Pharmacological Reports 71 (2019) 1001–1005

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

Expression of CYP2S1 and CYP2W1 in breast cancer epithelial cells and modulation of their expression by synthetic methoxy stilbenes

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ARTICLE INFO

Article history:
Received 5 February 2019
Received in revised form 8 August 2019
Accepted 13 August 2019
Available online 14 August 2019

Keywords:
Methoxy stilbenes
Breast cancer epithelial cells
CYP2S1
CYP2W1

ABSTRACT

Background: "Orphan" cytochromes are a new group of P450 cytochromes without a fully recognized biological role. The expression of these CYPs in tumors is higher than that in normal tissues, which makes them attractive as chemopreventive and/or therapeutic targets. In this study, we compared the effect of synthetic methoxy stilbenes and resveratrol on the expression of two orphan cytochromes, CYP2S1 and CYP2W1, in breast cancer cells.

Methods: Breast cancer cells, lines MCF7 and MDA-MB-231, were treated for 72 h with tested compounds. The expression of CYP2S1 and CYP2W1 was evaluated at the transcript and protein levels by RT-PCR and Western blot, respectively.

Results: The constitutive expression of both isoforms was confirmed at the mRNA and protein levels. *CYP2S1* and *CYP2W1* showed higher expression in MDA-MB-231 cells. In MCF7 cells treated with stilbenes, the expression of both CYPs was increased at the mRNA level, whereas at the protein level this effect was confirmed for CYP2S1 alone. In contrast, in estrogen receptor-negative MDA-MB-231 cells treated with stilbenes, the expression of both CYPs decreased, but mostly at the transcript level.

Conclusions: The results of the present study confirmed the constitutive expression of CYP2S1 and CYP2W1 in breast cancer cells, although their relatively low level of expression suggests that they may be less involved in the transformation of therapeutic agents in these types of tumors. Stilbenes, particularly 3MS and 4MS, can modulate the expression of "orphan" CYPs more efficiently than resveratrol.

Introduction

Resveratrol and its natural derivatives possess pleiotropic activity allowing them to interfere with all stages of carcinogenesis [1]. Because of the pharmacokinetic and bioavailability limitations of resveratrol, searching for its new synthetic analogs with better properties is still topical [2]. Our and many other *in vitro* and *in vivo* studies showed that one of the most promising analogs of resveratrol are methoxy derivatives [3–5]. We found that the new synthetic methoxy stilbenes, namely 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), affected estrogen metabolism in nontumorigenic breast epithelial MCF10A cell line through modulation of the basal expression of some enzymes and receptors related to estrogen homeostasis. This effect was less pronounced in breast cancer MCF7 and MDA-MB-231 cell lines [6,7]. The reduced expression of aryl hydrocarbon receptor (AhR) was the most significant change resulting from the treatment with these compounds particularly in MCF10A cells. AhR is a ligand-dependent transcription factor that is activated by xenobiotics such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAHs) [8,9]. Concomitant with other receptors, AhR protects organisms from exogenous and endogenous toxic chemicals by regulating the genes involved in xenobiotic metabolism and elimination, including cytochrome P450 (CYP) genes [10,11]. The latter may also comprise, CYPs 2S1 and 2W1 representing the so-called "orphan" CYPs [12].

These cytochrome P450 isoforms are expressed in extrahepatic tissues and often upregulated in cancer cells, including breast cancer. In this regard, 37.5% of CYP2S1 immune-positive cells were seen in breast cancer clinical samples [13]. Human CYP2S1 oxidizes a number of carcinogens through the peroxide shunt. CYP2S1 was proposed to be regulated by AhR [14]. CYP2S1 is induced by dioxin and coal tar, an abundant source of polyaromatic hydrocarbons (PAHs), which further confirms the involvement of AhR in its expression [15]. Elevated expression of CYP2W1 was found in intensively proliferating tissues. In this regard, a high level of *CYP2W1* mRNA was observed in fetal and tumor tissues, whereas it

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http://dx.doi.org/10.1016/j.pharep.2019.08.005
1734-1140/

was absent or found in negligible amounts in normal cells [16]. CYP2W1 catalyzes the oxidation of indole and certain lipids including lysolecithin and their stereoisomers. Furthermore, it shows monooxygenase activity toward 3-methylindole and chlorzoxazone [17,18]. Moreover, this isoform was found to be involved in the metabolism of several procarcinogens, such as PAHs [19]. The breast tissue is particularly susceptible to PAH-induced carcinogenesis. Several studies have shown that PAHs are ultimately metabolized to their most potent and deleterious products in breast epithelial cells [20]. These data suggest that "orphan" cytochromes may be involved in breast cancer development and that their reduced expression may diminish the risk or change the phenotype of breast cancer cells.

