Inhibition of 17β-estradiol activation by CYP1A1: Genotype- and regioselective inhibition by St. John's Wort and several natural polyphenols

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ABSTRACT

Several epidemiological studies associate certain CYP1A1 genotypes, alone or in combination, with an increased risk of estrogen-related cancers. Previously we demonstrated that metabolic activation of estrogens by CYP1A1 is a genotype-dependent reaction with the CYP1A1.2 (Ile462Val) variant being the most efficient catalyst (Kisselev et al.). To answer the question whether genotype-dependent inhibition of activation of estrogens by CYP1A1 could also contribute, we studied the inhibition of hydroxylation activity of the most common allelic variants of human CYP1A1 towards 17β-estradiol. We expressed and purified CYP1A1.1 (wild-type), CYP1A1.2 (Ile462Val), and CYP1A1.4 (Thr461Asn) and performed inhibition assays by natural polyphenols of our diet and drugs of NADPH-dependent estradiol hydroxylation in reconstituted CYP1A1 systems. From the polyphenols studied, a St. John’s Wort (Hypericum perforatum) extract, some of its main single constituents hypericin, pseudohypericin, and quercetin, as well as the flavonols kaempferol, myricetin and the phytoestrogens resveratrol and tetramethyl-stilbene exhibited strong inhibition. For the St. John’s Wort extract and its single constituents hypericin, pseudohypericin, and quercetin, inhibition exhibited a remarkable dependency on the CYP1A1 genotype. Whereas (wild-type) CYP1A1.1 was most inhibited by the whole crude extract, the variant CYP1A1.2 (Ile462Val) was significantly stronger inhibited by the constituents in its pure form: IC50 values for 2-hydroxylation was more than two times lower compared with the wild-type enzyme and the variant CYP1A1.4 (Thr461Asn). Besides this, the inhibition exhibited a remarkable regioselectivity. The data suggest that risk of estrogen-mediated diseases might be not only influenced by CYP1A1 genotype-dependent activation but also its inhibition by natural polyphenols of our diet and drugs.

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1. Introduction

Drug interactions have always been a major concern in medicine. The most important enzyme system for drug metabolism is the CYP system. To date, at least 17 CYP families have been identified in mammals. Three main CYP gene families, CYP1, CYP2, and CYP3, are responsible for most drug metabolism. Thereby inhibition and induction are probably the most common causes for drug interaction, and the large interindividual variation in response to enzyme inhibition and induction is one of the major complicating factors [1,2]. The present paper dealt with inhibition of CYP1A1, which has been generally considered as one of the major cytochrome P450 (CYP) isoforms responsible for the 2-hydroxylation of 17β-estradiol (E2) in extrahepatic tissues including breast [3]. This reaction leads to the formation of the catechol estrogen 2-hydroxy-E2 (2-OH-E2). Subsequent oxidation leads to quinones which are putative tumor initiators [4,5]. Although 2-OH-E2 is known to have less toxicity caused by DNA adduct formation than 4-OH-E2 (which is the major product of CYP1B1), recent results suggest that, at least in several cell lines, 2-OH-E2 is likely to exert more cytotoxicity by generating a larger...
amount of reactive oxygen species during redox cycling between semiquinones and quinones [6]. Secondly, metabolism of catechol estrogens can involve O-methylation by catechol-O-methyltransferase (COMT) [7] to 2-MeO-E2 which has emerged as a promising cancer therapy because of its growth-inhibitory and proapoptotic effects, its protective activities in the cardiovascular and renal systems [8–11] and, as recently demonstrated, as a promising agent to fight metastatic breast cancer [12].

Simultaneously, the production of 2-MeO-E2 may be reduced by feedback inhibition on the CYP1A1-catalyzed estrogen reactions [13]. The methoxy-estrogens also act as inhibitors of CYP1B1-catalyzed 4-hydroxylation leading to 4-OH-derived semiquinones and quinones with their known higher potential of carcinogenicity. Thirdly, 2-OH-E2 can undergo conjugation by other phase II enzymes. Therefore the expression and function of CYP1A1 appears to be a critical determinant of the metabolism and toxicity of estrogens.

