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# Anti-tumor properties of methoxylated analogues of resveratrol in malignant MCF-7 but not in non-tumorigenic MCF-10A mammary epithelial cell lines

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ABSTRACT

Resveratrol is a plant-derived polyphenol that is known for its anti-inflammatory and anti-tumorigenic properties in in vitro and in vivo models. Recent studies show that some resveratrol analogues might be more potent anti-tumor agents, which may partly be attributed to their ability to activate the aryl hydrocarbon receptor (AHR). Here, the anti-tumorigenic properties of resveratrol and structural analogues oxyresveratrol, pinostilbene, pterostilbene and tetramethoxystilbene (TMS) were studied in vitro, using in the malignant human MCF-7 breast cancer cell line and non-tumorigenic breast epithelial cell line MCF-10A.

Cell viability and migration assays showed that methoxylated analogues of resveratrol are more potent anti-tumorigenic compounds than resveratrol and its hydroxylated analogue oxyresveratrol, with 2,3',4,5'-tetramethoxy-*trans*-stilbene (TMS) being the most potent compound. TMS decreased MCF-7 tumor cell viability with 50% at 3.6 μM and inhibited migration with 37.5 ± 14.8% at 3 μM. In addition, TMS activated the AHR more potently (EC50 in a reporter gene assay 2.0 μM) and induced AHR-mediated induction of cytochrome P450 1A1 (CYP1A1) activity (EC50 value of 0.7 μM) more than resveratrol and the other analogues tested. Cell cycle analysis showed that TMS induced a shift in cell cycle status from the G1 to the G2/M phase causing a cell cycle arrest in the MCF-7 cells, while no effect of TMS was observed in the non-tumorigenic MCF-10A mammary epithelial cell line. Gene expression analysis showed that 3 μM TMS increased gene expression of CYP1A1 (289-fold), CYP1B1 (5-fold) and Nqo1 (2-fold), and decreased gene expression of IL-8 (3-fold) in MCF-7 cells. In MCF-10A cells, 10 μM TMS also increased gene expression of CYP1A1 (5-fold) and CYP1B1 (2-fold), but decreased gene expression of Nqo1 (1.4-fold) in contrast to MCF-7 cells.

TMS displays more potent anti-tumorigenic properties and activates the AHR more effectively than resveratrol. In addition, this is the first study to show that TMS, but not resveratrol, selectively inhibits the cell cycle of breast tumor cells and not the non-tumorigenic cells. Our study provides more insight in the anti-tumor properties of the methoxylated analogues of resveratrol in breast cells in vitro.

## 1. Introduction

Resveratrol is a phytoalexin naturally occurring in grapes, peanuts and pines, and is present in many dietary products. It was proposed to be an anti-inflammatory agent in the late 90s, and has received great scientific attention since (Jang, 1997). Next to its anti-inflammatory properties, resveratrol has been shown to possess anti-cancer properties and act as cancer chemopreventive (i.e. inhibit tumor initiation) and anti-tumor agent (i.e. inhibit tumor progression) in various in vitro and in vivo cancer models, including the human breast cancer cell line MCF-7 (Baur and Sinclair, 2006; Ko et al., 2017; Lu and Serrero, 1999).The exact anti-cancer mechanism of action of resveratrol remains unclear, but there are several studies that suggest that part of resveratrol action is mediated via the aryl hydrocarbon receptor (AHR) (Aggarwal et al., 2004; Casper et al., 1999).

The AHR is a cytoplasmic ligand-dependent transcription factor that mediates a wide range of biological and toxic events in response to endogenous and exogenous ligands. When activated by ligand binding, the receptor translocates to the nucleus and initiates the transcription of, among others, cytochrome P450 (CYP) 1A1 and CYP1B1 expression (Denison et al., 2011). These phase I metabolizing enzymes are expressed in many tissues, including cancer cells. CYP enzymes metabolize both exogenous and endogenous substrates, including the endogenous steroid hormone estradiol (E2). Here, especially CYP1B1-

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mediated metabolism of estrogens is of concern, as this may lead to genotoxic estrogen metabolites (Cavalieri and Rogan, 2011). CYP1B1 is also able to metabolize resveratrol to piceatannol, which is known for its anti-cancer properties from in vitro and in vivo studies and may partly explain the potency of resveratrol to protect cells against tumorigenic effects (Aggarwal et al., 2004; Potter et al., 2002). In addition to the regulation of some CYP enzymes, activation of the AHR is involved in a wide range of cellular responses. These include, among others, the modulation of tumor suppressing or promoting factors such as interaction with the Nrf2 pathway (No et al., 2014; Wakabayashi et al., 2010) and modulation of interleukin-8 (IL-8) expression (Freund et al., 2004; Yamashita et al., 2018).

