

CYP1A1 is overexpressed upon incubation of breast cancer cells with a polyphenolic cocoa extract

Carlota Oleaga · Miriam García · Anna Solé ·
Carlos J. Ciudad · Maria Izquierdo-Pulido ·
Véronique Noé

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Abstract

Purpose To evaluate the effect of cocoa flavonoids in breast cancer cells at the molecular level, a functional genomic analysis was performed using a polyphenolic cocoa extract (PCE) in MCF-7 and SKBR3 cell lines.

Methods The expression profile of 84 genes included in the Stress & Toxicity PathwayFinderTM PCR Array was analyzed after PCE incubation for 24 h. mRNA and protein levels were analyzed by RT-PCR and western blot, respectively. Gel shift assays were used to evaluate DNA–protein complexes. Protein complexes were identified by co-immunoprecipitation. Cell viability was evaluated by MTT assays.

Results Upon PCE incubation, 7 genes were overexpressed and 1 underexpressed in MCF-7 cells, whereas 9 genes were overexpressed in SKBR3 cells. Among the differentially expressed genes in both cell lines, cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) was chosen for further study. CYP1A1 mRNA and protein levels and enzymatic activity increased upon PCE incubation. CYP1A1 transcriptional activation by PCE was mediated through AhR binding to XRE elements within the CYP1A1 promoter in MCF-7 cells. A protein complex including AhR and ER α

was detected. The combination of PCE with tamoxifen caused a synergistic cytotoxicity in both cell lines and was due to an increase in apoptosis in MCF-7 cells.

Conclusions The interaction between ER α and AhR upon incubation with PCE leads to CYP1A1 induction in breast cancer cells. The synergy between PCE and non-cytotoxic tamoxifen concentrations opens the possibility for a combination therapy based on polyphenols from cocoa that increased tamoxifen efficacy.

Keywords AhR · Cocoa · CYP1A1 · ER α · Tamoxifen · Breast cancer

Abbreviations

AhR	Aryl hydrocarbon receptor,
Arnt	Aryl hydrocarbon receptor nuclear translocator
BSA	Bovine serum albumin
CHX	Cycloheximide
CI	Combination index
CYP1A1	Cytochrome P450 family 1 subfamily A polypeptide 1
DEPC	Diethyl pyrocarbonate
EGCG	Epigallocatechin-3-gallate
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
GS	Gel shift
NE	Nuclear extract
OVN	Overnight
PCE	Polyphenolic cocoa extract
PI	Propidium iodide
RT-PCR	Reverse transcription-polymerase chain reaction
SE	Standard error
STP	Staurosporine
TAM	Tamoxifen
XRE	Xenobiotic response element

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C. Oleaga · M. García · A. Solé · C. J. Ciudad · V. Noé (✉)
Department of Biochemistry and Molecular Biology,
School of Pharmacy, University of Barcelona,
Av. Diagonal 643, 08028 Barcelona, Spain
e-mail: vnoe@ub.edu

M. Izquierdo-Pulido
Department of Nutrition and Food Science,
School of Pharmacy, University of Barcelona,
Av. Diagonal 643, 08028 Barcelona, Spain

Introduction

Worldwide, more than a million women are diagnosed with breast cancer every year, accounting for a tenth of all new cancers and 23% of all female cases of cancer. Breast cancer incidence rates vary considerably, with the highest rates in the developed world and the lowest rates in Africa and Asia [1]. Around 430,000 new cases of breast cancer occur each year in Europe and an estimated 212,920 cases in the USA [2].

Estrogens are implicated in the initiation and promotion stages of breast cancer, and lifetime estrogen exposure is a major risk factor for breast cancer [3, 4]. Estrogens exert their carcinogenic effects by both estrogen receptor (ER)-dependent and independent mechanisms [3, 5, 6]. The ER-dependent mechanism underlying mammary carcinogenesis involves the activation of the ER by estrogens, leading to the expression of estrogen-responsive genes and stimulation of cell proliferation [3, 5–7]. The ER-independent pathway involves the synthesis of toxic estrogen metabolites that are highly reactive and damage DNA, protein, and lipids [3, 5, 6, 8, 9]. Most human breast cancers are initially positive for ER, and their growth can be stimulated by estrogens and inhibited by antiestrogens such as tamoxifen [10]. Tamoxifen is a selective ER modulator, which acts as an antiestrogen in breast, but as an estrogen in the uterus, the cardiovascular system, and bone. Its antitumor effects are directly related to ER levels in breast tumors [11], although it can also be used as therapy in ER-negative tumors to prevent a second tumor development [12].

Dietary or therapeutic agents interfering with receptor-mediated pathways or reducing the production of genotoxic estrogen metabolites could be effective in modulating estrogen-induced breast carcinogenesis. In this direction, green tea contains polyphenolic compounds, known as catechins, such as epigallocatechin-3-gallate (EGCG) with proven anticarcinogenic effect [13]. EGCG has shown cytotoxic activity in both ER α + and ER α – breast cancer cells [14, 15], and induction of apoptosis is one of its mechanisms of action [15, 16]. Cocoa is rich in polyphenols, similar to those found in green tea. In fact, cocoa has the highest flavanol contents of all foods on a per-weight basis and is a significant contributor to the total dietary intake of flavonoids [17]. The main subclasses of flavonoids found in cocoa are flavanols, particularly the flavanol monomers catechin and epicatechin, and their oligomers, also known as procyanidins [18]. Many examples of the health benefits of cocoa consumption can be found in the literature [18–21].

The aim of our study was to determine the effect of nontoxic concentrations of cocoa polyphenols at the molecular level using as a model two human breast cancer cell lines, MCF-7 and SKBR3, that show different ER α

status. Additionally, we sought to evaluate a possible synergism between cocoa polyphenols and tamoxifen, a drug widely used for breast cancer treatment, similar to that described for EGCG.

Materials and methods

Cocoa powder phenolic extract

Natural Forastero cocoa powder from Malaysia was employed for this study. Ten grams of cocoa was subjected to an extraction of phenols [22]. The total phenolic content in the extract was determined according to the Folin-Ciocalteu method [23] and expressed in mg per mL of catechin equivalents. The phenolic composition of PCE is indicated in Online Resource 1. A stock solution was prepared in 20% DMSO.

Cell culture

Human breast cancer cell lines MCF-7 and SKBR3, and human embryonic kidney immortalized cell line HEK293T were used. Cells were grown in F-12 medium (Gibco) supplemented with 7% (v/v) fetal bovine serum (Gibco), sodium penicillin G, and streptomycin. The concentrations of PCE (150–250 ng/ μ L) used in cell incubations were not cytotoxic (Online Resource 1). MG-132 (25 μ M, Calbiochem) was incubated overnight either alone or in combination with PCE. Cycloheximide (CHX, 50 μ g/mL, Sigma) was incubated for 48 h either alone or in combination with PCE. Tamoxifen citrate (TAM, Sigma) was incubated for 48 h at concentrations ranging from 10^{-6} to 10^{-3} M, either alone or in combination with PCE. Staurosporine (STP, 10^{-7} – 10^{-6} M, Sigma) was incubated for 24 h. The amount of DMSO in these incubations was always less than 0.4%.