The human gene for CYP1A1 is polymorphic. Apart from the wild-type (CYP1A1*1), many alleles have been described in different populations; however, several are very rare and of unknown functional significance (see http://www.imm.ki.se/CYPalleles/cyp1A1.htm). The most common alleles resulting in amino acid substitutions are CYP1A1*2 (Ile462Val) and CYP1A1*4 (Thr461Asn). Several epidemiological studies discuss an association of CYP1A1 genotypes with an increased risk of certain types of cancer including cancers possibly related to CYP1A1-mediated estrogen activation such as breast cancer [14,15], prostate cancer [16,17] and ovarian cancer [18]. Interestingly, when the human body burden of polychlorinated biphenyls, known as potent inducers of CYP1A1, was high, an increased risk of breast cancer associated with the presence of the CYP1A1*2 allele was reported [19].

Because CYP1A1 catalyzes the formation of primarily 2-OH-E2, the CYP1A1 polymorphism may affect disease risk by both modifying activation and its inhibition caused by environmental exposure such as natural polyphenols of our diet and drugs. Recently we found that all CYP1A1 variants catalyzed the formation of 2-, 4-, 6α-, and 15ß-hydroxylated estrogen metabolites from E2, yet with varying catalytic efficiency and distinct regiospecificity. For instance, the variant CYP1A1.2 (Ile462Val) had a significantly higher catalytic activity for all hydroxylation sites, but it was most pronounced for 2-hydroxylation. Catalytic efficiency for the formation of 2-OH-E2 was 5.7-fold higher, compared with the wild-type enzyme [20]. With regard to inhibition of estradiol activation there are only few reports; dependence on the CYP1A1 genotype has not been reported up to now. To address this question, we expressed and purified recombinant CYP1A1.1 (wild-type), CYP1A1.2 (Ile462Val) and CYP1A1.4 (Thr461Asn) proteins and compared how they were inhibited by several natural polyphenols of our daily diet and drugs in their capacity to metabolize the endogenously occurring 17ß-estradiol: (i) St. John’s Wort extract, an increasingly popular preparation in the treatment of depression [21] but with serious drug interactions [22–24], and its main single constituents hypericin, hyperforin, pseudo-hypericin, quercetin, caffeic acid, chlorogenic acid, rutin [25], and (ii) selected polyphenols which represent major dietary flavonoids. All of these were selected, because they exerted antioxidant, anticancer, antiinflammatory, antiproliferative, and apoptotic effects largely analyzed in vitro, in cell culture and animal models.

2. Materials and methods

2.1. Chemicals

E2, 2-OH-E2, 4-OH-E2, 6α-OH-E2, 16α-OH-E2 and the inhibitors quercetin, kaempferol, myricetin, rutin, resveratrol, and epigallocatechin gallate were purchased from Sigma (Deisenhofen, Germany). Hypericin, hyperforin, pseudohypericin, epigallocatechin gallate, caffeic acid, chlorogenic acid were purchased from Roth GmbH (Karlsruhe, Germany), and tetramethyl-stiblene from Tocris (Bristol, UK). [4-14C]E2 (specific activity: 1998 MBq/mmol; 1.85 mM in ethanol) was purchased from Hartmann Analytik (Braunschweig, Germany). As basis for the St. John’s Wort preparation commercially available Jarsin™ coated tablets (Lichtwer-Pharma, Berlin, Germany) were used. The tablets were ground and dissolved in DMSO to a concentration of 50 mg/ml and tested directly for inhibition of CYP1A1 as described below.

2.2. Expression, purification, reconstitution and analysis of recombinant enzymes

The modified CYP1A1 cDNAs for expression of the CYP1A1 variants were constructed according to Chernogolov et al. [26,27]. Enzymes were expressed in Spodoptera frugiperda (SF9) insect cells as C-terminal His-tag proteins and purified by Ni-affinity chromatography to a specific content of about 11 nmol CYP/mg protein as described [26]. Reduced CO-difference spectroscopy proved the expressed variants to be free any inactive cytochrome P420; homogeneity was proven by SDS-PAGE. Human P450 reductase was expressed in SF9 cells and purified to a specific catalytic activity of 18.2 units/mg protein according to Tamura et al. [28]; homogeneity was confirmed by SDS-PAGE.