The aim of this study was to assess the expression of CYP2S1 and CYP2W1 in the epithelial cancer breast cell lines MCF7 and MDA-MB-231, and to evaluate the effect of resveratrol and new synthetic methoxy stilbenes on their expression.

Materials and methods

Chemicals

The methoxy-*trans*-stilbenes 3MS, 4MS, and 5MS were provided by the Department of Chemical Technology of Drugs, PUMS, and synthesized as previously mentioned [6]. Primary antibody against β-actin was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA), while antibodies against CYP2S1 and CYP2W1 by Proteintech Group (Chicago, IL, USA). Secondary antibodies were obtained from Santa Cruz Biotechnology. The Western blotting detection system and SDS-PAGE gels (10%) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Protease inhibitor tablets were delivered by Roche Diagnostics GmbH (Penzberg, Germany). Most of other chemicals were purchased from Sigma-Aldrich GmbH (Buchs, CH). For details, see our previous publications [6,7]. The dimethyl sulfoxide 100 mM stock solutions of methoxy derivatives and resveratrol were stored at - 20 °C.

Cell culture and treatment

Human breast carcinoma cell lines MCF7 and MDA-MB-231 were supplied by the European Collection of Cell Cultures (Salisbury, Wiltshire, UK). The cells were maintained in DMEM media supplemented with 10% fetal bovine serum and 1% antibiotic solution at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. In experiments performed to determine the effect of stilbenes, 5% FBS was used in the cell medium. After 24 h of preincubation, the cells were treated with the tested compounds at the concentration based on the viability assay as previously described [7]. The incubation was continued for subsequent 72 h, and the cells were trypsinized from subconfluent monolayers. Control cells were treated with vehicle (DMSO) at a concentration of $\leq$ 0.1%.

Transcript levels of CYP2S1 *and* CYP2W1

Total RNA was isolated using the Gen MATRIX Universal RNA Purification Kit (EURx Ltd., Gdańsk, Poland). Subsequently, the first-strand cDNA was synthesized using the dART RT-PCR kit (EURx Ltd., Gdańsk, Poland) according to the instructions supplied. The real-time PCR reaction was carried out on a ready plate - RealTime ready Custom Panel 96 (Roche, Mannheim, Germany) using SG qPCR Master Mix (EURx Ltd., Gdańsk, Poland), according to the manufacturer's instructions. The primers were suitably designed by the manufacturer and placed directly on the plates. The SYBR Green quantitative real-time PCR was performed in triplicates on the LightCycler96 (Roche Diagnostics GmbH, Penzberg, Germany). The reaction mixture was composed of 1 μL of cDNA, 1 μL of tRNA, 10 μL of the SG qPCR Master Mix (EURx Ltd., Gdańsk, Poland), and up to 20 μL of RNAse-free water. The protocol included the following steps: enzyme activation at 95 °C for 10 min, 40–50 cycles (95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s), and elongation at 72 °C for 5 min.

Protein levels of CYP2S1 and CYP2W1

To determine CYP2S1 and CYP2W1 protein levels, an immunoblot assay was performed. The whole cell lysates were obtained using the RIPA buffer and protease inhibitors. The protein concentration was measured with albumin as a standard. The samples were separated on 10% SDS-PAGE gels with 100 μg of proteins per loading and transferred to nitrocellulose membranes. Skimmed milk (10%) was used as a blocking solution. Subsequently, the primary rabbit polyclonal CYP2S1, rabbit polyclonal CYP2W1, and rabbit β-actin antibodies were probed with the analyzed proteins. Then, the alkaline phosphatase-labeled anti-rabbit IgG antibody was used as the secondary antibody. The β-actin protein was used as an internal control. After densitometric scanning of membranes, we determined the amount of immunoreactive product in each lane using Bio-Rad GS710 Image Densitometer.