PCR arrays

Total RNA was prepared from 3×10^6 cells following the procedure recommended by Qiagen. Gene expression was analyzed using specific PCR arrays (Stress & Toxicity PathwayFinderTM, RT2ProfilerTM PCR Array, SuperArray), containing gene-specific primer sets for 84 relevant genes and 5 housekeeping genes, whose Ct values were used as a normalization factor. Fold changes in gene expression were calculated using the standard $\Delta\Delta$ Ct method. The expression of each gene was reported as the fold change obtained after each treatment relative to control after normalization of the data. A cutoff of 2-fold was chosen since small changes in gene expression may represent important changes downstream those differentially

expressed genes. Lists of differentially expressed genes, with a p value <0.05 , were generated from three independent experiments.

RT-PCR

Total RNA was extracted using Ultraspec (Biotex) in accordance with the manufacturer's instructions. cDNA was synthesized in a total volume of 20 μ L from RNA samples as described in Selga et al. [24]. CYP1A1 and ER α mRNA levels were determined in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 3 μ L of the cDNA reaction and the assays-on-demand Hs00153120_m1 for CYP1A1, Hs01045818_m1 for ER α and Hs00356991_m1 for APRT (all from Applied Biosystems). APRT mRNA was used as an endogenous control. Fold changes in gene expression were calculated using the standard $\Delta\Delta C_t$ method.

Western blot

Whole extracts were obtained from 3×10^6 control or PCE-treated cells according to Selga et al. [25]. Total extracts (50–150 μ g) were resolved on SDS-polyacrylamide gels and transferred to PVDF membranes (Immobilon P, Millipore) using a semidry electroblotter. The membranes were probed with CYP1A1 antibody H-70 (sc-20772), ER α antibody G-20 (sc-544), or AhR antibody H-211 (sc-5579) (all from Santa Cruz Biotechnology Inc). Signals were detected with secondary horseradish peroxidase-conjugated antibody and enhanced chemiluminescence, as recommended by the manufacturer (Amersham, Millipore). Blots were reprobed with antibodies against β -actin (A2066, Sigma) or tubulin (Cp06, Calbiochem) to normalize the results.

CYP1A1 enzymatic activity

The p450-Glo Assay (Promega) luminescent method was used to measure CYP1A1 enzymatic activity. Cells (9×10^3) were seeded in 96-well dishes and incubated with PCE (250 ng/ μ L) for 48 or 72 h. The medium was renewed either 8 h (MCF-7 cells) or 2 h (SKBR3 cells) before the end of the incubation with PCE, and 100 μ M of Luciferin 6' chloroethyl ether per well was added. At the end of the incubation period at 37 °C, the luciferin detection reagent was added, and the resulting lysates were transferred to an opaque white 96-well dish. Luminescence was read in a Modulus Microplate luminometer (Tuner Biosystems technology).

Co-immunoprecipitations

Total extracts were obtained from 3×10^6 control or PCE-treated cells following Tapias et al. [26]. Co-immunoprecipitations were performed by using either 5 μ g of ER α

antibody or unspecific IgGs (I-5006, Sigma). The specific detection of the co-immunoprecipitated AhR protein was performed by western blot using 1:200 dilution of the AhR antibody.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from control cells or cells treated with PCE (250 ng/ μ L) were prepared according to Noé et al. [27]. Binding reactions were performed as described in Tapias et al. [26] using a [γ - 32 P]-labeled specific XRE probe that was generated by PCR using a pair of CYP1A1-specific primers and HeLa genomic DNA as template. The amplified fragment of 327 bp contained four XRE binding sites and two putative Sp1 binding sites (Fig. 2a).

CYP1A1-FOR 5' tcaagtcaggctagcCATGCCAAATGGC ACTGGGGC 3'

CYP1A1-REV 5' cagtgtgcctcgagGTTGCGTGAGAAG GAACCGGAG 3'

The protein–DNA complexes were resolved on a 5% polyacrylamide-glycerol gel using 0.5 $\times$ TBE buffer (89 mM Tris boric acid, 2.5 mM EDTA, pH 8.0). The shifted bands were quantified by Phosphorimaging using the ImageQuant software v 5.2 (Molecular Dynamics). For competition experiments, the reaction mixture was incubated with different amounts of the unlabeled XRE fragment for 15 min prior to the addition of the radiolabeled probe. In the supershift experiments, 4 μ g of the antibody against AhR or unspecific IgGs (Sigma) was incubated OVN at 4 °C with the nuclear extracts before performing the binding reactions.

Cell viability assay

Cells (30,000) were plated in 2 mL of F-12 medium and incubated with increasing concentrations of PCE (50–250 ng/ μ L), or increasing concentrations of TAM (10^{-7} – 10^{-3} M), either alone or in combination with PCE (250 ng/ μ L). MTT assay was performed 24 h or 72 h later after the addition of PCE [28].

Analysis of synergism

The synergism between TAM and PCE was assessed following the Chou-Talalay method, which combines the median effect equation of Chou where dose–effect curves (from the drugs either alone or in combination) are transformed into linear equations, with the combination index (CI) equation and plot of Chou-Talalay [29]. The analysis was performed using the CalcuSyn V2 software (Biosoft).

Apoptosis assay

The levels of apoptosis in cells treated with STP, PCE, and/or TAM were determined as described in Blasco et al. [30].

Statistical methods

For the RT-PCR and western blot analyses, values are expressed as the mean $\pm$ SE. Data were evaluated by unpaired Student's *t* test when analyzing the difference between two conditions, control and treated. One-way ANOVA followed by Bonferroni post hoc multiple range test was used for different conditions that differ in one parameter (i.e. time). Both analyses were performed using the PASW Statistics v 18.0.0. software. Differences with *p* values <0.05 were considered significant.

Results

Differential gene expression analysis using PCR arrays

The expression profile of the 84 genes included in the Stress & Toxicity PathwayFinderTM PCR Array was analyzed in MCF-7 and SKBR3 cells, both control and treated with a polyphenolic cocoa extract (PCE) for 24 h. Treatment with PCE decreased the expression of *serpine 1* and up-regulated the expression of the *CYP1A1*, *GADD45A*, *GDF15*, *GPX1*, *RAD23A*, *TP53*, and *XRCC2* genes in MCF-7 cells (Online Resource 2). Upon incubation with PCE, 9 genes were overexpressed in SKBR3 cells: *CAT*, *CYP1A1*, *FM05*, *GADD45A*, *GDF15*, *HSPA5*, *IL18*, *LTA*, and *PTGS1* (Online Resource 3). All changes were statistically significant (*p* < 0.05). The *CYP1A1* gene was chosen from the three differentially expressed genes in common between both cell lines for further validation because *CYP1A1* mRNA showed the highest increase upon incubation with PCE, 17.5-fold in MCF-7 cells and 155-fold in SKBR3 cells, respectively.

CYP1A1 mRNA and protein levels and enzymatic activity upon incubation with cocoa extract

The differential expression of *CYP1A1* mRNA in control versus treated cells was validated by RT-PCR to confirm the changes obtained in the screening with the PCR arrays. It is worth mentioning that the basal levels of *CYP1A1* mRNA in SKBR3 cells were 18-fold higher than in MCF-7 cells (Fig. 1a). Upon incubation with PCE for 24 h, mRNA levels for *CYP1A1* were increased by 32-fold compared to control in MCF-7 cells. In SKBR3 cells treated with PCE, *CYP1A1* mRNA was increased by 969-fold compared to

the MCF-7 control and 54-fold compared to SKBR3 control (Fig. 1a).

