source of circulating estrogens. For example, in postmenopausal women, in whom estrogen production from the ovaries has ceased, these estrogens might contribute significantly to estrogen levels of 10-30 pg/ml (37-110 pM) [136].

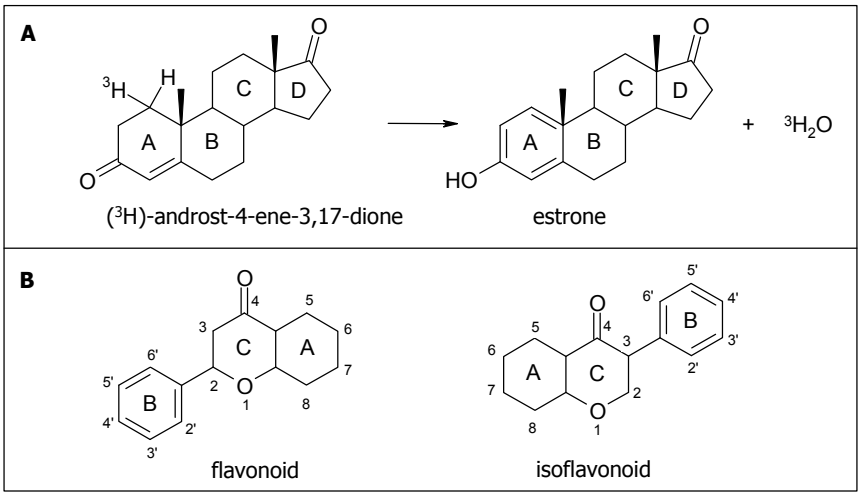


Fig 1.2. Structure of ^{3}H-androstenedione converted to estrone and tritiated water, catalyzed by aromatase (**A**) and the general structure of flavonoids and isoflavonoids (**B**).

Estrogenicity & Estrogen receptor

According to a definition first pronounced by Hertz [55] "the primary effect of an estrogen is the stimulation of mitotic activity in the tissues of the female genital tract. A substance which can directly elicit this response is an estrogen; one that cannot is not".
Another definition followed and states that estrogenicity "refers to the ability of a chemical to mimic a principal *in vivo* action of the hormone estradiol, such as stimulating cornification of the vaginal epithelium, evoking estrus behavior or "heat" (the receptivity of a female animal for a male), or inducing uterine enlargement" [14].

These two definitions do not specify the mediator(s) which carry the estrogenic signal to the estrogenic response. However, it is generally accepted that major players are the estrogen receptors (ER). Estrogen receptors are members of the nuclear receptor (NR) superfamily, class I (steroid receptors), subfamily 3 (estrogen-like receptors).The existence of estrogen receptors was discovered in the late 1950s, though only since 1996 two subtypes (ERα and ERβ) are being distinguished [74]. Both types mediate estrogenic responses in various types of mammalian tissue such as the central nervous system as well as bone, cardiovascular, liver, urogenital, gastrointestinal and mammary tissue. The ratio ERα : ERβ is tissue dependent [47, 75]. More recently a third type (ERγ) was discovered in fish (Atlantic croaker) [50]. All types have a high affinity for 17β-estradiol (E_2), and to a lesser extent for estrone (E_1) and estriol (E_3). Briefly, in the classical and simplified mechanism the signal "estrogen" is mediated via ERs localized in the cytosol or nucleus. Estrogens, lipophilic as all steroids, migrate through the cell (and nuclear) membrane and bind the binding domain at the C-terminus receptor (fig 1.3). Due to binding of the steroid, conformational changes in the receptor occur through which heat shock proteins (hsp70, hsp90) are being released and dimerization is facilitated with another activated ER. Hetero- (ERα-ERβ) or homo- (ERα-ERα or ERβ-ERβ) dimers have a high affinity for specific DNA sequences, the so called Estrogen Responsive Elements (ERE). Many genes responding to estrogens contain an ERE. These EREs vary in exact DNA sequence and are part of the promoter, up-stream from the target gene. ER-dimers have highest affinity for a perfect ERE (5'-(CA)GGTCAnnnTGACC(TG)-3'). This binding results in the strongest stimulation of transcription of the downstream gene. Imperfect EREs have minor differences in this palindrome and will result in lower affinity and less transcription. In contrast to activation processes of the estrogen receptor by an agonist, binding of an antagonist will not induce gene transcription. There are several possible modes of action to prevent gene transcription. Firstly, a molecule may prevent binding of the agonist. Secondly, binding of a molecule may activate the cascade equal to when an agonist binds, except that

the conformational change of the receptor will be such that recruitment of specific regulator proteins is prevented. A third possibility occurs when binding impairs the formation of dimerization and nuclearization [60].

Although ERα and ERβ are encoded by separate genes, their structure is very similar (fig 1.3.). Six domains in the human protein are distinguished (A, B, C, D, E and F) each having a distinct function. The A/B domain contains the activation function 1 (AF-1) and modifies gene transcription independently from ligand binding. Gene transcription is regulated in a cell-specific manner and depended on phosphorylation of specific aminoresidues, hence is referred to as the regulatory domain (RD). The C domain contains a zinc finger pair, capable of binding DNA (DBD). The D domain or hinge region (HR), a sequence of amino acids determining the nuclear localization, is involved in receptor dimerization and interaction with co-repressor proteins. The E domain contains the ligand binding domain (LBD), but is also thought to play a role in nuclear localization, receptor dimerization and contains the activation function 2 (AF-2) that ligand dependently interacts with transcription factors. The F domain interacts with cell-specific factors and is able to differentiate between an agonistic and antagonistic ligand. The structures of the two ER subtypes closely resemble each other. For example, the DBDs of ERα and ERβ are 97% homologous. On the other hand the RDs show around 16% homology and the LBDs differ at some critical amino acids [47, 72]. Differences in amino acid sequences in the RD or LBD may explain subtle differences in the proteins that ERs interact with or, respectively, in the affinity for ligands. Nevertheless, in general the structure of steroids receptors is highly conserved in the evolution of vertebrates. This is again illustrated by the fact that the integrated estrogen-receptor system is presumed to be the most ancient of all steroid-receptor controlled mechanisms. Hence, the ER and its mechanism direct maturation and reproduction in a wide spectrum of animal species from lower vertebrate species such as echinodermata to higher species such as mammals. Thus it is obvious that many species will be sensitive to compounds mimicking estrogens or interfering with ER complex forming. Not surprisingly, interference with this delicate, highly conserved endocrine signalling system may affect development and homeostasis in many vertebrates. In this perspective it is necessary to mention that the list of compounds showing affinity, though lower than E_2, for ERα or ERβ is increasing every day: e.g. synthetic estrogens (4,4'-dihydroxydiethylstilbene = DES; ethynyl estradiol = EE), naturally occurring compounds (plant (phyto) or fungi (myco) derived) (genistein, zearelone), synthetic compounds (alkylphenols, benzophenones, bisphenol A, musks, organochlorides, parabens, phthalates). Each compound has its binding affinity preference for ERα or ERβ, and is capable of activating the ER to a certain extent. The type of ligand that induced the cascade to complexed ERs may affect recognition of the ERE, which may determine the attraction of transcription factors [65]. Besides the palindrome and the ligand type, the intracellular context very much determines the transcription process - presence or absence of specific co-activator proteins and co-repressor proteins play an important role in the response to a ligand. More general, any ligand or so called Selective Estrogen Receptor Modulators (SERMs) may exert mixed agonist-antagonist effects by specifically activating or inactivating ERα or ERβ. This may lead to gene specific induced or reduced transcription, which is dependent on intracellular context (presence or absence of specific co-activator proteins and co-repressor proteins), but also tissue type. In case of some drugs it is a very desirable feature to display antagonistic as well as agonistic effects [82]. In the search for novel pharmaceuticals it is a challenge to find so called perfect SERMs; a drug used in breast cancer treatment that ideally would fully induce the beneficial effects of estrogens and fully diminish all harmful effects of estrogens. Since the 1970s tamoxifen is approved in breast cancer treatment. Its mechanism of action is to bind to the ER, which causes dimerization and activation of the ER. In this process, the AF1 remains functional, while only the AF2 site is hindered to recruit regulatory factors. In mammary tissue, a tissue exhibiting much AF2 activity, tamoxifen acts as an antagonist [34]. Initially, it was hypothesized that for women with high risk for breast cancer, the preventive intake of tamoxifen would reduce the breast cancer incidence. Although tamoxifen indeed reduces breast cancer risks and

displays beneficial effects on bone metabolism and cardiovascular processes, it increases the uterine cancer risk. In the uterine tissue tamoxifen was found to be estrogenic, illustrating that the cellular context is also of great importance on the exerted effect [66].

With respect to SERM capacities, compounds originating from natural sources have been increasingly investigated. For example polyphenols extracted from plant material (phyto-chemicals) have been investigated [76]. In general plant derived polyphenols appear to have a higher binding affinity for ERβ than for ERα [128]. As a consequence, some phytochemicals have been implicated for prevention or treatment of breast cancer or as replacements of hormone replacement therapy (HRT) (in postmenopausal women) [10, 99].

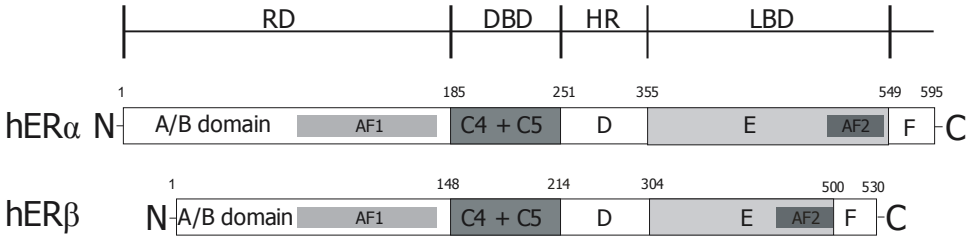


Fig 1.3. Functional domains of human estrogen receptors α and β. The regulatory domain (RD) recruits co-factors and initiates gene transcription, the DNA binding domain (DBD) exists of two zinc fingers that recognize and bind estrogen response elements in the DNA located upstream the target genes, the hinge region (HR) determines the location of the receptor and is involved in the dimerization process, the ligand binding domain (LBD) binds estrogens and is involved in the nuclear location and dimerization process. (Adapted from [72])

The classical mechanism as described above is regarded as a relatively slow and long lasting way of action of steroids. Although the cellular response to induce specific gene transcription after an estrogen signal is relatively quick, the full onset of the physiological response may take hours to days. This also takes into account that it will take hours to produce estrogens in specified cells (e.g. in ovaries, testis and adipose tissue) before their release in the circulation. However evidence for another, fast (within minutes) cellular responding mechanisms is emerging. It has been shown that membrane bound estrogen receptors, which expose their ligand binding domain on the outside of the cell, activate a cascade of signalling molecules such as cyclic adenosine monophosphate (cAMP) [139-141, 147].

Endocrine disruptors

Numerous terms for compounds that may interfere with any endocrine system emerged (such as xeno-estrogens, environmental estrogens, environmental hormones, endocrine disrupting chemicals, endocrine modulators and endocrine disruptors). In 1996 the (U.S.A.) Environmental Protection Agency (EPA) charged the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to develop a screening program for endocrine disruptors. In this process, the term 'endocrine disruptor' was defined. Initially, the definition for endocrine disruptors by Kavlock *et al.* was acknowledged:

"An exogenous agent which interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance or homeostasis, reproduction, development or behavior" [69].

However, this definition did not meet needs for regulatory operations and a working definition was developed:

" *An exogenous substance that changes endocrine function and causes adverse effects at the level of the organism, its progeny, and/or (sub)populations of organisms."*

In this definition the word [adverse] was under debate. In 1998 consensus in the committee was reached on a description that would suit the screening and testing program:
" *An exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle."* [37]

The European Commission (EC) and World Health Organisation (WHO) differentiate between an endocrine disruptor and a potential endocrine disruptor. For endocrine disruptors, the definition accepted at the Weybridge-workshop, UK, 1996 is maintained:
" *An exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations"* [36]

While for potential endocrine disruptors this is:
" *A substance that posses properties that might be expected to lead to endocrine disruption in an intact organism."*
These definitions or descriptions allow interference of compounds with one or more endocrine systems. Interference could occur by numerous possible modes of action and at different levels within an organism. To cover possible mechanisms and effects as much as possible, in this thesis a more general term, endocrine active compounds (EACs), will be used. Two mechanisms of action for EACs will be discussed in this thesis. Firstly, an EAC (directly) binds to the endogenous receptors in target tissue (e.g. ER in ovaries or breast tissue). Binding to such receptors may block or induce further responses. Secondly, an EAC (indirectly) affects an endocrine organ in general, the hormone synthesis or metabolism specifically and thus modulates the hormone production of the organ. A growing number of mechanisms sensitive to EAC is emerging in scientific literature. Some of these mechanisms are critical for the availability of E_2 in the circulation of endogenous hormones, for example hampering binding to Sex Hormone Binding Globulin (SHBG) or albumin in serum [59], inhibition of sulfotransferase (e.g. phthalates) or methyl catecholtransferase (e.g. PCBs) [131, 138]. Other mechanisms point towards EACs modulating the expression of a panel of receptors (such as ER, PR, AR)[31, 32], possibly via crosstalk (such as the Arylhydrogen Receptor (AhR) with the ER [106]. Effects of certain EACs (e.g. bisphenol A and methoxychlor) have been shown to occur even at the neurobehaviour level of perinatal exposed mice and rats, but also birds [45, 86]. The most widely studied class of compounds in this respect is the one that mimic actions of endogenously produced estrogens, or that can inhibit their function.

Since the 1990s there is public awareness that EACs could be of great concern for human health. Science had recognized such phenomena much earlier. In the 1940s it was first recognized in Australia that cattle (partly) fed on subterranean clover (*Trifolium sp.*) developed an impaired reproduction and infertility [11]. It took a decade before specific phytochemicals were identified as the responsible xeno-estrogen. This was found to be mainly formononetin, which is biotransformed to equol via daidzein by bacterial metabolism in the digestive tract. Also Biochanin A was identified, which is the precursor of genistein. Numerous examples followed of cattle being affected by dietary exposure to so called phyto-estrogens. In the 1960s, with her book "Silent Spring" R. Carson drew first public attention to the fact that also synthetic chemicals could have endocrine disrupting properties [19]. This book reviews scientific proof that at that time the commonly used insecticide dichloro-diphenyl-trichloroethane (DDT) had deleterious reproductive effects on wildlife [29, 116]. Another book entitled " Our Stolen Future" is the most important follow-up and was meant to revive the public awareness of endocrine disruptors again [24].

The described above led to speculations about the effects of EAC on human health and in 1958 Dr. R. Hertz may have been the first to describe concerns of consequences for the environment. In addition, human consumption of products of cattle treated with hormones raised concern for

human health [44]. Nowadays, there is a continuing debate on whether and how EACs are involved in decreased sperm quality, impaired fertility, disruption of the development of the male reproductive system and increase the incidence of breast cancer. Indeed, some of the described effects have also been observed in controlled experimental settings at relatively high exposure levels to animals, but scientific evidence for (adverse) effects on human health at human relevant exposure levels is still scarce and remains controversial. Clearly, (extremely) high exposure levels tested in laboratory settings or observed at highly polluted sites can affect the endocrine system and cause reproductive, developmental and oncological effects in various species, including humans. Presently, there is no general agreement on whether these effects also occur at low, more realistic exposure levels.

A great number of experimental approaches have emerged to study effects of EAC. Some study the molecular structure of an undetermined compound and look for similarities in compounds known to establish effects, called quantitative structure activity relationships (QSARs). Some laboratory assays investigate the binding of substances to ERs in the (*in situ*) competitive binding affinity assay. However, it has been shown that binding affinity itself is not very predictive for biologic potency (e.g. proliferative response in cells or complement C3 production) [126]. Other assays study affinity to and activation of ER in e.g. yeast cells with transfected (human) ER YES-assay. Activations studied in human adenoma carcinoma mammary MCF-7 cell (ERα+) proliferation assay (E-screen) [121, 122] or rat endometrial adenocarcinoma RUCA-I cell line (expression complement C3) [115, 134] are based on the same principle, but in a more relevant (mammalian) context. In addition, the uterotrophic assay conducted in prepuberal or ovariectomized adult female rat or mouse studies the *in vivo* activation of ERα. All these assays mainly focus on direct effects of xeno-estrogens with the ERα.

Furthermore, several systems have been developed that investigate more indirect effects of xeno-estrogens. A key enzyme in the synthesis of estrogens is aromatase. Its activity can be measured indirectly using the tritium-water-release-assay [81]. These *in vitro* assays may involve aromatase isolated from (human) placental or ovary tissue, but also whole cell systems have been used such as human adenocarcinoma cells (H295R), human choriocarcinoma cells (JEG-3) and human embryonic kidney cells (HEK293) transfected with the human aromatase gene [2, 70, 104, 108]. More realistic assays were developed in the last decade and use aromatase in primary cells derived from human tissue such as subcutaneous adipose tissue [18, 90]. The cells primarily responsible for this extra-glandular conversion of androgens to estrogens are the fibroblast [1, 16, 51].

Recently, (*in vitro*) models were developed that can be used to study the direct and indirect effects of compounds. An example is the co-culture system in which MCF-7 cells are cultured on top of human primary cultured mammary fibroblasts [43, 52].

Besides the panel of assays that can be used to investigate short term effects of EACs, one of the most challenging features of EACs that remains to be elucidated are their effects that may not show immediately but with a delay after exposure.

Endocrine Active Chemicals

The list of (potential) EACs is large and increasing. The following groups of compounds have been identified as possible EACs that can affect estrogen homeostasis and a number of these have played an important role in the research done for this thesis: alkylphenols, benzophenones, parabens, phthalates, phytochemicals, polycyclic musks and organochlorines (pesticides). A common, and important, structural feature of compounds that bind ERs is the possession of a hydroxy-group on an aromatic ring.

Alkylphenols

Alkylphenol polyethoxylates are applied as non-ionic surfactants in industrial and domestic detergents, paints, pesticides, cosmetics and antioxidants in plastics. Breakdown products, free alkylphenols, are formed in by bacteria in the environment. Alkylphenols formed in sewage treatments are then drained

into the surface water and consequently have been detected therein, in sediment and aquatic organisms. Another exposure route of alkylphenols may be their formation by dermal bacteria from leftovers of detergents present in clothing. Although suspicion of estrogenic effects by alkylphenols was already raised in 1938, direct interaction with ERs was first shown by Mueller and colleagues [93]. Soto *et al.* reported cell proliferative effects in MCF-7 breast cancer cells of these compounds [120].

Benzophenones and UV-screens

Benzophenones primarily block ultraviolet radiation type B (UV-B: 280-320 nm) and to a minor extent UV-A (320-400 nm). These compounds are used to prevent damage induced by UV-radiation. One of the most widely used benzophenones is the derivate 2,2',4,4'-tetrahydroxybenzophenone (BP-2), but also 3-Benzylidene camphor (3-BC) and 4-Methylbenzylidene camphor (4-MBC) are frequently added as UV-screen. Nowadays, these compounds are used in sunscreens, cosmetics, clear plastic or glass food packaging, perfumes and soaps. Usually, several UV-filters are combined in one product. Human exposure is mainly via dermal use of a product. Other sources of exposure are food in which benzophenones leak from the wrapping material. Due to their lipohilic character these benzophenones can also bioaccumulate in fatty tissue, thus also the fatty tissue meant for human consumption. As a result, parent compounds as well as metabolites (dihydroxybenzophenones) have been found in urine, human milk and wildlife samples. Initially, the toxicity of benzophenones was considered to be low. Recently, UV filters have been shown to be estrogenic in several *in vitro* assays [89, 111]. In addition, *in vivo* studies showed that in rats exposed to 1.5 mg benzophenone/kg or higher estrogenic responses were induced [110-112]. Human (dermal) exposure was estimated to be 100 mg UV-screens/day per person, primarily in summer periods [15]. *In vitro* experiments have also shown that these UV-filters can act in an additive way [53].

Bisphenol A

Bisphenol A (BPA), member of a group of bisphenol analogues, is also known to be estrogenic and was one of the first recognized synthetic estrogens [33]. Nowadays BPA is used primarily as raw material for polycarbonate plastics and epoxy resins (adhesives, coatings and primers) and is added as plasticizer or antioxidant in many plastic types (e.g. polyvinyl chloride (PVC)). Exposure of humans to BPA occurs primarily via food that has been in contact with food packaging containing BPA. In addition, exposure occurs via the environment and some dental resins that contain BPA [67]. Bisphenol A migrates into food, for example in canned food and beverages that has been heated inside the package. In general, exposure levels in the European Union (EU) are calculated to be about 0.4-1.6 µg/kg body weight which is below the acceptable daily intake (ADI) of 10 µg/kg body weight. The presence of (increasing) levels of BPA in human and wildlife samples is reason for concern. These levels possibly derive from exposure levels close to levels of 0.025-0.2 µg/kg bw per day that were found to cause various (adverse) effects in laboratory animals [22, 67, 85]. The binding affinity of bisphenol is higher for ERβ than for ERα, as is the case for most xeno-estrogens [103]. *In vitro* as well as *in vivo*, estrogenicity has been demonstrated [68, 97]. In addition, BPA is suspected to affect also other endocrine systems such as the thyroid, androgen, immune and central nervous system [142].

Parabens

These esters of *p*-hydroxy benzoic acids are lipophillic compounds, highly resistant to hydrolysis, effective as an antiseptic in a wide temperature and pH range, colorless, odourless and with low acute toxicity. Therefore parabens are very suitable as bactericide and fungicide and commonly used as preservatives in cosmetics, food and pharmaceutics [105]. To obtain higher efficiency several parabens are combined in one product. Parabens that are approved as food additives in Europe are methyl-, ethyl- and propylparaben (E218, E214, E216) as well as their sodium salts (E219, E215, E217). The use of parabens is considered relatively safe, since chronic exposure in rats did not show any adverse

health effects [38]. In addition, parabens are considered to be rapidly absorbed, metabolized and excreted and not to accumulate in the body [119]. However, more recent studies demonstrated that parabens weakly bind and activate ERs and that their potency increases with the size of the alkyl group [17, 101, 102]. *In vivo* (uterotrophic assay) studies found that orally administrated parabens were inactive, in contrast to subcutaneous administration. Another *in vivo* study showed that the main metabolite of parabens (*p*-hydroxybenzoic acid) was estrogenic [80]. Two recent studies detected parabens in human breast tissue [46, 64]. As a result it was suggested that these parabens are correlated with breast cancer [25, 26]. However, this strong suggestion has been criticized [46, 49, 64].

Pharmaceuticals

Commonly used human and veterinarian pharmaceuticals, such as cimetidine, fenofibrate, furosemide, paracetamol, phenazone and tamoxifen, were reported to exert weak *in vitro* estrogenic effects in the mM range [40]. However, their relevance for *in vivo* and environmental situation is unclear.

Phthalates

Phthalate esters or phthalates are a large group of compounds formed by esterification of 1,2-benzenedicarboxylic acid with alkyls or aryls. The most widely used phthalates are di-2-ethyl hexyl phthalate (DEHP), diisodecyl phthalate (DIDP) and diisononyl phthalate (DINP). Other phthalates such as di-ethyl-phthalate (DEP), di-n-butyl phthalate (DBP), butyl benzyl phthalate (BBP), and di-n-octyl phthalate (DnOP) are also widely used. Due to their lipophilic properties, phthalates tend to migrate from their matrix into fatty tissues [107]. Bioaccumulation of phthalates is not likely to occur [56]. Long branched phthalates, such as di-(2-ethylhexyl) phthalate (DEHP), are especially suitable as plasticizers and used since the 1950s to increase the flexibility of PVC. Shorter branched phthalates are suitable to retain odors in perfumes or pesticide solvents. As a result, nowadays humans are exposed to phthalates from numerous sources such as nail polish, paint, medical devices, pharmaceuticals, personal care products, soft baby toys and food wrappings. Exposure primarily occurs via oral uptake (food), but also via dermal uptake (direct product contact) and inhalation uptake (air) [143]. Concern was raised in the public media by non-governmental organisations such as Greenpeace, as young children can be exposed significantly to phthalates via leaking from toys [7]. In general, exposure levels are expected to be below the ADI, which varies between 0.02-7 mg/kg/day dependent on the phthalate and the country, even for those children that have intense contact with toys containing phthalate. Yet, the concern remains, since human samples have been found to contain phthalates [56, 88, 96]. For DEHP, calculated internal levels in some children pointed towards levels that exceeded the ADI 20-fold [73]. The (adverse) health effects of exposure to phthalates are still controversial. Since long, *in vitro* tests measuring estrogenicity show that phthalates might induce estrogenic effects in hormone sensitive cells [48]. In addition, recently it was demonstrated that phthalates inhibit sulfotransferases which normally inactivate endogenous estrogens [137] and thus potentially cause an indirect estrogenic effect. Nevertheless, phthalates have been found to be not or weakly anti-estrogenic *in vivo* [68]. Other effects of phthalates have been observed such as abnormal development of genital and renal tract in boys from exposed mothers, resulting in hampered reproduction [87, 127].

Phytochemicals

Non-steroidal polyphenolic compounds produced by plants that act biologically similar to estrogens are called phyto-estrogens. These phytochemicals are divided into four main groups: lignans, flavonoids, isoflavonoids and stilbenes. Flavonoids are further divided into subclasses: coumestans, flavanones, flavanols, isoflavones, flavones, flavonols and prenylated flavonoids. Their chemical structures show similarities to that of 17β-estradiol (E_2) - especially those of isoflavonoids are striking (fig 1.2 and table 1.1).

It has been speculated that some of these phytochemicals have a role in the plant defence system against pathogens and moreover may be part of a defensive strategy to control the reproduction systems of herbivores [62, 144].

Since the late 1980s, numerous publications have emerged suggesting beneficial effects of phytochemicals on human health. This was initiated by epidemiological studies that linked regions with isoflavonoid rich diets (e.g. Asian countries) with a lower incidence of hormone-dependent cancers (such as breast cancer and prostate cancer) [3, 79, 125]. Young, prepubertal, Asian people migrating to Western countries and switching to a Western diet had a breast and prostate cancer prevalence similar to the local population, while Asian people migrating at older age had a cancer prevalence similar to the original population [28, 146]. This illustrates that the lower cancer incidence has a dietary component in addition to a genetic component. At the end of the 1990s similar suggestions were made for the consumption of lignans [4].

Various mechanisms of action for the beneficial effects of phytochemicals have been postulated, both ER dependent and independent. Indeed, phyto-estrogens bind to both forms of the ER, some with a preference for the ERβ [75] either as agonist or antagonist dependent on the dose level. In general, the estrogenic potency of these phytochemicals decreases from prenylated isoflavonoids, coumestans, isoflavonoids to flavonoids. However, some flavonoids (flavones) interact with steroidgenesis e.g. by inhibiting CYP19 (aromatase) or 17 β-hydroxysteroid dehydrogenase [71, 78]. These interactions could be considered as anti-estrogenic effects. Another estrogen related effect is the binding of phyto-estrogens to SHBG and a related increase in SHBG production, thereby influencing clearance of endogenous estrogens [84, 94]. Furthermore, non-estrogen related effects of phyto-estrogens include inhibition of tyrosine kinases, angiogensis and antioxidant properties or reduction of cholesterol levels. In addition, phyto-estrogens can induce maturation of the undifferentiated mammary gland cells to their final stage [77], well before the first pregnancy/lactation, possibly preventing formation of cancerous cells at later age. Additionally, phyto-estrogens affect the normal menstrual cycle and have been proven to prevent bone loss (osteoporosis). The many *in vitro*, *in vivo* and epidemiological studies have shown inconsistent results and the mechanisms underlying these beneficial effects is still under debate. At present there appears to be agreement on the fact that consumption of (high amounts) isoflavonoids may be preventive for breast cancer only when consumed before and during adolescence. Thus, there seems to be a relatively small time window in which exposure is beneficial from an anti-carcinogenic point of view [6, 41].

The intake of phyto-estrogens is dependent on numerous factors such as type of diet, season, plant growing conditions and food processing [42]. The main sources in the human diet for isoflavonoids (genisten, daidzein) are tofu, and to a minor extent processed products derived thereof (e.g. soy sauce), chickpea (biochanin A), and alfalfa sprouts (formononetin). Coumestans dominantly occur in veterinary foods such as alfalfa and clover (coumestrol), but are also used in the preparation of certain medical over-the-counter preparations. The potent estrogen in the group of prenylated flavonoids (prenylnaringenin) is a trace ingredient of hop (*Humulus lupulus*) [91]. Flaxseed contains the highest known level of lignans, but in a regular (European) diet the main sources are cereals, grains and whole grain products, green or black tea, berries, nuts, seeds and legumes [42]. The production of flavanones is restricted to the family of citrus fruits [39].

During the last decades (iso)flavonoids became widely available via food supplements or medicinal plant preparations containing high doses of the chemicals. These "natural" supplements are taken because of their proposed cancer chemopreventive potential, relief of postmenopausal symptoms, improvement of memory and a potential breast enhancing effect. In general, elevated exposure of humans to phyto-estrogens via an unnatural matrix form (aglycones) has raised concern [5, 63]. Especially unborn and new born infants may be more susceptible to exposure to (phyto)estrogens as they do not produce estrogens themselves. Indeed, it was found that genistein established long-term effects in target organs of mouse and rat at all developmental ages that were

probably ER-mediated [61, 92]. It is not proven, however, that continuous exposure to phyto-estrogens may increase the breast cancer risk or hormone dependent cancers.

Polycyclic musks

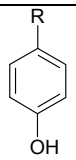
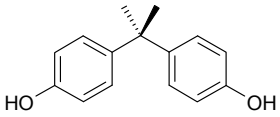
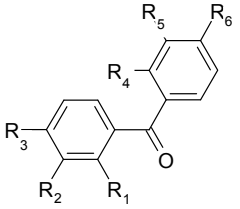
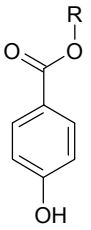
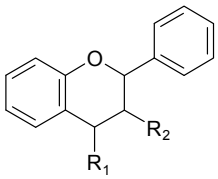
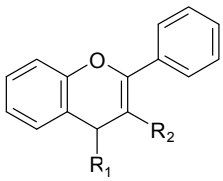
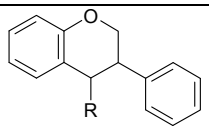
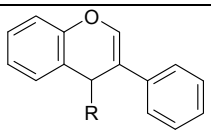
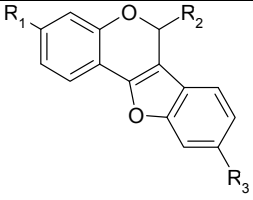
Polycyclic musks are artificial musks used in perfumes. Besides the musk odor itself, they also fixate other more volatile odors. They were discovered in 1950s and fully replaced the nitro musks in the 1980s, when it was discovered that the latter group including musks ketone and xylene, was found to bioaccumulate in the environment due to their lipophilic properties. Traces of these nitro musks, but also their amino metabolites, were detected in marine and fresh water, fish, human adipose tissue and breast milk. However, more recently polycyclic musks and their metabolites have also been found to accumulate in various aquatic organisms, but also samples of human origin contain polycyclic musks.

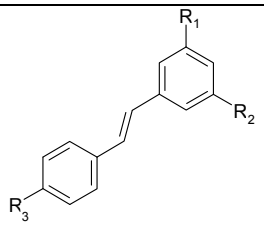
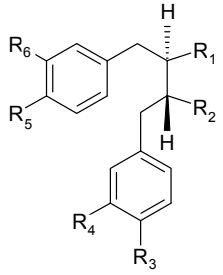
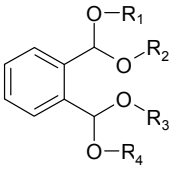
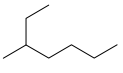
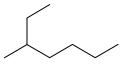
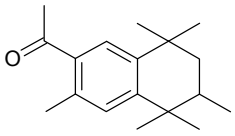
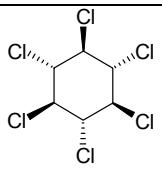
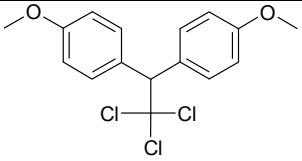
Polycyclic musks have been suggested to be possible xeno-estrogens due their polycyclic characteristics. Indeed, recent studies by Schreurs *et al.* illustrated that the polycyclic musks Tonalide (AHTN) and Galaxolide (HHCB) activate human ERs transfected in HEK293 and U2-OS, but their *in vivo* effects in a transgenic zebrafish model were predominantly antiestrogenic at internal concentrations of 15-200 mg/kg bw fish and suppressed responses of estradiol [113, 114]. These antiestrogenic levels are higher than the highest reported concentration in Dutch serum of 9.2 ng HHCB/L plasma and 11 ng AHTN/L plasma [88].

Organochlorine pesticides

Organochlorines have wide applications as pesticides. Dichloro-diphenyl-trichloroethane (DDT) was one of the first used as such, but has been banned almost everywhere since the 1960s. Due to its lipophilic properties DDT (bio)accumulates in organisms and biomagnifies in ecosystems and caused harmful effects in ecological systems [19, 109, 135]. Today, DDT has only limited use as lice or malaria mosquito killing agent in some developing countries. The mechanisms of action of DTT is to cause leakages in cell membranes which aims to disturb signal transduction of nerve cells in insects. Nevertheless, research has revealed that a particular isomer, *p,p*-DDT, as well as its breakdown product *p,p*-DDE, induces anti-androgenic responses in mice and rats. Another isomer, *o,p*-DDT and its metabolite *o,p*-DDE, have been found to be estrogenic [95]. Other organochlorines such as methoxychlor (MXC) and hexachlorocyclohexane (HCH) share their endocrine disruptive properties and are still in use as pesticides in some countries. For instance, one of the metabolites of MXC, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), has been shown to be estrogenic [117]. Some other organochlorine pesticides such as cyclodienes and chlordecone have also been found estrogenic, but their use has been banned in most countries. Many recent studies investigate the correlation between (human) exposure to DDT and the breast cancer incidence. The correlation and its underlying mechanism are still under debate, but a recent study by Cohn *et al.* convincingly points to a positive correlation of exposure to *p,p*-DDT in younger aged women and breast cancer incidence [23].

Table 1.1. Chemical structures of endocrine active compounds.

Group	Structure	R:
Alkylphenols		R: C_8H_{17} is octyl phenol [II, III] R: C_9H_{19} is nonyl phenol [II,III]
Bispehnol analogues		Bisphenol A (4,4'-Isopropylidenediphenol) [II, III]
Benzophenones		R_2: OH is 3-hydroxybenzophenone R_3: OH is 4-hydroxybenzophenone R_{1+3}: OH is BP-1 $R_{1+3+4+6}$: OH is BP-2 R_4: OH; R_6:OCH_3 is BP-3 R_5: SO_3H; R_6:OCH_3 is HMBS
Parabens		R: OH is *p*-hydroxy benzoic acid [IV] R: C_1H_3 is methyl parabens [IV] R: C_2H_5 is ethyl paraben [IV] R: C_3H_7 is propy paraben [IV] R: is isopropyl paraben [IV] R: C_4H_9 is butyl paraben [IV] R:  is isobutyl paraben [IV] R:  is benzyl paraben [IV]
Phytochemicals		R_{1+2}: H is flavan R_1: =O is flavanone (naringenin [II, III,V,VI], hesperetin) R_2: OH is flavan-3-ol (catechin [II, III], epicatechin [II, III]) R_1: =O; R_2: OH is flavanonol (taxifolin)
		R_{1+2}: H is flavonone R_1: =O is flavone (apigenin [V, VI], chrysin [V, VI], luteolin) R_2: OH is flavonone-3-ol R_1: =O; R_2: OH is flavonol (quercetin [II, III, VI], kaempferol)
		R: H is isoflavan R: =O is isoflavanone
		R: H is isoflavonone R: =O is isoflavone (genistein [II, III, VI], daidzein, glycitein)
		R_{1+3}: OH; R_2:=O is coumestrol [II,III]

		R_{1+2+3} = OH is resveratrol (stilbene)
		R_{1+2}: OH; R_{3+5}:OH; R_{4+6}: methoxy is matairesinol $R_{(1+2)}$:  ; R_{3+5}:OH; R_{4+6}: methoxy is secoisolariciresinol R_{1+2}:  ; R_{4+6}: OH is enterolactone $R_{(1+2)}$:  ; R_{4+6}: OH is enteriol
Phthalates		R_3: C_2H_5 is monoethyl phthalate R_{2+3}: C_2H_5 is diethyl phthalate [IV] R_3: C_4H_9 is monobutyl phthalate [IV] R_{2+3}: C_4H_9 is dibutyl phthalate [II, III,IV] R_1:  ; R_4: C_4H_9=butylbenzyl phthalate R_2:  is mono-(2-ethylhexyl) phthalate [IV] R_{1+4}:  is di-(2-ethylhexyl) phthalate [IV]
Polycyclic Musks	 Example: tonalide (AHTN)	Tonalide (AHTN: cas#=1506-02-1/21145-77-7) [IV] Galaxolide (HHCB: cas#= 1222-05-5) [IV] Cashmeran (DPMII: cas#= 33704-61-9) Celestolide (ADBI: cas#=13171-00-1) Phantolide (AHMI: cas#= 15323-35-0) Treasolid (ATII: cas#=68140-48-7) Versalide (AETT: cas#=88-29-9)
Organochlorine pesticides		Hexachlorocyclohexane [II, III]
		Methoxychlor [II, III]

When R is not indicated an H-atom is bound
Roman numerals in superscript point to the Chapter number that studied this compound.

Breast cancer

The most common type of cancer in women and also the major cause of cancer related death is breast cancer. Worldwide 10% of the female population encounters breast cancer during their life. In the Netherlands every year around 110/100000 women encounter breast cancer, which is one of the highest incidences in Europe [100]. Statistics demonstrate a global trend of increased breast cancer incidences, but a decrease in mortality as well (fig 1.4).

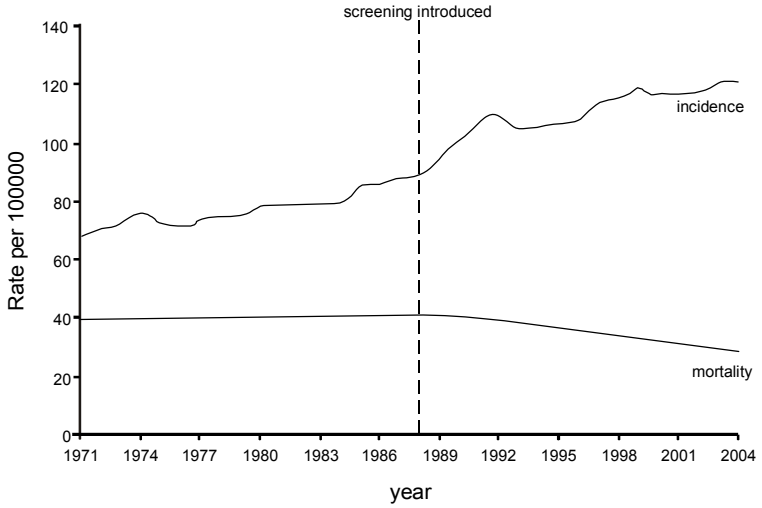


Fig 1.4. Age-standardized incidence of and mortality from female breast cancer, England, 1971-2004. Graph adapted from www.statistics.gov.uk/cci/nugget.asp?id=575.

Prepubertal mammary tissue contains undifferentiated ductal epithelial cells. These cells are under control of hormones and start to mature from the menarche onward and continue maturation until late adolescence. Proliferation and differentiation is induced at the duct termini that form the terminal end bud, which will be the origin for further elongation and branching of the ducts. The maturation process ends with a final differentiation during the first pregnancy in preparation for lactation. The undifferentiated structures are sensitive to estrogen signalling and contain potential precursor cells for mammary tumors. Many scientists hypothesize that this period of maturation is a critical time window in which developing malignant breast tumors in later life might at least partly be determined [41, 57].

It has been determined that loss of function of the BRCA1 and BRCA2 genes is responsible for only 5-10% of breast cancer cases [35]. Normally these genes are part of a DNA-repair mechanism. For other breast cancers, the etiology is complex and understanding of important regulatory factors is far from complete. Certain risk factors correlating with the occurrence of breast cancer are known. In order of importance these are age, family history, duration of premenopausal period (age of first/last menarche), age at first completed pregnancy, breast feeding, obesity, dietary intake of (fat) animal products and certain life-styles (alcohol consumption, smoking, use of cosmetics) [133]. Factors that correlate inversely with breast cancer occurrence are socioeconomic status and life-style (exercise). Some of these factors support the strong link between breast cancer and endogenous estrogens.

Indeed in the past decades, estrogens and their metabolites have been recognized as tumor promoters. They may initiate tumors as genotoxic estrogen metabolites via the formation of DNA adducts [20, 58]. Whether estrogenic activity reduces or increases breast cancer risk remains speculative [129]. However, around 50-80% of the breast tumors express ERα and are dependent on estrogens for their growth [124]. Strikingly, the majority of these estrogen sensitive tumors occur in postmenopausal women, whose endogenous production by ovaries has ceased. Androgens, however, are still produced

by the adrenal gland and ovaries and are further aromatized by peripheral tissues such as muscle and adipose tissue. This estrogen synthesis plays an important role in the postmenopausal period and this local production was found to contribute to E_2 levels in breast tissue similar to those found in premenopausal women due to local production [132]. Aromatase expression and activity in malignant breast tumors or surrounding tissues were found elevated compared to healthy breast tissue [21]. Reed *et al.* proposed a breast cancer model in which adipocytes, stromal cells, mammary epithelial tumor cells are interacting, as well as with infiltrated macrophages and lymphocytes [98]. Cells in the stroma, including fibroblasts, are recruited and activated by the tumor. In healthy fibroblasts, the gene CYP19 (coding for aromatase) is expressed via promoter region I.4, which is regulated by glucocorticoids and class I cytokines such as TNF-α and IL-6. However, in malignant mammary tumors a promoter switch occurs from I.4 to I.3 and pII [118, 145]. The two latter promoter regions are regulated by prostaglandins. Prostaglandines are excreted by tumor cells and induce production of cAMP (via protein kinase A) and phorbol esters (via protein kinase C). Estrogens excreted from the fibroblasts in the stroma cells stimulate ERs in the hormone sensitive epithelium cells. Taken together, these factors form a local positive feedback loop within breast tissue promoting tumor cell proliferation.

Earlier, several *in vitro* and *in vivo* models were introduced and discussed that investigate estrogenic potencies of compounds. The *in vitro* MCF-7 proliferation assay is regarded as a breast cancer cell model, as it involves human breast epithelial ERα positive cells. Obviously, this cell line represents only one part of the proposed *in vivo* breast cancer model. More recent studies involved fibroblasts isolated from healthy primary mammary tissue that were co-cultured with MCF-7 cells. This model mimics actual interaction between tumor cells and surrounding cells more closely than MCF-7 cells alone. Therefore, this co-culture model is suitable for investigating direct and indirect estrogenic effects of compounds [52].

In the treatment of estrogen sensitive breast cancer an endocrine therapy can be applied as a more convenient alternative to chemotherapy. Two types of endocrine therapy exist: (i) ER-blockage with ERα antagonists, and (ii) aromatase inhibition. Since the early 1970s, endocrine therapy with tamoxifen (a non-steroidal ERα antagonist), has been used as a first-line therapy for breast cancer as it acts anti-estrogenic in breast tissue. However, tamoxifen also has agonistic properties in endometrial tissue and bone tissue. As a result this SERM increases endometrial cancer risk, but is protective against osteoporosis [30]. Raloxifen (another non-steroidal compound) was developed more recently and is also used for the treatment of osteoporosis in postmenopausal women. Since 1997 this compound is allowed for treatment of breast cancer – it acts anti-estrogenically in several mammary tumor models and does not exert negative side effects such as increased incidence of uterine or endometrial cancer [54]. However, raloxifen has proven to be less effective than tamoxifen in some cases. Another novel compound, ICI 182,780 (faslodex/fulvestrant; a steroidal analogue) also strongly binds to ERs and hampers dimerization, resulting in rapid degradation and reduced receptor levels in the cell. ICI is regarded as a pure anti-estrogen that merely elicits ER antagonistic effects, while it lacks ER agonistic effects which may increase the risk of osteoporosis [60]. The use of fulvestrant has been approved since 2002 in the USA and since 2004 in the EU as a second-line treatment of breast cancer in postmenopausal women.

