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Resveratrol and its methoxy derivatives modulate the expression of estrogen metabolism enzymes in breast epithelial cells by AhR down-regulation

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Abstract Our earlier studies have shown that compared to resveratrol, its analogs with *ortho*-methoxy substituents exert stronger antiproliferative and proapoptotic activity. Since estrogens are considered the major risk factors of breast carcinogenesis, the aim of this study was to evaluate the effect of 3,4,2′-trimethoxy (3MS), 3,4,2′,4′-tetramethoxy (4MS), and 3,4,2′,4′,6′-pentamethoxy (5MS) *trans*-stilbenes on the constitutive expression of the enzymes involved in estrogen metabolism, as well as receptors: AhR and HER2 in breast epithelial cell line MCF10A. The results showed different effect of resveratrol and its methoxy derivatives on the expression of genes encoding key enzymes of estrogen synthesis and catabolism. Resveratrol at the doses of 1 and 5 μmol/L increased the level of *CYP19* transcript and protein level, while 5MS reduced mRNA transcript of both *CYP19* and *STS* genes. Resveratrol and all its derivatives reduced also *SULT1E1* mRNA transcript level. The reduced expression of AhR, CYP1A1, and 1B1 was also found as a result of treatment with these compounds. The most significant changes were found in the case of AhR. The most potent inhibitor of *CYP1A1* and *1B1* genes expression was 5MS, which reduced the levels of mRNA transcript and protein of both CYPs from 31 to 89% of the initial levels. These results indicate that methoxy derivatives of resveratrol might be efficient modulators of estrogen metabolism. Moreover, the number of methoxy groups introduced to stilbene structure may play a certain role in this effect.

Keywords Methoxy-*trans*-resveratrol · AhR · CYP450 · STS · SULT1E1 · Breast cancer

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Introduction

Estrogens exert diverse biological effects such as sexual differentiation and development, arterial vasodilation, and maintenance of bone density [1, 2]. However, estrogens are also known as an important risk factor of human breast and the other hormone-dependent cancers. These steroid hormones may contribute to breast cancer development in two ways: (1) acting as promoters by stimulating cell proliferation (ER-dependent mechanism), (2) inducing genotoxicity through the reaction of their active metabolites with DNA, thus acting as tumor initiators [3].

The biosynthesis of estrogens involves a series of enzymatic steps catalyzed by cytochromes P450 CYP11A, CYP17, and CYP19. The latter is responsible for converting testosterone into estradiol or androstenedione into estrone. Estrone is transformed to estradiol by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) or it is sulfonated to inactive estrone sulfate by steroid sulfotransferase (SULT1E1). In turn, steroid sulfatase (STS) hydrolyzes estrone sulfate into estrone. Therefore, SULT1E1 and STS play very important roles in maintaining biologically active estrogens in several hormonal-dependent tissues [4, 5]. In post-menopausal women, the ovaries cease to produce estrogens and the circulating inactive steroids synthesized in non-gonadal sites, such as adipose tissue, are considered to be major precursor substrates of local estrogen production [6, 7].

Estradiol is mainly metabolized to 2- and 4-hydroxyestradiol. These catechol metabolites can be subsequently *O*-methylated to monomethoxy estradiol metabolites. While 2-methoxyestradiol appears to be non-carcinogenic, 4-hydroxyestradiol undergoes metabolic redox cycling to generate superoxide and the reactive semiquinones and quinones free radicals. The labile quinones form DNA adducts such as the apurinic 4-hydroxyestradiol-1(α,β)-N^7-guanine and 4-hydroxyestradiol-1(α,β)-N^3-adenine [5, 8–10]. The oxidative metabolism of estrogens is mainly catalyzed by cytochromes P450: CYP1A1, CYP1A2, and CYP1B1. The latter is highly expressed in estrogen target tissues, including mammary gland, and specifically catalyzes the 4-hydroxylation of estradiol [5].

The aryl hydrocarbon receptor (AhR) protein, which is the member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family functioning as a ligand-activated transcription factor, controls critical steps in both estrogen synthesis and metabolism pathways [11]. AhR activates the expression of genes metabolizing estrogens and initiates degradation of ER by accelerating its ubiquitination [12]. Based on this observation, it was suggested that AhR is a tumor suppresser in human breast cancer. AhR has been shown to inhibit invasive and metastatic features of human breast cancer stem-like cells. Moreover, exogenous AhR agonists were able to promote cell differentiation regardless of ER or HER2 status [13].

The human epidermal growth factor receptor 2 (HER2) gene is overexpressed in 10–40% of breast cancers [14]. HER2 activation can lead to subsequent activation of signaling components via Ras-Raf-Akt-PKC pathways, which may contribute to tumor proliferation and drug resistance [15, 16]. In HER2, overexpressing breast cancer epithelial cells (MCF7) enhanced expression of AhR was observed suggesting the interaction between these two receptors [17]. Thus, modulation of AhR and HER2 as well as enzymes involved in the estrogen synthesis and catabolism by specific inhibitors is considered a useful way to prevent and/or treat breast cancer patients, particularly postmenopausal women.

Resveratrol (3,5,4′-trihydroxystilbene), a naturally occurring phytoalexin, has been reported to show a variety of beneficial properties including antioxidative and anticarcinogenic ones [18, 19]. It was shown that resveratrol is a competitive antagonist of several classic AhR ligands such as dioxin or benzo[a]pyrene [20]. Resveratrol promotes AhR translocation to the nucleus and binding to DNA at dioxin-responsive elements, but subsequent transactivation does not take place. Resveratrol inhibits the transactivation of several dioxin-inducible genes including cytochrome P450 1A1, both ex vivo and in vivo [20]. Moreover, it was shown that resveratrol prevents estrogen-DNA adduct formation and neoplastic transformation in MCF10F breast epithelial cells [21].

The promising data on the biological activity of resveratrol have encouraged the search for its derivatives showing enhanced activity as well as improved pharmacokinetic parameters. Compared to resveratrol, its analogs with *ortho*-methoxy substituents, such as *trans*-3,4,5,4′-tetramethoxystilbene (DMU-212), have been found to be more potent in some in vitro and in vivo studies including ovarian cancer cells culture [22]. Thus, such derivatives may serve as a useful starting point for the design of improved chemopreventive or cancer therapeutic agents [23]. Moreover, our earlier studies showed that 3,4,2′-trimethoxy-*trans*-stilbene was a potent inhibitor of human recombinant CYP1B1 activity [24].