Many studies have reported that AHR activators can inhibit the growth of a wide variety of tumor cells, like breast and endometrial tumor cells (Hsieh et al., 2011; Safe, 1995; Wormke et al., 2000). Although the anti-tumorigenic properties of resveratrol have also been observed in hormone independent breast tumor cells, these effects are generally greater in hormone dependent breast tumor cells. Such tumor cells express various steroid receptors and tumor progression can be stimulated by E2 and progesterone (P4). AHR ligands have been shown to modulate the function of steroid receptors and inhibit the tumorigenic response to steroid hormones. This cross-talk between the AHR and ER responsive pathways indicate another mechanism via which resveratrol can exert its anti-tumorigenic action in hormone-responsive tumor cells (Aggarwal et al., 2004; Casper et al., 1999). The AHR is therefore often suggested to be a potential target for cancer treatment, as it is higher expressed in many tumors compared to normal tissues (Koliopanos et al., 2002; Lin et al., 2003; Powell et al., 2013; Safe et al., 2017).

Although resveratrol itself displays anti-cancer properties, adding hydroxyl or methoxy groups or substitutions to the stilbene backbone of resveratrol may result in analogues that may be more active than resveratrol. These methoxylated analogues are assumed to be more biologically stable with a higher bioavailability and an increased transport of the compound into the cell (Kapetanovic et al., 2011; Tsai et al., 2017; Walle, 2009). Indeed, several in vitro and in vivo studies have indicated that other synthetic and natural derivatives of resveratrol can have a higher anti-cancer potency than resveratrol in various cancer models (Aiyar et al., 2010; Christiansen et al., 2012; Fulda, 2010; Kim et al., 2011; Li et al., 2010; Licznerska et al., 2017; McCormack et al., 2012; Paul et al., 2010; Schneider et al., 2010; Wakimoto et al., 2017).

We studied the anti-tumor properties of resveratrol and several analogues with varying hydroxy and/or methoxy substitutions in the human breast cancer cell line MCF-7 (Fig. 1). The effects of these compounds were studied on tumor cell viability and migration. In addition, we assessed the potency of these compounds to activate the AHR using a reporter gene assay and subsequent CYP1A1 enzyme activity. The anti-tumor properties of resveratrol and its most potent analogue tetramethoxystilbene (TMS) were further studied in human breast cancer cells (MCF-7) and compared with effects in non-tumorigenic (MCF-10A) mammary epithelial cells. For that, cell cycle status and gene expression patterns of selected genes that are linked to carcinogenicity and AHR activation were assessed in both cell lines.