In postmenopausal breast cancer patients, estrogen synthesis can be blocked through the inhibition of aromatase. This is achieved by treatment with suicidal aromatase substrate analogues (steroidal structured, type I) or competitive aromatase binders (non-steroidal structured, type II). Most type I inhibitors are compounds that need to be activated by aromatase. The intermediate formed binds specifically and covalently to the enzyme, which then becomes unavailable for androgens. The possible side effects of type I inhibitors, such as 4-OH androstenedione and exemenstane, are concurrent binding to ER and the AR inducing androgenic effects. Type II, non-steroidal structured inhibitors lack such side effects. The first type II inhibitor, aminoglutethimide (AG), has low specificity and requires such high and frequent pharmacotherapeutical dosages that

also other steroidogenic pathways are inhibited (medical andrenalectomy), which causes many side effects [9]. The second generation type II inhibitor, fadrozole, has an improved specificity but is less effective. The third generation aromatase inhibitors, include anastrozole (second-line therapy), letrozole and vorozol, which are trizole compounds having a high inhibitory potency [9]. Both type I and type II aromatase inhibitors have been found to reduce E_2 levels in tumor breast and its surrounding tissue and locally inhibit biosynthesis of E_2 [27]. One of the noticeable disadvantages of AIs, compared to tamoxifen, is that depletion of E_2 in the circulation may increase risk of osteoporosis.

Mixtures & mixture effects

Many compounds have been identified to have endocrine or, more specifically, estrogenic activity. In case of estrogenic compounds and ER activation, biologically active concentrations are 10^3 to 10^6 times higher than that of E_2. Although their potency is much lower than that of the natural ligand, the occurrence of (adverse effects) will depend on the systemic concentration of a compound. Obviously, humans and other living organisms generally are exposed to a large variety of chemicals. Several were described here to act via ERs. Nevertheless, experimental information on the effects of combined exposure to estrogenic chemicals is scarce, let alone at exposure levels relevant for humans.
There are three major theoretical concepts for the prediction of effects of chemicals in a mixture on the basis of the effects of its single constituents: (i) effect summation, (ii) effect multiplication and (iii) concentration addition.

In effect summation, it is assumed that two compounds (A and B) at concentrations C_A and C_B induce a similar type of effect but quantitatively different (E_A and E_B). The predicted effect of a mixture A and B (E_{A+B}) is the sum of the effects (E_A + E_B). Generally, effect summation is only applicable when individual compounds exert linear dose-response relations [12].

With effect multiplication, or independent action, E_{A+B} is predicted by multiplication of E_A and E_B (E_A x E_B) [13]. This concept assumes that the mixture constituents mediate their effect via different modes of action. This is valid when individual compounds exert simple exponential dose-response relations. However, this approach is of no use for mixtures that mediate their effect via the same receptor. The mathematical formulation of this concept:

$$E(Cmix) = 1 - \prod_{i=1}^{n} E(Ci) \qquad (1.1)$$

where $E(C_{mix})$ is the predicted effect of the mixture, C_i is the concentration of the i^{th} mixture constituent and $E(C_i)$ is the effect of this constituent when applied alone.

The basics of the concentration addition concept originates from Loewe and Muischnek [83]. In this concept, C_A inducing an effect E is compared to C_B inducing an equally strong effect E and therefore C_A and C_B are named iso-effective concentrations. Studies described in this thesis used two approaches to predict effects of mixtures, both derived from the concentration addition concept namely the method of isoboles and the estrogen equivalent (EEQ) approach. If the observed response exceeds the predicted response, this is referred to as a synergistic interaction, while a response smaller than predicted is referred to as an antagonistic interaction. In case the observed response meets the predicted response, this is referred to as zero interaction or additive effect. The algebraic notation of the method of isoboles is the equation:

$$\sum_{i=1}^{n} C_{(i)} / EC_{x(i)} = 1 \qquad (1.2)$$

where *n* is the number of mixture constituents, $C_{(i)}$ is the concentration of the *i* th mixture constituent at the (total) concentration of the mixture inducing *x*% of the maximum response. $EC_{x(i)}$ is the concentration of the *i* th mixture constituent singly inducing *x*% of the maximum response. If the outcome has the value one, the mixture constituents act in an additive manner. Values of this summation smaller than one indicate synergistic interaction, while values larger than 1 point to antagonistic affects. As an example, imagine a mixture of two compounds (A and B), tested at various ratios all inducing x% effect. When C_A is plotted against C_B on a linear scale, all ratios will be positioned on a straight line connecting C_A with C_B inducing x% effect (fig 1.5, line I). In case of an antagonistic interaction this line will be concave up (fig 1.5, line II), while in case of synergistic interactions this line will be concave down (fig 1.5, line III). The processing of (virtual) experimental data is illustrated for a mixture composed of compound A and B for the method of isoboles (table 1.2 and fig 1.5).

Table 1.2. Interaction calculated with the isoboles-method for a combination of compound A+B at their EC_{50}.

			Line I		Line II		Line III	
	conc. in mix[1] nM	D EC_{50} nM	d($mix_{additive}$) conc. at EC_{50} nM	d/D	d($mix_{antagonistic}$) conc. at EC_{50} nM	d/D	d($mix_{synergistic}$) conc. at EC_{50} nM	d/D
compound A	5	1	0.5	0.5	5	5.0	0.1	0.05
compound B	50	10	5	0.5	50	5.0	0.5	0.05
				sum		sum		sum
$mix_{additive}$	55	5.5		1.0				
$mix_{antagonistic}$	55	55				10.0		
$mix_{synergistic}$	55	0.55						0.1

[1] = concentrations of the compounds in the stock-mixture.

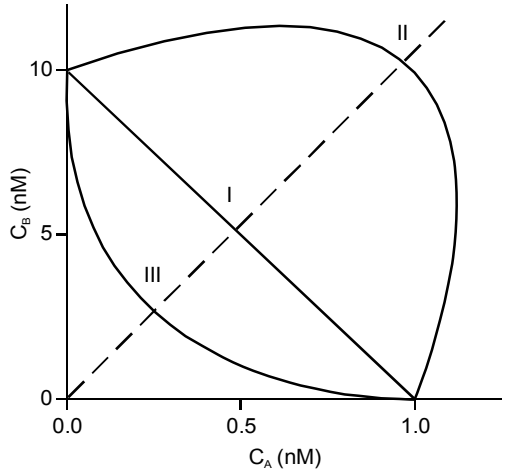


Fig 1.5. The concentration of compound B plotted against the concentration of compound A that induce 50% of their maximal effect (iso-effective concentrations) at three different interactions: I) no interaction, II) antagonistic interaction and III) synergistic interaction.

The second approach, the EEQ concept, was developed and first applied in a study calculating the estrogen body burden due estrogenic compounds in human serum [123] and is analogous to the TEQ (toxic equivalents) concept which was developed to calculate the toxicity of combined dioxin like compounds [130].

The EEQ approach principally facilitates the calculation of the response of a biological system to simultaneous exposure of similar acting chemicals, but exerting differences in potencies in the biological system. This overall response is then calculated by the summation of the potency of each

compound multiplied by its concentration. The algebraic notation to calculate the potency of compounds is the equation:

$$EEF_{(i)} = EC_{x(i)} / EC_{x(E2)} \quad (3)$$

where $EC_{x(i)}$ is the concentration of the i^{th} mixture constituent singly inducing x% of the maximum response and $EC_{x(E2)}$ is the concentration of E_2 singly inducing x% of the maximum response. The EEQ (estrogen equivalent) is then calculated by the equation:

$$EEQ_{(i)} = EEF_{(i)} \times C_{(i)} \quad (4)$$

where $C_{(i)}$ is the concentration of the i^{th} mixture constituent at the (total) concentration of the mixture inducing x% of the maximum response. The estrogen equivalents for combined compounds calculated from *individual compounds* is written in the equation:

$$EEQ_{calculated\_of\_mix} = \sum_{i=1}^{n} EEF_{x(i)} \times C_{(i)} \quad (5)$$

were n is the number of mixture constituents, $C_{(i)}$ is the concentration of the i^{th} mixture constituent at the (total) concentration of the mixture inducing x% of the maximum response. $EC_{x(i)}$ is the concentration of the i^{th} mixture constituent singly inducing x% of the maximum response.

The estrogen equivalents for combined compounds calculated from *observed data* is written in the equation:

$$EEQ_{observed\_of\_mix} = EC_{x(mix)} / EC_{x(E2)} \quad (6)$$

The processing of (virtual) experimental data is illustrated for a mixture composed of compound A and B for the EEQ concept (table 1.3 and fig 1.6).

The EEQ approach requires the constituents of the mixture to exert equally shaped dose response curves. This comprises curves with a similar efficacy being parallel to each other and the reference compound. In contrast, the method of isoboles lacks these prerequisites.

Table 1.3. EEFs and EEQs calculated for compound A and B and for a combination of A+B.

	conc. in mix	EC_{50}	EEF		EEQ	
	nM	nM				
E_2	0	10^{-3}	1.0		$5*10^{-3}$	
compound A	5	1	10^{-3}		$5*10^{-3}$	
compound B	50	10	10^{-4}			
				EEF x conc.	sum EEQ	
$mix_{additive,calculated}$	55				10^{-2}	
$mix_{additive,observed}$		5.5	$1.8*10^{-4}$	10^{-2}		Line I
$mix_{antagonistic,observed}$		55	$1.8*10^{-5}$	10^{-3}		Line II
$mix_{synergistic,observed}$		0.55	$1.8*10^{-3}$	10^{-1}		Line III

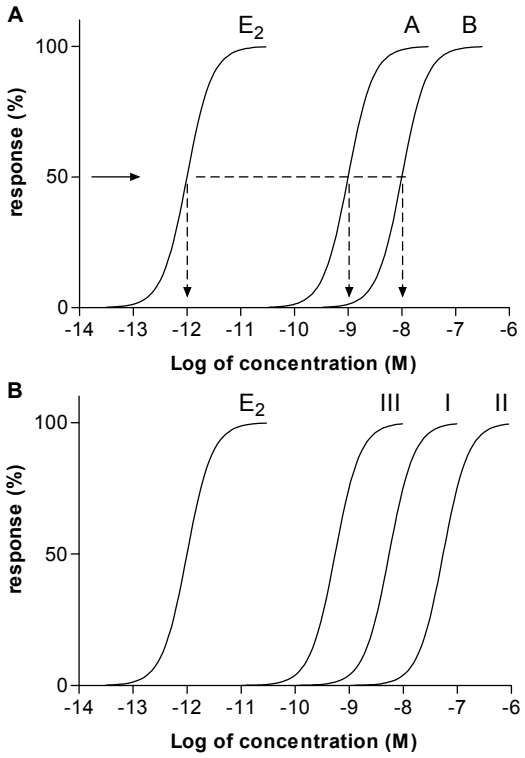


Fig 1.6. Individual dose response curves of Compound A, B and the reference compound (E_2) with arrows indicating how EC_{50}s are determined (**A**) and dilutions of compound A and B combined at a fixed ratio with additive (I), antagonistic (II) or synergistic (III) interaction (**B**).

This thesis

This thesis describes a series of *in vitro* and *in vivo* experiments, which are aimed at an estimation of the risk for humans of regular exposure to (mixtures of) exogenous (anti-)estrogens.

In **Chapter 2**, ingredients of a regular diet were combined and tested *in vitro* for mixture effects with estrogenicity as an endpoint, measured through cell proliferation and pS2 gene expression in MCF-7 cells. Combinations tested were mixtures of phytochemicals or synthetic chemicals, additionally tested in combination with 17β-estradiol (E_2). Results were analyzed with the two concentration addition approaches, namely the isoboles and estrogen equivalency methods.

Chapter 3 describes an *in vivo* study with a similar approach as chapter two, but using the blotted uterus weight of immature rats as endpoint for estrogenicity.

In **Chapter 4**, additives commonly used in cosmetics were tested *in vitro* for estrogenic effects in MCF-7 cells. Additionally, several mixtures of two parabens were composed, as well as combinations of individual parabens with 17β-estradiol (E_2), tested and analyzed for mixture effects in concentration

addition models. Furthermore, the aromatase inhibiting properties of parabens in human placental microsomes are described. The results obtained, supplemented with data on exposure and estrogenic potencies from literature, were used to assess (total) human exposure to estrogen equivalences.

Chapter 5 compares aromatase inhibition induced by synthetic lactones, some flavonoids or a pharmaceutical aromatase inhibitor used in breast cancer treatment (fadrozole) in human placental microsomes with that in fibroblasts isolated from healthy human mammary tissue. This comparison allowed an evaluation of possibilities of using microsomes as a fast tool to study aromatase inhibition in human target cells, such as connective tissue surrounding breast tumor cells.

Chapter 6 investigates the overall effect on estrogen sensitive human mammary adenocarcinoma cells of the dualistic character of some phytochemicals. In separate systems, estrogenic effects in MCF-7 cells, as well as the aromatase inhibiting effect of these compounds in healthy human primary mammary fibroblasts were tested. Ultimately, estrogenic and aromatase inhibiting properties were evaluated in a co-culture system with both cell systems.

Chapter 7 combines an overview of conclusions of previous chapters with a general discussion on total estrogen burden of humans by combined exposure to (anti-)estrogenic compounds.

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Chapter 2

Mixture effects of estrogenic compounds on proliferation and pS2 expression of MCF-7 human breast cancer cells

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Abstract

Humans are exposed to a variety of food-borne phytochemicals (PC) as well as synthetic chemicals (SC). Some of these compounds have been reported to have estrogenic or anti-estrogenic properties and are therefore suspected endocrine disruptors. Until now it remains unclear if non-additive effects occur in combinations with endogenous estrogens, such as 17β-estradiol (E_2). To investigate such interactions, several PC and SC were tested individually, in mixtures and as combinations of mixtures with E_2 for effects on ERα receptor mediated cell proliferation and estrogen regulated pS2 expression level in MCF-7(bus) cells. PCs (coumestrol, genistein, naringenin, catechin, epicatechin, quercetin) or SCs (4-nonylphenol, octylphenol, β-hexachlorocyclohexane, bisphenol A, methoxychlor, dibutyl phthalate) were mixed (PCmix and SCmix) either in concentrations reflecting human serum concentrations or at equipotent concentrations for estrogenicity. EC_{50} values were applied in two approaches of the concentration-addition model (the method of isoboles and the cumulative estrogen equivalency method) to assess mixture effects. In both models PCmix and SCmix or combinations of the mixtures with E_2 showed no departure from additivity. In conclusion, the tested PCs and SCs appeared to act as (full) agonists for the estrogen receptor and interacted in mixtures and with estradiol in an additive way. In addition it is concluded that the possible contribution of food-borne PCs to the estrogenic effect of xenobiotics is likely to be more significant than that caused by food-borne SCs.

Introduction

A normal diet exposes its consumer to a wide variety of chemicals that can be partly natural (e.g. phytochemicals) as well as anthropogenic. During the last decades many studies using estrogen receptor (ER) dependent *in vitro* and *in vivo* assays identified chemicals that can elicit estrogen like effects [43, 47]. Collectively, these chemicals are known as xenoestrogens. Binding of these estrogenic xenobiotics to ERs can result in complex cellular (anti-)estrogenic responses (e.g. cell proliferation) depending on both structure and concentration of the compound [28, 45, 49, 50, 55, 73].

Interest in phytochemicals (PCs), such as genistein, started in the 1980's after findings of epidemiological studies that compared Western and Asian diets. These results indicated that a diet with high PC content may give some prevention against predominant Western diseases, such as cardiovascular diseases, hormone-related diseases (breast and prostate cancer) and postmenopausal symptoms in women (osteoporosis, hot flashes) [1, 12]. At present several mechanisms underlying this apparent beneficial effect of PCs have been suggested, amongst which that some PCs may (i) specifically bind and activate ERβ more than ERα, although generally with less affinity than 17β-estradiol (E_2) [36-38] (ii) act as a SERM (selective estrogen receptor modulator) [20] (iii) reduce ER expression [15, 44, 59], (iv) compete as agonist with endogenous estrogens on the ER, (v) induce apoptosis [48], (vi) lower estrogen levels in circulation [31], (iv) reduce angiogenesis and tumor invasiveness [15, 42] and (vii) scavenge radicals [8, 46, 76].

As a consequence of these suggested beneficiary effects, the availability of phytochemical-containing food supplements has increased on the market with applications ranging from 'natural' alternative treatment to prevent Western diseases to natural methods of breast enhancement [17, 24, 71]. This phytochemical load comes on top of phytochemicals present in regular diet (vegetables, fruits and diary products).

Concern that chemicals of industrial origin may elicit endocrine disrupting effects has been raised as early as the 1960s [11, 16] and since then a wide variety of synthetic chemicals (SCs) have been shown to be weakly estrogenic [66]. More recently, the proposed endocrine disrupting properties of these chemicals have been suggested to be involved in an increased prevalence of hormone-dependent diseases [72].

The major routes of exposure for humans to these estrogenic synthetic chemicals are assumed to be via the regular diet of food containing added antioxidants, compounds leaking from food wrapping materials and residues of pesticides (vegetables, fruits and cow products) [4, 26, 58].

Thus, a normal human diet already results in exposure to a complex mixture of xenoestrogens resulting in systemic circulation in the body. With respect to possible mixture effects some studies suggest that non-additive effects might occur for combinations of synthetic estrogens. In the human breast carcinoma MCF-7 cells, with cell proliferation used as a measure for estrogenicity, synergistic effects have been reported for combinations of benzylbutyl phthalate, bisphenol A (BPA), dichlorodiphenyldichloroethylene (DDE) and hexachlorobenzene (HCB) [67] and mixtures of E_2 with BPA or pentachlorophenol [69]. In contrast, antagonistic effects have been reported from mixture experiments with nonyl- and octylphenol [54] and E_2 with benzylbutyl phthalate [69] in the same *in vitro* cell system. Suzuki and coworkers find additive effects for pentachlorophenol and methoxychlor. Also studies using recombinant yeast estrogen screen (YES), utilizing yeast expressing human ERα, show additive effects for multi-component mixtures of certain xenoestrogens (phenols, genistein, parabens etc.) [63]. Additivity was also found for mixtures of E_2, diethylstilbestrol (DES), and ethynyl estradiol (EE) in a MCF-7 system transfected with an ERα-responsive reporter gene [13].

Thus the effects of mixtures, especially combinations of PCs and SCs at relevant human levels, remain inconclusive. However, in view of the suggested adverse effects of estrogenic chemicals to human and wildlife health it is important for the risk assessment process to establish the types of mixture interactions that may occur among these "dietary" chemicals.

In our present study with mixtures of phyto- and synthetic estrogens, the simple and sensitive *in vitro* assay with MCF-7(bus) cells highly expressing ERα [27] was used to assess the type of mixture

interaction between these two groups of compounds close to concentrations found in human blood. Two markers for estrogenicity (cell proliferation and pS2 expression) that are commonly used were applied to measure effects of individual compounds and mixtures of PCs, SCs and E_2 at various ratios. Mixtures of PCs (PCmixes) consisted of genistein, catechin, epicatechin, coumestrol, naringenin and quercetin. Mixtures of SCs (SCmixes) consisted of 4-nonylphenol, β-hexachlorocyclohexane, bisphenol A, 4-octylphenol, methoxychlor and dibutyl phthalate. Two approaches of the concentration addition model [40] were applied to analyze mixture interactions and potencies: The method of isoboles [35] and the cumulative estrogen equivalency (EEQ) method [67].

Materials and Methods

Chemicals & Plastics

The test compounds 17β-estradiol (E_2), (+,-)catechin (CAT), (-,-)epicatechin (EPC), quercetin (QUE), genistein (GEN), (+,-)naringenin (NAR), bisphenol A (4, 4'-isopropyl-idenediphenol) (BPA) and dibutyl phthalate (DBP) were purchased from Sigma (St. Louis, MO, USA). Coumestrol (COU) was purchased from Fluka (Zwijndrecht, The Netherlands). β-hexachlorocyclohexane (HCH) and methoxychlor (MXC) were purchased from Riedel-de-Haën (Zwijndrecht, The Netherlands). 4-Octylphenol (OP) was purchased from Aldrich (Zwijndrecht, The Netherlands). 4-Nonylphenol (NP) was purchased from Acros Organics (Den Bosch, The Netherlands). We tested the direct interaction of these compounds with ERα by combining these chemicals with the synthetic ER down-regulator fulvestrant (ICI 182780, faslodex), obtained from Tocris (Bristol, UK) and the ERα antagonists raloxifen (kindly provided by R. Schreurs, Utrecht, The Netherlands) and tamoxifen (Sigma). Compounds were dissolved in DMSO (Sigma) except OP, NP, HCH, DBP, ICI 182780 and raloxifen which were dissolved in ethanol (Riedel-de Haën), and stored at -20°C prior to use. Dilutions from stocks were used in our experiments in which the vehicle concentration did not exceed 0.2%. Tissue culture plastics were purchased from Greiner (Alphen a/d Rijn, The Netherlands). Dulbecco's modified Eagles medium (DMEM), fetal calf serum (FCS) and supplements were from Gibco (Breda, The Netherlands), except sodium pyruvate ($C_3H_3NaO_3$), which was purchased from Sigma.

Mixtures

Seven mixtures were prepared for the experiments and their compositions are given in tables 2.1 and 2.2. Four PCmixes were composed by mixing several phytoestrogens (COU, GEN, NAR, CAT, EPC and QUE) known to be present in various amounts in a regular human diet and human plasma. A first mix (PCeq-mix) was composed with estrogenic compounds in equipotent concentrations based on the relative estrogenicity of the individual PCs, while concentrations of non-estrogenic PCs where based on human plasma concentrations of these PCs estimated from literature values. In this way none of the individual compounds contributed disproportionately to the overall estrogenic effect of the mixture. Then three PCpl-mixes were composed. PCpl-mix(1) was based on human plasma concentrations of COU, GEN, NAR, QUE, CAT, EPC that were estimated from literature values (table 2.1), except for COU. The concentration of COU was extrapolated from 0.001 mg/d intake [6] to a corresponding concentration of 10 pM, based on comparable data about intake and resulting plasma concentration for NAR (10 mg/d; ± 100 nM) and assuming similar kinetics. PCpl-mix(2) was made of proliferative compounds (COU, GEN, NAR), while PCpl-mix(3) was made of non-proliferative compounds (QUE, CAT, EPC) (table 2.1).

Three SCmixes were composed by mixing several synthetic estrogens (NP, HCH, BPA, OP, MXC, DBP). SCeq-mix(1) was composed in such a way that each estrogenic compound was present in an equipotent concentration based on the relative estrogenicity of the individual SCs.

Table 2.1. Data of the experimental conditions, composition of the mixtures and reported human intake or serum levels of the phytochemicals tested.

compound (MW)	test range MCF-7 cell proliferation assay	[1x mix][a]	[1x mix][a]	[1x mix][a]	[1x mix][a]	reported humane intake (mg/d)	reported human serum conc.
endogenous 17β-estradiol (E_2) (272.39 g/mol)	0.3 – 1E3 pM						
PCmixes		PCeq-mix	PCpl-mix(1)	PCpl-mix(2)	PCpl-mix(3)		
Coumestrol (COU) (268.23 g/mol)	1E-5 – 1E-2 µM	100 pM	10 pM	10 pM	0	0.001[6]	10 pM(estimated)
Genistein (GEN) (270.24 g/mol)	1E-3 – 1E0 µM	30 nM	500 nM	500 nM	0	0.16 – 0.42[6]; 31.4[75]	<15[19] – 1039 nM[25]
(+,-)naringenin (NAR) (272.26 g/mol)	1E-2 – 3E1 µM	300 nM	100 nM	100 nM	0	3 – 29 [23]	4.8 [23] – 112.9 nM
Quercetin (QUE) (338.27 g/mol)	1 – 100 µM	50 nM	50 nM	0	50 nM	16[29]; 11[23]; 3 – 24[30]	<26.8[23] – 1340 nM[5, 60]
(+,-)catechin (CAT) (290.27 g/mol)	1 – 1E3 µM	70 nM	70 nM	0	70 nM	3 – 34[60]	0.072 µM[60]
(-,-)epicatechin (EPC) (290.27 g/mol)	1E-2 – 1E2 µM	250 nM	250 nM	0	250 nM		0.27 µM[60]
total concentration		700.10 nM	970.01 nM	600.01 nM	370.00 nM		
test range MCF-7 cell proliferation assay		0.1 – 100x	0.01 – 10x	0.1 – 10x	0.003 – 10x		

[a] = concentration of a compound in the 1x mixture when diluted in SF-medium.

Two additional SCmixes were made of (i) compounds that generally did not attain a maximum proliferate effect (SCeq-mix(2)) and (ii) compounds that generally did attain a maximum effect (SCeq-mix(3)) (table 2.2).

Table 2.2. Data of the experimental conditions and composition of the mixtures of the synthetic estrogenic chemicals tested.

compound (MW)	test range MCF-7 cell proliferation assay	[1x mix][a]	[1x mix][a]	[1x mix][a]
endogenous 17β-estradiol	0.3 – 1E3 pM			
SCmixes		SCeq-mix(1)	SCeq-mix(2)	SCeq-mix(3)
4-octylphenol (OP) (272.4 g/mol)	0.001 – 1 µM	0.5 nM	0.5 nM	0
Methoxychlor (MXC) (345.65 g/mol)	0.01 – 10 µM	2 nM	2 nM	0
dibutyl phthalate (DBP) (278.35 g/mol)	0.1 – 100 µM	0.5 nM	0.5 nM	0
4-nonylphenol (NP) (220.35 g/mol)	1E-6 – 1E1 µM	0.01 nM	0	0.01 nM
bisphenol A (BPA) (228.3 g/mol)	0.01 – 3 µM	1 nM	0	1 nM
β-HCH (HCC) (290.83 g/mol)	1E-4 – 1E1 µM	1 nM	0	1 nM
total concentration		5.01 nM	3.00 nM	2.01 nM
test range MCF-7 cell proliferation assay		1 – 10000x	1 – 3000x	1 – 10000x

[a] = concentration of a compound in the 1x mixture when diluted in SF-medium.

Cell culture

Estrogen receptor α-positive (ERα+) MCF-7(bus) human breast adenocarcinoma cells were kindly provided by Dr. A.M. Soto (Tufts University School of Medicine, Boston). MCF-7 cells were received at passage number 114 and routinely maintained in 25 cm^2 or 75 cm^2 plastic flasks on DMEM with or without phenol red, containing 5% heat inactivated fetal calf serum (FCS) (Gibco) and supplemented with 1 U/ml penicillin-1 µg/l streptomycin (pen/strep) at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity. DMEM without phenol red was additionally supplemented with 1 mM sodium pyruvate. Tryps/EDTA (Gibco) was used to detach cells from plastics. All experiments used cells within passage numbers ranging from 118 to 138. Within this range no changes in EC_{50} or efficacy of E_2 were detected. In experiments steroid free medium (SF-medium) was used, consisting of DMEM without phenol red, containing 5% heat inactivated and charcoal/dextran (Sigma) treated-FCS or commercially available charcoal/dextran treated FCS (Hyclone, Etten-Leur, The Netherlands), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and 1 U/ml pen/strep.

MCF-7 cell proliferation assay

The MCF-7 proliferation assay (E-screen) was performed following conditions published earlier [56]. In short, 96-well cell culture microtiter plates were used, which on day 0 cells were seeded at a density of 4500 cells/200 µl SF-medium and starved of steroids. On day 2 SF-medium containing the test compound(s) was added and every concentration-response experiment was performed at least in triplicate. The final vehicle concentration of maximally 0.2% DMSO or EtOH had no effect on cell proliferation and served as solvent control. On day 5 SF-medium with test compound(s) was refreshed. On day 8 cell proliferation was determined by measuring the capacity of the cells in each

well to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma). Briefly, cultures were carefully washed with PBS to remove most of the test compounds [9] and incubated with 100 µl DMEM without phenol red, containing 1 mg/ml MTT for 1h at 37°C. The absorbance at wavelength 595 nm (Fluostar, BMG labtechnologies, Germany) served as a measure of formation of the reduction product of MTT (formazan), and was considered to be a relative measure of the number of cells. This absorbance was found to be linear with the number of cells per well within our experimental range up to $8*10^4$ cells/well. Absorbance minus background (MTT without cells) was used as the corrected absorbance in further calculations.

E_2 and each PC or SC and mixtures were tested at least three times in the concentration range specified in tables 2.1 and 2.2. Relative concentrations of the mixtures are indicated with a concentration factor (see PCpl-mix(1)) in which '1x' stands for an exposure that simulates the average and normal human *in vivo* internal exposure to PCs (table 2.1).

In additional two-component combination-experiments E_2 was combined with PCeq-mix, PCpl-mix(1) or one of the SCmixes at ratios varying from 0% to 100% of their experimentally derived EC_{50} values. For all the combinations used, the expected response was that of the EC_{50} of E_2 in our experiments.

To control if the proliferative effects of the test compounds were ER mediated, sub maximal cell proliferation was induced by a test compound or mixture and proliferative inhibiting effects of an ER antagonists (ICI 182780, raloxifen, tamoxifen) in the range of 10 pM – 0.1 nM were examined.

Cytotoxicity

Cytotoxicity was assessed using MTT and lactate dehydrogenase (LDH).

For MTT measures, MCF-7(bus) cells were seeded at $1*10^5$ cells/well (24 wells plate) in SF-medium. After 24h incubation at 37°C cells where exposed to test compounds for 48h. Cell number per well was determined by measuring the capacity of the cells in each well to reduce MTT as described above. Absorbencies of cultures lower than that of solvent treated cultures where considered cytotoxic.

For LDH (Sigma TOX-7), MCF-7 cells were seeded at $12.5*10^3$ cells/well (96 wells plate) in SF-medium. After 24h incubation at 37°C cells where exposed to test compounds for 24h. LDH released in the medium and total LDH were determined following the enclosed guidelines. LDH activity in the medium relative to the total available LDH per well served as a measure for cell membrane integrity. Relative LDH release higher than that of solvent treated cultures was considered cytotoxic.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

The expression of the pS2 gene was used as an additional measure for estrogenicity because its expression is estrogen-receptor mediated and transcription is induced in MCF-7 cells by estrogenic compounds. Results in literature indicate that increased expression starts after 30 minutes and peaks after 48h [22, 57]. MCF-7(bus) cells were seeded in 6-well ($3*10^5$ cells/2 ml/well) or 12-well ($3*10^4$ cells/1 ml/well) tissue culture plates. The cells were grown for several days until confluency was approximately 70%. On day 0 of the experiment the cells were washed twice with PBS and set on SF-medium. On day 2 medium was replaced by SF-medium containing test compound(s) (per concentration in duplicate). The final vehicle concentration of maximally 0.2% DMSO or EtOH had no effect on basal pS2 expression level and served as control. On day 3, 24h after exposure, RNA was isolated using RNA instapure (Eurogentec, Liège, Belgium) following instructions of the supplier. Of each sample 5 µl containing 10 ng of total RNA was added to a 20 µl RT-PCR reaction mixture (final $MgSO_4$ concentration of 1 mM) (Promega RT-PCR System, Madison, WI, USA). The sequences of pS2 primers (Invitrogen Co., Carlsbad, CA, USA) were 5'-GCG AAG CTT GGC CAC CAT GGA GAA CAA GG-3' and 5'-GCG GAT CCA CGA ACG GTG TCG TCG AA-3'. The PCR product for pS2 using this primer is 189 bp [39]. The RT-PCR reactions were initiated by 45 min synthesis of single strand cDNA at 48°C,

followed by 2 min enzyme inactivation at 94°C. Then amplification was performed for 25 cycles of 45 sec at 94°C, 45 sec at 55°C, 45 sec at 72°C. After a 7 min final extension at 72°C, amplification was terminated by soaking at 4°C. The PCR products were separated by size on a 2% denaturing agarose gel (Invitrogen Co.), and visualized by ethidium bromide (Bio-Rad, Veenendaal, The Netherlands) staining. The band intensity was measured and quantified using a FluorImager 595 (Amersham Bioscience, Piscataway, NJ) with ImageQuant software (version 5.0, Molecular Dynamics). E_2 and PCpl-mix(1) were tested several times in the concentration range 0.1 – 1 nM and 0.01 – 10x respectively. In additional two component combination-experiments E_2 was combined with PCpl-mix(1) at ratios varying from 0% to 100% of their experimental derived EC_{75} values. The reason for using the EC_{75} instead of EC_{50} was that the expression level of pS2 showed a large standard deviation at 50% or lower response levels.

To control if the induction of the pS2 expression level, in cells treated with E_2 or PCmix, was ER mediated, sub maximal cell expression was induced by E_2 or a mixture and inhibiting effects of ICI 182780 at a final concentration in the SF-medium of 10 nM were examined.

Calculations & models

Corrected formazan absorbance (MTT) or pS2 expression were plotted against logarithmically transformed concentrations and EC_{50} values were determined from concentration-response relationships described using a 'best-fit' approach according to formula (2.1) EC_{50} values:

$$y = \text{min.}+(\text{max.} - \text{min.})/(1+10^{(EC50-C(TC))*\text{hill slope}}) \quad (2.1)$$

where y is response and C(TC) the concentration of the test compound.

Interaction within the mixtures was determined by analyzing the data (corrected for background) from proliferation assays with the concentration addition-approach (CA) [34, 40] by the isoboles-method and the cumulative estrogen equivalencies (EEQs)-method [67]. Concentration addition assumes that an estrogenic compound activates ER and leads to an equal response as E_2 (efficacy and slope of concentration response curve) and excludes other mechanisms concerning cell proliferation that could be modulated. As the MCF-7 cells are considered to show estrogen-specific responses (e.g. cell proliferation and induction of pS2 expression level) in presence of ER agonists and all estrogenic tested compounds are ER agonists, other non-ER mediated mechanisms were not considered.

In the isoboles-method average EC_{50} values of individual test compounds and mixtures were applied:

$$\Sigma(C_{(TC \text{ in mix at } EC50)}/EC_{50(TC)}) \quad (2.2)$$

where TC are phytochemicals or synthetic chemicals. In this method a result of 1 indicates zero interaction within the compounds of the mixture. A result smaller or larger then 1 indicates synergism or antagonism, respectively [34]. To interpret the outcome of the isoboles method, a distribution for outcomes of formula 2 was determined in S-PLUS (S-PLUS 6.0 Professional, Insightful Corp, Seattle WA, USA). This distribution was generated from the distributions of the calculated concentrations for each constituent of the mixture at the EC_{50} of the mixture and of the estimated EC_{50} for each individually tested mixture constituent. Herein it was assumed that concentrations were conform a log-normal distribution. From this distribution, 90% of the possible outcomes is expected between the P_{05} and P_{95} values. If this P_{05} value is smaller and this P_{95} value is larger than 1, the isoboles method is supposedly not deviating from zero interaction.

In the EEQ-method estrogen equivalency factors (EEFs) were calculated for each individual compound or mixture (per experiment) with the formula:

$$EEF_{(TC \text{ or mix})} = EC_{50(E2)}/EC_{50(TC \text{ or mix})} \quad (2.3)$$

For each compound the geometrical mean of EEF from several experiments was multiplied with the concentration of the compound in the mix, resulting in estrogen equivalents (EEQs):

$EEQ_{(TC)} = EEF_{(TC)} * C_{TC\ (in\ mix)}$ (2.4)

The sum of the EEQs for each compound results in a calculated EEQ for the mix:

Calculated $EEQ_{(mix)} = \Sigma\ EEQ_{(TC)}$ (2.5)

This can then be compared with the observed EEQ of the mix:

Observed $EEQ_{(mix)} = EEF_{(mix)} * C_{1x\ mix}$ (2.6)

Statistically significant differences between observed estrogenic potencies and calculated estrogenic potencies of the mixtures were determined by the Mann-Whitney test. The acceptable level of significance for these analyses was set at $P<0.05$.
For two component combination-experiments the expected response, based on an additive model, for all ratios were calculated by applying the formulas 2.7 and 2.8:

$Response_{(expected)} = \Sigma\ (Fraction_{(E2\ or\ mixture)} * Response_{(E2\ at\ EC50\ or\ mix\ at\ EC50)})$ (2.7)

$\%\ of\ Response_{(expected)} = (Response_{(observed)}/Response_{(expected)}) * 100\%$ (2.8)

Results

17β-estradiol

E_2 caused a maximum increase in MCF-7 cell proliferation at 30 pM, with the number of cells increasing 8 to 9 fold compared with control. The average EC_{50} for E_2 was 5.1 pM. Maximum cell proliferation at 30 pM E_2 was inhibited concentration-dependently and completely by the ER antagonists tamoxifen (IC_{50} = 54 nM), raloxifen (IC_{50} = 1.7 nM) or ICI 182780 (IC_{50} = 1.0 nM) (data not shown).

Levels pS2 RNA increased in MCF-7 cells that were treated for 24h with E_2, with a maximum increase of 2.3 to 5 fold compared with control levels at 100 pM. The average EC_{50} of E_2 was 2.2 pM. E_2-induced (30 pM) pS2 expression was decreased to control levels or lower when co-treated with 10 nM ICI 182780 (data not shown).

Individual phytochemicals and synthetic chemicals

In cytotoxicity tests, phytochemicals showed no cytotoxic effects at concentrations up to 100 µM except for GEN, which showed cytotoxicity at 10 µM in the MTT assay. For SCs no cytotoxic effects were found at concentrations up to 1 µM. In the MTT assay, cytotoxicity was found at 10 µM for OP, while the other SCs were found cytotoxic at 100 µM in the MTT as well as in the LDH assay. (Data not shown).

Individual PCs that produced full concentration-response curves in the proliferation assay were COU (EC_{50} = 0.18 nM), GEN (EC_{50} = 74 nM) and NAR (EC_{50} = 707 nM) (fig 2.1). QUE only slightly induced MTT reduction at 100 µM. Concentrations of QUE higher than 100 µM were considered biologically irrelevant. CAT and EPC did not cause cell proliferation (fig 2.1). No cytotoxic effects were observed within the range of concentration-response curves of individually PCs or at levels present in the mixtures. EC_{50} values of COU, GEN, NAR were converted to their relative estrogenic potencies (formulas 2.3 to 2.6), which resulted in the following order: $E_2(1) >> COU(2*10^{-2}) > GEN\ (1*10^{-4}) > NAR(6*10^{-6}) >>> QUE \approx CAT \approx EPC$.

Individual SCs that produced full concentration response curves in the MCF-7 cell proliferation assay were NP (EC_{50} = 2.5 nM), BPA (EC_{50} = 170 nM) and HCH (EC_{50} = 200 nM) (fig 2.2). SCs that did not induce 100% (relative to E_2) cell proliferation in the test range were OP (EC_{50} = 380 nM, max. 75%), DBP (EC_{50} = 170 nM, max. 60%) and MXC (EC_{50} = 510 nM, max. 45%) (2.2). No cytotoxic effects were observed within the range of concentration-response curves of individually SCs or at

levels present in the mixtures. The relative estrogenic potencies of individual SCs were calculated and followed the order: E_2 (1) >> NP ($1*10^{-3}$) > HCH ($1*10^{-5}$) > DBP≈BPA ($2*10^{-5}$) > MXC ($5*10^{-6}$) > OP ($7*10^{-6}$).

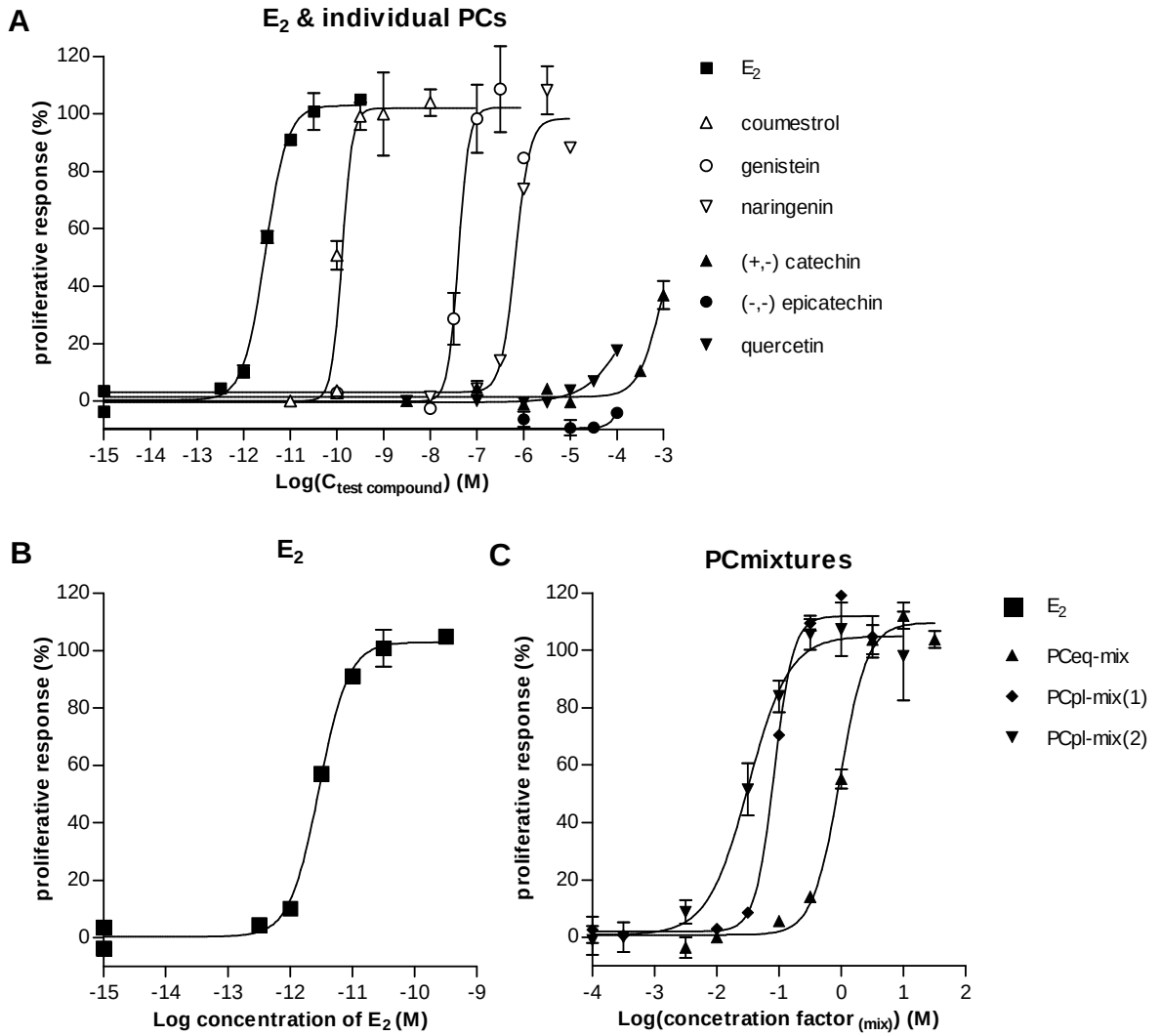

Fig 2.1. Cell proliferative effects of E_2, six different phytochemicals (**A**), E_2 (**B**) and three mixtures (**C**) in the MCF-7(bus) cell proliferation assay measured as MTT reduction. Concentrations of individual compounds are presented as Log($C_{(test\ compound)}$), while concentration factors of mixtures are presented as Log(concentration factor$_{(mix)}$).