The aim of this study was to evaluate the effect of three new synthetic methoxy stilbenes: 3,4,2′-trimethoxy-*trans*-stilbene (3MS), 3,4,2′,4′-tetramethoxy-*trans*-stilbene (4MS), and 3,4,2′,4′,6′-pentamethoxy-*trans*-stilbene (5MS) on the expression of AhR, HER2, and the enzymes involved in synthesis and catabolism of estrogens in breast epithelial cells MCF10A. This cell line represents benign stage of breast cancer development and is a well-developed cell culture model for studying carcinogenesis through non-estrogen receptor-mediated pathways [25, 26].

Materials and methods

Chemicals

Methoxy stilbenes: 3,4,2′-trimethoxy-*trans*-stilbene (3MS), 3,4,2′,4′-tetramethoxy-*trans*-stilbene (4MS), and 3,4,2′,4′,6′-pentamethoxy-*trans*-stilbene (5MS) were synthesized in the Department of Chemical Synthesis of Drugs PUMS as described previously [24]. Diethyl (3,4-dimethoxybenzyl)phosphonate was transformed in Horner–Wadsworth–Emmons reaction with proper aromatic aldehydes (2-methoxybenzaldehyde or 2,4-dimethoxybenzaldehyde or 2,4,6-trimethoxybenzaldehyde) into three investigated polymethoxy-*trans*-stilbenes. Figure 1 outlines the synthesis of these compounds.

Resveratrol, dithiothreitol, antibiotics solution (10^4 U penicillin, 10 mg streptomycin, 25 μg amphotericin B), bovine serum albumin, dimethylsulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), hydrocortisone, 10 mg/mL insulin, 100 μg/mL EGF, horse serum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RIPA buffer, trypsin, Tris, and tRNA from *E. coli* were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Primary antibodies against CYP1B1 and HER2 were supplied by Bioss (Woburn, MA, USA).

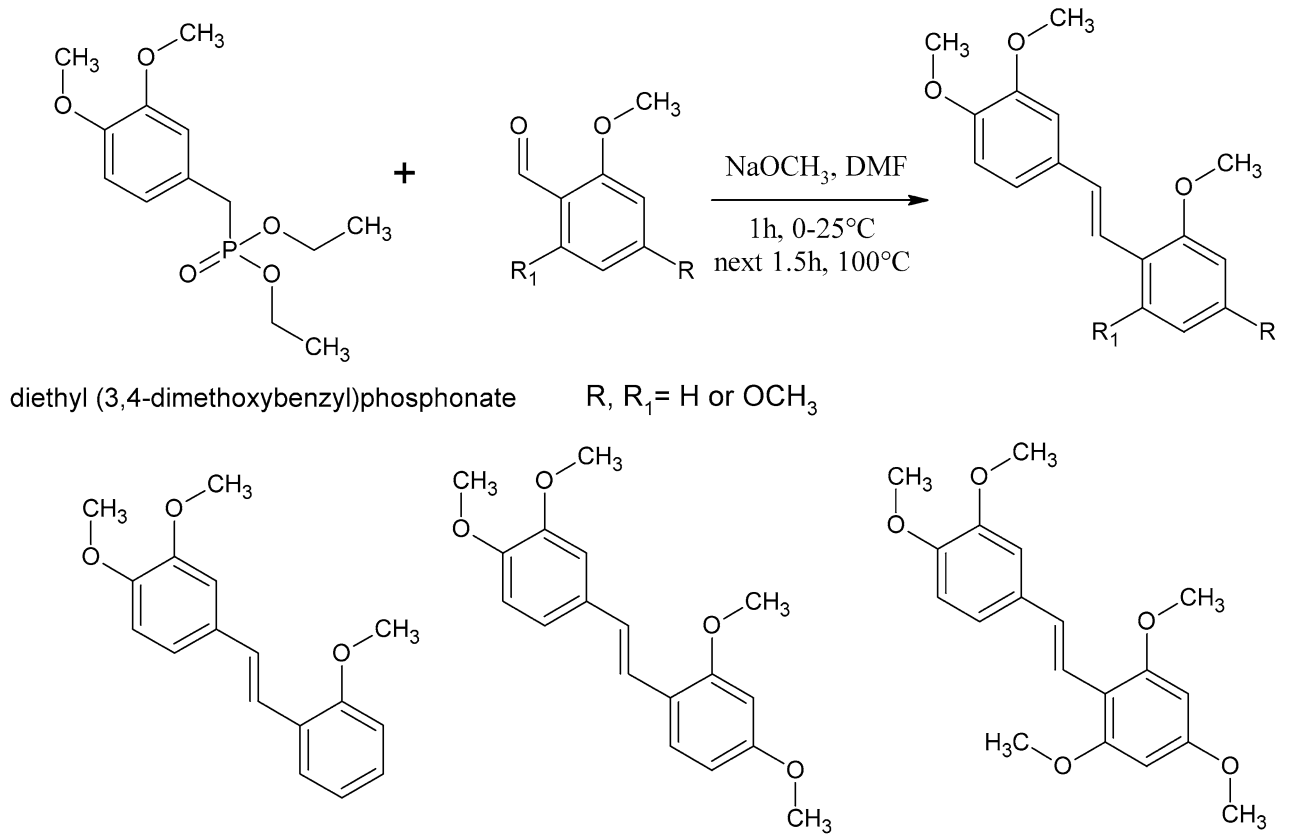

Fig. 1 A scheme of tri (3MS), tetra (4MS), and penta (5MS) methoxy-trans-stilbene synthesis and their structures

Primary antibodies against CYP1A1 and CYP19 were obtained from Abgent (San Diego, CA, USA). Primary antibodies against AhR, β-actin and secondary antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary and secondary antibodies against STS and SULT1E1 were obtained from Proteintech (Chicago, USA). Protease inhibitor tablets were obtained from Roche Diagnostics GmbH (Penzberg, Germany). SDS-PAGE Gels (7.5, 10 and 12%) and the Western blotting detection system were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other compounds were readily available commercial products. Resveratrol and its methoxy derivatives were dissolved in DMSO at the concentration of 100 mmol/L and stored at −20 °C.