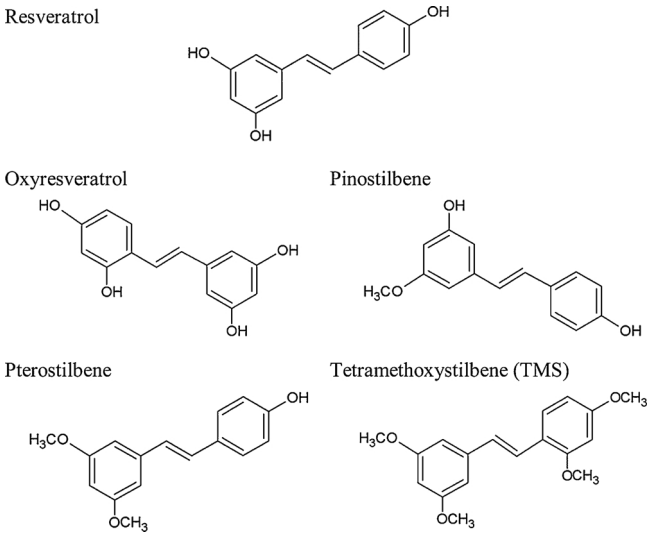


**Fig. 1.** Structure of resveratrol and four of its analogues used in this study.

## 2. Material and methods

### 2.1. Chemicals

Resveratrol (3,4',5-trihydroxy-trans-stilbene), and its derivatives oxyresveratrol (2,3',4,5'-tetrahydroxy-*trans*-stilbene), pinostilbene (3,4'-dihydroxy-5-methoxy-*trans*-stilbene hydrate) and pterostilbene (3,5-dimethoxy-4'hydroxy-*trans*-stilbene) were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands. Tetramethoxystilbene (2,3',4,5'-tetramethoxy-*trans*-stilbene) was obtained from Enzo Life Sciences, Antwerp, Belgium. All other chemicals used were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands unless stated otherwise.

### 2.2. Cell culture

Human breast carcinoma MCF-7 and human breast epithelial MCF-10A cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) The MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) supplemented with 5% fetal bovine serum (Life Technologies) and 0.01 mg/mL insulin (Life Technologies). The MCF-10A cells were cultured in Dulbecco's Modified Eagle's Medium/ Ham's F12 Medium (Life Technologies) (1:1) supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 0.01 mg/mL insulin and 500 ng/mL hydrocortisone (Life Technologies). Both culture media contained 100 U/mL penicillin and 100 µg/mL streptomycin (pen/strep). The cells were cultured in a humidified atmosphere with 5% $CO_2$ at 37 °C, and subcultured twice a week at a cell density of 70–80%.

### 2.3. Cell viability

3 days prior to the experiment, the MCF-7 culture medium was replaced with assay medium that contained 5% charcoal-stripped FBS (Hyclone) to remove hormones from the medium. MCF-7 cells were seeded at a density of 8000 cells/well on a 96-wells plate. After a 2-day incubation, the cells were exposed to ranges of resveratrol and analogues in concentrations up to 30 µM. 0.1% DMSO was used as a vehicle control. Medium containing the compounds was refreshed after 4 days and the experiment was finished after a total of 10 days. The viability of the cells was determined by using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. 1 mg/ml MTT was added to each well and incubated for 30 min to allow the cells to take up the MTT. Isopropanol was used to lysate the cells and absorbance could be measured at 595 nm. The viability assay was repeated 3 times with a replicate of 3 in each experiment.

### 2.4. Migration assay

A wound healing assay was performed to assess the migration of MCF-7 cells in the presence of resveratrol and analogues. MCF-7 cells were seeded at a density of $1.5*10^6$ cells/well in a 12-wells plate. After 24 h, a scratch was made in the monolayer of cells using a 200 µL pipet tip. Loose cells were washed away with medium after which the cells were exposed to 3 µM resveratrol and analogues. Photographs of the wound were taken at t = 0 and t = 48, and the wound area was calculated using ImageJ. The migration assay was repeated 3 times with a

replicate of 3 in each experiment.

### 2.5. AHR CALUX assay

The human HG2L7.5C1 CALUX cell line was created by a stable transfection of an AHR responsive firefly luciferase reporter gene plasmid pGudLuc7.5 and was a kind gift from Prof. M.S. Denison (University of California, Davis, CA). The HG2L7.5C1 cells were cultured in MEM alpha medium (Invitrogen) supplemented with 10% FBS and 1% pen/strep. The cells were maintained in a humidified atmosphere with 5% $CO_2$ at 37 °C, and subcultured twice a week. The cells were seeded at a density of $4*10^4$ cells/well in a 96-well plate. Cells were exposed to ranges of resveratrol and analogues for 24 h with and without 10 μM AHR antagonist CH-223191 (CAS 301326-22-7, Calbiochem), and 0.1% DMSO was used as a negative control for AHR activation. The experiment was finished by lysing and mixing the cells with luciferine reagent consisting 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.7 mM $MgSO_4.7H_2O$, 0.1 mM EDTA, 22.3 mM DTT, 261 μM coenzyme A, 470 μM luciferine and 530 μM ATP (pH 7.8). Luminescence was measured directly after as a proxy for AHR activation. MTT cell viability assays were performed to identify cytotoxicity. The AHR CALUX assay was repeated 3 times with a replicate of 3 in each experiment.