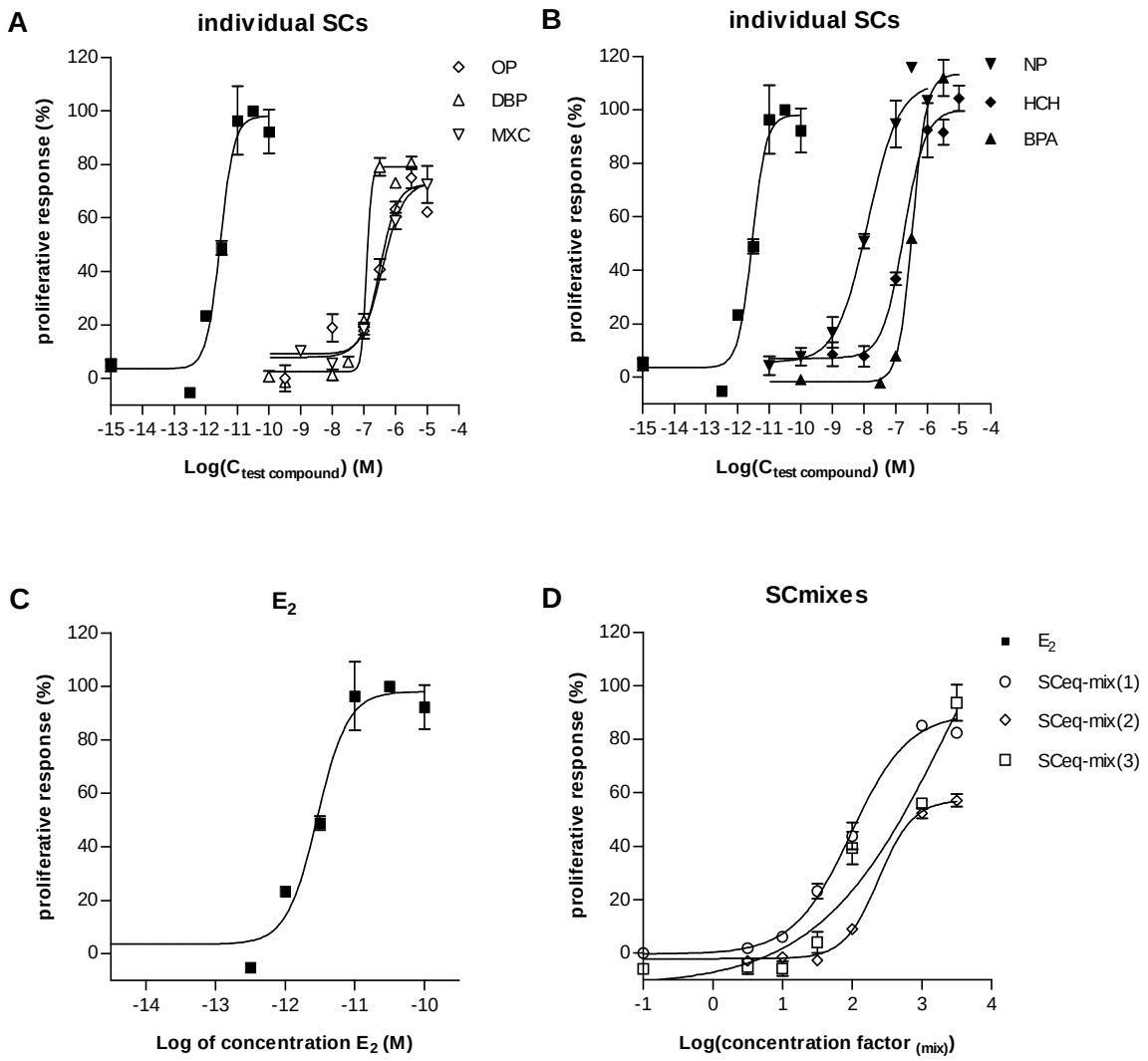

Fig 2.2. Cell proliferative effects of E_2, six different synthetic estrogens (**A and B**), E_2 (**C**) and four mixtures (**D**) in the MCF-7(bus) cell proliferation assay measured as MTT reduction. Concentrations of individual compounds are presented as Log($C_{(test\ compound)}$), while concentration factors of mixtures are presented as Log(concentration factor$_{(mix)}$).

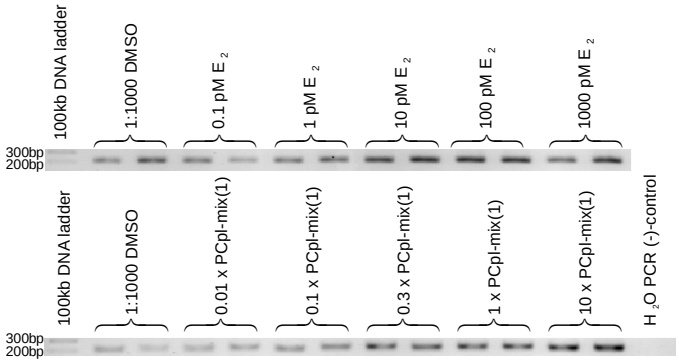

Fig 2.3. Induction of pS2 expression in MCF-7(bus) cells after 24h exposure to E_2 (upper gel) or PCpl-mix(1) (lower gel).

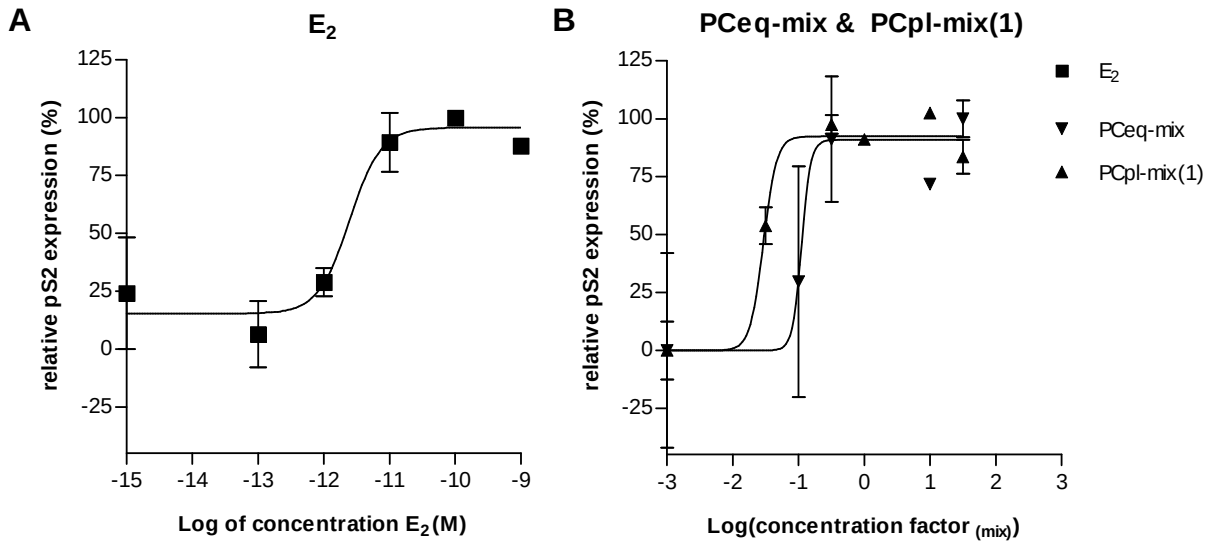

Fig 2.4. Relative pS2 expression measured as band intensity for E_2 (**A**) and mixtures of phytochemicals (**B**). Concentration of E_2 is presented as Log(concentration), while concentration factors of mixtures are presented as Log(concentration factor$_{(mix)}$).

Mixtures of phytochemicals

In the MTT assay, applied to assess cytotoxicity, PCmixes showed no cytotoxic effects at concentration factors up to of 10x and only PCeq-mix showed cytotoxicity at 100x.

The PCeq-mix, PCpl-mix(1) and PCpl-mix(2) produced full concentration-response curves, while PCpl-mix(3) did not show any proliferative effect (fig 2.1). Average EC_{50} value based on cell proliferation were 0.11x PCpl-mix(1), 0.93x PCeq-mix and 0.07x PCpl-mix(2) (table 2.3). EC_{50} values of PCmixes were converted to their relative estrogenic potencies compared to E_2 (formulas 2.3 to 2.6) (table 2.3).

Cell proliferation induced by treatment with 1x PCpl-mix(1) or 10x PCeq-mix was inhibited concentration-dependently and completely by the ER antagonists tamoxifen, ICI 182780 ($IC_{50(ICI\ 182780)}$ = 0.61 nM; 0.89 nM) or raloxifen ($IC_{50(raloxifen)}$ = 0.46 nM; 0.53 nM) (data not shown), which confirmed that the observed estrogenic effect caused by the PCmixes was ER mediated.

Beside cell proliferation, increased pS2 levels were observed after exposure of the cells to PCeq-mix or PCpl-mix(1), with a maximum increase of 2 to 4 fold control observed at 3x PCeq-mix or 1x PCpl-mix(1). The EC_{50} value was found at 0.11x PCeq-mix and 0.37x PCpl-mix(1) (figs 2.3 and 2.4). These EC_{50} values for pS2 were in the same order of magnitude as those for cell proliferation. Expression levels of pS2 induced by 3x PCeq-mix or 0.3x PCpl-mix(1) were completely inhibited when co-treated with ICI 182780 (10 nM) (data not shown), which confirmed that the observed stimulation of cell proliferation by the PCmixes was ER-mediated.

Calculated EEQs derived from cell proliferation data were similar to the observed experimental EEQs for PCeq-mix, PCpl-mix(1) and PCpl-mix(2) (table 2.3). Calculated interaction of these PCmixes with the isoboles-method were 1.3 for PCeq-mix, 0.7 for PCpl-mix(1) and 0.5 for PCpl-mix(2) (table 2.3), thus close to one and suggesting only weak, if any, departure from additivity. Yet, the method of isoboles does not include a measure for statistical significance. We attempted to use standard deviations of EC_{50}s in S-PLUS to interpret the statistical meaning of the result of the calculated interaction deviating from 1. This analysis indicated that our calculated interaction was not deviating from the range of results to be expected (tables 2.3 and 2.4). Thus both approaches to investigate mixture effects, which relied on the same data, showed to interact in a way very close to additivity for all PCmixes.

Table 2.3. EC_{50} values and estrogenic equivalency factors (EEFs) of selected phytochemicals and their mixtures in the MCF-7 proliferation assay and probit 'best fit' analysis for each phytochemical or their mixtures. Measured values were applied to calculate interactions using the estrogen equivalency (EEQ) and isoboles methods.

EEQ-method			PCeq-mix	PCpl-mix(1)	PCpl-mix(2)
Compound	**EC_{50}** Log(GM±GSD)[3] (M)	**EEF[1]** Log(GM±GSD)[3]	**EEQ[2]**	**EEQ[2]**	**EEQ[2]**
17β-estradiol	-11.3±0.3	0			
coumestrol	-9.75±0.20	-1.65±0.18	-11.65±0.18	-12.65±0.18	-12.65±0.18
genistein	-7.13±0.35	-3.96±0.23	-11.49±0.23	-10.26± 0.23	-10.26±0.23
(+,-)naringenin	-6.2±0.35	-5.21±0.15	-11.74±0.15	-12.21±0.15	-12.21±0.15
quercetin	>-6	-	-	-	-
(+,-)catechin	>-6	-	-	-	-
(-,-)epicatechin	>-6	-	-	-	-
			sum[4]	**sum[4]**	**sum[4]**
PCeq-mix (observed)	-0.03±0.08x	-11.30±0.12 (5.1E-12)			
PCeq-mix (calculated)			-11.13±0.68 (7.3E-12)		
PCpl-mix(1) (observed)	-0.97±0.26x	-10.27±0.32 (5.4E-11)			
PCpl-mix(1) (calculated)				-10.26±0.68 (5.5E-11)	
PCpl-mix(2) (observed)	-1.19±0.32x	-10.16±0.25 (7.0E-11)			
PCpl-mix(2) (calculated)					-10.26±0.68 (5.5E-11)
Method of isoboles			**[PC]/EC_{50}[5]**	**[PC]/EC_{50}[5]**	**[PC]/EC_{50}[5]**
coumestrol			5.3E-1	6.1E-3	3.7E-3
genistein			3.8E-1	7.3E-1	4.4E-1
(+,-)naringenin			4.0E-1	1.5E-2	9.2E-3
quercetin			-	-	-
(+,-)catechin			-	-	-
(-,-)epicatechin			-	-	-
			sum[6]	**sum[6]**	**sum[6]**
Distributions:			1.3	0.7	0.5
P_{05}			0.8	0.2	0.1
P_{95}			2.9	3.5	2.6

[1] = EEF = $EC_{50(E2)}/EC_{50(TC)}$ (calculated per experiment)
[2] = EEQ = $C_{(TC\ in\ mix)}$ x EEF
[3] = logarithm of the geometric mean and standard deviation
[4] = the sum EEQs of all components in the mixture.
[5] = the concentration of each phytochemical at the EC_{50} of the mixture [PC] divided by the EC_{50} of the individual test compound.
[6] = the sum of the decrease of all components in the mixture.
>1E6 = no proliferative effect in the tested range (10nM – 100μM) and were considered not to contribute to the proliferative effects of mixtures and not further processed in calculations. - = not calculated
P_{05} and P_{95} values were determined from distribution of possible outcomes of formula 2.2

Mixtures of synthetic chemicals

In the MTT assay, applied to assess cytotoxicity, SCmixes showed no cytotoxic effects at concentration factors up to of 1000x SCmix. SCeq-mix(1) and SCeq- mix(3) showed cytotoxicity at 3000x SCeq-mix(1) and 10000x SCeq-mix(3).

The SCeq-mix(1), SCeq-mix(3) produced full concentration-response curves for MCF-7 cells proliferation (fig 2.2 and table 2.4). The SCeq-mix(2) did not give a full concentration-response curve. Average EC_{50} values were found to be 48.4x SCeq-mix(1), 170.2x SCeq-mix(2) and 54.4x SCeq-mix(3). EC_{50} values of SCmixes were converted to their relative potencies (table 2.4). Calculated EEQs derived from these data were similar to the experimental EEQs for all SCeq-mixes (table 2.4). Calculated interaction with isoboles-method was 1.1 for SCeq-mix(1), 1.4 for SCeq-mix(2) and 0.8 for SCeq-mix(3), thus close to one and suggesting only weak, if any, departure from additivity. Yet, the method of isoboles does not include a measure for statistical significance. We attempted to use

standard deviations of EC_{50}s in S-PLUS to interpret the statistical meaning of the result of the calculated interaction deviating from 1. This analysis indicates that our calculated interaction were not deviating from the range of results to be expected (tables 2.3 and 2.4). Thus both approaches to investigate mixture effects, which relied on the same data, showed to interact in a way very close to additivity for all SCmixes.

Table 2.4. EC_{50} values and estrogenic equivalency factors (EEFs) of selected synthetic estrogenic chemicals and their mixtures in the MCF-7 proliferation assay and probit 'best fit' analysis for each chemical or their mixtures. Measured values were applied to calculate interactions using the estrogen equivalency (EEQ) and isoboles methods.

EEQ-method			SCeq-mix(1)	SCeq-mix(2)	SCeq-mix(3)
Compound	**EC_{50}** Log(GM±GSD)[3] (M)	**EEF[1]** Log(GM±GSD)[3]	**EEQ[2]**	**EEQ[2]**	**EEQ[2]**
17β-estradiol	-11.6±0.32	0			
4-octylphenol	-6.42±0.30	-5.17±0.33	-14.47±0.33	-14.47±0.33	0
methoxychlor	-6.29±0.17	-5.32±0.39	-14.02±0.39	-14.02±0.39	0
dibutyl phthalate	-6.78±0.28	-4.78±0.37	-14.08±0.37	-14.08±0.37	0
4-nonylphenol	-8.61±0.40	-2.9±0.45	-13.90±0.45	0	-13.90±0.45
bisphenol A	-6.78±0.21	-4.78±0.20	-13.78±0.20	0	-13.78±0.20
β-HCH	-6.70±0.08	-4.96±0.39	-13.96±0.39	0	-13.96±0.39
			sum[4]	**sum[4]**	**sum[4]**
SCeq-mix(1) (observed)	1.69±0.50x	-13.27±0.40 (5.4E-14)			
SCeq-mix(1) (calculated)			-13.21±0.89 (6.1E-14)		
SCeq-mix(2) (observed)	1.74±0.30x	-13.32±0.29 (4.8E-14)			
SCeq-mix(2) (calculated)				-13.40±0.63 (4.0E-14)	
SCeq-mix(3) (observed)	2.23±0.21x	-13.78±0.16 (1.7E-14)			
SCeq-mix(3) (calculated)					-13.67±0.63 (2.1E-14)
Method of isoboles			**[SC]/EC_{50}[5]**	**[SC]/EC_{50}[5]**	**[SC]/EC_{50}[5]**
4-octylphenol			6.4E-2	2.3E-1	0
methoxychlor			1.9E-1	6.7E-1	0
dibutyl phthalate			1.5E-1	5.1E-1	0
4-nonylphenol			2.0E-1	0	2.2E-1
bisphenol A			2.9E-1	0	3.2E-1
β-HCH			2.4E-1	0	2.7E-1
			sum[6]	**sum[6]**	**sum[6]**
Distributions:			1.1	1.4	0.8
P_{05}			0.7	0.8	0.4
P_{95}			6.2	3.4	2.6

[1] = EEF = $EC_{50(E2)}/EC_{50(test\ compound)}$ (calculated per experiment)
[2] = EEQ = $C_{(test\ compound\ in\ mix)}$ x EEF
[3] = logarithm of the geometric mean and standard deviation
[4] = the sum EEQs of all components in the mixture
[5] = the concentration of each synthetic chemical at the EC_{50} of the mixture [SC] divided by the EC_{50} of the individual test compound
[6] = the sum of the decrease of all components in the mixture
P_{05} and P_{95} values were determined from distribution of possible outcomes of formula 2.2

Combinations of E_2 and mixtures

To investigate potential non-additive effects between E_2 and PCs or SCs, binary equipotent combinations of E_2 and PCeq-mix, PCpl-mix(1), SCeq-mix(1), SCeq-mix(2), or SCeq-mix(3) were tested at various ratios. Ratios were chosen to produce a response equal to the EC_{50} (cell proliferation) or EC_{75} (pS2 expression) of E_2 alone, assuming addivity (table 2.5). The responses in

table 2.5 illustrated that these combinations interact in an additive way in MCF-7 cells, based on either ER-dependent cell proliferation or pS2 expression.

Table 2.5. Cell proliferative and pS2 response in the MCF-7(bus) after exposure to binary combinations of E_2 with PCmixes or SCmixes at various equipotent ratios in relation to estimated EC_{50} (in proliferative assay) or EC_{75} (in pS2 expression assay) responses. The response is expressed as percentage of the calculated expected response (see formulas 2.7 & 2.8 in Materials and Methods section).

	Proliferation [1] PCeq-mix	**Proliferation [2] PCpl-mix(1)**	**pS2 [3] PCpl-mix(1))**	**Proliferation [4] SCeq-mix(1)**	**Proliferation [5] SCeq-mix(2)**	**Proliferation [6] SCeq-mix(3)**
ratio E_2: mix	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
1 : 0	100±7	100±29	100±20	100±23	100±8	100±20
0.8 : 0.2	103±8	97±38	n.a.	112±17	116±13	105±18
0.75 : 0.25	n.a.	n.a.	102±5	113±15	107±9	125±28
0.7 : 0.3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
0.5 : 0.5	109±10	101±45	98±1	104±11	108±8	98±31
0.3 : 0.7	96±8	90±20	n.a.	n.a.	n.a.	n.a.
0.25 : 0.75	n.a.	n.a.	96±7	n.a.	n.a.	n.a.
0.2 : 0.8	110±4	96±19	n.a.	108±15	126±11	107±33
0 : 1	100±6	100±18	100±11	100±12	100±12	100±6

[1] = Applied EC_{50} values were 6 pM E_2 and 1.13x PCmix(eq).
[2] = Applied EC_{50} values were 6 or 7.1 pM E_2 and 0.17x PCpl-mix(1).
[3] = Applied EC_{75} values were 8 pM E_2 and 0.06x PCpl-mix(1).
[4] = Applied EC_{50} values were 5 pM E_2 and 100x SCeq-mix(1).
[5] = Applied EC_{50} values were 5 pM E_2 and 200x SCeq-mix(2).
[6] = Applied EC_{50} values were 5 pM E_2 and 80x SCeq-mix(3).
n.a. = not available

Discussion

Analysis of data from proliferation assays and pS2 expression showed an EC_{50} value in the same range for E_2, PCeq-mix and PCpl-mix(1). For E_2 this value is in accordance with previous reports of MCF-7(bus) systems in which estrogens were tested [51, 61, 65]. This indicates that both markers have similar sensitivity, which is in agreement with an earlier study by Jorgensen [32]. The maximum efficacy in the proliferation assay, however, is approximately twice that of the pS2 assay. As a result, cell proliferation in the MCF-7 cells was preferred as a quantitative marker for estrogenicity and tool for measuring mixture effects.

To calculate (estrogenic) interactions within mixtures two models (the method of isoboles and EEQ-method) were applied assuming that all measured endpoints are mediated through the same receptor type, ERα. We confirmed that all compounds or mixtures were acting via the ERα by showing that their estrogenic responses (cell proliferation and pS2 expression level) were inhibited by ERα antagonists or a down regulator and that the hill slopes of their concentration-response curves for estrogenic responses were approximately equal to that of E_2. For most estrogenic test compounds the maximum induction of cell proliferation was similar to that of E_2, except for OP, DBP and MXC, which generally showed less cell proliferation inducing capacities. Therefore these models may not be valid for certain compounds, although they will have approximate value.

It has been shown that the phyto- and synthetic chemicals used in our experiments interact with ERα [10, 21] and exhibit estrogenic properties. Combined exposure to compounds interacting with the ERα additive effects seems to prevail [14, 63]. Nevertheless, non-additive interactions have been reported in literature. Suzuki *et al.* exposed MCF-7 cells to a combination of E_2 and bisphenol A and reported a synergistic cell proliferative effect 2.5 fold higher than expected [69], while Rajapakse *et al.* reported an antagonistic effect caused by nonyl and octyl phenol in a mixture of these, E_2, ethynylestradiol, genistein and bisphenol A [54].

To our knowledge our *in vitro* study is the first to investigate the effects of mixtures of plant-derived (estrogenic) compounds at concentrations and in ratios of human exposures via a regular diet, as well as equipotent ratios. Our study included various (equipotent) mixtures of synthetic estrogens at concentrations close to those measured in human plasma, except for NP and DBP that have been reported to occur at 100 – 400 times higher levels in human plasma (NP: 0.25 ng/ml = 1.13 nM [33], DBP: 0.06 mg/L = 215.6 nM [74]). The isoboles-method and EEQ-method both indicated additivity for the PCmixes and SCmixes as the isoboles-method gave numbers close to one and the calculated EEQs were similar to the observed EEQs. Thus, these findings focusing on interaction between estrogenic compounds within a mixture, confirm that estrogenic compounds predominantly act in an additive way.

In the second part of our experiments we studied the mixture interactions between E_2 and different PC an SC mixtures at medium concentrations that were considered to be representative for the human systemic situation. In this approach it was assumed that medium concentrations approached those measured in blood of postmenopausal women (1 pM [64] or 13 – 26 pM [62]). Mixture interaction between E_2 and PCmixes or SCmixes were tested at various ratios and realistic systemic concentrations to mimic the possible impact of these xenoestrogens on the endogenous estrogens in the human body using cell proliferation and pS2 expression as endpoints. Our combination-experiments with E_2 and either PCmixes or SCmixes at ratios that would be either an $EC_{50(E2)}$ or $EC_{75(E2)}$ based on expected additivity did confirm an additive mechanism of action and found no evidence of either synergistic or antagonistic effects. Assuming that concentrations in cell medium serve as an approximation for human plasma or tissue concentrations, these results indicate that PCs and SCs probably act additively with the circulating endogenous estrogens. From a quantitative point of view, based on the observed estrogenic potencies of SCs and PCs, we suggest that the synthetic estrogens contribute significantly less to the total systemic estrogenicity than the phytochemicals, again assuming that medium concentrations reflect plasma concentrations. Predictions of estrogenicity in addition to the contribution of circulating estrogens and ultimately human health should be made with care because endogenous hormones and xenobiotics mimicking estrogens intrinsically differ in tissue distribution, metabolism and possibly ER binding behavior.

The interpretation and extrapolation of the results of our study for the human situation clearly have their limitations and several aspects should be taken into consideration. One of the limitations is the composition of the mixtures, which for the phytochemical mixtures (PCpl-mix 1, 2, 3) were based on, and for synthetic mixtures close to, the reported plasma concentrations after consumption of a normal human diet (tables 2.1 and 2.2). These compositions and concentrations clearly do not cover all possible divergences that can occur in human plasma due to inter-individual variations in absorption, metabolism [7, 52], gut flora and dietary habits. Another aspect that should be taken into consideration is that translating human plasma concentrations into medium concentrations seems realistic, but may differ from actual concentrations at the site of action due to kinetic differences per compound. In addition, it can be questioned to which extent the medium concentrations of phytochemicals and synthetic chemicals used in our *in vitro* experiments are a realistic reflection of human plasma concentrations. The free concentration of these chemicals *in vivo* may be lower than those in our *in vitro* system. The bioavailability of these chemicals might be less *in vivo* than *in vitro* due to nonspecific binding to an abundance of proteins (e.g. albumin) in human whole blood. At the same time xenoestrogens are found to bind with lower affinity than E_2 [3, 70]. Beside nonspecific binding, the bioavailability of endogenous hormones is disrupted by phytoestrogens as it is found that they increase transcription of SHBG [41] and that certain synthetic chemicals decrease binding of endogenous hormones [18]. A final aspect that should be considered is that the MCF-7 *in vitro* system itself leaves out aspects other than (anti-)estrogenic activity such as interaction with ERβ, possible non-genomic pathways resulting in estrogenic effects [53, 68], steroidogenesis [2], metabolism or kinetics of these compounds.

In conclusion the results of our study with MCF-7 cells show that mixtures of phytochemicals and synthetic estrogens with or without estradiol act in an additive way. Thus, it seems likely that both phytochemicals and synthetic estrogens can contribute in an additive way to the total estrogenicity present in human plasma. However, it should be taken into consideration that *in vitro* medium concentrations can not be fully compared with human plasma concentrations or concentrations at the site of action due to differences in protein levels and free available concentrations and kinetic characteristics of these compounds. In this respect it is possible that quantitative results from our experiments may somewhat overestimate estrogenicity compared with the human *in vivo* situation. Finally, our results also indicate that the contribution of phytochemicals to total estrogenicity significantly exceeds that of the synthetic estrogens.

References

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Chapter 3

Estrogenic effects of mixtures of phyto- and synthetic chemicals on uterine growth of prepubertal rats

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Abstract

Through the diet humans are exposed to many weak estrogenic phytochemicals (PCs) and synthetic chemicals (SCs), but most experimental studies used individual compounds rather than mixtures. Estrogenic effects were determined in the rat juvenile uterotrophic assay using a predefined phytochemical mixture (PCmix) containing coumestrol, genistein, naringenin, (+,-)catechin, (-,-)epicatechin and quercetin, and a predefined synthetic chemical mixture (SCmix) containing nonyl-, and octylphenol, β-hexachlorocyclohexane, methoxychlor, bisphenol A and dibutylphthalate. The mixture composition was based on human dietary uptake and actual ratios in serum. 17β-Estradiol and genistein were also tested individually. It was found that combinations of phytoestrogens and exogenous 17β-estradiol act additive. In contrast SCmix, inactive by itself even at high dose levels relative to human exposure, caused no synergistic or antagonistic uterotrophic effect with E_2 and/or the PCmix. Based on ED_{05} and ED_{01} values of the PCmix the margin of exposure in regular human diet for a uterotrophic effect is estimated many orders of magnitude. However, food supplements with phytochemicals might bring individual exposure around ED_{05} and ED_{01} values of the PCmix. Based on the results of our study the contribution of SCs to total estrogenicity in human diet can probably be neglected.

Introduction

The diet is an important source of human exposure to a large variety of chemicals, both from natural as well synthetic origin. Many of these chemicals, commonly called endocrine active compounds (EACs), are potentially capable of interfering with normal endocrine regulation through various pathways, as established in a large number of *in vitro* and *in vivo* experiments [6, 30, 45, 67]. Among others, these EACs include anthropogenic compounds that can interfere with the estrogen homeostasis and associated processes, otherwise called xenoestrogens. In addition, many plant synthesized chemicals have similar interfering properties and are usually defined as phytoestrogens. The mechanisms whereby xeno- or phytoestrogens can act are highly diverse, but interactions with both estrogen receptors α or β (ERs) are normal [37, 38]. Numerous other properties for these estrogenic compounds have also been reported that may be mediated apart from estrogen receptors, involving reduction of ER expression, changes in circulating estrogen levels, reduction in angiogenesis, tumor invasiveness and induction of radical scavenging or apoptosis [5, 10, 27, 44, 46, 48, 50, 53, 70]. With respect to the possible impact of phytoestrogens the common view appears to be that these can be beneficial for human health [1, 18], whereas the synthetic xenoestrogens are suspected to be involved in the increase of hormone dependent diseases including reduced fertility and certain types of cancer [14]. Although humans are simultaneously exposed to a large number of phytoestrogens and xenoestrogens through the diet, most experimental studies have focused on individual compounds rather than on mixtures [62]. The limited number of mixture studies focusing on human relevant concentrations and combinations are noticeable, since these are a prerequisite to properly assess the combined risks of these xeno- and phytoestrogens for humans.

A classical *in vivo* model to investigate the potency of estrogenic compounds with ERα is the rat or mouse uterotrophic assay [39, 56]. In this assay the increase in uterine weight in juvenile or ovariectomized females is used as a degree for estrogenicity of a chemical [49].

In the present study predefined mixtures of six phytochemicals (PCmix) or six synthetic chemicals (SCmix) with low estrogenic properties were used based on human exposure data. Both mixtures with or without 17β-estradiol (E_2) were tested in the rat uterotrophic assay and combinations of these mixtures with E_2 at various concentrations were prepared based on the concentration addition model [3, 17, 35, 36, 42]. Estrogenic potencies of these mixtures relative to E_2 were determined. In addition, the relative contribution with respect to estrogenicity of these xeno- and phytoestrogens for human exposure was estimated.

Materials and Methods

Chemicals

17β-estradiol (E_2; purity ≥ 98%) and the phytochemicals (PCs) (+,-)catechin (CAT), (-,-)epicatechin (EPC; purity ≥ 90%), quercetin (QUE; purity ≥ 98%) and (+,-)naringenin (NAR; purity ~ 95%) were purchased from Sigma (St. Louis, MO, USA). Genistein (GEN; purity ~ 97%) was purchased from Lancaster Synthesis (Muhlheim am Main, Germany) and coumestrol (COU; purity > 95%/98%) from Fluka (Zwijndrecht, The Netherlands). Dibutylphthalate (DBP) and 4,4'-isopropylidenediphenol (= bisphenol A, BPA; purity ≥ 95%) were purchased from Sigma. β-Hexachlorocyclohexane (HCH; purity ~ 98.1%) and methoxychlor (MXC) were bought from Riedel-de-Haën (Zwijndrecht, The Netherlands). 4-Nonylphenol (NP; purity ~ 99%) was purchased from Acros Organics ('s-Hertogenbosch, The Netherlands) and 4-octylphenol (OP; purity ~ 99%) from Aldrich (Zwijndrecht, The Netherlands). Corn oil (Sigma) was used as vehicle for subcutaneous dosage.

Composition of phytochemical and synthetic chemical mixtures

In order to imitate human *in vivo* exposure to phytochemicals (PCs) a PCmix was prepared that contained CAT, EPC, QUE, NAR, GEN and COU. These PCs are all known to be present in the normal human diet. The level of each compound in this mixture was similar to that reported for daily human exposure levels (see table 3.1). In order to compose this mixture and relate our subcutaneous dosage to an equivalent human oral dosage, a conversion had to be made to an estimated oral dose for the rat. In order to convert a subcutaneous to an oral dose, a comparison had to be made between the areas under curves (AUCs) of GEN for both methods of administration in the juvenile female rat. This was determined in an earlier study and it was reported that an oral dose of 40 mg GEN/kg bw was equivalent to a subcutaneous dose of 4 mg/kg. This concurred with an initial plasma concentration of 0.25 µg/ml (925 nM) that is in the same range as observed in humans [41]. For this study it was derived that the human daily intake of GEN is 0.3 mg/kg bw (23.4 – 31.4 mg/d)[69], a subcutaneous dose of 0.03 mg GEN/kg/d subcutaneous would result in a similar estimated serum concentration for rats and human via oral intake. However, such an assumption is only valid when the GEN kinetics of rat and human are comparable. This appears to be true, as half-life of GEN in rats ranges between 3.0 and 8.8 hours [8, 33], while for humans it ranges from 5.7 to 12.9 hours [7, 25, 34, 55, 66]. Additionally, for our mixture experiments it was assumed that the kinetics of other PCs are close to GEN and comparable for humans and rats. Information about these PCs is much more limited than for GEN, but the few studies available reported half-lives of e.g. NAR and QUE that are in the same range for rats and humans [11, 16, 20, 22, 43]. Human intakes for the phytochemicals included in the PCmix, were estimated from literature [4, 16, 23, 24, 54, 69] (see table 3.1).

Synthetic chemicals (SCs) were combined in a SCmix. The dose level of each compound in the SCmix (NP, OP, DBP, BPA, MXC, HCH) is given in table 3.1. The dose levels of these estrogenic chemicals were significantly higher than average human exposure levels to ensure a uterotrophic response for a least one dose level. Based on the administered dose levels in the rat it could be estimated these would be three to five or orders of magnitude higher than those reported for human exposure. Thus, from a quantitative point of view the experiments with the SCmix have limited value for the human situation, but combinations with the PCmix were done to study the model of additivity under these experimental conditions. It should be noticed that the ratios of SCs in the SCmix were close to those reported in human exposure situation (table 3.1). It should me noted that composing the PCmix as well as the SCmix did not aim to represent all possible endocrine active compounds humans may be exposed to.

All test compounds were suspended and homogenized in corn oil one day before administration as indicated in table 3.1. E_2 was tested at doses from 0.1 µg/kg bw to 100 µg/kg bw and all mixture experiments included a positive control group that was administered E_2 only. GEN was also individually administered at dose levels of 3, 20, 60 mg/kg. The PCmix and SCmix mixtures were tested at various higher concentrations that included 100x to 3000x PCmix or 1000x to 100000x SCmix.

The type of mixture effect was investigated by dosing different combinations of E_2, PCmix and SCmix. Combinations were based on the concentration addition (CA) model and assumed that an estrogenic compound can lead to an equal estrogen receptor mediated response compared to E_2 in terms of efficacy [17, 35, 36, 42].

Animals and dosing

Immature female HsdCpb:WU rats (19 to 20 days old), obtained from Harlan Laboratories (Horst, The Netherlands). Animals were sorted in groups by weight to have an approximately similar mean and standard deviation for all groups (Latin square). Groups dosed with corn oil (control), E_2, PCmix and SCmix contained six to eight animals. Animals were kept at a maximum of four per cage containing sawdust (Technilab-BMI bv, Someren, The Netherlands); water and pelleted γ-radiation sterilized

SRM-A food (Hope Farms, Woerden, The Netherlands) were available *ad libitum* and a 12h/12h light-dark cycle was maintained. The animals were allowed to acclimatise for 24h before dosing. At the age of 21 days the animals were dosed subcutaneously on three successive days (days 1, 2 and 3) with 5 ml/kg bw corn oil containing E_2 or the mixtures. Subcutaneous dosing was preferred over oral dosing to avoid biotransformation of the parent compounds by intestinal flora and first pass effects.

Tissue sampling and Uterotrophic assays

On day 4 (24 hour after the final dose) animals were weighed and anaesthetised using O_2/CO_2 or isoflurane (A.U.V., Cuijk, The Netherlands). Vaginal opening was determined before necropsy and only observed in animals treated with 10 µg E_2/kg or higher (in 7 out of 379 rats in total), but not in any of the animals administered with GEN, the PCmix or SCmix.

The uteri were dissected and blotted uterine weights were determined as described earlier [49]. Briefly, uterine horns and ovaries, cervix and fat, were removed, and uteri were pierced to release excessive fluids before blotted weight was determined.

Calculations & Statistics

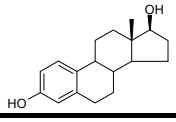
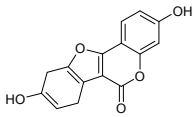
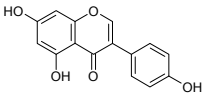
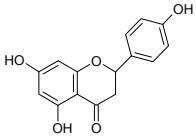
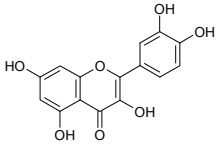
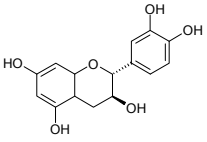
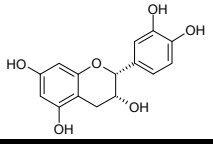
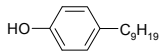
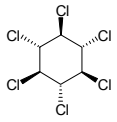
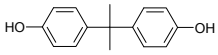
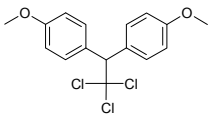
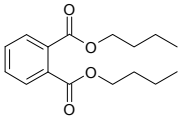
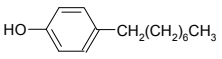
Uterus weights were analyzed for best-fit with exponential curve fitting to determine dose-response relationships in Prism 3.0 (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA). The 95% confidence intervals (CI) of the uterus weight were determined for all experiments with combinations of E_2 with PCmix and/or SCmix using Excel (Microsoft Excel 2000, NY, USA). Furthermore, for the three combinations of E_2 and PCmix, the expected uterus weight (R_{exp}) was based on the proportions of E_2 and PCmix and calculated by the equation:

$$R_{exp} = \sum_{i=E2 \text{ or } PCmix} F_{(i)} \times R_{obs\,(i)} \qquad (3.1)$$

where $F_{(i)}$ is the fraction of E_2 or PCmix in the combination to which animals were exposed and $R_{obs\,(i)}$ was the observed blotted uterus weight of animals exposed to the ED_{50} set for E_2 or PCmix. For combined exposure to E_2+PCmix and SCmix, the expected uterus weight equalled that of E_2+PCmix alone since SCmix did not induce uterotrophy by itself. The values for R_{exp} are represented by a dotted line (fig 3.2 and 3.3).

If in these experiments the expected uterus weight was within the 95% CI of the experimental determined values this was considered not statistically different and a confirmation of the additive model.

Table 3.1. Structure, human intake, blood/serum concentrations and composition of the mixtures of phytochemicals and synthetic chemicals used in the rat uterotrophic assay.

compound (MW in g/mol)	chemical structure	reported humane intake	reported human serum concentration	exposure 1x mix (*in vivo*)
endogenous				
17β-estradiol E_2 (272.4)		n.i.	n.i.	0
PCmix				
Coumestrol COU (268.23)		0.001 mg/d [4]	10 pM (estimated)	0.001 µg/kg
Genistein GEN (270.24)		0.16 – 0.42 mg/d [4] 23.4 – 31.4 mg/d [69]	<15 [15] – 1039 nM [19]	30.8 µg/kg
(+,-)naringenin NAR (272.3)		3 – 29 mg/d [16]	4.8 – 112.9 nM [16]	6.21 µg/kg
Quercetin QUE (338.3)		3 – 24 mg/d [16, 23, 24]	<26.8 [16] – 1340 nM [2, 54]	3.86 µg/kg
(+,-)catechin CAT (290.3)		3 – 34 mg/d [54]	0.072 µM [54]	4.63 µg/kg
(-,-)epicatechin EPC (290.3)		0.165 µg/kg/d (estimated)	0.27 µM [54]	16.55 µg/kg
SCmix				
4-nonylphenol NP (220.35)		7.5 µg/d [21]	0.2 – 0.3 ng/ml [31] (=0.9 – 1.4 nM)	0.0502 µg/kg
β-HCH HCH (290.83)		0.004 µg/kg/d [13]	1.7 – 4.1 ng/ml [63] (=5.8 – 14.1 nM)	0.99 µg/kg
bisphenol A BPA (228.3)		0.75 - 6.3 µg/person [12] 9 µg/kg/d [64]	0.64 – 1.49 ng/ml [61] (=2.8 – 6.5 nM)	0.32 µg/kg
Methoxychlor MXC (345.65)		0.1 – 0.8 µg/d [40]	0.53 nM (estimated)	0.0394 µg/kg
Dibutylphthalate DBP (278.35)		7.4 – 31 µg/kg/d [32, 65]	0.02 – 0.10 mg/l [65] (=72 – 359 nM)	12.69 µg/kg
4-octylphenol OP (206.3)		1.57E-5 – 1.57E-2 µg/kg/d [57]	0.1 – 0.2 ng/ml [31] (=0.37 – 0.73 nM)	0.0235 µg/kg

PCmix contains phytochemicals in realistic ratios and at 1x is intended to represent reported human exposure levels.
SCmix contains synthetic estrogenic compounds in ratios and at 1x is roughly the daily human exposure.
n.i. = not indicated

Results

For completeness per dose group the mean and standard deviation of animal weight (AW) on D0 and D4 as well as blotted uterus weight and blotted uterus weight per g AW are given in table 3.2. Further presentation of the results is with blotted uterus weight only.

Uterotrophic effect of E_2, GEN, PCmix & SCmix

The dose-response relationship for E_2 and uterotrophy was determined from six independent experiments and an average ED_{50} of 2.1 µg/kg bw was found (fig 3.1A). Starting from around 50 µg/kg bw a maximal response was found that produced an approximate fourfold increase in uterine weight compared to the controls. To determine a lowest observed effect level (LOEL) an ED_{05} and ED_{01} were calculated of respectively 0.063 µg/kg and 0.009 µg/kg bw, which indicates a LOEL in this range.

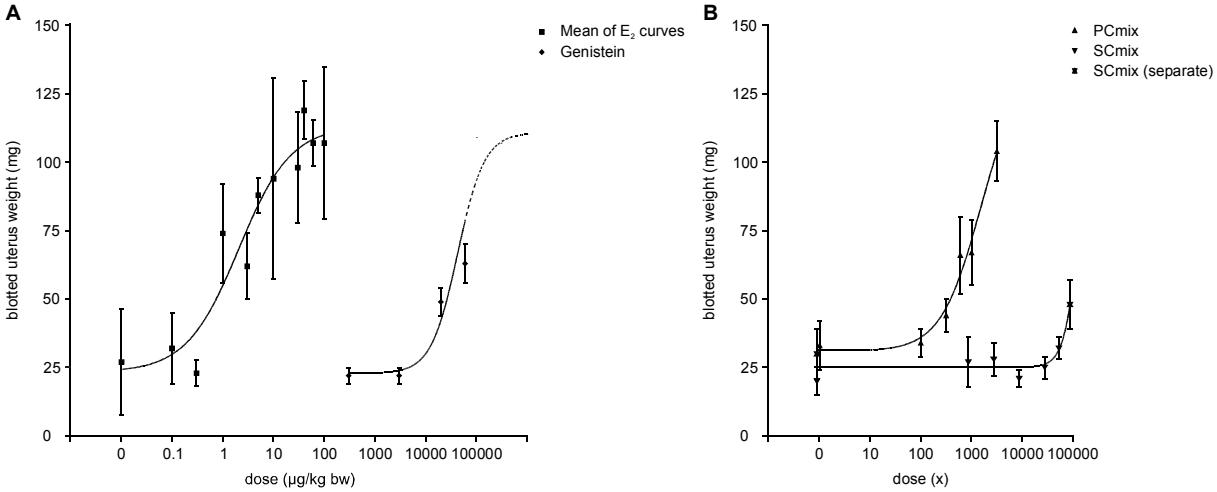


Fig 3.1. Dose-effect relationships for rat uterus weight of E_2 or genistein in µg/kg bw **(A)**, PCmix or SCmix in dose factor (1x dose see table 3.1) **(B)**. Error bars indicate standard deviation (group size n = 5 – 8). SCmix (separate) is the mean of a single group exposed to 100000x SCmix obtained from study 7.