Cell culture and treatment

MCF10A (ATTC CRL-10317) cell line was purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK). The cells were cultured in DMEM supplemented with 5% horse serum, 0.1% insulin solution, 0.05% hydrocortisone solution, 0.02% EGF solution, and 1% antibiotics solution. Experiments were conducted at a cell density of 70% confluence at standard conditions (5% CO_2/95% air). After a 24 h preincubation period, the cells were treated with the tested compounds at the doses selected based on viability assay: 0.5, 1, or 5 μmol/L. The incubation was continued for subsequent 72 h. Control cells were treated with vehicle (DMSO). The concentration of DMSO did not exceed 0.1%.

Cell viability assay

The effect of resveratrol and its methoxy derivatives on cell viability was assessed with the MTT assay, according to standard protocols. The cells were seeded in a 96-well culture plate. After a 24 h preincubation period, 1–100 μmol/L of resveratrol or its derivatives in the culture medium were added and the cells were incubated for 72 h. Subsequently, the culture medium was removed and a fresh PBS buffer containing MTT salt (0.5 mg/mL) was added to the wells. After a 4 h incubation, the formazan crystals were dissolved in acidic isopropanol and the absorbance was measured at 570 and 690 nm. All of the experiments were repeated three times, with at least three measurements per assay. In all of the subsequent experiments, non-toxic

concentrations of methoxy stilbenes and resveratrol (viability level above 70%) were used ranging from 0.5 to 5 μM, depending on compound.

Measurements of AhR, HER2, STS, SULT1E1, and CYP450 mRNA transcripts (quantitative real-time PCR)

Total RNA was isolated, using the GeneMATRIX UNIVERSAL DNA/RNA/protein kit (EURx Ltd., Gdańsk, Poland), and the first-strand cDNA was generated from total RNA using the dART RT-PCR kit (EURx Ltd., Gdańsk, Poland) according to the manufacturers' recommendations. Primer pairs capable of hybridization with unique regions of the appropriate gene sequence were designed in Beacon Designer (PREMIER Biosoft Intern.) as follows: forward and reverse-HER2 (5′-TCCCTACAACTACCTTTCTAC-3′ and 5′-CAGACCATAGCACACTCG-3′); STS (5′-TGTCTTGTCCTACCTCCAC-3′ and 5′-GTATCATTAGCCAATCTCAGC-3′); SULT1E1 (5′-GGTTCCTTATGGTTCCTG-3′ and 5′-GTATGATGTATAATCCTGTCC-3′), or as mentioned previously [27–29]. The quantitative real-time PCR was performed in triplicates using SybrGreen on the Chromo4 (Bio-Rad Laboratories, CA, USA) or the LightCycler96 (Roche Diagnostics GmbH, Penzberg, Germany). The final reaction mixture contained 80–250 nM of each primer, 0.5 μL of cDNA, 1 μL of tRNA, and 4 μL of the 5× HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), with RNAse-free water up to 20 μL. All reactions were run in triplicate. The protocol started with a 15-min enzyme activation at 95 °C, followed by 40-50 cycles of 95 °C for 15 s; 60 °C for 20 s; 72 °C for 20 s, and the final elongation at 72 °C for 5 min. The melting curve analysis was used for product size verification. Experiments were normalized for the expression of TATA box-binding protein (*TBP*). The Pfaffl relative method was used for fold-change quantification.

Measurements of AhR, HER2, STS, SULT1E1, and cytochromes P450 protein levels (Western blot)

Whole cell lysates were prepared using the RIPA buffer. Samples containing 100 μg of proteins were separated on 7.5 (AhR, HER2), 10 (CYP19, CYP1A1, CYP1B1, STS, and β-actin), or 12% (SULT1E1) SDS-PAGE gels and transferred to nitrocellulose membranes [28]. After blocking with skimmed milk (5% for STS and SULT1E1; 10% for CYP19, CYP1A1, CYP1B1, AhR, HER2, and β-actin), the proteins were probed with goat polyclonal AhR, rabbit polyclonal CYP19, rabbit polyclonal CYP1A1, rabbit polyclonal CYP1B1, rabbit polyclonal HER2, rabbit polyclonal STS, rabbit polyclonal SULT1E1, and rabbit β-actin antibodies. As the secondary antibodies, the alkaline phosphatase-labeled anti-goat IgG or anti-rabbit IgG was used. Protein contents were measured using albumin as a standard and the β-actin protein as an internal control. The amount of immunoreactive product in each lane was determined by densitometric scanning using a Bio-Rad GS710 Image Densitometer (Bio-Rad Laboratories, Hercules, CA, USA). The values were calculated as relative absorbance units (RQ) per mg protein.

Quantification of estradiol in culture medium

The concentration of estradiol in culture medium after 72 h of MCF10A cells incubation with methoxy stilbenes or resveratrol was assessed by an enzymatic immunoassay according to Jin et al. [30] using commercial kit (Estradiol EIA Kit, Cayman Chemical Company, Ann Arbor, MI, USA) and following the manufacturer's instructions. Briefly, the assay was based on the competition between estradiol and an estradiol-acetylcholinesterase conjugate for a limited amount of estradiol antiserum. The antiserum-estradiol and antiserum-acetylcholinesterase complexes bound to mouse monoclonal anti-rabbit IgG, previously attached to ELISA microplate. Then substrate for acetylcholinesterase was added, and the absorbance of the yellow product of the enzymatic reaction was measured at 405 nm. The concentration of estradiol in medium samples was expressed in pg/mL, using standard curve on each run. The data from three independent experiments were analyzed.

Statistical analysis

Statistical analysis was performed by one-way ANOVA. The statistical significance between the experimental groups and their respective controls was assessed by Tukey's post hoc test, at $P < 0.05$.