Next, we investigated whether the changes at the RNA level were translated into protein. PCE treatment for 24 h led to a very modest increase in *CYP1A1* protein levels (1.2-fold). A time course incubation during 24, 48, 72 and 96 h led to an increase in *CYP1A1* protein in MCF-7 cells of 3.9-fold after 48 h (Fig. 1b), whereas in SKBR3 cells, *CYP1A1* protein was increased 17.7-fold after 72 h (Fig. 1c). The difference between mRNA levels and the corresponding protein levels may indicate that many of the mRNA molecules do not reach the translational machinery, probably because the translation mechanism is saturated in these conditions.

Finally, *CYP1A1* activity was determined upon incubation with PCE. An increase in *CYP1A1* activity in good correlation with the observed increase in *CYP1A1* protein levels was determined for both cell lines (Fig. 1d).

Effect of cocoa extract on the aryl hydrocarbon receptor pathway

Xenobiotic-responsive element (XRE)-binding protein, a heterodimer of the aryl hydrocarbon receptor (AhR) and its nuclear translocator, regulates the transcription of *CYP1A1* through XRE in response to xenobiotic inducers [31, 32]. SKBR3 and MCF-7 cells were treated with PCE for 24 h, and their nuclear extracts were subjected to EMSA. Binding to a probe corresponding to four XREs present in the human *CYP1A1* promoter produced a pattern of three bands (Fig. 2b) probably due to AhR binding to XRE, as previously described [33]. The identity of AhR binding was confirmed by competition with increasing amounts of the unlabeled probe that completely abolished the binding pattern observed (Fig. 2d) and by supershift experiments with an AhR antibody that revealed that AhR contributed to this binding pattern (Fig. 2e). Incubation with PCE caused an increase in the DNA-binding capacity of nuclear AhR in MCF-7 cells, whereas in SKBR3 cells, binding to XRE was decreased, although its binding in basal conditions was higher than in MCF-7 cells (Fig. 2b, c).

Effect of PCE on AhR and ER α protein levels

AhR and ER α protein levels were analyzed from total extracts from MCF-7 and SKBR3 cells by western blot. In MCF-7 cells, the highest induction of *CYP1A1* at 48 h of incubation with PCE took place simultaneously with the down-regulation of ER α and an increase in AhR levels (Fig. 3a). Levels of ER α in SKBR3 cells were lower than in MCF-7 cells, in accordance with their respective ER statuses, and increased in a time-dependent manner upon incubation with PCE. In these cells, overexpression of

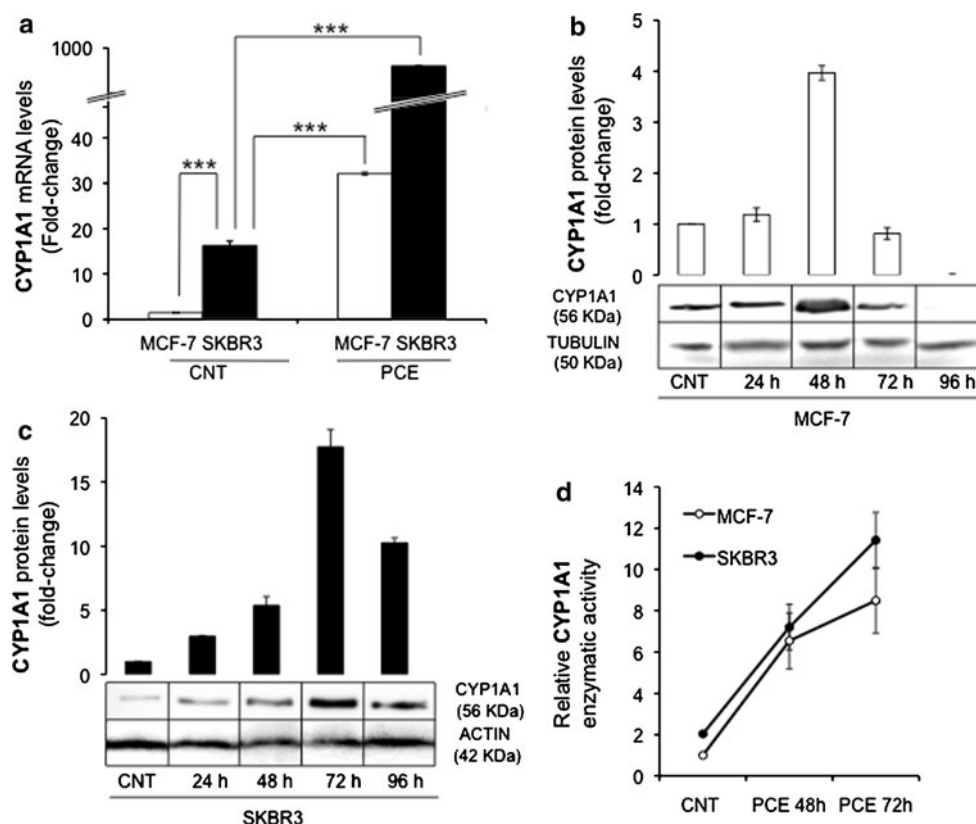


Fig. 1 CYP1A1 overexpression in cells treated with PCE. **a** Determination of CYP1A1 mRNA levels. Empty bars indicate CYP1A1 mRNA levels in MCF-7 cells, filled bars correspond to the mRNA levels in SKBR3 cells, either control (0.12% of DMSO) or treated with PCE (250 ng/ μ L) for 24 h for both cell lines. Results are expressed in fold changes compared to MCF-7 control and are the mean $\pm$ SE of 3 different experiments. *** p < 0.001 compared with the corresponding control situation. Determination of CYP1A1 protein levels. Empty bars indicate CYP1A1 protein levels in MCF-7 cells (**b**), filled bars correspond to the protein levels in SKBR3 cells (**c**), either control (0.12% of DMSO) or treated with PCE (250 ng/ μ L)

CYP1A1 protein was maximal at 72 h and correlated with an increase in both ER α and AhR levels (Fig. 3b). We next analyzed whether AhR and ER α were able to interact at the protein level. Co-immunoprecipitations were performed with a specific antibody against ER α , and the presence of AhR was analyzed in the resulting co-immunoprecipitate. As can be seen in Fig. 3c, d, AhR and ER α were part of a protein complex in both cell lines, either control or PCE-treated.

We analyzed the mechanism by which PCE triggered ER α protein levels in SKBR3 cells. This increase in protein was not due to transcriptional activation as ER α mRNA levels remained unchanged (Fig. 4a). CHX, an inhibitor of protein synthesis, was able to block ER α induction in response to PCE, indicating that the increase in ER α protein levels was the result of an increase in protein synthesis (Fig. 4b). Furthermore, the increase in ER α levels upon PCE incubation was dependent on proteasome processing

for the indicated times. Results represent the mean $\pm$ SE of 3 different experiments. Significant differences at all time points were evaluated by ANOVA plus post hoc Bonferroni comparison. **d** Determination of CYP1A1 activity. Empty circles indicate CYP1A1 enzymatic activity in MCF-7 cells, whereas filled circles correspond to SKBR3 cells, either control (0.12% of DMSO) or treated with PCE (250 ng/ μ L) for the indicated times. Results are expressed relative to the activity of the control and represent the mean $\pm$ SE of 3 different experiments. Significant differences at all time points were evaluated by ANOVA, plus post hoc Bonferroni comparison

as it was blocked in the presence of MG132, a proteasome inhibitor (Fig. 4b). In addition, PCE also appeared to modify ER α stability. A time course of ER α degradation was performed, using CHX to inhibit new protein synthesis and examining the decay of the remaining ER α protein in control and PCE-treated SKBR3 cells. ER α protein half-life in control cells was about 11.7 h, whereas in cells treated with PCE, it was decreased down to 2.1 h (Fig. 4c).