The dose-response relationship of GEN was estimated with 3 dose levels and using the maximum response observed of E_2 (308 mg/kg bw). Consequently, the estimated ED_{50} of GEN was calculated to be 43 mg/kg bw giving this phytochemical a relative uterotrophic potency of 5E-5 compared to E_2 (fig 3.1A). The dose-response relationship of GEN was also used to calculate an ED_{05} and ED_{01} of respectively 7.2 and 2.7 mg/kg bw, indicating a LOEL is in this dose range. Relative potencies of GEN based on ED_{05} and ED_{01} were also calculated and found to be respectively 8.8E-6 and 3.4E-6.

The PCmix was given to the rats in a range of 100 to 3000 times the estimated daily intake (PCmix). The maximum increase in uterus weight was a factor 3 to 4 compared to the controls. This indicates that at approximately 3000x PCmix a comparable maximum efficacy existed as for 10 to 100 µg E_2/kg bw (fig 3.1B). Based on this experiment it could be calculated that an ED_{50} for uterotrophy could be found at approximately 850 times the estimated human uptake levels (PCmix) (table 3.1). The dose-response relationship of the PCmix also allowed a calculation of ED_{05} and ED_{01} with values of respectively 50 and 138 times the PCmix, indicating a possible LOEL for uterotrophy.

Based on these ED_{50}, ED_{05} and ED_{01} values it can be derived that the relative estrogenic potency of the PCmix was respectively 2.4E-3, 4.6E-4 and 1.8E-4 of E_2. Overall, this indicates that the potency of the PCmix, reflecting human diet concentrations, is 3 to 4 orders of magnitude lower than that of E_2.

The SCmix containing xenoestrogens at concentrations approximately present in the human diet was significantly less potent than the PCmix. Although some uterotrophic effect could be observed at five orders of magnitude higher than the original SCmix, an ED_{50} value could not be reached (fig 3.1B). Due to this very weak uterotrophic response of the SCmix it was considered not feasible to construct a full dose-response curve. Consequently, no ED_{50}, ED_{05} and ED_{01} values were determined for the SCmix.

Uterotrophic effect of combinations E_2 & PCmix or SCmix

In order to test for interactions between E_2 and the investigated phytochemicals, a series of combinations of E_2 and the PCmix were prepared with an estrogenic potency around their experimental ED_{50} values (fig 3.1A and 3.1B). In these combinations of E_2 and PCmix both constituents were mixed proportionally (fig 3.2). The selected dose of 3 µg E_2 /kg bw induced a uterotrophic response that was somewhat higher than expected based on our first E_2 experiments (fig 3.1A). As a consequence, the projected line of additivity shown in fig 3.2 is not horizontal, as it ideally should have been. However, this does not influence a comparison between the experimental outcomes of the E_2:PCmix combinations (ratios 75:25, 50:50 and 25:75) and their expected theoretical outcome, which could be derived by interpolation on the line of additivity. As can be observed none of these three combinations significantly deviated from this line thereby supporting the model of additivity for combinations of E_2 and these phytoestrogens (fig 3.2).

Similar combination experiments were made with E_2 and the SCmix at dose levels of 1 µg E_2/kg bw and 3000 to 60000 times SCmix. However, within this dose range and combinations with the SCmix no influence on E_2 induced uterus weight was found (fig 3.3).

One other combination experiment was done with a fixed combination of E_2 (0.5 µg/kg) and the PCmix (500 times human uptake), which evoked a uterotrophic response similar to the ED_{50} of E_2. To this combination (0.5 µg/kg E_2 + 500x PCmix) a variable dose of SCmix was added ranging from 3000 to 100000x SCmix. This experiment was done to study if an excessive amount of xenoestrogens would have either an effect on the combined E_2+PCmix induced uterotrophy. Under these experimental conditions no interactive effect could be found by the synthetic chemicals (SCmix) (fig 3.4).

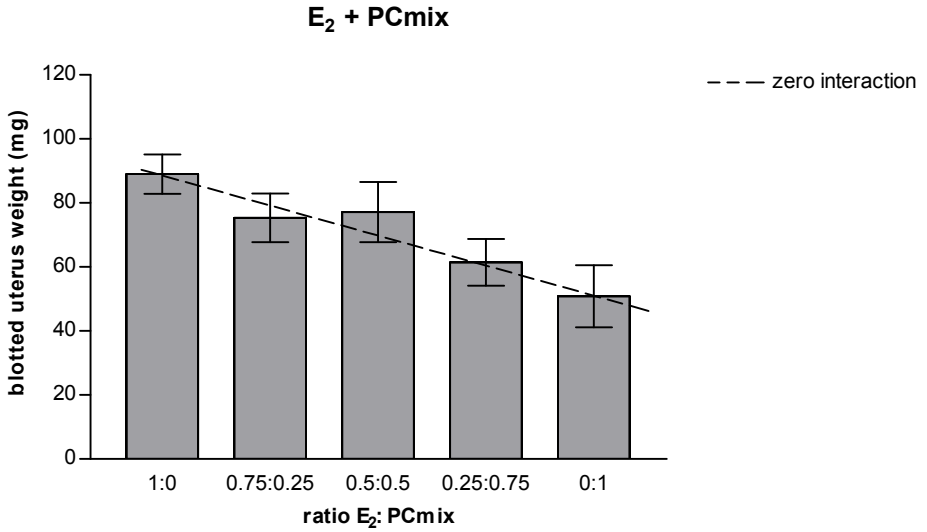


Fig 3.2. Dose-effect relationships for rat uterus weight of E_2 with PCmix at various equipotent ratios based on expected ED_{50} response (ED_{50} was set to 3 µg/kg for E_2 and 1000x for PCmix). The dotted line indicates the expected uterotrophic effect based on additivity. Error bars indicate 95% confidence intervals (group size= 4 - 8).

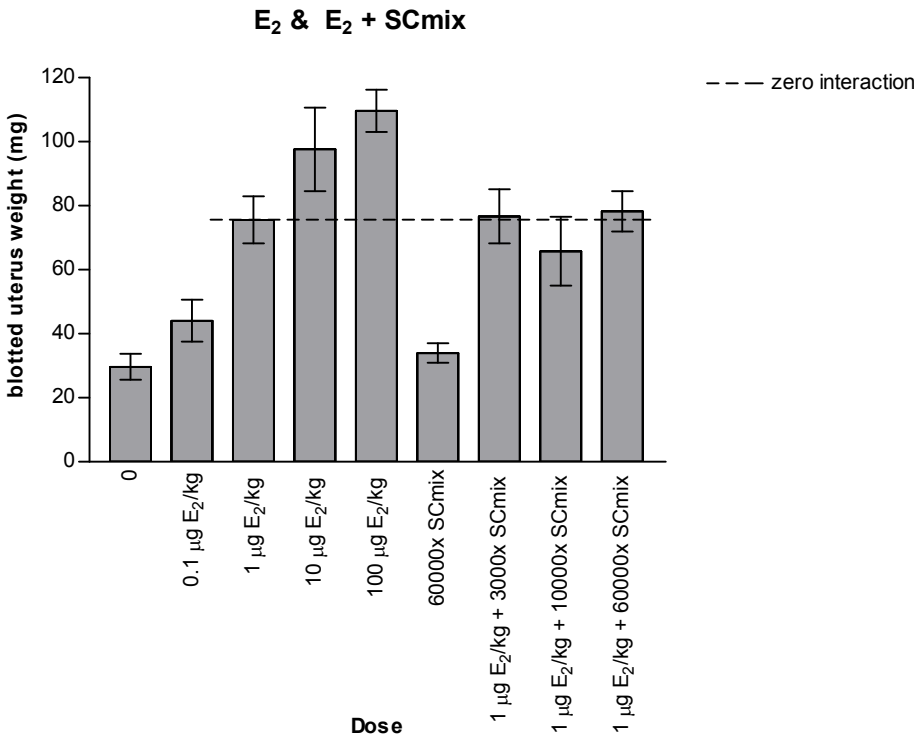


Fig 3.3. Blotted uterus weight in response E_2 and binary combinations of E_2 (1 µg/kg bw) with SCmix at various dose levels. Error bars indicate 95% confidence intervals. (group size = 6 for E_2 and 8 for the other groups).

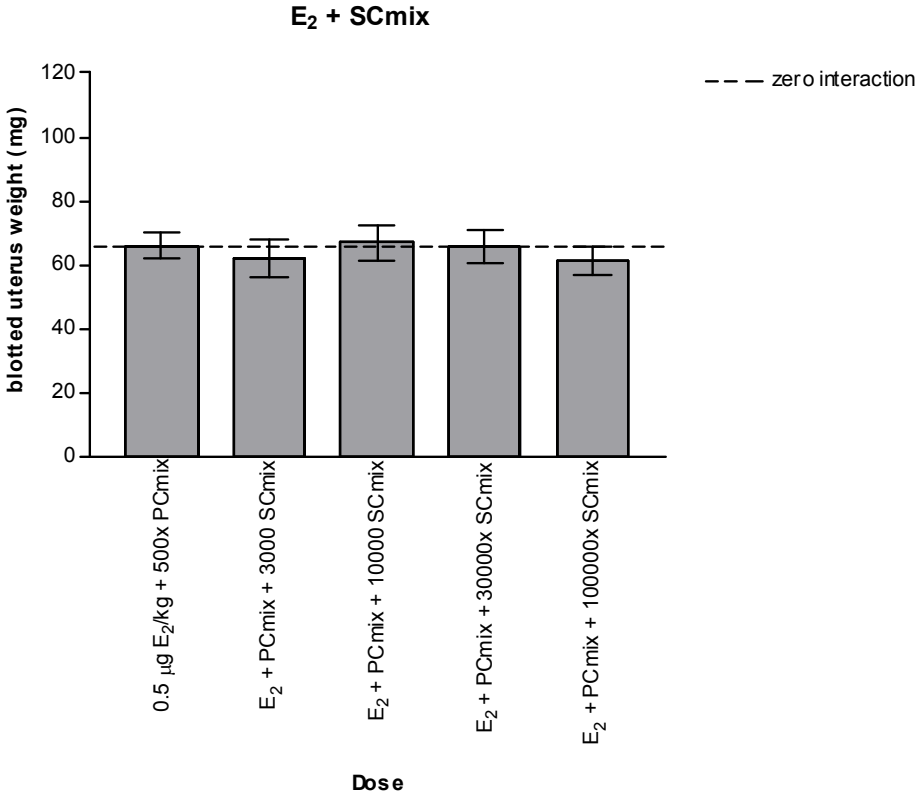


Fig 3.4. Dose-effect relationships for rat uterus weight of E_2 (0.5 µg/kg bw) + 500x PCmix combined with various dose levels of the SCmix. The dotted line indicates expected uterotrophic effect based on additivity. Error bars indicate 95% confidence intervals. (group size = 6 – 8).

Discussion and Conclusions

Humans are exposed to a wide variety and mixture of natural and synthetic chemicals that may influence various endocrine processes. From a hazard and risk assessment point of view it is also important to know if exposure to mixtures results in additive, antagonistic or synergistic interactions. Many of these synthetic endocrine active compounds or xenoestrogens are present in the human diet and can interact with the estrogen receptors and suggested to be associated with adverse health effects [14, 26, 68]. In real life, human exposure involves both xenoestrogens and phytoestrogens. Thus, both groups can potentially interact with endogenous estrogens. Very few studies addressed the estrogenic effects of combined exposure to both groups of chemicals. In a recent study, Tinwell and Ashby studied the effect of a multicomponent mixture of six synthetic estrogens and one phytoestrogen. This study showed that the combined exposure to this mixture of estrogenic compounds resulted in a positive effect on the uterus, which was otherwise absent for the individual components when administered on individual basis [62]. The present study describes the uterotrophic effects in pubertal rats by combinations of mixtures of common xeno- and phytoestrogens relative to E_2. In our experiments the individual compounds in the mixtures had ratios that were close to those observed in the human diet, but applied dose levels were two to five orders of magnitude higher than in the diet. Note also that the composition of the PCmix as well as the SCmix did not aim to represent all possible endocrine active compounds humans may be exposed to.

The dose-response relationship for E_2 observed in our experiments is similar as that reported earlier [28, 49] and indicates comparable estrogen sensitivity for chemicals in our rat study.

For our study with the mixture of phytochemicals (PCmix) a subcutaneous dose for the rat was related to the human uptake situation. Genistein was initially used as a model compound since sufficient kinetic information from rat and humans is available (see materials and method section). Although the accuracy of this method can be discussed, the derived dose should be considered as an order of magnitude estimate and also interpreted in view of the large inter-human variation that exist for dietary uptake of PCs (table 3.1).

The *in vivo* estrogenic effect of genistein has been studied earlier from which an ED_{50} of 25 – 28 mg/kg bw can be derived [29], which is comparable to the ED_{50} of 43 mg/kg bw in our study. It was found that the relative estrogenic potency of GEN was dependent on the dose-response relationship, e.g. ED_{50}, ED_{05} or ED_{01}. When comparing the relative estrogenic potencies based on ED_{50}, ED_{05} or ED_{01} it was observed that these could vary one order of magnitude. Clearly, this finding has implications for the risk assessment of these mixtures for which an estrogenic equivalency factor approach has been suggested [52, 59].

The PCmix in our study also induced uterotrophy. Assuming that a LOEL is in the ED_{01} to ED_{05} range an *in vivo* estrogenic effect can be expected at 50 to 138 times the PC concentrations in the mixture. For human exposure this implicates that possible estrogenic effects by PCs might be caused at levels that are approximately two orders of magnitude higher than that used in the PCmix. For GEN the ED_{01} to ED_{05} values ranged from 2.7 to 7.2 mg/kg bw. If these values are compared with the ED_{01} – ED_{05} range (50 to 138x PCmix) that is equivalent with 1.5 to 4.3 mg GEN/kg bw, it can be concluded that GEN is the phytoestrogen predominantly responsible for the estrogenic effect of this mixture.

Based on the results of our experiments with GEN and the PCmix the margin of exposure (MOE) for humans can also be estimated for a possible effect of the PCs involved. As GEN was clearly the PC mostly responsible for a uterotrophic effect of the PCmix an MOE can be focused on this PC. A regular Western or regular Asian diet results in an exposure of 0.002 – 0.006 GEN mg/kg/d [4] and 0.33 – 0.45 mg GEN/kg/d (23.4 or 31.4mg/day/70 kg) [69] respectively. Based on the ED_{01} to ED_{05} values of GEN a LOEL can be expected in the range from 2.7 to 7.2 mg/kg bw subcutaneous dose, which would be equivalent to a ten times higher oral dose of GEN. If it is assumed that the rat uterotrophic assay is representative for the human situation, this indicates a MOE around four and two orders of magnitude for respectively the regular Western and Asian diet. In addition, it should be

recognized that humans are regular taking food supplements that can contain grams of certain phytoestrogens, including genistein [58]. For these individuals it can not be excluded that exposure levels are in the range that caused uterotrophic effects in our rat study. However, apart from possible adverse effects higher intake levels of isoflavonoids by Asian people have also been correlated with beneficial and health promoting effects e.g. a low breast cancer incidence [1, 47, 51].

The mixture with synthetic xenoestrogens (SCmix) produced only a weak uterotrophic response at the highest dose level, approximately five orders of magnitude higher than estimated human exposure. Our results are in agreement with those from earlier studies concluding very little uterotrophic effect from these xenoestrogens could be expected at relevant human exposure levels [29]. Thus, our experiments again confirm the weak *in vivo* estrogenic potency of these synthetic xenoestrogens compared to some phytochemicals [60].

In our study a number of combinations of E_2, phytoestrogens and xenoestrogens were tested at dose levels that allowed interactions between individual components. However, all these combinations resulted in uterotrophic responses that did not deviate from additivity. Even excessive dose of levels of synthetic xenoestrogens, inactive by itself, did not cause any synergistic or antagonist effect. These results confirm several other *in vitro* and *in vivo* studies with mixtures of estrogenic compounds in which non additive estrogenic responses have not been found either [9].

In conclusion, the results of our study indicate that combinations of phytoestrogens and exogenous 17β-estradiol act additive, while synthetic xenoestrogens, inactive by itself, caused no synergistic or antagonistic uterotrophic effect in the rat. Based on ED_{05} and ED_{01} values determined in our rat study the margin of exposure in the regular human diet for a uterotrophic effect is estimated to be many orders of magnitude. Uptake of food supplements with phytochemicals might bring individuals to exposure levels that are around the ED_{05} and ED_{01} values observed in our study. The contribution of synthetic xenoestrogens used in our study to the total estrogenicity in the human diet can probably be neglected based on the results of our study.

Acknowledgments

We thank Drs. John Ashby and Helen Tinwell (Syngenta Central Toxicology Laboratory, Alderly Park, Cheshire, United Kingdom) for their hospitality and commitment to demonstrate the uterotrophic assay.

Table 3.2. Animal weight on day 0 and day 4 as well as and blotted uterus weight and blotted uterus weight per g animal weight of the uterotrophic assay per dose group. Immature rats were subcutaneously exposed on a daily bases for three subsequent days from postnatal day (PND) 21 – 23 to corn oil (CO), E_2, PCmix, SCmix or combinations.

Study	Treatment[gr.size]	Dose	AW-D0[1]	AW-D4[2]	BUW[3]	BUW/AW[4]
1	CO $^{n=5}$	5 ml/kg	46.8 ± 3.1[5]	58.9 ± 4.6	32 ± 12	0.54 ± 0.18
	E_2 $^{n=5}$	1 µg/kg	47.7 ± 3.3[5]	59.1 ± 3.5	73 ± 12	1.24 ± 0.15
	E_2 $^{n=5}$	5 µg/kg	48.9 ± 2.2[5]	58.9 ± 2.4	88 ± 6	1.51 ± 0.16
	E_2 $^{n=5}$	10 µg/kg	51.8 ± 3.2[5]	62.6 ± 3.1	96 ± 14	1.55 ± 0.29
	E_2 $^{n=5}$	40 µg/kg	45.9 ± 2.9[5]	56.7 ± 3.7	119 ± 11	2.10 ± 0.20
	GEN $^{n=5}$	0.4 mg/kg	46.9 ± 2.2[5]	58.4 ± 3.1	35 ± 7	0.59 ± 0.10
	GEN $^{n=5}$	20 mg/kg	47.4 ± 4.0[5]	59.0 ± 3.5	36 ± 3	0.61 ± 0.06
2	CO $^{n=5}$	5 ml/kg	46.1 ± 2.9	59.3 ± 3.4	22 ± 3	0.37 ± 0.06
	E_2 $^{n=5}$	0.3 µg/kg	45.4 ± 1.6	57.5 ± 3.2	23 ± 5	0.41 ± 0.06
	E_2 $^{n=5}$	3 µg/kg	45.3 ± 1.3	55.2 ± 1.7	62 ± 12	1.12 ± 0.20
	E_2 $^{n=5}$	30 µg/kg	46.1 ± 2.3	56.4 ± 2.2	90 ± 6	1.60 ± 0.05
	E_2 $^{n=5}$	60 µg/kg	45.7 ±2.1	56.8 ± 2.7	107 ± 8	1.89 ± 0.18
	GEN $^{n=5}$	3 mg/kg	45.4 ± 2.3	56.8 ± 1.9	22 ± 3	0.39 ± 0.06
	GEN $^{n=5}$	20 mg/kg	45.5 ±2	57.5 ± 3.6	49 ± 5	0.85 ± 0.09
	GEN $^{n=5}$	60 mg/kg	45.5 ± 3.3	57.5 ± 4.7	63 ± 7	1.10 ± 0.119
3	CO $^{n=8}$	5 ml/kg	42.7 ± 7.8	56.6 ± 10.2	33 ± 9	0.59 ± 0.12
	E_2 $^{n=8}$	10 µg/kg	43.3 ± 7.1	54.2 ± 7.9	93 ± 24	1.71 ± 0.34
	E_2 $^{n=8}$	30 µg/kg	44.2 ± 7.9	53.9 ± 7.8	102 ± 19	1.89 ± 0.24
	E_2 $^{n=8}$	100 µg/kg	44.4 ± 7.8	54.7 ± 7.3	105 ± 12	1.95 ± 0.28
	PCmix $^{n=8}$	1000x	45.0 ± 8.0	57.1 ± 9.9	34 ± 5	0.61 ± 0.14
	PCmix $^{n=8}$	3000x	45.5 ± 7.8	56.6 ± 9.7	44 ± 6	0.78 ± 0.17
	PCmix $^{n=8}$	6000x	45.7 ± 7.9	58.8 ± 8.8	66 ± 14	1.13 ± 0.20
	PCmix $^{n=8}$	10000x	46.7 ± 7.4	60.4 ± 9.4	67 ± 12	1.13 ± 0.19
	PCmix $^{n=8}$	30000x	47.3 ± 8.7	60.6 ± 9.5	104 ± 11	1.73 ± 0.24
4	CO $^{n=8}$	5 ml/kg	44.4 ±3.4	57.3 ± 5.0	26 ± 4	0.46 ± 0.09
	E_2 $^{n=6}$	0.1 µg/kg	46.9 ± 3.3	60.5 ± 5.6	30 ± 9	0.49 ± 0.14
	E_2 $^{n=6}$	1 µg/kg	47.0 ± 2.6	60.0 ± 2.8	74 ± 7	1.25 ± 0.08
	E_2 $^{n=6}$	10 µg/kg	45.9 ± 3.0	58.0 ± 3.1	92 ± 14	1.62 ± 0.26
	E_2 $^{n=6}$	100 µg/kg	46.5 ± 2.4	56.8 ± 2.7	107 ±8	1.89 ± 0.19
	E_2 + PCmix $^{n=8}$	2.25 µg/kg + 250x	44.5 ± 2.7	56.6 ± 4.5	75 ± 11	1.33 ± 0.12
	E_2 + PCmix $^{n=8}$	1.50 µg/kg + 500x	46.4 ± 2.8	57.4 ± 4.4	77 ± 13	1.33 ± 0.19
	E_2 + PCmix $^{n=8}$	0.75 µg/kg + 750x	47.6 ± 2.4	60.7 ± 2.3	61 ± 11	1.01 ± 0.18
	E_2 + PCmix $^{n=8}$	0 µg/kg + 1000x	46.6 ± 4.0	59.7 ± 4.2	51 ± 12	0.85 ± 0.21
5	CO $^{n=8}$	5 ml/kg	43.1 ± 2.7	56.2 ± 4.4	20 ± 5	0.36 ± 0.08
	E_2 $^{n=6}$	0.1 µg/kg	45.1 ± 3.5	57.7 ± 5.2	20 ± 4	0.35 ± 0.06
	E_2 $^{n=6}$	1 µg/kg	46.2 ± 3.2	58.9 ± 5.0	64 ± 4	1.09 ± 0.17
	E_2 $^{n=6}$	10 µg/kg	45.3 ± 3.7	57.8 ± 4.0	90 ± 9	1.56 ± 0.14
	E_2 $^{n=6}$	100 µg/kg	45.1 ± 3.2	55.3 ± 5.7	104 ± 14	1.90 ± 0.33
	SCmix $^{n=8}$	1000x	44.2 ± 3.1	58.7 ± 2.6	27 ± 9	0.48 ± 0.16
	SCmix $^{n=8}$	3000x	44.5 ± 2.2	59.7 ± 2.3	28 ± 6	0.47 ± 0.10
	SCmix $^{n=8}$	10000x	45.4 ± 2.3	57.9 ± 3.1	21 ± 3	0.36 ± 0.06
	SCmix $^{n=8}$	30000x	44.4 ± 2.8	57.8 ± 3.2	25 ± 4	0.42 ± 0.07
	SCmix $^{n=8}$	60000x	45.5 ± 2.0	60.3 ± 3.4	32 ± 4	0.54 ± 0.07
6	CO $^{n=8}$	5 ml/kg	45.6 ± 3.7	61.0 ± 5.4	30 ± 6	0.49 ± 0.11
	E_2 $^{n=6}$	0.1 µg/kg	43.6 ± 3.1	58.3 ± 3.5	44 ± 8	0.75 ± 0.12
	E_2 $^{n=6}$	1 µg/kg	45.4 ± 3.1	61.2 ± 4.4	76 ± 9	1.25 ± 0.23
	E_2 $^{n=6}$	10 µg/kg	45.9 ± 3.4	60.7 ± 4.0	97 ± 16	1.61 ± 0.23
	E_2 $^{n=6}$	100 µg/kg	46.2 ± 2.3	59.5 ± 3.2	110 ± 8	1.85 ± 0.20
	SCmix $^{n=8}$	60000x	46.6 ± 2.9	63.0 ± 4.7	34 ± 4	0.54 ± 0.09
	E_2 + SCmi $^{n=8}$x	1 µg/kg + 3000x	45.9 ± 3.1	61.1 ± 4.3	77 ± 12	1.25 ± 0.15
	E_2 + SCmix $^{n=8}$	1 µg/kg + 10000x	46.6 ± 3.2	62.3 ± 3.2	66 ± 16	1.05 ± 0.22
	E_2 + SCmix $^{n=8}$	1 µg/kg + 60000x	47.4 ± 1.7	64.3 ± 2.9	78 ± 9.0	1.22 ± 0.15
7	CO $^{n=6}$	5 ml/kg	46.2 ± 2.8	61.4 ± 4.8	30 ± 9	0.48 ± 0.12
	E_2 $^{n=6}$	1 µg/kg	46.0 ± 2.3	61.6 ± 3.7	81 ± 6	1.32 ± 0.07
	E_2 $^{n=6}$	100 µg/kg	46.1 ± 2.2	58.3 ± 2.6	110 ± 17	1.89 ± 0.33
	PCmix $^{n=7}$	1000x	45.2 ± 2.5	60.7 ± 2.2	67 ± 17	1.11 ± 0.30
	SCmix $^{n=7}$	100000x	46.7 ± 4.2	63.2 ± 4.6	48 ± 9	0.76 ± 0.18
	E_2 + PCmix + SCmix $^{n=8}$	0.5 µg/kg + 500x + 0	48.1 ± 2.0	63.1 ± 2.5	66 ± 17	1.05 ± 0.26
	E_2 + PCmix + SCmix $^{n=8}$	0.5 µg/kg + 500x + 3000x	48.7 ± 2.7	62.3 ± 3.8	62 ± 7	1.00 ± 0.11
	E_2 + PCmix + SCmix $^{n=8}$	0.5 µg/kg + 500x + 10000x	48.2 ± 2.7	63.0 ± 3.3	67 ± 9	1.06 ± 0.13
	E_2 + PCmix + SCmix $^{n=8}$	0.5 µg/kg + 500x + 30000x	47.8 ± 3.4	62.3 ± 4.5	66 ± 9	1.07 ± 0.21
	E_2 + PCmix + SCmix $^{n=8}$	0.5 µg/kg + 500x + 100000x	47.7 ± 2.4	63.4 ±3.2	61 ± 14	0.96 ± 0.19

[1] = Animal weight (g) on D0 (PND 20); [2] = Animal weight (g) on D4 (PND 24); [3] = Blotted Uterus Weight (mg); [4] = Blotted Uterus Weight per Animal weight on D4 (mg/g); [5] = Animal weight (g) on D1 (PND 21).

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Chapter 4

Aromatase inhibiting and combined estrogenic effects of parabens and estrogenic effects of other additives in cosmetics

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Abstract

There is concern widely on the increase in human exposure to exogenous (anti)estrogenic compounds. Typical are certain ingredients in cosmetic consumer products such as musks, phthalates and parabens. Monitoring a variety of human samples revealed that these ingredients, including the ones that generally are considered to undergo rapid metabolism, are present at low levels. In this *in vitro* research individual compounds and combinations of parabens and endogenous estradiol (E_2) were investigated in the MCF-7 cell proliferation assay. The experimental design applied a concentration addition model (CA). Data were analyzed with the estrogen equivalency (EEQ) and method of isoboles approach. In addition, the catalytic inhibitory properties of parabens on an enzyme involved in a rate limiting step in steroid genesis (aromatase) were studied in human placental microsomes. Our results point to an additive estrogenic effect in a CA model for parabens. In addition, it was found that parabens inhibit aromatase. Noticeably, the effective levels in both our *in vitro* systems were far higher than the levels detected in human samples. However, estrogenic compounds may contribute in a cumulative way to the circulating estrogen burden. Our calculation for the extra estrogen burden due exposure to parabens, phthalates and polycyclic musks indicate an insignificant estrogenic load relative to the endogenous or therapeutic estrogen burden.

Introduction

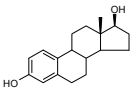
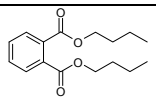
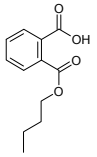
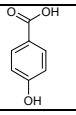
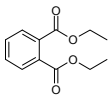
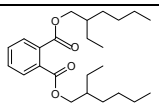
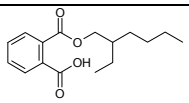
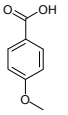
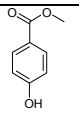
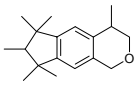
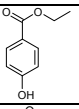
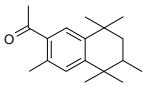
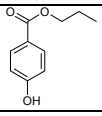
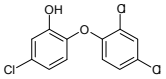
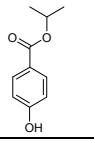
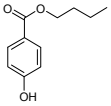
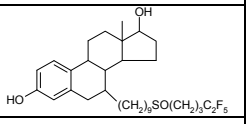
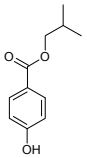
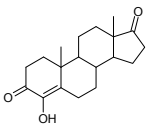
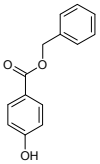
There is concern widely on the increase of exposure of humans to endocrine active compounds [9]. In this respect, several estrogenic cosmetic additives recently have been suggested to pose a possible risk to humans and concern has been raised because of a suggested relationship with breast cancer [15, 18]. However, these studies were seriously criticized by other authors pointing out a methodological flaw in the experimental design [25, 30]. In addition, other scientists have brought forward that it is doubtful that the low level of exposure to humans of these cosmetic additives would be related to adverse effects [55, 56]. Many consumer products however, such as wash off products that are used regularly, often daily, still contain weakly estrogenic compounds such as (polycyclic) musks, phthalates and parabens, or potentially weakly androgenic compounds such as triclosan [23] in spite of the discussions mentioned above. These products often contain multiple of the above ingredients and consequently human topical exposure may be combinatorial. In addition, human exposure to parabens can also occur via food and certain pharmaceuticals [20, 54], while phthalates leak out of food-wrapping materials into food products [2, 57]. The purposes these additives serve are various with e.g. musks giving an odor to the product [24], phthalates being a fragrance fixate, skin moisturizer, skin softener, skin penetration enhancer, anti foaming agent and solvent [35]. Furthermore, parabens and triclosan preserve the product and triclosan acts as an antiseptic [1, 20].

Clearly, the predominant route of exposure to these compounds is skin absorption, but other routes of exposure such as absorption through mucosa and intestines can also occur. Although individual chemical properties and biotransformation rates of these cosmetic ingredients differ widely, recent chemical analysis of human blood showed the presence of polycyclic musks [3, 28], parabens [17], phthalates [43] at low levels (table 4.1). An earlier toxicological report on parabens with focus on regular toxicological studies (lacking endpoints measuring endocrine related parameters) did not indicate that these background levels could be a potential hazard for humans [20]. However, more recent and mostly *in vitro* studies using various systems point towards potential endocrine disrupting and (anti-)estrogenic properties for some of these cosmetic additives. Polycyclic musks were found to be ERβ receptor antagonists in reporter cell lines, but on the other hand induced cell proliferation in the MCF-7 breast tumor cells via the ERα receptor [6, 59, 60]. Certain parabens can also bind to the ERα and activate this receptor protein transfected in yeast cells, induce proliferation in MCF-7 cells and induce uterotrophy in subcutaneously exposed mice or rats [9, 14, 16, 39, 54]. Certain phthalates were also found to activate ERα in a recombinant yeast cell line and induce cell proliferation in MCF-7 cells [26, 64], however phthalates did not induce an estrogenic response *in vivo* [31, 75]. These studies focused on individual effects. Exposure to mixtures of cosmetic additives on the other hand has not been investigated yet, although human samples illustrate the presence of a mixture of these chemicals at low levels. A first aspect of this study was to investigate estrogenic effects of mixtures of parabens.

As a second aspect of this study, we investigated effects of parabens on aromatase. Aromatase is the rate limiting enzyme responsible for converting androgens to estrogens. When a compound binds and inhibits aromatase this causes a decreased conversion of testosterone into estrogens and can thus be interpreted as an anti-estrogenic effect. Aromatase inhibitory effects of parabens could be expected based on their chemical structure. Parabens have a hydroxy- and/or keto-group in their aromatic structure, which in essence is similar to known aromatase inhibitors. To our knowledge, aromatase interference of parabens has not been experimentally investigated yet.

In this study estrogenic and aromatase inhibitory effects of several cosmetic additives were investigated. Estrogenicity was studied in the MCF-7 proliferation assay (E-screen). In this assay a range of cosmetic additives was tested individually, comprising several parabens, their common metabolite 4-hydroxybenzoic acid (4HBA or pHBA) and two of its structural isomers (3HBA and 2HBA), several phthalates, two polycyclic musks and triclosan. Ultimately, combinations of parabens were tested and the results were analyzed with two concentration addition approaches: the EEQ-method (estrogen equivalency) [65] and isoboles-method [5, 36].

Table 4.1. Test compounds, chemical structure and reported concentration in human samples.

compound name abbreviation cas#	chemical structure	reported conc. in human sample ng/g [a]	compound name abbreviation cas#	chemical structure	reported conc. in human sample ng/g [a]
Endogenous estrogen			**Phthalates**		
17β-estradiol E_2 cas# 50-28-2			dibutyl phthalate DBP cas# 84-74-2		2.6 – 257 [43, 49]
Parabens			monobutyl phthalate MBP cas# 131-70-4		7.2 – 30.9 [67]
p-hydroxy benzoic acid pHBA cas# 99-96-7		44 [48]	diethyl phthalate DEP cas# 84-66-2		2.2 – 19 [43, 49]
m-hydroxy benzoic acid 3HBA cas# 99-06-9		-	di-(2-ethylhexyl) phthalate DEHP cas# 117-81-7		28 – 5863 [43, 49]
o-hydroxy benzoic acid 2HBA cas# 69-72-7		-	mono-2-ethylhexyl phthalate MEHP cas# 4376-20-9		1.3 – 9.0 [67]
4-methoxybenzoic acid MeOxPB cas# 100-09-4		-	**Polycyclic musks**		
methyl paraben MePB cas# 99-76-3		-1.8 – 29.3 [17]	galaxolide (HHCB) cas# 1222-05-5		0.2 – 9.2 [43, 49] 0.274 [3]
ethyl paraben EtPB cas# 120-47-8		-0.9 – 7.4 [17]	tonalide (AHTN) cas# 1506-02-1 or cas# 21145-77-7		0.1 – 11 [43, 49] 0.722 [3]
propyl parabens ProPB cas# 94-13-3		-4.3 – 10.4 [17]	triclosan cas# 3380-34-5		
isopropyl paraben IsoproPB cas# 4191-73-5		-1.1 – 5.2 (5)	**Reference compounds**		
butyl paraben BuPB cas# 94-26-8		-3.7 – 11.5 [17]	ICI 182,780 ICI cas# 129453-61-8		
isobutyl paraben IsobuPB cas# 4247-02-3		-1.1 – 5.2 [17]	4OH-androstenedione 4-OH A cas# 566-48-3		
Benzylparaben BenzPB cas# 94-18-8		0.0 [17]			

[a] In the conversion from reported levels in human samples to ng/g it is assumed that 1 ml = 1 g.
[43]: Human serum concentration
[49]: Human maternal serum concentration
[3] : German human blood samples
[48]: Human samples from adults such as blood, umbilical cord and milk, while parabens could not be detected [47]
[17]: Concentrations measured in extract of breast tumors
[67]: Prenatal urinary concentration of pregnant women.

Based on earlier studies with mixtures of estrogenic compounds, it was hypothesized that a combination of two estrogenic parabens might also act in an additive way [70, 71]. Aromatase inhibition was studied in human placental microsomes with the tritium water release assay. In this assay a range of several parabens, 4HBA, 3HBA and 2HBA was tested. As several of these additives in cosmetics posses a hydroxy- and/or keto-group in their aromatic ring it was hypothesized that aromatase inhibition could occur in analogy with e.g. 4 hydroxy-androstenedione.

Materials and Methods

Chemicals & Plastics

17β-estradiol (E_2), was purchased from Sigma (St. Louis, MO, USA). Galaxolide (HHCB, Cas nr: 1222-05-5) was obtained from International Flavors and Fragrances (I.F.F.) BV (Hilversum, The Netherlands) and Tonalid (AHTN, Cas nr: 1506-02-1 and 21145-77-7) from PFW Aroma Chemicals BV (Barneveld, The Netherlands). Mono-ethylhexyl-phthalate (MEPH), monobutyl-phthalate (MBP) and di-(2-ethylhexyl)-phthalate (DEHP) were purchased from (TCI, Zwijndrecht, The Netherlands). Diethylphthalate (DEP) and Igrasan (triclosan) were purchased from (Fluka, Zwijndrecht, The Netherlands). Di-butylphthalate (DBP), methylparaben (MePB), ethylparabene (EtPB), butylparaben (BuPB) and 4-hydroxy-androstendione (4-OH A) were obtained from Sigma (Zwijndrecht, The Netherlands). 2-Hydroxybenzoic acid (2HBA), 3-hydroxybenzoic acid (3HBA), 4-methoxybenzoic acid (MeOxPB), propylparaben (ProPB), isopropylparaben (IsoproPB), isobutylparaben (IsobuPB) and benzylparaben (BenzPB) were purchased from (Sigma-Aldrich, Schnelldorf, Germany). The ER antagonist faslodex (ICI 182780) (ICI) was obtained from Tocris (Bristol, UK). All compounds were prepared as 1000x stock solutions in ethanol (Riedel-de-Haën, Zwijndrecht, The Netherlands), stored at –20°C prior to exposure of the cells via the medium. The final solvent concentration did not exceed 0.3% (*v/v*) in the MCF-7 cell proliferation assay and 1% (*v/v*) in the aromatase assay. Tissue culture plastics were purchased from Greiner (Alphen a/d Rijn, The Netherlands). Phenol red free Dulbecco's modified Eagles medium (DMEM), the supplements fetal calf serum (FCS), L-glutamine (200 mM) and 10.000 U/ml penicilline-streptomycine (p/s) were purchased from Gibco (Breda, The Netherlands). Insuline from bovine pancreas (ins) and sodium pyruvate ($C_3H_3NaO_3$) were purchased from Sigma. Charcoal-dextran treated FCS (SF-FCS) was obtained from Hyclone (Etten-leur, The Netherlands).

Cell culture

Routinely MCF-7 human breast adenocarcinoma cells (No. HTB-22, ATCC-LGC Promochem, Teddington, United Kingdom) were maintained in 25 cm^2 or 75 cm^2 plastic flasks on DMEM, containing 5% FCS (Gibco) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1:1000 p/s at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity. To passage cells, cells were rinsed with PBS and detached by trypsine/EDTA (Gibco). In our experiments the MCF-7 cells were used within the passage number range 10-45. Within this range the EC_{50} and maximum efficacy of E_2 were constant. Steroid free medium (SF-medium) was used in experiments, consisting of DMEM, containing 5% SF-FCS supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and 1 U/ml pen/strep.

MCF-7 cell proliferation assay

The MCF-7 cell proliferation assay (also known as E-screen) makes use of the dominantly expressed estrogen receptor α (ERα), mediating a mitotic effect in response to estrogenic compounds [63]. Experimental conditions were similar as those published earlier [52, 70]. In short, on day 0, 4500 cells/200 µl SF-medium were seeded in 96-wells plates and starved of steroids for two days. Cultures were then exposed for 6 days to SF-medium containing the test compound(s) with a medium refreshment after three days and measurements at least in triplicate. Each experiment included control cultures that were cultured in SF-medium with and without 0.1 – 0.3% ethanol, both in triplicate. Then cell number per well was determined by measuring the capacity of the cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)(Sigma) as a measure for cell number per well. Briefly, cultures were carefully rinsed with PBS to remove most of the test compounds [8] and incubated with 100 µl DMEM, containing 1 mg/ml MTT for 1h at 37°C. After cell lysis, absorbance at 595 nm (Fluostar, BMG lab technologies, Germany) minus background (MTT without cells) was used as the corrected absorbance in further calculations.

Concentration ranges tested were chosen such that both a low and high effective proliferative response was covered. Thus, E_2 and each test compound were tested multiple times in a concentration range of 0.3 pM – 1 nM (E_2) and 1 nM – 10 mM (each test compound). In addition, three stock solutions in EtOH were composed of EtPB and ProPB (0.1 mM EtPB and 0.02 mM ProPB for PBmix 1, 0.05 mM EtPB and 0.05 mM ProPB for PBmix 2 and 0.02 mM EtPB and 0.1 mM ProPB for PBmix 3). These stock solutions were tested at several dilutions in the range $1.2x10^{-7}$ – $1.2x10^{-4}$ M for PBmix 1 and for PBmix 3, and $1x10^{-7}$ – $1x10^{-4}$ M for PBmix 2. The rational to have three different ratios EtPB : ProPB in three PBmixes was to be able to recalculate at what concentrations of individual constituents (EtPB and ProPB) an equal proliferative effect (50%) is reached (iso-effective concentration) for several combinations. Concentrations of PBmix are indicated as total concentration of both PBs. In addition, two-component combination-experiments were performed using a paraben with E_2 or one of the other investigated parabens at ratios varying from 0% to 100% of their experimentally derived EC_{50} values. For all combinations used, the response was expected to be the EC_{50} of E_2 in these combination experiments.

To control if cell proliferative effects of the test compounds were ER mediated, sub maximal cell proliferation was induced by a paraben and the inhibiting effects of an ER antagonist (ICI) in the range of 10 pM – 100 nM was examined. Phthalates and musks were not extensively tested under such conditions.

Cytotoxicity

Cytotoxicity was assessed using MTT again. MCF-7 cells were seeded at $1x10^5$ cells/well (24 wells plate) in SF-medium. After 24h incubation at 37°C cells were exposed to test compounds for 24h. Cell number per well was determined by measuring the capacity of the cells in each well to reduce MTT as described above. Absorbance lower than that of solvent treated cultures was considered cytotoxic.

Aromatase activity

Placental tissue was obtained from the St. Antonius Hospital (Nieuwegein, The Netherlands) after permission of the patient by informed consent. In all our experiments aromatase activity was determined in microsomes derived from one human placenta. This was done to assure a similar background aromatase activity throughout the study. The tissue was snap frozen in liquid nitrogen upon arrival and stored at –70°C until use. To homogenize the tissue, TRIS-HCL (50mM) : KCl (1.15%) buffer was added at 1ml per g tissue and homogenized on ice in a Potter-Elvehjem Teflon glass homogenizer. Homogenate was first centrifuged (Beckman L7-55) at 10000 g, 4°C for 25 min to remove cell debris and then centrifuged at 100000 g, 4°C for 75 min. The pellet was resuspended in sucrose solution (250 mM) 1 ml/g tissue and total protein content of the microsome suspension was

measured [42] using bovine serum albumin (BSA) in the range of 0 – 100 µg/ml as a protein standard. Aliquoted (1 mg/ml total protein) microsomes were stored at -70°C prior to further use. Catalytic aromatase inhibiting capacities of the compounds were tested mostly in triplicate (except BenzPB and MeOxPB) in the range of 100 nM – 100 µM in 24 wells plate. BuPB, pHBA and its two isomers were also tested at 1 mM. This range of concentrations was chosen to cover concentrations that have been found to be effective for other compounds such as certain phytochemicals. Wells contained microsomes (15 µg protein/250 µl final volume), HEPES/Mg_2Cl buffer (46.5 mM/4.65 mM), 1β-^{3}H-androstenedion (357 nM, New England Nuclear Research Products, Boston, MA, USA) and NADPH (0.5 mM; 0.42 mg/ml). In control wells no NADPH was added and wells without microsomes and NADPH served as blanks. In negative control wells ethanol was added to a final concentration of 1% (*v/v*). In positive control wells 4-OH A was added to a final concentration of 1 µM. Microsomes were incubated at 37°C for 45 min. After incubation, 200 µl medium from each well was used for chloroform (500 µl) extraction. From 100 µl of the aqueous layer unconverted androstenedione was removed by treatment with 100 µl dextran coated charcoal (5 µg active charcoal + 0.5 µg dextran/100 µl MQ). 125 µl of centrifuged samples was added to 3.5 ml liquid scintillation cocktail (Ultimate Gold, PerkinElmer, Wellesley, MA) and counted in a scintilator (Packard, Groningen, The Netherlands) to quantify the release of tritiated water. This value, that represents the release of tritiated water, was used to calculate aromatase activity in pmol/h/mg protein. The human derived placental microsomes in this study had an average aromatase activity (conversion rate) of 293 ± 169 pmol/h/µg protein.