Results

The effect of resveratrol and methoxy stilbenes on cell viability

The treatment with resveratrol or its methoxy derivatives reduced the viability of MCF10A cells, basically in dose-dependent manner (Fig. 2). Among the methoxy stilbenes, 3MS showed the highest cytotoxicity, while 5MS the lowest. At the highest doses, the cytotoxicity of the latter was even lower than that of parent compound, resveratrol. Based on these findings, all the subsequent studies were conducted at concentrations ranging from 0.5 to 5 μmol/L assuring viability above 70%.

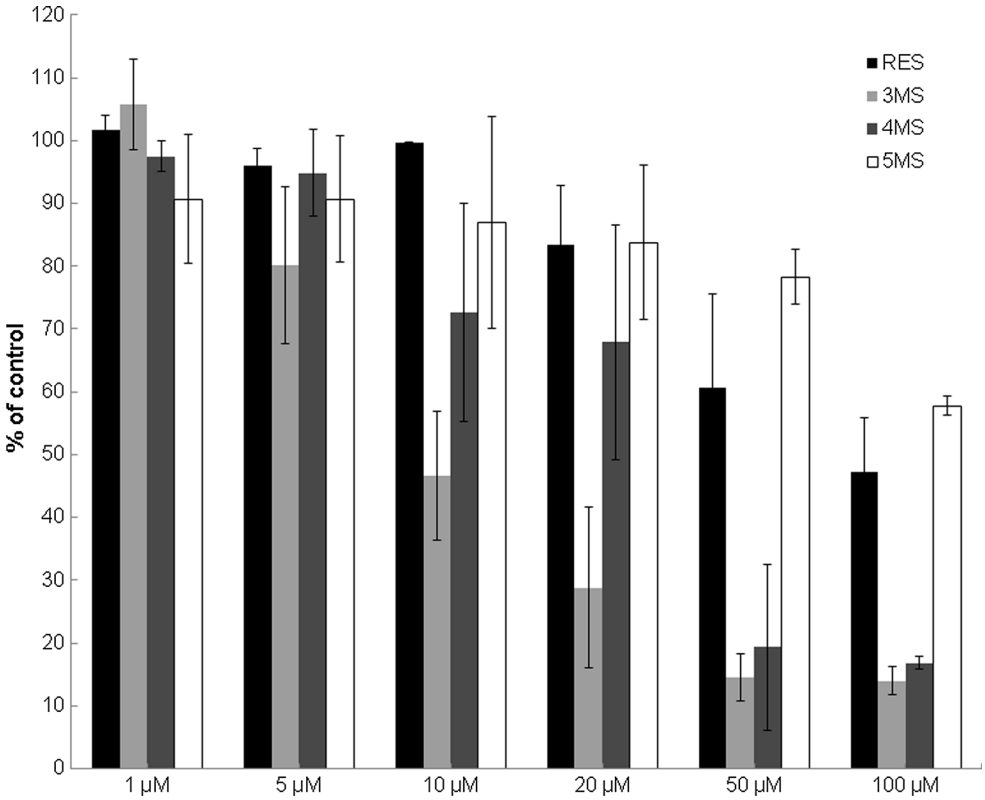

Fig. 2 The effect of resveratrol (RES) and synthetic tri (3MS), tetra (4MS), penta (5MS) methoxystilbenes on the MCF10A cell line viability. The mean values ±SEM from three independent experiments run in triplicate are presented

The effect of resveratrol and its methoxy derivatives on the expression of *CYP19, STS,* and *SULT1E1* genes

The constitutive expression of genes encoding enzymes involved in estrogen synthesis was determined at the transcript and protein levels. The values were calculated as a relative change in transcript or protein level in comparison to control cells (expression equals 1) and are shown in Figs. 3 and 4. The reduced levels of *CYP19* and *STS* mRNA transcripts were observed as a result of treatment with 5MS. In contrast to 5MS, resveratrol increased the *CYP19* and *STS* transcripts and/or proteins. All tested stilbenes reduced the *SULT1E1* mRNA transcript level. In case of resveratrol, however, the observed decrease of both transcript and protein levels was observed at the dose of 5 μmol/L.

The effect of resveratrol and its methoxy derivatives on the expression of *CYP1A1* and *CYP1B1* genes

As shown in Fig. 5, resveratrol at the dose of 5 μmol/L reduced the expression of CYP1A1 and CYP1B1, while in the lower dose of 0.5 μmol/L increased *CYP1A1* mRNA level was noted. The most potent inhibitor of the expression of these genes was found to be 5MS, which reduced the levels of mRNA and protein of both cytochromes P450 from 31 to 89% of the initial levels.

The effect of resveratrol and its methoxy derivatives on the expression of *AhR* and *HER2* genes

It is widely established that functional activation of AhR results in its translocation into the nucleus, which results in transcriptional activation of the phase I enzymes. Thus, the functional activation of AhR and its modulation by stilbenes were confirmed by determining the expression of *CYP1A1* and *CYP1B1*. In order to explain, if this effect is related to diminished level of AhR as well as HER2, their expression was determined.

As shown in Fig. 6, the *AhR* expression was significantly reduced in most cases at both mRNA and protein levels by resveratrol and its methoxy derivatives, and the most pronounced effect was observed in transcript level. There were no significant differences in effect on *AhR* expression between resveratrol and methoxy stilbenes. The *HER2* mRNA level was diminished as a result of cell treatment with 4MS and 5MS, but this reduction was not reflected in protein level (Fig. 6).

