Estrogen metabolizing enzymes

biomarkers of exposure, effect and susceptibility for carcinogenesis

Enzymen voor oestrogeenafbraak als biomarkers van blootstelling, effect en gevoeligheid voor borstkanker

met een samenvatting in het Nederlands

Proefschrift

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Majorie Beatrix Maria van Duursen

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Promotor: prof. M. van den Berg, Ph.D., Institute for Risk Assessment Sciences, Utrecht, The Netherlands

Co-promotor: J. T. Sanderson, Ph.D., Institute for Risk Assessment Sciences, Utrecht, The Netherlands

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The fact of evolution is the backbone of biology,
and biology is thus in the peculiar position of being
a science founded on an improved theory,
is it then a science or faith?

Charles Darwin (1809 – 1882)

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List of Abbreviations

17β-HSD	17β-hydroxysteroid dehydrogenase
2-MeOE2	2-methoxyestradiol
2-OHE2	2-hydroxyestradiol
3'-UTR	3' untranslated region
4-MeOE2	4-methoxyestradiol
4-OHE2	4-hydroxyestradiol
8-oxo-dG	8-oxo-2'-deoxyguanosine
Ade	adenine
AhR	aryl hydrocarbon receptor
Ala	alanine
Arg	arginine
Arnt	AhR nuclear translocator
Asn	asparagine
BMI	body mass index
BRCA	breast cancer gene
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CAT	catechin
CE	catechol estrogen
CHR	chrysin
COMT	catechol-O-methyltransferase
CYP1A1	cytochrome P450 1A1
CYP1A2	cytochrome P450 1A2
CYP1B1	cytochrome P450 1B1
DCM	dichloromethane
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DRE	dioxin responsive element
DTT	dithiothreitol
E1	estrone
E2	17β-estradiol
EDTA	ethylenediaminetetraacetic acid
EPI	(-)-epicatechin
ER	estrogen receptor
EROD	ethoxyresorufin-O-deethylase
EtOH	ethanol
FBS	fetal bovine serum
FLA	flavone
GEN	genistein
Gly	glycine
GSH	gluthatione
GST	gluthatione-S-transferase
Gua	guanine
HBEC	human breast epithelial cell
HS	horse serum
Ile	isoleucine
Leu	leucine
LMA	low melting agarose
MCF-10A	human breast epithelial cell line of nonmalignant origin (fibroadenoma)
MCF-7	neoplastic breast epithelial cell line derived from breast adenocarcinoma
MeOH	methanol
Met	methionine
mRNA	messenger ribonucleic acid

MTT	3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMA	normal melting agarose
OR	odds ratio
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-p-dioxin
PCR	polymerase chain reaction
PDCF	polychlorinated dibenzofuran
PGE2	prostaglandin E2
QUE	quercetin
Rb	retinoblastoma repressor protein
RFLP	restriction fragment length polymorphism
Ro 41-0960	2'-fluoro-3,4-dihydroxy-5-nitrobenzophenone
ROS	reactive oxygen species
RP	relative potency
RSF	resorufin
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
SD	standard deviation
SEM	standard error of the mean
Ser	serine
SULT	sulfotransferase
TCDD	2,3,7,8-tetrachloro-p-dibenzodioxin
TEF	toxic equivalent factor
TEQ	toxic equivalent
Thr	threonine
TMCS	trimethylchlorosilane
TNFα	tumor necrosis factor α
UGT	UDG-glucuronosyltransferase
UTR	untranslated region
UV	ultra violet
Val	valine

1

General Introduction

1 Estrogens

In 1929, two Americans Edgar Allen and Edward A. Doisy announced the isolation and characterization of the lipid soluble hydroxyketone and triol female sex hormones from hundreds of gallons of urine obtained from pregnant women. The compounds were named theelin[1] and theelol[2], after the Greek word "theelus", which means "female". Later, these compounds would be renamed to estrone and estriol. It was not until 1935 that the water-soluble estrogen, 17β-estradiol (dihydrotheelin) was isolated from pig follicular fluid. These findings opened the way for endocrine research.

Estrogens are steroid hormones that are usually called the female sex hormones, but they are also crucial for male fertility [3]. In females, estrogens play an important role in the maintenance of the female reproductive system (monthly preparation of the body for a possible pregnancy, participation in pregnancy) and the development of female secondary sex characteristics (development of breasts, further development of uterus and vagina, broadening of the pelvis, growth of pubic and axillary hair, increase in adipose tissue) [4]. They also play a role in non-reproductive events, such as minimizing the loss of calcium from the bones and promoting blood clotting. The body's main estrogen in non-pregnant, premenopausal women is 17β-estradiol.

1.1 Estrogen synthesis

Estrogens are produced via two main pathways. First, through *de novo* synthesis from cholesterol. This synthesis includes several rate-limiting steps, which makes fine-regulation of estrogen levels possible. The final step in estrogen synthesis is the aromatization of androgens to estrogens by cytochrome P450 19 (CYP19, aromatase) [5,6]. The second source of estrogens is through desulfatation of estrogen sulfates [7,8]. The most abundant circulating estrogen is estrone sulfate, which is locally taken up by target cells, enzymatically hydrolyzed to estrone by membrane-bound steroid sulfatase and then hydroxylated to 17β-estradiol by 17β-hydroxysteroid dehydrogenases [9].

In premenopausal non-pregnant women, estrogens are predominantly synthesized in the ovaries and, to a lesser extent, in peripheral tissues such as adipose tissues and the adrenal glands. In postmenopausal women, the source of estrogen production shifts toward the peripheral tissues, which is reflected in the plasma and tissue estrogen levels. In premenopausal women, the ratio of estrogens in tissue and plasma is 1:1 and in postmenopausal women this ratio is 10-50:1 [10]. In addition, the plasma levels of estrogen precursors (androgens such as testosterone) are higher than the level of circulating

estradiol in postmenopausal women (even higher than in men) [11]. Further, estrone sulfate concentrations are higher in blood and breast tissue of postmenopausal women than estrone or estradiol concentrations [12]. This indicates that estrogens can be produced in the target tissues and in this way, high concentrations can be reached locally.

1.2 Estrogen metabolism

1.2.1 Phase I metabolism

Oxidative metabolism of estrogens can occur at the A-ring or at the D-ring (figure 1.1). D-ring metabolism is mainly catalyzed by the CYP3A4/5 isoenzymes and results in the formation of 16α-hydroxyestrogens. A-ring metabolism of estrogens is the most important metabolic route and yields the catechol estrogens 2-hydroxy- and 4-hydroxyestrogen. In the liver, mainly 2-hydroxylation of estrogens occurs, which is catalyzed by cytochrome P450 1A2 [13]. A-ring metabolism in extrahepatic tissues, is predominantly cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1)-mediated [14-16]. CYP1A1 mainly converts estrogens to the 2-hydroxyestrogens but, due to a lack of specificity of the enzyme, about 15-20% to the 4-hydroxyestrogens [17]. CYP1B1 on the other hand, is a catalytically highly efficient and specific estrogen 4-hydroxylase, which is predominantly responsible for the extrahepatic 4-hydroxylation of estrogens [18].

CYP1A1 and CYP1B1 are co-expressed in many human tissues. Though constitutive extrahepatic CYP1A1 expression is generally extremely low, CYP1A1 can be found in tissues such as prostate, mammary gland, intestine, thymus, colon, adrenal, ovary, uterus, lung, and testis [19]. CYP1B1 on the other hand, is mainly expressed in extrahepatic tissues, and can be found ubiquitously with the highest constitutive levels of mRNA detected in tissues such as kidney, mammary gland and prostate [20], but also in brain, heart, placenta, lung, skeletal muscle, spleen, thymus, testis, ovary, small intestine, colon, peripheral blood leukocytes, adrenal, pituitary and in the uterus [17]. In addition, the CYP1B1 protein appears to be over-expressed in a variety of malignant tissues, including breast tumor tissue [21-23].

1.2.2 Phase II metabolism

1.2.2.1 Catechol-O-methyltransferase (COMT)

A second step in estrogen metabolism involves the detoxification of the catechol estrogens. A major route is the methylation of catechol estrogens catalyzed by catechol-*O*-methyltransferase (COMT) [24-27]. COMT is involved in the inactivation of endogenous catecholamines and catechol estrogens, by transferring a methyl group from S-adenosyl-

L-methionine (SAM) to the substrate and thus converting them into their methoxy derivatives (reviewed by Männistö et al.[27]).

The *COMT* gene codes for two isoforms of the COMT protein; the soluble form (S-COMT) and the membrane-bound form (MB-COMT), which can both be found ubiquitously in human tissues [28,29]. Mostly, S-COMT expression is higher in human tissues, except for in the brain, where MB-COMT is predominant [27]. In malignant breast tissue, COMT expression was shown to be higher than in normal breast tissue [30]. Weisz et al. identified COMT expression in the nuclei of both normal and malignant breast tissues of breast cancer patients and in healthy control breast tissues, suggesting the protective role of COMT against increased catechol levels [31].

1.2.2.2 Other enzymes involved in estrogen metabolism

Besides O-methylation, other routes of catechol estrogen inactivation are via sulfate or glucuronide conjugation, although little is known about these routes for catechol estrogens [9]. Only a few *in vitro* studies report the sulfate conjugations by human sulfotransferases (SULTs) [32,33] and the glucuronidation by UDP-glucuronosyltransferases (UGTs) [34,35] of catechol estrogens. In contrast with the methyl conjugates [36,37] and the sulfate conjugates [38] of catechol estrogens, there are no reports regarding the excretion of the glucuronide conjugates of catechol estrogens in human urine.

When not inactivated, catechol estrogens can undergo redox cycling and be converted to reactive quinones (see 2.1). These catechol estrogen quinones can be inactivated by conjugation with gluthatione (GSH) either non-enzymatically or catalyzed by a member of the gluthatione-S-transferase (GST) family. GSH-conjugation has been shown to occur both *in vitro* and *in vivo* [9].

2 Estrogens and breast cancer

During the last decades an increase has been observed in the occurrence of malignant neoplasms of the female breast in industrialized countries and presently, it is one of the major causes of death among these women. About 1 in 8 women in the industrialized countries will develop breast cancer sometime during her life. In the Netherlands, this results in about 10.000 cases a year of which approximately 3500 will die [39,40].

There are several known genetic factors that increase the risk of developing breast cancer, such as several breast cancer genes (BRCAs). However, only 5-10% of the breast cancer cases can be attributed to high risk genes such as BRCA [41,42]. In over 65% of the cases, women are diagnosed with sporadic breast cancer. The etiology of these cancers is

not yet completely understood, but several known factors that contribute to the development of breast tumors have been identified. These factors include the involvement of genetic polymorphisms (low penetrance genes; these will be described more extensively in paragraph 3.3), which might identify certain high risk populations, but also age, reproductive and menstrual history, use of hormones (hormone replacement therapy, oral contraceptives), radiation exposure, mammographic breast density, lifestyle factors, and history of breast disease. All these factors have in common that they influence the cumulative life-time exposure of a woman to estrogens.

Estrogens act locally in the mammary gland and it has been known for over a 100 years that exposure to high levels of estrogens contribute to the development of breast cancer. As early as in 1896, the Scottish surgeon George Beatson (1848-1933), reported that removing the ovaries of pre-menopausal women with breast cancer, resulted in a decrease of the breast tumor size [43]. However, it took several more years before more was known about the mechanism behind this finding. Generally, nowadays there are two suggested mechanisms that might explain the key role of estrogens in breast tumor development [44,45]. First, estrogens may act as a cancer promoter by activation of estrogen receptor (ER)-mediated processes. ER-binding of estrogens can result in the stimulation of oncogenes, causing cell transformation and proliferation. Enhanced proliferation, and thus DNA replication, increases the chances of errors in DNA replication and repair and thus the frequency of mutations. When they occur in critical genes, accumulation of these mutations can result in neoplastic growth. Secondly, estrogens can act as cancer initiators through their genotoxic metabolites, which can induce damage to cellular macromolecules such as DNA [46,47]. In this thesis, I will focus on this latter mechanism.

2.1 Estrogen metabolism and its involvement in breast cancer development

Evidence for the genotoxic role of catechol estrogens originate from studies performed with the male Syrian golden hamster kidney model. Male Syrian golden hamsters are susceptible to induction of renal carcinomas by estrogens. Several studies have shown in this model that estradiol and 4-hydroxyestradiol, but not 2-hydroxyestradiol, are carcinogenic [47-50].

An early study using *ex vivo* human breast tissue homogenates, described more 4-hydroxyestradiol formation than 2-hydroxyestradiol formation in breast tumor tissue [51]. Another study showed increased estrogen 2-hydroxylation in malignant breast tumors compared with normal breast tissue, but they did not look at the estrogen 4-hydroxylation [52]. The first studies that compared both estrogen 2- and 4-hydroxylation in

healthy and malignant breast tissues date from about 10 years later. Liehr and Ricci described that levels of 4-hydroxyestrogens were almost 4 times higher in microsomes from breast carcinomas than from adjacent healthy breast tissues after incubation with estradiol [53]. Two other studies report the tissue levels of 4-hydroxyestrogens to be 3 times higher than 2-hydroxyestrogen levels in breast tissues from women with breast cancer compared with women without breast cancer [54,55]. Although a causal association between catechol estrogens and breast carcinogenesis cannot be established from these *ex vivo* studies with human tissues, studies that describe impaired inactivation of the catechol estrogens support this hypothesis. The occurrence of estrogen-induced kidney tumors in male Syrian hamsters was increased when COMT activity was inhibited [56]. Further, the rates of O-methylation of catechol estrogens was lower in breast cancer tissues compared with normal breast tissue [54] and some studies describe an association between women with a low activity form of COMT (described in more detail in *3.3.3*) and a higher breast cancer incidence [57,58]

The carcinogenic potential of catechol estrogens is thought to be mediated by the quinone forms of the catechol estrogens. Unless methylated, catechol estrogens may undergo oxidation to reactive quinones which can covalently bind to the nucleophilic groups of purine bases (guanine or adenine) in the DNA [59,60]. The adducts of estrogen-3,4-quinones, formed by oxidation of 4-hydroxyestrogens, are unstable and can be spontaneously released from the DNA thus forming an apurinic site. Apurinic sites may generate mutations that can play a critical role in the development of cancer. In contrast, the DNA adducts of estrogen-2,3-quinones, formed by oxidation of 2-hydroxyestrogens, are chemically stable and remain bound to the DNA, unless they are removed during DNA repair [44,46,59,61,62]. The carcinogenic role of catechol estrogen-derived quinones was recently emphasized by the higher levels of conjugates of these quinones [54] and catechol estrogen quinone-derived DNA adducts [63] in breast tissue extracts from women with breast cancer compared with breast tissue extracts from women without cancer.

In addition to the catechol estrogen quinone-derived DNA adducts, the catechol estrogen-derived quinones can also cause oxidative DNA damage. During redox-cycling between the semiquinone and quinone-forms of the catechol estrogens, reactive oxygen species (ROS) are produced, which can cause oxidative damage to DNA and (sub)cellular membranes (lipid peroxidation). The production of ROS is supported by the *in vitro* studies showing free radical production during estrogen-3,4-quinone redox cycling in breast cancer cells [64] and the findings that in breast cancer tissue 4-hydroxyestrogens, and to a lesser extent 2-hydroxyestrogens, estrone and estradiol, increased the 8-oxo-2'-

deoxyguanosine (8-oxo-dG, an oxidized DNA base and marker of hydroxyl radical action) contents significantly compared with controls [65,66]. Furthermore, increased levels of 8-oxo-dG have been detected in human breast cancer tissues compared with healthy breast tissues [67,68].

3 Altered estrogen metabolism

Mounting evidence suggests that estrogen metabolites play a role in breast carcinogenesis. This implies that altered estrogen metabolism is associated with increased breast cancer risk. Estrogen metabolism can be affected by exposure to exogenous compounds. Exogenous compounds can up- or downregulate enzyme levels through receptor-mediated pathways (paragraph 3.1) or can compete for the same enzyme as the natural substrate and thus inhibit enzyme activity (paragraph 3.2). In addition, genetic polymorphisms in the estrogen metabolizing enzymes may influence either the catalytic activity or expression level of an enzyme thereby influencing the catechol estrogen levels (paragraph 3.3).

3.1 Aryl hydrocarbon receptor (AhR) pathway

The aryl hydrocarbon receptor (AhR) is found ubiquitously in vertebrate tissues. However, the physiological role of the AhR remains to be elucidated and so far no endogenous ligands have been identified that bind with high affinity to the receptor [69]. Still, a broad range of chemical structures, both synthetic and naturally occurring, have been identified that bind to the AhR and either up- or downregulate the AhR-mediated pathway. Synthetic ligands include several environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). In addition, a large group of naturally occurring ligands have been found that occur in our daily diet and comprise vegetable-derived chemicals such as indole-3-carbinol (I3C) and its main metabolites, indolo-(3,2,-b)-carbazole (ICZ) and 3,3'-diindolylmethane (DIM), and also certain plant-derived flavonoids.

Activation of the AhR-mediated pathway, results in a diverse spectrum of biological and toxic responses, which are species and tissue specific [70,71]. The most consistent response to AhR agonism is the upregulation of CYP1A1, which is commonly used as an indication that the AhR-mediated mechanism of toxicity has been activated [72]. Upon binding of a ligand to the cytosolic AhR, the complex is translocated into the

nucleus where it associates with a protein called Arnt (AhR nuclear translocator) before the complex binds with high affinity to a dioxin responsive element (DRE) in the DNA [69,73]. The DREs are located upstream in the promoter regions of AhR-responsive genes, such as *CYP1A1* and *CYP1B1*. Consequently, binding of the ligand-AhR-Arnt complex leads to stimulation or inhibition of gene transcription.

CYP1A1 and CYP1B1 are members of the cytochrome P450 superfamily of enzymes that catalyze the oxidation and sometimes reduction of a large number of xenobiotic chemicals such as drugs, toxic chemicals, carcinogens and endogenous compounds such as steroids, fatty acids, vitamins, and prostaglandins. Metabolism of these compounds generally results in detoxification by forming polar metabolites that are easily excreted from the body. However, in some cases metabolism results in the bioactivation of the parent compound. This is the case for PAHs, but appears also to be the case for estrogens by metabolic activation to reactive catechol estrogens (see paragraph 2.1).

AhR agonists have also shown to inhibit estrogen-induced responses *in vivo* and *in vitro*. TCDD and structurally-related compounds decreased the incidence of spontaneous hormone-dependent tumors in Sprague-Dawley rats, inhibited estradiol-induced responses on the immature, ovariectomized rodent uterus and in ER-positive breast cancer cell lines (reviewed by Safe et al. [74]). The extent of these antiestrogenic effects were dependent on the affinity of the compounds for the AhR, suggesting an AhR-mediated effect on the ER pathway. Several mechanisms have been suggested, which might all to some extent contribute to the inhibitory AhR-ER crosstalk. These mechanisms involve increased estrogen metabolism, a direct interaction between the AhR and promoter regions of estrogen-responsive genes, competition for nuclear coregulatory proteins and degradation of the ER by proteasomes [74,75].

3.2 Enzyme inhibition

Enzyme inhibition can occur in several ways. Extreme pH or temperature can irreversibly disrupt the protein structure and thus enzyme function. Also, compounds which can covalently bind to side chains critical for enzyme activity can irreversibly inhibit the enzyme function. In addition, non-covalent binding of compounds that can either reversibly change the conformation of the enzyme or compete for the substrate binding site can inhibit enzyme activity. Non-covalent enzyme inhibition can either be competitive, non-competitive or uncompetitive. Competitive inhibition occurs when the substrate and the compound compete for binding to the same active site of an enzyme.

Then, occupation of the active enzyme can occur, thereby lowering the activity of an enzyme for its natural substrate. With non-competitive binding, the inhibitor binds to a site other than the active site, thereby causing a change in the structure of the enzyme and reducing its catalytic efficiency toward the substrate. With uncompetitive inhibition, the compound also binds elsewhere on the enzyme molecule, but only when the substrate is bound.

3.3 Genetic polymorphisms in estrogen metabolizing enzymes

Polymorphisms in the estrogen metabolizing enzymes may define subpopulations of women that are more susceptible to breast cancer development. A polymorphism is a common mutation in the gene that is present in at least 2% of the population. The contribution of one single gene containing a polymorphism might not increase the relative risk for breast cancer, but a combination of polymorphisms might be associated with a small to moderate increased risk for breast cancer. Therefore, these genes are also called low penetrance genes [76,77]. Effects of the polymorphisms are difficult to investigate since a large study population is needed to filter out subtle effects on, for instance, breast cancer risk. In addition, comparison of the various studies are further complicated because polymorphisms are mostly ethnically related [77].

There are several studies and review articles that describe the polymorphisms in *CYP1A1*, *CYP1B1* and *COMT* and their possible relation to increased breast cancer risk. Here, a short overview is given.

Table 1.1. Genetic polymorphisms in CYP1A1, CYP1B1 and COMT genes[78]

Enzyme	Alternate name	Nucleotide change	codon	Remarks
CYP1A1	*2A	3801T → C	3'-UTR	
	*2C	2455A → G	462Ile → Val	In haem-binding domain, exon 7
	*3	3205T → C	3'-UTR	African-American only
	*4	2453C → A	461Thr → Asp	Exon 7
CYP1B1		142C → G	48Arg → Gly	Exon 2
		355G → T	119Ala → Ser	Exon 2
		1294G → C	432Val → Leu	In haem-binding domain, exon 3
		1358A → G	453Asn → Ser	In haem-binding domain, exon 3
COMT	L	1947G → A	108/158Val → Met	

3.3.1 CYP1A1

CYP1A1 polymorphisms are not very common in Caucasians in contrast to Asian and African-American populations. The most frequently studied polymorphisms are described in Table 1. The *CYP1A1*3* allele is a rare African-American specific polymorphism. The *CYP1A1*2A* and **2C* polymorphisms usually occur together in Caucasians [79].

Although extensively described in case-control studies, little is known about the functional effects of the *CYP1A1* polymorphisms. Some describe an association with higher enzyme activity and induciblity of mRNA expression levels for the *CYP1A1*2A* or *CYP1A1*2C* genotypes [80,81], while others describe no such effect [82,83].

Most studies describe *CYP1A1* polymorphisms in relation to lung cancer, because CYP1A1 is involved in the activation of PAHs, which are present in e.g. cigarette smoke. Several studies have investigated the association between *CYP1A1* polymorphisms and breast cancer risk. The *CYP1A1*2A* polymorphism was associated with increased breast cancer risk in African-American [84] and Asian women [85], but not in Caucasians [86]. The risk associated with the *CYP1A1*2A* allele appears to be slightly increased in smoking women in the Nurses' Health Study (ethnicity unknown) [87,88]. For the *CYP1A1*2C* polymorphism the reports are also inconclusive; one study reports a decrease in breast cancer risk in Japanese women associated with the *CYP1A1*2C* [89]. Another study described an association between the *CYP1A1*2C* allele and increased breast cancer risk in Caucasian women with above median PCB serum levels [90]. Two studies report a slight increased breast cancer risk associated with smoking and the *CYP1A1*2C* allele [91,92]. No association with breast cancer risk was found for *CYP1A1*4* [88,93]. A large case-control study described no association between smoking, either of the *CYP1A1*2C* or *CYP1A1*4* polymorphisms and increased breast cancer risk [94]. A slight increase of breast cancer risk was observed in smoking African-American women who had at least one *CYP1A1*3* allele [88].

3.3.2 CYP1B1

There are several polymorphisms in the *CYP1B1* gene that result in an amino acid transition, but the most frequently studied polymorphisms in relation to breast cancer risk are those in codon 432 and 453 (Table 1). Both these polymorphisms occur in the haem-binding domain of the CYP1B1 enzyme. The valine (Val) to leucine (Leu) amino acid substitution in codon 432 and an asparagine (Asn) to serine (Ser) substitution in codon 453, alone or in combination, of the *CYP1B1* gene are correlated with a lower catalytic activity of the enzyme [95-97]. In addition, some found an association with the

Leu/Leu genotype and higher CYP1B1 mRNA expression levels [98], while others described no effect of *CYP1B1* Val432Leu polymorphism on expression levels [96].

So far, no studies have reported a significant relationship between *CYP1B1* Asn453Ser and breast cancer risk [99,100]. Several studies have investigated the association with the *CYP1B1* Val432Leu polymorphism. No association was found in Caucasian [100], Korean [101] or Japanese women [102] and in the Nurses' Health Study (ethnicity unknown) [99]. One study found that the Leu/Leu genotype was associated with an increased breast cancer risk in postmenopausal Chinese women [103] and one study reported a correlation between the presence of the *432Val* allele and increased breast cancer risk in obese, Turkish women [104]. Another study reported the association between the *432Val* allele and breast cancer risk in woman who lived next to a waste incinerator for more than 10 years [105]. In addition, the Val/Val genotype has been associated with estrogen- and progesterone receptor-positive breast cancers in Caucasian women [99,100].

3.3.3 COMT

A base pair change (G to A) in the *COMT* gene leads to a Val to methionine (Met) amino acid change in codon 108 (soluble-form) or codon 158 (membrane-bound form) of the enzyme. This change has been associated with a 3- to 4-fold decrease in enzyme activity [106]. Therefore, the alleles are also called the low activity allele, COMT-L (*108/158Met* allele) and the high activity allele COMT-H (*108/158Val* allele).

Thompson et al. found an association between COMT-L and increased breast cancer risk in premenopausal Caucasian women and a decreased risk in postmenopausal women [107]. This association was more pronounced in both groups when the body mass index (BMI) was taken into account. In contrast, Mitrunen et al. found a decreased breast cancer in premenopausal Finnish women associated with COMT-L and an increased risk in postmenopausal women, but only when hormone replacement therapy, BMI and early age of menarche were taken into account [108]. Lavigne et al. also described that an increased breast cancer risk in postmenopausal women was correlated with the COMT-L allele in combination with a high BMI (> 24.5 kg/m^2) [57]. These studies all suggest a strong modifying effect of BMI on breast cancer risk associated with the COMT-L allele. Further, an increased breast cancer risk was shown in postmenopausal Taiwanese [109] and pre- and postmenopausal Korean [58] women with the COMT-L allele. Several other studies found no association between the COMT genotype and breast cancer risk in a study among African-American and white women [110], nor among Swedish [111], Japanese [112]

and Turkish women [104]. One study reported an association of the COMT-L allele and the clinical stage and the extent of metastasis of breast cancer in Japanese women [113].

4 In this thesis

In the etiology of breast cancer, estrogens and its metabolites play a key role as tumor initiators and promoters. Co-expression of estrogen synthesizing enzymes (aromatase and steroid sulfatase [114,115]) and estrogen metabolizing enzymes (CYP1A1 and CYP1B1 [116,117]) in breast tissue makes it plausible that locally formed estrogens and estrogen metabolites may reach concentrations sufficiently high to evoke tumorigenic effects. Exposure to exogenous compounds can alter estrogen metabolism by affecting enzyme levels or catalytic activity. In addition, several functional polymorphisms have been described for the estrogen metabolizing enzymes that can result in different catechol estrogen levels.

The aim of this thesis was to study altered estrogen metabolism and the implications for breast carcinogenesis. Phase I metabolism of estrogens is described in chapter two. We describe the influence of several dioxin-like compounds on estrogen 2-hydroxylase (CYP1A1) and estrogen 4-hydroxylase (CYP1B1) activity. Special emphasis is placed on the effects on the estrogen 4-/2-hydroxylation ratio, which is suggested to provide a marker for neoplastic breast tissue [53]. A tumorigenic (MCF-7) and a non-tumorigenic (MCF-10A) human mammary epithelial cell line were used in this study and the usefulness of the estrogen 4-/2-hydroxylation ratio as biomarker for elevated breast cancer risk is discussed. Chapter three and four focus on the phase II metabolism of estrogens by COMT. In chapter three, we investigated the effects of several naturally occuring compounds (phytochemicals) on COMT activity in cytosol from healthy mammary tissues obtained from reduction mammoplasty. Some phytochemicals have a catechol structure, which makes them potential substrates for the COMT enzyme. As a result, competition between catechol estrogens and phytochemicals with a catechol structure, and decreased inactivation of catechol estrogens can occur. Implications of decreased COMT activity caused by phytochemicals for catechol estrogen-induced DNA damage were studied in the MCF-7 cell line. Chapter four describes the effects of impaired COMT activity on cell growth, catechol estrogen-induced DNA damage and cell cycle status in the MCF-7 and MCF-10A cell lines. COMT appears to play a crucial role in determining the genotoxic potential of the catechol estrogens. We showed that

this effect is largely attributed to 2-MeOE2, which is known to exert a variety of anti-tumor actions.

Another aim of this thesis was to investigate the use of CYP1A1 and CYP1B1 in human peripheral blood lymphocytes as biomarkers of exposure to dioxins and dioxin-like compounds. This is described in the last two chapters. In chapter five, we studied the interindividual differences in constitutive and induced catalytic activity and gene expression of CYP1A1 and CYP1B1 in human lymphocytes of ten healthy female volunteers. Lymphocytes were cultured and exposed to TCDD or the less potent dioxin-like PCB126. In addition, the possible influence of the CYP1A1*2A and CYP1B1 Val432Leu polymorphisms was studied. Furthermore, the suitability of CYP1A1 and CYP1B1 expression levels in human lymphocytes as biomarkers of exposure to environmental factors was addressed by comparing the effects we found *in vitro*, with the concentrations of dioxins and dioxin-like compounds that can be found in human blood. In chapter six we studied the use of CYP1B1 as biomarker of exposure in a human population as part of a European project (PCBRISK). This study reports the effect of the CYP1B1 Val432Leu polymorphism on the correlation between CYP1B1 gene expression level in peripheral lymphocytes and exposure to environmental factors within a human population exposed to environmental pollution as a consequence of the 25-year long production of PCBs in eastern Slovakia.

A summary and general discussion of the results and implications of these studies is given in chapter seven.