Synergistic effect between PCE and tamoxifen

A synergistic effect for the combination of tamoxifen with EGCG in breast cancer cells has been described [34–36]. In this direction, we wanted to test whether cocoa polyphenols would also exert a synergistic effect in combination with tamoxifen (TAM). MCF-7 and SKBR3 cells were incubated with increasing concentrations of TAM (10^{-6} – 10^{-3} M) either alone or in combination with PCE

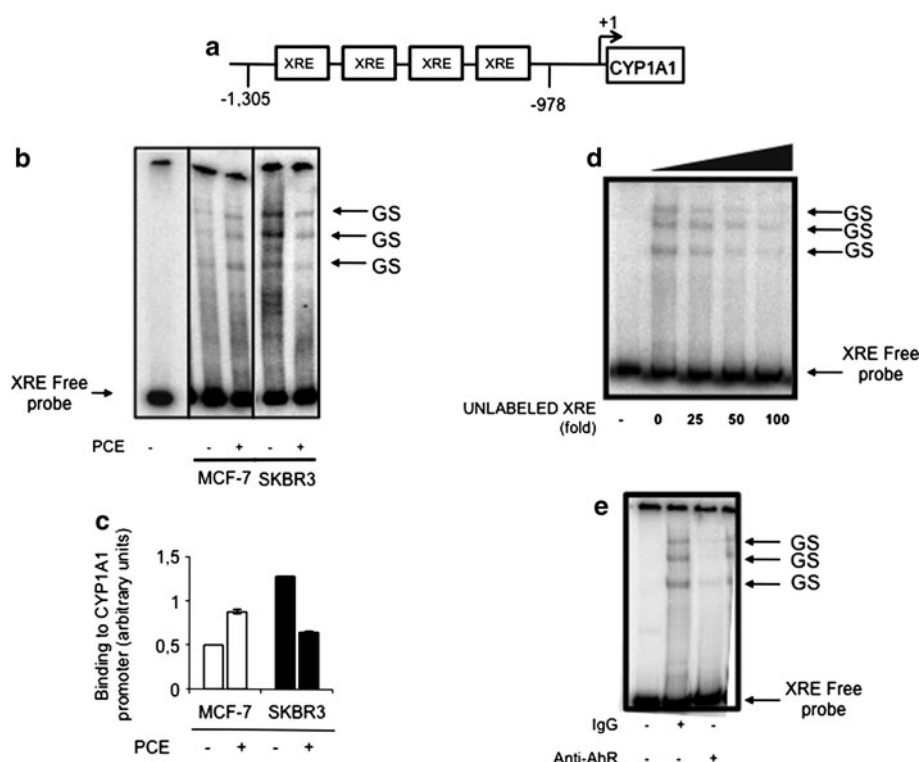


Fig. 2 Binding to the XRE probe. **a** Diagram corresponding to the 327-bp probe used for the binding assays generated by PCR from the CYP1A1 promoter and containing four XRE boxes. **b** Gel shift produced by the binding of nuclear extracts (NE) prepared from MCF-7 and SKBR3 cells cultured in the absence or in the presence of PCE (250 ng/μL for 24 h) to an XRE probe. Arrows indicate the shifted bands (GS). **c** The global quantification of the shifted bands in control versus PCE-treated cells was performed using the ImageQuant

software v 5.2 (Molecular Dynamics) and was plotted as absolute values (arbitrary units). **d** Competition assays. Increasing amounts of the unlabeled XRE probe were added to the binding reaction 15 min before the addition of the labeled probe. **e** Supershift experiment. The AhR antibody was added to the nuclear extracts and incubated OVN at 4 °C before the addition of the probe. IgGs were added in parallel as a control

(250 ng/μL), and cell viability was determined after 48 h. The presence of PCE, which did not cause significant cell death by itself (Online Resource 1), increased the cytotoxic effect of TAM in both cell lines (Fig. 5a, b). The reduction in cell viability was more evident in MCF-7 cells, reaching an increase of 44% when combined with 10^{-6} M TAM. Synergism ($CI < 1$) was confirmed for the combination of 10^{-6} M TAM with PCE in both cell lines and for 10^{-5} M TAM with PCE in SKBR3 cells as well (Online resource 4). As a control, we used HEK293 cells, in which the cytotoxic effect of tamoxifen alone was reduced by 20% when added in combination with PCE (Fig. 5c).

Levels of apoptosis upon incubation with PCE and tamoxifen

To get further insight into the mechanism by which the combination of PCE and TAM increased cytotoxicity, the levels of apoptosis were determined. The level of basal apoptosis in SKBR3 cells was twice than in MCF-7 cells. Incubation with PCE at 150 or 250 ng/μL showed no induction of apoptosis in MCF-7 cells, whereas in SKBR3,

it did increase apoptosis around 2-fold over the control. TAM at 10^{-6} M did not induce apoptosis by itself in any cell line, but when combined with 150 ng/μL of PCE, it was able to increase the levels of apoptosis in MCF-7 cells (Fig. 5d). The combination of PCE with TAM in SKBR3 cells (with higher basal apoptotic levels) did not cause an increase in the apoptosis levels, as compared to each condition alone (Fig. 5e).

Discussion

In this work, we analyzed the gene expression profile of human breast cancer cells treated with purified cocoa polyphenolic extract, used as representative of the wide flavonoid spectrum (monomers and oligomers) present in cocoa. Using PCR arrays, we described the differential expression of several genes involved in stress and toxicity pathways from which the CYP1A1 gene was chosen for further study for several reasons: (1) it was the most overexpressed gene in both cell lines analyzed upon incubation with PCE, (2) its overexpression in response to

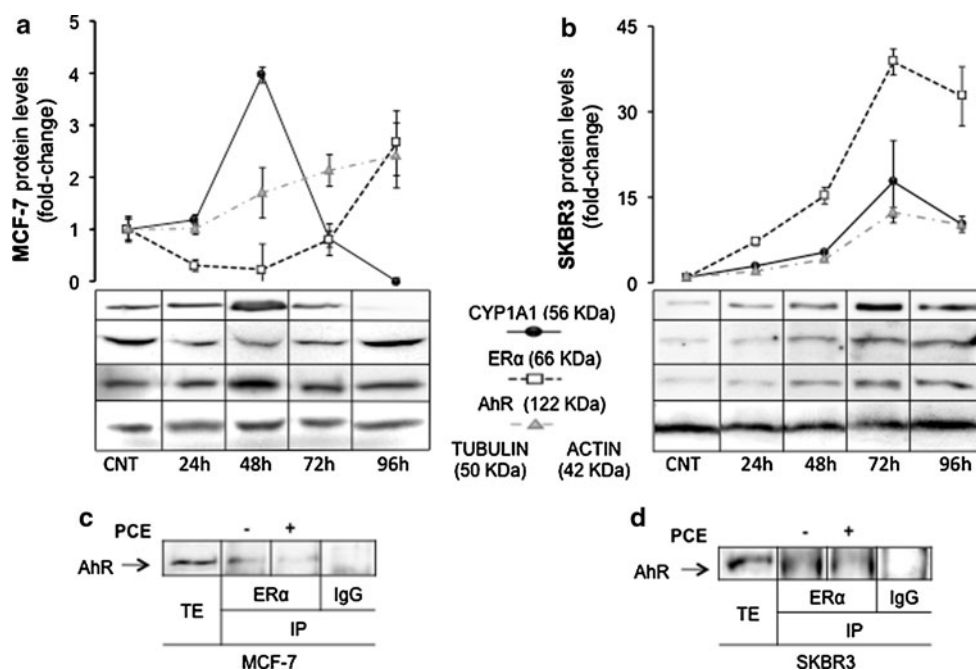


Fig. 3 Protein levels of AhR, ER α , and CYP1A1 in cells extracts. **a** and **b** Levels of AhR, ER α , and CYP1A1. Cells were treated with PCE (250 ng/ μ L) for the indicated periods of time, and the levels of CYP1A1 (circles), AhR (triangles), and ER α (squares) were determined by western blot from total cell extracts from either control (0.12% of DMSO) or PCE-treated MCF-7 (**a**) and SKBR3 (**b**) cells. Representative blots and the plots corresponding to the quantification of the results after normalization are shown. Results