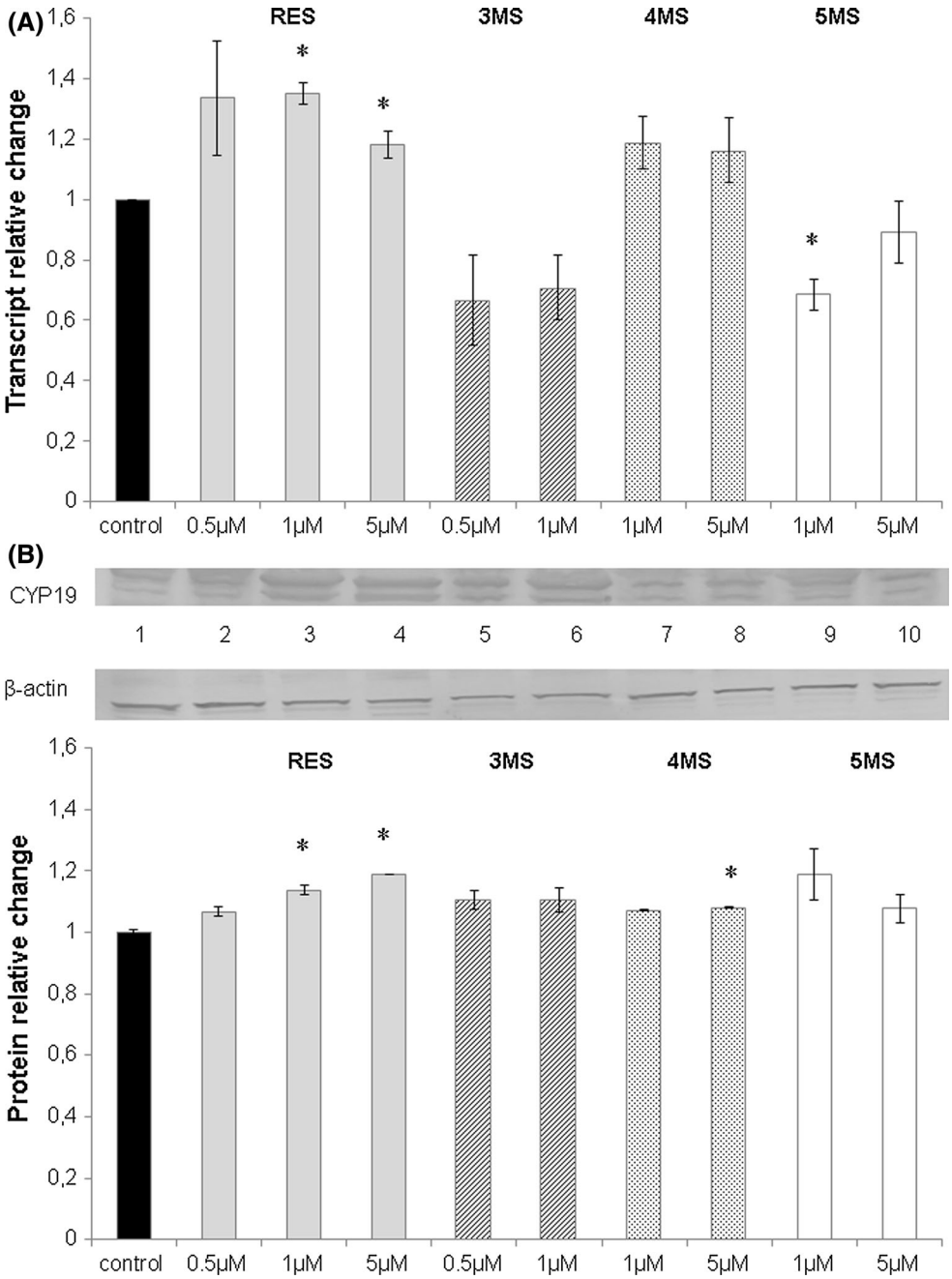

Fig. 3 The effect of 72 h incubation with resveratrol (RES) and synthetic methoxy-*trans*-stilbenes on the level of the *CYP19* transcript (**A**) and protein (**B**) in MCF10A cells. These values were calculated as a relative change in transcript or protein level in comparison to control cells (expression equals 1). The mean values ± SEM from 3 independent experiments run in triplicate are presented. *Mean values were significantly different from the control group ($P < 0.05$). Western blot analysis: representative blot is shown: (*Lane 1*) control; (*Lanes 2, 3, 4*) resveratrol; (*Lanes 5, 6*) 3,4,2′-trimethoxy-*trans*-stilbene (3MS); (*Lanes 7, 8*) 3,4,2′,4′-tetramethoxy-*trans*-stilbene (4MS); (*Lanes 9, 10*) 3,4,2′,4′,6′-pentamethoxy-*trans*-stilbene (5MS)

Estradiol concentrations in culture medium

Estradiol was detected in culture medium of both untreated MCF10A cells as well as cells treated with resveratrol or methoxy derivatives. However, the estradiol concentrations were very low, actually only slightly above the level of detection. Thus, no statistical differences have been found between untreated and treated cells with analyzed compounds (data not shown).

Discussion

Estrogens are considered the major risk factor of breast cancer [31]. Besides the expression of the major forms of estrogen receptor (ERα and ERβ), determinants affecting the amount and activity of estrogen in breast tissue include enzymes involved in estrogen synthesis and catabolism [32, 33].

In this study, we evaluated the effect of resveratrol and its three methoxy derivatives on the expression of genes