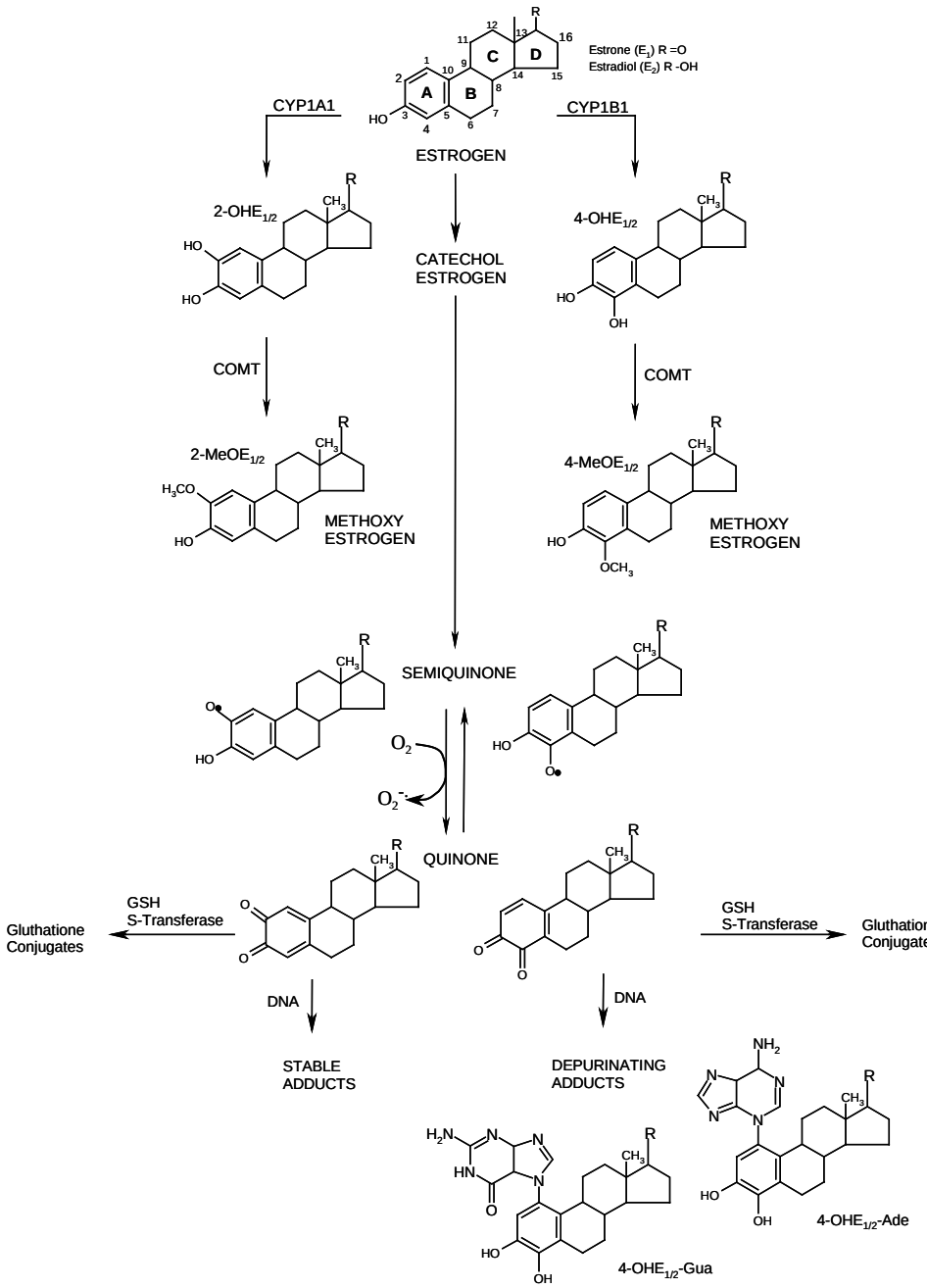

Figure 1.1 Schematic overview of estrogen metabolism and DNA adduct formation in breast tissue (adapted after Cavalieri et al. [46]).

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Effects of Several Dioxin-like Compounds on Estrogen Metabolism in the Malignant MCF-7 and Non-tumorigenic MCF-10A Human Mammary Epithelial Cell Lines

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Majorie B. M. van Duursen
J. Thomas Sanderson
Marieke van der Bruggen
Jeroen van der Linden
Martin van den Berg

Abstract

In human breast tissue, estrone (E1) and estradiol (E2) are mainly hydroxylated by cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1) to 2-hydroxyestrogens (2-OHE1/2) and 4-hydroxyestrogens (4-OHE1/2), respectively. Several studies show that 4-OHE1/2, but not 2-OHE1/2, may act as a carcinogen and a high estrogen 4-/2-hydroxylation ratio appears to be a marker for the presence of neoplasms. In this study, we investigated the effects of several dioxin-like compounds on estrogen 2- and 4-hydroxylation in a malignant (MCF-7) and a non-tumorigenic (MCF-10A) human mammary epithelial cell line. 2- and 4-methoxyestrogen (MeOE1/2) formations were used as measures of the 2- and 4-hydroxylation pathways, respectively. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PCDF), 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and 3,3'4,4',5,5'-hexachlorobiphenyl (PCB 169) concentration-dependently induced 2-MeOE1/2 formation and ethoxyresorufin-O-deethylation (EROD) activity through induced CYP1A1 expression in MCF-7 and MCF-10A cells. 2,3',4,4',5-pentachlorobiphenyl (PCB 118) had no such effect. Effects on CYP1B1 expression and 4-MeOE1/2 formation were less pronounced; only TCDD caused an induction, whereas PCB 169 was a potent and selective inhibitor of 4-MeOE1/2 formation (IC50 0.7 and 2.2 nM PCB 169 in MCF-7 and MCF-10A cells, respectively). MCF-10A cells were less responsive toward dioxin-like compounds and the apparent EC50 values for CYP1A1 and CYP1B1 induction in this study were 10-100 fold higher than in MCF-7 cells. The constitutive 4-/2-MeOE1/2 ratios were 2.99 ± 0.78 and 0.93 ± 0.40 in MCF-7 and MCF-10A, respectively. Incubation with dioxin-like compounds resulted in a concentration-dependent decrease in the 4-/2-MeOE1/2 ratio, but an increase in potentially carcinogenic estrogen metabolites in both MCF-7 and MCF-10A cells. This indicates that even though the 4-/2-OHE1/2 ratio may be used as indicator for the presence of neoplasms, it is readily lowered by dioxin-like compounds and its value as a prognostic parameter for cancer risk should be further examined.

INTRODUCTION

Dioxin-like compounds comprise a group of widespread environmental pollutants, including polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins- and furans (PCDDs/PCDFs), that exert a broad spectrum of biological and toxic effects similar to that of the most potent congener 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [1,2]. Most dioxin-like effects are aryl hydrocarbon receptor (AhR) mediated and include reproductive and developmental toxicities, endocrine disruption, carcinogenesis and altered expression of cytochrome P450 enzymes [2-4].

Numerous epidemiological studies describe a possible correlation between dioxin-like compounds, including some PCBs, and increased breast cancer risk, but the results are ambiguous. Epidemiological studies with women exposed to TCDD in a plant accident near Seveso, Italy, suggest that exposed women had a decreased risk of breast cancer in the ten years following the accident [5,6]. In contrast, a recent follow-up study with these women showed a significant relation with high TCDD serum levels and increased breast cancer incidence [7]. Other studies also show a higher breast cancer incidence and mortality among women after long-term exposure to TCDD [8,9]. Several studies have investigated the correlation between PCB exposure and breast cancer incidence. Studies that concern the sum of all PCB congeners present in breast adipose tissue (BAT) or plasma, generally find no elevation in breast cancer risk [10-13]. However, when the individual PCB congeners are taken into account the results are more ambiguous. For example, two studies found a strong association between high levels of PCB 118 and increased breast tumor incidence with odds ratio OR, 2.85; 95% CI, 1.24-6.52 [14] and OR, 1.60; 95% CI 1.01-2.5 [15], while several other studies found no correlation for this congener [11,13,16,17]. Even though the association between dioxin-like compounds and breast cancer is questionable, the ability of these compounds to alter the expression of certain cytochrome P450 enzymes might play a role in the etiology of breast cancer. Some cytochrome P450 enzymes (CYPs) are involved in the oxidative metabolism of the estrogens 17β-estradiol (E2) and estrone (E1). A major estrogen metabolizing pathway involves hydroxylation on the A-ring to form the catechol estrogens (CEs) 2- and 4-hydroxyestrogen, catalyzed by CYP1A1 and CYP1B1, respectively [18-20]. The CEs may be further converted by catechol-*O*-methyltransferase (COMT) to their methoxy derivatives [21-24], but CEs can also undergo oxidation to reactive quinones [25]. Quinones of the 2-hydroxyestrogens (2-OHE1/2) can form stable DNA adducts that remain in the DNA unless repaired, but quinones of the 4-hydroxyestrogens

(4-OHE1/2) can form depurinating DNA adducts, a potential tumor-initiating event in human cancers [25-29]. The carcinogenic effects of the CEs have been studied both *in vitro* and *in vivo*. In Syrian hamster kidney and rat models, E2 and 4-OHE1/2, but not 2-OHE1/2 are carcinogenic [27,30,31]. In some studies, 2-OHE1/2 appear to protect tissues from tumor formation [32,33]. In *ex vivo* studies higher ratios of 4-OHE2/2-OHE2 are found after incubation of E2 in microsomes from neoplastic tissues than from normal, healthy mammary and uterine tissues [34-36]. In addition, high mRNA levels for CYP1B1, estrogen 4-hydroxylase, are found in malignant mammary tissues [37-39].

In this study, we investigated the influence of several dioxin-like compounds on estrogen 2-hydroxylase (CYP1A1) and estrogen 4-hydroxylase (CYP1B1) activity and special emphasis was placed on the effects on the estrogen 4-/2-hydroxylation ratio. A tumorigenic (MCF-7) and a non-tumorigenic (MCF-10A) human mammary epithelial cell line were used in order to obtain a better insight in the usefulness of this ratio as biomarker for elevated breast cancer risk.

MATERIALS AND METHODS

Chemicals The chemicals used were obtained from the following companies: 2,3,7,8-TCDD (> 99% pure), Cambridge Isotope Laboratories (Woburn, MA); 2,3,4,7,8-pentachlorodibenzofuran (PCDF), 2,3',4,4',5-pentachlorobiphenyl (PCB 118), 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169) all had a purity of >98% and were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany); estrogen standards, Steraloid Inc (Newport, RI). The cell culture media were purchased from Gibco BRL (Breda, The Netherlands) and N,O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA/TMCS) was purchased from Supelco (Zwijndrecht, Belgium). All other chemicals were obtained from Sigma (St. Louis, MO).

Cell lines and cell culture MCF-7 and MCF-10A cell lines were obtained from the American Type Culture Collection (Rockville, MD). MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 0.01 mg/ml insulin and 5% fetal calf serum. MCF-10A cells were cultured in Dulbecco's Modified Eagle's Medium and Ham's F12 Medium (1:1) supplemented with 20 ng/ml epidermal growth factor, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone and 5% horse serum. Both media contained 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C.

Cell viability The cell viability was determined by measuring the capacity of the cells to reduce MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to the blue-colored formazan [40]. Cell cultures of MCF-7 and MCF-10A were exposed to TCDD (10 nM), PCDF (100 nM), PCB 126 (1 μM), PCB 169 (5 μM) or PCB 118 (1 μM) for 72 hours. Then, serum free medium containing 1 mg MTT/ml was added for 1 hour. The medium containing MTT was then removed and the cells were washed twice with warm PBS. Formazan was extracted by adding 1 ml isopropanol at room temperature. Formazan formation was measured spectrophotometrically at an absorbance wavelength of 560 nm and cell viability was calculated using DMSO treated cells as 100% viable control cells.

EROD activity assay Ethoxyresorufin-O-deethylation (EROD) activity was determined in MCF-7 and MCF-10A cell cultures as a measure of CYP1A1 activity (adapted after [41]). Cells were plated onto 12-well plates and exposed to the test compound or DMSO (0.1 % *v/v*) the next day. After 72 hours, the media were replaced with 50 mM Tris buffer (pH 7.8) containing 0.9% NaCl (w/v), 6.25 mM $MgCl_2$, 5 μM 7-ethoxyresorufin and 10 μM dicumarol. The fluorescence was determined at an excitation wavelength of 530 nm and an emission wavelength of 590 nm every five minutes until an optimum was reached. EROD activity was linear for approximately 20 minutes in MCF-7 and for at least 75 minutes in MCF-10A cells.

Estrogen metabolism assay To determine the effect of several dioxin-like compounds on estrogen metabolism, the cell cultures were exposed to various concentrations of the test compounds or the solvent vehicle (0.1% v/v DMSO) for 72 hours (adapted after [42]). Then, the media were replaced with serum free medium containing 1 μM E2 for 6 hours. A 1-ml aliquot of the medium was adjusted to pH 5 with 10% v/v acetic acid and incubated with β-glucuronidase/aryl sulphatase (3000 U and 225 U, respectively) at 37°C for 18 hours. The internal standard (200 pmol equilin) was added and the estrogen metabolites were extracted from the medium by adding 6 ml dichloromethane (DCM). This mixture was vortexed for 30 seconds and then centrifuged for 15 minutes at 3000 rpm. The DCM phase was evaporated at 37°C under nitrogen. Trimethylsilyl derivatives of the estrogen metabolites were prepared with 50 μl BSTFA/TMCS (99:1) and 10% v/v pyridine. The samples were incubated at 60 °C for 30 minutes and then evaporated. The residue was dissolved in 20 μl cyclohexane and 1 μl was injected and analyzed by GC/MS. Gas chromatography was performed on a 30 m x 0.25 mm DB-5MS column with a film thickness of 0.25 μm (J&W Scientific, Folsom, CA). The temperature of the injector, ion source and interface were set at 275, 225 and 300°C, respectively. The oven temperature was kept at 175°C for 1 minute, then it increased with 25°C/min to 280°C, where it was

kept for 10 minutes before increasing it (25°C/min) to the final temperature of 300°C where it was kept for 5 minutes. Selected ion monitoring was performed with an ionization beam of 70 eV. Concentrations of estradiol, estrone, methoxyestradiols (4- and 2-MeOE2) and methoxyestrones (4- and 2-MeOE1) were calculated using the peak area at *m/z* 416, 342, 446 and 372, respectively, and corrected with the peak area of the internal standard (equilin) at *m/z* 340. Peak identification was performed by using the corresponding standards.

mRNA isolation and quantitation of yield MCF-7 and MCF-10A cells were cultured in 12-well plates and exposed to various concentrations of dioxin-like compounds the next day. After a 72 hour induction, RNA was extracted from the cells using the RNA isolation kit from Promega (Madison, WI) and RNA samples were stored at –70 °C until analysis. The absorbance ratio A260 nm/A280 nm was determined spectrophotometrically and used as indication of the purity of the sample. RT-PCR conditions, primers and amplification parameters are described elsewhere [43]. RT-PCR amplification products were detected using agarose gel (1%) electrophoresis and ethidium bromide staining. Intensity of the ethidium bromide staining was quantified using a FluorImager (Molecular Dynamics, Amersham Biosciences Corp, Piscataway, NJ).

Other assays Cellular protein contents were measured with the method of Lowry [44] using BSA as protein standard. Protein contents were used to normalize the rates of metabolite formation. Graph processing was performed using Prism 3.0 (GraphPad Software, San Diego, CA). Satistical significance was determined by Student's t test.

RESULTS

Cell viability

MTT reduction, an indicator of mitochondrial function, was used as marker for cytotoxicity. In MCF-7 and MCF-10A cells, exposure of TCDD (10 nM), PCDF (100 nM), PCB 126 (1 μM), PCB 169 (5 μM) or PCB 118 (1 μM) for 72 hours had no significant effect on MTT reduction (data not shown).

EROD activity in MCF-7 and MCF-10A cells

Constitutive EROD activities were very low in both cell lines, 0.30 ± 0.02 pmol resorufin/min/mg protein in MCF-7 and 0.19 ± 0.04 pmol resorufin/min/mg protein in MCF-10A cells, but EROD activity was readily inducible. The effects of TCDD, PCDF, PCB 126 and PCB 169 on EROD activity in MCF-7 and MCF-10A cells are shown in

figure 1. In MCF-7 cells, EC50 values were 0.04, 0.02 and 2.3 nM for TCDD, PCDF and PCB 126, respectively (Figure 2.1A). In MCF-10A cells these values were approximately 10-100 fold higher (0.5, 0.4 and 140 nM, respectively. Figure 2.1B).

PCB 169 did cause a significant induction of EROD activity at concentrations higher than 1 μM in MCF-7 and 5 μM in MCF-10A cells, but no EC50 values could be obtained. PCB 118 did not induce EROD activity in both cell lines at concentrations up to 1 μM (data not shown).

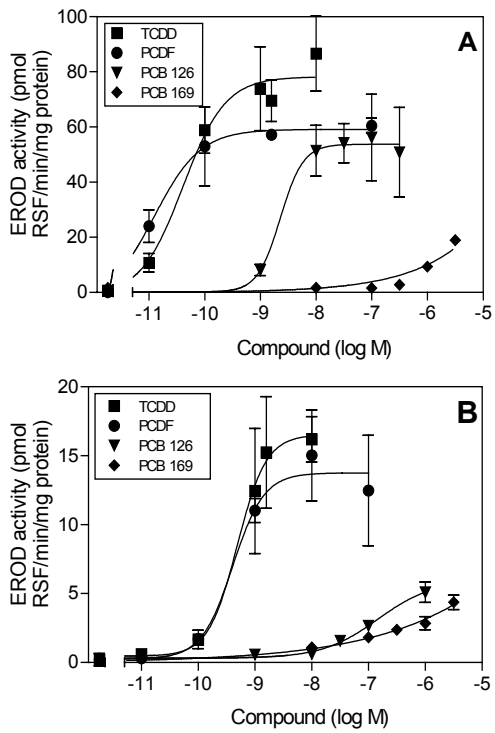

Figure 2.1 Ethoxyresorufin-*O*-deethylase (EROD) activity in (A) MCF-7 and (B) MCF-10A cells after induction with various concentrations of TCDD, PCDF, PCB 126 or PCB 169. Data (in pmol resorufin (RSF)/min/mg protein) are represented as means ± SEM (N=3).

Methoxyestrogen formation in MCF-7 and MCF-10A cells

Methoxyestrogen formation, as indicator of the estrogen 2- and 4-hydroxylation pathways, was calculated by determining both the estradiol metabolites and the estrone metabolites after incubation with 1 μM E2. The sum of the concentrations of 2-MeOE1 and 2-MeOE2 or 4-MeOE1 and 4-MeOE2 were used to calculate the rates of 2-MeOE1/2 and 4-MeOE1/2 formation, respectively.

In MCF-7 cells, estradiol was mainly metabolized to 2-MeOE2 and 4-MeOE2, but some estrone metabolites could also be detected. The constitutive rates of 2-MeOE1/2 and 4-MeOE1/2 formation in MCF-7 cells were 1.06 ± 0.29 and 3.20 ± 0.93 pmol/h/mg protein, respectively. Incubation with dioxin-like compounds resulted in a concentration-dependent increase of 2-MeOE1/2 formation with EC50 values of 0.05, 0.1 and 7.6 nM for TCDD, PCDF and PCB 126, respectively (Figure 2.2A). PCB 169 induced 2-MeOE1/2 formation at concentrations above 500 nM, but an EC50 value could not be determined because the induction did not reach a maximum at the highest tested concentration of 5 μM. TCDD, PCDF and PCB 126 induced 4-MeOE1/2 formation at concentrations on the lower end of the concentration-curve with no effect on 2-MeOE1/2 formation (Figure 2.2B). This induction subsequently declined upon a strong increase of 2-MeOE1/2 formation. PCB 169 acted as an inhibitor of 4-MeOE1/2 formation. PCB 118 had no effect on estrogen metabolism at concentrations up to 1 μM (data not shown).

MCF-10A cells showed to be active in estrone formation. After incubation with estradiol predominantly estrone and estrone metabolites were recovered. The constitutive rates of metabolite formation were 1.27 ± 0.29 pmol/h/mg protein for 2-MeOE1/2 and 1.02 ± 0.26 pmol/h/mg protein for 4-MeOE1/2. TCDD, PCDF, PCB 126 and PCB 169 concentration-dependently increased 2-MeOE1/2 formation, but their efficacies and relative potencies were about 10-100 fold lower than in MCF-7 cells (Figure 2.3A). EC50 values for 2-MeOE1/2 formation induction were 1.2, 0.4 and 446 nM for TCDD, PCDF and PCB 126, respectively. In MCF-10A, 4-MeOE1/2 formation was hardly affected by incubation with dioxin-like compounds; only TCDD and PCB 169 caused a significant effect (Figure 2.3B). TCDD concentration-dependently induced 4-MeOE1/2 formation with an EC50 value of 0.3 nM, while PCB 169 inhibited the 4-MeOE1/2 formation at concentrations higher than 1 nM. PCB 118 had no effect on estrogen metabolism (data not shown).

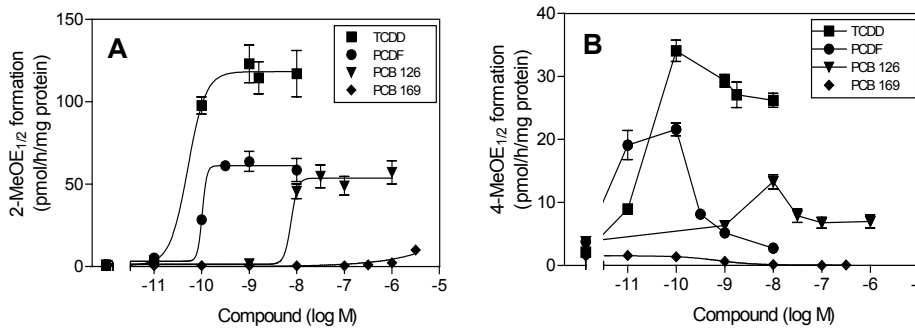

Figure 2.2 Methoxyestrogen formation in MCF-7 cells after induction with various concentrations of TCDD, PCDF, PCB 126 or PCB 169. (A) 2-MeOE1/2 formation, (B) 4-MeOE1/2 formation in pmol 2- or 4-MeOE1/2/h/mg protein after 6 hours incubation with 1 μM E2. Data are represented as means ± SEM (N=4).

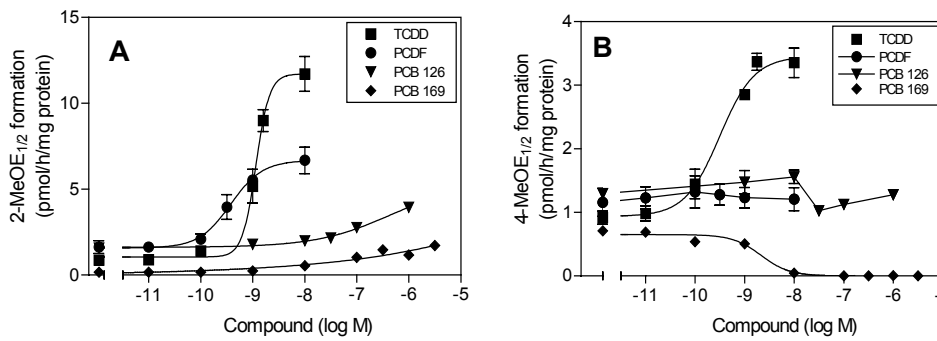

Figure 2.3 Methoxyestrogen formation in MCF-10A cells after induction with various concentrations of TCDD, PCDF, PCB 126 or PCB 169. (A) 2-MeOE1/2 formation, (B) 4-MeOE1/2 formation in pmol 2- or 4-MeOE1/2/h/mg protein after 6 hours incubation with 1 μM E2. Data are represented as means ± SEM (N=4).

Inhibitory effects of PCB 169

PCB 169 showed to be a strong inhibitor of 4-MeOE1/2 formation with IC50 values of 0.7 nM and 2.2 nM in MCF-7 and MCF-10A, respectively (Figure 2.4). At these concentrations, PCB 169 had no effect on 2-MeOE1/2 formation.

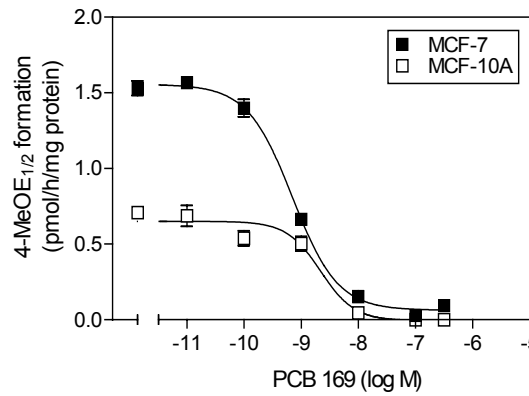

Figure 2.4 4-MeOE1/2 formation in MCF-7 and MCF-10A cells after 72 hours incubation with various concentrations of PCB 169. Data are represented as means ± SEM (N=4).

The selectivity of this inhibitory effect of PCB 169 was shown by its ability to inhibit TCDD induced 4-MeOE1/2 formation at concentrations where no effect on 2-MeOE1/2 formation could be detected (Figure 2.5). MCF-7 and MCF-10A cell cultures were incubated with 10 nM TCDD for 72 hours. Then the media were replaced with serum free medium containing estradiol and various concentrations of PCB 169 or the solvent vehicle (0.1 % v/v DMSO). In MCF-7 cells, TCDD induced 2- and 4-MeOE1/2 formation to 120.2 and 36.4 pmol/h/mg protein, respectively. PCB 169 inhibited TCDD induced 4-MeOE1/2 formation with an IC50 value of 4.2 nM, while at this concentration 2-MeOE1/2 formation was not affected (Figure 2.5A). In MCF-10A cells, control cultures had rates of 33.2 and 4.5 pmol/h/mg protein for 2 and 4-MeOE1/2 formation, respectively. Although the selective inhibitory effects of PCB 169 were less pronounced, a strong inhibitory effect on 4-MeOE1/2 formation could be seen (IC50 2.9 nM). 2-MeOE1/2 formation was inhibited with an IC50 of 5.5 nM (Figure 2.5B).

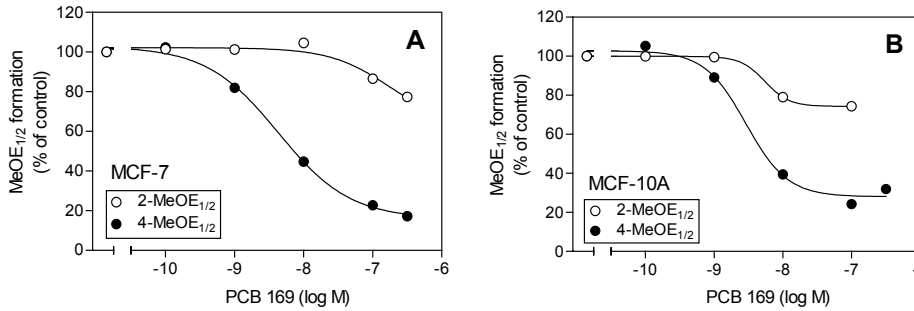

Figure 2.5 Effect of PCB 169 on TCDD induced 2-MeOE1/2 and 4-MeOE1/2 formation in (A) MCF-7 and (B) MCF-10A cells. Cell cultures were exposed to 10 nM TCDD for 72 hours. Then, media were replaced with 1 μM E2 and various concentrations of PCB 169. Data is represented as the means of two measurements compared with control cultures.

The 4-/2-methoxyestrogen ratio (4-/2-MeOE1/2)

The methoxyestrogen ratio was not different from the hydroxyestrogen ratio after 6 hours of incubation with estradiol (data not shown). However, the greater stability of the methoxyestrogens and their lower detection limits made them a more suitable choice for the determination of the estrogen 4- and 2-hydroxylation pathways.

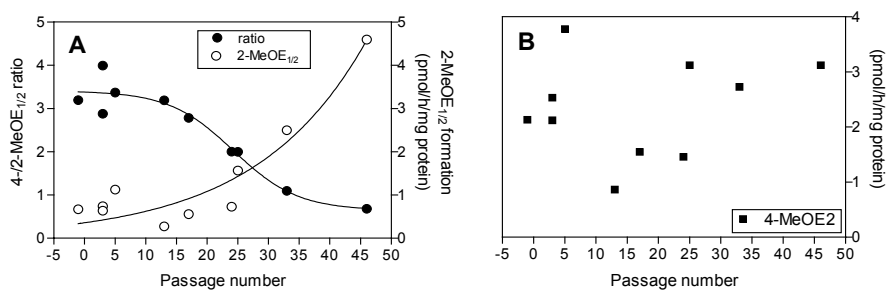

Figure 2.6 Effect of passage number on (A) 4-/2-MeOE1/2 ratio, 2-MeOE1/2 formation and (B) 4-MeOE1/2 formation in MCF-7 cells. Each data point represents the average of 4 measurements. Correlation coefficients (R2) are 0.90, 0.93 and 0.03 for 4-/2-MeOE1/2 ratio, 2-MeOE1/2 formation and 4-MeOE1/2 formation, respectively.

In this study, the constitutive 4-/2-MeOE1/2 ratio in MCF-7 cells was 2.99 ± 0.78, but this ratio decreased with increasing passage number. This decrease appeared to be caused by an increase in 2-MeOE1/2 formation rather than a change in 4-MeOE1/2 formation (Figure 2.6). This change in metabolism was not seen in MCF-10A cells. The constitutive 4-/2-MeOE1/2 ratio in MCF-10A cells was 0.93 ± 0.40. All experiments described here were performed with MCF-7 and MCF-10A cells with passage numbers under 20.

A 72-hour incubation with dioxin-like compounds caused a concentration-dependent decrease of the 4-/2-MeOE1/2 ratio in MCF-7 and MCF-10A cells (Figure 2.7). Only PCB 118 had no significant effect on the ratio (data not shown). In MCF-7, a slight increase in ratio could be seen at low concentrations of PCDF and PCB 126 because of the induction of 4-MeOE1/2 before 2-MeOE1/2 formation was affected. However, this increase was statistically not significantly different from the control cultures. In the case of TCDD, PCDF and PCB 126 the subsequent decrease in ratio was caused mainly by a strong increase of 2-MeOE1/2 formation. In the case of PCB 169, a decrease in 4-/2-MeOE1/2 ratio was caused by direct catalytic inhibition of 4-MeOE1/2 formation, as described previously. The EC50 values for the decrease in 4-/2-methoxyestrogen ratio in MCF-7 are 0.01, 0.05, 2.0 and 0.8 nM for TCDD, PCDF, PCB 126 and PCB 169, respectively (Figure 2.7A). The same effects on the ratio could be seen in MCF-10A cells, although less pronounced than in MCF-7 cells, with EC50 values of 0.8, 0.2, 21.5 and 0.7 nM for TCDD, PCDF, PCB 126 and PCB 169, respectively (Figure 2.7B).