### 2.6. CYP1A1 activity

CYP1A1 activity in the MCF-7 cells was measured by a CYP1-mediated 7-ethoxyresorufin *O*-deethylation (EROD) activity assay. The MCF-7 cells were seeded at a density of $3*10^4$ cells/well in a 96-well plate. After 2 days, cells were exposed to ranges of resveratrol and its analogues for 24 h with and without 10 μM AHR antagonist CH-223191 (CAS 301326-22-7, Calbiochem), and 0.1% DMSO was used as a negative control. The experiment was finished by washing the cells and adding DMEM medium supplemented with 50 mM $MgCl_2$, 50 μM 7-ethoxyresorufin, and 10 μM dicoumarol. Fluorescence was measured at an excitation wavelength of 530 nm and emission wavelength of 590 nm. A resorufin calibration curve was used for quantification of the CYP1A1 activity (van Duursen et al., 2005). The EROD assay was repeated 3 times with a replicate of 3 in each experiment.

### 2.7. Cell cycle

The MCF-7 and MCF-10A cells were seeded with a density of $4*10^5$ cells/well on a 12-well plate. Cells were exposed to 10 μM (MCF-7) and 30 μM (MCF-10A) resveratrol and TMS. 10 μM 2-methoxyestradiol (2-$MeOE_2$) was used as a positive control for a cell cycle arrest in the G2/M phase (Sangjun et al., 2009). After 20 h of incubation, the cells were fixated with 70% ethanol until further analysis. Cell cycle analysis was conducted by staining the cells with propidium-iodide (PI, Sigma, the Netherlands). PI content was measured with the use of flowcytometry. FlowJo was used to analyze the distribution of cells in each phase of the cell cycle, set at < G1, G1, S, and G2/M phase. Multinucleate cells or otherwise aberrant cells were excluded from gates (> G2).

### 2.8. mRNA isolation and RT-qPCR

The MCF-7 and MCF-10A cells were seeded with a density of $2*10^5$ cells/well on a 12-well plate. Cells were exposed for 48 h to 3 μM (MCF-7) and 10 μM (MCF-10A) resveratrol and TMS. RNA was isolated with the phenol-chloroform extraction method using RNA Instapure (Eurogenetec, Liege, Belgium). Purity and concentration of the isolated RNA were determined were determined spectrophotometrically at an absorbance wavelength of 260 and 280 nm. Complementary DNA (cDNA) was synthesized with 1 μg RNA using the iScript cDNA Synthesis Kit (BioRad), according to the manufacturer's instructions. The obtained cDNA was diluted 10 times and stored at 4 °C until further analysis. Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) was performed in a 96-well green shell PCR plate (Bio-Rad, Veenendaal, the Netherlands) using the iQ5 cycler (Bio-Rad, Veenendaal, the Netherlands). A PCR mastermix was made containing 12.5 μL of IQ SYBR Green Supermix, 1 μL of a forward and reverse primer (each with a concentration of 10 μM) and 0.5 μL of water per sample. For the qPCR, 10 μL of cDNA of each sample was added to 15 μL of the PCR mastermix. Oligonucleotide sequences of the primers were; β-actin, FW TTGTTACAGGAAGTCCCTTGCC, RV ATGCTATCACCTCCC CTGTGTG; CYP1A1, FW CAGAAGATGGTCAAGGAGCA, RV GACATTG GCGTTCTCATCC; CYP1B1, FW CGGCCACTATCACTGACATC, RV CTC GAGTCTGCACATCAGGA; Nqo1, FW GGATTGGACCGAGCTGGAA, RV AATTGCAGTGAAGATGAAGGCAAC; IL-8, FW CTCTTGGCAGCCTTCCT GATT, RV TATGCACTGACATCTAAGTTCTTTAGCA. Primer-specific annealing temperatures were 60 °C for all primers used. Gene expression was determined using the ΔΔCq method of relative quantification where gene expression in the co-cultures was normalized to β-actin.