Reconstitution of enzymatic activity of CYP1A1 and P450 reductase was achieved by incubation with insect cell control microsomes as described recently [29]. They contained no CYP and no P450 reductase and exhibited no activity towards several substrates such as ethoxyresorufin, benzo[a]pyrene, arachidonic acid, and the estrogens studied here. CYP content was measured by reduced CO-difference spectroscopy [30]. Protein concentration was determined using the Coomassie Plus protein assay (Pierce, Rockford, IL, USA). P450 reductase concentration was determined spectrally using an extinction coefficient of 21 mM−1 cm−1 (at 450 nm). P450 reductase activity was determined as NADPH-cytochrome c reductase activity [31].

2.3. Inhibition assays of estradiol hydroxylation

For NADPH-mediated reactions, 20 pmol CYP1A1 were reconstituted with a 5-fold molar excess of recombinant human P450 reductase and 80 μg of control microsomes (incubation for 10 min on ice) and added to 50 mM Tris/HCl buffer (pH 7.5) containing 100 mM NaCl and 2 mM ascorbate. 1 μl containing the appropriate amount of inhibitor were added followed by addition of 1 μl DMSO containing the substrate E2 (in a final concentration of 15 μM). The reactions were started after short preincubation (2 min at 37 °C) by addition of NADPH (final concentration 1 mM) in a final volume of 100 μl. Reactions proceeded 20 min at 37 °C under gentle shaking in a water bath and were stopped by addition of 1 ml ethyl acetate. Metabolites and substrate were extracted twice in 1 ml ethyl acetate. Extracts were combined and, after evaporation, reconstituted in 80 μl of methanol/water (80/20, v/v). Usually 45 μl were injected for HPLC analysis.

Substrate stock solutions were prepared by solution of the original [14C]E2 to 1.5 mM in ethanol. The solvent ethanol was evaporated and estradiol dissolved in DMSO so, that 1 μl per assay (100 μl) resulted in an E2 concentration of 15 μM (and approximately 200 000 cpm per assay). All inhibition experiments were done at a E2 concentration of 15 μM, only little above the Km of the 2-hydroxylation of E2 by CYP1A1 (all three CYP1A1 variants exhibited similar Km values of about 10 μM; [20]). Whenever possible, we worked with inhibition stock solutions of 10 mM in DMSO which were further diluted so, that addition of 1 μl to the assay volume resulted in the desired inhibitor concentration.
2.4. HPLC analysis

A HPLC system (LC-10Avp, Shimadzu, Japan) equipped with a radioactivity detector (LB509, Berthold, Germany) and a reversed-phase column Nucleosil 100-5C18HD (250 × 4 mm) (Machery-Nagel, Dueren, Germany) was used for separation and analysis of the metabolites. HPLC method used for separation and analysis was described before [20] with the only modification, that the final volume of the reaction assay was reduced from earlier 250 to 100 μl, here. Shortly, HPLC was performed at a flow rate of 0.8 ml/min beginning with a 10-min isocratic elution with 30% B (acetonitril/0.5% acetic acid):70% A (water/0.5% acetic acid). This was followed by a 10-min linear gradient from 30% B/70% A to 40% B/60% A and a 15-min gradient from 40% B / 60% A to 100% B. Elution was complete after another 15 min of 100% B. Metabolites were identified by comparing their retention times with authentic standards.

2.5. Data analysis

IC50 values represent the mean of two separate determinations using 8 different concentration of the inhibitor (usually between 0.1 and 100 μM). They were determined fitting the data with non-linear regression using Sigma Plot 2001 using the Enzyme Kinetics Module (SPSS Science Software, Erkrath, Germany). Statistical significance of results was assessed using one-way ANOVA software (GraphPad Software, San Diego, CA, USA).

3. Results

Wild-type and variant CYP1A1 catalyzed E2 hydroxylations mainly at C-2, C-6, and C-15 [18]. First we analyzed inhibition by SJW extract, its major constituents and several other natural polyphenols of the main hydroxylations by wild-type CYP1A1. IC50 values for the three hydroxylation sites are represented in Table 1.

To compare the inhibitory potency for the major hydroxylation site C-2, we calculated inverse IC50 values and represented it in Fig. 1. Comparison of the values showed quercetin, pseudohypericin, and hypericin to be the most potent inhibitors among the constituents of SJW extract. The other constituents, hyperforin, rutin, caffeic acid, and chlorogenic acid showed almost no inhibition capacity, at least not up to a concentration of about 100 μM. The whole extract also inhibited potently 2-hydroxylation with an IC50 of about 19.5 μg/ml. Among the other polyphenols studied, the flavonols kaempferol and myricetin were most inhibiting, even more than the most potent SJW inhibitors.