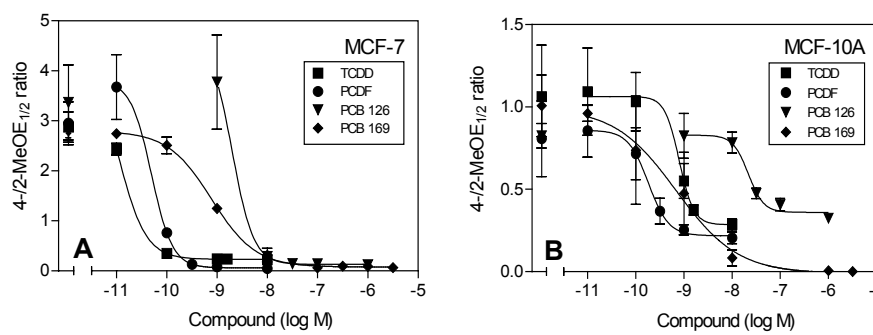

Figure 2.7 4-/2-methoxyestrogen ratio in (A) MCF-7 and (B) MCF-10A cells after induction with various concentrations of TCDD, PCDF, PCB 126 or PCB 169. Data are represented as means ± SEM (N=4).

mRNA levels of CYP1A1 and CYP1B1 after induction.

In breast tissue, estradiol 2- and 4-hydroxylation is mainly catalyzed by CYP1A1 and CYP1B1. In order to study whether the effects of dioxin-like compounds on estrogen metabolism can be attributed to altered CYP1A1 and CYP1B1 expression, mRNA levels of these enzymes were determined in MCF-7 and MCF-10A cells (Figure 2.8). Beta-actin mRNA was not affected by incubation with the dioxin-like compounds and could therefore be used as a reference amplification response. Constitutive mRNA levels of CYP1A1 and CYP1B1 were higher in MCF-7 than in MCF-10A cells. In both cell lines, CYP1A1 mRNA expression was very low at constitutive levels but was more readily induced by TCDD, PCDF and PCB 126 than CYP1B1. PCB 169 did not affect CYP1A1 and CYP1B1 mRNA levels significantly in MCF-7 cells compared with the control levels (Figure 2.8A). In MCF-10A cells PCB 169 caused a slight decrease of CYP1A1 and CYP1B1 expression, but only CYP1B1 mRNA levels were significantly lower than control levels (Figure 2.8B). After incubation with PCB 118, mRNA levels of CYP1A1 and CYP1B1 were slightly lower than control levels in both cell lines.

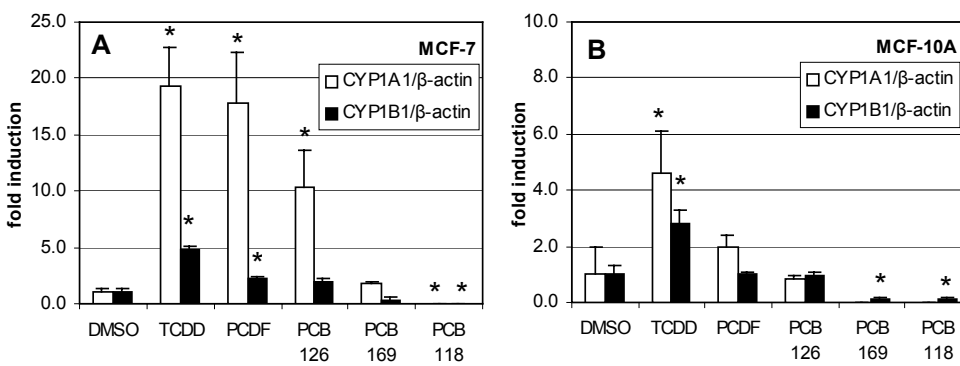

Figure 2.8 CYP1A1 and CYP1B1 mRNA in (A) MCF-7 and (B) MCF-10A cells after 72 hours incubation with various dioxin-like compounds. Bars represent the means of three measurements (response ratio CYP/beta-actin) ± SD compared with the DMSO treated control. * Significantly different from control ($p < 0.05$).

DISCUSSION

The effects of AhR-mediated CYP induction on estrogen metabolism has often been studied in MCF-7 cell cultures, but little is known about MCF-10A cells. The MCF-10A cell line is a human mammary epithelial cell line derived from a fibrocystic tissue and has the characteristics of non-tumorigenic normal breast epithelium [45,46]. Spink et al. showed that MCF-10A cells express the estrogen receptor and the AhR [47]. In the present study, CYP1A1 and CYP1B1 enzymes in MCF-10A cells showed to be inducible by dioxin-like compounds although their responsiveness was much lower than in MCF-7 cells. Further, estradiol was rapidly converted to estrone by MCF-10A cells, suggesting a high activity of 17β-hydroxy steroid dehydrogenase (17β-HSD), the enzyme responsible for the interconversion of estradiol and estrone.

CYP1A1 and CYP1B1 induction by dioxin-like compounds in MCF-7 and MCF-10A
The tested dioxin-like compounds all exerted AhR-mediated effects on CYP1A1 and CYP1B1 mRNA levels, EROD activity, and estrogen metabolism in MCF-7 and MCF-10A cells. In this study, the effects were similar in MCF-7 and MCF-10A cells, though in MCF-10A cells the effects were less pronounced. It is unclear why MCF-10A cells are less responsive to dioxin-like compounds. A low expression level of the AhR may play a role [47]. In risk assessment of dioxin-like compounds, the potency of a dioxin-like compound to induce an effect is usually compared with the potency of TCDD to induce the same effect [1,48]. The potencies of the tested compounds to induce 2-MeOE1/2 formation, EROD activity and CYP1A1 mRNA levels, were in the order TCDD ≈ PCDF > PCB 126 > PCB 169 > PCB 118, which is in line with the relative toxicological potencies of these compounds established by the World Health Organization (WHO) [1]. Although the effects on 4-MeOE1/2 formation and CYP1B1 mRNA were less distinct, the same order of induction by dioxin-like compounds could be seen. Apparently, CYP1A1 is more inducible than CYP1B1 upon treatment with dioxin-like compounds.

PCB 169 showed to be very potent in affecting estrogen metabolism in both MCF-7 and MCF-10A cells. Regarding the ability of PCB 169 to inhibit TCDD induced 4-MeOE1/2 formation, this effect seems to be caused by direct CYP1B1 inhibition rather than an AhR-mediated effect on mRNA level. This conclusion is supported by the small effect PCB 169 appeared to have on CYP1B1 mRNA expression. This direct inhibitory effect of PCB 169 seen in this study is in good agreement with a study performed by Pang

et al. [49], who also found a selective inhibitory effect of PCB 169 on TCDD induced 4-MeOE1/2 formation in MCF-7 cells with an EC50 value of 1.6 nM.

As expected based on its relative potency determined by the WHO, PCB 118 had no effect on estrogen metabolism and EROD activity in both MCF-7 and MCF-10A cell lines. The apparent decrease in mRNA levels caused by PCB 118 in both MCF-7 and MCF-10A cells is probably due to the poor quality of the RNA samples. The low yield of total mRNA made it difficult to quantify the mRNA expression of CYP1A1 and CYP1B1.

Constitutive estrogen metabolism

In this study we used the methoxyestrogen levels as measure for estrogen 4- and 2-hydroxylation. This means that an additional enzymatic step, the methylation of the CEs by COMT, must be taken into consideration. COMT activities can differ greatly among the cell lines and human tissues, since COMT is polymorphic which results in a high and low activity isoform of the enzyme [23,50]. MCF-7 cells appear to have a low catalytic activity form [23] and MCF-10A cells appear to have a high catalytic activity isoform of COMT [51]. We found that the methoxyestrogen ratio was not different from the hydroxyestrogen ratio after six hours of estrogen incubation. Still, it is more accurate, from a mechanistic point of view, to determine the hydroxyestrogen levels to determine a possible cancer risk because those are the potentially genotoxic metabolites.

The constitutive 4-/2-MeOE1/2 ratios in MCF-7 and MCF-10A cells were 2.99 ± 0.78 and 0.93 ± 0.40, respectively. These ratios were comparable with the 4-OHE2/2-OHE2 ratios described by Liehr et Ricci for *ex vivo* mammary tissues [34]. The 4-OHE2/2-OHE2 ratios were 3.8 in adenocarcinomas, 1.3 in normal breast tissues from breast cancer patients and 0.7 in healthy breast tissue from reduction mammoplasty surgeries. This suggests that the cell lines MCF-7 and MCF-10A might be good models to compare estrogen metabolism in a malignant and a non-tumorigenic human mammary cell type. However, other studies describing the estrogen metabolism in MCF-7 or MCF-10A cells found different constitutive estrogen 4-/2-hydroxylation ratios. Spink et al. compared estrogen metabolism in various cell lines and found ratios of 0.728 ± 0.013 and 0.286 ± 0.024 for MCF-7 and MCF-10A, respectively [47]. Another study found constitutive rates of 2- and 4-MeOE1/2 formation in MCF-7 cells of 0.318 ± 0.014 and 0.165 ± 0.014, respectively, [49] resulting in a constitutive 4-/2-MeOE1/2 ratio of 0.52 ± 0.05. These differences in metabolic rates might be caused by the fact that cell lines are not as uniform as believed. We showed in this study that MCF-7 cells had an altered estrogen metabolism, as was seen by a decline in 4-/2-MeOE1/2 ratio after several

passages of the cells (ratio of 2.88 at passage 8 compared with 0.68 at passage 32). These results indicate that there are variations between MCF-7 cell cultures which might make it difficult to compare studies performed with this cell line. This study was performed with MCF-7 cells with low passage numbers, freshly derived from ATCC, which might explain the differences in ratios with other publications. We found no time-dependent decrease in estrogen 4-/2-hydroxylation ratio in MCF-10A cells.

Effect of dioxin-like compounds on estrogen metabolism

Dioxin-like compounds, including PCBs, are often studied in association with breast cancer risk. Some epidemiological studies show an association between increased dioxin-like compound levels in serum or BAT and increased breast cancer risk. However, the possible role of these compounds in the etiology of breast cancer remains unclear. It has been proposed that some of these compounds increase breast cancer risk because they might alter immune response [52], cell differentiation [53] and enzyme expression and because some of these compounds have the potential to stimulate cell growth [54]. We hypothesized that their ability to affect estrogen metabolizing enzymes might result in changed ratio of potentially genotoxic CEs and possibly to an increased cancer risk. The tested dioxin-like compounds, except for PCB 118, concentration-dependently decreased the 4-/2-MeOE1/2 ratio with relative potencies in the order of TCDD ≥ PCDF > PCB 169 > PCB 126 in MCF-7 and MCF-10A cells. Supposing the 4-/2-MeOE1/2 ratio can be used to estimate the cancer risk through DNA damage by CEs, a decrease in ratio suggests a protective effect of the dioxin-like compounds against the development of neoplasms. However, despite the decrease in ratio, the absolute levels of 4-MeOE1/2 increased upon incubation with the dioxin-like compounds. This raises the question whether an absolute increase of 4-OHE1/2 might be more informative, because these are the suspected carcinogenic compounds. On the other hand, the estrogen 2-hydroxylation pathway appears to be important in overall risk estimation of the CEs, because several ways in which 2-OHE1/2 can protect against tumor formation have been suggested. First, 2-OHE1/2 appear to be less harmful than the 4-OHE1/2 because they can form more stable DNA adducts [25] and more 2-hydroxylation of estradiol means less potentially harmful 4-hydroxylation of estradiol. Second, 2-OHE1/2 possess less cell growth stimulatory activity than estradiol and other metabolites such as 4-OHE1/2 and 16α-hydroxyestrone [55,56]. Third, 2-MeOE1/2 seem to downregulate the estrogen synthesizing enzyme aromatase [57] resulting in a lower estradiol concentration and subsequently lower concentrations of circulating harmful metabolites. In addition,

2-MeOE1/2 can inhibit cell proliferation and induce cell apoptosis [58,59]. These data indicate that both the estrogen 2- and 4-hydroxylation pathways determine the final potential of the CEs to increase carcinogenic risk. Even though it appears that the 4-/2-OHE1/2 ratio might be used as a marker for the presence of neoplasms, we showed in the present study that this ratio is easily lowered by dioxin-like compounds, which has implications for the usefulness of the 4-/2-OHE1/2 ratio as prognostic parameter for breast cancer risk. Further studies will be necessary to determine the quantitative and qualitative importance of the CEs in the prediction of estrogen dependent cancer-initiating events.

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3

Phytochemicals Inhibit Catechol-O-Methyltransferase Activity in Cytosolic Fractions from Healthy Human Mammary Tissues: Implications for Catechol Estrogen-Induced DNA Damage

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Majorie B. M. van Duursen[1],
J. Thomas Sanderson[1]
Paul Chr. de Jong[2]
Marlies Kraaij[1]
Martin van den Berg[1].

[1]Institute for Risk Assessment Sciences (IRAS), Universiteit Utrecht, Utrecht, The Netherlands.
[2]St Antonius Hospital, Department of Internal Medicine, Nieuwegein, The Netherlands

Abstract

Phytochemicals are natural dietary constituents of fruits and vegetables. Some of these phytochemicals are known to affect estrogen metabolizing enzymes. In breast tissue, estradiol can be metabolized to the catechol estrogens 2- and 4-hydroxyestradiol (2-OHE2 and 4-OHE2). Catechol estrogens are suspected carcinogens potentially involved in the etiology of breast cancer. Catechol-O-methyltransferase (COMT) converts the catechol estrogens to their inactive methoxy derivatives (2-MeOE2 and 4-MeOE2). In this study we investigated the effects of several phytochemicals on COMT activity in cytosolic fractions of seven healthy human mammary tissues from reduction mammoplasty. Large interindividual variations were observed in the constitutive levels of COMT activity. However, in all cytosol samples the catalytic efficiency of COMT was greater for 2-MeOE2 formation than for 4-MeOE2 formation. The known COMT inhibitor Ro 41-0960 and several phytochemicals with a catechol structure (quercetin, catechin and (-)-epicatechin) concentration-dependently inhibited COMT activity, while phytochemicals without a catechol structure (genistein, chrysin and flavone) showed no effect up to 30 μM. Distinct interindividual variations were observed in sensitivity toward COMT inhibition among the various tissue samples as was shown by the range in IC50 values for Ro 41-0960 (5 – 42 nM). The toxicological relevance of COMT inhibition and the effect of reduced inactivation of catechol estrogens was studied by determining the amount of catechol estrogen-induced DNA damage in MCF-7 cells using the comet assay. Catechol estrogens alone caused no increase of DNA damage compared with control treated cells. However, both Ro 41-0960 and quercetin caused a decrease of methoxy estradiol formation and an increase of catechol estrogen-induced DNA damage in MCF-7 cells. This suggests that phytochemicals with a catechol structure have the potential to reduce COMT activity in mammary tissues and may consequently reduce the inactivation of potentially mutagenic estradiol metabolites and increase the chance of DNA damage.

INTRODUCTION

During the last decades an increase has been observed in the occurrence of malignant neoplasms of the female breast in industrialized countries. Presently, it is one of the major causes of death among women in western countries. In the etiology of breast cancer, estrogens play a key role in tumor development. The role of estrogen in carcinogenesis has been suggested to be dual; it may act by stimulating cell transformation and cell proliferation, and it may act as a tumor initiator through its metabolites by inducing damage to cellular macromolecules such as DNA [1-5]. The tumor initiating action of estrogens is believed to be a result of hydroxylation of the main estrogen in premenopausal women, 17β-estradiol (E2), to the catechol estrogens 2- and 4-hydroxyestradiol [6-8]. Unless inactivated, catechol estrogens can undergo oxidation to reactive quinones [2]. Quinones of 2-hydroxyestradiol (2-OHE2) can form stable DNA adducts that remain in the DNA unless repaired, but quinones of 4-hydroxyestradiol (4-OHE2) can form depurinating DNA adducts, a potential tumor-initiating event in human cancers [2-4,9]. Catechol-O-methyltransferase (COMT) plays an important role in the inactivation of catechol estrogens [10,11]. COMT is a phase II enzyme involved in the inactivation of many endogenous catechol substrates by transferring a methyl group from S-adenosyl-L-methionine (SAM) to the substrate and thus converting them into their methoxy derivatives (reviewed by [12]).

Low COMT activity has been associated with increased breast cancer risk (reviewed by Yue et al [13]). There are several ways in which COMT activity might be altered. Lachman et al. has described a low activity form of COMT resulting from a genetic polymorphism [14]. A single nucleotide substitution in codon 108 causes an amino acid transition (Val → Met) which results in a high (Val/Val) or low activity (Met/Met) form of the COMT enzyme with a three-to-four-fold difference in activity. COMT activity can also be inhibited by substrate competition for the enzyme. There are many naturally occurring substrates for COMT in the body, but some exogenous compounds have also been identified as substrates for the enzyme. For example, certain catechol metabolites of PCBs have been shown to inhibit COMT activity [15]. In addition, many dietary catechols, such as phytochemicals, can be a substrate for COMT. Phytochemicals are a diverse group of chemicals which can be found in fruits and vegetables. This group of biologically active compounds occurs in high concentrations in our diet and the daily intake can comprise a few hundreds of milligrams per day [16]. As a result, submicromolar plasma levels can be reached [17-19]. Phytochemicals have been shown to possess

antioxidant, anticancer and antiviral properties. Because of these properties, they are generally regarded as safe and many phytochemicals are sold in high concentrations as dietary supplements with recommended intake levels that exceed normal daily intake up to a 100-fold. However, besides the beneficial properties phytochemicals may also affect various enzyme activities. For example, Zhu et al. described the effect of quercetin, a phytochemical found in many food items, on COMT activity in male Syrian hamsters [20]. In hamsters fed with quercetin, a decreased COMT activity was found which resulted in increased catechol estrogen concentrations in the kidneys and subsequent enhancement of estradiol-induced tumorigenesis.

In the present study, we investigated COMT activity in healthy mammary tissues, where COMT plays an important role in the inactivation potentially genotoxic catechol estrogens. We studied the constitutive rates of O-methylation of catechol estrogens and the effects of phytochemicals on this activity in healthy human mammary tissue cytosol. We hypothesized that phytochemicals with a catechol structure, like quercetin, catechin and (-)-epicatechin (chemical structures in Figure 3.1), make a suitable substrate for the COMT enzyme and thus potentially inhibit the formation of methoxy estrogens. We also investigated the effects of several phytochemicals without a catechol structure (genistein, chrysin and flavone) on COMT activity. Ro 41-0960, a known selective COMT inhibitor, was used as a standard positive control. COMT inhibition results in decreased inactivation of catechol estrogens which in turn may lead to increased DNA damage. Therefore, we studied the implications of decreased COMT activity caused by phytochemicals on catechol estrogen-induced DNA damage by performing the alkaline comet assay using the malignant human mammary tumor cell line MCF-7.

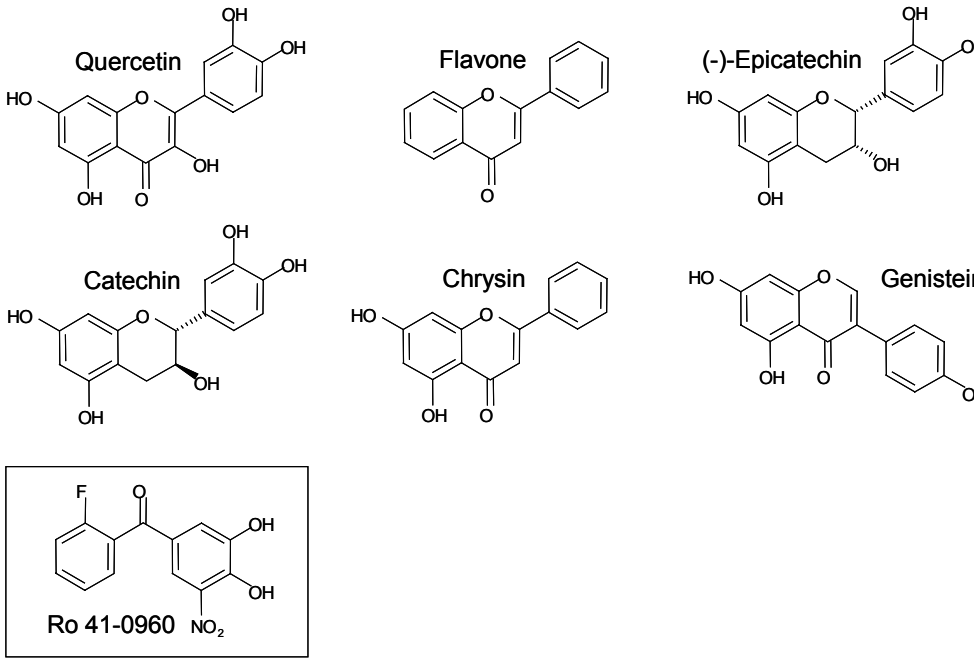

Figure 3.1 Chemical structures of the compounds used in this study; phytochemicals with a catechol structure (quercetin, catechin and (-)-epicatechin), without a catechol structure (flavone, chrysin and genistein) and the known COMT inhibitor Ro 41-0960.

MATERIALS AND METHODS

Chemicals and reagents S-adenosyl-L-methionine (SAM), dithiothreitol (DTT), 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Ro 41-0960 (2'-fluoro-3,4-dihydroxy-5-nitrobenzophenone), quercetin, catechin, (-)-epicatechin, chrysin, genistein and flavone were obtained from Sigma (St. Louis, MO, USA). Estrogen standards were obtained from Steraloid Inc. (Newport, RI, USA), and N,O-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA/TMCS) was purchased from Supelco (Bellefonte, PA, USA).

Sample preparation Tissues from reduction mammoplasty (N=7) were obtained from the Antonius Hospital (Nieuwegein, the Netherlands). The study was approved (number TME/Z-02.09) by the medical ethical committee of the hospital. All women gave permission for the removed tissue to be used by an informed consent. Tissues were

diagnosed as histologically normal breast tissue by a pathologist. Upon arrival in our laboratory, fresh tissues were snap frozen in liquid nitrogen and stored at -70 °C until use. Before preparation of cytosolic fractions, the tissues were thawed at 4 °C and kept on ice. Adipose tissue was removed with a surgical knife and the remaining parenchyma was cut into small pieces. The tissue pieces were weighed and 3 ml cold phosphate buffer (50 mM, pH 7.6 containing 0.1 mM EDTA) was added per g tissue. This mixture was homogenized with a Potter-Elvehjem teflon-glass homogenizer. Cytosolic fractions were prepared through ultracentrifugation (Beckman L7-55). Homogenates were first centrifuged at 10.000 g for 15 minutes at 4 °C to remove the cell debris and remaining adipose tissues. Subsequently, the supernatant was centrifuged at 100.000 g for 75 minutes at 4 °C to separate the cytosolic (supernatant) from the microsomal (pellet) fractions. Aliquots of the cytosolic fractions were stored at -70 °C until analysis. Protein contents of the fractions were determined by the method of Lowry et al. [21] using bovine serum albumine (BSA) as protein standard.

COMT activity In order to study the O-methylation activity, cytosolic protein (300 µg) was incubated with 50 mM phosphate buffer (pH 7.6), 5 mM $MgCl_2$, 150 µM SAM, 1 mM DTT and various concentrations of a phytochemical or the solvent vehicle (0.1% v/v MeOH) to a final volume of 492.5 µl. Reaction mixtures were incubated at 37 °C for 5 minutes before the reaction was started by adding 2-OHE2 and 4-OHE2 (3.75 µM each). After 30 minutes, the reaction was stopped by putting the reaction tubes on ice. The metabolite extraction procedure was adapted from Spink et al. and performed as described previously [22,23]. Briefly, the internal standard (20 µl equilin, 10 µM) was added and 2- and 4-MeOE2 were extracted with dichloromethane. Trimethylsilyl derivatives of the estrogens were prepared and analyzed by GC/MS. Peak areas were determined at m/z 446 and 340 for 2- and 4-MeOE2 and equilin, respectively. Peak identification and quantification was performed with the corresponding standards.

Cell lines and cell culture MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle's Medium supplemented with 0.01 mg/ml insulin, 5% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C.

Cell viability The cell viability was determined by measuring the capacity of the cells to reduce MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to the blue-colored formazan [24]. Cell cultures of MCF-7 were exposed to the catechol estrogens (7.5 µM of 2- and 4-OHE2), Ro 41-0960 (10 µM) or quercetin (30 µM) for 6 hours. Then,

serum-free medium containing 1 mg MTT/ml was added for 1 hour. The medium containing MTT was then removed and the cells were washed twice with warm PBS. Formazan was extracted by adding 1 ml isopropanol at room temperature. Formazan formation was measured spectrophotometrically at an absorbance wavelength of 560 nm and cell viability was calculated using solvent vehicle treated cells (ethanol, methanol and DMSO, total of 0.17% v/v) as 100% viable control cells.

Alkaline single-cell gel electrophoresis (comet) assay The effect of COMT inhibition on DNA damage caused by catechol estrogens was determined using the single-cell gel electrophoresis (comet) assay as described by Singh et al. with some modifications [25]. For this assay, 5 x 10^5 MCF-7 cells were plated onto 12-well plates and placed in a humidified atmosphere with 5% CO_2 at 37°C. The next day, cells were exposed for 5 hours to serum free medium containing the solvent vehicles (ethanol, methanol and DMSO, total of 0.17% v/v) and catechol estrogens (7.5 µM 2-OHE2 and 4-OHE2), Ro 41-0960 (10 µM) and quercetin (10 µM or 30 µM), alone or in combination. Then, media were removed and analysed for methoxy estradiol concentrations as described above. The cells were washed with PBS and 100 µl trypsin was added. As soon as the cells detached, one ml of warm medium containing 5% FBS was added and the cells were suspended and transferred to a 1.5-ml eppendorf cup. The cell suspension was briefly centrifuged and 1000 µl of the supernatant was removed. The remaining cells were gently resuspended and a 10 µl aliquot was added to 90 µl warm 0.5% low melting agarose. This mixture was spread onto a frosted slide covered with 1.5% normal melting agarose and placed on a ice-cold glass plate to solidify. Then, the slides were placed in freshly prepared cold lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris containing 1% Triton X-100 and 10% DMSO, pH 10) for one hour at 4°C. After this, the slides were kept in the dark to prevent DNA damage by exposure to direct light. Subsequently, the slides were placed in a horizontal slide holder in an electrophoresis unit containing cold electrophoresis solution (0.3 M NaOH, 0.001 M EDTA) for 25 minutes and then electrophoresed for 25 minutes at 25 V, 290-310 mA. Then, the slides were washed three times with a sterile neutralization buffer (0.4 M Tris/HCl, pH 7.5) and dehydrated for 10 minutes in 100% ethanol. The slides were kept in a dark box at 4°C until analysis. Prior to analysis, the slides were stained with ethidium bromide (20 µg/ml). Analysis was performed under a fluorescence microscope using a 20x objective and a filter of 450-490 nm equipped with a digital camera. Of each treatment 175-200 cells (four slides per treatment, 40-50 cells per slide) were analyzed and the tail moment (comet tail length x % tail DNA) was determined using the PC image-analysis program Casp described by Konca et al. [26].

Data analysis Enzyme kinetic parameters (Vmax and Km values in pmol/min/mg protein and μM, respectively) were calculated with Prism 3.0 (GraphPad Software, San Diego, CA, USA). Statistical significance of difference of the mean was determined by the Student's t-test. Variance and differences among the means were determined by a one-way ANOVA with a Tukey-Kramer Multiple Comparisons test using GaphPad InStat 3.06 (GraphPad Software Inc., San Diego, CA).

RESULTS

Constitutive COMT activity

Enzyme kinetics were studied in samples 1 and 4 by determining the MeOE2 formation at various equimolar concentrations of 2- and 4-OHE2. The concentration of SAM, the methyldonor for O-methylation, in the incubation mixture was 150 μM, which was a saturating concentration (data not shown). After 30 minutes, MeOE2 concentrations were determined. In both samples the catalytic efficiency of COMT was higher for 2-MeOE2 formation than for 4-MeOE2 formation. In sample 1, catalytic efficiencies (Vmax/Km) were 3.0/5.0 = 0.6 and 1.3/9.5 = 0.1 for 2- and 4-MeOE2 formation, respectively. In sample 4, catalytic efficiencies were 16.2/9.4=1.7 and 4.2/19.7=0.2 for 2- and 4-MeOE2 formation, respectively. In both samples, the rate of methoxy formation was linear up to a concentration of 25 μM for at least 45 minutes (data not shown). An incubation of 30 minutes with 7.5 μM catechol estrogens was chosen to study the constitutive COMT activity and the effects of phytochemicals.

The constitutive rates of methylation of catechol estrogens in the various tissue samples are shown in Table 3.1. The mean 2-MeOE2 formation of seven tissues was 8.12 ± 1.32 pmol/min/mg protein with a range of activity of 2.14-19.03 pmol/min/mg protein. For 4-MeOE2, the mean metabolite formation and range of activity were 1.83 ± 0.29 and 0.37-3.81 pmol/min/mg protein, respectively. ANOVA analysis showed significant differences in 2- and 4-MeOE2 formation between the tissue samples (P=0.0004 and P=0.0044, respectively). Further analysis showed that cytosol from tissue sample 3 had substantially higher rates of methoxy estradiol formation compared with other tissue samples. However, despite the variation in rates of methylation, the ratio of 4- MeOE2/2-MeOE2 formation at 7.5 μM 2- and 4-OHE2 was not significantly different statistically between the tissues (ANOVA analysis, P=0.125). The average 4-/2-MeOE2 ratio in seven tissue samples was 0.26 ± 0.02.

Table 3.1 2- and 4-MeOE2 formation, 4-MeOE2/2-MeOE2 ratio and IC50 values for Ro 41-0960 inhibition in cytosolic fractions of healthy mammary tissues after incubation with an equimolar concentration of 7.5 μM 2- and 4-OHE2.