represent the mean $\pm$ SE of 3 different experiments. Significant differences for all time points were established by ANOVA, and post hoc Bonferroni confirmed significant differences for CYP1A1, as well as ER α for SKBR3. **c** and **d** Co-immunoprecipitations. MCF-7 (**c**) and SKBR3 (**d**) cells were treated with PCE (250 ng/ μ L). Total extracts were immunoprecipitated with either ER α antibody or IgGs. The presence of the AhR protein in the immunoprecipitates was assessed by western blot. Total extracts were used as a positive control

polyphenols had already been described, and (3) it plays an important role in the oxidative metabolism of estrogens.

CYP1A1 is a candidate gene for low-penetrance breast cancer susceptibility because it plays an important role in the metabolism of xenobiotics or carcinogens as well as in the oxidative metabolism of estrogens [37]. CYP1A1 encodes aryl hydrocarbon hydroxylase (AHH) that catalyzes a hydroxylation reaction in Phase I metabolism as a first step to increase the polarity of different molecules. Some of these metabolites can be more active than the initial molecules and behave as electrophilic compounds, thus initiating or promoting tumorigenic processes. Additionally, other metabolites may behave as chemoprotectors, such as the result of 2-hydroxylation in E1 and E2 metabolism [38]. CYP1A1 expression occurs predominantly in extrahepatic tissues; its mRNA has been detected in normal and cancerous breast tissue and can be induced in human breast-derived cell lines [39]. In humans, CYP1A1 is under the regulatory control of the aryl hydrocarbon receptor (AhR) [31, 40, 41]. Previous studies reported that quercetin induces time- and dose-dependent increases in both CYP1A1 mRNA levels and enzyme activity in MCF-7 cells [32]. The ability of quercetin to modulate CYP1A1 expression is probably mediated by its AhR-binding activity [32, 42].

In our model, treatment with cocoa extract induces CYP1A1 expression in breast cancer cells. The involvement of AhR signaling pathway in CYP1A1 induction by PCE was evaluated by gel shift assays using an XRE probe. In MCF-7 cells, the accumulation of CYP1A1 mRNA following PCE treatment for 24 h was paralleled by the ability of nuclear extracts to increase the form of AhR/DNA complexes, suggesting that some polyphenols present in the cocoa extract may act as activators of the AhR signaling pathway. However, in SKBR3 cells, XRE binding was decreased in the presence of PCE, indicating a different signaling pathway, probably XRE-independent, leading to CYP1A1 overexpression. The existence of a protein complex including AhR and ER α in both cell lines suggests that both proteins could be contributing to the transcriptional activation of the CYP1A1 promoter. In ER(–) cells (SKBR3), PCE behaves as an ER antagonist, such as TAM in MCF-7 cells, that increases ER α protein levels [43], whereas in ER(+) cells (MCF-7), PCE incubation decreases ER α levels, acting as an ER agonist. Cocoa polyphenols are inducing ER α protein synthesis and processing by the proteasome in SKBR3 cells. The ER α precursor needs to be activated by a chymotrypsin-like activity of the proteasome [44]. Additionally, ER α half-life in

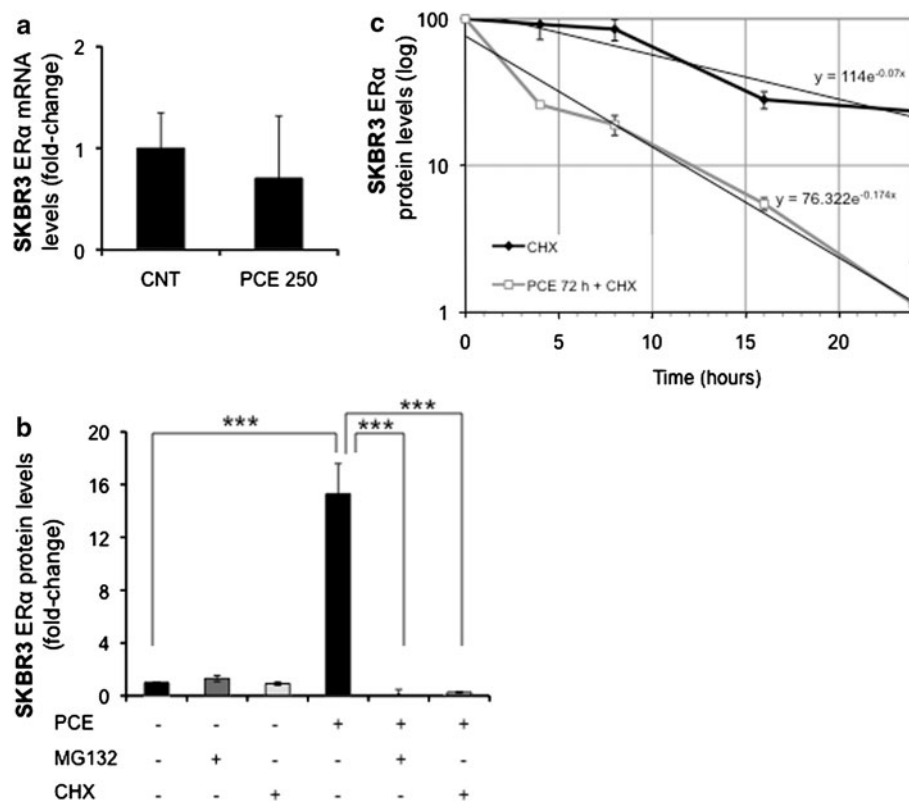


Fig. 4 Mechanism of ER α induction in SKBR3 cells. **a** Determination of ER α mRNA levels. Cells were treated with PCE (250 ng/ μ L) for 48 h, total RNA was extracted, and ER α mRNA levels were determined by RT-PCR as described. Results represent the mean $\pm$ SE of 3 different experiments. **b** Effects of CHX and MG132. SKBR3 cells were treated with PCE (250 ng/ μ L) for 48 h alone, or in combination with CHX (50 μ g/mL, 30 min before PCE incubation) or MG132 (25 μ M, overnight), and ER α protein levels were analyzed in total extracts. Results are expressed as fold changes compared to the control, incubated only with DMSO, and represent the mean $\pm$ SE of 3 different experiments. *** p < 0.001 compared