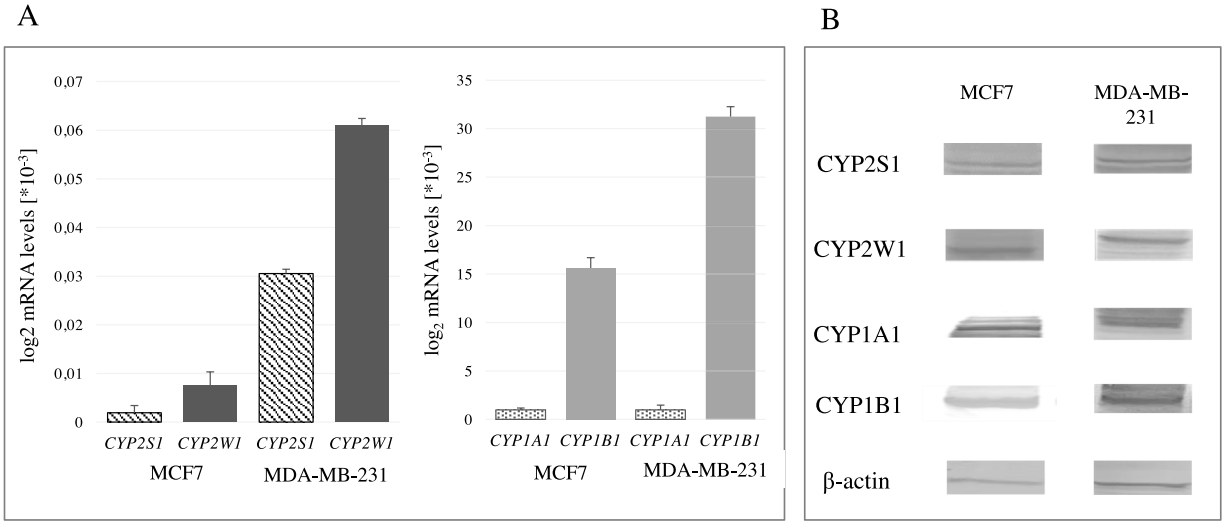

Fig. 1. The basal mRNA transcript (A) and protein (B) levels of CYP2S1, CYP2W1, CYP1A1, and CYP1B1 in MCF7 and MDA-MB-231 cells.