	2-MeOE2 formation (pmol/min/mg protein)	4-MeOE2 formation (pmol/min/mg protein)	4-/2-MeOE2 ratio	IC50 (nM) for Ro 41-0960
Sample 1	5.38 ± 1.45[a]	1.13 ± 0.14	0.21 ± 0.06[b]	33.6 ± 5.8 [c]
Sample 2	2.17 ± 0.31	0.37 ± 0.05	0.17 ± 0.03	11.4 ± 5.0
Sample 3	19.03 ± 4.81[d]	3.81 ± 1.21[e]	0.20 ± 0.08	5.1 ± 2.7
Sample 4	5.38 ± 2.35	1.16 ± 0.24	0.22 ± 0.10	42.1 ± 14.5[f]
Sample 5	2.14 ± 0.47	0.67 ± 0.11	0.30 ± 0.02	27.5 ± 9.8
Sample 6	9.10 ± 1.34	2.31 ± 0.30	0.27 ± 0.03	8.6 ± 1.5
Sample 7	9.36 ± 1.30	2.53 ± 0.41	0.27 ± 0.04	5.8 ± 2.0

[a]Mean ± standard error of the mean of 4 to 7 determinations.

[b]4-MeOE2 formation (pmol/min/mg protein) divided by 2-MeOE2 formation (pmol/min/mg protein)

[c]Mean IC50 value (nM) of two duplicate determinations ± SEM

[d]Significantly different from sample 1 and 4 ($P < 0.05$) and sample 2 and 5 ($P < 0.01$)

[e]Significantly different from sample 2 and 5 ($P < 0.05$)

[f]Significantly different from sample 3 and 7 ($P < 0.05$)

COMT inhibition by Ro 41-0960

Because substantial interindividual variation in methylation rates was observed, a concentration-response curve was made with Ro 41-0960, a known COMT inhibitor, for every tissue sample. IC50 values for every tissue sample are listed in Table 3.1. Large variations between the tissue samples in responsiveness to Ro 41-0960 were observed. Sample 4 was the least responsive to Ro 41-0960 with an IC50 value for COMT inhibition of 42.1 nM and this IC50 value was significantly different from sample 2 and 5. Although COMT has a higher constitutive activity with 2-OHE2 than with 4-OHE2 as substrate, inhibition of methylation occurred in the same order of magnitude for both 2- and 4-MeOE2 formation (Figure 3.2).

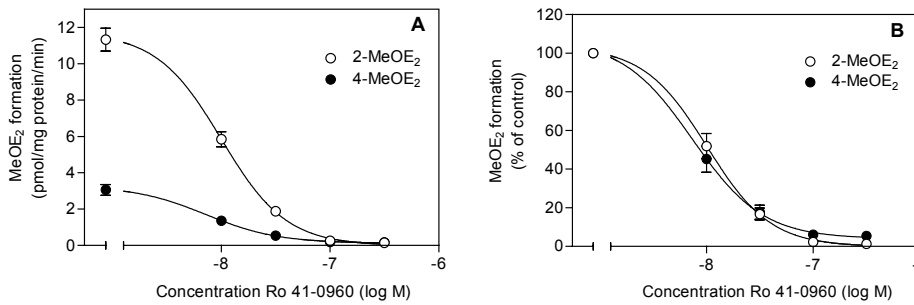

Figure 3.2 Inhibition of COMT by Ro 41-0960 in tissue sample 6. COMT activity is represented as (A) absolute MeOE2 formation (pmol/mg protein/min) and (B) as percentage of control activity. Cytosolic fractions from human mammary tissue sample 6 was incubated with an equimolar concentration of 7.5 μM 2- and 4-OHE2. After 30 minutes methoxy estradiols were extracted and analysed by GC/MS. Data are represented as mean and range of two determinations.

Effects of phytochemicals on COMT activity

The *O*-methylation of 7.5 μM 2- and 4-OHE2 in cytosolic fractions of human mammary tissues was concentration-dependently inhibited by quercetin, catechin and epicatechin as was expected based on their catechol structure (Figure 3.3). While Ro 41-0960 fully inhibited COMT activity at the highest concentration tested (0.3 μM) in all tissue samples, the phytochemicals decreased COMT activity to about 30-40% of the control activity at the highest concentrations tested (10-100 μM). IC50 values for COMT inhibition were 0.48 μM (tissue #1), 1.64 μM (tissue #1) and 1.96 μM (tissue #3) for quercetin, catechin and epicatechin, respectively (Table 3.2). ANOVA analysis showed that only the differences in IC50 values for quercetin and flavone were statistically significant ($P < 0.05$). Phytochemicals without catechol structure were less potent COMT inhibitiors. Genistein and chrysin appeared to reduce COMT activity slightly at the highest concentration tested (30 μM). Flavone, on the other hand, decreased COMT activity in tissue sample 3 to 40% of the control levels at the highest concentration tested (100 μM) with an IC50 value of 5.5 μM. However, in tissue sample 5, which was less responsive to COMT inhibition by Ro 41-0960, no inhibitory effect on COMT activity could be detected (data not shown).

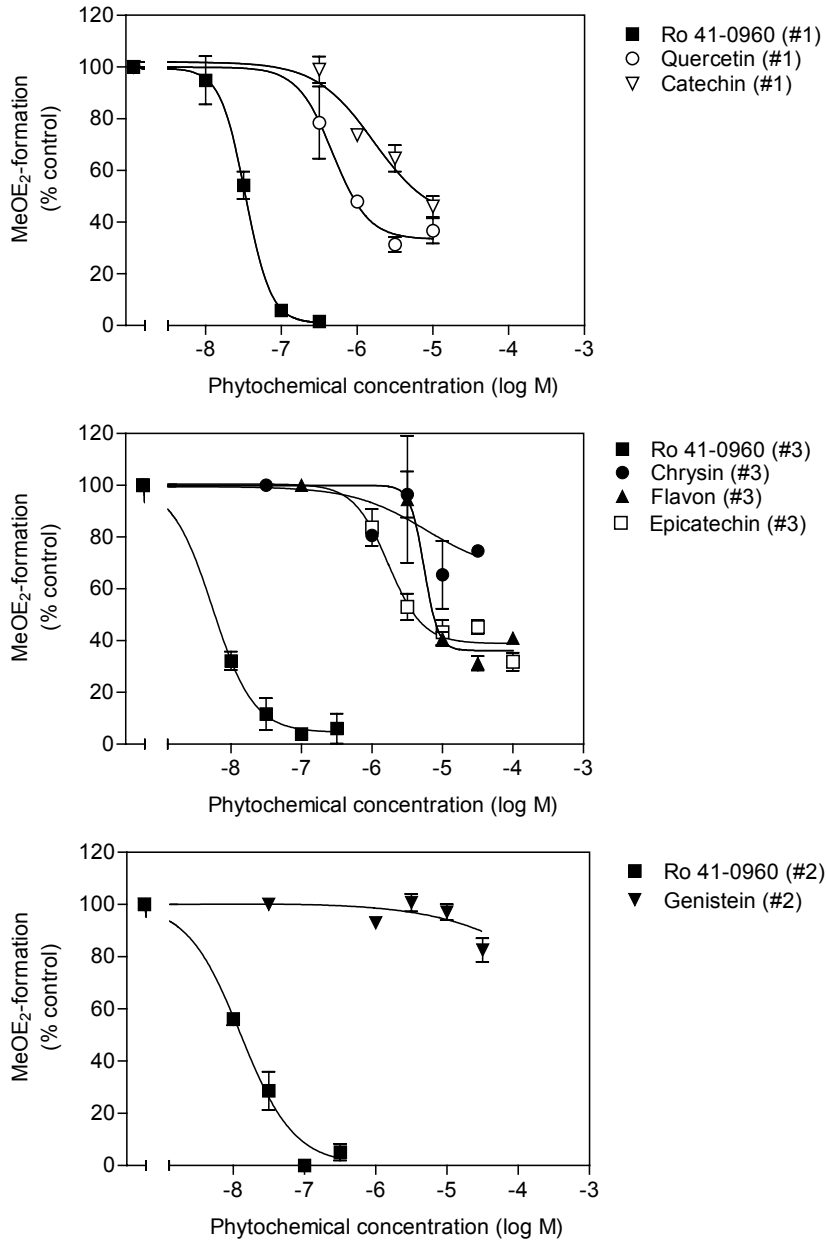

Figure 3.3 Effect of various phytochemicals on COMT activity in cytosolic fractions from human mammary tissues. Cytosolic fractions were incubated with an equimolar concentration of 7.5 μM 2- and 4-OHE2. After 30 minutes methoxy estradiols were extracted and analysed by GC/MS. Data are represented as means and range of two duplicate determinations. The numbers in the legend refer to the tissue sample numbers. Open and closed symbols are phytochemicals with and without a catechol structure, respectively.

Because of the large variations among the tissue samples in responsiveness to COMT inibition, the potencies of the phytochemicals to inhibit COMT activity were calculated relative to the inhibitory potency of Ro 41-0960 in the same tissue sample (Table 3.2). For quercetin, catechin, epicatechin and flavone the relative potencies were 0.07 (tissue #1), 0.021 (tissue #1), 0.0026 (tissue #3) and 0.0009 (tissue #3), respectively.

Table 3.2 IC50 values and relative potencies (RP) for COMT inhibition by quercetin (QUE), catechin (CAT), epicatechin (EPI), chrysin (CHR), genistein (GEN) and flavone (FLA) in human mammary tissue cytosol.

	QUE	CAT	GEN	EPI	CHR	FLA
Tissue sample number	1	1	2	3	3	3
IC_{50} (μM) phytochemical[a]	0.48 ± 0.19*	1.64 ± 0.84	NA	1.96 ± 0.44	NA	5.49 ± 1.92
IC_{50} (nM) Ro 41-0960	33.6 ± 5.8	33.6 ± 5.8	11.4 ± 5.0	5.1 ± 2.7	5.1 ± 2.7	5.1 ± 2.7
RP (Ro 41-0960 =1) [b]	0.070	0.021	NA	0.0026	NA	0.0009

[a]Mean IC50 value of two duplicate determinations ± SEM

[b]RPs are calculated by dividing the IC50 value for COMT inhibtion by Ro 41-0960 by the IC50 value of the phytochemical in cytosol from the corresponding tissue sample

*Significantly different from IC50 value for flavone

Catechol estrogen-induced DNA damage in MCF-7 cells

To investigate the implications of possible COMT inhibition by quercetin in whole cells, the effects of quercetin on catechol estrogen-induced DNA damage were studied. MCF-7 cells were exposed to catechol estrogens with or without Ro 41-0960 and quercetin. Then, the amount of DNA damage was determined using the comet assay and methoxy estrogen levels were determined in the culture medium by GC/MS analysis.

An equimolar concentration of catechol estrogens (7.5 μM), catechol estrogens together with quercetin (30 μM) or Ro 41-0960 (10 μM) or a combination of the two COMT inhibitors, did not cause cytotoxicity in MCF-7 cells, as determined by the MTT test (data not shown). Incubation of MCF-7 cells with catechol estrogens, Ro 41-0960 or quercetin alone did not cause a significant increase of DNA damage compared with the solvent vehicle treated cells (Figure 3.4). The extent of background DNA damage was significantly increased (by about 200%) in catechol estrogen exposed cells when COMT was inhibited by 10 μM Ro 41-0960 ($P < 0.05$). When quercetin was added to the cells

together with Ro 41-0960, catechol estrogen-induced DNA damage increased even further in an apparent concentration-dependent manner, compared with Ro 41-0960 treated cells. A concentration-dependent increase of catechol estrogen-induced DNA damage was also seen after incubation with catechol estrogens and quercetin alone. Catechol estrogen-induced DNA damage levels increased 76 and 160% when cells were co-incubated with 10 and 30 μM quercetin, respectively, compared with vehicle treated control cells.

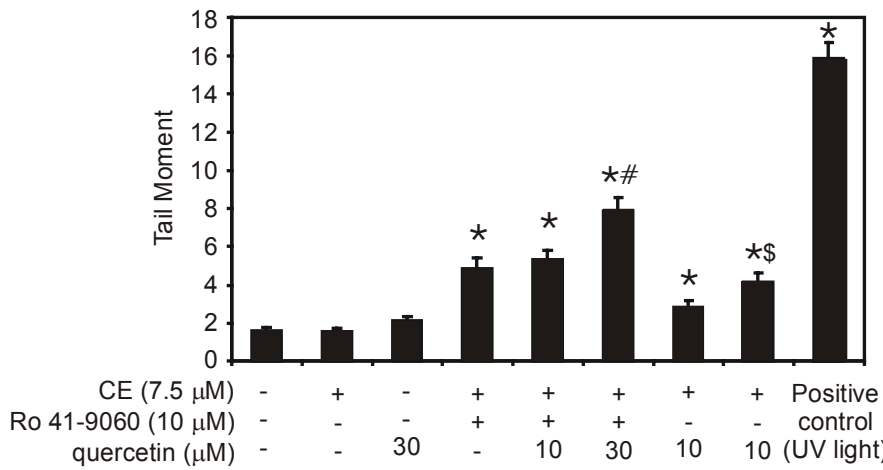

Figure 3.4 Catechol estrogen-induced DNA damage in MCF-7 cells and the effect of COMT inhibition. MCF-7 cells were incubated with 7.5 μM catechol estrogens (CE), 10 μM Ro 41-0960 and/or quercetin (10 or 30 μM). Exposure of the cells to UV light (1 minute) was used as a positive control. The experiment were performed twice in duplicate and 40-50 comets per slide were analysed. Data are represented as mean Tail Moment (comet tail length x % of total DNA in the comet tail) of four slides ± SEM. * significantly different from vehicle control-treated and CE-treated cells ($P < 0.05$). # significantly different from CE+Ro 41-0960 and CE+Ro 41-0960+ quercetin (10 μM)-treated cells ($P < 0.01$). $ significantly different from CE+ quercetin (10 μM)-treated cells ($P < 0.01$) .

To study whether the increase of catechol estrogen-induced DNA damage could be attributed to decreased COMT activity, methoxy estrogen levels were determined in the culture media of the MCF-7 cells used for the comet assay. Constitutive methoxy estradiol formation in MCF-7 cells was 148.1 ± 5.4 and 85.5 ± 4.9 pmol/h/10^6 cells for 2-MeOE2 and 4-MeOE2, respectively (Figure 3.5). Incubation with 10 μM Ro 41-0960 inhibited methoxy estradiol formation by about 98% compared with control cells. Incubation with both Ro 41-0960 and quercetin did not significantly change methoxy estradiol formation compared with Ro 41-0960 alone (data not shown). In culture media of MCF-7 cells incubated with catechol estrogens and quercetin, a concentration-dependent decrease in methoxy estradiol formation compared with control cells was seen. Incubation with 10 μM quercetin resulted in a 46% and 18% decrease in 2- and 4-MeOE2 formation, respectively. For 30 μM the decrease was 82% and 73% for 2- and 4-MeOE2 formation, respectively.

These data indicate that the increase in catechol estrogen DNA damage in MCF-7 cells shown in the comet assay, was due to COMT inhibition resulting in a decreased inactivation of the catechol estrogens as shown by decreased methoxy estradiol formation.

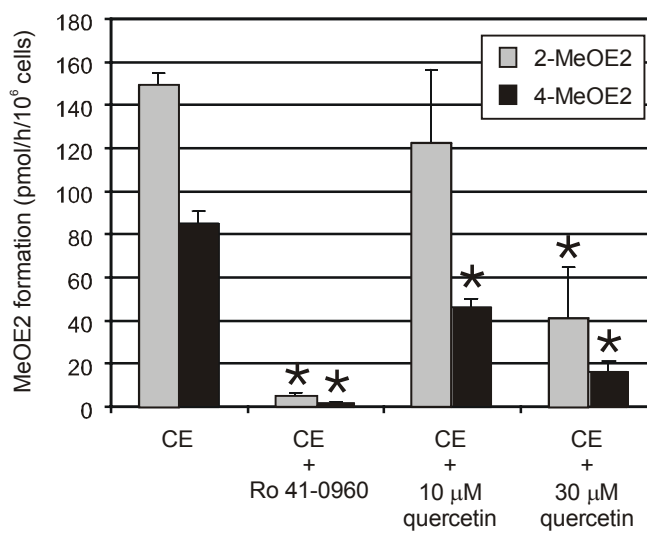

Figure 3.5 Methoxy estradiol formation (pmol/h/10^6 cells) in MCF-7 cells after incubation with 7.5 μM catechol estrogens (CE) or co-incubation with CE and Ro 41-0960 (10 μM) or quercetin (10 or 30 μM). Data are represented as means ± SD (N=3). * significantly lower than CE-treated cells.

DISCUSSION

Constitutive COMT activity and inhibition by Ro 41-0960

In this study we compared constitutive COMT activities in seven healthy human mammary tissue samples. In line with other studies, we found a higher catalytic activity of COMT for 2-MeOE2 formation than for 4-MeOE2 formation [12,27]. As expected for human tissues, there was a large inter-individual variation in constitutive COMT activity. One other study found the rates of 2-MeOE2 formation by COMT in healthy human breast tissues (N=12) to be 6.65 pmol 2-MeOE2/min/mg protein (range of 0.8-14.6 pmol 2-MeOE2/min/mg protein) [28]. These values are in good agreement with the rates of methoxy formation that we found in our study with a mean 2-MeOE2 formation of 8.3 ± 2.0 pmol/min/mg protein and a range of activity of 2.1-19.0 pmol/min/mg protein. In addition to a large variation in constitutive activity, a large variation in inter-individual responsiveness to the selective inhibitor Ro 41-0960 was observed. Generally, in samples with a high constitutive COMT activity, the activity was most readily inhibited by Ro 41-0960. This might be caused by difference in cytosolic expression of COMT. Although we corrected for protein content of the cytosolic fraction, we did not determine the expression level of the COMT enzyme. Another possibility for the large variation in activity, might be a genetic conformational difference between the COMT enzymes. It has been described that a genetic polymorphism of COMT (Val108Met) can result in a high and a low activity isoform of the enzyme [14]. However, several studies have described that reduced activity in the low-activity isoform of COMT is mainly caused by a change in thermostability of the enzyme and not as much by a change in catalytic activity of the enzyme [12,14,27].

Effect of phytochemicals on COMT activity

In this study we showed that phytochemicals with a catechol structure are capable of inhibiting COMT activity in cytosolic fractions of healthy human mammary tissues. Zhu and Liehr described that quercetin acted as inhibitor of COMT activity in hamster kidney cytosol with IC50 values of 8 μM and 2 μM at substrate concentrations of 10 μM 2-OHE2 and 4-OHE2, respectively [20]. We found in this study lower IC50 values for COMT inhibition with quercetin (0.5 μM for both 2-OHE2 and 4-OHE2). This is probably due to differences in study design; we added both 2- and 4-OHE2 together to the cytosol and we used human COMT. Zhu and Liehr concluded that quercetin acted as a non-competitive inhibitor of COMT activity by competing for SAM. Quercetin, and

other phytochemicals containing a catechol structure, have shown to be a substrate for COMT and thus compete for cofactors necessary for *O*-methylation of the substrate, such as SAM [29]. Upon *O*-methylation, a methyl group from SAM is transferred to the catechol substrate resulting in S-adenosyl-L-homocysteine (SAH). An increasing concentration of SAH was shown to (non-competitively) inhibit the association of the methyl donor SAM with COMT. This might also explain why phytochemicals reduced COMT activity to 60% of the control activity while Ro 41-0960 fully inhibited methylation. Ro 41-0960 is a poor substrate for COMT but it binds tightly to the catalytic site of the enzyme thus inhibiting methylation of other substrates without depletion of cofactors [30,31].

The tested phytochemicals with a catechol structure, quercetin, catechin and (-)-epicatechin all reduced COMT activity, but large differences in inhibitory potency were found. This might be a result of structural differences of these phytochemicals, but interindividual variations among the tissue samples in COMT activity and responsiveness toward inhibition might also play a role. Interindividual variation between the tissue samples was especially apparent with flavone which showed COMT inhibition in sample 3, but not in sample 5. In an attempt to correct for interindividual variations in sensitivity toward COMT inhibition, we calculated the potency of a phytochemical relative to the potency of Ro 41-0960 to inhibit COMT activity in the same tissue sample. The RPs varied less than a 100-fold with the RPs of the three phytochemicals with a catechol structure, e.g. quercetin, catechin and epicatechin, being higher than the RP of flavone, the phytochemical without a catechol structure. However, we did not study the potencies of all phytochemicals in all the tissue samples. As a result, it is not clear whether the RPs represent the differences between individuals or differences between the potencies of the phytochemicals. Therefore, the calculated inhibition potencies of the phytochemicals, both absolute and relative to Ro 41-0960, should be considered with care.

Catechol estrogen-induced DNA damage in MCF-7 cells

Although COMT activity was inhibited by the phytochemicals in cytosol from healthy mammary tissues, the question was raised if this inhibition is relevant in a more complex system such as whole cells. We showed that incubation with Ro 41-0960 or quercetin caused a significant increase in DNA damage by catechol estrogens compared with catechol estrogens alone. Chen et al. also showed by the comet assay a low potency of another catechol estrogen, 4-hydroxyestrone (4-OHE_1), to induce DNA damage in

MCF-7 cells [32]. They mainly attributed this low potency to the fact that 4-OHE_1 does not auto-oxidizes and requires oxidative enzymes to generate the highly reactive quinone. However, our study suggests that the inactivation of catechol estrogens plays an important role in the potential of these compounds to cause DNA damage. We showed that a decrease in inactivation of the potentially genotoxic catechol estrogens by COMT inhibition caused a significant increase in catechol estrogen-induced DNA damage. Our data concur with the results described by Lavigne et al. [33]. They found a clear association between catechol estrogen levels and 8-oxo-dG levels in MCF-7 cells after treatment with estradiol and the COMT inhibitor Ro 41-0960. These data show that catechol estrogens have the potencial to induce DNA damage, but that this is strongly dependent on the cellular capacity for inactivation by COMT.

Implications for breast cancer development

Phytochemicals are often studied in relation with hormone-dependent cancers such as breast cancer. The low breast cancer incidence in Asian countries is often attributed to the soy-rich diet which contains high concentrations of isoflavones like genistein. On the other hand, some studies describe a deleterious effect of certain phytochemicals in women with breast cancer [34,35]. Our study shows that phytochemicals with a catechol structure have the potential to reduce COMT activity in cytosol of healthy mammary tissues at concentrations which are well within the range of plasma levels that are reached by regular daily intake. We found for quercetin an IC50 value of 0.5 μM for COMT inhibition. Hollman et al. found plasma levels up to 0.74 μM quercetin after consumption of a meal rich in plant products [19]. It is not unlikely that higher levels can be reached since quercetin plasma levels return to basal levels after about 20 hours, so repeated consumption of high levels of quercetin can result in accumulation in the blood. Furthermore, the COMT inhibiting properties of quercetin also resulted in decreased inactivation of potentially genotoxic catechol estrogens and an increase in catechol estrogen-induced DNA damage. Yet, it is difficult to predict the effects of excessive phytochemical intake in individuals, as large variations in COMT activity and responses to COMT inhibition between the various breast tissue samples were found. Nevertheless, this study shows that adverse effects of high levels of certain phytochemicals are not unlikely. Therefore, high intake of phytochemicals, for example through dietary supplements, should be considered with care.

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4

Dual Role of Catechol-O-Methyltransferase Determines the Genotoxic Potential of Catechol Estrogens in the Malignant MCF-7 and Non-tumorigenic MCF-10A Human Mammary Cell Lines

Manuscript in preparation

Majorie B. M. van Duursen
J. Thomas Sanderson
Sandra Nijmeijer
Martin van den Berg

Abstract

In breast tissue, estradiol is mainly metabolized to the potentially genotoxic catechol estrogens 2-hydroxyestradiol (2-OHE2) and 4-hydroxyestradiol (4-OHE2) by cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1), respectively. A major inactivation step for the catechol estrogens is conversion to their methoxy derivatives 2-methoxyestradiol (2-MeOE2) and 4-methoxyestradiol (4-MeOE2) by catechol-O-methyltransferase (COMT). In this study we investigated the implications of impaired COMT activity on the tumorigenic properties of the catechol estrogens in the malignant MCF-7 and the nontumorigenic MCF-10A human mammary epithelial cell lines. In both cell lines, 4-OHE2 had no significant effects on cell proliferation, cell cycle status and apoptosis up to 10 μM. It did, however, concentration-dependently induced DNA damage, but only when COMT was inhibited by Ro 41-0960. On the other hand, 2-OHE2 inhibited cell growth, caused only a slight induction of DNA damage and induced cell cycle arrest and apoptosis. However, all these effects were attenuated when COMT was inhibited by Ro 41-0960, indicating that these effects were mediated by 2-MeOE2. This was confirmed by adding 2-MeOE2 directly to the cells. 2-MeOE2 concentration-dependently inhibited cell growth with IC50 values of 1.5 μM and 2.7 μM in the MCF-7 and MCF-10A cell line, respectively. In MCF-7 cells, 10 μM 2-MeOE2 decreased catechol estrogen-induced DNA damage by 80%, but this protective effect could not be explained by the induction of cell cycle arrest and apoptosis. In addition, the effects on cell cycle were more pronounced in MCF-7 than in MCF-10A cells, indicating a cell type-specific mechanism of 2-MeOE2 action.

These results show that COMT plays a crucial and dual role in the determination of the genotoxic potential of the catechol estrogens; it detoxifies the catechol estrogens and it converts 2-OHE2 to the anti-tumorigenic 2-MeOE2. Further studies should be performed to clarify the molecular, cell type-specific actions of 2-MeOE2 and to investigate the role of COMT in breast carcinogenesis.

INTRODUCTION

Estrogens play a key role in breast tumor development. The role of estrogens in breast carcinogenesis has been suggested to be dual [1]. Estrogens may act as a direct carcinogen through estrogen receptor (ER)-mediated processes. ER-binding of estrogens can result in the onset of oncogenes and causes cell transformation and proliferation. Enhanced proliferation and DNA replication inceases the chances of errors in the DNA during replication. This results in increased accumulation of mutations, which can ultimately lead to neoplastic changes and tumor development. Estrogens may also act as tumor initiators through their oxidated metabolites, which can induce damage to cellular macromolecules such as DNA. Epidemiological evidence suggests that alterations in estrogen metabolism are associated with breast cancer risk. In breast tissue, estradiol can be metabolized to the catechol estrogens 2-hydroxyestradiol (2-OHE2) and 4-hydroxyestradiol (4-OHE2) by cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1), respectively. Catechol estrogens are potentially genotoxic compounds. Unless inactivated, catechol estrogens may undergo oxidation to reactive quinones that can form DNA adducts [2]. Quinones of 2-OHE2 can form stable DNA adducts that remain in the DNA unless repaired, but quinones of 4-OHE2 form depurinating DNA adducts, a potential tumor-initiation event in human carcinogenesis [1-3]. The carcinogenic effects of 4-OHE2, but not 2-OHE2, have been shown both *in vitro* and *in vivo* [3-5]. *Ex vivo* studies showed that estrogen 4-hydroxylation was favored over 2-hydroxylation in neoplastic tissues but not in normal, healthy mammary tissues [6-8].

A major inactivation step for catechol estrogens is conversion to their non-genotoxic methoxy derivatives by catechol-O-methyltransferase (COMT). COMT activity is not easily up- or downregulated [9], but may be activity impaired by catalytic inhibition. Several compounds such as catechol metabolites of PCBs [10] and certain phytochemicals [11,12], have been shown to compete with natural substrates for the enzyme and thus inhibit COMT activity. Variations in COMT activity can also be a result of a genetic polymorphism. A base pair change (G to A) in codon 158 of the *COMT* gene leads to a valine to methionine amino acid change in the enzyme. This change has been associated with a 3- to 4-fold decrease in enzyme activity [13]. Recently, the decreased activity of the low activity form of COMT (COMT-L) has been suggested to result from lower enzyme levels of COMT [14]. Several studies have described an association between COMT-LL and increased breast cancer risk [15,16], while others found no correlation [17,18]. This discrepancy

might be caused by the ethnic differences in genotype distribution and the fact that the impact of a single polymorphism is difficult to establish in an epidemiological study [19-21].

Theoretically, it is not unlikely that COMT activity plays a significant role in the etiology of breast cancer. A study with *ex vivo* breast tissue showed that less *O*-methylation of the catechol estrogens occurred in tissue of breast cancer patients compared with breast tissue from healthy women [8]. Impaired COMT activity is expected to result in a decreased detoxification and subsequent accumulation of potentially genotoxic catechol estrogens. The accumulation of catechol estrogens was shown to increase catechol estrogen-induced DNA damage *in vitro* but also in *in vivo* studies [12,22]. Another important role for COMT appears to be the conversion of 2-OHE2 to 2-MeOE2. In some studies, 2-OHE2 appears to protect against tumor formation [23,24], although this effect is most likely mediated by 2-MeOE2 since *in vivo* and *in vitro* 2-OHE2 is rapidly converted to 2-MeOE2 [25,26]. Furthermore, 2-MeOE2 exerts a variety of antitumor actions such as inhibition of cell proliferation, induction of apoptosis and inhibition of angiogenesis and is evaluated as a potential anticancer agent [27,28].

In this study we investigated the implications of impaired COMT activity on the genotoxic potential of the catechol estrogens in the malignant MCF-7 and the non-tumorigenic MCF-10A human mammary epithelial cell lines. Further, we studied the effect of 2-MeOE2 on cell cycle status in both cell lines and its protective role against catechol estrogen-induced DNA damage in MCF-7 cells.

MATERIALS AND METHODS

Cell culture MCF-7 and MCF-10A cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 0.01 mg/ml insulin and 5% fetal calf serum. MCF-10A cells were cultured in Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) supplemented with 20 ng/ml epidermal growth factor, 0.1 mg/ml insulin, 500 ng/ml hydrocortisone and 5% horse serum. Both media contained 100 U/ml penicillin and 100 µg/ ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

Cell growth Cell growth was determined by measuring the capacity of the cells to reduce MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to the blue-colored formazan [29]. MCF-7 or MCF-10A cells (1 x 10^5) were seeded onto 24 well plates. The next day (t=0), the medium was replaced with medium containing various concentrations of

estrogen metabolites (2-OHE2, 4-OHE2, 2-MeOE2 or 4-MeOE2) or the sovent vehicle (methanol, 0.1% v/v). Cell growth was determined at t = 0 and after 5, 24, 48, or 72 hours of incubation with the estrogen metabolites. Then, the incubation medium was replaced with medium containing 1 mg MTT/ml. After 30 minutes, the medium containing MTT was removed and the cells were washed twice with warm PBS. Formazan was extracted by adding 1 ml isopropanol at room temperature. Formazan formation was measured spectrophotometrically at an absorbance wavelength of 595 nm. Cell growth was calculated using the vehicle control treated cells with the same incubation time as 100% growth.