Table 1

IC50 values for inhibition of estradiol (E2) hydroxylation by CYP1A1: regioselective inhibition.

<table>
<thead>
<tr>
<th>Polyphenol</th>
<th>Class</th>
<th>IC50 (μM) *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2-15α-OH-E2</td>
</tr>
<tr>
<td>SJW extract</td>
<td>Hypericin perf.extract</td>
<td>19.5</td>
</tr>
<tr>
<td>Hypericin</td>
<td>Naphthodianthrone</td>
<td>9.8</td>
</tr>
<tr>
<td>Pseudohypericin</td>
<td>Naphthodianthrone</td>
<td>6.8</td>
</tr>
<tr>
<td>Hyperforin</td>
<td>Phloroglucinol</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Flavonol</td>
<td>3.2</td>
</tr>
<tr>
<td>Rutin</td>
<td>Flavonoid glycoside</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>Phloroglucinol</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>Phenylpropane</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>Flavonol</td>
<td>2.2</td>
</tr>
<tr>
<td>Myricetin</td>
<td>Flavonol</td>
<td>2.4</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Phytosterol</td>
<td></td>
</tr>
<tr>
<td>Stilbene</td>
<td>Phytosterol</td>
<td></td>
</tr>
<tr>
<td>TMS</td>
<td>Stilbene</td>
<td>4.8</td>
</tr>
<tr>
<td>(−)Epigallocatechin gallate</td>
<td>Tea catechin</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

* For SJW extract IC50 values are given in μg/ml.

Table 1 showed that for several polyphenols the inhibition exhibited a remarkable regioselectivity. The inhibition capacities of the whole SJW extract and its single constituents pseudohypericin and hypericin, but not quercetin, were more than twofold more effective for inhibition of 2-hydroxylation of estradiol than those for 15α- and 6α-hydroxylation which were approximately the same. Also the phytoestrogens resveratrol and TMS inhibited 2-hydroxylation much more effectively than hydroxylation at other sites. Kaempferol and myricetin did not show any regiospecific inhibition, although they were the most potent inhibitors at all.

Earlier we found a CYP1A1 genotype-dependent inhibition by quercetin of benz(a)pyrene activation [32]. Therefore, it would be interesting to ask, whether activation of estradiol by CYP1A1 also exhibits genotype-selective inhibition. Therefore we analyzed inhibition of estradiol 2-hydroxylation by the different CYP1A1 alleles for whole SJW extract and its constituents (hypericin, pseudohypericin, quercetin, hyperforin, rutin, caffeic acid, chlorogenic acid). Fig. 2 shows the effect of the whole SJW extract and of several constituents on 2-OH-E2 formation by the three CYP1A1 variants. IC50 values were determined and represented in Table 2.

Clearly, the figures and the table show the CYP1A1.2 variant to be the most inhibited catalyst for the pure SJW constituents hypericin, pseudohypericin, and quercetin. All other constituents studied showed no inhibition and no differences between the CYP1A1 variants. Contrary to the behaviour of pure SJW constituents, inhibition by the whole SJW extract was most effective for the (wild-type) CYP1A1.1 variant. Inhibition of CYP1A1.2 and CYP1A1.4 was at least two times less effective than that for the wild-type variant.

The data with regard to a genotype-dependent inhibition of 15α- and 6α-hydroxylation go in line with the genetic variability of inhibition in 2-hydroxylation (data not shown). Hypericin, pseudohypericin, and quercetin inhibited about two times more 15α- and 6α-hydroxylation by the CYP1A1.2 variant, whereas the whole crude SJW extract inhibited most (wild-type) CYP1A1.1 variant. Interestingly, hyperforin, a relative weak inhibitor at all with a IC50 > 100 μM, exhibited the same tendency. However, the few data taken at such high inhibitor concentration do not justify any significant results for hyperforin (Fig. 3).