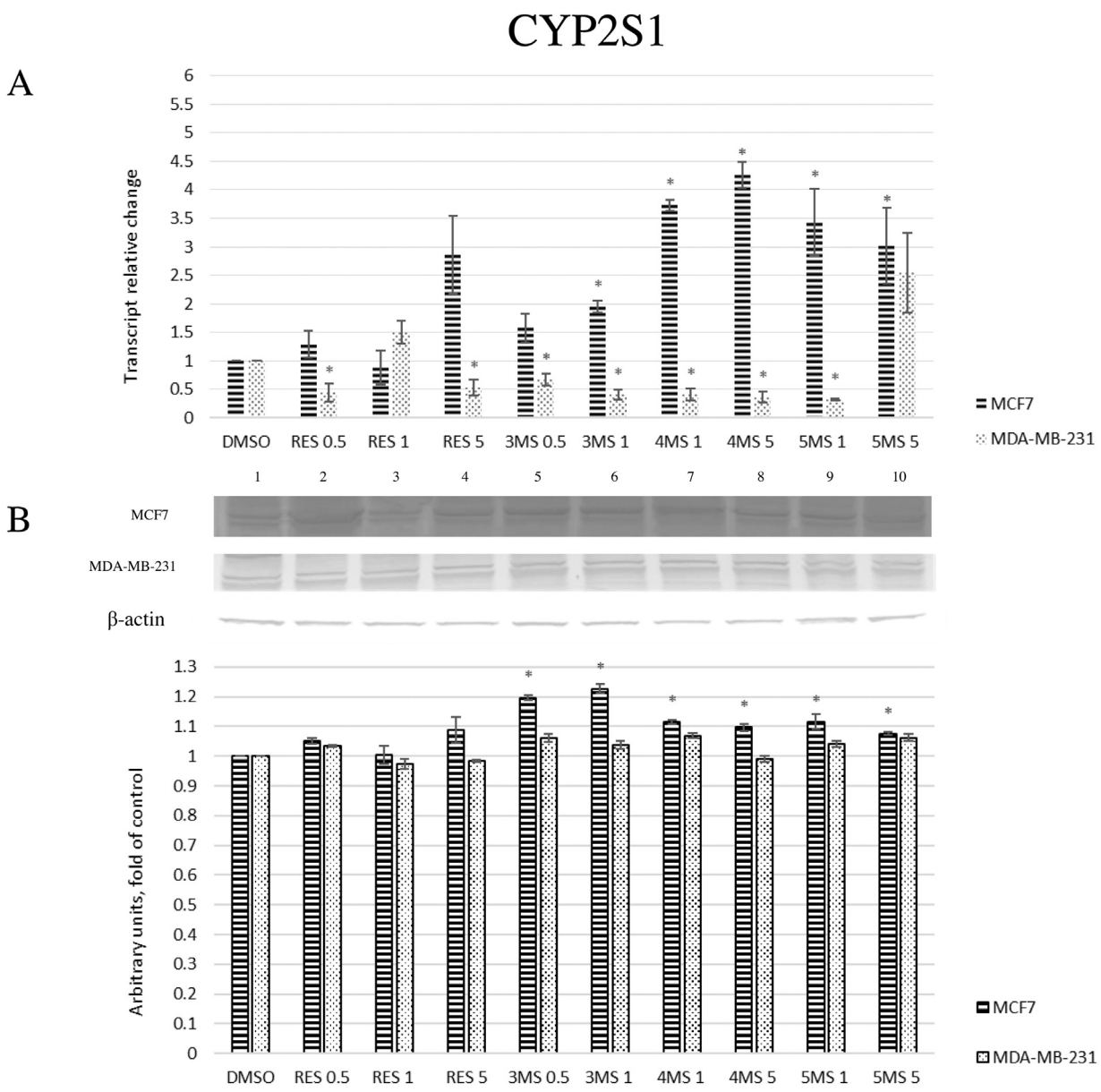

Fig. 2. The effect of 72-h incubation with resveratrol (RES) and synthetic methoxy-*trans*-stilbenes on the level of *CYP2S1* transcript (A) and protein (B) in MCF7 and MDA-MB-231 cells. The values were calculated as a relative change in the transcript or protein level in comparison with control cells (expression equals 1). The mean values ± SEM from three independent experiments performed in triplicate are presented. *Mean values were significantly different from the control group ($p < 0.05$). Western blot analysis - Representative blots are 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).

The results were expressed as relative absorbance units (RQ) per milligram protein and showed as fold of the control.

Statistical analysis

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

Results

CYP2S1 and CYP2W1 expression in breast cancer cell lines and its comparison with CYP1A1 and CYP1B1 expression

The constitutive mRNA level of cytochromes *CYP2S1* and *CYP2W1* was detected in both MCF7 and MDA-MB-231 cell lines. However, higher *CYP2S1* and *CYP2W1* expression levels were observed in MDA-MB-231 (Fig. 1A). The obtained results were confirmed at the protein level, which is illustrated in Fig. 1B. For comparison, the expression of CYP1A1 and CYP1B1 is also presented (Fig. 1A, 1B). In contrast to *CYP2S1* and *CYP2W1*, the constitutive expression level of these isoforms was similar in both breast cancer cell lines but significantly higher than that of "orphan" CYPs.

Modulation of CYP2S1 expression by resveratrol and methoxy stilbenes

In MCF7 cells, treatment with stilbenes mostly increased the expression of CYP2S1. At both tested doses, methoxy stilbenes caused a significant effect at the transcript and protein levels. The only exception was the effect of 3MS at the lower dose

(Fig. 2). In contrast, in MDA-MB-231 cells, resveratrol and all methoxy stilbenes mostly decreased *CYP2S1* transcript levels. However, this effect was not confirmed at the protein level (Fig. 2).