Single-cell gel electrophoresis (comet) assay Catechol estrogen induced DNA damage was determined using the single-cell electrophoresis (comet) assay as described by Singh et al. with some modifications as described elsewhere [11,30]. Briefly, cells were exposed for 5 hours to the test compounds. Then, the medium was removed, the cells were trypsinized and resuspended in medium containing 5% fetal calf serum. A 10-µl aliquot of the cell suspension was mixed with 0.5% low melting agarose and spread onto a frosted slide covered with 1.5% normal melting agarose. The slides were placed in freshly prepared cold lysis solution for one hour at 4°C, then placed horizontally in an electrophoresis unit containing cold electrophoresis solution for 25 minutes and electrophoresed for 25 minutes at 25 V, 290-310 mA. Finally, the slides were washed with a sterile neutralization buffer and dehydrated in 100% ethanol. The slides were kept in a dark box at 4°C until analysis. Prior to analysis, the slides were stained with ethidium bromide. Analysis was performed under a fluorescence microscope using a 20x objective and a filter of 450-490 nm equipped with a digital camera. Of each treatment 175-200 cells (four slides per treatment, 40-50 cells per slide) were analyzed and the tail moment (comet tail length x % tail DNA) was determined using the PC image-analysis program Casp described by Konca et al. [31].

Cell cycle status MCF-7 or MCF-10A cells (4 x 10^5 cells/well in a 12-well plate) were incubated with estrogen metabolites for 5, 20 or 48 hours. After incubation, cells were trypsinized and washed twice with PBS. Then, the cells were fixed by incubating the cells with 500 µl ice-cold ethanol (70%) for 30 minutes at room temperature and subsequently treated with RNase A (0.1 mg/ml) for 15 minutes at 37 °C. Finally, the cells were washed with PBS and stained with propidium iodide (PI, 0.05 mg/ml) for 10 minutes at room temperature. PI-elicited fluorescence was measured using a FACScan flow cytometer (Becton-Dickinson, Erembodegem, Belgium).

Data analysis Data were analysed using Prism 3.0 (GraphPad Software, San Diego, CA, USA). Statistical significance of differences of the means were determined by one-way ANOVA analysis followed by a Tukey-Kramer multiple comparisons test or Student's t-test. Differences were considered statistically significant with $P < 0.05$.

RESULTS

Cell growth

The number of MCF-7 and MCF-10A cells continued to increase compared with t = 0 up to 72 hours, regardless of their treatment, indicating that a decrease in cell growth was not due to cytotoxicity (data not shown). The effects of the tested estrogen metabolites on cell proliferation were similar in the MCF-7 and MCF-10A cell lines. 4-OHE2 had no significant effect up to 10 μM (Figure 4.1A and 4.2A), while 2-OHE2 decreased cell growth. The maximum decrease in cell growth by 10 μM 2-OHE2 was about 35% an 48% in MCF-7 and MCF-10A cells, respectively. The growth inhibiting effect of 2-OHE2 in MCF-7 and MCF-10A cells was attenuated when COMT activity was inhibited by Ro 41-0960 (Figure 4.1A and 4.2A, grey squares), suggesting that this effect of 2-OHE2 was attributable to 2-MeOE2. This was confirmed by exposing the cells directly to the methoxyestradiols (Figure 4.1B and 4.2B). Cell growth was concentration-dependently decreased by 2-MeOE2 with IC_{50} values of 1.5 μM and 2.7 μM 2-MeOE2 in the MCF-7 and MCF-10A cell line, respectively. 4-MeOE2 had no significant effect on cell growth in either cell line up to 10 μM.

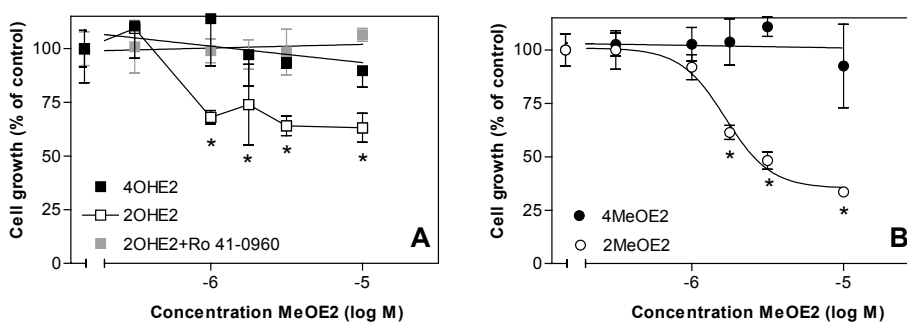


Figure 4.1 MCF-7 cell growth at various concentrations of (A) 4-OHE2 (black squares), 2-OHE2 (white squares) or 2-OHE2 with Ro 41-0960 (grey squares) or (B) 4-MeOE2 (black circles) or 2-MeOE2 (white circles). Cell growth was determined by MTT assay after 72 hours of incubation. Data are represented as mean ± SD (N=4). * Significantly different from solvent vehicle-treated cells ($P < 0.05$).

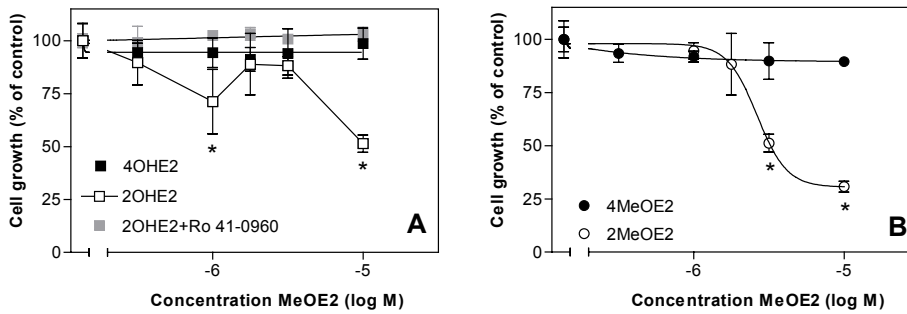


Figure 4.2 MCF-10A cell growth at various concentrations of (A) 4-OHE2 (black squares), 2-OHE2 (white squares) or 2-OHE2 with Ro 41-0960 (grey squares) or (B) 4-MeOE2 (black circles) or 2-MeOE2 (white circles). Cell growth was determined by MTT assay after 72 hours of incubation. Data are represented as mean ± SD (N=4). * Significantly different from solvent vehicle-treated cells ($P < 0.05$).

Catechol estrogen-induced DNA damage

The ability of catechol estrogens to induce DNA damage was determined by the comet assay. Without COMT inhibition, no differences were observed between DNA damage levels in vehicle control-, catechol estrogen- or Ro 41-0960-treated cells (data not shown). However, when COMT was inhibited by Ro 41-0960, increasing concentrations of 4-OHE2 caused a log-linear increase of the level of DNA damage in both MCF-7 (Figure 4.3A) and MCF-10A (Figure 4.3B) cells. In contrast, 2-OHE2 did not increase DNA damage significantly in MCF-7 cells. In MCF-10A cells, the level of DNA damage caused by 2-OHE2, was higher than in MCF-7, but was still less effective than that caused by 4-OHE2 (Figure 4.3B).

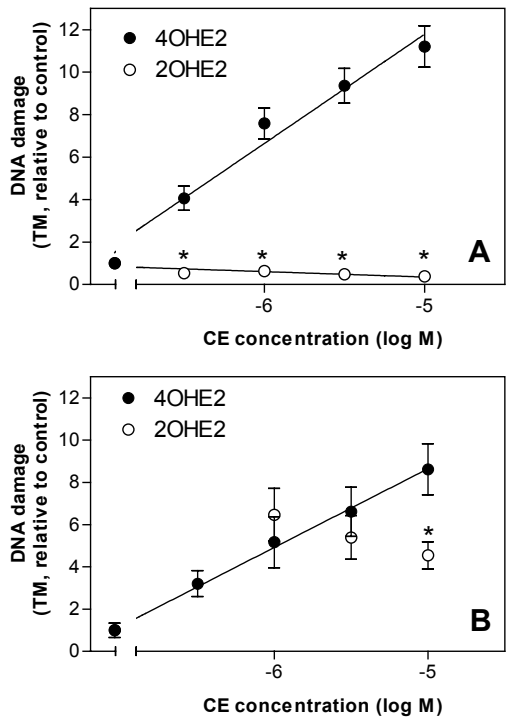


Figure 4.3 Catechol estrogen induced DNA damage in (A) MCF-7 and (B) MCF-10A cells. Cells were incubated with various concentrations of 2-OHE2 or 4-OHE2 for 5 hours. DNA damage was determined using the comet assay and is expressed as Tail Moment (TM, % of total DNA in tail x tail length), relative to vehicle control treated cells ± SEM of two duplicate experiments. * Significantly different from 4-OHE2-treated cells at the same concentration ($P < 0.05$).

We further aimed to investigate whether the protection COMT activity provides against catechol estrogen-induced DNA damage was mediated through the detoxification of the catechol estrogens alone or could also be an effect of the formed methoxyestrogens. MCF-7 cells were incubated with 2-OHE2 and 4-OHE2 (0.5 μM of each), Ro 41-0960 (10 μM) and 2- or 4-MeOE2 (5 or 10 μM). This showed that 2-MeOE2 exerted a direct protective effect against catechol estrogen-induced DNA damage (Figure 4.4). At 5 μM and 10 μM, 2-MeOE2 decreased the levels of catechol estrogen-induced DNA damage with 73.5 and 81.5 %, respectively, to background levels. 4-MeOE2 did not have a significant effect on catechol estrogen-induced DNA damage levels up to 10 μM.

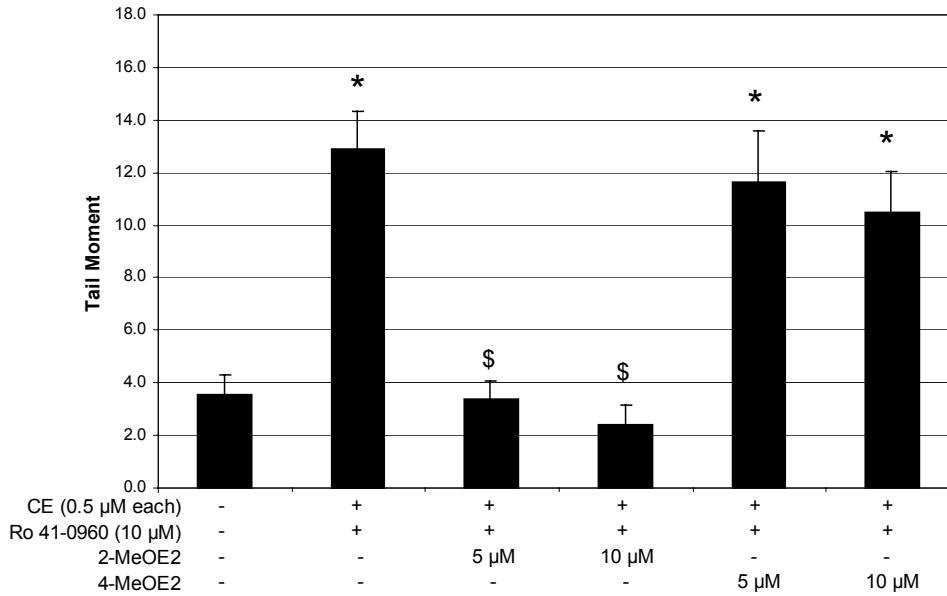
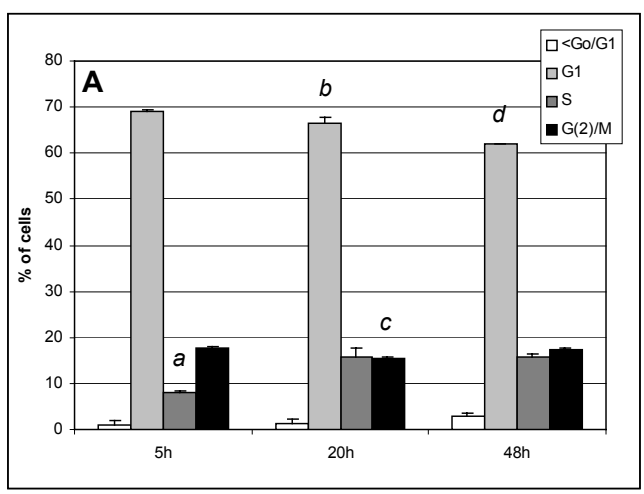


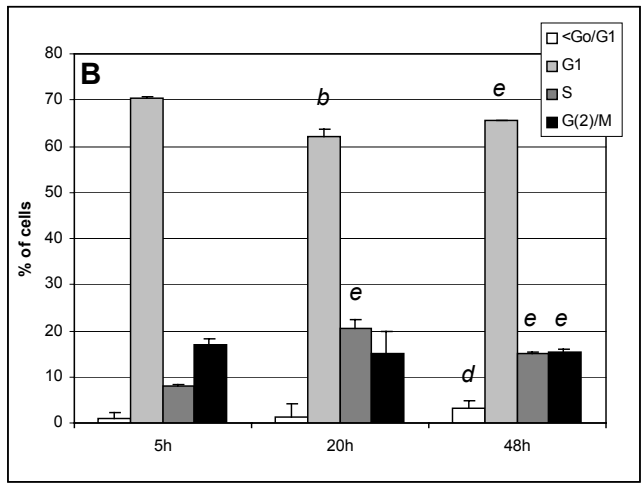
Figure 4.4 Protective effect of 2-MeOE2, but not 4-MeOE2, against catechol estrogen-induced DNA damage in MCF-7 cells. DNA damage (Tail Moment) in MCF-7 cells after a 5-hour treatment with the vehicle control (0.17 % v/v methanol), or a combination of catechol estrogens (CE, 0.5 µM of each catechol estrogen), Ro 41-0960 (10 µM) and 2-MeOE2 or 4-MeOE2 (5 or 10 µM) as stated in the figure. Data are represented as mean Tail Moment (TM, % of total DNA in tail x tail length) ± SEM of two duplicate experiments. * Significantly different from vehicle control treated cells ($P < 0.05$). $ Significantly different from catechol estrogen+Ro 41-0960 treated cells ($P < 0.05$).

Cell cycle status

To further investigate the protective effect of 2-MeOE2 against catechol estrogen-induced DNA damage, the cell cycle status of MCF-7 cells was determined. Since 2-MeOE2 causes cells to arrest in the G(2)/M phase in several cell lines [32-34], we hypothesized that this protects the cells from catechol estrogen-induced DNA damage.

MCF-7 cells were incubated with catechol estrogens and Ro 41-0960 and either methanol or 10 µM 2-MeOE2, identical to the treatment of the cells in the comet assay, for 5, 20 or 48 hours. The number of solvent vehicle treated cells in the G1 phase showed a small, but significant, decline from 69.1% after 5 hours to 66.4% and 62.0% after 20 and 48 hours, respectively (Figure 4.5A). The changes in the number of cells in the G1 phase were complemented by the percentage of cells in the <G1/G0, S or G(2)/M phase, but there was no clear trend apparent over time. Incubation of the cells with both catechol estrogens and Ro 41-0960 caused some variation in the number of cells in the cell cycle phases compared with solvent vehicle-treated cells and with incubation time, but again no clear trend was noticeable (Figure 4.5B). On the other hand, a minor, but significant effect of 2-MeOE2 on cell cycle status was observed after a 5-h incubation (Figure 4.5C). This effect became more pronounced after a longer incubation period. 2-MeOE2 caused a time-dependent decrease in number of cells in the G1 phase and consequently increased the amount of cells in the G(2)/M phase and the amount of apoptotic cells (<G1/G0).





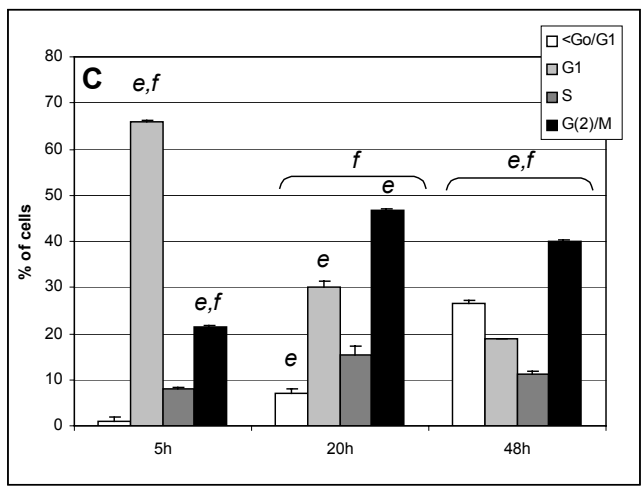


Figure 4.5 Time-dependent effect of 2-MeOE2 on cell cycle status catechol estrogen-treated cells. MCF-7 cells were incubated with (A) the solvent vehicle (0.14 % v/v methanol), (B) catechol estrogens (CE, 0.5 μM of each catechol estrogen) and Ro 41-0960 (10 μM) or (C) catechol estrogens, Ro 41-0960 and 2-MeOE2 (10 μM) for 5, 20 or 48 hours. Data are represented as mean % of total cells ± SD of three determinations. Significantly different ($P < 0.05$) from, *a,* 20-h and 48-h treatment; *b*, 5-h treatment; *c*, 5-h and 48-h treatment; *d*, 5-h and 20-h treatment; *e*, number of solvent vehicle treated cells in the same phase at the same incubation time; *f*, catechol estrogen and Ro 41-0960 treated cells in the same phase at the same incubation time. Note, % of cells was significantly different among all phases in the 2-MeOE2-treated cells at all three incubation times (Figure 4.5C).

To stress the dual role of COMT activity in the determination of the genotoxic potential of the catechol estrogens, MCF-7 cells were incubated with 2-OHE2, 2-MeOE2, 4-OHE2 or 4-MeOE2, separately, with or without Ro 41-0960. To investigate whether the induction of cell cycle arrest and apoptosis by 2-MeOE2 were cell-type specific, the same experiment was performed with MCF-10A cells. After an incubation of 20 hours, 4-OHE2 and 4-MeOE2 had no significant effects on the cell cycle status in MCF-7 cells or MCF-10A cells (data not shown). In contrast, a 20-h incubation of MCF-7 cells with 2-OHE2 or 2-MeOE2 alone significantly decreased the number of cells in the G1 phase (by about 20 %) and concomitantly increased the number (with about 15%) of cells in the G(2)/M phase and, to a lesser extent, the number of cells in the S phase (increase of about 5%) (Figure 4.6A). Only the number of cells in the S phase was slightly different between the 2-MeOE2 and 2-OHE2 treated cells. However, when COMT was inhibited by Ro 41-0960, the effect of 2-OHE2 on cell cycle status was no longer observed and the overall cell cycle status, exept for the minor differences in number of cells in the S phase, was not significantly different from the solvent vehicle treated cells. In MCF-10A cells, the effects of the estrogen metabolites on cell cycle were somewhat different from those in MCF-7 cells. The effects of 2-OHE2 with or without Ro 41-0960 on MCF-10A cell cycle were similar, though less effective, than in MCF-7 cells (Figure 4.6B). Incubation of MCF-10A cells with 2-OHE2 resulted in a decrease of 16.5% of cells in the G1 phase and S phase (3.5%) and concomitantly an increase in cells in the G(2)/M (14.3%) and <G1/G0 (4.9%) phase. The effects of 2-OHE2 were attenuated by COMT inhibition, but not fully abolished. In addition, 2-MeOE2 only caused a slight decline in cells in the G1 (3.3%) and S phase (2.9%) and a minor induction of cells in the G(2)/M phase (2.5%) apoptosis (3.7%).

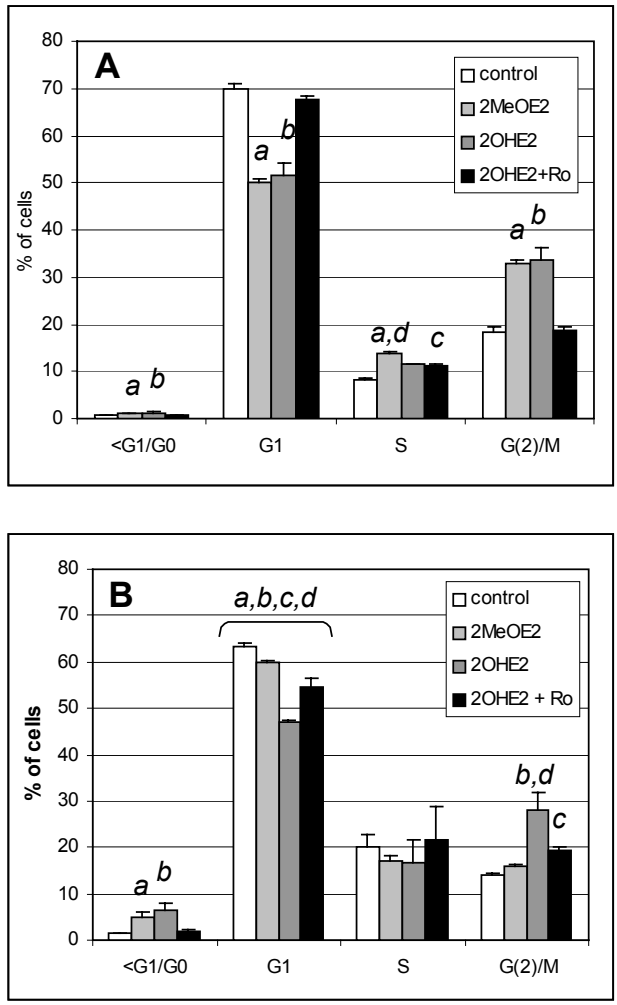


Figure 4.6 Effect of 10 μM 2-MeOE2 or 10 μM 2-OHE2 (with or without 10 μM Ro 41-0960, Ro) on cell cycle status in (A) MCF-7 cells or (B) MCF-10A cells after a 20-hour incubation. Bars represent mean % of total cells ± SD of three determinations. Significant differences ($P < 0.05$): *a,* 2-MeOE2 vs solvent vehicle control and 2-OHE2+Ro 41-0960; *b,* 2-OHE2 vs solvent vehicle control and 2-OHE2+Ro 41-0960; *c,* 2-OHE2+Ro 41-0960 vs solvent vehicle control; *d,* 2-MeOE2 vs 2-OHE2.

DISCUSSION

The 2-hydroxyestrogens have been called the 'good' estrogens and 4-hydroxyestrogens the 'bad' estrogens. However, mounting evidence suggest that the 'good estrogen'-properties of 2-hydroxyestradiol should be attributed to its methoxy conjugate. 2-MeOE2 has been shown to exert many anti-tumor actions in studies in vitro and in vivo [27,35] and it is currently under investigation as an anticancer agent [28,36]. This implicates that COMT, the enzyme responsible for the conversion of 2-OHE2 to 2-MeOE2, plays a key-role in estrogen metabolism and the determination of the genotoxic potential of the catechol estrogens.

MCF-7 and MCF-10A cell lines

We used a malignant and a nontumorigenic cell line in this study. MCF-7 is a widely used neoplastic breast epithelial cell line, the growth of which is estrogen-dependent. MCF-10A is a human breast epithelial (HBEC) cell line of nonmalignant origin [37]. MCF-10A cells, in contrast to MCF-7 cells, do not form colonies in agar-methocel and are not tumorigenic in severely immuno-comprised mice [38,39]. Furthermore, MCF-10A cells are said to be ER-negative [39,40], though some report low expression of the ER protein [40,41]. MCF-10A cell growth is glucocorticoid- and growth factor- (epidermal growth factor, insulin) dependent [39].

Another important difference between the MCF-7 and MCF-10A cell lines is the isoform of the COMT enzyme. The COMT enzyme is polymorphic and the 158Val → Met transition has been associated with a high and a low activity form of the COMT enzyme [13]. The MCF-7 and MCF-10A cell lines have previously been shown to contain the low and high activity form of COMT, respectively [42]. PCR-RFLP analysis in our lab confirmed these genotypes in the MCF-7 and MCF-10A cell lines (data not shown). However, Goodman et al. showed by Western blotting a lower expression of the COMT protein in MCF-10A cells compared with MCF-7, but they found no differences among the kinetic parameters of COMT of the two cell lines with respect to 2- and 4-OHE2 methylation [42].

Cell proliferation

MCF-7 cell growth is often used to study ability of xenobiotic compounds to elicit ER-mediated effects [43]. Usually, these experiments are performed with estrogen-deprived MCF-7 cells, in order to increase the response toward weak estrogen-like compounds. In our study, however, we determined the effect of the estrogen metabolites on cell growth in

MCF-7 cells that are cultured in normal medium containing fetal bovine serum. In other studies, 2-OHE2 appears to be a weak mitogen in estrogen-deprived MCF-7 cells compared with the parent compound 17β-estradiol [24,44]. In our study, we did not observe this suggested mitogenic effect of 2-OHE2. 2-MeOE2, on the other hand, is a known antiproliferative agent. Several studies have reported the growth inhibiting properties in MCF-7 cells of 2-MeOE2 with IC50 values between 0.81 μM and 52 μM [45-48]. No reports have been found that describe the effects of 2-MeOE2 on MCF-10A cell growth. In our study, MCF-10A cell growth was also concentration-dependently inhibited by 2-MeOE2. MCF-10A cells are believed to have no [39,40] or a very low [40,41] expression of the ER, which suggests no involvement of the ER in the 2-MeOE2-mediated growth inhibition in this cell line. This concurs with the findings of LaVallee et al., who described the anti-proliferative actions of 2-MeOE2 to be independent of the ER [47]. Liu and Zhu, on the other hand, reported ER-mediated mitogenic actions of 2-MeOE2 in estrogen-deprived MCF-7 cells at low (10 – 750 nM) concentrations [48]. This indicates that the background estrogen levels might be important in the determination of the actions of 2-MeOE2.

Catechol estrogen-induced DNA damage

The 4-hydroxyestrogens are suggested to be genotoxic because they can produce depurinating DNA adducts, in contrast with the stable adducts formed by 2-hydroxyestrogens. However, both 2-OHE2 and 4-OHE2 can undergo redox-cycling thus producing reactive oxygen species, which can cause oxidative damage to DNA. This explains why 2-OHE2 also causes DNA damage in our study, though to a lesser extent than 4-OHE2. Lavigne et al. showed a linear relationship between increasing catechol estrogen concentrations (2-OHE2 plus 4-OHE2) and increasing 8-oxo-2'-deoxyguanosine (8-oxo-dG) levels, an oxidized DNA base used as a marker of damage caused by hydroxyl radicals. 2-MeOE2 reduced 8-oxo-dG formation in MCF-7 cells as was expected based on this linear relationship [22]. It was suggested by Dawling et al. that methoxyestrogens act as noncompetitive inhibitors of CYP1A1 and CYP1B1 activity, which would result in decreased formation of potentially genotoxic catechol estrogens and a subsequent decrease in DNA damage levels [49]. However, we found no protective effect of 4-MeOE2 in our study. In addition, the protective effect by 2-MeOE2 against catechol estrogen-induced DNA damage was also observed when we added hydroxyestrogens directly to the cells, thus avoiding the oxidative metabolism of estradiol. Therefore, this protective effect of 2-MeOE2 appears not to be a result of decreased catechol estrogen formation, but to result from a direct action of 2-MeOE2. We hypothesized that the protection against

catechol estrogen-induced DNA damage by 2-MeOE2 might be a result of a shift in cell cycle phase. The G(2)/M phase in the cell cycle is very important for the maintenance of genomic stability because in this phase the DNA is condensed and protected against genotoxic compounds.

Cell cycle analysis

Several studies have described the arrest of human cancer cell lines, such as prostate, breast, bone and lung, in the G(2)/M phase after incubation with 2-MeOE2 [32-34]. This effect is probably due to the fact that 2-MeOE2 inhibits microtubule polymerization, a process involved in pulling the chromosomes to the opposite poles during mitosis [50]. The induction of apoptosis by 2-MeOE2 is a result of cells that are arrested in the G(2)/M phase and are ultimately eliminated. In addition, 2-MeOE2 can also induce apoptosis directly through several signaling pathways, such as p53- or caspase-mediated pathways [27,35,51]. Yet, most studies describe the effects of 2-MeOE2 after incubation times of at least 24 hours. We also showed a strong effect of 2-MeOE2 on cell cycle arrest and apoptosis after incubation times of 20 and 48 hours in MCF-7 cells. However after a 5-h incubation, 2-MeOE2 induced cell cycle arrest and apoptosis only slightly, while the protection by 2-MeOE2 against catechol estrogen-induced DNA damage in MCF-7 cells was already profound. This suggests that it is unlikely that 2-MeOE2-induced cell cycle arrest and apoptosis can fully account for the protection against DNA damage. Shimada et al. investigated the short-term actions of 2-MeOE2 in prostate cancer cells [52]. They described the suppressing effect of 2-MeOE2 within one hour of incubation on nuclear factor kappa B, a transcription factor involved in the regulation of immune response, inflammation and cell cycle. This shows that 2-MeOE2 does have the ability to rapidly affect cell signaling pathways, which might protect the DNA against genotoxic compounds, such as catechol estrogens. In addition, 2-MeOE2 might also act as an antioxidant and thus protect against catechol estrogen-induced DNA damage [53].

The actions of 2-MeOE2 were reported to occur only in malignant cell types and not in normal, non-tumorigenic cell types [35]. In the present study, we found this cell type-specific action also for the effects on the cell cycle.. We showed a 2-MeOE2-mediated arrest in the G(2)/M phase of the cell cycle and an induction of apoptosis in the malignant MCF-7 cells, but not in the non-tumorigenic MCF-10A cells. Alternatively, this could be attributed to the high activity of 17β-hydroxy steroid dehydrogenase (17β-HSD) in MCF-10A cells. 17β-HSD converts estradiols (including methoxyestradiol) to estrones (methoxyestrones) [54] and 2-MeOE1 does not have the same growth inhibiting effects as

2-MeOE2 [55]. On the other hand, the cell type-specificity was not observed for the 2-MeOE2-mediated antiproliferative activity. In our study, 2-MeOE2 inhibited cell proliferation in both MCF-7 and MCF-10A cell lines. Yet, it is difficult to explain the differences in 2-MeOE2 actions between the MCF-7 and MCF-10A cell lines, because of the differences in estrogen metabolism. In addition, the molecular mechanisms of 2-MeOE2 actions require further investigations, as they are broad and not yet completely understood [27].