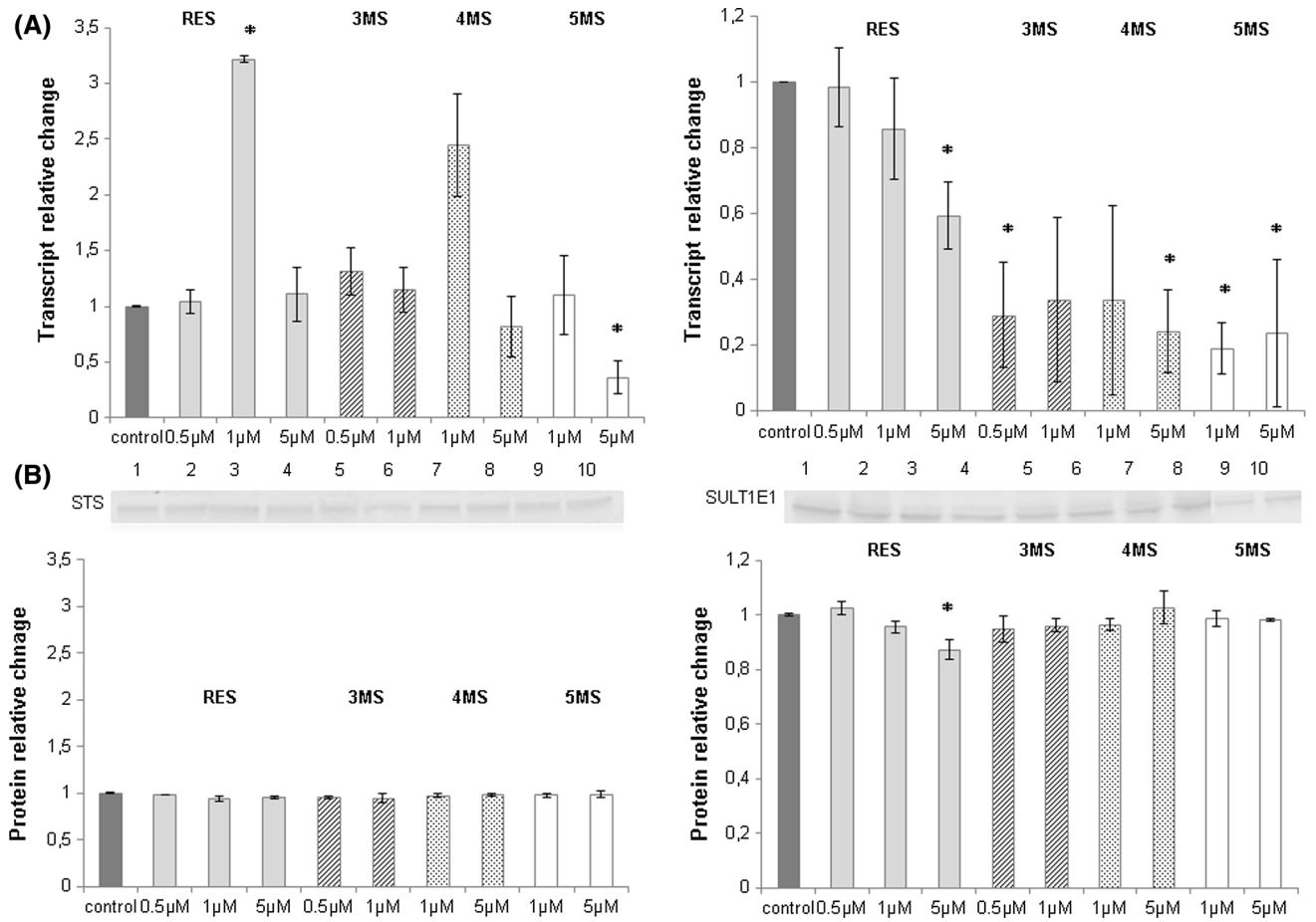

Fig. 4 The effect of 72 h incubation with resveratrol (RES) and synthetic methoxy-*trans*-stilbenes on the level of the *STS* and *SULT1E1* transcripts (**A**) and proteins (**B**) in MCF10A cells. These values were calculated as a relative change in transcript or protein level in comparison to control cells (expression equals 1). The mean values ± SEM from 3 independent experiments run in triplicate are presented. *Mean values were significantly different from the control group ($P < 0.05$). Western blot analysis: representative blot is shown: (*Lane 1*) control; (*Lanes 2, 3, 4*) resveratrol; (*Lanes 5, 6*) 3,4,2′-trimethoxy-*trans*-stilbene (3MS); (*Lanes 7, 8*) 3,4,2′,4′-tetramethoxy-*trans*-stilbene (4MS); (*Lanes 9, 10*) 3,4,2′,4′,6′-pentamethoxy-*trans*-stilbene (5MS)

encoding enzymes and receptors involved in the estrogen metabolism using MCF10A cell line as a model. This cell line was derived from spontaneously immortalized breast epithelial cells obtained from a donor with benign proliferative breast disease, and thus mimics the early stages of breast carcinogenesis [25]. The constitutive expression of *CYP19, STS, SULT1E1* and *CYP1A1, 1A2, 1B1* genes in these cells was demonstrated in earlier studies done by us and other authors, although on the lower level in comparison with the MCF7 breast cancer cell line [27, 28, 34]. It is important, that the low doses of resveratrol used in the study (1–5 μM) would be close to blood levels measured after in vivo administration.

The results of our current study showed the moderate, but different effect of resveratrol and its methoxy derivatives on the expression of genes encoding key enzymes of estrogen synthesis. In this regard, resveratrol at the doses of 1 and 5 μmol/L increased the level of *CYP19* transcript and protein level and showed a tendency to increase the expression of STS, while 5MS reduced the expression of *CYP19* and *STS* at mRNA level. Interestingly, resveratrol was shown previously to inhibit aromatase activity and expression in MCF7 cells stably transfected with *CYP19* (MCF-7aro) and SK-BR-3 cells expressing ERα and JEG-3 placental cells [35, 36]. This discrepancy may result from different regulations of key determinants of breast estrogen metabolism in MCF10A-derived lineage model and MCF7 cells [34].

Another interesting observation of our study is the fact that resveratrol and its all derivatives reduced the *SULT1E1* mRNA transcript level. In an earlier study, Otake et al. [37] demonstrated that resveratrol and even to more extent flavonoid quercetin inhibited the catalytic activity of recombinant human SULT1E1 and reduced the sulfation of estradiol by this enzyme in intact human mammary epithelial cells. Thus, the results of our study provide the