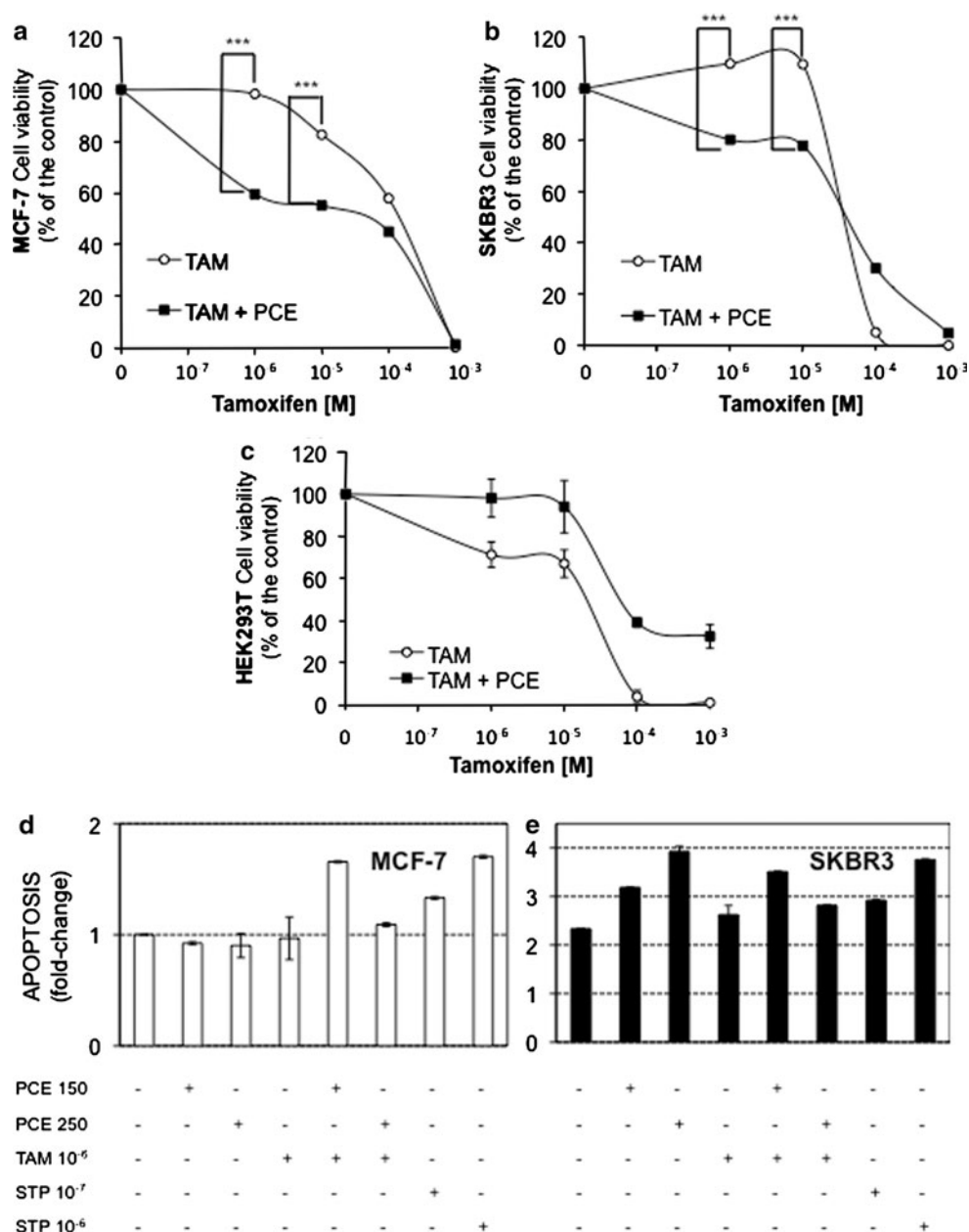
with the corresponding condition, control treated only with DMSO (0.32%) or PCE. **c** ER α half-life. Cells were incubated with 50 μ g/mL cycloheximide for the indicated times either alone or in combination with PCE for 72 h, and total extracts were prepared. The levels of ER α protein were determined by western blot as described. The quantification of the bands was performed by image analysis and plotted using a logarithmic scale. In *black* ER α half-life in control cells (treated only with 0.32% of DMSO) and in *gray* ER α half-life in PCE-treated cells. Results are the mean $\pm$ S.E. of 3 independent experiments

SKBR3 decreases to a value comparable to MCF-7 (3–5 h) in the presence of PCE [45]. Altogether, the increase in protein synthesis concomitant to a decrease in protein stability could be due to an increase in ER-precursor levels and processing upon the addition of PCE.

In addition to CYP1A1, the levels of GADD45A and GDF15 were also increased in MCF-7 and SKBR3 cell lines after PCE treatment. Growth arrest and DNA-damage-inducible alpha, GADD45A, is a member of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. GADD45A is also a candidate breast cancer susceptibility gene because its product participates in DNA repair, and it is a downstream gene of p53 and BRCA1, both of which are breast cancer susceptibility genes [46]. Its expression has been shown to be induced by polyphenols such as quercetin [47] and EGCG [48]. Growth differentiation factor 15, GDF15, also known as

macrophage inhibitory cytokine and nonsteroidal anti-inflammatory drug-activated 1 is a member of the transforming growth factor beta superfamily and regulates tissue differentiation and maintenance. A pronounced induction of GDF15 expression by oxLDL, C6-ceramide, tumor necrosis factor, and hydrogen peroxide has been described in cultured human activated macrophages [49]. GDF15 has been shown to have antitumorigenic activity, and it is up-regulated in resveratrol-treated cancer cells [50, 51]. In MCF-7 cells, treatment with tocotrienol-rich fraction from palm oil extract (PTRF) induces a significant increase in the expression of GDF15 mRNA and protein levels. The authors suggest that the effects of PTRF on gene expression modulation in MCF-7 cells are due in part to its interaction with ER transcriptional pathway [52]. All these observations indicate that the increase in GADD45A and GDF15 mRNA levels upon incubation with PCE could be linked to the antioxidant properties of PCE [53].

Fig. 5 Effects of tamoxifen plus PCE. Effect on cell viability. Cell viability was determined in MCF-7 (a) SKBR3 (b) and HEK293T (c) cells incubated with the indicated concentrations of Tamoxifen (TAM) either alone (empty circles) or in combination with PCE (250 ng/μL for 24 h, filled squares). Results are expressed as % of living cells compared to the control only with DMSO (0.22%) and represent the mean ± SE of 3 different experiments. *** $p < 0.001$. d, e TAM + PCE combination induces apoptotic levels. Levels of apoptosis are represented as fold changes compared to the level of apoptosis in control cells (MCF-7, empty bars; SKBR3, filled bars) for the different incubation conditions indicated in the figure (TAM; PCE; STP). The levels of apoptosis in the presence of staurosporine (STP) were determined as a positive control. Results represent the mean ± SE of 3 different experiments



Additionally, PCE could trigger an XRE-mediated transcriptional effect for the induction of GADD45A, taking into account that the GADD45A promoter contains an AhR element [54], and its expression is induced by TCDD [55].