Implications for breast cancer

In our study we showed that in the malignant MCF-7 cell line, the anti-tumor actions of 2-OHE2 (inhibition of growth proliferation, induction of cell cycle arrest and apoptosis and protection against DNA damage) are impaired when COMT is inhibited and can largely be explained by 2-MeOE2 actions. This shows that COMT activity plays an important role in determining the genotoxic potential of the catechol estrogens. It appears that COMT has a dual role in the protection against catechol estrogen-induced tumorigenic events. Firstly, it detoxifies potentially genotoxic catechol estrogens. Secondly, it produces the anti-tumor agent 2-MeOE2, a physiologically relevant, endogenous estrogen metabolite. This implies that reduced COMT activity, for example due to catalytic inhibition or a genetic polymorphism, is expected to play an important role in breast carcinogenesis.

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5

Cytochrome P450 1A1 and 1B1 in Human Blood Lymphocytes Are Not Suitable as Biomarkers of Exposure to Dioxin-like Compounds: Polymorphisms and Interindividual Variation in Expression and Inducibility

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Majorie B. M. van Duursen
J. Thomas Sanderson
Martin van den Berg.

Abstract

Cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1) are phase I enzymes the expression of which can be affected by many environmental compounds, including dioxins and dioxin-like compounds. Because CYP1A1 and CYP1B1 expression can easily be determined in peripheral blood lymphocytes, it is often suggested as biomarker of exposure to these compounds. In this study we investigated the interindividual differences in constitutive and induced CYP1A1 and, to a lesser extent, CYP1B1-catalyzed ethoxyresorufin-O-deethylase (EROD) activity and *CYP1A1* and *CYP1B1* gene expression in human blood lymphocytes in a group of ten non-smoking females. Freshly isolated lymphocytes were exposed to the most potent dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or the less potent dioxin-like polychlorinated biphenyl 126 (PCB126). In addition, we determined the occurrence of the *CYP1A1*2A* and *CYP1B1* Val432Leu polymorphisms. All individuals showed a concentration-dependent increase of EROD activity by TCDD, which was significantly correlated with an increase in CYP1A1, but not CYP1B1 expression. The maximum induced EROD activity by 10 nM TCDD was very different among the individuals, but the EC50 values were about the same. PCB126 also caused a concentration-dependent increase of EROD activity, but was a factor 100-1000 less potent than TCDD among the individuals. The allele frequencies for *CYP1A1*2A* and *CYP1B1* Val432Leu reflected a normal Caucasian population, but the polymorphisms had no apparent effect on the expression and activity of these enzymes in this study. Our study shows a large variability in constitutive and induced EROD activity, and CYP1A1 and CYP1B1 expression in human lymphocytes. In addition, dioxin concentrations at which effects were observed in our *in vitro* study are about 10-fold higher than the human blood levels found *in vivo*, indicating that EROD activity and CYP1A1 and CYP1B1 expression in human lymphocytes might not be applicable as biomarkers of exposure to dioxin and dioxin-like compounds

INTRODUCTION

Cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1) are phase I enzymes that can be found in many tissues including peripheral blood lymphocytes. They are involved in the metabolic activation of polycyclic aromatic hydrocarbons (PAHs), but also in the oxidative metabolism of estrogens to potentially genotoxic catechol estrogens [1]. CYP1A1 and CYP1B1 expression is regulated through the aryl hydrocarbon receptor (AhR)-mediated pathway. Several environmental contaminants including PAHs and persistent organochlorine pollutants, such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins (PCDDs), are AhR agonists and can affect the expression of CYP1A1 and CYP1B1. CYP1A1 and CYP1B1 expression can be readily determined in peripheral blood lymphocytes, making them potential candidates for use as non-invasive biomarkers of exposure to environmental compounds with dioxin-like properties. In order to use CYP1A1 and CYP1B1 expression for this purpose, several confounding factors must be considered, including age, gender and cigarette smoking. In addition, several genetic polymorphisms have been identified in the *CYP1A1* and *CYP1B1* genes that might affect gene expression or catalytic activity, thereby complicating the use of these genes as biomarkers of exposure. The *CYP1A1*2A* polymorphism, a T to C transition in the 3'-untranslated region (UTR) of the *CYP1A1* gene, is often studied in relation to CYP1A1 inducibility, but the studies are inconclusive about its effect [2-5]. An amino acid substitution in codon 432 (Val to Leu) of the *CYP1B1* gene is associated with a lower catalytic activity of the enzyme [6,7]. In addition, some studies report an association between the Leu/Leu genotype and higher expression levels [8], while others find no effect of this polymorphism on expression levels [7].

Although CYP1A1 and CYP1B1 expression are often investigated as biomarker of exposure, the polymorphisms of *CYP1A1* and *CYP1B1* are seldom considered. To complicate matters further, polymorphisms are ethnically related [9], which makes comparison of the various studies difficult. In a study of Hungarian workers (n = 161) from an aluminium production plant exposed to polycyclic aromatic hydrocarbons, the number of PAH-DNA adducts in lymphocytes was not different among the *CYP1A1* and *CYP1B1* genotypes [10]. Hanaoka *et al.* found a correlation between the presence of at least one *CYP1B1* 432Leu allele and higher expression of CYP1B1 in the lymphocytes from 37 Chinese coke oven workers [11]. Lin *et al.* studied CYP1A1 and CYP1B1 expression in human lymphocytes of 32 Taiwanese male and female subjects including both smokers and non-smokers [12]. They showed a large interindividual variation in induction of

CYP1A1 and CYP1B1 upon *in vitro* exposure of the lymphocytes to PAHs. Spencer et al. showed that CYP1B1 mRNA levels in human lymphocytes of ten North Carolina volunteers (male and female, both smokers and non-smokers), concentration-dependently increased upon *in vitro* exposure to 2,3,7,8-tetrachloro-p-dibenzodioxin (TCDD) [13].

Interindividual variations in mRNA expression patterns, catalytic activity and polymorphisms are important factors to consider when using CYP1A1 and CYP1B1 as biomarkers of exposure, yet little is known about these factors. The objective of this study was to investigate the interindividual differences in concentration-response curves for EROD activity and CYP1A1 and CYP1B1 gene expression in human lymphocytes upon induction with TCDD or the less potent dioxin-like polychlorinated biphenyl 126 (PCB126). We studied the possible influence of the *CYP1A1*2A* and *CYP1B1* Val432Leu polymorphisms on EROD activity and CYP1A1 and CYP1B1 expression levels. To minimize the role of confounding factors, we recruited ten healthy female Caucasian volunteers who were all non-smokers. Furthermore, the relevance of CYP1A1 and CYP1B1 in human lymphocytes as biomarker of exposure to environmental factors was addressed by comparing the concentrations at which we found effects on EROD activity and mRNA levels *in vitro*, with the concentrations of dioxins and dioxin-like compounds that can be found in human blood.

MATERIALS AND METHODS

Study subjects Venous blood was collected from 10 healthy female individuals in two 7-ml blood collection tubes containing EDTA (BD Vacutainer, Franklin Lakes, NJ, USA). All individuals were Caucasian female non-smokers currently living in Utrecht, The Netherlands, with an average age of 26.4 years (range 24.1-28.7 yr.).

Lymphocyte isolation Lymphocytes were isolated from fresh blood samples within 1 hour of collection using Ficoll-Paque isolation according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA). One ml of whole blood was stored at -70°C for DNA analysis. The lymphocytes were washed twice with warm PBS and then suspended in culture medium consisting of phenol red-free RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1.5% phytohaemagglutin (PHA) and 10% (v/v) fetal bovine serum (FBS). The lymphocyte concentration was determined using a coulter counter and the cell number was adjusted to $5x10^5$ cells/ml. A 300 µl aliquot of cell suspension was centrifuged to remove the

medium and the pellet was resupended in 250 µl RNA Instapure (Eurogentec, Maastricht, The Netherlands) and stored at -70°C (non-mitogen stimulated cells). Of the remaining cell suspension, 1-ml aliquots added to two 12-well plates. Then, 1 ml of culture medium was added containing twice the desired concentration of TCDD of solvent vehicle DMSO at a final concentration of 0.1% (v/v). If sufficient lymphocytes were obtained, a second 12-well plate was incubated with various concentrations of PCB126. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. After 72 hours, the culture medium of one plate was replaced with 500 µl RNA Instapure and stored at -70°C and the medium of the other plate was replaced with Tris-buffer for ethoxyresorufin-O-deethylase (EROD) activity determination.

Ethoxyresorufin-O-deethylase (EROD) activity Cells were exposed to 0.5 ml of a 50 mM Tris buffer (pH 7.8) containing 0.9% w/v NaCl, 6.25 mM $MgCl_2$, 5 µM 7-ethoxyresorufin and 10 µM dicumarol. Plates were placed in a 37°C pre-heated FluoStar plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) and fluorescence was measured at an excitation wavelength of 530 nm and emission wavelength of 590 nm every 3 minutes for half an hour. A calibration curve of resorufin was used for quantification of the activity. After determination of EROD activity, the plates were stored at -20°C until protein determination. Protein contents were measured by the method of Lowry *et al.* using BSA as protein standard [14].

RNA isolation and quantitation of yield RNA was isolated using phenol-chloroform extraction. Purity and concentration of the isolated RNA was determined spectrophotometrically at an absorbance wavelength of 260 and 280 nm. RNA samples were stored at -70°C until analysis. RT-PCR conditions, primers and amplification parameters are as described previously [15]. RT-PCR products were separated on a 2% agarose gel and bands were stained with ethidium bromide. Intensity of the ethidium bromide staining was quantified using a FluorImager (Amersham Biosciences, Piscataway, NJ, USA).

PCR and Restriction Fragment Length Polymorphism (RFLP) analysis Genomic DNA was isolated from 300 µl whole blood with a DNA isolation kit (Promega, Madison, WI, USA) according to the manufacturers instructions. Methods and primers for genotype analysis for CYP1A1 MspI and CYP1B1 Val432Leu were adapted from Kawajiri et al. and Tang *et al.* [16-18]. First, a PCR was carried out with 500 ng DNA using the Access RT-PCR System by Promega (Promega, Madison, WI, USA) in a 25 µl reaction volume containing 1x PCR buffer, Taq polymerase (5 U/µl), $MgCl_2$ (2 mM for CYP1A1 and 1.3 mM for CYP1B1), 200 µM dNTPs and 1 pmole of the reverse primer and 1 pmole of the forward

primer. The primers were obtained from Invitrogen (Invitrogen Co., Carlsbad, CA, USA). The PCR reactions started with a denaturing step at 94°C for 3 minutes followed by 30 cycles of 94°C for 1 minute (denaturing), annealing for 1 minute (at 66°C and 59°C for CYP1A1 and CYP1B1, respectively) and 72°C for 1 minute (extension) and ended with a final extension step at 72°C for 7 minutes. Subsequently, 10 µl of PCR product was digested for 3 hours at 37°C with 10 units of *Msp*I or *Eco57*I (Fermentas Inc., Hanover, MD, USA) for detection of the CYP1A1 and CYP1B1 polymorphisms, respectively. As positive control for endonuclease restriction 4.5 µg lambda DNA (Fermentas Inc., Hanover, MD, USA) was used. A volume of 10 µl unrestricted PCR product and 10 µl of restricted PCR product were loaded on a 2.5% non-denaturing agarose gel containing ethidium bromide (0.05 µg/ml). The bands were visualized with UV light and genotypes were determined by visual inspection of the restriction fragments. For CYP1A1, endonuclease digestion of the PCR product (340 bp) resulted in two DNA fragments (200 and 140 bp) when the mutated allele (T→C transition) was present. For CYP1B1, digestion of the PCR product (271 bp) with the mutated allele (G→C transition) yielded two fragments of 166 and 105 bp.

Data analysis Deviations from the Hardy-Weinberg equilibrium were calculated using the two-sided χ^2 test. Statistical significance of differences of the means and variances were determined by one-way ANOVA analysis followed by a Tukey-Kramer multiple comparisons test. Comparison of the concentration-response curves was performed using a F test. Differences were considered statistically significant if $P < 0.05$. Statistical calculations were performed using GraphPad InStat 3.06 (GraphPad Software Inc., San Diego, CA).

RESULTS

EROD activity in cultured lymphocytes

The catalytic activity of CYP1A1 in the cultured lymphocytes was determined by measuring EROD activity. Constitutive EROD activity was low (0.23 ± 0.11 pmol resorufin/min/mg protein), but detectable in all individuals and the activity was concentration-dependently increased by TCDD (Figure 5.1). Maximal induction of EROD activity by 10 nM TCDD was between 10 and 125-fold above constitutive levels in the ten individuals. Statistically significant differences among the minimum and maximum EROD activities were observed among the individuals (Table 5.1). However, the relative responses to TCDD were very similar; when the EROD activity was

calculated as % of the maximum activity, the concentration-response curves overlapped. Relatively little variation was found in the EC50 values for induction of EROD activity, which varied between 0.52 and 3.88 nM. Only the EC50 value in individual 8 was significantly higher than in all the others. This indicates that although wide variation was observed in the maximal response elicited by TCDD (efficacy), the concentration of TCDD necessary to produce 50% of the maximal EROD activity (potency) did not vary greatly among this relatively small and homogeneous group of women.

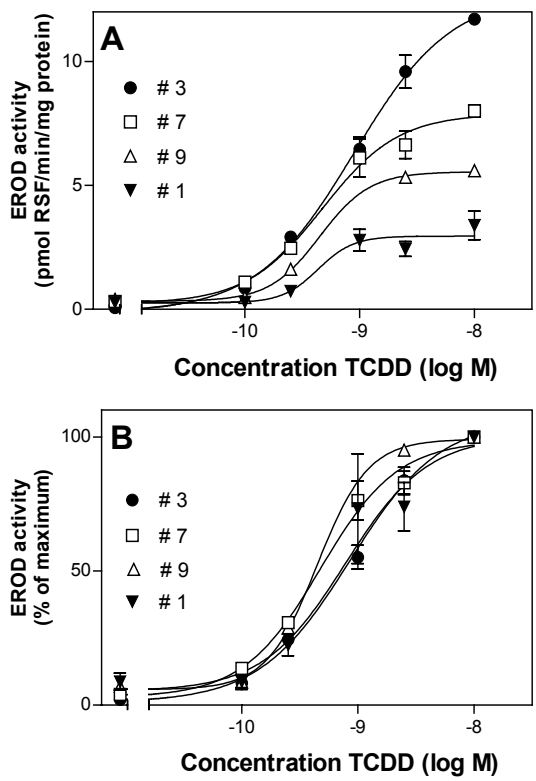

Figure 5.1 Representative concentration-response curves for EROD induction by TCDD in cultured lymphocytes of four individuals. A, EROD activity is expressed as pmole resorufin (RSF)/min/mg protein; B, EROD activity is represented as % of the maximum activity in each individual. Data are represented as means of two determinations and the range.

From five individuals sufficient lymphocytes were obtained and cultured to determine a concentration-response curve for PCB126, the most toxic PCB. In all individuals, PCB126 concentration-dependently induced EROD activity, but only in the lymphocytes of three individuals (number 1, 4 and 8) a maximum response was reached at the highest concentration tested (0.3 μM). In all three individuals, the maximum level of EROD induction reached by PCB126 was about 2-fold lower than the maximum induction caused by 10 nM TCDD (data not shown).

Where possible, the PCB126 concentrations at half maximum induction (EC50 values) were calculated and compared with the potency of TCDD in the same individual. These EC50 values were 59.8, 38.4 and 37.2 nM PCB126, for individual 1, 4 and 8, respectively, resulting in relative potencies (RPs) of 0.011, 0.054 and 0.104. However, considering the differences in maximum EROD induction between the TCDD and PCB126 concentration-response curves, the EROD activity at 25% induction by TCDD was calculated (EC25) and subsequently, the PCB126 concentration necessary to obtain the same level of EROD activity (C25) was determined. In this way, a RP could be calculated for the individuals where the concentration-response curves for PCB126 did not reach an maximum. The RPs for PCB126 calculated using the C25 values varied between 0.002 and 0.014 among the individuals (Table 5.1).

CYP1A1 and CYP1B1 expression in uncultured and cultured lymphocytes

The expression levels of CYP1A1 and CYP1B1 in the lymphocytes were calculated relative to the expression in the solvent vehicle-treated cultured lymphocytes of the same individual. In the uncultured (non-mitogen stimulated) lymphocytes, CYP1A1 transcript levels were too low to be detected. CYP1B1 expression on the other hand, was detectable. After 72 hours of culture in media containing the mitogen PHA, CYP1A1 expression was still lower than CYP1B1, but both were detectable. Only in individual 9, CYP1A1 expression in cultured solvent-vehicle treated lymphocytes was too low to be detected. Mitogen-stimulation increased the expression of CYP1B1 by about 60% (Figure 5.2). Induction by TCDD increased the expression of both genes even further. The induction of CYP1A1 expression by 10 nM TCDD (2.6 ± 1.1 fold) was statistically significantly higher than that of CYP1B1 (1.8 ± 0.5 fold). The differences in maximally induced EROD activity among the individuals was reflected by the CYP1A1 mRNA levels. The level of induction of EROD activity was positively correlated with the level of CYP1A1 expression ($r = 0.67$, $P = 0.04$, $n = 9$. Figure 5.3). For CYP1B1 this correlation was not significant ($r = 0.02$, $P = 0.95$, $n = 10$).

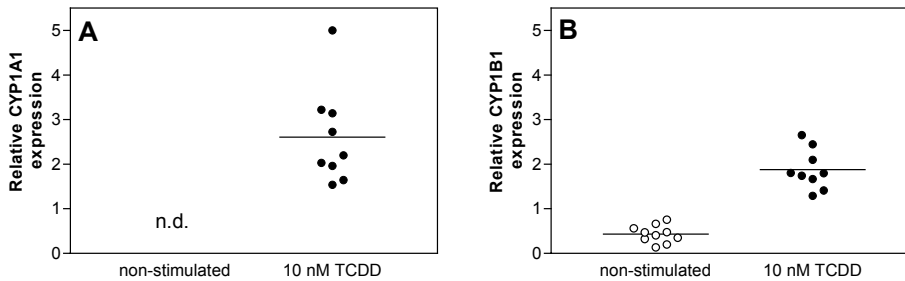

Figure 5.2 CYP1A1 (A) and CYP1B1 (B) mRNA expression in lymphocytes of ten individuals. Expression is calculated relative to CYP1A1 or CYP1B1 expression in lymphocytes cultured for 72 hours in media containing the mitogen PHA and 0.1% v/v DMSO. All data points represent the average expression of two determinations in one individual. The line indicates the mean expression.

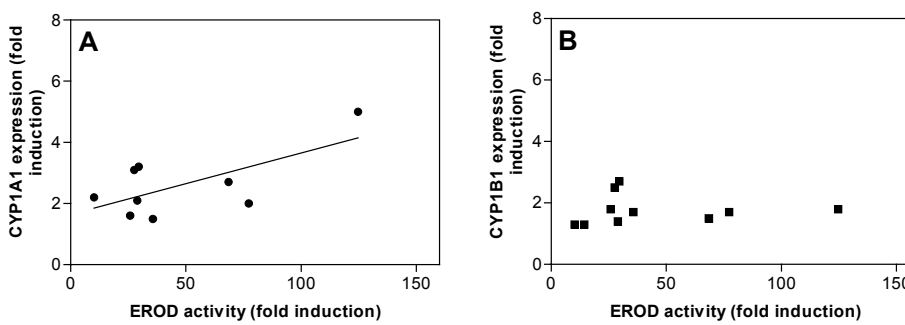

Figure 5.3 Correlation between induction of EROD activity and induction of CYP1A1 (A; $r = 0.67$, $P = 0.04$, $n = 9$) or CYP1B1 (B; $r = 0.02$, $P = 0.95$, $n = 10$) mRNA levels. Data are represented as fold induction at 10 nM TCDD compared with the vehicle control-treated cultured lymphocytes. Every data point represents one individual.

For the individuals 1 and 3, who displayed the lowest and the highest induction of EROD activity at 10 nM TCDD, respectively (Figure 5.1), an entire concentration-response curve was determined for CYP1A1 and CYP1B1 mRNA expression. When this curve was compared with the concentration-response curve for EROD induction in the same individual, a shift of the curve to the left was seen (Figure 5.4) indicating that increased mRNA expression occurred at lower concentrations of TCDD than EROD induction. For individual 1, the EC50 value was 0.10 nM TCDD for CYP1A1 mRNA induction and 0.63 nM for EROD induction. For individual 3, these values were 0.23 nM and 0.82 nM, respectively. This shift in concentration-response curve was also seen for CYP1B1 expression and the concentration-response curves for CYP1A1 and CYP1B1 induction completely overlapped (data not shown).

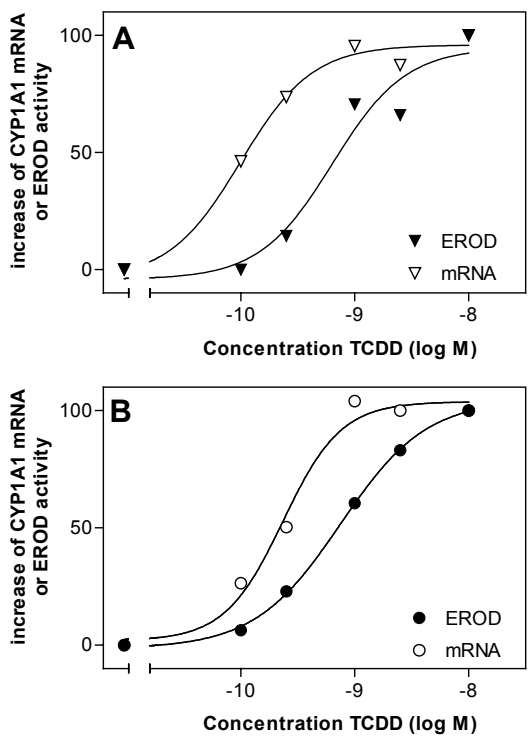

Figure 5.4 Increase of CYP1A1 mRNA and EROD activity of individual 1 (A) and 3 (B) after incubation with various concentrations TCDD. Data are represented as means of two determinations with control (mitogen-stimulated, DMSO) = 0 and maximum mRNA expression or EROD activity = 100.

*CYP1A1*2A and CYP1B1 Val432Leu Polymorphisms*

The *CYP1A1 Msp*I and *CYP1B1* Val432Leu genotypes of each individual were determined in DNA isolated from whole blood (Table 5.1). The allele frequencies were 0.95 for *CYP1A1*1* (wild-type) and 0.35 for *CYP1B1* 432Val. These frequencies did not deviate significantly from the Hardy Weinberg-equilibrium ($P = 0.99$ and $P = 0.95$ for *CYP1A1*1* and *CYP1B1* 432Val, respectively). The individuals shown in Figure 5.1 all had the *CYP1A1*1/1* genotype and for *CYP1B1* these genotypes were Val/Val (individual 1), Val/Leu (individual 3), Leu/Leu (individual 7) and Leu/Leu (individual 9).

Only one individual (number 6) had a mutant *CYP1A1* allele (*CYP1A1*1/2A*), which made it impossible to detect an effect of this polymorphism in this study. To study the effect of the *CYP1B1* Val432Leu polymorphism, the individuals were divided into two groups with the same *CYP1B1* genotype, excluding the one individual with the *CYP1A1*1/2A* genotype and the one individual with the *CYP1B1* Val/Val genotype. No effects were found of the *CYP1B1* Val432Leu polymorphism on the maximum fold induction of CYP1B1 mRNA, constitutive EROD activity and EC50 for EROD induction by TCDD. The only statistically significant difference observed was the maximum level of TCDD-induced EROD activity, which was higher in the Val/Leu-group (9.5 ± 0.2 pmole RSF/min/mg protein) than in the Leu/Leu-group (6.7 ± 0.8 pmole RSF/min/mg protein).

Table 5.1 Summary of EROD activity and gene expression and genotype of CYP1A1 and CYP1B1 in cultured lymphocytes of ten female healthy volunteers.

	EROD activity								Gene expression		Genotype	
		TCDD			PCB126							
Individual	Control (0.1% v/v DMSO)*	Maximally induced (10 nM TCDD)*	EC50 (nM)	EC25 (nM)	C25† (nM)	EC50 (nM)	RP‡ (C25)	RP‡ (EC50)	CYP1A1 (fold induction) §	CYP1B1 (fold induction) §	CYP1A1 *2A	CYP1B1 Val432Leu
1	0.29 ± 0.02	2.95 ± 0.16	0.64 ± 0.39	0.31	63.1	59.8	0.005	0.011	2.2	1.3	*1/1	Val/Val
2	0.07 ± 0.04	8.73 ± 0.82	0.65 ± 0.21						5.0	1.8	*1/1	Val/Leu
3	0.34 ± 0.03	9.38 ± 0.94	0.82 ± 0.14						3.1	2.5	*1/1	Val/Leu
4	0.22 ± 0.02	6.51 ± 0.04	2.07 ± 0.20	0.85	151.4	38.4	0.006	0.054	3.2	2.7	*1/1	Val/Leu
5	0.12 ± 0.07	8.22 ± 0.87	0.98 ± 0.11						2.7	1.5	*1/1	Val/Leu
6	0.28 ± 0.09	10.0 ± 1.31	0.59 ± 0.02						1.5	1.7	*1/2A	Val/Leu
7	0.31 ± 0.15	8.00 ± 0.24	0.50 ± 0.13						1.6	1.8	*1/1	Leu/Leu
8	0.06 ± 0.04	4.64 ± 0.21	3.88 ± 1.31	1.23	91.2	37.2	0.014	0.104	2.0	1.7	*1/1	Leu/Leu
9	0.39 ± 0.10	5.60 ± 0.06	0.52 ± 0.07	0.22	112.2		0.002		n.d.	1.3	*1/1	Leu/Leu
10	0.25 ± 0.06	7.23 ± 0.99	0.74 ± 0.09	0.29	173.8		0.002		2.1	1.4	*1/1	Leu/Leu
Average	0.23 ± 0.11	7.06 ± 2.71	0.80 ± 0.07	0.58 ± 0.44	118.3 ± 44.7	45.1 ± 12.7	0.006 ± 0.005	0.056 ± 0.047	2.6 ± 1.1	1.8 ± 0.5		
Allele frequency											CYP1A1*1 0.95	432Val 0.35

* Data are represented as average EROD activity (pmole resorufin/min/mg protein) ± SD of two measurements

† C25 is the PCB126 concentration necessary to obtain the EROD activity that was reached at 25% induction by TCDD

‡ Relative potency (RP) of PCB126 to induce EROD activity. RPs are calculated using the (E)C25 or EC50 values of TCDD and PCB126.

§ Expression after incubation with 10 nM TCDD for 72 hours, is calculated relative to CYP1A1 or CYP1B1 expression in lymphocytes cultured for 72 hours in media containing the mitogen PHA and 0.1% v/v DMSO

DISCUSSION

The purpose of this study was to obtain a better insight in the possibility to use human peripheral lymphocytes as biomarkers of exposure to environmental compounds such as dioxin and dioxin-like compounds. Peripheral lymphocytes provide an easily obtainable source of CYP1A1 and CYP1B1, the expression of which is affected by dioxin and dioxin-like compounds, including certain PCBs. However, little is known about the interindividual variability in constitutive expression and catalytic activity of CYP1A1 and CYP1B1, or their responses toward environmental contaminants. Therefore, we investigated *in vitro* responses toward TCDD and PCB126 in freshly isolated lymphocytes of healthy volunteers. In order to minimize the variability within the population in this study, we recruited a group of young female volunteers with little variation in factors known to affect CYP1A1 and CYP1B1 expression, such as smoking, domicile, ethnicity, age and gender.

Influence of the CYP1A1 m1 and CYP1B1 Val432Leu polymorphisms

With the limited number of individuals in our study, it is not possible to make a conclusive statement about a potential effect of the studied genetic polymorphisms on EROD induction or CYP1A1 and CYP1B1 gene expression levels. The individuals in this study did appear to reflect a normal Caucasian population. The allele frequencies for the studied genotypes did not deviate from the Hardy-Weinberg equilibrium and allele frequencies were similar to other published frequencies [9,17,19,20]. Although, we did not observe a clear-cut effect of the *CYP1A*2A* and *CYP1B1* Val432Leu polymorphisms, it should be noted that in the literature the opinions are not conclusive either. With regard to induction of EROD activity, some studies describe no effect of the *CYP1A1*2A* polymorphism on EROD activity in microsomes of smokers and non-smokers [21] or in induced lymphoctes of Caucasian volunteers [22]. Another study describes a higher basal and induced EROD activity in lymphocytes of individuals with at least one mutated *CYP1A1*2A* allele [23]. An effect of the *CYP1B1* Val432Leu polymorphism on EROD activity is not expected because of the limited contribution of CYP1B1 to EROD activity. This was also shown in another study that described no effect of the *CYP1B1* Val432Leu polymorphism on basal EROD activity [7]. In addition, the *CYP1B1* 432Val variant is believed to be the low-activity variant of CYP1B1 [6]. Therefore, the apparent association in this study between higher maximally induced EROD activity and the *CYP1B1* Val/Leu-genotype should be considered with care.

With regard to the *CYP1A1*2A* or *CYP1B1* Val432Leu polymorphisms, most studies report no effect on expression level [2,3,7,23]. Garte *et al.* on the other hand described a 3-fold decrease in expression associated with homozygous *CYP1A1*2A* genotype in lymphocytes of 177 healthy volunteers [5]. Hanaoka *et al.* showed an association between the *CYP1B1* Leu/Leu genotype and a higher expression level in lymphocytes of workers exposed to PAHs [8]. In our study, we found no statistically significant effects of either the *CYP1A1*2A* or *CYP1B1* Val432Leu polymorphisms om mRNA expression levels.

EROD activity in cultured human lymphocytes

EROD activity is commonly used as a marker for CYP1A1-mediated catalytic activity, although CYP1B1 also exerts some EROD activity. The constitutive EROD activity in the lymphocytes was very low, but detectable (average of 0.20 ± 0.14 pmole/min/mg protein) and varied substantially between the individuals in this study. EROD activity in the cultured lymphocytes was readily induced by TCDD, with maximum levels of induction at 10 nM TCDD between 10 and 150-fold above basal activity. Maximum induction of EROD activity by PCB126 was only about 2-fold and occurred at higher concentrations compared with TCDD. To estimate the potency of a dioxin-like compound, the TEF (toxic equivalent factor) concept was developed [24] and evaluated by the World Health Organization (WHO) [25]. TEF values are derived by evaluating the potency of a compound relative to that of TCDD. The WHO established the TEF value of PCB126 to be 0.1 [25]. In our study, the relative potency of PCB126 to induce EROD activity in human lymphocytes depended greatly on the method of calculation (see Table 5.1). However, the RPs for PCB126 that were calculated using the EC50 values, which is the standard method for RP calculation, were well within the range of the TEF established by the WHO.