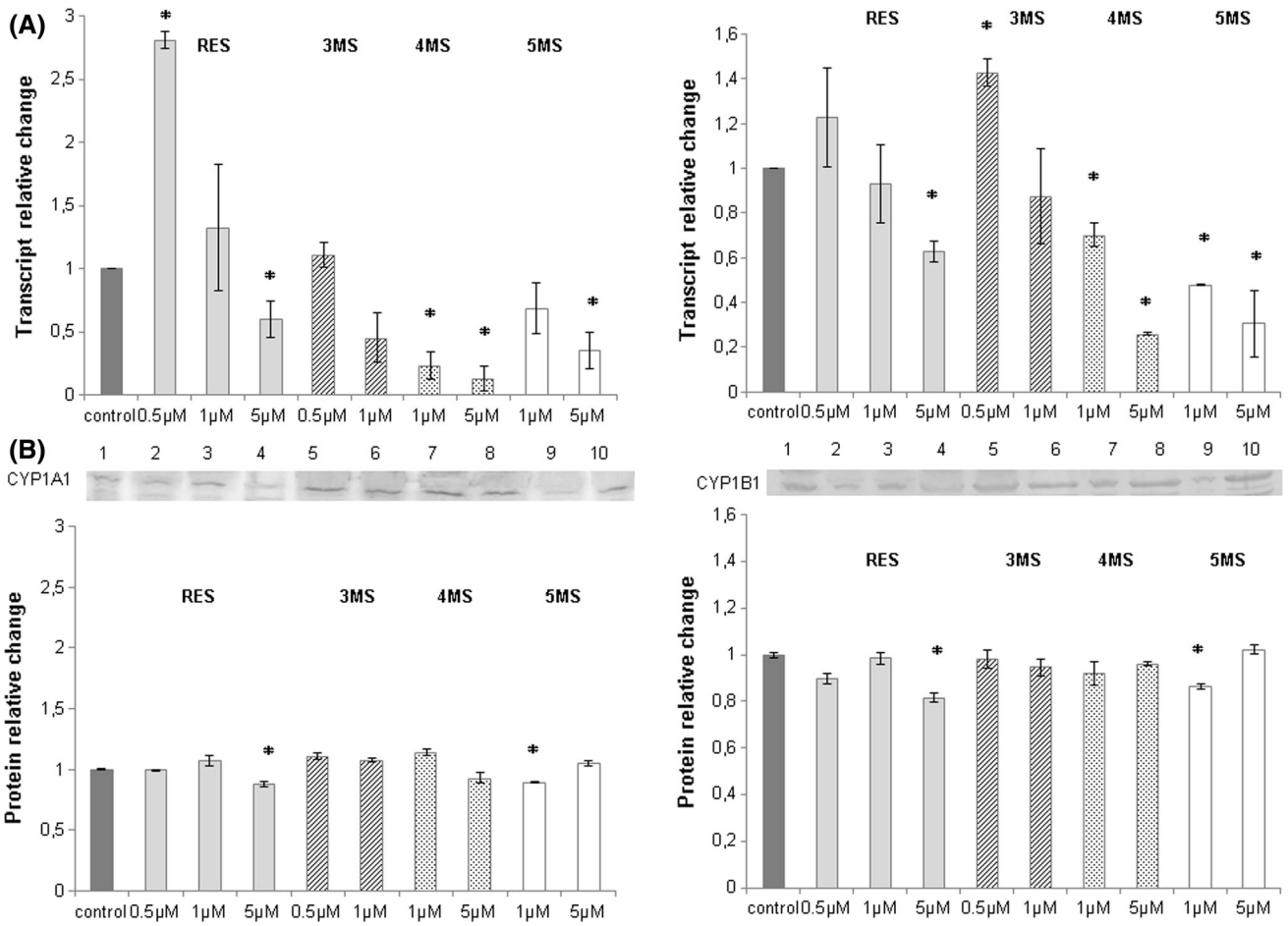

Fig. 5 The effect of 72 h incubation with resveratrol (RES) and synthetic methoxy-*trans*-stilbenes on the level of the *CYP1A1* and *CYP1B1* transcripts (**A**) and proteins (**B**) in MCF10A cells. These values were calculated as a relative change in transcript or protein level in comparison to control cells (expression equals 1). The mean values ± SEM from 3 independent experiments run in triplicate are presented. *Mean values were significantly different from the control group ($P < 0.05$). Western blot analysis: representative blot is shown: (*Lane 1*) control; (*Lanes 2, 3, 4*) resveratrol; (*Lanes 5, 6*) 3,4,2′-trimethoxy-*trans*-stilbene (3MS); (*Lanes 7, 8*) 3,4,2′,4′-tetramethoxy-*trans*-stilbene (4MS); (*Lanes 9, 10*) 3,4,2′,4′,6′-pentamethoxy-*trans*-stilbene (5MS)

evidence that resveratrol may affect not only SULT1E1 enzyme activity, but also the transcription of gene encoding the enzyme.

The inhibition of SULT1E1 expression in MCF10 cells, which mimics the early stages of breast carcinogenesis, may elevate estrogen hormone levels and thus should be considered as potentially harmful. On the other hand, SULT1E1 may catalyze the bioactivation of some cooked food-derived mutagens and their subsequent binding to genomic DNA [38]. Thus, inhibiting *SULT1E1* expression with resveratrol and its methoxy substituted analogs may be considered as a protective mechanism in breast cancer initiation. However, it has to be stressed that this effect was confirmed on protein level only for resveratrol at the highest 5 μM dose.

The difference between mRNA transcript and the corresponding protein levels may confirm the suggestion that a certain threshold level of mRNA must be achieved before the protein can be translated, or cell-specific posttranscriptional modifications including proteolytic degradation can modulate protein levels [39, 40].

Interestingly, it was shown also that resveratrol itself is sulfated in tumor breast cells by SULT isozyme 1A1. Moreover, at the higher concentrations (40–100 μmol/L) resveratrol acted as non-competitive inhibitor of this enzyme [41, 42]. Thus, it is possible that the reduced protein level of SULT1E1 found in our study as a result of resveratrol treatment may also lead to the enzyme activity inhibition.

It was suggested that resveratrol may protect against the initiation effect of estrogen through reduced expression of *CYP1A1* and *CYP1B1*, which are involved in estrogen catabolism pathway, often leading to genotoxicity [43]. In this regard, the studies of Chen et al. [44] have shown that