Calculations & models

To determine cell proliferative effects of test compounds, the corrected formazan absorbance (MTT) was plotted against logarithmically transformed concentrations. EC_{50} values were estimated from these concentration-response relationships, which were described using a 'best-fit' approach according to formula (4.1):

$$y = \text{min.}+(\text{max.} - \text{min.})/(1+10^{(\text{Log(EC50)-Log(C(TC))} \times \text{hill slope})}) \qquad (4.1)$$

where y is response and $C_{(TC)}$ the concentration of the test compound.

To determine aromatase inhibitory effects of test compounds, the relative aromatase activity was plotted against logarithmically transformed concentrations. IC_{50} values were estimated from concentration-response relationships that were described using a 'best-fit' approach according to formula (4.1).

Interaction within the mixtures (PBmix 1, PBmix 2, PBmix 3) were determined by analyzing the data (corrected for background) from cell proliferation assays with the concentration addition-approach (CA) ([5, 36, 40], by the isoboles-method and the cumulative estrogen equivalency (EEQ)-method [65]. The concentration addition approaches applied to our data assumes that a response is solely induced by an estrogenic compound via the ER and therefore excludes other mechanisms that could influence cell proliferation. In addition, a prerequisite to apply the EEQ-method is that the hill slope and the maximum response of the test compound equals that of E_2.

In the isoboles-method the geometrical mean of the estimated EC_{50} values for the individual parabens EtPB and ProPB and the PBmixes were applied in the formula:

$$\Sigma(C_{(\text{TC in mix at EC50})}/EC_{50(TC)}) \qquad (4.2)$$

where TC is the test compound. In this method a result of 1 indicates zero interaction of the compounds in the mixture. A result lower or higher than 1 indicates synergism or antagonism, respectively [5, 36].

In the EEQ-method estrogen equivalency factors (EEFs) were calculated for each individual compound or mixture (per experiment) with the formula:

$$EEF_{(TC\ or\ mix)} = EC_{50(E2)}/EC_{50(TC\ or\ mix)} \quad (4.3)$$

where $EC_{50(E2)}$ is the geometric mean (GM) of the corresponding experiment and the GM of EEFs obtained from all experiments was calculated and shown in table 4.2. For each compound the mean EEF of several experiments was multiplied with the concentration of the compound in the mix, resulting in estrogen equivalents (EEQs):

$$EEQ_{(TC)} = EEF_{(TC)} \times C_{TC\ (in\ mix)} \quad (4.4)$$

The sum of the EEQs for each compound results in a calculated EEQ for the mix:

$$\text{Calculated } EEQ_{(mix)} = \Sigma\ EEQ_{(TC)} \quad (4.5)$$

is then compared with the observed EEQ of the mix:

$$\text{Observed } EEQ_{(mix)} = EEF_{(mix)} \times C_{mix} \quad (4.6)$$

where C_{mix} is the total concentration of the components in the mixture.
In addition to EC_{50} values estimated from 'best-fit' analysis, EC_5s and EC_{10}s were calculated by formula (4.7):

$$EC_x = (x/(100-x))^{(1/hill\ slope)} \times EC_{50} \quad (4.7)$$

where x is the desired effect (5% or 10% of the maximum effect) and hill slope and EC_{50} were obtained from the 'best-fit' analysis in formula 1. These EC_5s and EC_{10}s were calculated for those test compounds that did not induce a maximum cell proliferative response as high as E_2 (MePB, MeOxPB, DEP, DBP and DEPH) to again calculate the estrogenic potency by formula (4.3). To determine concentration at which test compounds inhibited aromatase for 5% or 10% the EC_{95}s and EC_{90}s were calculated.
For two component combination-experiments calculation of the expected response (R_{exp}) for all ratios were based on the proportions of E_2 or a paraben and a paraben and based on an additive model. These R_{exp} were calculated by the equation:

$$R_{exp} = \sum_{i=E2\ or\ mix} F_{(i)} \times R_{obs\,(i)} \quad (4.8)$$

where $F_{(i)}$ is the fraction of E_2 or paraben in the combination to which cells were exposed and $R_{obs\ (i)}$ was the observed response of cells, measured as the MTT value corrected for background, exposed to the EC_{50} set for E_2 or the paraben. The percentage of the observed response was then related to this expected response by equation:

$$\%\ of\ R_{exp} = R_{obs} / R_{exp} \times 100\% \quad (4.9)$$

Aromatase inhibitory properties were determined for individually tested parabens. These were calculated by subtracting blank values from sample values and dividing by values of samples lacking NADPH.

Table 4.2. EC_{50} values and estrogenic equivalency factors (EEFs) of cosmetic additives and some metabolites and three mixtures of two parabens (EtPB and ProPB) in the MCF-7 proliferation assay and probit 'best fit' analysis for each test compound or mixture. Measured values were applied to calculate interactions using the estrogen equivalency (EEQ) and isoboles methods.

EEQ-method			**PBmix 1**	**PBmix 2**	**PBmix 3**
Compound	**EC_{50}** Log(GM ± GSD)[3] (M)	**EEF**[1] Log(GM ± GSD)[3]	**EEQ**[2] mean (Log(mean))	**EEQ**[2] mean (Log(mean))	**EEQ**[2] mean (Log(mean))
17β-estradiol	12E-3	1			
pHBA	n.m	n.c.			
2HBA	n.m	n.c.			
3HBA	n.m	n.c.			
MePB	-4.8 ± 0.03	-6.3 ± 0.43			
EtPB	-5.5 ± 0.3	-5.7 ± 0.36	-9.7 ± 0.36	-10.0 ± 0.36	-10.4 ± 0.36
ProPB	-5.8 ± 0.17	-5.1 ± 0.27	-9.8 ± 0.27	-9.4 ± 0.27	-9.1 ± 0.27
IsoProPB	-6.0 ± 0.06	-4.7 ± 0.09			
BuPB	-6.1 ± 0.09	-5.0 ± 0.24			
IsobuPB	-6.1 ± 0.25	-4.6 ± 0.20			
BenzPB	-6.1 ± 0.05	-4.7 ± 0.40			
MeOxPB	-5.60 ± 0.20	-5.2 ± 0.35			
DBP	-5.6 ± 0.31	-5.1 ± 0.56			
MBP	n.m.	n.c.			
DEP	-4.6 ± 0.46	-6.2 ± 0.42			
DEHP	-5.5 ± 0.23	-5.3 ± 0.19			
MEHP	n.m.	n.c.			
Galaxolide	-5.9 ± 0.34	-5.0 ± 0.37			
Tonalide	-6.3 ± 0.39	-4.6 ± 0.51			
			sum (EEQ)[4]	**sum(EEQ)**[4]	**sum(EEQ)**[4]
PBmix 1(calculated)			-9.4 ± 0.45		
PBmix 1(observed)	-5.2 ± 0.13 *	-5.8 ± 0.36	-9.7 ± 0.36		
PBmix 2(calculated)				-9.3 ± 0.45	
PBmix 2(observed)	-5.6 ± 0.15 *	-5.5 ± 0.34		-9.5 ± 0.34	
PBmix 3(calculated)					-9.1 ± 0.45
PBmix 3(observed)	-5.7 ± 0.03 *	-5.4 ± 0.25			-9.3 ± 0.25
Method of isoboles			**[TC] at EC_{50}(mix)**	**[TC] at EC_{50}(mix)**	**[TC] at EC_{50}(mix)**
EtPB			-5.3 ± 0.13	-5.9 ± 0.15	-6.5 ± 0.03
ProPB			-6.0 ± 0.13	-5.9 ± 0.15	-5.8 ± 0.03
			[TC]/EC_{50}[5]	**[TC]/EC_{50}**[5]	**[TC]/EC_{50}**[5]
EtPB			1.3	0.3	0.1
ProPB			0.6	0.7	1.0
			sum[6]	**sum**[6]	**sum**[6]
			2.0	1.0	1.1
Distributions:					
P_{05}			0.8	0.5	0.6
P_{95}			5.7	2.4	2.1

At the highest exposure the concentrations EtPB and ProPB in the SF-medium, respectively, were 0.1 mM and 0.02 mM in PBmix1, 0.05 mM and 0.05 mM in PBmix 2 and 0.02 mM and 0.1 mM in PBmix 3.

* = total concentrations, thus the sum of the concentrations for EtPB and ProPB in mixture at EC_{50} of the PBmix

(1) = EEF = $EC_{50(E2)}/EC_{50(TC)}$ (calculated per experiment)

(2) = EEQ = $C_{(TC\ in\ mix)}$ x EEF or for the mixture $C_{(total)}$ x EEF

(3) = logarithmic values for geometric mean and standard deviation, meaning the average of the log transformed values.

(4) = the sum EEQs of all components in the mixture.

(5) = the concentration of each test compound at the EC_{50} of the mixture [TC] divided by the EC_{50} of the test compound.

(6) = the sum of the decrease of all components in the mixture.

n.m. = no mitotic effect in the tested range (10 nM – 100 μM); n.c. = not calculated

P_{05} and P_{95} values were determined from distribution of possible outcomes of formula 4.2

Statistics

To interpret the out come of the isoboles method, a distribution for outcomes of formula 4.2 was determined in S-PLUS (S-PLUS 6.0 Professional, Insightful Corp, Seattle WA, USA). This distribution was generated from the distributions of the calculated concentrations for EtPB and ProPB at EC_{50} of the mixture and the estimated EC_{50} for individual tested EtPB and ProPB. From this distribution, 90% of the possible outcomes is expected between the P_{05} and P_{95} values. If this P_{05} value is smaller and P_{95} value is larger than 1, the isoboles method is assumed not to deviate from zero interaction [70].

Statistically significant differences between observed estrogenic potencies and calculated estrogenic potencies of the mixtures were determined by the *t*-test in S-PLUS. The acceptable level of significance for these analyses was set at $P<0.05$.

Statistical differences of aromatase activities were determined by Student's *t*-test and were performed with Excel (Microsoft Excel 2000, NY, USA). The acceptable level of significance was set at $P<0.05$.

Results

17β-estradiol

In the MCF-7 proliferation assay the EC_{50} for 17β-estradiol (E_2) was on average 12 pM and a maximum effect of six to eight fold the control level was observed at 1 nM. In addition, cell proliferation of 1 nM E_2 was concentration-dependently inhibited by ICI with an IC_{50} = 1.0 nM and fully blocked at 10 nM (fig 4.1A).

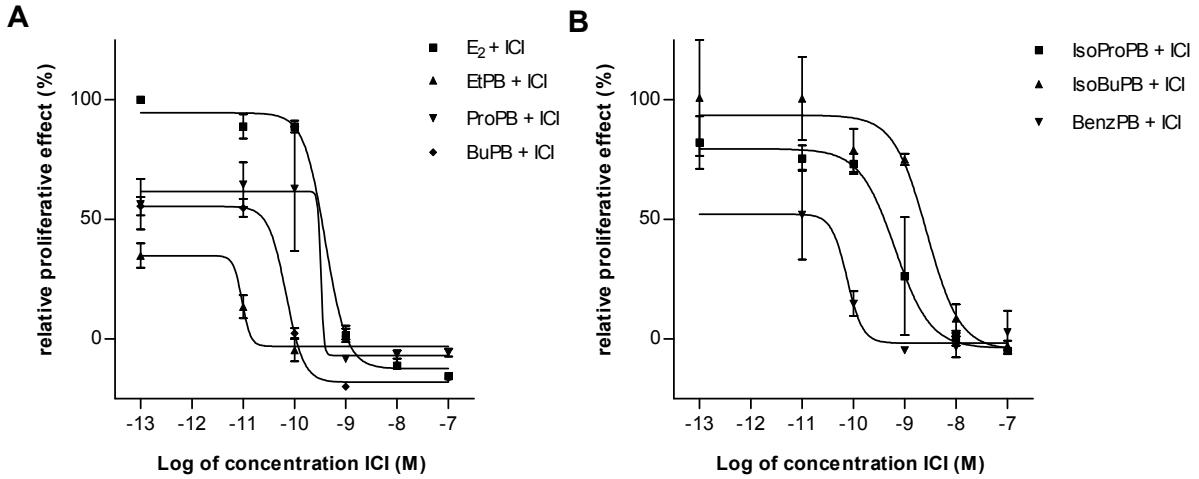


Fig 4.1. Cell proliferative effects of fixed concentrations E_2, EtPB, ProPB, BuPB (**A**), BenzPB, IsoProPB or IsoBuPB (**B**) inhibited by the anti-estrogen ICI 182780 (ICI) in the MCF-7 cell proliferation assay measured as MTT reduction. Concentrations of ICI are presented as Log($C_{(TC)}$), while at 10^{-13} M no ICI was added.

Individual additives in cosmetics

The tested parabens (MePB, EtPB, IsoprPB, BuPB, IsobuPB) did not show cytotoxic effects up to 1 mM. For tested phthalates (DBP, MBP, DEP) and the tested musk (HHCB) this was up to 0.1 mM. For triclosan this was up to 0.01 mM.

Individual parabens that produced full concentration-response curves in the MCF-7 cell proliferation assay were EtPB, ProPB, BuPB, BenzPB, IsoproPB and IsobuPB. EC_{50} values were in the range from 0.5 to 10 µM. Parabens that generally did not induce 100% (relative to E_2) cell proliferation were MePB (EC_{50} = 17 µM, max. 45%) and MeOxPB (EC_{50} = 3 µM, max. 75%) (fig 4.2A, 4.2B & table 4.2). None of the hydroxylated benzoic acid isomers did induce cell proliferation.

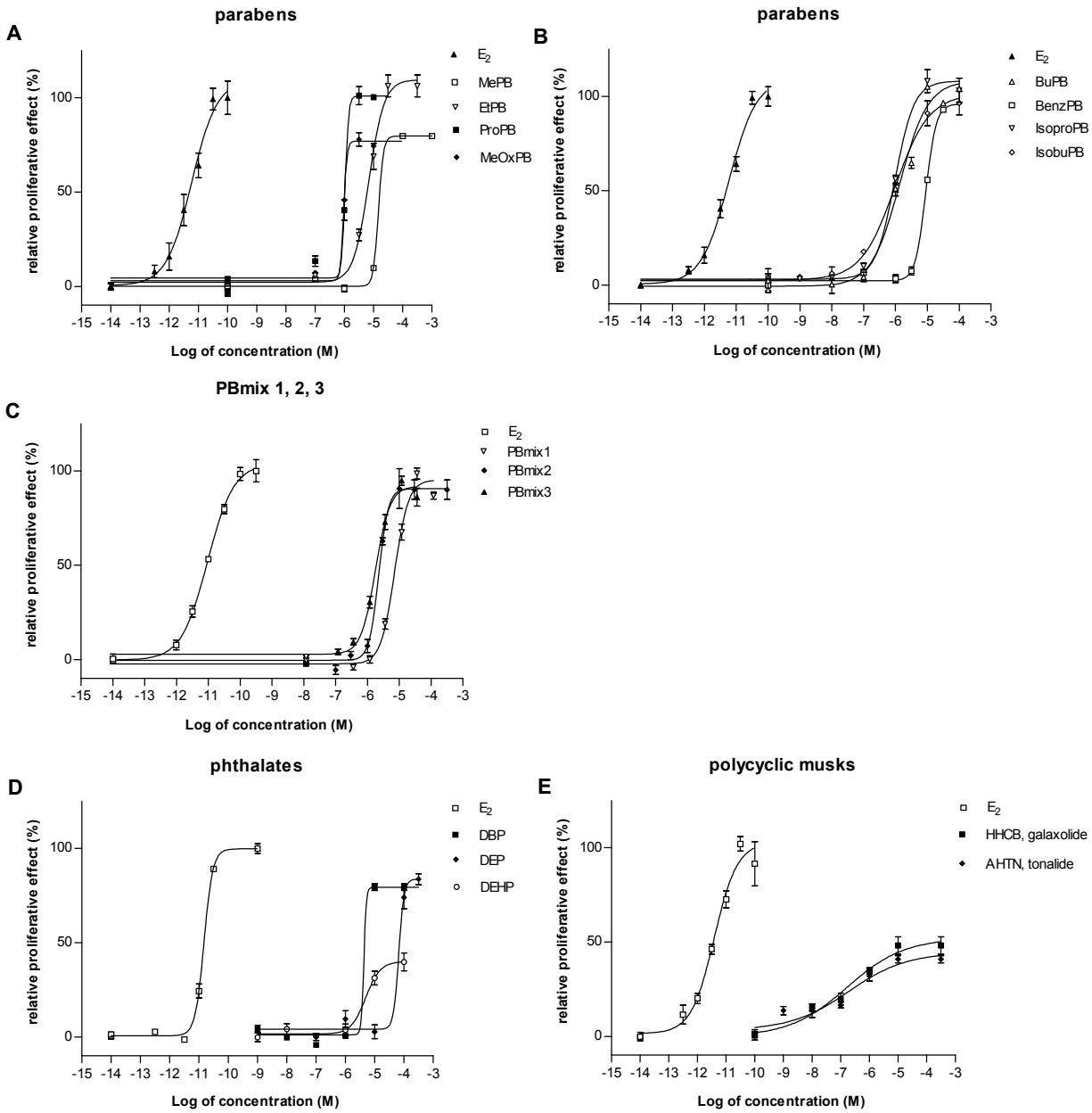


Fig 4.2. Cell proliferative effects of E_2 and six parabens (**A & B**), E_2 and PBmix 1, 2 and 3 (containing fixed ratios EtPB and ProPB) (**C**) and E_2 and three phthalates (**D**) and E_2 and two musks (**E**) in the MCF-7 cell proliferation assay measured as MTT reduction. Concentrations of individual chemicals are presented as Log($C_{(TC)}$), while mixtures are presented as Log(total concentration).

Phthalates that produced concentration-dependent cell proliferation in the MCF-7 cells were DEP, DEHP, DBP and EC_{50} values were in the range between 2 and 22 µM. In general, these phthalates could not induce more than 75% of the maximum cell proliferation induced by E_2 (fig 4.2D & table 4.2). MBP and MEHP did not induce cell proliferation in the MCF-7 cells, even at the highest concentrations.

The polycyclic musks HHCB and AHTN induced a concentration dependent cell proliferation in the MCF-7 cells with EC_{50} values around 1 µM, though maximal induced effect in the MCF-7 cells was less that 50% of E_2 (fig 4.2E & table 4.2). Triclosan did not induce any cell proliferation.

Like with E_2, sub maximally induced cell proliferation by parabens, phthalates and musks could be fully blocked by the ER antagonist ICI 182780 at 10 nM (for parabens see fig 4.1A & 4.1B).

It should be noted that calculating estrogenic potency factors relative to E_2 is only correct for those compounds that have a similar efficacy (maximal induction of cell proliferation) and a similar hill slope as E_2. Therefore if formula 4.3 (see materials and methods) is used for these compounds e.g. the phthalates and the musks, this will result in an overestimated potency (i.e. underestimation of the EEF). Estrogen equivalency factors for the parabens IsobuPB, BenzPB, BuPB and IsoproPB relative to E_2 (=1) could be reliably derived from their EC_{50} values and were around 10^{-5}, while for ProPB and EtPB these were around 10^{-6}. The potencies of the other parabens were $6x10^{-6}$ for MeOxPB and $5x10^{-7}$ for MePB when derived from their EC_{50}s. In addition, potencies of MeOxPB and MePB were also derived from their EC_5 and EC_{10} values and were $7x10^{-7}$ for MeOxPB and $1x10^{-7}$ for MePB (based on EC_5s) and $1x10^{-6}$ for MeOxPB and $2x10^{-7}$ for MePB (based on EC_{10}s). As these compounds did not induce a maximum response as high as E_2, potencies based on EC_{50}s are probably less reliable than these derived from EC_5s or EC_{10}s and to overestimate the compounds potency. Indeed, potencies based on EC_{50}s gave lower EEF values than these derived from EC_5s or EC_{10}s.

Potencies of phthalates derived from EC_{50}s were around $1x10^{-7}$. Then again, derived from EC_5s or EC_{10}s these EEFs were around $1x10^{-6}$. Potencies of musks derived from EC_{50}s were around $1.0x10^{-5}$, while potencies derived from EC_5s or EC_{10}s the EEF were $6x10^{-6}$ for HHCB and $2x10^{-5}$ for AHTN. (Table 4.2).

Mixtures of parabens

The PBmixes 1, 2 and 3, consisting of EtPB and ProPB, were composed to investigate combination effects of these two compounds. All PBmixes produced a full concentration-response curve in the test range of $1.2x10^{-7}$ – $1.2x10^{-4}$ M (PBmix 1 and PBmix 3) and $1x10^{-7}$ – $1x10^{-4}$ M (PBmix 2) (fig 4.2C). The mean EC_{50} value obtained from cell proliferation assays were 5.5 µM, 2.3 µM, 1.9 µM (total concentration) (fig 4.2C & table 4.2). EC_{50} values were converted to their relative estrogenic potencies compared to E_2 (formulas 4.3 and 4.6)(table 4.2). Calculated EEQs were similar to the observed experimental EEQs for the PBmixes (table 4.2).

The interaction was calculated with the isobolic-method by appliance of formula 4.2. For the three PBmixes the interaction was around 1 (table 4.2), suggesting no interaction between EtPB and ProPB. Yet, the method of isoboles does not include a measure for statistical significance. We attempted to use standard deviations of EC_{50}s in S-PLUS to interpret the statistical meaning of the result of the calculated interaction deviating from 1. This analysis indicates that the calculated interaction is not deviating from the range of results to be expected (table 4.2).

Binary combinations of E_2 and parabens

Interactive effects between E_2 and parabens were investigated in binary equipotent combinations of E_2 and MePB, EtPB, ProPB, BuPB or BenzPB and tested at various ratios. The results of these experiments confirm an additive effect between E_2 and the different parabens (fig 4.3). In addition, similar experiments were done to investigate possible interactive effects between the parabens MePB and BuPB, EtPB and BuPB, ProPB and BuPB. In this case ratios were chosen to produce a response in the MCF-7 cells that would have been similar to the EC_{50} of the individual parabens alone if straightforward addivity would occur. As with the previous experiments the responses to binary mixtures of different parabens again confirm additivity between these compounds (fig 4.4) in MCF-7 cells.

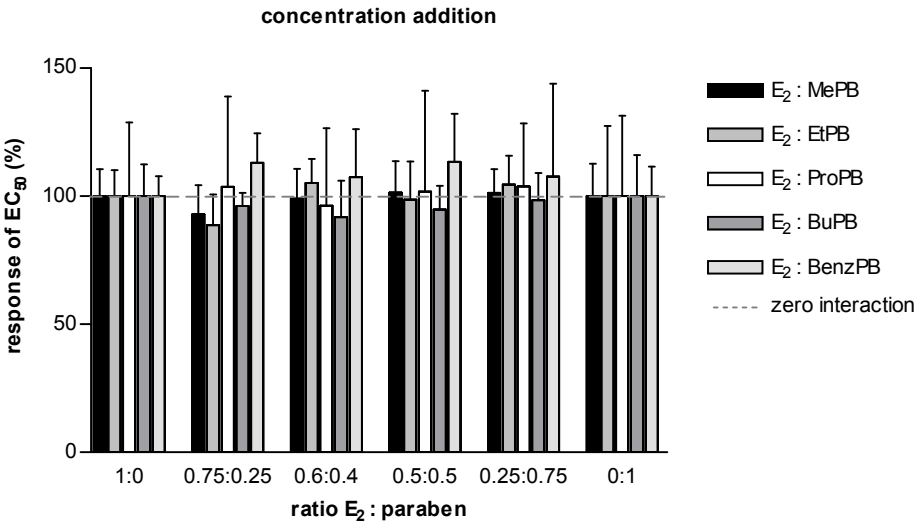


Fig 4.3. Cell proliferation in the MCF-7 proliferation assay after exposure to combinations of a paraben with another paraben at various equipotent ratios in relation to calculated EC_{50} responses. Applied EC_{50} values were 46 µM MePB, 2 µM EtPB, 1 µM ProPB, 0.5 µM BuPB and 0.9 µM BenzPB, which were calculated based on average experimental EC_{50} values of the individual compounds.

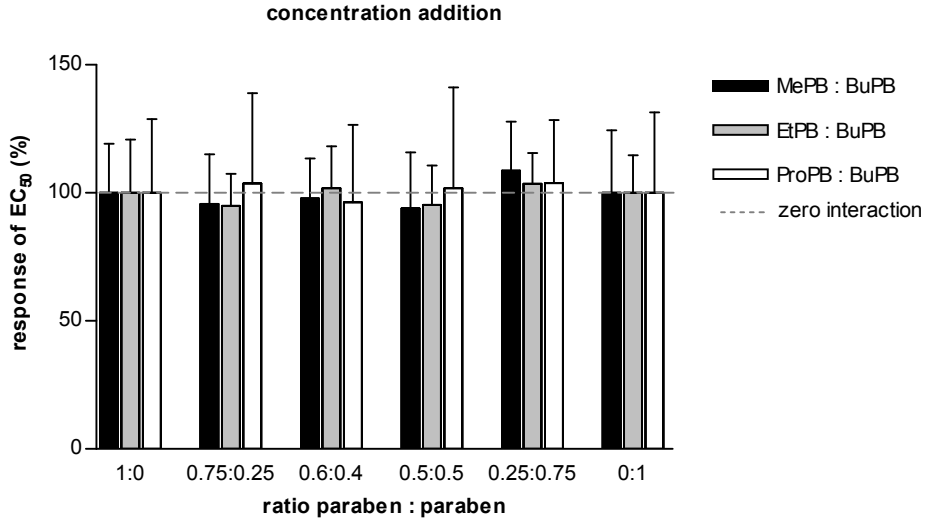


Fig 4.4. Cell proliferation in the MCF-7 proliferation assay after exposure to combinations of an E_2 with a paraben at various equipotent ratios in relation to calculated EC_{50} responses. Applied EC_{50} values were 20 pM E_2, 50 µM MePB, 50 µM EtPB, 1 µM ProPB and 0.5 µM BuPB, which were calculated based on average experimental EC_{50} values of the individual compounds.

Catalytic inhibition of aromatase by parabens

4-OH A (1 µM) completely blocked aromatase activity and was used as reference compound. Thus 4-OH A (1 µM) served as a positive control for aromatase inhibition and measures of these samples were set to 0% in further calculations.

Aromatase in human placental derived microsomes were concentration dependently and statistically significantly inhibited by the parabens ProPB, BuPB and IsoproPB at 10 µM, and EtPB, MePB and BenzPB at 100 µM. The parabens MePB, EtPB, ProPB and IsoproPB inhibited aromatase activity to 55% of the maximum aromatase activity at the highest tested concentration, while BuPB and BenzPB inhibited aromatase activity for 45% and 10% respectively at the highest tested concentration (fig 4.5A & 4.5B).The hydroxybenzoic acids pHBA, 2HBA, 3HBA, MeOxPB and IsobuPB did not inhibit aromatase statistically significant (data not shown). Estimated from their IC_{50} values aromatase inhibitory potencies decrease in the order: ProPB (IC_{50} = 3.5 µM) > EtPB (IC_{50} = 10.6 µM)

> MePB (IC_{50} = 11.3 µM) > IsoproPB (IC_{50} = 16.2 µM) > BenzPB (IC_{50} = 25.8 µM) > BuPB (IC_{50} = 26.4 µM). In addition, concentrations at which aromatase were inhibited for 5% or 10% were calculated. Calculated IC_5s (and IC_{10}s) were 0.1 µM (0.3 µM) for ProPB, 6.3 µM (7.2 µM) for EtPB, 6.0 µM (7.0 µM) for MePB, 7.4 µM (9.1 µM) IsoproPB, 0.4 µM (1.2 µM) for BenzPB and 0.4 µM (1.1 µM) for BuPB.

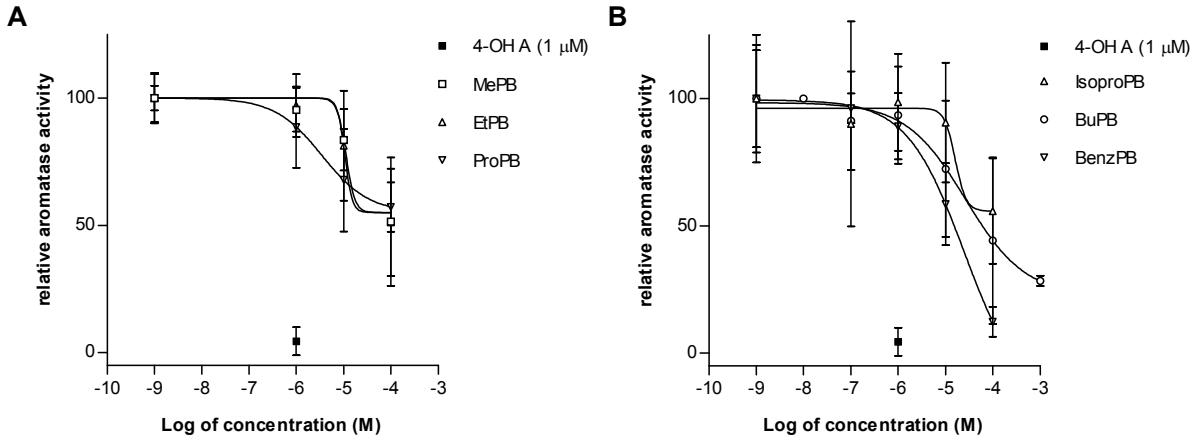


Fig 5. Relative catalytic inhibitory effects of the parabens MePB, EtPB and ProPB (**A**) and BuPB, BenzPB and IsoproPB (**B**) on the aromatase enzyme present in human placental microsomes measured as the release of tritiated water (^3H) reflecting the rate of conversion of ^3H-androstenedione to estrone. Microsomes treated with EtOH (1% (*v/v*)) are set to 100% and 1 µM 4-OH A to 0%.

Discussion and Conclusions

In this study we present results of estrogenic effects of several phthalates, polycyclic musks and estrogenic and aromatase inhibitory effects of parabens plus its main metabolite pHBA and some of its isomers. More over, mixtures of parabens and combinations of parabens with E_2 were tested to investigate combination effects on estrogenicity. These chemicals are common ingredients in various every day cosmetics. Although occurring at a low level exposure and exerting low biological potency, there is growing concern for possible adverse effects on human health.

According to existing estimates of human adult or infant exposure levels to parabens and phthalates, partly derived from cosmetics, safety margins such as the acceptable daily intake (ADI) or tolerable intake (TDI) for individual compounds are not expected to be exceeded at normal exposure related to regular use of cosmetics [11, 12, 19, 29, 34, 62]. Nevertheless combinations of these chemicals have been detected in human samples such as blood and adipose tissue [3, 17, 28, 43](table 4.1). To date, little information is available about the mixture effects of these additives present in cosmetics. Thus the major focus of our experiments was to assess mixture effects of e.g. parabens using estrogenicity as an endpoint. Results from our mixture studies with parabens and E_2 all confirm that the concentration addition model sufficiently explains the observed estrogenic effect. This is in agreement with earlier studies that reported an additive effect for dibutyl phthalate, benzyl-4-hydroxyparabene and other estrogenic compounds like benzophenone, bisphenol A and genistein with or without E_2 in the yeast screen [26, 51, 61]. However, a study by Suzuki *et al.* using a combination of butylbenzyl phthalate + E_2 indicated a weak antagonistic effect on the cell proliferation in MCF-7 cells [66].

Thus, results of these studies with respect to combination effects are equivocal and information is limited in spite of the common exposure of humans to these compounds and occurrence in blood may account for a cumulative body burden of estrogenic compounds. A recent estimation for estradiol equivalent (EEQ) dose due to dermal exposure of parabens was in the range between 73 – 2175 pg EEQ/g tissue/day, depending on absorption rate through the skin (10 – 30%)

and estrogenic equivalency factor (EEF) used [27]. In a similar calculation as applied by Harvey and Everett and considering potencies per paraben estimated in this paper (10^{-5}, except MePB that was around 10^{-7} – 10^{-6}) and assuming an additive effect for mixtures of parabens *in vitro*, the median tissue levels of parabens reported by Darbre *et al.* [17] sums up to a total EEQ of around 0.082 pg EEQ/g tissue. Similar calculations using potencies reported by Lemini and co workers derived from *in vivo* data (around 10^{-4}) [38] would lead to 2.1 pg EEQ/g tissue. Based on available information from literature the addition of 0.082 pg (EEQs) E_2 would lead to an increase of 0.15% in normal breast tissue (55.3 pg E_2/g tissue) and 0.024% in breast tumor tissue (348 pg E_2/g tissue) [10]. In this respect it should also be noticed that a medium concentration of 0.082 pg E_2/ml (= 0.3 pM) was not significantly inducing MCF-7 cell proliferation in our experiments. In conclusion, this indicates that parabens contribute only marginally if at all to the total circulating estrogens. Then again, concern remains that a main metabolite of parabens, *p*-hydroxybenzoic acid (pHBA), could substantially increase the estrogen load, since blood concentration of 44 ppb (= 44 ng/ml) have been reported [48]. Nevertheless, pHBA and the isomers 3OH and 2OH benzoic acid did not induce cell proliferation in our E-screen. This is in agreement with findings of Routledge *et al.* [54], but Pugahendi *et al.* observed cell proliferative effects at 1 µM and 10 µM when medium with exposure was refreshed daily [50].

In line with the estimation done by Harvey and Everett [27], we also estimated the total body burden due to dermal exposure of other estrogenic ingredients in cosmetics such as phthalates and polycyclic musks. As indicated earlier, conversion of EC_{50}s into EEFs for compounds with a cell proliferative induction lower than E_2, such as phthalates and polycyclic musks, might somewhat overestimate their potency (i.e. smaller EEFs). Therefore we proceeded our calculations for a total EEQ with potencies derived from EC_5s and EC_{10}s, as these may be closer to the real potency of these test compounds. Again assuming that established medium concentrations at least closely represent reported blood concentrations, internal concentrations (table 4.1) of phthalates (DEP, DEHP, DBP) sum up to 1 pg EEQ/ml serum and of polycyclic musks (HHCH and AHTN) to 0.03 pg EEQ/ml. Although we are not aware of any publications on monoesters in human blood samples, analysis of human urine samples found such monoesters [67] and makes it plausible that humans are indeed systemically exposed to mono-phthalates. However, the monoesters of phthalates included in this study did not demonstrate estrogenic effects. Thus, their contribution in our EEQ calculations is considered negligible.

Putting these numbers into perspective one should consider daily exposure to estrogens from other sources than cosmetics such as contraceptives and levels of endogenous estrogens. For example, EEQs were calculated for a common source of estrogenic compounds in our diet namely phytoestrogens, such as coumestrol, genistein, (prenylated) naringenin and chrysin. Depending on the constitution of the diet, plasma levels around 2 – 20 ng/ml are detected [21, 68] and potencies are around 10^{-6} – 10^{-5}. Thus a regular diet account for 2.2 pg EEQ/ml. (See for a detailed calculation of EEQs the Excel sheet, available online [72].) Orally taken contraceptives taken by women may cause an exposure to ethinyl estradiol (EE) of 20 – 40 µg/day leading to serum peak levels of around 125 pg EE/ml serum and a serum steady state level of 43.5 pg EE/ml serum, while application of dermal patches leads to 71 pg EE/ml serum [69]. It has been reported that the potency of EE is around twice that of E_2 [4, 22] so that 125 pg EE (i.e. 43.5 pg) and 71 pg EE are equivalent to 87 pg EEQ/ml and 142 pg EEQ/ml respectively. Endogenous levels of E_2 are in the range of 20 – 350 pg E_2/ml serum in adult women and 20 – 50 pg/ml serum in adult men [74]. Although exposure to estrogens are recognized as a risk factor for breast cancer [32, 73], there is a lot of debate whether contraceptives increase the risk for such cancers. Our study implies that the relatively small exposure of estrogen equivalents due to application of cosmetics is probably insignificant. On top of that, the difficulty to estimate adverse effects on a whole organism increases since some of these compounds and other compounds have been shown to exert indirect anti-estrogenic activity by acting on enzymes involved in the steroidgenesis and may interfere with normal binding of endogenous hormones to serum proteins,

thus changing their bioavailability [13, 33, 41]. In addition, it should be considered that compounds with estrogenic effects may act both agonistic or antagonistic depending on the tissue type and concentration. This dual effect on estrogenicity may be determined by the cellular context of the estrogen receptor (SERM concept).

To the best of our knowledge this is the first study that investigated aromatase inhibitory properties for parabens and benzoic acids. Benzoic acids and MeOxPB did not inhibit aromatase in microsomes from human placental tissue, while ProPB and BuPB statistically inhibited aromatase activity at 10 µM or higher and other tested parabens did so at 100 µM. Interestingly, aromatase inhibitory properties occur at concentrations that are within one order of magnitude where cell proliferation in MCF-7 cells occurs. Thus, at estrogenic effective levels of parabens, aromatase inhibition could also be expected to some minor extent. As aromatase is the enzyme responsible for conversion of androgens into estrogens, its inhibition could be considered as an opposite, thus anti-estrogenic effect, to the cell proliferation occurring in estrogen responsive tumor cells. Potentially, inhibition of aromatase could therefore diminish estrogenic effects in a human situation. However, reported concentrations of parabens in human samples were much lower than aromatase inhibitory effective concentrations in our *in vitro* assay. Dualistic stronger estrogenic potencies than aromatase inhibitory potencies are also known for some phytochemicals such as apigenin, chrysin, naringenin and 8-prenylnaringenin [7, 33, 37, 44-46, 53, 58]. Then again, the overall effect in the *in vivo* situation of exposure to such compounds is unknown, but at least should be questioned in the path to proper risk assessment.

In conclusion, it is obvious that actual concentrations found in human samples for single parabens (in the range of 10 – 80 nM) are 2 to 4 orders of magnitude lower than effective concentrations in *in vitro* systems such as cell proliferation and aromatase inhibition in placental microsomes. However, the list of chemicals exerting potential endocrine active effects grows as does the number of consumer products available containing these chemicals. On top of that, for such chemicals the evidence for an additive effect on endocrine sensitive endpoints accumulates. We have estimated cumulative estradiol equivalents for human internal systemic exposure to various compounds acting through the estrogen receptor and brought this total EEQ value into relation to endogenous and therapeutic levels of E_2 and EE. We conclude that this total EEQ value is unlikely to cause adverse effects in humans. Nevertheless, further research will have to elucidate whether exposure to potential endocrine active compounds, specifically cosmetic additives, is safe.

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Chapter 5

Aromatase inhibition by synthetic lactones and flavonoids in human placental microsomes and breast fibroblasts – A comparative study

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Abstract

Interference of exogenous chemicals with the aromatase enzyme can be useful as a tool to identify chemicals that could act either chemopreventive for hormone-dependent cancer or adverse endocrine disruptive. Aromatase is the key enzyme in the biosynthesis of steroids, as it converts androgens to estrogens. Certain flavonoids, plant derived chemicals, are known catalytic aromatase inhibitors. Various systems are in use to test aromatase inhibitory properties of compounds. Commonly used are microsomes derived from ovary or placental tissue characterized by high aromatase activity. To a lesser extent whole cell systems are used and specifically cell systems that are potential target tissue in breast cancer development. In this study aromatase inhibitory properties of fadrozole, 8-prenylnaringenin and a synthetic lactone (TM-7) were determined in human placental microsomes and in human primary breast fibroblasts. In addition, apigenin, chrysin, naringenin and two synthetic lactones (TM-8 and TM-9) were tested in human microsomes only. Comparison of the aromatase inhibitory potencies of these compounds between the two test systems showed that the measurement of aromatase inhibition in human placental microsomes is a good predictor of aromatase inhibition in human breast fibroblasts.

Introduction

The biosynthesis of steroids is increasingly studied as a target for endocrine disruptors as well as chemoprevention in breast cancer. A key enzyme herein is aromatase, of which the main action is demethylation at the C18-position and aromatization of the A-ring of C19 steroids. Thus, aromatase is responsible for converting androgens (C19-steroids such as testosterone and androstenedione) to estrogens (C18-steroids such as 17β-estradiol (E_2) and estrone (E_1)). In humans, CYP19, the gene coding for aromatase, is expressed in various tissue types such as bone, ovary, testis, brain, skin and adipose tissue, as well as placenta, although under the control of tissue-specific promoters [38]. Androgens and estrogens in their turn do not only play an important role in reproductive processes, but also in vascular function, lipid and carbohydrate metabolism, as well as bone mineralization and epiphyseal closure in both sexes [37]. On the other hand, exposure to estrogens in general, both to endogenously produced estrogens and exogenous estrogenic compounds, has been related to initiation and promotion of hormone dependent diseases such as breast cancer. Moreover, a specific class of exogenous estrogens, such as the plant derived phytoestrogens are also believed to act beneficial possibly due chemopreventive properties, but extensive debate about this is still ongoing [21, 25, 40]. In addition, certain lactone-containing secondary plant metabolites have been suggested to potentially interfere with steroid synthesis on the basis of structural similarities with endogenous steroid hormones.

Previous investigations into the mechanism of adverse and beneficial properties of (anti-)estrogenic compounds have often focused on direct interaction with the estrogen receptor (ER) and their induced response [39]. Other investigators studied indirect (anti-)estrogenic effects by focusing on interference with the biosynthesis of these compounds [44]. These studies primarily involved aromatase isolated from (human) placental or ovary tissue, but also whole cell systems have been used such as human adrenocortical carcinoma cells (H295R), human choriocarcinoma cells (JEG-3) and human embryonic kidney cells (HEK293) transfected with the human aromatase gene [1, 17, 33, 34]. Limited data is available that were obtained from primary cells derived from human tissue such as subcutaneous adipose tissue [5, 26]. Interestingly, breast cancer related research describes a potentially important role of the surrounding adipose tissue, and most importantly connective tissue (fibroblasts) herein, of (estrogen sensitive) epithelial breast carcinomas [9]. Interaction between these two types of tissue could arise from tumorous cells excreting prostaglandins that can induce a promoter switch for aromatase expression in connective tissue. Briefly, in healthy breast tissue expression of the gene CYP19, coding for the aromatase enzyme, is under the control of cytokines and glucorticoids via the promoter region I.4. In tumorous tissue aromatase expression is regulated by cytokines and prostaglandins excreted by the tumor cells via promoters I.3 and II [38, 47]. Overall the aromatase expression is significantly more induced in cancerous breast tissue compared to healthy tissue [7]. Especially in postmenopausal women, in whom the gonadal production of estrogens has ceased, circulating testosterone is locally converted in adipose tissue to estrogens, which may reach levels leading to tumor promotion [41]. The common strategies to treat estrogen-dependent breast cancer are to block estrogen receptor binding or to inhibit estrogen production. Estrogenic actions can be blocked by estrogen receptor antagonists such as fulvestrant and tamoxifen. Estrogen production can be reduced by aromatase inhibitors like aminoglutethimide, anastrozole, letrozole, exemestane and fadrozole. Inhibiting the estrogen production is nowadays part of breast cancer treatment, both in situations of adjuvant treatment as well as metastatic breast cancer. Experimental research showed that certain dietary ingredients, mostly plant derived polyphenols, might act via the latter mechanism and thus theoretically could contribute in a chemopreventive way to prevent breast cancer development. In this respect, the list of potentially interesting synthetic and phyto-chemicals is increasing and comprises members of various flavonoid-classes, but also metabolites formed by microflora in the human gut from matairesinol and daidzein such as enterolactone and equol respectively [18]. For example, a recent study from Monteiro *et al.* illustrated that 8-prenylnaringenin

(8-PN) is a potent aromatase inhibitor [28, 29]. This trace ingredient of hop and hop products had an IC_{50} of 65 – 80 nM, while IC_{50} values to inhibit aromatase of other phytochemicals, including some phytoestrogens, are in the range of 0.2 – 150 µM. The capacity to reduce aromatase activity strongly correlates to hydroxylation of the A ring and to a lesser extend to hydroxylation of the B ring [34]. In addition, three synthetic lactones that contain a lactone group with a keto group, have been shown to exert aromatase inhibitory properties in human adrenocarcinoma (H295R) cells [35].