The development of a combination therapy that increases the efficacy of tamoxifen has been evaluated. It has been described that the combination of tamoxifen and docetaxel synergistically inhibited the growth of MDA-MB-231, CEM-VBLr, and MCF-7Adr breast cancer cell lines [56]. Similarly, Shen et al. [57] demonstrated synergistic cytotoxicity when MDA-MB-435 cells were treated with tamoxifen and genistein. Synergism has also been reported in vivo, as complete inhibition of DMBA-induced mammary tumors in rats was achieved following treatment

with both tamoxifen and 6-MCDF, an aryl hydrocarbon receptor antagonist [58]. EGCG in combination with 4-OH-TAM is synergistically cytotoxic to MDA-MB-231 cells at low concentrations of both tamoxifen and EGCG [34]. The combination of tamoxifen and EGCG elicits an earlier and enhanced apoptotic response in MDA-MB-231 cells [36] and synergistically inhibits the growth of MDA-MB-231 xenografts [35]. The combination of green tea extract and tamoxifen is better than either drug alone at suppressing the growth of MCF-7 xenografts and correlates with increased levels of apoptosis and a suppression of angiogenesis [59]. In our conditions, the cytotoxic effect of tamoxifen was enhanced by the combination with PCE in MCF-7 and SKBR3 cell lines. The presence of PCE caused

a synergistic effect, confirmed by the Chou-Talay method, which led to a decrease in cell viability of up to 40% in MCF-7 cells at tamoxifen concentrations that did not affect cell viability by themselves. Our study also showed the induction of apoptosis in this cell line, as part of the mechanism by which this combination was lethal for breast cancer cells. Additionally, in non-tumor cells, the presence of PCE in combination with tamoxifen was able to reduce the cytotoxic effect of tamoxifen.

In summary, the changes in CYP1A1 expression upon incubation with PCE could explain the antioxidant effect of flavonoids at the molecular level since this gene is involved in different oxidative pathways. Additionally, CYP1A1 overexpression might interfere with estrogen metabolism and the production of estrogen metabolites in breast cells. The increase in CYP1A1 activity may shift estrogen metabolism toward the production of 2-OHE2, a relatively non-genotoxic metabolite [60]. Moreover, the increase in estrogen metabolism could lead to the reduction in the levels of estrogens in mammary tumors, thus contributing to the cytotoxic effect of tamoxifen. Further in vivo studies are necessary to analyze the synergism between tamoxifen and cocoa and to establish the possible benefits of cocoa polyphenol consumption during breast cancer therapy.

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References

1. Ferlay J, Bray F, Pisani P et al (2004) GLOBOCAN 2002: cancer incidence, mortality and prevalence worldwide. IARC Cancer-Base no. 5, Version 2.0. International Agency for Research on Cancer, Lyon
2. Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P (2007) Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol* 18:581–592
3. Clemons M, Goss P (2001) Estrogen and the risk of breast cancer. *N Engl J Med* 344:276–285
4. Key T, Appleby P, Barnes I, Reeves G (2002) Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. *J Natl Cancer Inst* 94:606–616
5. Bhat HK, Calaf G, Hei TK, Loya T, Vadgama JV (2003) Critical role of oxidative stress in estrogen-induced carcinogenesis. *Proc Natl Acad Sci USA* 100:3913–3918
6. Yager JD, Davidson NE (2006) Estrogen carcinogenesis in breast cancer. *N Engl J Med* 354:270–282
7. Cavalieri E, Chakravarti D, Guttenplan J, Hart E, Ingle J, Jan-kowiak R, Muti P, Rogan E, Russo J, Santen R, Sutter T (2006) Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. *Biochim Biophys Acta* 1766:63–78
8. Cavalieri EL, Rogan EG (2004) A unifying mechanism in the initiation of cancer and other diseases by catechol quinones. *Ann N Y Acad Sci* 1028:247–257
9. Patel MM, Bhat HK (2004) Differential oxidant potential of carcinogenic and weakly carcinogenic estrogens: involvement of metabolic activation and cytochrome P450. *J Biochem Mol Toxicol* 18:37–42
10. Dhingra K (1999) Antiestrogens–tamoxifen, SERMs and beyond. *Invest New Drugs* 17:285–311
11. Rutqvist LE, Cedermark B, Fornander T, Glas U, Johansson H, Nordenskjöld B, Rotstein S, Skoog L, Somell A, Theve T (1989) The relationship between hormone receptor content and the effect of adjuvant tamoxifen in operable breast cancer. *J Clin Oncol* 7:1474–1484
12. Port ER, Montgomery LL, Heerdt AS, Borgen PI (2001) Patient reluctance toward tamoxifen use for breast cancer primary prevention. *Ann Surg Oncol* 7:580–585
13. Ahmad N, Mukhtar H (1999) Green tea polyphenols and cancer: biologic mechanisms and practical implications. *Nutr Rev* 57:78–83
14. Kavanagh KT, Hafer LJ, Kim DW, Mann KK, Sherr DH, Rogers AE, Sonenshein GE (2001) Green tea extracts decrease carcinogen-induced mammary tumor burden in rats and rate of breast cancer cell proliferation in culture. *J Cell Biochem* 82:387–398
15. Vergote D, Cren-Olive C, Chopin V, Toillon RA, Rolando C, Hondermarck H, Le Bourhis X (2002) (-)-Epigallocatechin (EGC) of green tea induces apoptosis of human breast cancer cells but not of their normal counterparts. *Breast Cancer Res Treat* 76:195–201
16. Gupta S, Ahmad N, Nieminen AL, Mukhtar H (2000) Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. *Toxicol Appl Pharmacol* 164:82–90
17. Lee KW, Kim YJ, Lee HJ, Lee CY (2003) Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. *J Agric Food Chem* 51:7292–7295
18. Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Uribe C, Schmitz HH, Kelm M (2006) (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci USA* 103:1024–1029
19. Cooper KA, Donovan JL, Waterhouse AL, Williamson G (2008) Cocoa and health: a decade of research. *Br J Nutr* 99:1–11
20. Corti R, Flammar AJ, Hollenberg NK, Luscher TF (2009) Cocoa and cardiovascular health. *Circulation* 119:1433–1441
21. Visioli F, Bernaert H, Corti R, Ferri C, Heptinstall S, Molinari E, Poli A, Serafini M, Smit HJ, Vinson JA, Violi F, Paoletti R (2009) Chocolate, lifestyle, and health. *Crit Rev Food Sci Nutr* 49:299–312
22. Andrés-Lacueva C, Lamuela-Raventós RM, Jauregui O, Casals I, Izquierdo-Pulido M, Permanyer J (2000) An LC method for the analysis of cocoa phenolics. *LCoGC Eur* 13:902–904
23. Swain T, Hillis WE (1969) The total phenolic constituents of *Prunus domestica*. *J Sci Food Agric* 10:4292–4296
24. Selga E, Morales C, Noe V, Peinado MA, Ciudad CJ (2008) Role of caveolin 1, E-cadherin, Enolase 2 and PKC α on resistance to methotrexate in human HT29 colon cancer cells. *BMC Med Genomics* 1:35