Modulation of CYP2W1 expression by resveratrol and methoxy stilbenes

CYP2W1 expression tended to increase in MCF7 cells, but significant changes were observed only as a result of treatment with 4MS at a dose of 5 μM at the transcript level and with 3MS at both doses at the protein level (Fig. 3). In contrast, in MDA-MB-231 cells, a significant decrease in *CYP2W1* mRNA level was noted as a result of treatment with resveratrol at a dose of 0.5 μM and 5 μM and with 3MS and 5MS at a dose of 1 μM. Similarly, 5MS in lower dose decreased CYP2W1 expression at the protein level.

Discussion

CYP2S1 and CYP2W1 represent the so-called "orphan" P450 cytochromes, whose function is not clearly defined. It has been reported that CYP2W1 is overexpressed in human colon cancer tissue [21,22]. CYP2S1 expression has been found in epithelial cells that are targets for carcinogen exposure [15,23,24]. The data on the expression of these isoforms in breast cancer tissues or cell lines derived from breast cancer epithelium have not been so far conclusively documented. In the current study, we confirmed the observation of Tan et al. [25], indicating the high expression of CYP2W1 and low expression of CYP2S1 in noninvasive ER + MCF7 breast cancer cells. Moreover, we described for the first time the expression of these cytochromes in MDA-MB-231 metastatic cell line. The expression of CYP2W1 and CYP2S1 in MDA-MB-468 cell line has been previously reported [25]. Both lines are triple-negative cells (ER-, PR-, and HER2-), which differ in the expression