Besides therapeutic and nutritional purposes the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) proposed that screening for aromatase modulators could contribute to identify potential endocrine active or disruptive chemicals. Therefore it has recommended to include an *in vitro* aromatase assay in the Tier 1 Screening Battery Alternate Methods [10]. This should be a quick, easy and reliable *in vitro* screen for detecting aromatase inhibitory properties of chemicals. Microsomes isolated from (human) placenta has been suggested as useful test systems to measure aromatase inhibition, in view of its relatively high aromatase concentration. However, the validity of the results obtained with e.g. microsomal placental fractions for inhibitory properties in target cells in humans is unknown. Therefore, further comparative studies with different human cell systems, such as primary human fibroblasts, are necessary.

In this study, a comparison was made of the aromatase inhibitory properties of fadrozole, various phytochemicals and one synthetic lactone on human placental microsomes, which makes it possible to relate these results with other studies that involved placental microsomes, and cultured human mammary fibroblasts.

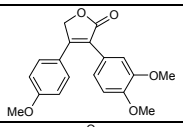
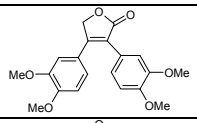
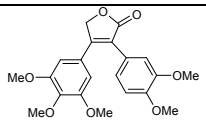
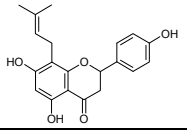
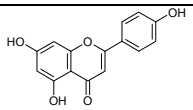
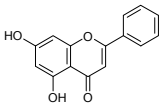
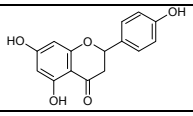
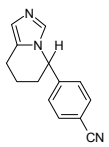
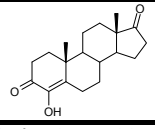
Materials and Methods

Chemicals & Plastics

Fadrozole (FAD) was kindly provided by Novartis Pharma AG (Basel, Switserland). Dexamethasone (DEX), 4-hydroxy-androstendione (4-OH A), chrysin (CHR), naringenin (NAR) and 4', 5, 7-trihydroxy-8-prenylnaringenin (8-PN) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Apigenin (APG) was purchased from Lancaster Synthesis (Muhlheim am Main, Germany). The synthetic lactones 3-(3,4-dimethoxyphenyl)-4-(4-methoxyphenyl)-2(5*H*)-furanone (TM-7), 3,4-bis(3,4-dimethoxyphenyl)-2(5*H*)-furanone (TM-8) and 3-(3,4-dimethoxyphenyl)-4-(4-trimethoxyphenyl)-2(5*H*)-furanone (TM-9) were by the Laboratory of Medicinal Chemistry, Chulabhorn Research Institute (Bangkok, Thailand)(table 5.1). All compounds were prepared as 1000x stock solutions in DMSO (Sigma) and stored at –20°C and added freshly to the medium previous to exposure. The final solvent concentration did not exceed 0.4%.

Tissue culture plastics were purchased from Greiner (Alphen a/d Rijn, The Netherlands). Roswell Park Memorial Institute 1640 medium (RPMI) , the supplements fetal calf serum (FCS), L-glutamine (200 mM), 10.000 U/ml penicillin-streptomycin (p/s) were purchased from Gibco (Breda, The Netherlands). Insulin from bovine pancreas (ins) was purchased from Sigma.

Table 5.1. Names, structures, test ranges and estimated aromatase inhibitory properties expressed as IC_{50} values of test compounds used in tritiated water release aromatase assays performed in microsomes derived from human placental tissue and/or in fibroblasts derived from human mammary tissue. Per compound, literature values for estrogenic properties expressed as EC_{50}s as well as aromatase inhibitory properties expressed as IC_{50}s or Ki-s are indicated.

	chemical structure	reported estrogenicity	reported aromatase inhibition	aromatase inhibition in microsomes (human placenta)		aromatase inhibition in fibroblasts (human breast adipose)	
test compound		**EC_{50}**	**IC_{50} or Ki [a]**	**test range**	**IC_{50}**	**test range**	**IC_{50}**
		µM	µM	µM	µM	µM	µM
synthetic lactone							
TM-7		n.i.	1.0 [a][35]	0.3 – 100	6.9	0.3 – 100	3.7
TM-8		n.i.	1.2 [a][35]	0.3 – 100	1.0	n.t.	
TM-9		n.i.	6.8 [a][35]	0.3 – 100	13.7	n.t.	
prenylflavonoid							
4',5,7-OH-8-prenylnaringenin (8-PN)		0.00082 [27] 0.0003 [24] 0.0000031 [43]	0.065 [28] 0.080 [29]	0.01 – 100	0.2	0.01 – 3	0.3
Flavones							
Apigenin 4',5,7-OH-flavone (APG)		0.4 [42] 0.5 [4] 1.0 [45]	1.2[17]; 2.9[19], 20 [34] 8.7 [32]	0.3 – 100	4.2	n.t.	
Chrysin 5,7-OH-flavone (CHR)		4 [42] 3.2 [4]	0.5 – 11 [5, 6, 17, 19, 33, 34] 2.6 [a][16] 1 [a][11] >>1000 [32]	0.1 – 100	1.1	n.t.	
flavanone							
naringenin 4',5,7-OH-flavanone (NAR)		0.3 [42] 1.0 [4]	9.2 [19] 85 [34] 0.3 [a][11]	0.1 – 100	2.9	n.t.	
imidazole							
fadrozole (FAD)		n.i.	0.059; 0.2 [2] 0.002 [26] 0.00643 [46] 0.007 [30] 0.001; 0.005 [3] 0.007 [30]	0.001 – 100	$3.8*10^{-3}$	$3*10^{-3}$ – 0.3	$0.8*10^{-3}$
steroidal aromatase inhibitor							
4-OH androstenedione (4-OH A)		n.i.	0.080 [46]	1		1	

n.i. = not indicated; n.t. = not tested; [(a)] = Ki-values converted from IC_{50}-values by the authors of the corresponding publications

Isolation and culture of fibroblasts

Fibroblasts were isolated from healthy mammary tissue following the method described by Heneweer *et al.* [13] and Van Meeuwen *et al.* [42]. Tissue was obtained from the St. Antonius Hospital (Nieuwegein, The Netherlands), were it was kept at 4°C directly after mammary reduction surgery on

pre-menopausal adult female patients. The tissue became available after pathologic research confirmed that it was healthy. All three involved patients gave informed consent. The study was approved by the Medical Ethical Committee of this hospital (TME/Z-02.09).

Briefly, to isolate fibroblasts tissue from one patient (approximately 150 ml) was minced followed by digesting cell-cell connections in vigorous shaking 100 ml Krebs buffer with 150 U/ml Collagenase (type I) (Sigma) at 37°C for 2½h. Disconnected cells were separated from undigested fragments by sieving and rinsing with PBS. The cell suspension was centrifuged at 800 rpm for 20 min. Fat on top was discarded and the pellet, mainly existing of erythrocytes and fibroblasts, was re-suspended in maintenance medium (RPMI with 10% FCS, 1 µg/ml ins and p/s) and divided over two to four culture flasks (25 cm^2). Fibroblasts were incubated at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity. After two days, cultures were rinsed with PBS and medium was refreshed. At 80% confluence the cells were put through in several culture flasks. Fibroblasts were cultured for 3 to 6 weeks until they had grown to sufficient numbers to perform experiments.

Isolation of human placental microsomes

Microsomes were derived from human placental tissue of one patient. Placental tissue was obtained from the St. Antonius Hospital (Nieuwegein, The Netherlands) after the patient gave informed consent. The tissue was snap frozen in liquid nitrogen upon arrival and stored at –70°C until use. To homogenize the tissue, TRIS-HCL (50mM) : KCl (1.15%) buffer was added 1 ml per g tissue and squeezed on ice in a Potter-Elvehjem Teflon glass homogenizer. Homogenate was first centrifuged (Beckman L7-55) at 10000 g, 4°C for 25 min to remove cell debris and then centrifuged at 100000 g, 4°C for 75 min. The pellet was resuspended in sucrose (250 mM) 1 ml/g tissue and total protein content of the microsome suspension was measured by the method of Lowry *et al.* [22] using bovine serum albumin (BSA) in the range of 0 – 100 µg/ml as a protein standard. Aliquoted (1 mg/ml total protein) microsomes were stored at –70°C until use.

Aromatase activity

Aromatase activity in placental microsomes and cultured mammary fibroblasts was measured by the amount of formed ^3H_2O, which was isolated from the culture medium by a method described earlier [20] with minor modifications. In this method it was assumed that formed ^3H_2O is a measure for the conversion rate of ^{3}H-androstenedione to estrone, which is catalyzed by aromatase. Under the chosen experimental conditions of our study it is assumed that this conversion was linear (Vmax conditions). Two reference compounds, 4-OH A and FAD, were considered to catalytically and fully inhibit aromatase.

Catalytic aromatase inhibitory capacities of test compounds were tested in each batch fibroblasts isolated from three different women in triplicate and three to four times in microsomes isolated from one patient in ranges as specified in table 5.1.

For the experiments with in fibroblasts, on day 0, cells were washed with PBS, trypsinized (tryps/edta, Gibco), counted (on a Bürker-Türk) and seeded in maintenance medium at cell densities of 90 – 150*10^3 cells/well in a 24 wells plate. On day 1, medium was refreshed with medium containing 30 nM DEX, where DEX served as an aromatase inducer with expression via the promoter region I.4. On day 2, 24h after exposure to DEX, wells were rinsed with PBS and cells were exposed to medium without supplements or containing DMSO, test compounds or 1 µM 4-OH A and 1β-^{3}H-androstenedione (54 nM, PerkinElmer, New England Nuclear Research Products, Wellesley, MA, U.S.) and incubated for 1.5h at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity.

For tests with placental microsomes, wells (24 wells plate) contained microsomes (15 µg protein/250 µl), HEPES/$MgCl_2$ buffer (46.5 mM/4.65 mM), 1β-^{3}H-androstenedione (357 nM), NADPH (0.5 mM; 0.42 mg/ml) and DMSO or test compound. Microsomes were incubated at 37°C for 45 min in an atmosphere of 5% CO_2/95% air under saturating humidity. Activity of wells without NADPH were

tested once in triplo and exerted no difference in activity compared to wells without microsomes and were therefore not included in further experiments.

After incubation, plates were placed on ice and tritiated water was isolated. Briefly, from each well 200 µl medium was used in chloroform (500 µl) extraction. From 100 µl of the aqueous layer unconverted androstenedione was removed by treatment with dextran-charcoal (100 µl of a solution of 5 g activated charcoal and 0.5 g dextran/100 ml MilliQ). To 3.5 ml liquid scintillation cocktail (Ultimate Gold, PerkinElmer, Wellesley, MA) 125 µl of centrifuged samples was added and counted in a scintillator (Packard, Groningen, The Netherlands) to quantify the release of tritiated water. Additionally, for fibroblasts the protein content of the well was determined after cell lysis with a NaOH solution.

The value for the release of tritiated water minus background (working solutions without fibroblasts or microsomes) was used to calculate aromatase activity in pmol/h/mg protein.

Cytotoxicity

Cytotoxicity of test compounds on fibroblasts was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reducing capacity per well after incubation in the aromatase assay. Cells were incubated with 1 mg/ml MTT at 37°C for 30 min and formed formazan was solved in isopropanol. When the absorbance was statistically significant lower than that of solvent treated controls the compounds where considered cytotoxic and excluded from further calculations.

Calculations & Statistics

Dose-response curves were estimated with 'best fit' analysis in Prism (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA) from aromatase activity (pmol/h/mg protein) plotted against logarithmically transformed concentrations by formula (5.1):

$$y = \text{min.} + (\text{max.}-\text{min.})/(1+10^{(EC50-C(TC))*\text{hill slope}}) \quad (5.1)$$

where y is aromatase activity (pmol/h/mg protein), min. is minimal aromatase activity, max. is the maximum aromatase activity and C(TC) is the logarithmically transformed concentration of the test compound. For fibroblasts and microsomes IC50s were estimated from pooling all available values for aromatase activity obtained from three or four individual experiments.

Statistical significance of the differences in maximum aromatase inhibition were determined by a Student's *t*-test and were performed using Excel (Microsoft Excel 2000, NY, USA). The acceptable level of significance was set at $P<0.05$.

To make possible comparison of the aromatase activities in (treated) fibroblasts between different women or between experiments with placental microsomes, values of samples treated with DMSO were set to 100% and these treated with 0.3 nM FAD were set to 0%. Formulas 5.2 and 5.3 were used to transform the aromatase activity (AA) into relative aromatase activity (RAA):

$$EA = AA(\text{sample})/AA(0.3\text{ nM FAD}) \quad (5.2)$$

$$RAA = (EA_{(\text{sample})}-1/EA_{(\text{solvent control})}-1) \times 100\% \quad (5.3)$$

where AA is the calculated aromatase activity in pmol/h/mg protein, EA the effect on aromatase activity.

Results

Various classes of naturally occurring flavonoids such as a prenylflavonoid, a flavone and two flavanones as well as a number of synthetic lactones and an imidazole were tested in this study for aromatase inhibitory effects in human placental microsomes. Three of the test compounds (FAD, TM-7 and 8-PN) were also tested in primary fibroblasts isolated from healthy mammary tissue pretreated with DEX to induce aromatase expression to measurable levels (table 5.1). MTT reducing capacities of

fibroblasts exposed to FAD, TM-7 or 8-PN were not significantly lower than solvent treated cells. Thus, test compound concentrations were below cytotoxic concentrations (data not shown).

Catalytic inhibition of aromatase in placental microsomes and breast fibroblasts

The batch of microsomes applied in experiments was obtained from placental tissue derived from one patient. The mean aromatase activity in these microsomes was 2346 ± 307 pmol/h/mg protein. The mean aromatase activity in fibroblasts obtained from three women was 4.3 ± 1 pmol/h/mg protein (3.3 ± 0.01; 4.9 ± 10.5; 4.4 ± 0.4 pmol/h/mg protein) after induction with DEX. The basal aromatase activity in these cultures have been reported in the range of 0.005 to 0.09 pmol/h/mg, while induction with DEX increased aromatase activity to 0.2 – 5.8 pmol/h/mg protein [14]. Aromatase activities were transformed to values where these samples treated with solvent were set to 100% and these treated with 0.3 µM FAD to 0%. In placental microsomes, the reference compound 4-OH at 1 µM reduced the aromatase activity to 5%, which is close to but significantly (P<0.05) higher than FAD at 0.1 µM or higher (table 5.2). However, in breast fibroblasts the inhibitory activity of these two compounds was similar (aromatase activities of 0.08 ± 0.04 pmol/h/mg protein and 0.06 ± 0.04 pmol/h/mg protein respectively). In placental microsomes the test compounds that inhibited aromatase to a similar degree, but at a higher concentration to that of 4-OH A, were TM-7 (7.4%), 8-PN (6.2%) and APG (6.7%). However, TM-8 (34.1%), TM-9 (14.9%), NAR (9.4%) and CHR (7.9%) did not achieve such a degree of inhibition at their highest concentrations. In breast fibroblasts TM-7 (4.6%) and 8-PN (10%) did reach similar aromatase inhibition as FAD or 4OH-androstenedione within the tested range, but these activities were significant (P<0.05) higher (table 5.2).

Estimated IC_{50} values gained from placental microsomes experiments were in de range of 0.2 – 13 µM for flavonoids and synthetic lactones, while that of FAD was around 4 nM. For these phytochemicals and the synthetic lactones the order of potency to reduce aromatase activity was: FAD >> 8-PN > TM-8 > CHR > NAR > APG > TM-7 > TM-9. IC_{50} values gained from breast fibroblasts were in the range of 0.3 – 4 µM for 8-PN and TM-7, while that of FAD was around 1 nM. The order of potency to reduce aromatase activity in the fibroblasts was: FAD >> 8-PN > TM-7. These results indicate that the tested compounds exerted similar aromatase inhibitory capacities in both test *ex vivo* systems (table 5.1, fig 5.1A, B & fig 5.2).

Table 5.2. Aromatase activity and relative aromatase activity in microsomes or fibroblasts exposed to the test compounds at the concentration that showed the highest reduction in aromatase activity. Aromatase activity was measured in tritiated water release aromatase assays and is expressed as pmol/h/mg protein and in %.

	microsomes[1]	**%[2]**	**fibroblasts[3]**	**%[2]**
DMSO	2346.0±306.9	100.0	3.3[s]; 4.9[s]; 4.4[s]	100.0; 100.0; 100.0
TM-7	187.0±18.7[s]	7.4	0.19[s]; 0.34[s]; 0.25[s]	5.2; 5.1; 3.5
TM-8	810.4±101.1[s]	34.1	n.t.	
TM-9	362.0±50.3[s]	14.9	n.t.	
8-PN	159.2±43.5[s]	6.2	0.32[s]; 0.41[s]; 0.72[s]	9.3; 6.5; 14.4
apigenin	170.6±16.4[s]	6.7	n.t.	
chrysin	199.5±29.0[s]	7.9	n.t.	
naringenin	234.3±27.5[s]	9.4	n.t.	
fadrozole	15.7±0.3	0.0	0.01; 0.10; 0.10	0.0; 0.0; 0.0
4-OH A	132±64.1[s]	5.0	0.03; 0.07; 0.09	0.5; -0.7; -0.3

n.t. = not tested
[s] = P<0.05 compared to the highest inhibition by FAD
[1] = average±SD aromatase activity obtained from microsome experiments expressed in pmol/h/mg protein
[2] = relative aromatase activity, where solvent treated samples (DMSO) were set on 100% and 0.3 µM FAD treated samples set on 0%
[3] = average aromatase activity per experiment using fibroblasts expressed in pmol/h/mg protein

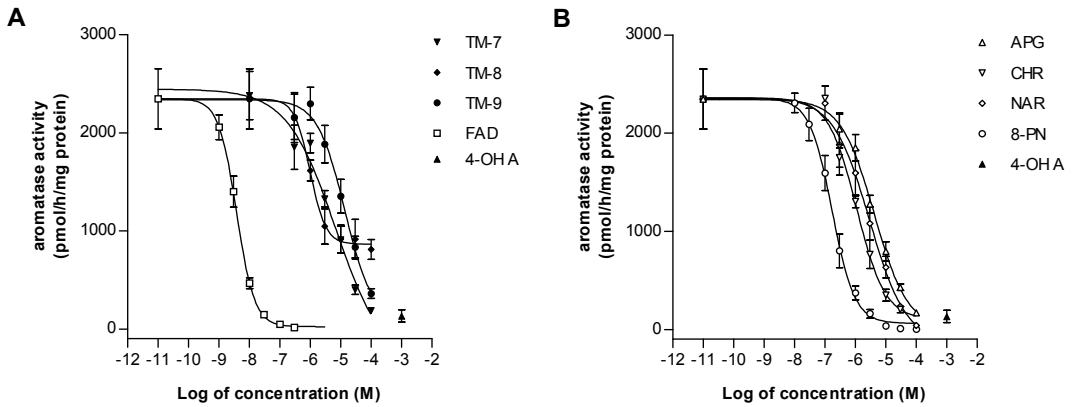


Fig 5.1. Aromatase activity in microsomes isolated from human placental tissue measured as conversion rate of ^{3}H-androstenedione expressed as pmol/h/mg total protein, plotted against logarithmically transferred concentrations synthetic lactones and FAD (fadrozole) (**A**) and phytochemicals APG (apigenin), CHR (chrysin), NAR (naringenin) and 8-PN (8-prenyl naringenin) (**B**).

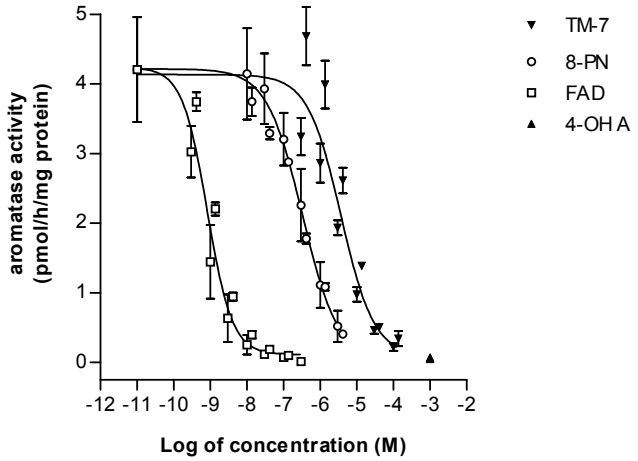


Fig 5.2. Aromatase activity in fibroblasts isolated from healthy human mammary tissue measured as conversion rate of ^{3}H-androstenedione expressed as pmol/h/mg total protein, plotted against logarithmically transferred concentrations the synthetic lactone TM-7, FAD (fadrozole) and the phytochemical 8-PN (8-prenylnaringenin).

Direct comparison of estimated IC_{50} values, obtained from aromatase activity measurements in placental microsomes, indicates that the potency of FAD to catalytically inhibit aromatase is three to four orders of magnitude higher than that of the flavonoids and synthetic lactones. Noticeably, 8-PN is about one order of magnitude more potent than the other test compounds (excluding FAD). This estimation is only approximate as some test compounds, such as TM-8, TM-9, CHR and NAR, were not able to fully block aromatase activity even at the highest concentration. A similar analysis of data obtained from fibroblasts, indicate that the potency of FAD to catalytically inhibit aromatase is also around three and two orders of magnitude higher than that of the synthetic lactone TM-7 and 8-PN respectively.

Discussion and Conclusions

Aromatase is a key enzyme in the biosynthesis of steroids. Therefore, compounds that interfere with aromatase transcription or activity can be endocrine active compounds and have adverse effects on

e.g. reproduction and development. On the other hand, some of these compounds may have therapeutic value in prevention or recurrence of breast cancer in postmenopausal women. In this study, effects were investigated of various classes of flavonoids, three synthetic lactones and fadrozole on aromatase activity in human placental microsomes and primary human breast fibroblasts derived from healthy individuals. These effects were transformed to IC_{50}s and used to estimate potencies to inhibit aromatase activity for these compounds. Comparison of these potencies shows that in both test systems the potency of FAD to catalytically inhibit aromatase was two to four orders of magnitude higher than that of flavonoids and synthetic lactones, except the flavonoid 8-PN. This latter flavonoid was one order of magnitude more potent than other test compounds (except FAD). In addition, it was also shown that three compounds (FAD, 8-PN and TM-7) were capable of strongly (>90%) reducing aromatase activity The IC_{50} values calculated for aromatase inhibition in placental microsomes were close to those observed in primary breast fibroblasts. Consequently, aromatase inhibitory properties in placental microsomes appear to be a good predictor for aromatase inhibitory properties in primary breast fibroblasts. This conclusion supports the further use of placental tissue for screening purpose of aromatase inhibition.

Compounds inhibiting aromatase may do so by binding to the whole enzyme (e.g. FAD) or the catalytic pocket of the enzyme (e.g. 4-OH A) [6, 46]. Flavonoids have been thought to bind competitively with the natural substrate (androgens) to the active site of aromatase in the orientation such that their A and C ring mimic the D and C ring of androgens [16] (fig 5.3). Koa and co-workers illustrated that the C4-keto group as well as the hydroxylation positions greatly determine aromatase inhibitory properties. In general, C5, C7 and C8 hydroxylated flavonoids are equally good aromatase inhibitors, independent of hydroxylation at the C4' position. These findings are in agreement with our IC_{50}s of ± 2 µM for CHR, NAR and APG (see table 5.1). These flavonoids possess hydroxy groups on the C5 and C7 positions. Basically 4', 5, 7-trihydroxy-8-prenylnaringenin (8-PN) is NAR or CHR with a prenyl-group at the C8 position. Opposite to what could have been expected from findings of Kao *et al.* the prenyl-group at the C8 increases the inhibitory effect. Recently, Monteiro *et al.* also found that 8-PN was a stronger aromatase inhibitor than chrysin and identified 8-PN as the most potent prenylflavonoid aromatase inhibitor in choriocarcinoma-derived (JAR) and breast adenocarcinoma-derived (Sk-Br-3) cells [28, 29]. The IC_{50} value of 65 – 80 nM [28] is interesting from a therapeutic point of view as this is in the same order of magnitude as values found for FAD in human placental microsomes and aromatase transfected in human embryonic kidney cells (HEK 293 cells) [2]. It differs about one order of magnitude from that found for FAD in human fibroblasts isolated from breast adipose tissue (IC_{50} = 0.002 µM) [26]. Although the in our present study estimated IC_{50} for 8-PN of 300 nM is significantly higher than 65 nM, it illustrates that some dietary compounds, might have aromatase inhibition potencies that come close those of therapeutics. Therefore some of these compounds could have beneficial value in the prevention or treatment of hormone dependent types of breast cancer.

Relevant to the results of our experiments it is also worth mentioning that some of these flavonoids tested are also known to bind and activate both estrogen receptors (ERα & ERβ), with a preference for ERβ. Molecular studies have generated information on structure-activity relationships to activate ERα or ERβ and showed that the orientation of flavonoids bound to the ER is such that their A and C ring mimic the A and B ring of estrogens (fig 5.3) [8, 23]. However, the orientation for aromatase inhibition by these flavonoids appears opposite.

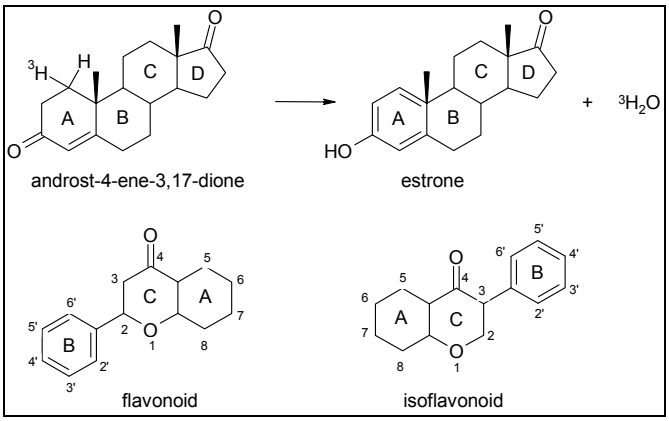


Fig 5.3. The structure formulas of ^3H labeled androstenedione and estrone, the substrate and product respectively in the aromatase assay, and the general structure of flavonoids. In the structures C-rings numbering is indicated with A, B, C or D and outside the structure of flavonoids the carbon numbering is indicated.

Thus, some flavonoids tested in our experiments have aromatase inhibitory as well as estrogenic properties, which can be considered as opposing effects with respect to estrogenicity. However, it is unknown what the overall effect of both mechanisms is on estrogen responsive tissue. By simply comparing effective concentrations of both properties, it can be concluded that for these flavonoids the IC_{50} values for aromatase inhibition are about one order of magnitude higher than EC_{50} values for inducing an estrogenic response (table 5.1). However, which effect prevails over the other in an *in vivo* situation remains speculative. An important factor is the local concentration near the ER receptor or aromatase enzyme in the cell. These concentrations directly depend on e.g. blood plasma or tissue concentrations. Several flavonoids plasma concentrations have been found in the nM or low µM range (5 to 110 nM for naringenin [12], 0.006 – 0.57 µM genistein [15, 31], 0.003 – 0.79 µM daidzein [31, 36]). Thus flavonoids concentrations might reach effective concentrations for estrogenic responses in hormone sensitive cells (e.g. breast tumor cells) as well as for aromatase expressing cells (e.g. connective tissue surrounding tumor cells), assuming *in vitro* medium concentrations are representative for plasma concentrations.

IC_{50} values obtained from our experiments with placental microsomes are close to those in primary breast fibroblasts. This comparison indicates that placental microsomes are useful to quickly identify possible aromatase inhibitory properties of a compound (therapeutic as well endocrine active chemicals) in the *in vivo* situation. Our results therefore support the use of microsomes from human placentas to identify catalytic aromatase inhibition of suspected endocrine disruptors as proposed by the EDSTAC. However, the use of these microsomes as a tool to analyze aromatase inhibitory properties also has its restrictions. Placental microsomes are only useful to detect direct catalytic inhibition of aromatase and are not suitable to measure possible down or up regulation through modulated transcription. Obviously, in whole cell systems as well as *in vivo* situations aromatase expression, enzyme synthesis and enzyme degradation might occur [46].

In conclusion, the aromatase inhibitory capacities of several phytochemicals and synthetic lactones were estimated to be two to four orders of magnitude lower than that of the therapeutic aromatase inhibitor fadrozole. For three test compounds the aromatase inhibitory properties assessed in placental microsomes was similar to that of primary human fibroblasts.

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Chapter 6

(Anti)estrogenic effects of phytochemicals on human primary mammary fibroblasts, MCF-7 cells and their co-culture.

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Abstract

In the public opinion, phytochemicals (PCs) present in the human diet are often considered beneficial (e.g. by preventing breast cancer). Two possible mechanisms that could modulate tumor growth are via interaction with the estrogen receptor (ER) and inhibition of aromatase (CYP19). Multiple *in vitro* studies confirmed that these compounds act estrogenic, thus potentially induce tumor growth, as well as aromatase inhibitory, thus potentially reduce tumor growth. It is thought that in the *in vivo* situation breast epithelial (tumor) cells communicate with surrounding connective tissue by means of cytokines, prostaglandins and estradiol forming a complex feedback mechanism. Recently our laboratory developed an *in vitro* co-culture model of healthy mammary fibroblasts and MCF-7 cells that (at least partly) simulated this feedback mechanism (M. Heneweer *et al.*, TAAP vol. 202(1): 50-58, 2005). In the present study biochanin A, chrysin, naringenin, apigenin, genistein and quercetin were studied for their estrogenic properties (cell proliferation, pS2 mRNA) and aromatase inhibition in MCF-7 breast tumor cells, healthy mammary fibroblasts and their co-culture. The proliferative potency of these compounds in the MCF-7 cells derived from their EC_{50}s decreased in the following order: estadiol ($4*10^{-3}$ nM) > biochanin A (9 nM) > genistein (32 nM) ≥ testosterone (46 nM) > naringenin (287 nM) > apigenin (440 nM) > chrysin (4 µM). The potency to inhibit aromatase derived from their IC_{50}s decreased in the following order: chrysin (1.5 µM) > naringenin (2.2 µM) > genistein (3.6 µM) > apigenin (4.1 µM) > biochanin A (25 µM) > quercetin (30 µM). The results of these studies show that these PCs can induce cell proliferation or inhibit aromatase in the same concentration range (1 – 10 µM). Results from co-cultures did not elucidate the dominant effect of these compounds. MCF-7 cell proliferation occurs at concentrations that are not uncommon in blood of individuals using food supplements. Results also indicate that estrogenicity of these PCs is quantitatively more sensitive than aromatase inhibition. It is suggested that perhaps a more cautionary approach should be taken for these PCs before taken as food supplements.

Introduction

Breast cancer is the predominant type of cancer in industrialized countries and the second leading cause of cancer-related deaths in women [32]. Breast cancer is diagnosed more frequent in postmenopausal women and the hormone sensitive tumor incidence is higher in postmenopausal women (60%) than premenopausal women [6].

Numerous risk factors for breast cancer etiology have been identified, including gender (women more than male), family history (heritability/loss of function of susceptibility genes BRCA-1 and BRCA-2), socioeconomic status, life-style (alcohol consumption, smoking, exercise, obesity) and steroid-hormonal status (including age of first/last menarche and first pregnancy, breast feeding), reviewed by Veronesi *et al.* [56].

Estrogen production in the ovaries of postmenopausal women ceases and plasma levels drop 80 – 90% compared to those of premenopausal women [29, 46]. In the postmenopausal period testosterone levels, produced in the adrenal cortex, increase and testosterone is converted to estrogens in adipose, muscle and connective tissue expressing CYP19 [28]. This conversion is catalyzed by aromatase, the protein product of CYP19, and the rate limiting enzyme converting androgens to estrogens. Peripheral produced estrogens are secreted from cells that express aromatase and can significantly increase the levels in surrounding tissue (e.g. in mammary tissue) to concentrations that are in the same range of those observed in premenopausal women [54]. These locally produced estrogens, e.g. in breast tissue, can potentially initiate, via catechol E_2, and stimulate the growth of estrogen sensitive breast tumors. Supporting evidence for this mechanism is provided by the fact that estradiol levels have been reported to be higher in breast tumors than in healthy tissue [6]. This observation is also mechanistically supported by observed elevated expression of aromatase activity in breast adipose tissue of breast cancer patients [59]. Aromatase expression can be induced by cytokines e.g. prostaglandin (PGE2) and interleukin-6 (IL-6) that are amongst others secreted by tumor cells, but also by infiltrating macrophages and lymphocytes [12, 36, 37, 47, 48]. In healthy tissue the expression of aromatase is under the control of the promoter region (pr) I.4, regulated by class I cytokines such as IL-6, IL-11, and Tumor Necrosis Factor-α (TNF-α), but also glucocorticoids (GREs) are required for its regulation. Noticeable, a promoter switch from pr I.4 to pr I.3 and pr pII is commonly found in breast tumor tissue and linked to elevated CYP19 expression [10]. In contrast these promoters I.3 and pII are under the control of a cAMP responsive element (CRE) and not by e.g. cytokines. [47].

Recently, this interaction between breast tumor cells and surrounding tissue has been studied in an *in vitro* model [17]. In these experiments the modulation by compounds such as dexamethasone, 17β-estradiol, diethylstilbestrol, fadrozole and fulvestrant (ICI 182780) were studied by measuring pS2 gene and CYP19 expression in different cell types. In literature the expression level of pS2 has been described to be positively correlated to exposure of MCF-7 cells to estrogens [13, 38]. Therefore in our co-culture experiments the pS2 expression level serves as a measure for exposure to estrogenic compounds. CYP19 expression, on the other hand, greatly determines aromatase activity in this co-culture model and occurs predominantly if not exclusively in the breast fibroblasts (fig 6.1).

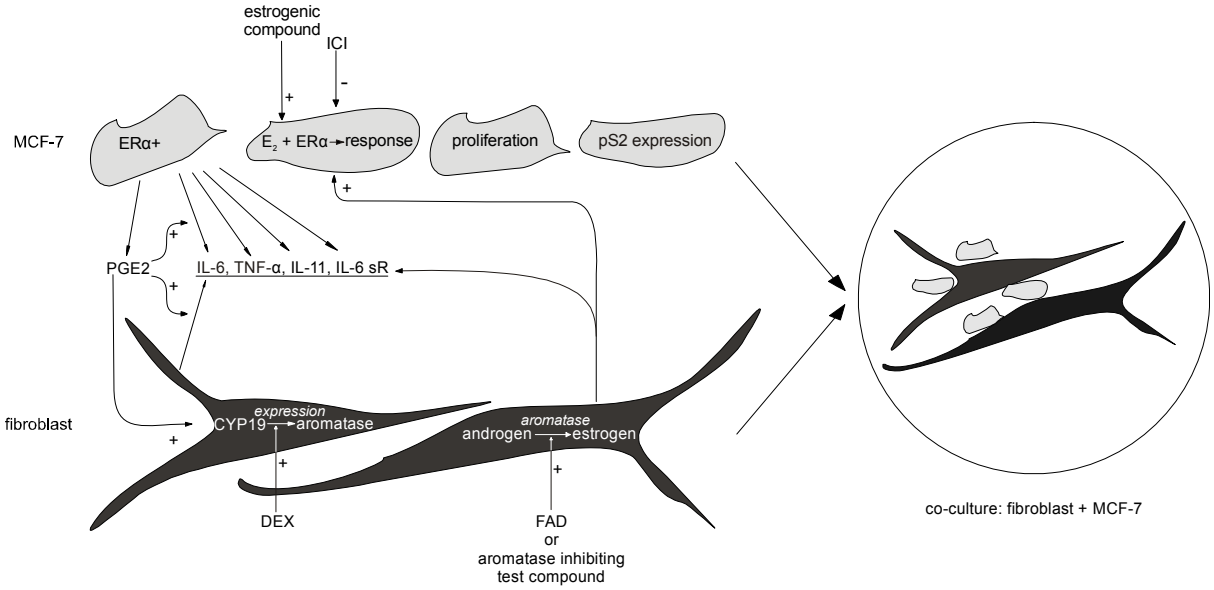


Fig 6.1. Schematic representation of the model where fibroblast-epithelial cell interactions occur and MCF-7 cells represent epithelial cells. Cytokines secreted by MCF-7 cells and glucocorticoids activate the promotor I.4 and thus transcription of the CYP19 gene. Additional, malignant epithelial cells (MCF-7) secrete PGE2 that stimulates pathways to phosphorylate CREB (c-AMP-response element binding protein) and controls CYP19 expression via the promoters I.3 and pII. PGE2 has also been found to stimulate aromatase activity by induction of IL-6. Testosterone added to the medium as substrate for aromatase, will be converted to estradiol in de fibroblasts and secreted in the medium. This conversion is inhibited by aromatase inhibiting test compounds. Estradiol will bind and activate estrogen receptors α (ERα) in the MCF-7 cells as will other estrogenic test compounds added to the medium. MCF-7 cells will start an estrogen dependent response including cell proliferation and induced expression of the pS2 gene. ICI 182780 (ICI) is an ER antagonist and fadrozole (FAD) is an aromatase inhibitor. Dexamethasone (DEX) is applied as a synthetic glucocorticoid. Notify that *in vivo* IL-6, IL-6 sR and TNF-α are secreted mainly by recruited cells of the immune response (TAMs = tumor associated macrophages; TILs (tumor-infiltrating lymphocytes)).

Certain dietary phytochemicals (PCs) like apigenin, biochanin A, chrysin and naringenin have been shown to act as competitive inhibitors in various *in vitro* systems [2, 8, 14, 22, 42]. The aromatase inhibitory properties of PCs have also been confirmed with computer analysis using structure activity relation for the pocket binding domain of this enzyme [9, 21]. Thus, these PCs act similar to pharmaceutical aromatase inhibitors such as fadrozole and could therefore have a chemo preventive role in breast cancer therapy. On the other hand, some of these PCs are also known to bind and activate the estrogen receptor (ER) [21, 23] in breast tumor cell lines, thereby causing growth stimulation of ER positive tumor cells. As these phytochemicals are part of a regular diet, the possible beneficial as well adverse effects on human health should be considered in conjunction. The objective of this study was to investigate possible counteracting effects of various PCs using ER dependent cell proliferation and aromatase inhibition as endpoints. In this study test compounds were investigated in two separate cells systems, human breast adenocarcinoma MCF-7 cells and primary fibroblasts isolated from healthy mammary tissue, as well as a co-culture system from both cell types as described above. The results from these experiments were used to evaluate the overall (anti-)estrogenic effect of these PCs in relation to human (internal) exposure.

Materials and Methods

Chemicals & Plastics

17β-estradiol (E_2), testosterone (T), 4-androstene-3,17-dione (A-dione), biochanin A (BioA), genistein (GEN), naringenin (NAR) and dexamethasone (DEX) were purchased from Sigma (St. Louis, MO, USA). Chrysin (CHR) was obtained from Aldrich (Zwijndrecht, The Netherlands). Apigenin (APG) was purchased from Lancaster Synthesis (Muhlheim am Main, Germany). The catalytic aromatase inhibitor fadrozole (FAD) was kindly provided by Novartis Pharma AG (Basel, Switzerland). The ER antagonist

fulvestrant (ICI 182780) (ICI) was obtained from Tocris (Bristol, UK). All compounds were prepared as 1000x stock solutions in DMSO (Sigma), except ICI of which solutions were prepared in EtOH, stored at –20°C and added freshly to the medium previous to exposure. The final solvent concentration did not exceed 0.3% (except in the aromatase assay where fibroblasts were exposed to BioA 10^{-4} M that was equivalent to a solvent concentration of 1%).

Tissue culture plastics were purchased from Greiner (Alphen a/d Rijn, The Netherlands). Phenol red free Roswell Park Memorial Institute 1640 medium (RPMI), fetal calf serum (FCS) and 10.000 U/ml penicillin-streptomycin (P/S) were purchased from Gibco (Breda, The Netherlands). Charcoal-dextran treated (considered steroid free) FCS (SF-FCS) was obtained from Hyclone (Etten-leur, The Netherlands). Insulin from bovine pancreas (INS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma.

MCF-7 cell culture

The estrogen receptor α-positive (ERα+) MCF-7 human breast adenocarcinoma cell line was obtained from the American Type Culture Collection (No. HTB-22, ATCC-LGC Promochem, Teddington, United Kingdom). MCF-7 cells were routinely maintained in 25 cm^2 or 75 cm^2 plastic flasks on RPMI containing 5% FCS, supplemented with 1% P/S and 1 µg/ml INS (culture medium) at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity. In all experiments steroid free medium (SF-medium) was used, consisting of culture medium without INS and with 5% SF-FCS added instead of FCS.

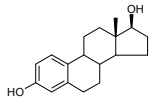
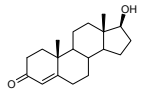
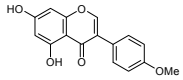
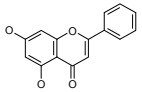
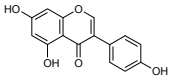
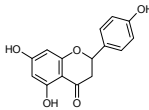
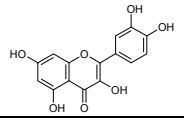
Proliferation assay

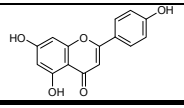
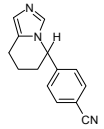
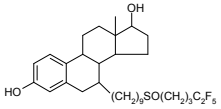
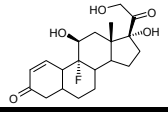
The proliferation assay (E-screen) was performed with MCF-7 cells following conditions published earlier [35, 55]. In summary, cells were seeded in 200 µl SF-medium in a 96-wells plate at a density of ~4500 cells/well. Medium with test compounds was refreshed after three days of exposure. After 6 days of exposure cell numbers were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reducing capacities as a measure for cell number per well. Absorbance at 595 nm (Fluostar, BMG lab technologies, Germany) minus background (MTT without cells) was used as the corrected absorbance in further calculations.

E_2 and each PC were tested in the concentration range specified in table 6.1. To control if proliferative effects of the test compounds were indeed ER mediated, inhibition by the ER antagonist ICI at 10 nM was examined at sub maximal cell proliferation induced by E_2 or a PC. For E_2 and all PCs that induced cell proliferation in the MCF-7 cells complete inhibition was confirmed.

Possible proliferative capacities of the mammary fibroblasts were studied separately under similar experimental conditions as used for MCF-7 cells, but with cell density of 9000 cells/well and without refreshing medium on day 5.