CYP1A1 and CYP1B1 mRNA expression

In contrast to CYP1B1 expression, extrahepatic CYP1A1 expression is low [26,27], which was also seen in this study. CYP1B1 mRNA could be detected readily in uncultured, non-mitogen stimulated lymphocytes, whereas CYP1A1 mRNA was undetectable, although our RT-PCR method may not have been sensitive enough to detect low levels of expression. Mitogen stimulation and TCDD incubation increased CYP1A1 and CYP1B1 expression substantially in the lymphocytes.

The mRNA levels appeared to be a more sensitive indicator of exposure to TCDD than EROD activity, because the effects on mRNA levels were detected at lower

concentrations. This effect might be explained by the fact that upon binding of dioxin or dioxin-like compounds to the AhR, the *CYP1A1* and *CYP1B1* genes are first transcribed into mRNA before translation to the functional enzymes occurs. Therefore, it can be expected to detect increased mRNA levels before increased catalytic activity.

CYP1A1 and CYP1B1 as biomarker of exposure

To make a statement based on this study concerning the use of CYP1A1 and CYP1B1 in human lymphocytes as biomarkers of exposure to dioxin and dioxin-like compounds, it is important to compare the concentration-response curves *in vitro* with human blood levels found *in vivo*. Human blood levels of a mixture of dioxin and dioxin-like compounds are expressed as toxic equivalents (TEQs), compound concentrations normalized with their WHO-TEF values. Background TEQs that are found in human plasma in industrialized countries are about 20 ng TEQ/kg fat [28]. This TEQ can be translated to a TCDD concentration in whole blood of about 0.8 pM TCDD[a]. In our study, the concentration that caused a significant induction of EROD activity in human lymphocytes was about 10-15 pM TCDD, but induction of CYP1A1 and CYP1B1 gene expression was detected at lower concentrations. This suggests that the concentration of TCDD necessary to evoke a statistically significant induction of CYP1A1 and/or CYP1B1 expression in human lymphocytes is about a factor 10 higher than the background levels that are currently present in human blood. The average TEQs in plasma of human populations accidentally exposed to high concentrations of dioxin and dioxin-like compounds can be as high as about 100 ng/kg fat which corresponds to about 4 pM TCDD in whole blood [28]. This is a concentration at which minor effects on CYP1A1 and/or CYP1B1 expression could occur. However, the constitutive variation in levels of CYP1A1 and CYP1B1 expression among individuals is very large, which makes it very difficult to filter out potential subtle effects on expression by environmental exposure. It should also be noted that in the above calculations, we assumed that all TEQs are bioavailable. However, the free concentration of the TEQs available to the lymphocytes may be considerably lower, since these highly lipophilic dioxins and dioxin-like compounds are dissolved in blood fat and bind to proteins. This makes it unlikely that dioxin and dioxin-like compounds in blood of both background and highly exposed human populations are significantly affecting CYP1A1 and CYP1B1 activity or expression.

[a] Parameters used for calculation: plasma weight = 1.02 kg/L, plasma fat = 2%, total blood volume in female = 4.7 L (of which 2.8 L is plasma), MW TCDD = 322.

Although the population in our study was limited, these findings provide some insight into interindividiual differences in basal CYP1A1 and CYP1B1 expression and induction upon exposure to dioxin and dioxin-like compounds. We showed a large interindividual variation in constitutive and induced EROD activity and mRNA levels of CYP1A1 and CYP1B1 in human peripheral blood lymphocytes. Furthermore, the concentration of dioxin or dioxin-like compounds necessary to evoke an *in vitro* effect on EROD activity or CYP1A1 or CYP1B1 mRNA levels are much higher than the human blood levels found *in vivo*. Therefore, we feel that EROD activity or CYP1A1 or CYP1B1 gene expression levels are not suitable to use as biomarkers of exposure to dioxin-like compounds. However, epidemiological studies investigating the correlation between exposure to dioxin-like compounds and CYP1A1 and CYP1B1 gene expression and catalytic activity should be performed to support or reject this statement. In addition, the role of polymorphisms in the *CYP1A1* and *CYP1B1* genes should be explored, because these might influence the interindividual sensitivity and responses to dioxin-like compounds.

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6

No Effect of CYP1B1 Val432Leu Polymorphism on CYP1B1 mRNA Levels in an Organochlorine Exposed Population in Slovakia

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Majorie B. M. van Duursen[1]
Rocío Fernández Cantón[1]
Anthony Kočan[2]
J. Thomas Sanderson[1]
Krijn Kieviet[1]
Martin van den Berg[1]

[1]Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
[2]National Reference Center for Dioxins and Related Chemicals, Institute of Preventive and Clinical Medicine, Bratislava, Slovakia

Null Results in Brief

INTRODUCTION

Cytochrome P450 1B1 (CYP1B1) is a phase I enzyme involved in the metabolic activation of many polycyclic aromatic hydrocarbons (PAHs). It is also involved in the hydroxylation of estradiol to 4-hydroxyestradiol, a potentially genotoxic metabolite that is suggested to play a role in carcinogenesis. CYP1B1 is expressed in many tissues including mononuclear peripheral blood cells and is regulated through the aryl hydrocarbon receptor (AhR)-mediated pathway, which can be induced by several environmental chemicals, including PAHs and persistent organochlorine pollutants such as polychlorinated biphenyls (PCBs). A single nucleotide polymorphism of the CYP1B1 gene (1294G→C) leads to an amino acid substitution in codon 432 (Val to Leu). This substitution results in a lower catalytic activity of the enzyme [1], but the effect of this CYP1B1 Val432Leu polymorphism on CYP1B1 gene expression and its inducibility is not clear. Gene expression and genotype of *CYP1B1* can easily be determined in blood lymphocytes, making it a potential candidate to be used as biomarker for exposure to AhR agonists. We studied the correlation between CYP1B1 gene expression level and exposure to environmental factors within the *CYP1B1* Val432Leu genotype groups. This study was part of a European project (PCBRISK) that investigates a human population exposed to environmental pollution as a consequence of the 25-year long production of PCBs in eastern Slovakia.

STUDY POPULATION AND METHODS

Blood samples were taken from two populations in two different areas in Slovakia, the Michalovce District (polluted area) and the Stropkov District (less polluted, reference area). RNA was isolated from the lymphocytes and the samples (n = 334) were stored at -80°C until analysis. Methods for blood collection, isolation of human lymphocytes and RNA isolation are described elsewhere [2]. Blood concentrations of PCBs were measured as sum of all PCBs or as ng TEQ/kg lipid, which were defined as the sum of PCBs normalized with the WHO toxic equivalent factors. *CYP1B1* genotype was determined by a PCR-RFLP method using 750 ng RNA, adapted after Tang et al. [3]. 4.5 µg lambda

DNA (Fermentas Inc., Hanover, MD, USA) was used as positive control for endonuclease restriction.

CYP1B1 mRNA expression was normalized by a log 10-transformation. Deviations from the Hardy-Weinberg equilibrium were calculated using the two-sided χ^2 test. One-way analysis of variance (ANOVA) was conducted and Pearson correlation coefficients were calculated to study associations between variables. Statistical calculations were performed using GraphPad InStat 3.06 (GraphPad Software Inc., San Diego, CA) and SAS (SAS Institute Inc. Cary, NC).

RESULTS AND DISCUSSION

Of the 334 RNA samples stored, we were able to determine the *CYP1B1* Val432Leu genotype of 114 samples (34%). Of the other samples, RNA concentrations were too low for genotyping. Of one sample no information on gender was available. In the total population, the allele frequency for Leu was 0.44 (Table 6.1). This allele frequency is similar to other observed frequencies in various healthy Caucasian populations and did not statistically significantly deviate from the Hardy-Weinberg equilibrium ($P = 0.1$). Genotype frequencies were not different within the male or female subgroups and they did not deviate from the Hardy-Weinberg equilibrium (male $P = 0.1$ and female $P = 0.5$).

Table 6.1 Characteristics of the Slovakian study population.

	Val/Val‡	*Val/Leu*	*Leu/Leu*	*P* value for difference
PCB level (sumPCB in ng/kg blood lipid)	2907.1 ± 6684.2	1768.6 ± 2329.0	2629.1 ± 5121.0	0.59
TEQ (ng/kg blood lipid)	88.6 ± 36.2	86.9 ± 64.3	82.1 ± 30.9	0.86
CYP1B1 expression (arbitrary units)	0.61 ± 2.49	0.96 ± 2.17	0.44 ± 3.56	0.72
Gender (male/female)	25/17	23/20	17/11	0.54
Smoking status (smoker/unknown)	28/14	22/22	18/10	0.40
Number of subjects (% of total)	42 (36.8%)	44 (38.6%)	28 (24.6%)	

Note Values are represented as mean ± SD or, in case of gender and smoking status, as number of individuals.
‡ *CYP1B1* Val432Leu genotype

No difference in total PCB levels and TEQs were observed between the population from the polluted area and the reference area (data not shown) and therefore the population was regarded as one, equally exposed, population. Within this entire population, there was no significant correlation between PCB levels or TEQs in blood lipid and CYP1B1 mRNA levels (R = 0.09, $P = 0.4$, $n = 114$ and R = 0.01, $P = 0.9$, $n = 114$, respectively). When we divided the entire population in the Val/Val, Val/Leu and Leu/Leu genotype groups, no statistically significant differences were found in CYP1B1 mRNA levels, PCB levels or TEQs between the genotypes (Table 6.1). There were no significant correlations between PCB levels in blood lipid and CYP1B1 expression levels in lymphocytes within the Val/Val (R = 0.09, $P = 0.6$, $n = 42$), Val/Leu (R = 0.10, $P = 0.5$, $n = 44$) or Leu/Leu (R = 0.10, $P = 0.6$, $n = 28$) genotype groups. This was also true for TEQs in blood lipid and CYP1B1 expression levels with correlations of 0.18 (Val/Val, $P = 0.28$, $n = 40$), 0.11 (Val/Leu, $P = 0.51$, $n = 38$) and –0.17 (Leu/Leu, $P = 0.43$, $n = 23$). Further, no statistically significant associations between CYP1B1 expression and PCB levels or TEQs in blood lipids were found using linear regression modeling with adjustment for smoking and gender, two possible confounding factors for CYP1B1 expression (data not shown).

Our data indicate that environmental exposure to PCBs in our study population had no statistically significant effect on mRNA expression of CYP1B1, regardless the *CYP1B1* Val432Leu genotype. This is inconsistent with the study described by Hanaoka *et al.* [4]. They found a higher expression level of CYP1B1 in peripheral blood lymphocytes upon exposure to PAHs in Chinese coke oven workers who had at least one *CYP1B1* 432Leu allele. However, the number of subjects in that study was small (37 cases and 13 control workers) and it concerned an Asian population. Two other studies reported a correlation between CYP1B1 expression levels in peripheral blood lymphocytes and exposure to AhR agonists. One found a correlation with dioxin exposure higher than 6.5 pg TEQ/g lipid in a Japanese population [5]; the other found no effect of PAH exposure in healthy volunteers of unknown ethnicity [6]. Spencer *et al.* have suggested using CYP1B1 expression as a biomarker for *in vivo* exposure to dioxin-like compounds [7]. However, despite the high blood levels of PCBs and TEQs in our study population and the separation of the population into genetically more homogenous groups with respect to *CYP1B1* Val432Leu genotype, no correlation was observed between PCB levels and CYP1B1 mRNA levels human lymphocytes. In addition, the large interindividual variability in CYP1B1 expression and PCB blood levels in the population makes it

unlikely that possible, subtle effects of PCB exposure on RNA expression can be detected. This makes the suitability to use CYP1B1 expression as biomarker for exposure questionable.

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7

General Discussion

1 Introduction

In the etiology of breast cancer, estrogens and their metabolites play a key role as tumor initiators and promoters. However, while the role of estrogen as a carcinogen is well-established, the involvement of estrogen metabolites is not yet completely understood and still the subject of ongoing research. The most commonly heard criticism of the hypothesis that estrogen metabolites are carcinogenic, is that estrogen levels are too low to produce a biologically relevant concentration of potentially genotoxic metabolites in the breast tissue. This criticism, however, is based on the plasma levels of estrogens, but plasma and tissue levels are not well correlated [1]. In addition, co-expression of estrogen synthesizing enzymes (aromatase and steroid sulfatase [2,3]) and estrogen metabolizing enzymes (CYP1A1, CYP1B1 [4,5]) in breast tissue, make it plausible that locally formed estrogens and estrogen metabolites may reach concentrations sufficiently high to evoke tumorigenic effects.

2 Exogenous compounds and altered estrogen metabolism

There is a difference in genotoxic potential between estrogen metabolites. Therefore, the balance among the estrogen metabolizing enzymes is very important in the determination of estrogen metabolite concentrations and their potential to induce DNA damage and ultimately tumor formation. A balance between phase I metabolism (for example by CYP1A1 and CYP1B1) and phase II metabolism (for example by COMT) of estrogens is crucial. In this thesis, we have described exogenous compounds that affected phase I metabolism (chapter two) and phase II metabolism (chapter three) of estrogens in mammary epithelial cell lines and *ex vivo* human breast tissues, respectively.

2.1 Phase I metabolism

Liehr and Ricci reported the 4-OHE2/2-OHE2 concentration-ratios in *ex vivo* mammary tissues to be 3.8 in adenocarcinomas, 1.3 in normal breast tissues from breast cancer patients and 0.7 in healthy breast tissue from reduction mammoplasty [6]. This decrease in 4-OHE2/2-OHE2 ratio can mainly be attributed to an increase in estrogen 4-hydroxylation in the tumorigenic tissue; the estrogen 2-hydroxylation was similar in tumor and healthy breast tissue. No CYP1A1 and CYP1B1 enzyme levels or activities were determined in this study and other studies investigating the enzyme levels do not conclusively describe an increase in CYP1B1 levels in tumorigenic breast tissues

compared with healthy tissues. Based on mRNA expression levels and immunohistochemical staining, it appears that in healthy breast tissue CYP1B1 enzyme levels are higher than CYP1A1 enzyme levels [5,7]. This is consistent with the constitutive mRNA expression levels found for CYP1A1 and CYP1B1 in several tumorigenic and healthy mammary epithelial cell lines [8]. Only a few studies compared the CYP1A1 and CYP1B1 expression patterns between healthy and tumorigenic breast tissues. Some describe CYP1B1 expression to be tumor-specific and higher CYP1B1 levels in breast tumor tissue than in adjacent non-tumor tissue [9,10], while others find lower levels [7]. CYP1A1 expression appears to be higher in healthy tissue than in tumor breast tissue of the same patient [7,10]. This inconsistency between levels of estrogen metabolizing enzymes and concentrations of estrogen metabolites might be partially explained by the aspecificity of CYP enzymes. To some extent CYP1A1 and CYP1B1 are both responsible for 4-OHE2 and 2-OHE2 formation [11-13]. Another explanation may be the higher inactivation rate of the 2-hydroxyestrogens than 4-hydroxyestrogens due to the catalytic preference of COMT (see also paragraph 2.2.1). Also, the involvement of polymorphisms in the estrogen metabolizing enzymes, especially of CYP1B1, may play a role. Two polymorphisms in CYP1B1, Asn453Ser and Val432Leu (also see chapter one, paragraph 3.3), occur in the haem-binding domain of the enzyme and have been shown to affect enzyme function [13,14]. This indicates that when a possible correlation between mRNA expression or protein levels of CYP1B1 and estrogen 4-hydroxylation activity of the enzyme is being investigated in breast tissue, the differences in catalytic activity between the CYP1B1 isoforms should also be taken into account. Until now, no studies have been performed to investigate this.

There are many known AhR agonists that induce the expression of CYP1A1 and CYP1B1. *In vitro* studies with mammary epithelial cells, derived from primary cultures or cell lines, show that CYP1A1 expression is more readily induced than that of CYP1B1 upon incubation with dioxin or dioxin-like compounds [5,8,15-17]. Induction of CYP1A1 and CYP1B1 by AhR agonists consequently results in increased estrogen metabolism (chapter two). Further, we showed that incubation of mammary epithelial cells with several dioxin-like compounds resulted in a concentration-dependent decrease in estrogen 4-/2-hydroxylation ratio. This ratio or the CYP1B1/CYP1A1 expression is suggested to provide a marker for neoplastic breast tissue [6], which implies that a decrease in ratio indicates a protective effect of the dioxin-like compounds against the development of neoplasms. This concurs with the antiestrogenic properties that are described for dioxin-like compounds. TCDD and structurally-related compounds have been shown to inhibit

several estrogen-induced responses in cell lines and animals, resulting from the inhibitory cross-talk between the AhR and ER pathways [15-22]. However, incubation of mammary epithelial cells with dioxin-like compounds also results in an increase in absolute levels of potentially carcinogenic estrogen metabolites due to increased estrogen metabolism in these cells (chapter two). Both the quantitative and qualitative importance of the catechol estrogens should therefore be considered in the prediction of estrogen dependent cancer-initiating events. Furthermore, the overall lack of knowledge concerning the expression of CYP1A1 and CYP1B1 and estrogen metabolism in healthy and tumorigenic breast tissues, makes it difficult to use the estrogen 4-/2-hydroxylation ratio or CYP1B1/CYP1A1 expression as a prognostic marker for cancer risk.

2.2 Phase II metabolism by COMT

Inactivation of catechol estrogens is mainly mediated by methylation catalyzed by COMT [23]. This means that COMT plays a crucial role in the determination of the genotoxic potential of catechol estrogens. This role of COMT appears to be two-fold. Firstly, it detoxifies potentially genotoxic catechol estrogens (described in 2.2.1) and secondly, it produces 2-MeOE2, which exerts a variety of antitumor actions, from 2-OHE2 (described in 2.2.2).

2.2.1 Detoxification of catechol estrogens

Catechol estrogens are formed rapidly in tissues such as breast tissue, but they are also rapidly inactivated [24]. The kinetic profile of 2- and 4-methoxyestradiol formation by COMT appears to be dependent on the levels of CYP1A1 and CYP1B1 [25]. However, generally it is reported that COMT displays a higher efficiency toward 2-MeOE2 formation than 4-MeOE2 formation from 2-OHE2 and 4-OHE2, respectively. This was described for COMT in human brain [26], epithelial breast cell lines [27], kidney cytosol [28], purified porcine COMT [29] and in healthy human breast tissue cytosol (chapter three). On the other hand, a study with recombinant human COMT showed the catalytic efficiency of human COMT to be highest toward 4-MeOE2 formation, subsequently followed by 4-MeOE1, 2-MeOE2 and 2-MeOE1 [30].

Several studies have investigated the differences in catalytic activity between the COMT-L and COMT-H isoforms of the enzyme. Lachman et al. described a 3 to 4-fold lower activity of the COMT-LL compared with the COMT-HH form in human liver biopsy samples toward methylation of 3,4-dihydroxybenzoic acid, a known catechol substrate for COMT. Dawling et al. found a 2 to 3-fold lower methoxyestradiol formation

in cell lines expressing COMT-LL compared with COMT-HH [30]. However, Goodman et al. found no differences in kinetic parameters (Km and Vmax) between the high and low activity forms of COMT with respect to 2- and 4-OHE2 methylation [27]. It appears that the reduced activity of the low-activity isoform of COMT is mainly caused by a change in thermostability of the enzyme and not as much by a change in catalytic activity of the enzyme [26,27,31]. Recently, it has been suggested that the catalytic differences between COMT-H and COMT-L are caused by a difference in enzyme protein levels [32]; the high COMT activity of COMT-H is a result of a higher protein level of the enzyme. This, however, does not concur for the MCF-7 and MCF-10A cell lines. Goodman et al. described lower protein levels for COMT in MCF-10A cells (containing COMT-HH) compared with MCF-7 cells (containing COMT-LL) [27].

COMT activity is not readily up- or downregulated [26], although, some studies have described the down-regulation of COMT gene expression by estradiol through the ER-dependent pathway [33,34]. COMT activity can also be inhibited through catalytic inhibition. Synthetic COMT inhibitors are used in the treatment of hypertension, asthma, and Parkinson disease. These synthetic COMT inhibitors are generally poor substrates for COMT but they bind tightly to the catalytic site of the enzyme thus inhibiting methylation of other substrates without depletion of cofactors [35-37]. Further, several exogenous compounds such as catechol metabolites of PCBs [29] and certain phytochemicals [38], have been shown to compete with natural substrates for the enzyme and thereby inhibiting COMT activity. This type of inhibition is usually both competitive and non-competitive. Quercetin, for example, has shown to be a substrate for COMT and thus compete for cofactors necessary for *O*-methylation of the substrate, such as S-adenosyl-L-methionine (SAM) [39]. Upon *O*-methylation, a methyl group from SAM is transferred to the catechol substrate resulting in S-adenosyl-L-homocysteine (SAH). An increasing concentration of SAH was shown to (non-competitively) inhibit the association of the methyl donor SAM with COMT.

2.2.2 Anti-tumor actions of 2-MeOE2

COMT converts the catechol estrogens to their non-genotoxic methoxy derivatives. The methylation of 4-OHE2 results in 4-MeOE2. Methylation of 2-OHE2 mainly yields 2-MeOE2, but a small proportion of 2-OHE2 is methylated on the C-3 position, yielding the metabolite 2-OH-3-MeOE2 [27,30]. All methoxyestrogens have shown to exert feedback inhibition on CYP1A1- and CYP1B1-mediated hydroxylation of estrogens [40].

Of special interest is the methoxyestrogen 2-MeOE2. It exerts a variety of antitumor actions which makes it an interesting compound to be used as anticancer agent [41] and it is currently undergoing clinical phase I/II trials [42]. At physiologically relevant concentrations, 2-MeOE2 has been shown to inhibit cell growth, induce apoptosis and inhibit angiogenesis in various cell lines and *in vivo* in mice, rats and dogs [43-46]. The mechanisms of these actions are not yet fully understood, but research on 2-MeOE2 has revealed several effects contributing to the anti-tumor properties. The antiproliferative actions of 2-MeOE2 are not mediated through the ER directly [47]. 2-MeOE2 does, however, reduce estrogen production through inhibition of constitutive and induced aromatase activity, and inhibition of aromatase activity stimulation by TNFα, IL-6 or PGE2 [48]. This indicates that 2-MeOE2 may reduce estrogen-dependent cell growth through inhibition of estrogen production. A more direct contributor to the antiproliferative effect of 2-MeOE2 appears to be its ability to cause disruption of microtubule function. Microtubules are involved in chromosome movement during mitosis. A cell cycle check-point in the M-phase ensures that the chromatids are aligned correctly so they can be pulled to the opposite poles of the dividing cell during mitosis (see Box 1). It is believed that this checkpoint is regulated at the level of microtubule attachment to the centromere and disruption of microtubules prevents the completion of mitosis [49]. 2-MeOE2 can bind to β-tubulin, thereby inhibiting tubulin polymerization [50]. This effect results in the ability of 2-MeOE2 to arrest cell cycle in the G(2)/M phase in cell lines of various origins (prostate, lung, breast) thus inhibiting cell growth [51-53]. Another target for 2-MeOE2 action appears to be the tumor suppressor gene p53. The p53 gene is activated when DNA damage is detected and blocks cell cycle progression (in the G1 phase check point) until repair is completed. In normal cells, p53 levels are low but transcription is increased by DNA damage. Most studies describe the *in vitro* increase of p53 expression after incubation of prostate, breast or lung cancer cells with 2-MeOE2 [54-56]. However, the p53-upregulation appears not to occur in every cell type [52,57]. Further, 2-MeOE2 inhibits superoxide dismutase (SOD, enzyme involved in the elimination of the superoxide radical $O_2^{\bullet-}$) activity which results in an increase of free-radical-mediated damage to mitochondrial membranes [58]. Both p53 upregulation and mitochondrial damage results in the release of cytochrome *c* and subsequent apoptosis of the cell [59]. Inhibition of angiogenesis by 2-MeOE2 is most likely mediated by the inhibition of HIF-1α (hypoxia-inducible factor α) translation. HIF-1α is a transcription factor that is involved in the activation of transcription of over 40 genes at low oxygen levels in the cell [60-63]. Downregulation of many of those genes has been directly tied with

inhibition of angiogenesis, proliferation, survival and metastasis of tumors [62,64,65]. HIF-1α action is dependent on its translocation to the nucleus where it heterodimerizes with HIF-1β. HIF-1β is also known as Arnt (AhR nuclear translocator) [63,66], involved in AhR-mediated pathway in binding of the ligand-AhR complex to the DNA (see also chapter one, 3.1 AhR pathway). This suggests the involvement of 2-MeOE2 in the regulation of Arnt levels and subsequent AhR functioning.

Lavigne et al. showed a linear relationship between increasing catechol estrogen concentrations (2-OHE2 plus 4-OHE2) and increasing 8-oxo-dG levels, an oxidized DNA base and marker of hydroxyl radical action. 2-MeOE2 reduced 8-oxo-dG formation in MCF-7 cells as was expected based on this linear relation [67]. This effect was discussed by Dawling et al. to result from a non-competitive catalytic inhibition of CYP1A1 and CYP1B1 by methoxy estrogens, leading to a decreased formation of hydroxyestrogens from estradiol and a subsequent decrease in catechol estrogen-induced DNA damage [40]. However, in chapter four we showed that the protection of 2-MeOE2 against DNA damage was a direct effect that was demonstrated by adding hydroyestrogens and 2-MeOE2 directly to the cells. The mechanism of this DNA protection by 2-MeOE2 could not be explained by its ability to arrest the cells in the G(2)/M phase, because this effect was detected after a much longer incubation with 2-MeOE2. Cell cylcle arrest, growth inhibition and induction of apoptosis by 2-MeOE2, as described above, were performed after an incubation period of at least 24 hours, while protection against DNA damage was already detected after a 5-hour incubation (chapter three and four). This suggests a fast, direct action of 2-MeOE2 in the protection against DNA damage, which is important to the investigation of 2-MeOE2 as a therapeutic agent in the treatment of cancer.

2.3 Altered estrogen metabolism and the implications for breast cancer development

Estrogen synthesis and metabolism is tightly regulated and many enzymes are involved (figure 7.1). However, all these enzymes can be up- or downregulated by exogenous compounds. In this thesis, we have shown upregulation of CYP1A1 and CYP1B1 by dioxins and dioxin-like compounds (chapter two) and COMT inhibition by phytochemicals (chapter three). We have also showed that COMT inhibition results in the accumulation of catechol estrogens and a subsequent increase of catechol estrogen-induced DNA damage *in vitro* (chapter three and four). Yet, it is difficult to study the involvement of changes in estrogen metabolism in the development of breast cancer in

individuals, since its impact is probably much lower than the established risk factors such as early menarch and late menopause that contribute to the cumulative life-time exposure to estrogens. However, high cumulative life-time exposure to estrogens also implicates high cumulative life-time exposure to estrogen metabolites and numerous *in vitro* and *ex vivo* studies demonstrate the genotoxic potential of catechol estrogens. Furthermore, studies that investigate the association between polymorphisms in estrogen metabolizing enzymes and breast cancer risk imply the involvement of these enzymes in breast cancer susceptibility, especially when multiple estrogen metabolizing enzymes are taken into account [68,69].

Taken together, this indicates that activity of estrogen metabolizing enzymes, and thus estrogen metabolite patterns, could entail a certain breast cancer risk. This implies that a woman's susceptibility to breast cancer may be determined by the isoforms of the polymorphic estrogen metabolizing enzymes and that exposure to exogenous compounds could modify a woman's risk to develop breast cancer.

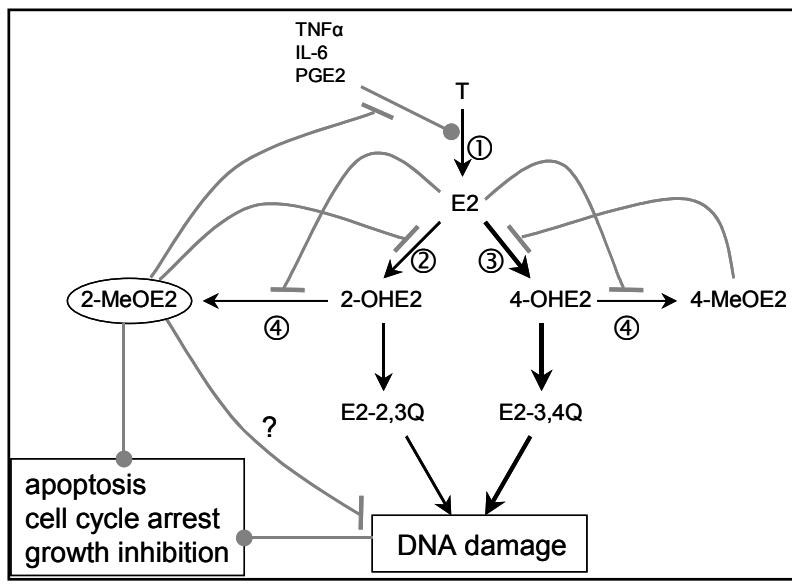


Figure 7.1 Schematic overview of the feedback regulation of estradiol synthesis and metabolism and its contribution to the induction of DNA damage in human breast cells. T, testosterone; E2, estradiol; 2-OHE2, 2-hydroxyestradiol; 2-MeOE2, 2-methoxyestradiol; 4-OHE2, 4-hydroxyestradiol; 4-MeOE2, 4-methoxyestradiol E2-2,3Q, estradiol-2,3-quinone; TNFα, tumor necrosis factor alpha; IL-6, interleukine-6; PGE2, prostaglandine E2. Enzymes involved are ① aromatase (CYP19); ② CYP1A1; ③ CYP1B1; ④ COMT. Grey lines with a round end indicate a stimulatory effect and grey lines with a flat end indicate an inhibitory feedback loop.