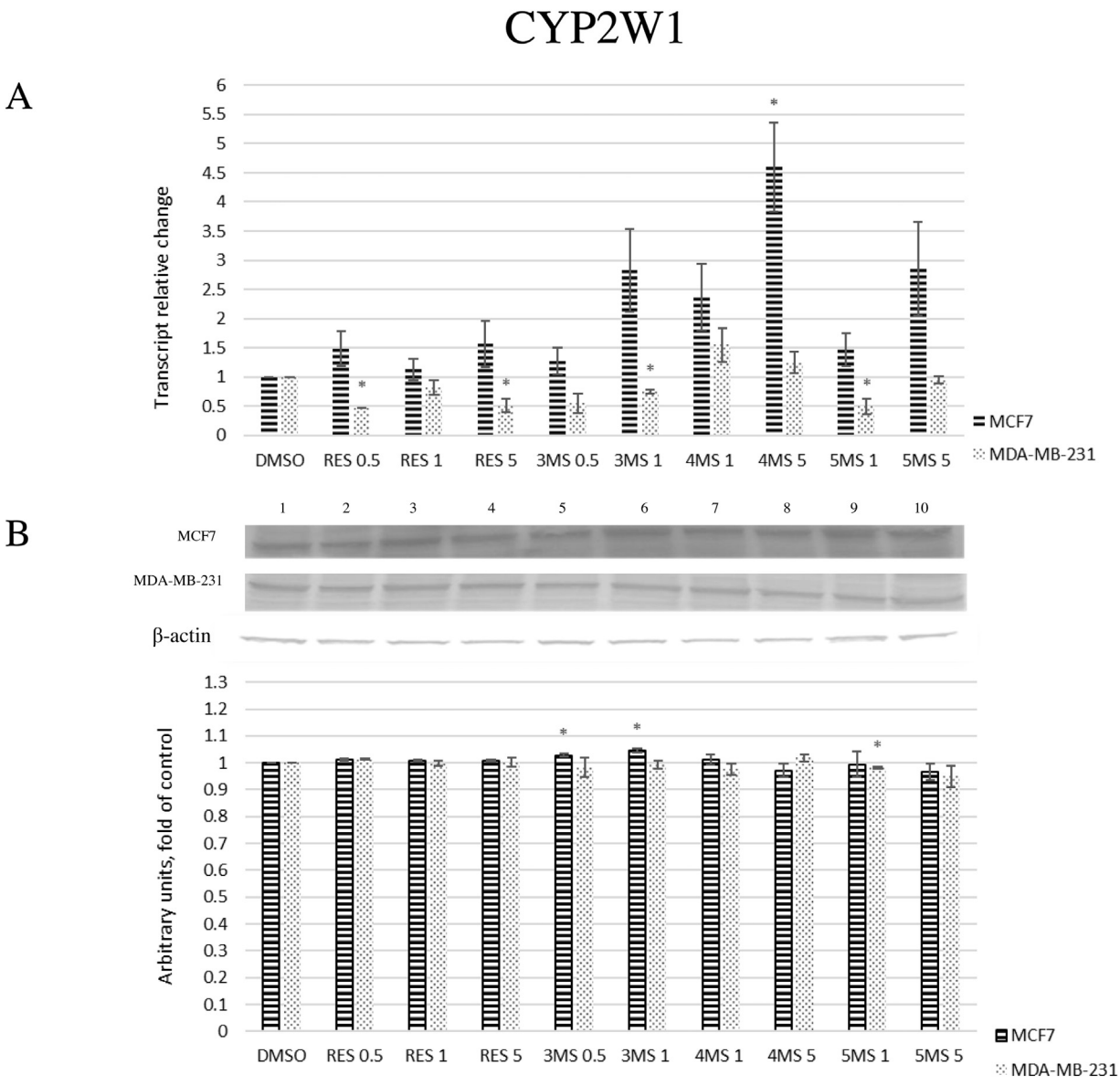

Fig. 3. The effect of 72-h incubation with resveratrol (RES) and synthetic methoxy-*trans*-stilbenes on the level of *CYP2W1* transcript (A) and protein (B) in MCF7 and MDA-MB-231 cells. The values were calculated as a relative change in the transcript or protein level in comparison with control cells (expression equals 1). The mean values ± SEM from three independent experiments performed in triplicate are presented. *Mean values were significantly different from the control group ($p < 0.05$). Western blot analysis - Representative blots are 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).

of proliferation markers such as Ki67 and E-cadherin or claudin-3 and in the response to chemotherapy [26]. In this regard, MDA-MB-468 cells are often responsive to chemotherapy, whereas MDA-MB-231 cells show intermediate susceptibility to treatment. In contrast to the constitutive expression levels of CYP2S1 and CYP2W1, no significant difference was found between the expression of CYP1A1 and CYP1B1 in both tested breast cancer cell lines, which is in accordance with the observations of other authors [27]. The expression of *CYP1A1* and *CYP1B1* is controlled by AhR. This receptor can act as a transcription factor that induces not only the expression of the *CYP1* family but also the *CYP2* family of genes or may initiate the degradation of estrogen receptor and suppress estrogen signaling [28,29].

Our previous studies showed that resveratrol, a naturally occurring phytoalexin, interferes with AhR expression and activity [6,7]. Moreover, its methoxy derivatives reduced the expression of AhR in the breast epithelial MCF10A cell line. This effect was much less pronounced in the breast cancer cell lines used in this study.

Assuming that *CYP2S1* and *CYP2W1* expression is controlled by AhR, the results of our current study indirectly confirmed our earlier observations. The expression of CYP2S1 in MCF7 cells increased as a result of treatment with all tested methoxy stilbenes. The effect of tested stilbenes on CYP2W1 in this cell line was less pronounced, but an increased level of its protein was observed after treatment with 3MS. It is worthy to note that in our previous study, the same compounds also increased the expression of *CYP1B1* in MCF7 cells [7]. In MDA-MB-231 cells, *CYP2S1* and *CYP2W1* transcript levels decreased when treated with resveratrol or methoxy stilbenes; however, this result was not observed at the protein level. The steady state protein level is determined by transcription, mRNA decay, translation, and protein degradation [30]. Thus, several reasons may contribute to the discrepancy between the effect of stilbenes on mRNA and protein levels.

Overall, the results of our current study showed relatively low level of CYP2S1 and CYP2W1 in both, less and more aggressive breast cancer cell lines. This is an important information since the data about the expression of these P450 isoforms in cancer cells are scarce, similarly, as their modulation by potential chemopreventive or chemotherapeutic agents. CYP2W1 is responsible for biotransformation of pro-drugs used in cancer therapy, what additionally, emphasizes the significance of our research [31]. The results of our current study also indicate that stilbenes are moderate modulators of the expression of cytochromes P450, at least those controlled by AhR. Further studies are required to confirm this conclusion.

Declaration of Competing Interest

None.

Acknowledgment

The present study was supported by research funding from Poznan University of Medical Sciences, Poznań, Poland.

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