Table 6.1. Phytochemicals and reference compounds used in human breast adenomacarcinoma MCF-7 cells and primary mammary fibroblasts, cytotoxicity measured as MTT or LDH and EC_{50}s estimated from the MCF-7 cell proliferation assay and IC_{50}s estimated from the aromatase assay in fibroblasts.

compound (Cas#)	chemical structure	test range (e-screen) nM	test range (co-culture) nM	cytotoxicity MCF-7 [1] nM	cytotoxicity MCF-7 [2] nM	cytotoxicity fibroblasts [3] nM	EC_{50} [4] nM	IC_{50} [5] nM
Endogenous hormones								
17β-estradiol (E_2) Cas# 50-28-2		$3*10^{-4}$ – 1	1	> $1*10^2$	> 10	n.a.	$4*10^{-3}$	
Testosterone (T) Cas# 58-22-0		20 – $2*10^4$	20	n.a.	> $2*10^4$	> $2*10^3$	46 *	
Androstenedione (A-dione) Cas# 63-05-8		$1*10^{-3}$ – $1*10^5$	20	n.a.	n.a.	> $1*10^3$	n.p.	
Phytochemicals								
Biochanin A (BioA) Cas# 491-80-5		$1*10^{-2}$ – $1*10^4$	$1*10^{-3}$ – $1*10^4$	> $1*10^3$	> $1*10^4$	> $3*10^4$	9	$25*10^3$
Chrysin (CHR) Cas# 480-40-0		10 – $3*10^4$	10 – $1*10^6$	> $1*10^5$	> $1*10^5$	≥ $3*10^4$	$3*10^3$ [a]	$1.5*10^3$ [b]
Genistein (GEN) Cas# 446-72-0		1 – $1*10^3$	n.a.	≥ $1*10^5$	> $1*10^5$	> $1*10^5$	32	$3.6*10^3$
(+,-)Naringenin (NAR) Cas# 67604-48-2		10 – $1*10^4$	10 – $1*10^5$	> $1*10^5$	> $1*10^5$	> $1*10^5$	287	315
Quercetin (QUE) Cas# 6151-25-3		n.a.	n.a.	n.a.	> $1*10^5$	> $1*10^5$	n.a.	$30*10^3$

Apigenin (APG) Cas# 520-36-5		10 - $3*10^3$	n.a.	n.a.	n.a.	≥ $1*10^5$	440 *	444
reference compounds								
Fadrozole (FAD) Cas# 102676-47-1		1 – $1*10^4$	$3*10^4$	n.a.	> $1*10^6$	> $1*10^5$		0.6
ICI 182780 (ICI) Cas# 129453-61-8		$1*10^{-2}$ – $1*10^2$	10	> $1*10^6$ nM	> $1*10^3$	> $1*10^2$		
Dexamethasone (DEX) Cas# 50-02-2						> $1*10^2$		

[1] = MCF-7 cells were exposed to test compounds for 48h. Cytotoxicity was measured with MTT.
[2] = MCF-7 cells were exposed to test compounds for 24h. Cytotoxicity was measured with LDH.
[3] = Fibroblasts were exposed to test compounds for 24h. Cytotoxicity was measured with MTT.
[4] = Geometrical mean of EC_{50}s estimated from log-transformed concentration-response curves (MCF-7 proliferation assay).
[5] = Mean of IC_{50}s estimated from log-transformed concentration-response curves (aromatase assay in fibroblasts).
n.a. = not available
n.p. = not proliferative
[a] = maximum proliferative effect is lower than E_2.
[b] = maximum aromatase inhibition is lower than FAD

Fibroblast isolation and co-cultures with MCF-7

Fibroblasts were isolated from healthy mammary tissue following the method described by Heneweer *et al*. [18]. Tissue was obtained from the St. Antonius Hospital (Nieuwegein, The Netherlands), were it was kept at 4°C directly after mammary reduction surgery on pre-menopausal adult female patients. The tissue became available when pathologic research confirmed that it was healthy. Patients orally permitted with an informed consent as approved by the Medical Ethical Committee of the hospital (TME/Z-02.09).

Isolation of fibroblasts was done by mincing the tissue (approximately 150 ml) followed by digesting cell-cell connections in vigorous shaking 100 ml Krebs buffer with 150 U/ml Collagenase (type I)(Sigma) at 37°C for 2½h. Disconnected cells were separated from undigested fragments by sieving and rinsing with PBS. The cell suspension was centrifuged at 800 rpm for 20 min. Fat on top was discarded and the pellet, mainly existing of erythrocytes and fibroblasts, was re-suspended in culture medium with 10% FCS and divided over two to four culture flasks (25 cm^2). Fibroblasts were incubated at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity. After two days, cultures were rinsed with PBS and medium was refreshed. At 80% confluence the cells were passaged in several culture flasks. Fibroblasts were cultured for 1 to 2 months before they had grown to sufficient quantity to perform experiments (co-cultures, aromatase, cell proliferation, cell toxicity). Before starting the experiments cells were washed with PBS, trypsinized (tryps/edta, Gibco), counted (Bürker-Türk) and seeded in the appropriate cell density. For co-culture experiments cells were seeded in 6 wells plates at a density of $8*10^4$ cells/well in 2 ml culture medium (day 1). On day 4, 72h steroid deprived cultures of MCF-7 cells were washed with PBS, trypsinized, counted and seeded on top of the fibroblasts at a density of $8*10^4$ cells/well in 2 ml SF-medium. On day 5, SF-medium was replaced with SF-medium containing test compounds in the range specified in table 6.1 and incubated for 72h. Each PC was tested in triplicate in at least two separate experiments with fibroblasts isolated form different individuals. Because fibroblasts from different individuals were used in every experiment the following six reference tests were included: (i) DMSO as solvent control, (ii) a CYP19 up regulator DEX at 30 – 100 nM, (iii) E_2 at 1 nM to generate a maximal response, (iv) T at 20 nM to set the response produced by basal conversion of aromatase in fibroblasts, (v) FAD at 30 µM to completely inhibit aromatase and (vi) ICI at 10 nM to completely block the ER mediated response. Additionally, T at 2 mM and A-dione at 20 nM were included in several tests as substrate for aromatase.

mRNA isolation, RT-PCR and pS2& CYP19 quantification

mRNA was isolated from co-cultures to investigate expression levels of pS2 and 36B4. This approach was chosen, as it seemed impractical to separate and analyze individual cell types once grown in co–culture. In this co-culture MCF-7 cells express pS2, whereas the fibroblasts do not as reported earlier [17]. The expression level of the pS2 gene has been described to be positively correlated to the exposure to estrogenic compounds in MCF-7 cells. pS2 expression is ER-mediated and increases after 30 minutes and peaks after 48 hours of exposure [13, 38]. The expression level of 36B4, on the other hand, has been used earlier as a control to correct for loading discrepancies [34].

After 72h exposure, the experiment was terminated on day 8. Wells were rinsed with PBS and mRNA was isolated applying RNA Instapure (Eurogentec, Liège, Belgium) following guidelines of the supplier. RNA quantity and purity was determined by measuring A260 and A280 (Smartspec Spectrophotometer Plus, Bio-Rad, California, USA). From each sample total RNA stocks of 2 ng/µl were prepared. From each RNA stock 5 µl was added to 20 µl RT/PCR reaction mixture (Promega RT-PCR System, Madison, WI, USA). Amplified target genes were 36B4 and pS2. The primer pairs had a sequence of 5'-AAA CTG CTG CCT CAT ATC CG-3' and 5'-TTT CAG CAA GTG GGA AGG TG-3' for 36B4 [34] and 5'-GCG AAG CTT GGC CAC CAT GGA GAA CAA GG-3' and 5'-GCG GAT CCA CGA ACG GTG TCG TCG AA-3' for pS2 [26]. These primers were obtained from Invitrogen (Paisley, UK). PCR products were

respectively, 562 bp and 189 bp. The reverse transcription (RT) PCRs were initiated by 45 min synthesis of single strand cDNA at 48°C, followed by 2 min enzyme inactivation at 94°C. Then, amplification was performed for 25 cycles of 45 sec at 94°C, 45 sec at 60°C (36B4) or 55°C (pS2), 45 sec at 72°C. After a 7 min final extension at 72°C, amplification was terminated by soaking at 4°C. The PCR products were analyzed by size separation on a 2% denaturing agarose gel, and visualized by ethidium bromide staining. The band intensity was measured by a FluorImager (Amersham Bioscience, Piscataway, NJ) and quantified using ImageQuant (version 5.0). As a control for added RNA quantity per RT-PCR, total RNA was amplified using a 36B4 primer set. Because 36B4 expression was found to be constitutive and at the same level independent from exposure to estrogenic compounds and we experienced an increase in standard deviation when the pS2 level was corrected for the 36B4 level, the uncorrected band intensity of pS2 was used in calculations and measuring 36B4 expression was omitted in later experiments.

Aromatase assay

Aromatase activity was determined in MCF-7 cells and primary fibroblasts culture following the method of Lephart and Simpson [27] with minor modifications. In short, MCF-7 cells or fibroblasts were seeded in 0.5 ml culture medium or SF-medium at a density of $2*10^5$ cells/well (24 wells plate). One day before the aromatase activity was determined, medium was replaced with SF-medium containing DMSO (1:1000), FAD (30 µM) or DEX (3-30-100 nM) and cultures were incubated for 24h at 37°C. Assays in which inhibitory capacities of FAD and individual phytochemicals (APG, BioA, CHR, GEN, NAR and QUE) were tested, used fibroblasts that were preincubated with 30 nM DEX for 24h. To study effects of these phytochemicals (in a range of 10 – 10^5 nM) a preincubation of 2 to 3h was done before the aromatase assay. As a reference FAD was also tested in the range of 0.3 – 100 nM, but without preincubation time. On the day of the aromatase assay, medium was replaced with 250 µl RPMI without supplements or compounds, except the PCs, but containing 54 nM ^{3}H-androstenedione (New England Nuclear Research Products [PerkinElmer] Wellesley, MA). Cells were then incubated for 1.5h at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity. After incubation from each well 200 µl medium was used in a chloroform (500 µl) extraction. Unconverted androstenedione from 100 µl of the aqueous layer was treated with 100 µl of a dextran-charcoal suspension. To 3.5 – 4 ml liquid scintillation cocktail (Ultimate Gold, PerkinElmer, Groningen, The Netherlands) 125 µl of centrifuged samples was added and counted in a Tri-Carb LS counter (Packard Biosciences, Groningen, The Netherlands) to quantify release of tritiated water. Total protein content per well was measured with the Lowry assay to determine enzyme activity/h/mg total protein [30, 39]. Briefly, NaOH (5 mM) was added (and stored at –20°C until measurement) to each well (1 ml/well (24 wells plate). Added was 1 ml Lowry A+Lowry B (50:1)/well and incubated at room temperature for 10 minutes. Then 100 µl folin+MQ (1:1) was added, mixed and incubated at room temperature in the dark. Absorption at 750 nm (Smartspec spectophotometer, Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) was used to measure protein content (µg/ml). A range of BSA (Bovine Serum Albumin) concentrations up to 100 µg/ml was used to make a standard-curve.

Cytotoxicity

Cytotoxicity was determined using MTT reduction and lactate dehydrogenase (LDH) activity. For MTT measures in MCF-7 cells, MCF-7 cells were seeded at 10^5 cells/well (24 wells plate) in SF-medium. After 24h incubation at 37°C the cells were exposed to test compounds for 48h. For MTT measurements in fibroblasts, fibroblasts were seeded at 10^4 cells/well (96 wells plate) in culture medium. After 24h of incubation at 37°C, the cells where exposed to test compounds for 24h. Cell number per well was determined by measuring the capacity of the cells in each well to reduce MTT as a degree of cytotoxicity. For LDH measurement, MCF-7 cells were seeded at $12.5*10^3$ cells/well (96 wells plate) in SF-medium. After 24h of incubation at 37°C, cells where exposed to test compounds for

24h. LDH released in the medium and total LDH were determined as described earlier (Sigma TOX-7). LDH activity in the medium relative to the total available LDH per well served as a measure for cell membrane integrity and degree of cytotoxicity.

Calculations & Statistics

In order to calculate EC_{50} values of the individual test compounds in the MCF-7 cell proliferation assay, the corrected formazan absorbance (MTT) was plotted against logarithmically transformed medium concentration and analyzed in Prism 3.0 (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA) using a 'best-fit' approach according to the equation (6.1):

$$y = \text{min.}+(\text{max.} - \text{min.})/(1+10^{(EC50\text{-}C(TC)*\text{hill slope})}) \quad (6.1)$$

where y is the response and the concentration of the test compound $C_{(TC)}$.
The relative proliferative effect was calculated using the formulas:

$$PE = \text{Absorbance}_{(\text{sample})}/\text{Absorbance}_{(\text{solvent control or medium control})} \quad (6.2)$$

$$RPE = (PE_{(\text{sample})}\text{-}1/PE_{(\text{max response E2})}\text{-}1) \times 100\% \quad (6.3)$$

where PE is proliferative effect and RPE is relative proliferative effect.
The relative pS2 expression (RPE) was calculated using the formulas:

$$PE = \text{pS2 intensity}_{(\text{sample})}/\text{pS2 intensity}_{(\text{solvent control})} \quad (6.4)$$

$$RPE = (PE_{(\text{sample})}\text{-}1/PE_{(\text{max response E2})}\text{-}1) \times 100\% \quad (6.5)$$

The aromatase activity (AA) and relative aromatase activity (RAA) were calculated using the formulas:

$$AA = \text{dpm1}_{(\text{sample})}/\text{dpm1}_{(30\ \mu\text{M FAD})} \quad (6.6)$$

$$RAA = (AA_{(\text{sample})}\text{-}1/AA_{(\text{solvent control})}\text{-}1) \times 100\% \quad (6.7)$$

where dpm1 is a measure for the amount of released tritium. In order to calculate an IC_{50} of a compound, the average RAA value of several experiments was analyzed with Prism 3.0 using a one-site competition model approach. Statistically significant differences between RAA values of untreated fibroblasts and treated fibroblasts were determined in a two tailed *t*-test (Microsoft Excel 2000, NY, USA). Significance levels of 0.05 ($P<0.05$) were considered statistically significant.

Results

Cell proliferation experiments with MCF-7 cells and mammary fibroblasts

The MCF-7 cell proliferation assay was used as a measure for estrogenicity and to determine estrogenic properties of the test compounds. In this assay the individual reference compounds (E_2, T, A-dione, DEX, FAD and ICI) and the PCs (APG, BioA, CHR, GEN and NAR) were tested. As a control for later co-culture experiments, E_2, BioA, CHR, GEN and NAR, were tested in a cell proliferation assay using fibroblasts instead of MCF-7 cells.

E_2, T, APG, BioA, CHR, GEN and NAR produced a concentration dependent cell proliferation in the MCF-7 cells (fig 6.2A, C, D). EC_{50} values for the individual compounds are given in table 6.1. A-

dione, DEX, FAD and ICI did not cause cell proliferation within the tested concentration range. The proliferative potency of these compounds in the MCF-7 cells decreased in the following order: E_2 (EC_{50} = $4*10^{-3}$ nM) > BioA (EC_{50} = 9 nM) > GEN (EC_{50} = 32 nM) ≥ T (EC_{50} = 46 nM) > NAR (EC_{50} = 287 nM) > APG (EC_{50} = 440 nM) > CHR (EC_{50} = 4 µM). NAR, GEN and BioA showed a maximal efficacy that was close to E_2, but for APG, CHR and T this was generally 70%, or less from that of E_2.

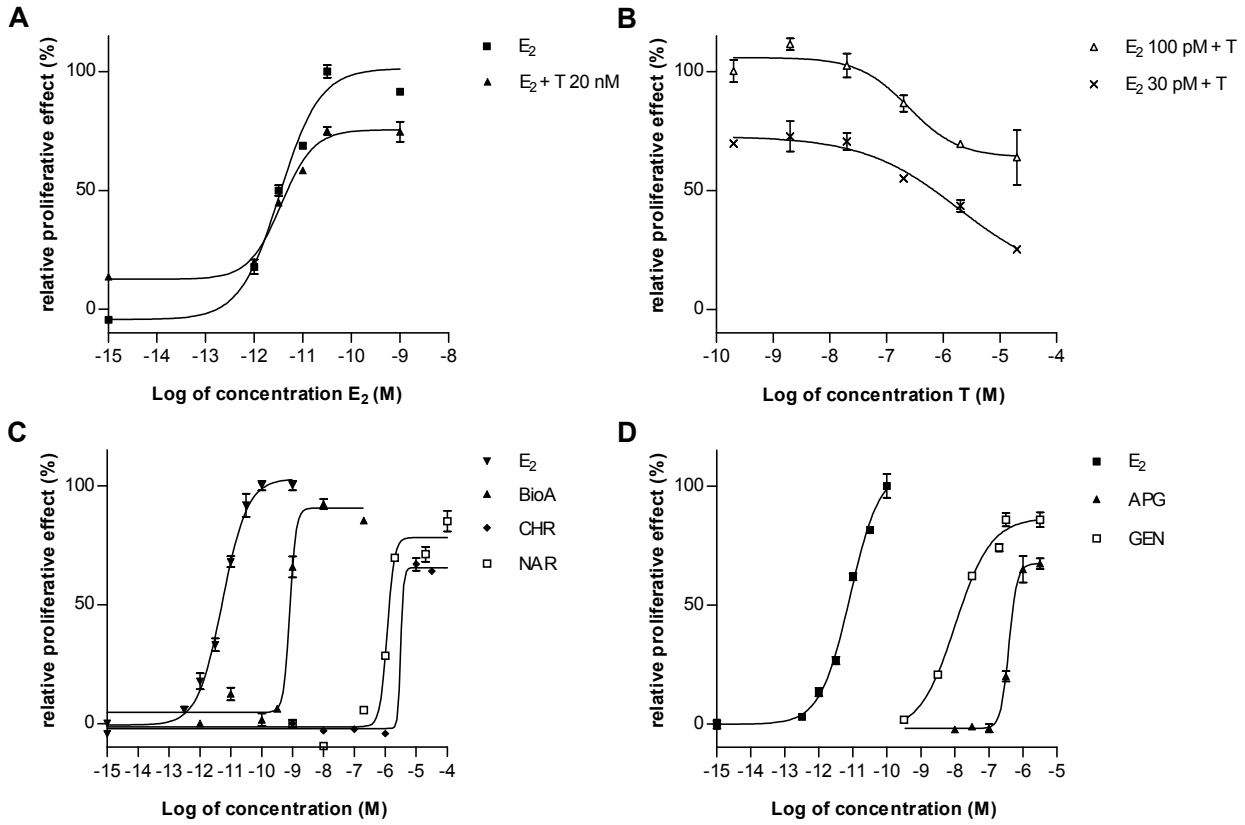


Fig 6.2. MCF-7 cell proliferative effect of 17β-estradiol (E_2) and E_2 in combination with 20 nM T (**A**), 30 nM and 100 nM E_2 in combination with increasing concentrations T (**B**), BioA, CHR, NAR (**C**), APG and GEN (**D**) in the MCF-7 proliferation assay.

As a control for later co-culture experiments the proliferative effect of T was determined. The proliferative effect induced by T in our MCF-7 cells at concentrations higher than 20 nM was noticeable (fig 6.3). To elucidate the mechanism behind this estrogenic effect of T the cell proliferation induced by 2 µM T was studied in combination of either FAD (aromatase inhibitor) or ICI (ER antagonist). Cell proliferation by T was not reduced when co-treated with FAD up to 10 µM, but concentration dependently inhibited by ICI (IC_{50} = 0.1 nM; maximal ≥ 1 nM). The latter results confirm that cell proliferation by T in MCF-7 cells is ER mediated.

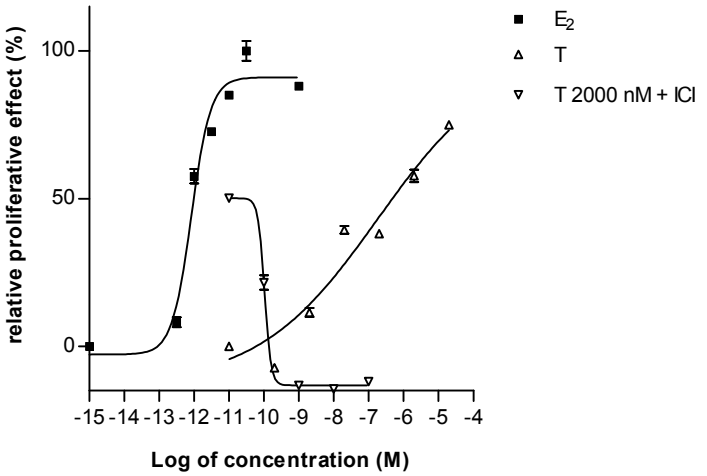


Fig 6.3. MCF-7 cell proliferative effect of increasing concentrations testosterone (T), a concentration inducing sub maximal proliferation (2000 nM) combined with increasing concentrations ICI 182780 in the MCF-7 proliferation assay.

As the preceding experiments confirmed that T can interact with ER mediated processes in the MCF-7 cells, further studies with this cell line and mixtures of E_2 and T were performed. It was found that a fixed concentration of 20 nM T did reduce the efficacy of E_2 (fig 6.2A). In addition, cell proliferation of either 30 or 100 pM E_2 was concentration dependently inhibited by T (fig 6.2B). Note that at 20 nM, the relevant concentration for later co-culture experiments, the response is not inhibited noticeably.

The mammary fibroblasts did not show cell proliferation after 6 days of exposure to either E_2, BioA, CHR, GEN or NAR (data not shown), thus in the following co-culture experiments ER dependent cell proliferation was only expected from MCF-7 cells. In addition, none of the compounds showed cytotoxic effects in the MCF-7 cells (table 6.1) at estrogenic concentrations.

Aromatase activities in mammary fibroblast and MCF-7 cells

To investigate which cell types in the co-culture system, were capable of converting androgens into estrogens, aromatase activity was measured in fibroblast and MCF-7 cell cultures individually. These results show that aromatase activity was clearly present in the fibroblasts, but very little aromatase activity, if at all, could be measured in MCF-7 cells (fig 6.4A, B). Using DEX as an inducer of aromatase via pr 1.4 it was studied if this enzyme activity could be induced in either of both cell types. In cultured fibroblasts that were pre-incubated for 24h with 30 nM DEX an aromatase induction of about one order of magnitude could be observed. This induction was highest after five weeks of culturing compared to a seven week culturing period, indicating that aromatase activity may vary over culture time. In MCF-7 cells no significant induction of aromatase could be observed (fig 6.4A). The implications from these results are that in the following co-culture experiments the conversion of T to E_2 occurs predominantly in fibroblasts.

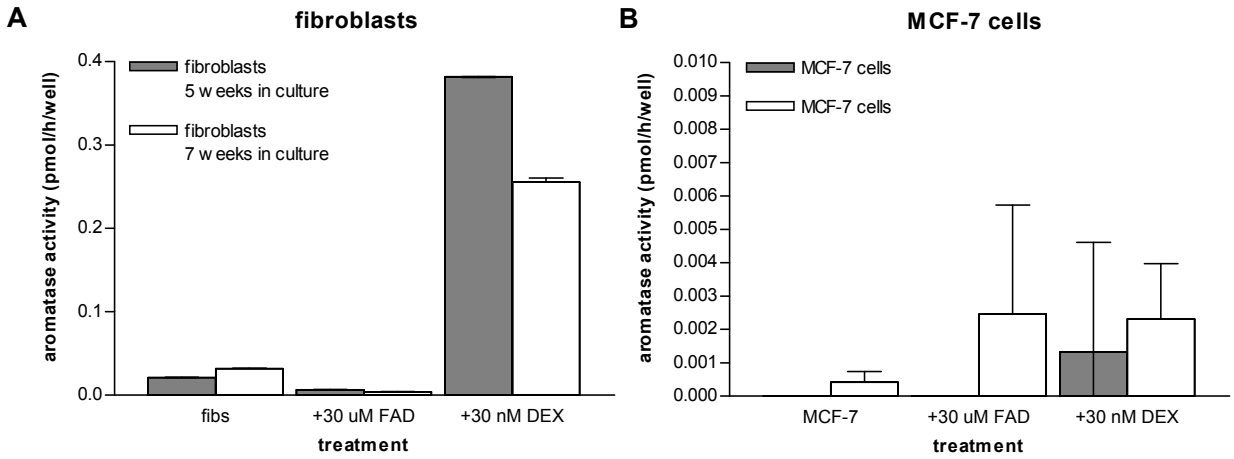


Fig 6.4. Aromatase activity measured in separately cultured primary isolated fibroblasts (**A**) and MCF-7 cells (**B**) after 24h preincubation with DMSO (1:1000), DEX (30 nM) or DEX (30 nM) + FAD (30 µM). Fibroblasts were tested 5 and 7 weeks after cell isolation. Error bars indicate standard deviations (n=2).

Inhibition of aromatase by fadrozole and phytochemicals in fibroblasts

To investigate possible aromatase inhibition by these PCs, monocultured fibroblasts were treated with various concentrations of PCs. FAD was used as a positive control for aromatase inhibition. The IC_{50} for inhibition of aromatase by FAD was 0.6 nM. The PCs were dosed to the cells 2 - 3h before aromatase activity was measured and all PCs showed a concentration-dependent inhibition. The potency for aromatase inhibition decreased in the following order: CHR (IC_{50} = 1.5 µM) > NAR (IC_{50} = 2.2 µM) > GEN (IC_{50} = 3.6 µM) > APG (IC_{50} = 4.1 µM) > BioA (IC_{50} = 25 µM) > QUE (IC_{50} = 30 µM). It was also observed that in the concentration range of 10 to 100 µM the degree of aromatase inhibition by APG, BioA, CHR, NAR and QUE was comparable to that of 10 nM or higher FAD (≥ 75%). This indicates that the *in vitro* potency of these PCs as aromatase inhibitors is approximately three orders of magnitude weaker than that of FAD. In addition, aromatase inhibition by GEN was at least one order of magnitude less potent than that of the other PCs (fig 6.5A,B).
Statistically significant (P<0.05) aromatase inhibition was found at the concentration of ≥ 1 µM for CHR and NAR, ≥ 10 µM or higher for APG and GEN, ≥ 30 µM for BioA, and 100 µM for QUE. The highest concentrations CHR (30 µM and 100 µM) and APG (100 µM) showed cytotoxicity after 24h as measuered with the LDH assay (table 6.1). In the latter situation, reduced aromatase activity of these compounds may therefore partly be explained by cytotoxicity, although exposure was only 2 to 3h in this assay.

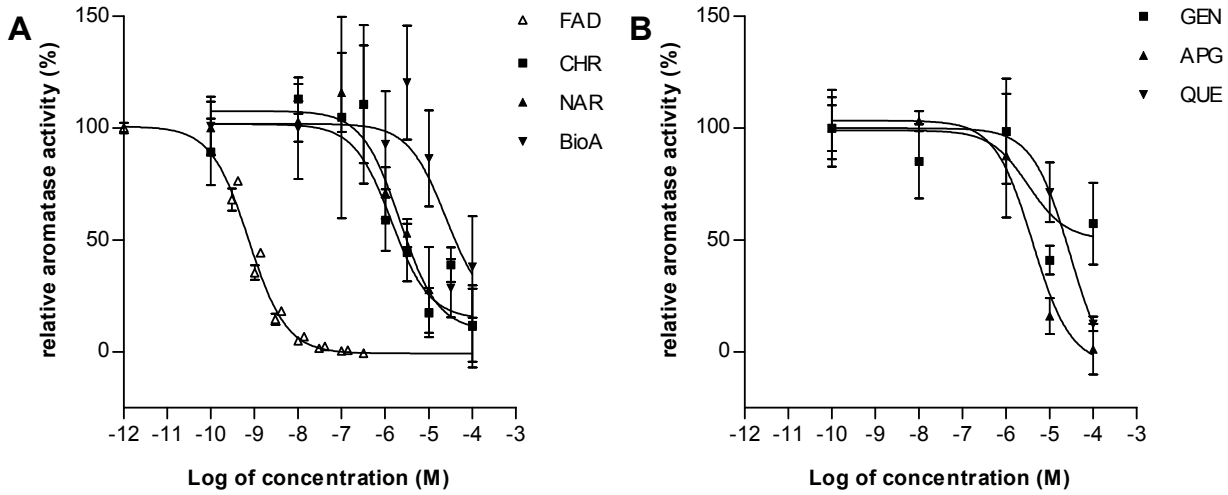


Fig 6.5. Aromatase activity measured in fibroblasts after 24h preincubation with DEX (30 nM) and, except for FAD, 2 - 3h preincubation with increasing concentrations FAD, BioA, CHR, NAR (**A**) APG, GEN or QUE (**B**). Mean of relative aromatase activity for BioA, CHR and NAR were determined in three experiments in triplo, while APG and GEN were tested in two separate experiments in triplo and QUE was tested once in trilpo at $1*10^4$ and $1*10^5$ nM. Notice that at the highest test concentration of BioA and GEN inhibition is substantially above the minimum.

MCF-7 and fibroblasts co-culture experiments

To investigate the resultant effect of PC induced MCF-7 cell proliferation and aromatase inhibition in fibroblasts, co-cultures of both cell types were exposed to a PC and/or T. pS2 expression was used as a measure for estrogenic response in MCF-7 cells. The co-culture experiments required various *in vitro* techniques and for practical reasons fibroblasts from different women had to be used. Therefore every expriment included a number of reference samples. Reference compounds were E_2 (ER agonist), ICI (ER antagonist), DEX (aromatase inducer), FAD (aromatase inhibitor), A-dione and T (aromatase substrates). Concentrations used for these compounds were based on results of earlier studies [17]. In the co-culture experiments pS2 expression was used as a marker for ER mediated effects instead of MCF-7 cell proliferation as it is not feasible to assess cell numbers for both cell types separately. As expected, in the co-cultures E_2 as well as T and A-dione induced pS2 expression, which was blocked by ICI. FAD could also block T induced pS2 expression (fig 6.6), indicating that in the co-culture aromatase in the fibroblasts was required to convert androgens to estrogens. In line with this observation, combined treatment of T with DEX induced a higher pS2 expression than T alone. This indicates that DEX can induce basal aromatase activity in fibroblasts when in co-culture. Here it should be noticed that Heneweer and co workers could not find significant pS2 expression in MCF-7 cells exposed to 20 nM T [17], although in our proliferation assay the positive response to 20 nM T was very low but noticeable. However, our results obtained with T and FAD show that the fibroblasts indeed interact with the MCF-7 cells in a way that can be explained sufficiently by the proposed paracrine model (fig 6.1).

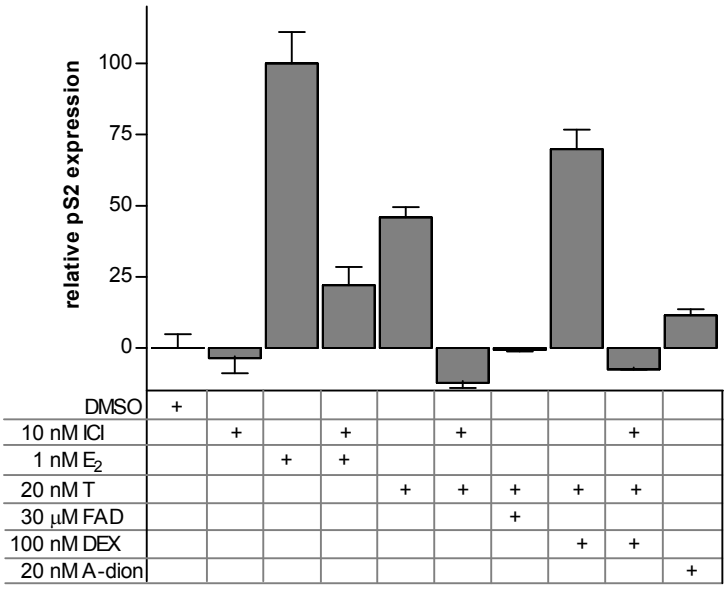


Fig 6.6. The markers pS2 expression level and cell proliferation as a measure for indirect and direct estrogenicity of reference compounds (DMSO, E_2, ICI 182780, T, A-dione, DEX, FAD) in co-cultures of fibroblasts and MCF-7 cells after a 72h exposure. DMSO treated cultures are set to 0%, while 1 nM E_2 treated cultures represent 100%.

BioA, CHR and NAR were also tested individually or in the presence of 20 nM T (aromatase substrate) (fig 6.7A-C). Without T, these PCs showed a concentration-dependent up-regulation of pS2 expression, which occurred in the same concentration range as found for cell proliferation in MCF-7 cells only. To study possible effects on aromatase activity in the fibroblasts by these PCs present in the co-culture, different concentrations of BioA, CHR and NAR were tested in combination with T. In the experiment done with 20 nM T alone the estrogenic pS2 response must have originated from constitutive aromatase activity in the fibroblasts. In relation to this basal formed estrogen, none of the three PCs showed a significant and consistent effect on pS2 expression that might have been caused by a reduced or increased estrogen production via modified aromatase activity in the fibroblasts (fig 6.7A-C). In general, these results indicate that aromatase inhibition in the fibroblasts was not occurring at concentrations that could induce estrogenic effects in MCF-7 cells, measured either as pS2 expression or cell proliferation (fig 6.2A-D).

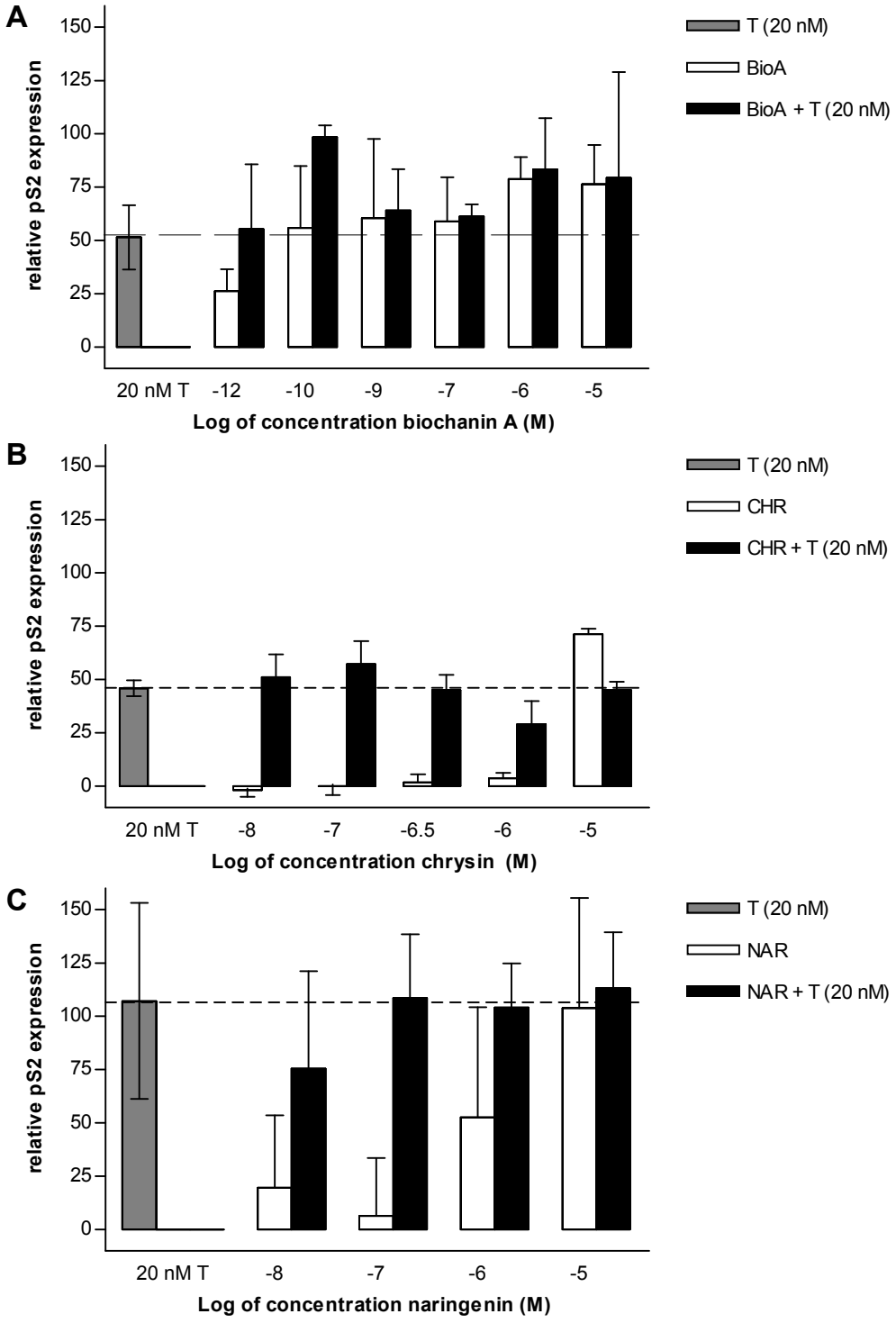


Fig 6.7. pS2 expression level as a measure for indirect and direct estrogenicity of BioA (**A**), CHR (**B**) or NAR (**C**) in co-cultures of fibroblasts and MCF-7 cells after a 72h exposure. DMSO treated cultures are set to 0%, while 1 nM E_2 treated cultures represent 100%.

Discussion

In this paper results are presented from experiments with primary fibroblasts from healthy human mammary tissue, human epithelial estrogen sensitive breast tumor cells (MCF-7) and co-cultures of both cell types. The effects of APG, BioA, CHR, GEN, NAR and QUE on aromatase activities in

mammary fibroblasts and/or cell proliferation in MCF-7 cells were studied. These PCs are known dietary components and can induce cell proliferation in ER positive breast tumor cell lines like MCF-7, and T47D [5, 53]. In addition, some PCs have been found to inhibit aromatase activity in various human *in vitro* systems, including those from placenta [1, 31], ovaria [22], adrenocortical carcinoma cells [42], endometrial stromal cells [14] and breast pre-adipocytes [8]. With respect to the formation or recurrence of estrogen dependent tumors, e.g. breast cancer, the observed aromatase inhibition and ER mediated cell proliferation by PCs can be considered respectively beneficial or adverse. Both effects might concur at the same dose level and time, but presently it is unclear which type of effect could prevail at relevant human exposure levels. Our present co-culture study allows aromatase inhibition and estrogenicity to be measured in conjunction and is a further step towards clarifying the relevance of both effects for the human situation. However, to evaluate these opposing effects in the co-cultures it was also necessary to study the effects of these PCs in MCF-7 cells or mammary fibroblasts only.

In the MCF-7 cells these PCs were found to stimulate cell proliferation with EC_{50} values for APG, CHR and NAR in the range from 0.1 to 1 µM, while BioA and GEN were approximately one order of magnitude more potent. These results are in agreement with other studies using MCF-7 cells and PCs [5] and again confirm that phytochemicals like BioA and GEN can be ERα agonists although with potencies that are at least three orders of magnitude lower than E_2.

In addition to the effects observed for these PCs in MCF-7 cells it was observed that T also caused cell proliferation, but with a potency that was four orders of magnitude less than E_2 and less maximum efficacy. The question is if this cell proliferation is by T activating the ER or originates from its conversion to E_2 by aromatase in different MCF-7 cell lines [2, 7, 40, 43, 44, 50]. However, in our experiments it was found that MCF-7 cells were not or only at very low rate exhibiting aromatase activity, which observation is in concordance with other studies [4, 20, 41, 49]. In addition, co-exposure of our MCF-7 cells in our experiments with an aromatase inhibitor (FAD) did not inhibit this induced cell proliferation by T, which supports a direct sone agonistic effect of this androgen on the ERα.

Although mammary fibroblasts have been found to interact actively with adjacent tumor tissue for breast tumors to our knowledge no studies have been done so far with primary fibroblasts originating from healthy women. For a number of PCs used in our studies with mammary fibroblasts IC_{50} values for aromatase inhibition were found around 1 to 10 µM. These results are in good agreement with the aromatase inhibition by PCs observed in other human primary tissues, cell lines and microsomes [8, 22, 25, 42]. In view of the suggested chemo preventive properties suggested for some PCs it should be noticed that the inhibitory potencies of some of these PCs in primary mammary fibroblasts are three orders of magnitude lower than that of the therapeutical aromatase inhibitor fadrozole which has an IC_{50} of around 1 nM.

The results of our experiments using separate cell systems as well as co-cultures, show that effective concentrations of PCs for cell proliferation and aromatase inhibition can overlap. It can be questioned if there is a small, but significant, medium concentration range where aromatase inhibition dominates over estrogenic effects. If these *in vitro* medium concentrations could be used as a first approach to model human serum concentrations the existence of a dominating preventive effect for breast tumor growth could be suggested. However, based on our results with separate cell systems (fibroblasts and MCF-7 cells), only CHR could exert significant aromatase inhibition at non-estrogenic concentrations (1 µM). However, this difference between both opposing effects could not be reproduced in the co-culture system. A possible reason for this discrepancy between single cell systems and their co-culture could be that the method for pS2 measurement (i.e. RT-PCR) was too insensitive to pick up subtle differences in E_2 production by aromatase in the fibroblasts.

Our co-culture model is based on the positive feedback loop (fig 6.1) between estrogen production in the fibroblasts and ERα related properties in the breast tumor cells [17]. Control experiments with A-dione and T with or without DEX used as an aromatase inducer did cause a significant increase in pS2 expression, while co-treatment with FAD decreased the response in pS2

expression (fig 6.6). This proves that this part of the feedback system was indeed functioning in our co-culture system of mammary fibroblasts and MCF-7 tumor cells. It should be noted that the intensity of this part of the feedback loop may vary for each individual batch of mammary fibroblast or even with the length of culturing period. Interindividual differences in (inducibility of) aromatase activity in (tumorous) mammary fibroblasts has been reported earlier [11, 18]. In addition culture conditions of cells are also known to change gene number copies as well as gene expression levels [33]. In our experiments we also experienced differences in basal aromatase activity within the same batch of fibroblasts depending on the length of culture time.

We did not investigate the feedback system from MCF-7 cells to fibroblasts, e.g. by PGE2 that could lead to aromatase induction. However, an earlier study with MCF-7 cells and mammary fibroblasts failed to detect this part of the feedback system between both cell types [17]. It has been suggested that this interaction could indeed be absent from this *in vitro* system, but insufficient sensitivity of either the aromatase or pS2 assay could be other reasons [17]. Another explanation for this apparent lack of aromatase inhibition in the co-cultures with concurrent decrease in pS2 RNA level in the MCF-7 cells could come from the presence of T as aromatase substrate. In our experiments T was found to cause cell proliferation in MCF-7 cells by itself as well reduce effect of E_2 (fig 6.2A, B). An earlier study found no increased pS2 expression at 20 nM T [17]. However, in view of our own results it is important to establish which mechanism is responsible for the induced pS2 expression in MCF-7 cells. This is especially important for further validation of the co-culture system in which T is essential as substrate for aromatase. Androgens have been reported earlier to reduce cell proliferation in MCF-7 cells and T47D cells [3] and MCF-7 cells with transfected androgen receptors (AR) [51]. Szelei *et al.* suggests that this is androgen receptor mediated or that androgens stimulate the cells to synthesize a protein that prevents cells going into the next cell-cycle. In this respect it should be recognized that 20 nM T was used as an effective amount of substrate for aromatase in the co-cultures, which is also around the EC_{50} value for cell proliferation in our MCF-7 cells. Thus, it might very well be that T might have interfered with pS2 expression in the co-cultures modulating estrogenic response in the MCF-7 cells. However, it could be questioned if this effect is also not occurring at normal human exposure situations as serum testosterone levels are in the range 0.3 – 2.4 nM for pre- and postmenopausal women [24, 57].

The question arises if normal human dietary exposure to the investigated phytochemicals can lead to levels from which based on our experiments either effects on cell proliferation or aromatase can be expected. For some of these PCs blood concentrations have been reported such as 60 nM for biochanin A [19], 5 to 110 nM for naringenin [15], 15 to 900 nM for genistein [52, 58] and 27 nM to 50 nM for quercetin [15, 16]. Assuming that these blood concentrations can be used as a first comparison with nominal medium concentrations in our *in vitro* systems, it can not be excluded that this might lead to cell proliferation in breast tumor cells because EC_{50} values for e.g. BioA and GEN are in the same range as those observed in blood. However, when using this approach also for inhibition of aromatase in mammary fibroblast this seems less likely, as IC_{50} values for these PCs were above µM levels. Nevertheless, it is easy to estimate that individuals who take food supplements with these PCs can exceed their normal dietary intake easily with one to two orders of magnitude (comparing daily intake and contents of commercially available food supplements [45]). Thus, for these specific situations it can not be excluded that effects on aromatase as well as induction of cell proliferation in breast tumor cells can be realistic under the assumptions of this preliminary risk and exposure assessment. Therefore caution with food supplements of these PCs might be advised based on our results with types of breast cells, especially for those groups that are already at higher risk, because of e.g. inherited risk or previous history of a breast tumor.