4. Discussion

Our earlier results showed that all CYP1A1 variants are actively involved in the oxidative metabolism of E2, but with a varying degree of efficiency and regiospecificity. Most remarkably, catalytic efficiency
of CYP1A1.2 (Ile462Val) for the formation of the major metabolite 2-OH-E2 was 5.7-fold higher than those of the wild-type enzyme, clearly demonstrating that this polymorphismus is functionally significant. In the present *in vitro* study, we characterized the inhibition properties of polyphenols in the NADPH-dependent oxidation of E2 by three most common allelic variants of human CYP1A1. The studies revealed that whole extracts of SJW, its single constituents hypericin, pseudohypericin, quercetin, the flavonols kaempferol and myricetin, as well as the phytoestrogens resveratrol and TMS are potent inhibitors of CYP1A1-mediated estradiol activation.

It is interesting that all the constituents studied in its pure form most effectively inhibited the CYP1A1.2 variant (or showed no inhibition), whereas the crude whole SJW extract inhibited most wild-type CYP1A1.1. SJW preparations are very complex mixtures of a huge variety of compounds. Therefore, it is difficult to single out the inhibitor which mainly determined the properties of the whole extract. It might be a compound not considered in our study: either a constituent with high inhibitory potency and a relative low abundance or a less potent inhibitor with a much higher concentration. The results obtained for hyperforin seem to indicate that hyperforin may play a role: hyperforin, although a relative weak inhibitor may be present in a relative high concentration, exhibited the same behaviour as the whole SJW extract, i.e. most potent inhibited the wild-type CYP1A1. With regard to the antidepressant activity of constituents of SJW extracts, earlier hyperforin,
a phloroglucinol, has been identified as a major antidepressant [33]. More recently it has been shown in the forced swimming test—an animal model for antidepressant activity—that flavonoids, especially rutin, are essential constituents of a “therapeutic extract”—pointing to the importance of flavonoids in the antidepressant treatment [34,35]. Additional data are required for a more meaningful analysis of in vitro data which, it is hoped, should enable us to identify the active principle/compound(s) and its target in the brain.

The in vivo situation of CYP1A1 inhibition is much more complicated as pharmacokinetic properties (e.g. distribution, bioavailability, intracellular distribution and clearance) must be taken into account. Data regarding pharmacokinetic distribution of St. John’s Wort constituents have not been published until now—to our knowledge. With regard to this, we refer to our discussion in [32,36]. Shortly, it is suggested that the drug is expected to come in contact with potential target tissues where CYP1A1 is expressed and/or induced, e.g. in the lung and in the gastrointestinal tract (oesophagus and small intestine) as well as the breast [37]. With regard to pharmacokinetic properties such as plasma concentration and clearance, hypericin, pseudohypericin and hyperforin have been studied in healthy volunteers and these constituents were identified.
as relatively low clearance drugs with long half-life times (10–16 h) [38]. Their plasma concentrations can reach steady state concentrations up to about 3 µM. With regard to the intracellular concentration it is likely that such highly hydrophobic substances as hypericin are localized in the different cellular membrane systems [39]. Indeed for hypericin, localization experiments in human glioblastoma and carcinoma cell lines proved its predominant localization at micro-molecular concentrations, mainly the endoplasmic reticulum, the site where CYP1A1 is also localized. With regard to other flavonoids, quercetin as one of the most abundant flavonoids of our daily diet, it was reported that quercetin could be readily absorbed and can reach micromolecular concentrations in the plasma and urine [40]. Moreover, quercetin exhibited a long elimination half-life (~24 h), suggesting that repeated intake would lead to a build-up to even higher levels [41]. These data suggest that the concentration of at least some of St. John’s Wort constituents taken with the therapeutically recommended doses can reach plasma and/or intracellular concentrations roughly in the range of IC₅₀ values for inhibition determined in the present study. This has been also reported for other polyphenols studied here [40,42]. In this way, the hypothesis is supported that SJW, single constituents of it and other polyphenols can modify estradiol activation and its inhibition in a genotype-dependent process.