3 CYP1A1 and CYP1B1 as biomarkers of exposure to environmental compounds

Beside their involvement in estrogen metabolism, CYP1A1 and CYP1B1 are involved in the phase I metabolism of many PAHs. Furthermore, their expression levels are upregulated by AhR agonists, including PAHs, dioxins and dioxin-like compounds (described in Chapter One, 3.2 AhR pathway). CYP1A1 and CYP1B1 are expressed in peripheral blood lymphocytes, which is an easily obtainable source. Therefore, CYP1A1 and CYP1B1 expression in human lymphocytes are often used as biomarkers of exposure to dioxins and dioxin-like compounds. However, several important factors should be kept in mind when applying CYP1A1 and CYP1B1 expression as biomarker. Firstly, several confounding factors have been identified that may affect CYP1A1 and CYP1B1 expression, such as age, gender and cigarette smoking [70,71]. Secondly, genetic polymorphisms in the *CYP1A1* and *CYP1B1* genes might affect expression levels. For example, decreased CYP1A1 expression appears to be associated with homozygous *CYP1A1*2A* genotype [72]. However, other studies found no such association [73,74]. As a result of these two factors, a large interindividual variability in constitutive and induced CYP1A1 and CYP1B1 expression levels can be found [75,76], which complicates the use of CYP1A1 and CYP1B1 expression in lymphocytes as biomarkers of exposure. In addition, the *in vivo* exposure of an individual should be high enough to evoke an effect on CYP1A1 and CYP1B1 expression in lymphocytes. Still, a number of studies have been performed that use CYP1A1 and/or CYP1B1 expression in peripheral blood lymphocytes as biomarker of exposure to environmental compounds suchs as PAHs [70,77,78] and dioxins [79,80].

Yet, little has been reported about the confounding factors that influence the suitability of CYP1A1 and CYP1B1 expression as biomarkers. Spencer *et al*. reported that CYP1B1 mRNA levels in human lymphocytes of ten North Carolina volunteers (male and female, both smokers and non-smokers), increased concentration-dependently upon *in vitro* exposure to 2,3,7,8-tetrachloro-p-dibenzodioxin (TCDD) [81]. They suggested using CYP1B1 expression as a biomarker for *in vivo* exposure to dioxin-like compounds. However, even in a group of volunteers in which we tried to minimize the influence of confounding factors, we still found a large variablity in constitutive and TCDD-induced EROD activity and CYP1A1 and CYP1B1 expression in lymphocytes (chapter five). In addition, the dioxin concentrations at which effects were observed in our *in vitro* study were about 10-fold higher than human blood levels found *in vivo*. To further investigate the use of CYP1B1 as biomarker, we studied a large human population (n = 114), exposed

to PCBs in eastern Slovakia (described in chapter six). In this non-homogenous population, again we found a large variation in CYP1B1 expression. And despite the high blood levels of PCBs and TEQs in some individuals of the study population and the separation of the population into the *CYP1B1* Val432Leu genotype groups, no significant correlation was observed between PCB levels in plasma fat and CYP1B1 mRNA levels in lymphocytes. Unfortunately, we only had access to RNA samples, not DNA samples, of the population. Therefore, we could not study the occurrence and effect of the *CYP1A1*2A* polymorphism, since this polymorphism is located in the 3'-UTR of the gene, which is not present in the mRNA.

These two studies indicate that it is unlikely that CYP1A1 or CYP1B1 mRNA expression in human peripheral lymphocytes can be used as biomarkers of exposure, either background or occupationally, to dioxins or dioxin-like compounds.

4 The use of biomarkers

In this thesis, special attention was given to the use of biomarkers. Three types of biomarkers are distinguished in risk assessment; biomarkers of effect, exposure and susceptibility [82]. In chapter two, the estrogen 4/2-hydroxylation ratio as marker for breast cancer (biomarker of effect) was investigated and discussed. In chapter five and six, the use of CYP1A1 and CYP1B1 expression in human lymphocytes as biomarkers of exposure to dioxin-like compounds was evaluated. We briefly discussed the use of estrogen metabolizing enzymes as biomarkers of susceptibility in chapter four and in this chapter (paragraph 2.3). Biomarkers of susceptibility describe genetic conditions, such as polymorphisms, which potentially enhance the susceptibility of an individual toward a compound.

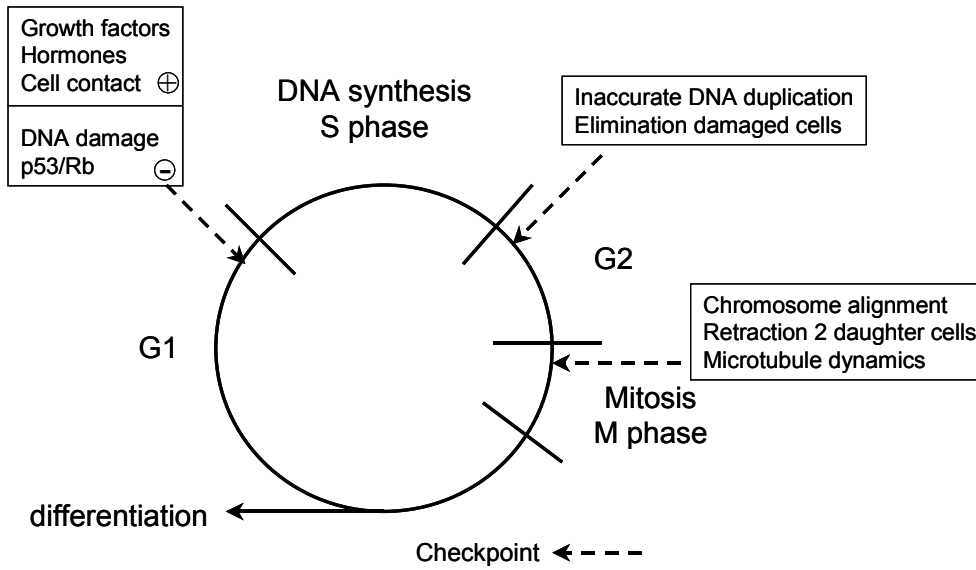
Regardless of the type, a biomarker should always be measurable and it should be predictive for a biologically relevant effect. This implies that a good biomarker should involve three factors. First, a biomarker should be based on knowledge of the dose-response relationship and mechanism of action of a compound, which is crucial for developing a specific and sensitive method. Secondly, one should understand how perturbations in the biomarker are associated with the onset of a disease or toxic effect, so the *additional* risk of exposure to a certain compound on top of the background risk can be estimated. Thirdly, one should understand the influences of various confounding factors on the biomarker, taking into account genotype, life style and interindividual differences in exposure or health risk.

Typically, studies that use biomarkers do not include all these factors. This is partially due to the complexity of the study it would involve. But also due to the fact that researchers can lose sight of the bigger picture; one enzyme is only a small step in the complex mechanism of biochemical processes that occur in the cell. Furthermore, in case of carcinogenesis one specific biochemical change elicited by a compound does not necessarily mean an increase in cancer risk, since carcinogenesis comprises multiple steps. However, biomarkers can be very useful in gaining understanding of biochemical processes, reducing uncertainties in risk assessment and in monitoring exposed populations to discover trends and possible health effects. This holds true for both exogenous compounds, such as dioxins, dioxin-like compounds and natural flavonoids, as well as endogenous compounds, such as catechol estrogens as described in this thesis.

5 Main conclusions in this thesis

- Estrogen 4-/2-hydroxylation ratio in breast tissue might be used as biomarker for the presence of neoplasms, but not as a prognostic marker
- COMT plays a crucial and dual role in the determination of the genotoxic potential of catechol estrogens
- The molecular mechanisms and cell type-specific actions of 2-MeOE2 should be elucidated before it can be used as anticancer agent
- There is a lack of knowledge concerning the association between the polymorphisms, expression and activity of CYP1A1, CYP1B1 and COMT in healthy and tumorigenic breast tissues
- Clustering genetic polymorphisms in estrogen metabolizing enzymes might identify women with different susceptibilities for developing catechol estrogen-initiated breast cancer
- Exposure to exogenous compounds might affect estrogen metabolism in breast tissue, which might modify a woman's risk to develop breast cancer
- CYP1A1 and CYP1B1 expression in peripheral blood lymphocytes are not suitable as biomarkers of exposure to dioxins and dioxin-like compounds
- Biomarkers (of exposure, effect or susceptibility for carcinogenesis) are helpful to understand biological processes of exposure and possible health effects, but should not be used as categorical risk assessors

BOX 1. The Cell Cycle



The cell cycle, the process of cell division, consists of several phases. The cells grow and amplify their synthetic machinery (Gap 1, G1, duration ~ 8-30 hours) in preparation for DNA synthesis (S phase, duration ~ 8 hours) and then reorganize their chromatin into chromosomes (Gap 2, G2, duration ~ 3 hours) prior to mitosis (M phase, duration ~ 1 hour). Sometimes the cells will leave the cell cycle, temporarily or permanently, in the G1 phase. Usually these cells (G0) are then terminally differentiated: they never reenter the cell cycle, but instead carry out their specific function in the organism until they die. The cell has three main checkpoints for interrupting the cell cycle if something goes wrong. Several proteins in the cytoplasm, such as cyclins, p53 and Rb (retinoblastoma repressor protein), growth stimuli, such as hormones, and DNA integrity control the passage of a cell through the cell cycle [49,83]

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Nederlandse Samenvatting

1 Inleiding

1.1 Oestrogenen en borstkanker

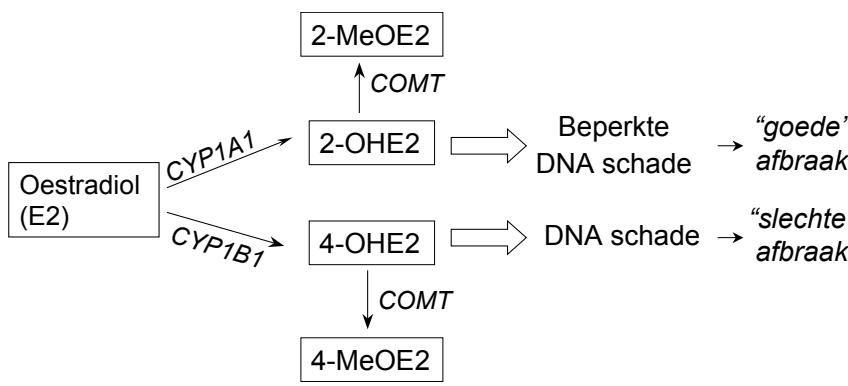
Over de afgelopen jaren is er een toename te zien in het aantal gevallen van borstkanker in de westerse wereld. Tegenwoordig krijgt gemiddeld 1 op de 8 vrouwen borstkanker. In Nederland komt dit neer op ongeveer 10 000 vrouwen per jaar, van wie er ongeveer 3 500 sterven aan de gevolgen ervan. Er zijn verschillende genetische risicofactoren bekend, zoals het borstkankergen BRCA, maar in het overgrote deel van de borstkanker gevallen is de oorzaak niet zo duidelijk aan te wijzen. Er is wel een aantal factoren die het risico op borstkanker kunnen vergroten. Dit zijn bijvoorbeeld op jonge leeftijd de eerste menstruatie krijgen, op late leeftijd in de menopauze komen, geen kinderen krijgen of op late leeftijd een eerste kind krijgen, al eerder borstkanker hebben gehad en ook verschillende levensstijlfactoren kunnen een rol spelen. Al deze factoren hebben invloed op de hoeveelheid oestrogenen waar een vrouw gedurende haar leven aan wordt blootgesteld. Oestrogenen, de vrouwelijke hormonen, spelen een belangrijke rol tijdens maandelijkse voorbereiding van het lichaam op een mogelijke zwangerschap, tijdens een zwangerschap, en bij de ontwikkeling van de vrouwelijke secundaire geslachtskenmerken (borstontwikkeling, verdere ontwikkeling van baarmoeder en vagina, verbreding van het bekken, groei van schaamhaar, toename van vetweefsel). Ze spelen ook een rol bij onder andere beperking van calcium verlies uit de botten en stimulatie van bloedstolling. Er zijn drie oestrogenen: oeston, oestriol en oestradiol. Oestradiol is het voornaamste oestrogeen in niet-zwangere, premenopausale vrouwen.

Oestrogenen kunnen borstkanker veroorzaken. Al in 1896 ontdekte de Schotse chirurg George Beatson (1848 – 1933) dat het verwijderen van de eierstokken bij een vrouw met borstkanker er voor zorgde dat de tumor kleiner werd. Pas veel later werd wat meer duidelijk over de rol van oestrogenen in het ontstaan van borstkanker. Er zijn waarschijnlijk twee manieren waarop ze dat doen. Ten eerste stimuleren oestrogenen borstweefsel te delen. Hoe sneller borstweefsel gaat delen, hoe grote de kans wordt dat daarbij fouten gemaakt worden bij het kopieren van het DNA. Als die fouten in het DNA zich opstapelen en niet meer gerepareerd kunnen worden, kan een cel zich gaan ontwikkelen tot tumorcel en uiteindelijk tot een tumor. Een andere manier waarop oestrogenen borstkanker kunnen veroorzaken is via de afbraakprodukten. Naar deze laatste manier heb ik onderzoek gedaan.

1.2 Oestrogeenafbraak en de rol in het ontstaan van borstkanker

Oestrogeenconcentraties worden goed gecontroleerd en gereguleerd in het lichaam. Oestrogenen worden gemaakt van cholesterol. In premenopausale vrouwen worden oestrogenen voornamelijk gemaakt in de eierstokken en een beetje in andere weefsels, zoals het borstweefsel en vetweefsel. Na de menopause vindt de voornaamste oestrogeenproductie niet meer plaats in de eierstokken, maar juist in die andere weefsels. Oestrogenen worden gemaakt, maar ook afgebroken in organen waar ze hun werk moeten doen. Afbraak van oestrogenen gebeurt in twee stappen. Eerst wordt oestradiol door de enzymen cytochroom P450 1A1 (CYP1A1) en cytochroom P450 1B1 (CYP1B1) omgezet in respectievelijk 2-hydroxyoestradiol (2-OHE2) en 4-hydroxyoestradiol (4-OHE2) (Figuur A). Daarna worden 2-OHE2 en 4-OHE2 gemethyleerd door catechol-O-methyltransferase (COMT) en kunnen ze worden uitgescheiden via de urine.

Als 2-OHE2 en 4-OHE2 niet allemaal door COMT worden omgezet, bijvoorbeeld omdat de concentraties te hoog zijn zodat COMT het niet aankan, kunnen ze verder omgezet worden in reactieve stoffen, zgn. quinonen. Deze quinonen kunnen binden aan bijvoorbeeld DNA. Quinonen van 2-OHE2 zijn niet zo schadelijk. Ze blijven gebonden aan het DNA totdat ze worden opgeruimd door de cel. Maar quinonen van 4-OHE2 kunnen na binding het DNA stuk maken. Zo onstaat er een gat in het DNA wat niet door de cel gerepareerd kan worden en bestaat de kans dat de cel zich gaat ontwikkelen tot een tumorcel. Dit betekent dus dat de manier waarop oestrogenen worden afgebroken de kans bepaalt op (borst)tumorvorming.



Figuur A Schematisch overzicht van oestradiol afbraak in borstweefsel. 2-OHE2, 2-hydroxyoestradiol; 2-MeOE2, 2-methoxyoestradiol; 4-OHE2, 4-hydroxyoestradiol; 4-MeOE2, 4-methoxyoestradiol; CYP1A1, cytochroom P450 1A1; CYP1B1, cytochroom P450 1B1; COMT, catechol-O-methyltransferase (voor structuren zie Figuur 1.1 in hoofdstuk 1, General Introduction).

In borstweefsel worden oestrogenen voornamelijk omgezet door de enzymen CYP1A1, CYP1B1 en COMT. Als de activiteit van deze enzymen verandert, verandert ook de hoeveelheid en verhouding van de oestrogeenafbraakproducten. Lichaamsvreemde stoffen, zoals dioxinen maar ook stoffen uit planten, kunnen enzymactiviteiten veranderen als ze door het lichaam worden opgenomen. Ze doen dit bijvoorbeeld door een cel aan te zetten meer enzymen te maken of door op het enzym gaan zitten waardoor het niet meer kan werken. Er zijn ook genetische variaties (polymorfismen) bekend in enzymen die zorgen voor verschillende enzymactiviteiten in verschillende mensen.

2 Het gebruik van biomarkers bij risicoschattingen

Bij het maken van een risicoschatting wordt een inschatting gemaakt van de kans dat een bepaalde ziekte kan optreden als gevolg van blootstelling aan omgevingsfactoren. Deze omgevingsfactoren kunnen lichaamsvreemde stoffen zijn zoals milieuvervuilende stoffen en natuurlijke (planten)stoffen, maar ook lichaamseigen stoffen, zoals oestrogenen.

Als je een risicoschatting maakt, is het handig om biomarkers te hebben. Een biomarker is een parameter (bijvoorbeeld een afbraakproduct of een enzymactiviteit) die je meet in het lichaam aan de hand waarvan je een voorspelling kan doen over de kans dat een ziekte optreedt. Er zijn drie soorten biomarkers. Biomarkers die een effect voorspellen, biomarkers die de gevoeligheid voorspellen en biomarkers die een maat geven van de blootstelling aan lichaamsvreemde stoffen. In dit proefschrift zijn deze drie biomarkers onderzocht en beschreven.

2.1 Biomarkers van effect

Effect biomarkers geven de mate van een biologische reactie weer na blootstelling aan omgevingsfactoren. Een voorbeeld van een effect biomarker is de verhouding tussen 4-OHE2 (“slechte” afbraakproducten) en 2-OHE2 (“goede” afbraakproducten) in borstweefsel. Uit enkele studies blijkt deze verhouding in borsttumoren 3 te zijn en in gezond weefsel 1. Sommige onderzoekers stellen daarom dat de verhouding 4-OHE2:2-OHE2 de aanwezigheid van een borsttumor aangeeft. Maar het is nog maar de vraag of de verhouding ook kan voorspellen wat de kans is om borstkanker te krijgen.

De enzymen die voor de vorming van 4-OHE2 en 2-OHE2 zorgen (CYP1B1 en CYP1A1) kunnen beïnvloed worden door dioxinen en dioxine-achtige stoffen (zie Box A). Borstweefselcellen die zijn blootgesteld aan dioxinen en dioxine-achtige stoffen, gaan meer CYP1A1 en CYP1B1 enzymen maken, al is het effect veel groter op CYP1A1. Dit

gebeurt zowel in normale borstweefselcellen als in borsttumorcellen. Als gevolg vindt er meer afbraak van oestrogenen plaats (er zijn immers meer enzymen) en verandert de verhouding 4-OHE2:2-OHE2 (er is immers veel meer CYP1A1 dan CYP1B1).

Wat precies schadelijker is, meer afbraakproducten of een andere verhouding van afbraakproducten, is moeilijk te zeggen. Blootstelling van borstweefselcellen aan 4-OHE2 en 2-OHE2 is op zich niet schadelijk, omdat ze normaal snel door COMT worden omgezet, zodat ze geen schade kunnen aanrichten. Pas als COMT geremd wordt, ontstaan er problemen. Zo kan COMT geremd worden door sommige fytochemicaliën (zie Box B) die zo erg lijken op 4-OHE2 en 2-OHE2, dat ze ook door COMT worden herkend en omgezet. Daardoor is er minder COMT beschikbaar om 4-OHE2 en 2-OHE2 om te zetten wat leidt tot DNA schade. Maar COMT speelt nog een belangrijke rol bij de bescherming van borstweefselcellen. 2-OHE2 wordt door COMT omgezet in 2-methoxyoestradiol (2-MeOE2). 2-MeOE2 heeft een aantal anti-kanker werkingen. Het remt groeien van cellen, het zet cellen aan tot apoptose (geprogrammeerde celdood) en het beschermt cellen tegen DNA schade. Dus een lagere COMT activiteit betekent ook minder 2-MeOE2 vorming, dus minder anti-kanker stof. Alleen kijken naar de verhouding van 4-OHE2 en 2-OHE2 in borstweefsel lijkt in dus niet voldoende om een uitspraak te kunnen doen over een mogelijk borstkankerrisico.

2.2 Biomarkers van blootstelling

Blootstellings biomarkers worden gebruikt om blootstelling aan bepaalde omgevingsfactoren vast te stellen. Zo worden de enzymen CYP1A1 en CYP1B1 vaak gebruikt om de blootstelling aan dioxinen en dioxine-achtige stoffen te bepalen. CYP1A1 en CYP1B1 komen ook voor in bloedcellen en kunnen daarin makkelijk gemeten worden. Net als bij borstweefselcellen, gaan witte bloedcellen die worden blootgesteld aan dioxinen en dioxine-achtige stoffen in een laboratorium experiment, meer CYP1A1 en CYP1B1 enzymen maken. Hoe meer dioxine en dioxine-achtige stoffen je toevoegt, hoe meer enzymen een cel maakt. Het idee achter CYP1A1 en CYP1B1 als blootstellings biomarker is dat hoe meer iemand wordt blootgesteld aan dioxine en dioxine-achtige stoffen, hoe meer CYP1A1 en CYP1B1 enzymen in witte bloedcellen gemeten zullen worden.

Het probleem is alleen dat de hoeveelheid CYP1A1 en CYP1B1 die personen in witte bloedcellen hebben nogal kan verschillen. Verder zijn er vrij hoge concentraties dioxine en dioxine-achtige stoffen nodig om een goed waarneembaar effect op CYP1A1 en CYP1B1 te krijgen. Hele subtiele effecten van dioxine en dioxine-achtige stoffen op de

hoeveelheid CYP1A1 en CYP1B1 vallen daardoor niet op. Dat blijkt uit een kleine studie met gezonde vrouwen, waarbinnen weinig variatie was te verwachten in blootstelling aan dioxine en dioxine-achtige stoffen. Maar ook uit een grote EU-studie met twee groepen mensen uit Slowakije. Eén groep mensen is jarenlang blootgesteld aan hoge concentraties PCBs, doordat die daar werden geproduceerd. De andere groep mensen was niet blootgesteld aan zulke hoge concentraties. De hoeveelheid PCBs in het bloed en de hoeveelheid CYP1A1 en CYP1B1 in witte bloedcellen was in beide groepen hetzelfde. Er bleek ook geen verband te zijn tussen de hoeveelheid PCBs die mensen in hun bloed hadden en de hoeveelheid CYP1A1 en CYP1B1 in de witte bloedcellen. Dit betekent dat CYP1A1 en CYP1B1 in witte bloedcellen niet gebruikt kunnen worden als blootstellings biomarker voor dioxine en dioxine-achtige stoffen.

2.3 Biomarkers van gevoeligheid

Er zijn grote verschillen hoe mensen op blootstelling aan omgevingsfactoren kunnen reageren. Genetische verschillen (polymorfismen) zorgen veelal voor die verschillen in vatbaarheid. Deze polymorfismen kunnen in cruciale genen zitten die bijvoorbeeld betrokken zijn bij de regulatie van celdeling, DNA reparatie of tumorvorming (zoals het borstkankergen BRCA). Polymorfismen kunnen ook in minder cruciale genen voorkomen, waardoor bijvoorbeeld de vatbaarheid voor borstkanker verandert. Polymorfismen zijn een voorbeeld van biomarkers van gevoeligheid. Biomarkers van gevoeligheid geven dus een bepaalde vatbaarheid aan die mensen kunnen hebben voor effecten van een omgevingsfactor.

Er is een aantal polymorfismen bekend van CYP1A1, CYP1B1 en COMT, waarvan niet altijd de effecten bekend zijn. Een polymorfisme kan bijvoorbeeld gevolgen hebben voor de hoeveelheid enzym in een cel. Zo kan een persoon met een bepaalde variant van CYP1B1 na blootstelling aan dioxine en dioxine-achtige stoffen veel meer CYP1B1 enzym hebben dan een persoon zonder die variant. In Slowakije-populatie was echter geen effect te zien van een CYP1B1 polymorfisme. Sommige polymorfismen veranderen de aktiviteit van een enzym. Dat betekent dat bijvoorbeeld een vrouw een variant van CYP1A1 en COMT kan hebben met een lage aktiviteit en een variant van CYP1B1 kan hebben die zorgt voor een hogere enzymaktiviteit. Deze combinatie betekent dus veel “slechte” oestrogeenafbraakproducten die niet snel onschadelijk gemaakt worden. Deze vrouw heeft dus van zichzelf een verhoogde kans heeft op borstkanker.
Om de effecten van polymorfismen in CYP1A1, CYP1B1 en COMT op bijvoorbeeld borstkankerrisico vast te stellen zijn grote, uitgebreide experimenten nodig. De effecten

van polymorfismen zijn subtiel in vergelijking met bijvoorbeeld de "standaard" borstkankerrisicofactoren zoals beschreven in paragraaf 1.1. Toch, de invloed van één polymorfisme is misschien gering, maar het effect van meerdere polymorfismen kan aanzienlijk zijn. Je hebt daarom een hele grote groep mensen en heel veel metingen nodig om de effecten van polymorfismen te kunnen zien.

3 Kort samengevat

Oestrogenen, vrouwelijke hormonen, kunnen borstkanker veroorzaken. Dit gebeurt onder andere doordat de 'slechte' afbraakproducten van oestrogenen borstweefsel kunnen beschadigen. Lichaamsvreemde stoffen zoals dioxinen, dioxine-achtige stoffen en bepaalde plantenstoffen kunnen de aktiviteit van de enzymen die zorgen voor de oestrogeenafbraak veranderen. Hierdoor kan de verhouding 'goede'-'slechte' afbraakproducten te veranderen of worden de afbraakproducten niet onschadelijk gemaakt. De verhouding en de hoeveelheid oestrogeenafbraakproducten is goed te voorspellen aan de hand van de aanwezige enzymen in borstweefsel. Het probleem is echter dat het gevolg van verandering in oestrogeenafbraak voor borstkankerrisico moeilijk in te schatten is. We weten niet of de hoeveelheid enzymen in borstweefsel en bloed hetzelfde zijn en er is geen duidelijk verband tussen de hoeveelheid enzymen voor oestrogeenafbraak in het bloed en de hoeveelheid lichaamsvreemde stoffen in het bloed. Blootstelling aan bepaalde lichaamsvreemde stoffen kan dus *in principe* leiden tot een verhoogde kans op borstkanker door veranderde enzymaktiviteiten, maar de enzymen op zich zijn hiervoor geen goede biomarkers voor.

Verder moet in het achterhoofd worden gehouden dat de studies die zijn beschreven in dit proefschrift allemaal zijn uitgevoerd *in vitro*, in een schaaltje in het laboratorium. Het is niet zeker dat vrouwen die zijn blootgesteld aan dioxinen en dioxine-achtige stoffen ook een hele andere oestrogeenafbraak hebben en een verhoogde kans op borstkanker. Van fytochemicaliën wordt over het algemeen gezegd dat ze gezond zijn en het borstkankerrisico verlagen. Maar het is niet uit te sluiten dat sommige ook negatieve effecten hebben. Dit zal niet zo zeer gebeuren bij normale inname van groenten en fruit door gezonde mensen, maar bijvoorbeeld wel bij extreem hoge inname door bijvoorbeeld voedingssupplementen. Het motto van Philippus Aureolus Theophrastus Bombastus von Hohenheim (1493-1541), beter bekend als Paracelsus, de grondlegger van de moderne toxicologie, blijft daarbij nog altijd overeind: "*dosis sola facit venenum*". Het is de dosis dat iets een gif maakt.

BOX A. Dioxine en dioxine-achtige stoffen

Dioxine en dioxine-achtige stoffen zijn overal in het het milieu te vinden: in de lucht, grond, water, voedsel en in dier en mens. Ze lossen gemakkelijk op in vet. Verder zijn het erg stabiele verbindingen die goed bestand zijn tegen afbraak. Ook zie je accumulatie in de voedselketen; een dier (of een mens) dat een dier opeet die dioxinen bevat, krijgt behalve de dioxinen die al in het lichaam waren ook nog eens de dioxinen erbij van het opgegeten dier.

Er zijn drie verschillende klassen te onderscheiden: de polychlorinated dibenzo-p-dioxinen (PCDDs), polychlorinated dibenzofurans (PCDFs) en de polychlorinated biphenyls (PCBs). PCDDs en PCDFs zijn ongewenste bijproducten van chemische en thermische processen en hebben geen economisch belang. Ze kunnen vrijkomen bij industriele processen zijn, maar ook bij natuurlijke processen zoals een vulkaan uitbarsting of bosbrand. TCDD is de meest potente dioxine. De PCBs kennen wel een brede toepassing en werden veelvuldig geproduceerd, totdat de productie werd verboden in de jaren 80. Ze werden onder andere gebruikt als vlamvertragers, verf toevoeging en als wax.

BOX B. Fytochemicaliën

Fytochemicaliën zijn chemische stoffen die door planten worden gemaakt. Deze chemicaliën geven planten hun kleur, smaak, geur en textuur. De fytochemicaliën zijn in de evolutie ontstaan als bescherming tegen toenemende zuurstof concentraties in de atmosfeer. Ze helpen ook een plant bij de bescherming tegen bacteriën, schimmels, virusen en vraat door dieren. Tegenwoordig wordt de term fytochemicaliën voornamelijk gebruikt voor plantenstoffen die een effect hebben op de gezondheid, maar geen voedingsstoffen (eiwitten, koolhydraten, vetten, mineralen en vitaminen) zijn.

Dankwoord

There's no simple explanation
for anything important
any of us do
The Tragically Hip – Courage

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Curriculum Vitae

Majorie Beatrix Maria van Duursen was born in Leiden on 9 mei 1977. In 1995, she graduated from high school at the Hertog Jan College in Valkenswaard. That same year she began her study Biology at the Utrecht University. She participated in an excellent tracé program on the role of *Hox*-genes in the early development of *Drosophila Melanogaster*. In the final part of her study she investigated the effects of phthalate esters on human reproduction at the Science Shop for Biology under supervision of prof. M. van den Berg, Ph.D. (Institute for Risk Assessment Sciences, IRAS) and mrs. M. Lürsen, M.Sc. (SSB). After that she developed an analytical method for identification and quantification of heroin metabolites in human plasma (under supervision of mrs. I.J. Bosman, Ph.D., Utrecht University, Faculty of Pharmacy, section Human Toxicology). She received her Masters degree in 2000, and started working at the Health Council of the Netherlands in the Committee on the Safety Assessment of Novel Foods. The end of that year, she began her Ph. D. project at the IRAS. Her Ph. D. project focused on the use of estrogen metabolizing enzymes as biomarkers of exposure, effect and susceptibility for breast cancer. She also participated in the EU project PCBRISK that evaluates human health risks of low-dose long-term exposure to a group of persistent organic pollutants in a Slovakian population. She will continue her research work at the IRAS.