In conclusion, the results of our *in vitro* studies with MCF-7 cells, primary mammary fibroblasts and their co-culture have shown that these phytochemicals can induce cell proliferation or inhibit aromatase activity in the same concentration range. This cell proliferation can occur at

concentrations that are not uncommon in blood of individuals using food supplements and appears quantitatively more sensitive than aromatase inhibition. Based on the results with these two types of human breast cells perhaps a more cautionary approach should be taken for these phytochemicals before taken as food supplements.

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Chapter 7

General Discussion and Conclusions

Introduction

Human exposure to endocrine disruptors is of interest for a wide range of disciplines such as fields within science, medical doctors, commercial organizations and policy makers. More specifically, of concern are exogenous compounds that mimic estrogens or interfere with the normal function of estrogens, called xeno-estrogens. At least three aspects of xeno-estrogens contribute to this wide interest – (i) estrogens are steroids that function in the development of primary and secondary sex characteristics, (ii) exposure to endogenous as well as exogenous estrogens have been linked to hormone dependent diseases (i.e. breast and prostate cancer) [22] and (iii) (human) exposure to xeno-estrogens is assumed to occur widespread [5, 27, 31, 32]. Additionally, there is not much known about the effects of human exposure to combinations of e.g. xeno-estrogens and phytoestrogens. In fact, very few studies have addressed this topic of mixture toxicity in the recent past [34, 36, 37].

This thesis

Responses to estrogenic compounds in biological systems are primarily estrogen receptor (ER) mediated and effects can be either estrogenic or anti-estrogenic [3]. In addition, certain xenobiotics including phytochemicals have the ability to interfere with the steroidgenesis because of interaction with aromatase [19]. In this thesis, effects of various xeno-estrogens on both ER-isoforms (α and β), as well as on aromatase, were further investigated. Specifically, interactions of combinations of xeno-estrogens in ER mediated effects were also included in these studies. The aim of this thesis was to provide further data contributing to the estimation of human risks induced by exposure to individual members as well as mixtures of these compounds, with specific emphasis on estrogenic compounds.

Restrictions of the studies

Several aspects restrict the direct translation of our *in vitro* and *in vivo* experiments to the human situation, the most important one being simplification. As a consequence, findings in this doctoral thesis, even when combined with available data from literature, should not be considered as a comprehensive hazard assessment that can be used for a definitive risk assessment of human exposure to endocrine active compounds (EACs), but can only be taken as a first estimate. Some simplifications applicable in our research are mentioned below.

In both our *in vitro* and *in vivo* models that use estrogenicity as endpoints, the effects are predominantly mediated via the ERα. However, in whole organisms these compounds can also interact with another ER isoform (ERβ) or with totally different (steroid) receptors, which have a promiscuous character with respect to binding affinities (e.g. androgen receptor, human steroid and xenobiotic receptor (SXH) or pregnane X receptor (PXR)) [18, 33]. In our *in vitro* studies, a compound is considered to act via the ER, if an antiestrogen (i.e. tamoxifen, faslodex or raloxifen) suppresses the estrogenic response.

Our *in vitro* models measuring aromatase activity were either microsomes isolated from human placental tissue or primary cultures of human breast fibroblasts. Placental microsomes are cellular organelles that contain cytochrome P450 enzymes such as aromatase. Breast fibroblasts express the CYP19 gene (coding for aromatase) and play a key role in an estrogen sensitive breast cancer model. In contrast to fibroblasts, placental microsomes are not capable to synthesize the aromatase enzyme *de novo*. In co-cultures between MCF-7 cells and fibroblasts, fibroblasts were used as the aromatase containing cells, being the androgen converting system. The endpoints in this model were estrogen-induced pS2 mRNA [23, 25], representing the overall effect of xeno-estrogens on aromatase expressed in fibroblasts, and ER-mediated proliferation of MCF-7 cells. Clearly this model should be considered as a better hormone dependent breast cancer *in vitro* model than MCF-7 cells alone. However, this chosen co-culture model does not represent other important factors for cell-cell

communication such as interleukin 6 (IL-6) and the soluble receptor (IL-6sR) of infiltrating macrophages and lymphocytes, which may be important factors in breast cancer development [28, 30].

In our *in vitro* models we did not take into account specific physico–chemical characteristics of the test compound. Properties such as water solubility (related to protein binding), tissue accumulation and conversion to more biologically active metabolites may further affect the concentration at the target site (e.g. ER or aromatase enzyme) and influence the overall observed effect. The assumptions about the (exact) free concentration of test compounds in both cell cultures could be responsible for errors in calculated potencies for estrogenicity, as well as aromatase inhibition calculated in this thesis [16, 17].

In some cases, the relative concentrations of individual compounds within mixtures of xeno-estrogens used in our *in vitro* studies are based on average concentrations and do not represent the wide individual concentration range that can be found in humans [1, 26]. Furthermore, these mixtures are limited to the major xeno-estrogens identified and do not fully reflect exposure to the wide array of estrogenic compounds to which humans can actually be exposed. In the future these limitations could possibly be overcome for example by using extracts derived from human (e.g. serum), animal or environmental samples and to determine their estrogenic potency [11].

A last limiting issue of consideration would be the difference of exposure in experimental models versus that of long term, low level, exposure to EACs in real life, while the time span of *in vitro* as well as *in vivo* studies to investigate i.e. xeno-estrogens is usually relatively short. Bearing this in mind it can not be excluded that EACs might have their effects across generations [12, 41].

Regardless the uncertainties inherent with the *in vitro* models, it is felt that the research described in this thesis does improve our knowledge basis for risk assessments of (mixtures of) xeno-estrogens. These *in vitro* test systems have been developed as alternatives to animal experiments, and in this respect, it should be realized that perfect *in vitro* models for endocrine effects are a utopia due to the complex interactions that take place in the whole body.

Combined xeno-estrogens act additively

In **chapter 2 and 3**, two sets of multi-component mixtures (three or six individual compounds) were composed of human relevant phytoestrogens (coumestrol, genistein, naringenin, quercetin, cathechin and epicatechin) or synthetic estrogens (4-nonylphenol, octylphenol, β-hexachlorocyclohexane, bisphenol A, methoxychlor, dibutyl phthalate). These mixtures were tested in either the MCF-7 cell proliferation assay (E-screen) or the prepubertal rat uterotrophic assay. Responses in both the E-screen and the uterotrophic assay are inducible by (xeno)estrogens which are mediated through the ER and thus these responses were considered as measures for estrogenicity. Results obtained from the *in vitro* model showed that estrogenic potencies of xeno-estrogens are around 10.000 to 100.000 times lower than that of 17β-estradiol (E_2). For genistein tested individually *in vivo*, the potency was around 500.000 times lower that that of E_2. In both models, interactions between xeno-estrogens and between xeno-estrogens and E_2, the latter used as reference estrogen, were calculated with the concentration addition (CA) model. The outcomes of both *in vitro* and *in vivo* studies point toward zero interaction, which indicates additive effects, for all combinations. As these studies included mixtures representing actual internal human exposure, the observed additive effects implicate that the contribution of xeno-estrogens to the actual total human body burden of estrogenic activity is related solely to the internal concentration and estrogenic potency of individual compounds. Additionally, the results from these studies suggest that from an estrogenic point of view, and considering systemic human exposure levels, the human daily exposure to phytoestrogens is probably of more relevance than that of synthetic xeno-estrogens.

Chapter 4 investigated *in vitro* estrogenic effects of additives commonly used in cosmetic products such as phthalates, polycyclic musks, triclosan, parabens and their major metabolite (para-hydroxybenzoic acid). Most of these compounds induced proliferation in the MCF-7 cells, except

triclosan. However, compared with E_2 the relative estrogenic potencies were all 100.000 to 1.000.000 times lower. In addition, the polycyclic musks did not induce proliferation in the MCF-7 cells to the same extent (efficacy or Y_{max}) as E_2 even at the highest concentration tested. Such a difference hampers the practical use of e.g. EC_{50} values for exact comparison of estrogenic potencies with E_2. This study focused also on interactions between ethyl- and propylparaben and between various parabens and E_2. Again, interactions were evaluated in the CA model. All combinations of ethyl- and propylparaben and various individual parabens combined with E_2 indicated additive effects. Consequently, this strict additive mixture effect was used to transform internal concentrations of parabens found in literature [8] to EEQs by using the estrogenic potencies estimated in this study. The total EEQ for all parabens summed up to around 0.082 pg EEQ/g tissue. Relative to the endogenous burden in premenopausal women, of around 350 pg E_2/ml plasma [42], this is only 0.023% and should clearly be considered as only a marginal contribution to the total estrogen burden in the human body. When compared to levels reported in normal breast tissue (55.3 pg E_2/g tissue) or breast tumor tissue (348 pg E_2/g tissue) this contribution is only marginal [7].

Xeno-estrogens inhibit aromatase

Chapter 4 investigated the catalytic inhibition of aromatase by parabens. Aromatase was isolated from human placental microsomes and determined with the tritium water release assay. The results from these experiments showed that methyl, ethyl, propyl, isopropyl, butyl and benzyl paraben can all inhibit aromatase activity.

The potency to inhibit aromatase was around 1.000 to 10.000 times lower than the reference compound fadrozole (**chapter 5:** IC_{50} = 3.8 nM). The latter compound is a pharmacotherapeutical aromatase inhibitor nowadays used to prevent breast cancer recurrence. For an intact organism, the interference with aromatase activity by parabens could imply that steroidgenesis might be affected. From an estrogenic point of view, inhibiting the conversion of androgen to estrogens is an anti-estrogenic effect. Interestingly, aromatase inhibitory properties of parabens occurred close to concentrations at which an estrogenic response was found in the MCF-7 cell system. Taken into consideration the possibility that both effects can occur jointly in, for instance, breast tissue, it remains difficult to estimate the overall effect, while both effects should be included in a thorough risk assessment of these compounds.

Chapter 5 investigated aromatase inhibition by three synthetic lactones (TM-7, TM-8, TM-9), four phytoestrogens (apigenin, chrysin, naringenin and 8-prenylnaringenin) and fadrozole. Aromatase activity in both placental microsomes and fibroblasts isolated from healthy human mammary tissue was studied. Noticeable, the synthetic lactone TM-7, fadrozole and 8-prenylnaringenin inhibited aromatase activity at similar concentrations in either system. Interestingly, the aromatase inhibitory potency of 8-prenylnaringenin was only one order of magnitude lower than that of fadrozole in both systems. In comparison, the other test compounds had aromatase inhibitory potencies that were around two to four magnitudes lower than that of fadrozole. These results implicitly show that inhibition by xenobiotics of aromatase in human placental microsomes has a good predictive value for inhibition of aromatase in mammary breast fibroblasts. These breast fibroblasts play a key role in a model of estrogen dependent mammary cancer [9] and from this perspective aromatase inhibition is generally considered beneficial. It was calculated that due to intake of a regular diet, either Western or Asian, concentrations of the phytoestrogens might reach concentrations effective to: (i) induce (anti)estrogenic responses in hormone sensitive cells (e.g. breast tumor cells) (**chapter 2 & 6**), as well (ii) inhibit aromatase in aromatase expressing cells (e.g. connective tissue surrounding tumor cells) (**chapter 5 & 6**).

Phytoestrogens are stronger estrogens than aromatase inhibitors

Previous chapters investigated either estrogenic or aromatase inhibition in different experimental systems. From an estrogenic point of view these effects could be considered as opposite. In **chapter 6** both effects were tested in separate models. Estrogenic responses of five phytoestrogens (biochanin A, genistein, naringenin, apigenin and chrysin) were followed in the MCF-7 cell proliferation assay. Furthermore, inhibition by six phytoestrogens (chrysin, naringenin, genistein, apigenin, biochanin A, quercetin) of aromatase in fibroblasts were also tested. The effective concentrations that inhibited aromatase in the fibroblasts were found to be higher than those producing estrogenic effects in the MCF-7 cells. In addition, chapter 6 combined both the MCF-7 cell and breast fibroblasts to investigate estrogenic and aromatase inhibitory effects in one system. In this co-culture system a feedback loop between MCF-7 cells and mammary fibroblast has been established and is regarded as a more advanced breast cancer model than a monoculture MCF-7 cells only [15]. In this model, the expression levels of pS2 in MCF-7 cells (cultured together with primary fibroblasts) served as a measure of estrogenicity. Results obtained with this co-culture model showed that biochanin A, chrysin and naringenin alone induced pS2 expression levels in MCF-7 cells. Similar co-culture conditions in the presence of testosterone, which is a substrate of aromatase in fibroblasts, showed that the aromatase inhibitory effect was not significant at low, non-estrogenic concentrations, while at higher concentrations the estrogenic effects were found to be dominant over aromatase inhibition. This again confirms that estrogenic effects of these phytochemicals are more potent than aromatase inhibitory capacities. Clearly, the biological significance of compounds being " more estrogenic than aromatase inhibiting" has to be elucidated, more in relation to the overall endocrine effect on the whole organism. Those people taking foodsupplements or herbal preparation, that can contain high amounts of phytochemicals, consequently increase their exposure levels to these compounds. Some of these supplements claim to be anticarcinogenic, relief from postmenopausal complains or are bust-enhancing. Based on our results, it might be considered that besides the claimed beneficial effects these supplements could also be harmful and increase estrogen sensitive breast cancer growth.

Contribution of xeno-estrogens to estrogenicity in the human body

In chapter 2, 3, 4 and 6 it was found that various xenobiotics to which humans are daily exposed, exhibit estrogenic properties. From our results that focused on combinations of compounds, it was concluded that these estrogenic compounds show zero interaction according to the CA model. So far, only a few findings by others suggest mixture effects other than additive [29, 36]. Thus, our results with more complicated estrogenic mixtures and inclusion of endogenous E_2 are fully in line with the majority of findings reported in literature [6, 10, 13, 34]. Based on this it can be concluded that the total estrogenic burden can be calculated using the EEQ system that consists of the multiplication of the internal concentration and the estrogenic potency of each compound. However, due to the short half-lives of many phyto- and synthetic estrogens [4, 21, 24, 38], it can be questioned if such an EEQ system could also be applied for uptake situations as is done for e.g. dioxin like compounds [39, 40]. Due to the large differences in half-live for xeno-estrogens it is very likely that the composition of the mixture determined for uptake is qualitatively and quantitatively largely different from that observed systemically, e.g. in blood [2].

Chapter 4 included also an assessment of the internal exposure to estrogenic compounds, expressed as estrogen equivalents EEQs. It was estimated that the estrogenic contribution of cosmetic additives may be low and irrelevant compared to the circulating endogenous estrogens. For instance, polycyclic musks were estimated to contribute 0.008% (0.029 pg EEQ/g tissue), parabens contribute 0.023% (0.082 pg EEQ/g tissue) and phthalates contribute 0.311% (1.09 pg EEQ/g tissue), in which the endogenous E_2 level in breast tissue was set at 100% (350 pg EEQ/g tissue). In addition, further calculations of the contribution to total daily exposure per xeno-estrogen group point towards UV-screens as the most prominent source of external EEQs using a calculation model/data from Harvey *et*

al. 2006. Our calculations then point towards a contribution of 1.633% (5.7 pg EEQ/tissue)[14]. It should be noted, however, that this calculated value for internal EEQ-exposure via UV-screens might be excessive relative to regular exposure situations. Such a high exposure to these UV screens is expected only to occur occasionally, as sun blockers are primarily used for a short period during the year. The second highest source of internal EEQs/day was calculated to be phytochemicals with a contribution of 0.64% (2.24 pg EEQ/g tissue), followed by the organochlorines that contribute for 0.315% (1.1 pg EEQ/g tissue). Exposure to alkylphenols and bisphenol A results in a contribution of 0.103% (0.36 pg EEQ/g tissue). For comparison, the estrogen contribution of contraceptives (as ethinylestradiol) was calculated to be around 41% (142 pg EEQ/g tissue) (fig 7.1). However, relative to the burden of adult males (50 pg E_2/g tissue), the relative contributions of xeno-estrogens are seven times higher. In addition, prepubertal children have systemic E_2 levels of 10 pg/ml serum or lower. Consequently, relative body burdens of xeno-estrogens are 35 times higher.

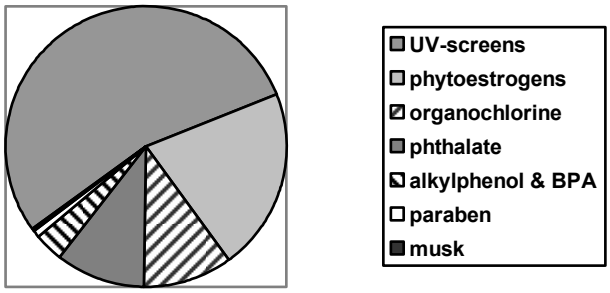

Fig 7.1. Pie chart of estimated estrogen contribution per group of xeno-estrogens.

Concluding remarks

Regular risk assessment is executed in four steps: hazard identification (characteristics of the potential hazardous source), effect assessment (levels inducing adverse health effects), exposure assessment (actual exposure level) and risk characterization (considering effects and levels). Conducting such a traditional risk assessment for xeno-estrogens is hampered by several confounding factors. From an estrogenic point of view, findings of epidemiological studies point to positive correlations between the use of postmenopausal estrogen therapy (1-2 mg estrogen esters/day combined with progestogens,) and hormone dependent breast, endometrial and cervix cancers [42]. A possible relationship between the use of contraceptives (ethinyl)estradiol in the range of µg/day combined with progentogens and breast cancer is also indicated [42]. From a xeno-estrogens point of view, the EACs are compounds that may exert their endocrine effect only after a (life) long exposure and sometimes even in the next generation.

Obviously, life long exposure of humans to xeno-estrogens on a regular level is much different from any experimentally controlled situation. However, if regular human exposure levels and internal concentrations of xeno-estrogens are considered, the majority of the present scientific information, including that presented in this thesis, indicate that these are most likely too low to be harmful. The preliminary risk assessment performed in chapter 4 indicates that the contribution of synthetic and phytoestrogens is only marginal compared to endogenously produced estrogens or even contraceptives. When aromatase inhibition is also taken into account for these xenobiotics or phytochemicals, it must be concluded that regular exposure of humans is probably too low to reach effective levels.

However, reasons for concern might originate from innovations in the food sector and shifts in peoples dietary habits [20]. The use of food supplements containing large amounts of phytochemicals is becoming more and more popular over the last decade [35]. In addition, the number of food products enriched with phytochemicals is increasing. Weighing the possible beneficial effects of (anti-)estrogenic phytochemicals against possible adverse effects is extremely complex and scientific evidence for either effect is ambiguous. In this perspective, possible sensitive groups in the population are postmenopausal women and (unborn) children. Therefore it is worrisome that until today there is no adequate regulation of food products or additives containing high levels of (anti-)estrogenic phytochemicals and adequate human risk assessment is not possible due to the lack of scientific data.

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Nederlandse samenvatting

Introductie

Ieder gezond organisme is in staat homeostase binnen het lichaam te handhaven. Deze evenwichtige situatie wordt door vele, o.a. externe factoren beïnvloed. Zo worden mensen in het dagelijkse leven blootgesteld aan een groot aantal verschillende stoffen. Enkele daarvan zijn in staat binnen in het lichaam te komen en de endocrine homeostase uit evenwicht te brengen. Deze groep stoffen, de zogenaamde hormoon verstorende stoffen of hier endocrien actieve chemicaliën (EAC), kunnen hormonen nadoen, normaal functioneren belemmeren, maar ook het productie process van hormonen beïnvloeden.

Al decennia lang maakt men zich zorgen over blootstelling van mensen aan een bepaald gedeelte van de EAC's namelijk degene die lijken op een bepaald hormoon (estrogenen) en worden aangeduid als xeno-estrogenen. Zowel binnen in het lichaam aangemaakte estrogenen als ook xeno-estrogenen worden geassocieerd met bepaalde ziektes zoals borst- en prostaatkanker. Anderzijds wordt van sommige natuurlijke xeno-estrogenen gedacht dat deze gezond zijn voor de mens.

Zoals aangegeven komen xeno-estrogenen voor in de omgeving van mensen en in monsters van menselijke oorsprong. Dit roept twee vragen op: (i) vormen de aangetroffen blootstellingsniveaus een gevaar voor de gezondheid en (ii) wat zijn de risico's van blootstelling aan een combinatie van deze stoffen? Er bestaat vooral onvoldoende kennins om de tweede vraag bevredigend te beantwoorden, terwijl dit juist een relevante bloostellingssituatie is voor mensen. Een deel van dit proefschrift concentreert zich dan ook op gecombineerde effecten van xeno-estrogenen die middels estrogeenreceptoren (ER) gereguleerd worden.

Tegelijkertijd kan een xeno-estrogeen ook effecten opwekken die niet middels de ER gereguleerd zijn. Bijvoorbeeld sommige, uit planten afkomstige chemicaliën kunnen de ER, en daarmee bepaalde processen, activeren en tegelijkertijd het productie process van steroïden (b.v. estrogenen) remmen. Dit zijn twee schijnbaar tegengestelde effecten. Een ander gedeelte van dit proefschrift gaat in op effecten op aromatase, een belangrijk enzym in de synthese van estrogenen.

Een laatste deel van dit proefschrift bekijkt het uiteindelijke effect van zulk soort tegengestelde werkingsmechanismen van een stof in één systeem. Daarnaast wordt een schatting gemaakt van de totale belasting estrogen stoffen bij mensen.

In deze samenvatting zullen een aantal belangrijke begrippen worden toegelicht. Daarna worden resultaten beschreven die in dit proefschrift verkregen zijn met daarbij de belangrijkste conclusies. Vervolgens worden de resultaten bediscusseerd.

Hormonen

Hormonen zijn de signaal stoffen van communicatie systemen die van vitaal belang zijn voor het handhaven van homeostase in een organisme. Een groep hormonen, de steroïden, functioneren als signaalstof in processen als groei en reproductie. Ze worden gesynthetiseerd in een proces dat de steroïdgenesis wordt genoemd. De uitgangsstof cholesterol is een lipide en heeft derhalve een vetminnend (lipofiel) karakter, evenals de geproduceerde steroïden. Voor dit proefschrift zijn de "mannelijke" steroïden (androgenen) en de "vrouwelijke" steroïden (estrogenen) van belang. Eenmaal geproduceerd in specifieke cellen, worden steroïden afgescheiden aan de bloedcirculatie. Dankzij hun lipofiele eigenschappen dringen steroïden middels diffusie weefsels binnen en bereiken daarmee vrijwel alle cellen.

Slechts cellen die beschikken over specifieke receptoren, b.v. estrogeenreceptoren (ER), herkennen een specifiek steroïdsignaal, b.v. een estrogeen, en zullen een respons generen. Het biologisch meest active estrogeen is 17β-estradiol (E_2) en wordt in zowel vrouwen als mannen aangemaakt in respectievelijk de ovaria en de testes. Weefseltypen waarin estrogeenreceptoren tot expressie worden gebracht zijn het centraal zenuwstelsel en bot-, cardiovasculair-, lever-, urogenitaal-, gastrointestinaal- en mammaweefsel. Er zijn twee typen of isovormen van estrogeenreceptoren (ERα en ERβ) die afhankelijk van het weefseltype in een bepaalde verhouding tot expressie worden gebracht.

Estrogeenreceptoren maken deel uit van de familie van nuclaire receptoren. Indien een E_2 molecuul deze receptor bindt, treedt er een cascade van reacties op waarbij de receptor als transcriptie factor fungeert en direct DNA-transcriptie moduleert (gentranscriptie activatie of repressie). De verandering in genexpressie patroon van een cel vormt de basis voor een biologische respons op cellulair, weefsel- en organismaal niveau. Dit proces is in de evolutie sterk geconserveerd, met als gevolg dat lagere en hogere diersoorten steroïdhormoon signalen overbrengen middels deze principes.

Niet lichaamseigen stoffen die op estrogenen lijken (xeno-estrogenen) kunnen op vele niveaus normale signaal overdracht en de respons beïnvloeden. Ondermeer kunnen dat de volgende zijn: verhindering van binding van endogene hormonen aan de receptor, een niet normale activatie van de receptor met als gevolg een niet normale herkenning van specifieke nucleotide volgorde in het DNA, genaamd estrogeen responsieve elementen (ERE's). Overigens kan één van de twee ER-isovormen gevoeliger zijn dan de andere ER-isovorm voor dergelijke fenomenen die veroorzaakt kunnen worden door een xeno-estrogeen. Daarnaast kan een stof interferen met de steroïdgenesis of met hormoon-transport eiwitten (albumine en sex hormone binding globuline) die zich in de circulatie bevinden. Of kan het de expressie van andere receptoren in een cel beïnvloeden. Daarbij wordt de respons die in gang gezet wordt na interactie met estrogeenreceptoren bepaald door de cellulaire context.

Hormone verstorende stoffen

De officiële definitie(s) voor een EAC is (ook) na discussie veel omvattend gebleken. Twee voorbeelden van EAC's die van belang waren voor de toenemende aandacht voor het onderwerp hormoonverstorende stoffen zijn biochanin A en DDT. Biochanin A en formononetin komen voor in rode klaver. Nieuw Zeelandse schapen ondervonden fertiliteitsproblemen na consumptie van rode klaver. DDT werd veelvuldig gebuikt als pesticide en in de 50'er jaren van de vorige eeuw werden eerste waarnemingen gedaan van (schadelijke) verandering in de natuur ten gevolge van DDT. DDT, vooral een bepaalde isomeer en afbraak product daarvan (*o,p*-DDT), bleek estrogene eigenschappen te vertonen.

Sindsdien zijn er vele andere stoffen bekend geworden die een bewezen of verdacht hormoon actief zijn. Enkele groepen zijn van belang voor dit proefschrift: van planten afkomstige chemicaliën (phyto-estrogenen), oppervlakte active stoffen in reinigingsmiddelen (alkylphenolen), conserveringsmiddelen in cosmetica (parabenen), UV-absorberende stoffen in cosmetica of plastics (UV-screens/benzophenonen), geurstoffen in cosmetica (synthetische polycyclische musken), geurstof-fixativen in cosmetica en weekmakers in plastics (phthalaten), grondstof en bescherming voor plastics (bisphenol A) en pesticides (organochlorines).

Hormoon afhankelijk kanker

Naast nuttige effecten, worden estrogenen ook in verband gebracht met de initiatie en groei van borstkanker. Ook wordt gedacht dat ze een rol spelen bij prostaatkanker. In de Westerse wereld komt bij 1 op de 10 vrouwen borstkanker voor, waarvan ongeveer tweederde een hogere expressie van ERβ en vooral ERα heeft. Dientengevolge laten deze tumoren hormoonafhankelijke groei zien. Hoe een dergelijke verandering precies ontstaat is onduidelijk. Wel is een model opgesteld hoe de tumor

autonoom kan functioneren. Hierin interacteren de tumor en omliggend weefsels met elkaar. Factoren die uitgescheiden worden door tumor cellen (PGE2, IL-6, IL-6 sR, TNF-α) zetten bindweefselcellen (fibroblasten) in het omliggende, (stroma)weefsel aan om androgenen om te zetten in estrogenen, m.a.w. het enzym aromatase, dat hierbij een hoofdrol speelt, wordt verhoogd tot expressie gebracht in de fibroblasten. De geproduceerde en uitgescheiden estrogenen stimuleren vervolgens op een paracrine wijze de tumor cellen tot groei en het aan maken van meer factoren. Deze vorm van borstkanker komt minder voor in landen waar de consumptie van phyto-estrogenen hoog ligt. Hierdoor is er veel aandacht voor de relatie tussen voeding met phyto-estrogenen. Een bekend phyto-estrogeen is genisteïne dat voorkomt in soja en afgeleide producten. Zoals aangegeven kunnen phyto-estrogenen estrogeenreceptoren activeren en zijn het dus hormoon actieve stoffen. Het mechanisme waarmee phyto-estrogenen tegelijkertijd ook beschermend zouden kunnen zijn tegen borstkanker is nog onduidelijk. De leeftijd waarop (eerste) blootstelling plaatsvindt, kan een cruciale rol spelen in deze paradox.

Mengsels

Zoals aangeven is gecombineerde blootstelling relevant voor de humane situatie. Om hier een riscoschatting van te kunnen maken is het van belang te weten hoe deze stoffen interacteren. Er zijn drie mogelijke interacties gesuggereerd voor xeno-estrogenen, namelijk dat ze elkaar tegen werken (antagonisten), elkaar versterken (synergisten) of individueel werken (additief). In het laatste geval spreekt van nul-interactie.

Met behulp van een aantal modellen kan berekend worden welke van deze drie interacties de meest waarschijnlijke is. Een veel gebruikte benadering is die van de concentratie additie (CA). Het CA concept gaat ervan uit dat de een stof een dosis-respons relatie heeft die beschreven wordt met een logistische functie/sigmoid functie, wat in een grafiek een S-curve oplevert (zie figuur 1). Indien voor een mengsel en de individuele componenten de karakteristieken van deze curve bekend zijn kan een berekening uit gevoerd worden om de interactie tussen de componenten te bepalen.

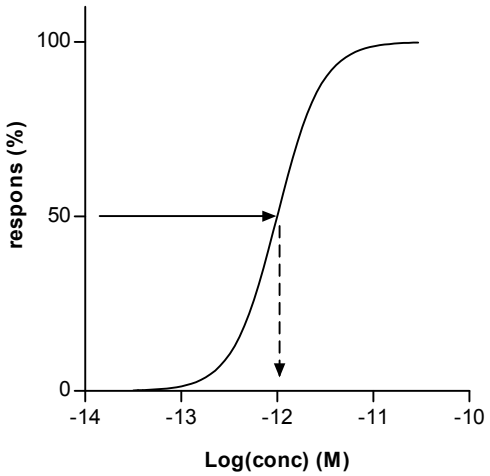

Fig 1. Concentratie-respons relatie: een hogere concentratie teststof (x-as) induceert een hogere respons (y-as) van een bepaald effect.

Twee typen berekening zijn gebruikt in dit proefschrift. Beiden maken gebruik van de concentratie waarbij een teststof 50% van de maximale respons van het systeem (EC_{50}) induceert, omdat hierrondom een klein concentratie verschil relatief het grootste verschil in respons geeft (fig 1). Het eerste type, de isobole-methode, bestaat uit het vaststellen van de concentratie om een bepaalde

respons te induceren (i) van iedere component in het mengsel en (ii) van iedere component apart. Daarna kan de optelsom van de i/ii bepaald worden. De concentratie van de componenten binnen een mengsel wordt dus vergeleken met de optelsom van de concentratie van iedere component apart. Het tweede type, de estrogeen equivalentie quefficiënt (EEQ)-methode, rekent de concentratie van een component in een mengsel en de potentie van dat component t.o.v. een referentie stof (estrogeen equivalentie factor, EEF) om naar een hoeveel van die referentie stof, b.v. E_2. De experimenteel waargenomen potentie van het mengsel wordt dan vergeleken met de voorspelde potentie op basis van de opgetelde EEQ's van de individuele componenten.

Methoden & resultaten

Xeno-estrogenen in een mengsel interacteren additief

In **hoofstuk 2 en 3** zijn interacties van xeno-estrogenen onderzocht in een *in vitro* systeem (hoofdstuk 2) en een *in vivo* systeem (hoofdstuk 3). De samenstelling van de geteste mengsels was zodanig dat zowel de gebruikte xeno-estrogenen als de verhouding daartussen reflecteert waaraan mensen worden blootgesteld. Mengsels bestonden uit phyto-estrogenen (coumesterol, genisteïne, naringenine, quercetine, catechine en epicathechine) of synthetische xeno-estrogenen (hexachlorocyclohexane, bishenol A, nonylphenol, octylphenol, methoxychloor en dibutylphthalate).

In dit proefschrift is als *in vitro* estrogeniteitstest de MCF-7 proliferatie assay, ofwel E-screen, ingezet. Resultaten verkregen m.b.v. deze assay laten zien dat de interactie tussen xeno-estrogenen onderling en de interactie met E_2 volgens het concentratie additie model additief zijn.

Als *in vivo* estrogeniteitstest is de uterotrophe assay, uitgevoerd in prepuberale ratten, ingezet. Resultaten verkregen m.b.v. deze assay bevestigen dit voor de interactie tussen een mengsel phyto-estrogenen en E_2.

De potenties van de voor mensen relevante mengsels suggereert dat de bijdrage van phyto-estrogenen aan de totale blootstelling aan estrogenen mogelijk groter is dan die van sythetische xeno-estrogenen.

Hoofstuk 4 laat zien dat additieven in cosmetica, zoals parabenen, phthalaten en synthetische polycyclische musken een positieve respons geven in de E-screen. Tevens is nul-interactie gevonden voor combinaties van de paraben ethylparabeen en propylparabeen en combinaties van parabenen en E_2.

Xeno-estrogenen remmen de steroidgenesis

In **hoofdstuk 4 en 5** is onderzocht of xeno-estrogenen de steroidgenesis kunnen remmen. Een belangrijk enzym in de steroïdgenesis is aromatase. Tevens is dat een van de enzymen die door xeno-estrogenen wordt aangegrepen. De geteste xeno-estrogenen (phyto-estrogenen, een aantal additven in cosmetica en een aantal synthetische lactonen) zijn relevant voor de blootstelling van mensen.

Om effect van een teststof op de activiteit van aromatase te bepalen werd de aromatase assay gebruikt. Hierin wordt de conversie gemeten van een aromatase substraat (androstenedion, dat in dit geval radioactief gelabeled was). De gebruikte aromatase was afkomstig van twee verschillende bronnen: (i) gewonnen uit microsomen van menselijk placentaweefsel en (ii) aromatase aanwezig in fibroblasten geïsoleerd uit gezond menselijk mammaweefsel.

De resultaten met deze aromatase assays laten een aromatase inhiberend effect zien van een aantal phyto-estrogenen en parabenen.

Hoofdstuk 5 en 6 bevestigen de aromatase inhiberende effecten van synthetische lactonen en phyto-estrogeen op aromatase in fibroblasten geïsoleerd uit gezond menselijk borstweefsel. In hoofdstuk 5 worden de potenties vergeleken van een synthetisch lacton (TM-7) en twee phyto-estrogenen (chrysine en 8-prenylnaringenine) om aromatase in de placenta en aromatase in

fibroblasten te inhiberen. Deze vergelijking impliceert dat studies die placenta microsomen gebruiken om aromatase inhibitie te onderzoeken van voorspellende waarde zijn voor potenties in complete cellulaire systemen.

Phyto-estrogenen zijn potentere estrogenen dan aromatase remmers

Zoals aangegeven worden mensen blootgesteld aan phyto-estrogenen en kunnen deze stoffen zowel estrogene effecten bewerkstellingen, als ook aromatase, en dus de steroidgenesis verstorende effecten. Dit zijn twee tegengestelde effecten. In **hoofdstuk 6** is onderzocht wat het uiteindelijke effect kan zijn als men aan een dergelijke stof wordt blootgesteld.

Als *in vitro* borstkankermodel is de E-screen gecombineerd met aromatase in fibroblasten, geïsoleerd uit gezond menselijk mammaweefsel. Dit is een zogenaamde co-cultuur assay. In plaats van estrogeniteit van stoffen in het groeimedium van de cellen te bepalen door cel proliferatie te meten (zoals in de E-screen) is dat bepaalt door van een estrogeen gevoelig gen (pS2) het expressie niveau te meten.

De resultaten verkregen m.b.v. deze co-culturen suggereren dat de geteste phyto-estrogenen bij lagere concentraties overwegend estrogeen zijn, terwijl hogere concentraties ook aromatase remmend zijn. De uiteindelijke resultante van humane blootstelling aan phyto-estrogeen, op basis van dit *in vitro* model, blijft speculatief. Echter, concentraties die relevant zijn voor interne blootstelling worden verwacht overwegend een estrogeen effect te induceren.

Xeno-estrogenen dragen minimaal bij aan totale estrogeen blootstelling

Hoofdstuk 4 probeert inzicht te geven in de estrogene bijdrage aan de totale estrogene belasting per groep xeno-estrogenen. Zoals aangetoond is in hoofstuk 2, 3,en 4 interacteren xeno-estrogenen additief in het concentratie additie model. Hieruit volgt dat EEQ's van verschillende stoffen optelbaar zijn.

Hiertoe zijn uit wetenschappelijk literatuur EEF's en concentraties in monsters afkomstig van mensen (of aannames daaromtrent) gebruikt van xeno-estrogenen. Vermenigvuldiging van deze twee waardes leveren EEQ's die vervolgens opgeteld zijn per groep xeno-estrogenen.

De berekende bijdrage (uitgedrukt in EEQ's) geven aan dat vergeleken met estrogenen geproduceerd in het lichaam zelf de bijdrage van een individuele xeno-estrogeen verwaarloosbaar is. Indien endogeen E_2 in borstweefsel niveau van 350 EEQ/g weefsel op 100% gesteld wordt, levert de groep UV-filters de grootste bijdrage van 1.633% (5.7 pg EEQ/g weefsel per dag), echter blootstelling afkomstig van deze bron wordt aangenomen voornamelijk plaats te vinden gedurende een korte periode in het jaar. De op een na grootste bijdrage komt van phyto-estrogenen met 0.64% (2.24 pg EEQ/g weefsel per dag), gevolgt door organochlorines met 0.315% (1.1 pg EEQ/g weefsel per dag en alkylphenolen en bisphenol A met 0.103% (0.36 pg EEQ/g weefsel per dag). Hierbij dient opgemerkt te worden dan de opgestelde lijst met stoffen die (potententieel) estrogeen zijn en bijbehorende concentraties niet compleet is. Voor mannen, met 50 pg E_2/g weefsel, verzevenvoudigd de relatieve bijdrage. Voor kinderen, met < 10 pg E_2/ml serum, zijn de relatieve bijdragen 35 keer hoger.

Discussie

Bij ieder onderzoek zijn kanttekeningen te plaatsen. De volgende zijn van toepassing op de studies verricht voor en beschreven in dit proefschrift.

Een directe vertaling van *in vitro* en *in vivo* resultaten naar de humane situatie is beperkt mogelijk. Dit komt doordat de gebruikte modellen om estrogeniteit, aromatase remming of een resultante daarvan te bepalen een sterke vereenvoudiging van de werkelijkheid zijn. Enkele relevante vereenvoudigingen worden hier kort vermeldt. Ten eerste is er geen rekening gehouden met sommige physisch-chemische eigenschappen van teststoffen, die wel bepalend kunnen zijn voor de concentratie

op de interactie-locatie, b.v de estrogeenreceptor. Enkele van die eigeschappen zijn wateroplosbaarheid, accumulatie en afbreekbaarheid van de stof in de gebruikte modellen. Ten tweede, richtten assays zich vaak op een bepaald aspect. Zo bepaalt de estrogeniteitstestest interactie met ERα en laten interactie met de ERβ buiten beschouwing. Het is bekend dat een aantal xeno-estrogenen juist een hogere affiniteit heeft voor de ERβ. De aromatase assay met microsomen is in staat remming van het enzym te bepalen en laat synthese van nieuw enzym buitenbeschouwing, terwijl die wel plaats kan vinden in organismen. Ten derde, specifiek betrekking hebbende op de mengselstudies, zijn de samengestelde mengsels gesimplificeerde mengsels vergeleken met extracties gemaakt van monsters afkomstig van mensen, dieren of uit de omgeving. Daarbijkomt dat risico('s) door blootstelling aan xeno-estrogenen in het algemeen lastig te beoordelen zijn. Al dan niet schadelijk effecten manifesteren zich mogelijkerwijs pas na een leven lang bij lagere blootstellingsniveaus, of in de volgende generatie. Desalniettemin heeft dit proefschrift een bijdrage geleverd om risico analyses van (mengsels) xeno-estrogenen uit te voeren.

Samenvattend, leidt dit proefschrift tot de conclusie dat xeno-estrogenen concentratie additief werken en dus dat belasting aan estrogeniteit door blootstelling aan xeno-estrogeen optelbaar is. Berekening daarvan geeft aan dat normale blootstelling van mensen aan xeno-estrogenen afkomstig van verschillende bronnen hoogstwaarschijnlijk niet schadelijk is voor de humane gezondheid. De grootste bron xeno-estrogenen vormen de UV-filters, maar omdat deze in het algemeen tijdelijk relevant is, zijn phyto-estrogenen de belangrijkste dagelijkse bron aan xeno-estrogenen. Berekeningen geven aan dat xeno-estrogenen in het algemeen slechts marginaal t.o.v. de belasting aan estrogeniteit geproduceerd in het lichaam of door contraceptieven. Daarnaast kunnen enkele xeno-estrogenen de steroïdgenesis verstoren door aromatase te remmen. Echter, voordat deze effecten bij mensen optreden, is vermoedelijk een hogere dan de huidige blootstelling nodig.

Bepaalde ontwikkelingen in de samenleving die ertoe lijden dat blootstelling aan phyto-estrogenen toeneemt, kunnen redenen voor voorzichtigheid zijn. Er is bijvoorbeeld een groeiende markt voor voedingssupplementen die hoge doseringen phytochemicaliën, inclusief phyto-estrogenen, bevatten in een niet natuurlijke matrix. De effecten van zulke voedingssupplementen zijn echter grotendeels onbekend. Een aantal groepen mensen zijn extra gevoelig voor blootstelling aan (hoge) niveaus xeno-estrogenen, zoals ongeborenen en pasgeborenen of postmenopausale vrouwen. Omdat estrogeen gevoelige borstkanker vooral bij postmenopausale vrouwen optreedt, zijn ook zij wellicht gevoeliger voor blootstellig aan hoge niveaus xeno-estrogenen. Er is tot op heden geen adequate regulering van dergelijke producten, mede omdat er onvoldoende wetenschappelijke informatie is om een adequate risico analyse uit te voeren.

Curriculum Vitae

Jeroen Alexander van Meeuwen was born on 23 August 1975 in Leidschendam, The Netherlands. He was educated at the Adelbert College in Wassenaar and graduated in 2000 for his Master of Science degree in Biology from Leiden University. During his study he did an internship at the Institute of Molecular Plant Sciences, Clusius Laboratorium, University Leiden under the supervision of Drs. I. Kuiper and at the Division of Medical Pharmacology, Sylvius Laboratory, University Leiden under the supervision of Dr. E. Vreugdenhil. In 2002 he received his degree for biology teacher from the Leiden University. He started in 2002 his Ph.D. project at the Cellular and Molecular Toxicology department, division Toxicology of the Institute for Risk Assessment Sciences, Utrecht University under the supervision of Prof. dr. Martin van den Berg, Prof. dr. Aldert Piersma and partly Dr. Thomas Sanderson. In this project he studied mixtures of xenoestrogens and possible beneficial and adverse effects on the Dutch population, which has resulted in this thesis.

Jeroen Alexander van Meeuwen werd geboren op 23 augustus 1975 te Leidschendam. Op het Adeltbert College te Wassnaar behaalde hij het VWO diploma en in 2000 rondde hij de studie Biologie af aan de Universiteit Leiden. Tijdens de studie vervulde hij een stageplaats bij het Instituut Moleculaire Plantkunde, Clusius laboratorium, Universiteit Leiden onder de supervisie van Drs. I. Kuiper en op de Divisie van Medische Pharmacologie, Sylvius laboratorium, Universiteit Leiden onder de supervisie van Dr. E. Vreugdenhil. In 2002 behaalde hij de eerste graad docent biologie aan de Universiteit Leiden. Daarna begon hij in 2002 aan het AIO-project bij de Cellulaire en Moleculaire Toxicologie afdeling, toxicologie divisie van het Institute for Risk Assessment Sciences, Universiteit Utrecht onder de supervisie van Prof. dr. Martin van den Berg, Prof. dr. Aldert Piersma en gedeeltelijk Dr. Thomas Sanderson. In dit project onderzocht hij mengsels van xenoestrogenen en mogelijke voordelige en nadelige effecten daarvan op de Nederlandse populatie, wat resulteerde in dit proefschrift.

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