Earlier we demonstrated that quercetin potently inhibited benzo[a]pyrene-diolepoxide 2 formation by CYP1A1—the terminal step in the bioactivation of the well-known lung carcinogen benzo[a]pyrene—in a genotype-selective manner [32]. The inhibition effect was greatest towards the (wild-type) CYP1A1.1 variant. In the present study the variant CYP1A1.2 is the most inhibited catalyst, supporting the hypothesis that inhibitory effects of a distinct polyphenol on CYP1A1 activity depend on both the CYP1A1 genotype and the reaction (substrate) used in the assay. Note that quercetin is not only a constituent of SJW, but also one of the major dietary flavonoid, found in a broad range of fruit, vegetables, and beverages such as tea and wine.

In the present study we report mainly results obtained with natural polyphenols as potential inhibitors. Preliminary experiments with environmental toxicants such as the polycyclic aromatic hydrocarbon benzo[a]pyrene and the xenoestrogen bisphenol A showed much higher inhibitory potential toward estradiol activation. For instance, benzo[a]pyrene inhibited 2-hydroxylation of estradiol with a IC₅₀ of less than 1 µM in a CYP1A1 genotype-dependent manner. Experiments are in progress to further analyze inhibition of CYP1A1-mediated estrogen metabolism by environmental toxicants and xenoestrogens.

A potential limitation of our in vitro study is the lack of data with regard to inhibition of CYP1A1 expression. Other studies demonstrate that at least several compounds investigated in the present study inhibit the formation of catechol estrogens by inhibiting expression of CYP1A1. For instance, resveratrol strongly inhibited the expression of CYP1A1 in cultured human mammary epithelial MCF-10A cells after CYP1A1. For instance, resveratrol strongly inhibited the expression of CYP1A1, in different target cells. As discussed in the Introduction, estrogen activation occurs via a multi-step pathway under participation of several enzymes, which almost all are polymorphic. It is not expected that single (CYP1A1) gene polymorphisms necessarily cause remarkable interindividual variability to be related to disease. Thus, future epidemiological studies should consider the whole range of polymorphic genes involved in estradiol expression and metabolism, and the influence of life style such as food and drugs on polymorphic enzymes in order to have the potential to design successful chemoprevention of cancer and other disease. We believe our data support the view that knowledge that directly leads to prevention is based on studies of functional parameters such as protein expression and functional assays [45].

5. Conclusions

In conclusion, the results suggest that SJW preparations, several of their major constituents such as hypericin, pseudohypericin, and quercetin as well as several other polyphenols of our diet potently inhibit the formation of 2-OH-E2 (and other hydroxylations products) from estradiol. Our in vitro data indicate inhibition by these compounds of estradiol activation as a CYP1A1 genotype-dependent mechanism. In dependence on the inhibitor, subjects carrying the CYP1A1.2 allele (hypericin, pseudohypericin, quercetin) or the wild-type CYP1A1.1 allele (whole SJW extract) will be mostly affected. It is too early to predict any clinical relevance, but it should be noted that ethnic differences in the frequency of CYP1A1 alleles are considerable: the CYP1A1*2 allele occurs with a much higher frequency in Asians (18–33%) than in Caucasians (2–10%). CYP1A1*4 occurred in about 2–6% of Caucasians, but has not been found in Japanese and Chinese. Notably, in several American populations living in South America exorbitantly high frequencies of 81–100% and 54–100% for CYP1A1*2A and CYP1A1*2C, respectively, were found [44]. Further studies will be necessary to elucidate the mechanism of inhibition and a possible inhibition of 2-OH-E2 formation due to reduction of the expression of CYP1A1.

In general, our studies provide further support that the effect of dietary (including drug) exposure may be modified by variation in metabolism-related genes. The studies suggest that as mechanism not only the activation but also the inhibition of the activity of such genes should be considered to play a role, even if the differences found in our study are not as big. The data presented in the present paper might contribute to an enhanced understanding of factors that influence metabolism of estrogens, particularly the function of CYP1A1, in different target cells. As discussed in the Introduction, estrogen activation occurs via a multi-step pathway under participation of several enzymes, which almost all are polymorphic. It is not expected that single (CYP1A1) gene polymorphisms necessarily cause remarkable interindividual variability to be related to disease. Thus, future epidemiological studies should consider the whole range of polymorphic genes involved in estradiol expression and metabolism, and the influence of life style such as food and drugs on polymorphic enzymes in order to have the potential to design successful chemoprevention of cancer and other disease. We believe our data support the view that knowledge that directly leads to prevention is based on studies of functional parameters such as protein expression and functional assays [45].

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