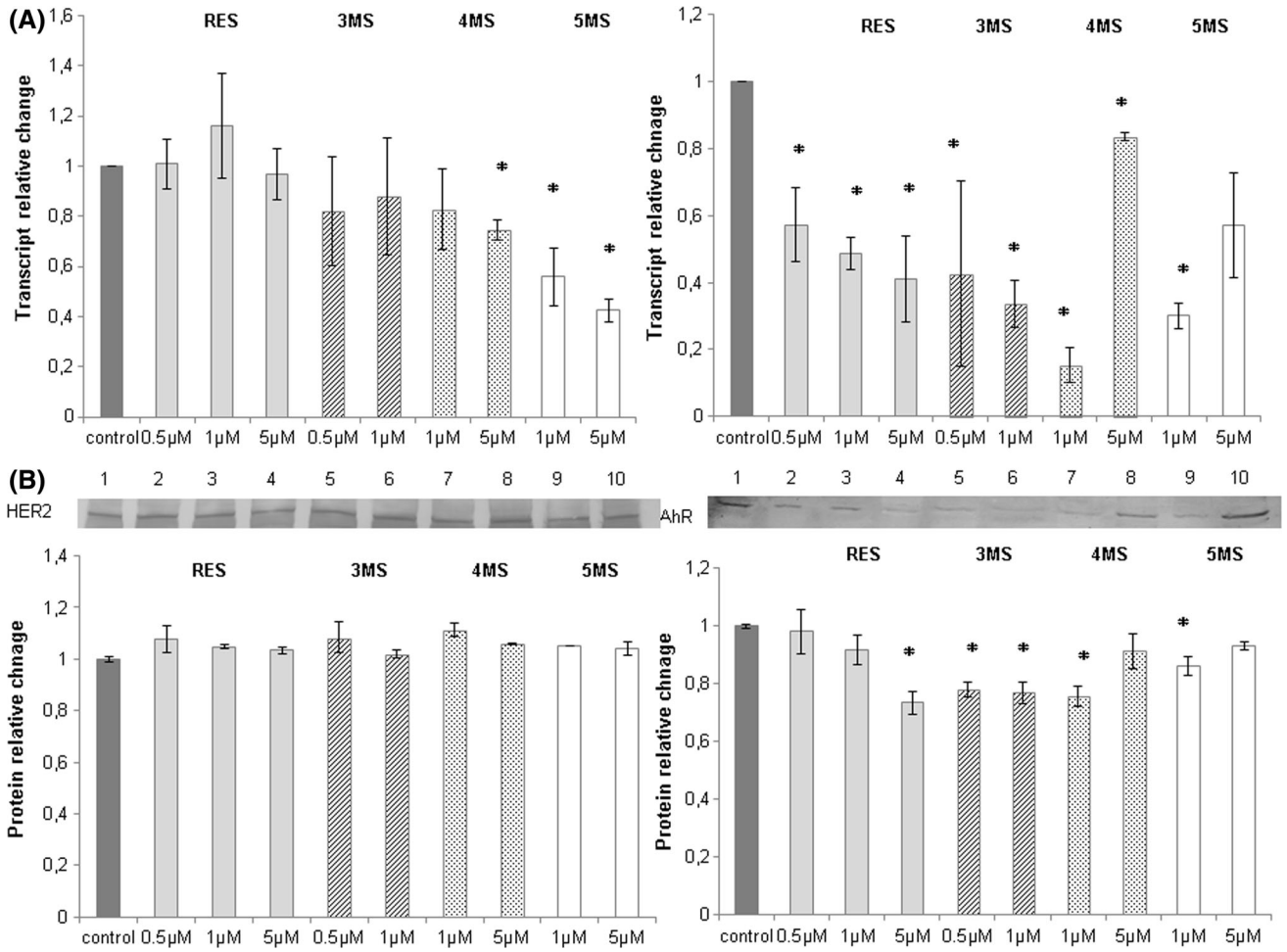

Fig. 6 The effect of 72 h incubation with resveratrol (RES) and synthetic methoxy-*trans*-stilbenes on the level of the *HER2* and *AhR* transcripts (**A**) and proteins (**B**) in MCF10A cells. These values were calculated as a relative change in transcript or protein level in comparison to control cells (expression equals 1). The mean values ± SEM from 3 independent experiments run in triplicate are presented. *Mean values were significantly different from the control group ($P < 0.05$). Western blot analysis: representative blot is shown: (*Lane 1*) control; (*Lanes 2, 3, 4*) resveratrol; (*Lanes 5, 6*) 3,4,2′-trimethoxy-*trans*-stilbene (3MS); (*Lanes 7, 8*) 3,4,2′,4′-tetramethoxy-*trans*-stilbene (4MS); (*Lanes 9, 10*) 3,4,2′,4′,6′-pentamethoxy-*trans*-stilbene (5MS)

resveratrol strongly inhibited the expression and the catalytic activity of CYP1A1 and CYP1B1 in 2,3,7,8-tetrachloro dibenzo-*p*-dioxin (TCDD)-induced MCF10A cells. This effect might have resulted from the simultaneous inhibition of TCDD-induced AhR DNA binding activity in these cells. Resveratrol prevented also TCDD-induced estrogen-DNA adduct formation and neoplastic transformation in MCF10F cells [21]. However, in the latter studies, constitutive expression of CYP1B1 was not affected as a result of treatment with resveratrol at the dose as high as 25 μmol/L.

In our study, the effect of resveratrol on *CYP1A1* and *CYP1B1* expression was tested at the doses selected based on the viability test. These doses were 5–50 times lower than that used in the above study. At the dose of 5 μmol/L, all tested stilbenes except 3MS reduced both *CYPs* transcript levels, but only resveratrol and 5MS decreased also their protein levels. Thus, our results confirm the suggestions that lower doses of chemopreventive agents, such as resveratrol, may be more efficient than higher doses [45].

Resveratrol and all of its methoxy derivatives reduced the expression of AhR. In the case of resveratrol and 5MS, the reduced expression of CYP1A1 and CYP1B1 basically correlated with the reduced expression of AhR, at least on the mRNA level, which suggests the possible effect of these stilbenes on activation of AhR pathway.

Interestingly, it was shown that SULT1E1 expression is MCF10A cells is transcriptionally regulated through a suppressive action of AhR [46]. Thus, we can speculate that reduced expression of SULTE1 found in our study may result from diminished expression of AhR.

In summary, our present study demonstrated that resveratrol and its methoxy derivatives modulate constitutive expression of enzymes and receptors involved in estrogen synthesis and catabolism in MCF10A cells. The reduced expression of AhR linked with reduced levels of CYP1A1 and CYP1B1 transcripts and proteins might be particularly important in prevention of tumorigenic transformation in breast epithelial cells. Among the analyzed methoxy derivatives of resveratrol, 5MS showed the highest activity. Since this compound also showed the lowest cytotoxicity, it may suggest its potential as chemopreventive agent in prophylaxis of estrogen-dependent cancers.

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