25. Selga E, Oleaga C, Ramirez S, de Almagro MC, Noe V, Ciudad CJ (2009) Networking of differentially expressed genes in human cancer cells resistant to methotrexate. *Genome Med* 1:83
26. Tapias A, Ciudad CJ, Noe V (2008) Transcriptional regulation of the 5'-flanking region of the human transcription factor Sp3 gene by NF-1, c-Myb, B-Myb, AP-1 and E2F. *Biochim Biophys Acta* 1779:318–329
27. Noe V, Alemany C, Chasin LA, Ciudad CJ (1998) Retinoblastoma protein associates with SP1 and activates the hamster dihydrofolate reductase promoter. *Oncogene* 16:1931–1938
28. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63
29. Chou TC (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 58:621–681
30. Blasco F, Penuelas S, Cascallo M, Hernandez JL, Alemany C, Masa M, Calbo J, Soler M, Nicolas M, Perez-Torras S, Gomez A, Tarrason G, Noe V, Mazo A, Ciudad CJ, Piulats J (2004) Expression profiles of a human pancreatic cancer cell line upon induction of apoptosis search for modulators in cancer therapy. *Oncology* 67:277–290
31. Nebert DW, Roe AL, Dieter MZ, Solis WA, Yang Y, Dalton TP (2000) Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochem Pharmacol* 59:65–85
32. Ciolino HP, Daschner PJ, Yeh GC (1999) Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. *Biochem J* 340(Pt 3):715–722
33. Trapani V, Patel V, Leong CO, Ciolino HP, Yeh GC, Hose C, Trepel JB, Stevens MF, Sausville EA, Loaiza-Perez AI (2003) DNA damage and cell cycle arrest induced by 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203, NSC 703786) is attenuated in aryl hydrocarbon receptor deficient MCF-7 cells. *Br J Cancer* 88:599–605
34. Chisholm K, Bray BJ, Rosengren RJ (2004) Tamoxifen and epigallocatechin gallate are synergistically cytotoxic to MDA-MB-231 human breast cancer cells. *Anticancer Drugs* 15:889–897
35. Scandlyn MJ, Stuart EC, Somers-Edgar TJ, Menzies AR, Rosengren RJ (2008) A new role for tamoxifen in oestrogen receptor-negative breast cancer when it is combined with epigallocatechin gallate. *Br J Cancer* 99:1056–1063
36. Stuart EC, Larsen L, Rosengren RJ (2007) Potential mechanisms for the synergistic cytotoxicity elicited by 4-hydroxytamoxifen and epigallocatechin gallate in MDA-MB-231 cells. *Int J Oncol* 30:1407–1412
37. Nebert DW, Dalton TP, Okey AB, Gonzalez FJ (2004) Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem* 279:23847–23850
38. Parl FF, Dawling S, Roodi N, Croke PS (2009) Estrogen metabolism and breast cancer: a risk model. *Ann N Y Acad Sci* 1155:68–75
39. Huang Z, Fasco MJ, Figge HL, Keyomarsi K, Kaminsky LS (1996) Expression of cytochromes P450 in human breast tissue and tumors. *Drug Metab Dispos* 24:899–905
40. Delescluse C, Lemaire G, de Sousa G, Rahmani R (2000) Is CYP1A1 induction always related to AHR signaling pathway? *Toxicology* 153:73–82
41. Okey AB, Riddick DS, Harper PA (1994) Molecular biology of the aromatic hydrocarbon (dioxin) receptor. *Trends Pharmacol Sci* 15:226–232
42. Ramadass P, Meerarani P, Toborek M, Robertson LW, Hennig B (2003) Dietary flavonoids modulate PCB-induced oxidative stress, CYP1A1 induction, and AhR-DNA binding activity in vascular endothelial cells. *Toxicol Sci* 76:212–219
43. Wijayarathne AL, McDonnell DP (2001) The human estrogen receptor- α is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J Biol Chem* 276:35684–35692
44. Balan KV, Wang Y, Chen SW, Pantazis P, Wyche JH, Han Z (2006) Down-regulation of estrogen receptor- α in MCF-7 human breast cancer cells after proteasome inhibition. *Biochem Pharmacol* 72:566–572
45. Alarid ET, Bakopoulos N, Solodin N (1999) Proteasome-mediated proteolysis of estrogen receptor: a novel component in autologous down-regulation. *Mol Endocrinol* 13:1522–1534
46. Yu KD, Di GH, Li WF, Rao NY, Fan L, Yuan WT, Hu Z, Wu J, Shen ZZ, Huang W, Shao ZM (2010) Genetic contribution of GADD45A to susceptibility to sporadic and non-BRCA1/2 familial breast cancers: a systematic evaluation in Chinese populations. *Breast Cancer Res Treat* 121:157–167
47. Yoshida T, Maeda A, Horinaka M, Shiraishi T, Nakata S, Wakada M, Yogosawa S, Sakai T (2005) Quercetin induces gadd45 expression through a p53-independent pathway. *Oncol Rep* 14:1299–1303
48. Suganuma M, Saha A, Fujiki H (2011) New cancer treatment strategy using combination of green tea catechins and anticancer drugs. *Cancer Sci* 102:317–323
49. Schlittenhardt D, Schober A, Strelau J, Bonaterra GA, Schmiedt W, Unsicker K, Metz J, Kinscherf R (2004) Involvement of growth differentiation factor-15/macrophage inhibitory cytokine-1 (GDF-15/MIC-1) in oxLDL-induced apoptosis of human macrophages in vitro and in arteriosclerotic lesions. *Cell Tissue Res* 318:325–333
50. Golkar L, Ding XZ, Ujiki MB, Salabat MR, Kelly DL, Scholtens D, Fought AJ, Bentrem DJ, Talamonti MS, Bell RH, Adrian TE (2007) Resveratrol inhibits pancreatic cancer cell proliferation through transcriptional induction of macrophage inhibitory cytokine-1. *J Surg Res* 138:163–169
51. Lim JH, Park JW, Min DS, Chang JS, Lee YH, Park YB, Choi KS, Kwon TK (2007) NAG-1 up-regulation mediated by EGR-1 and p53 is critical for quercetin-induced apoptosis in HCT116 colon carcinoma cells. *Apoptosis* 12:411–421
52. Comitato R, Leoni G, Canali R, Ambra R, Nesaretnam K, Virgili F (2010) Tocotrienols activity in MCF-7 breast cancer cells: involvement of ER β signal transduction. *Mol Nutr Food Res* 54:669–678
53. Katz DL, Doughty K, Ali A (2011) Cocoa and chocolate in human health and disease. *Antioxid Redox Signal*. doi:10.1089/ars.2010.3697
54. Balliet AG, Hollander MC, Fornace AJ Jr, Hoffman B, Liebermann DA (2003) Comparative analysis of the genetic structure and chromosomal mapping of the murine Gadd45g/CR6 gene. *DNA Cell Biol* 22:457–468
55. Fletcher N, Wahlstrom D, Lundberg R, Nilsson CB, Nilsson KC, Stockling K, Hellmold H, Hakansson H (2005) 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) alters the mRNA expression of critical genes associated with cholesterol metabolism, bile acid biosynthesis, and bile transport in rat liver: a microarray study. *Toxicol Appl Pharmacol* 207:1–24
56. Ferlini C, Scambia G, Distefano M, Filippini P, Isola G, Riva A, Bombardelli E, Fattorossi A, Benedetti Panici P, Mancuso S (1997) Synergistic antiproliferative activity of tamoxifen and docetaxel on three oestrogen receptor-negative cancer cell lines is mediated by the induction of apoptosis. *Br J Cancer* 75:884–891
57. Shen F, Xue X, Weber G (1999) Tamoxifen and genistein synergistically down-regulate signal transduction and proliferation in estrogen receptor-negative human breast carcinoma MDA-MB-435 cells. *Anticancer Res* 19:1657–1662

58. McDougal A, Wormke M, Calvin J, Safe S (2001) Tamoxifen-induced antitumorigenic/antiestrogenic action synergized by a selective aryl hydrocarbon receptor modulator. *Cancer Res* 61:3902–3907
59. Sartippour MR, Pietras R, Marquez-Garban DC, Chen HW, Heber D, Henning SM, Sartippour G, Zhang L, Lu M, Weinberg O, Rao JY, Brooks MN (2006) The combination of green tea and tamoxifen is effective against breast cancer. *Carcinogenesis* 27:2424–2433
60. Mense SM, Chhabra J, Bhat HK (2008) Preferential induction of cytochrome P450 1A1 over cytochrome P450 1B1 in human breast epithelial cells following exposure to quercetin. *J Steroid Biochem Mol Biol* 110:157–162