### 2.9. Data analysis

All data is expressed as means + standard deviation of three independent experiments (N) that were performed in triplicate (n). ImageJ was used to analyze particles in the snapshots taken to calculate the migration of the cells. Data was analyzed using GraphPad Prism 7. Student T-tests were performed to determine statistical difference of the mean between the treatments in the viability, migration, AHR CALUX, CYP1A1 activity, cell cycle and gene expression assays.

## 3. Results

### 3.1. Effects of resveratrol and its analogues on tumor cell viability

Human MCF-7 breast cancer cells were exposed to resveratrol and its analogues to assess their effect on tumor cell viability. The cells were exposed for 10 days to a concentration range up to 30 μM for all compounds, in combination with 20 pM E2 to induce proliferation. E2 concentration-dependently stimulated MCF-7 cell proliferation with an EC50 value of 20 pM E2 (van Meeuwen et al., 2007). Oxyresveratrol and resveratrol did not statistically significantly affect MCF-7 cell viability at concentrations up to 30 μM (Fig. 2, upper panel). In contrast, resveratrol analogues pinostilbene, pterostilbene, and tetramethoxystilbene (TMS) statistically significantly reduced MCF-7 tumor cell viability with a 50% reduction of cell viability at 14.2 μM for pinostilbene, 15.6 μM for pterostilbene, and 3.6 μM for TMS (Fig. 2, lower panel).

### 3.2. Effects of resveratrol and its analogues on MCF-7 tumor cell migration

A wound healing assay was used to assess the effects of resveratrol and its analogues on MCF-7 tumor cell migration. After inflicting a scratch in the MCF-7 monolayer, the cells were exposed for 48 h to 3 μM for all compounds or 0.1% vehicle control (DMSO) and the wound surface area was measured as marker for cell migration. After 48 h, the wound surface area in the vehicle-treated control monolayer was approximately 40% of the original wound surface area. Effects on cell migration of resveratrol and its analogues were calculated relative to the migration of the vehicle-treated control cells. No statistically significant difference in cell migration was observed between the control treatment and the resveratrol treatment. Oxyresveratrol reduced MCF-7 tumor cell migration with 12.1 ± 7.2%, pinostilbene with 15.2 ± 3.8% and pterostilbene with 13.2 ± 11.6%. Only TMS statistically significantly reduced MCF-7 tumor cell migration with 37.5 ± 14.8% (Fig. 3).

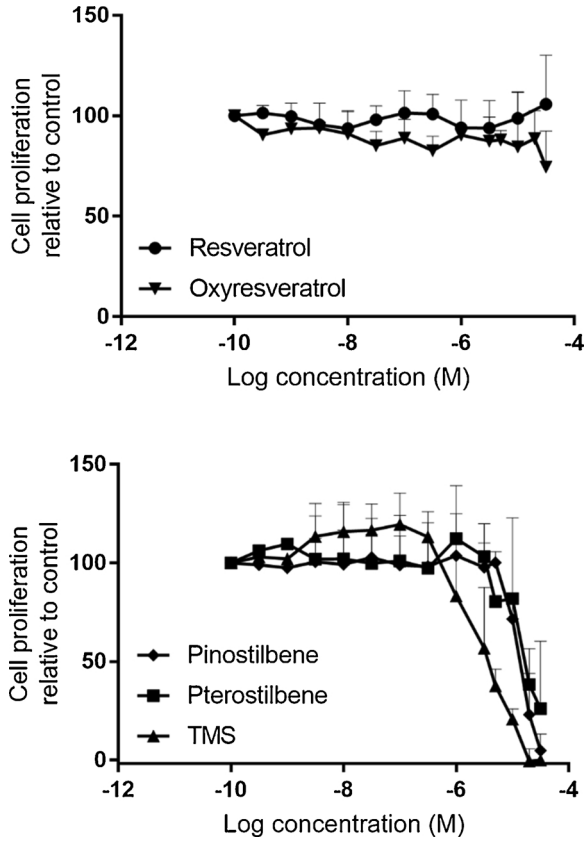


**Fig. 2.** Cell viability assay in MCF-7 cells. Cells were exposed to a concentration range (0–30 μM) of resveratrol and four of its analogues in combination with 20 pM E2. Data is expressed relative to the vehicle treated cells (0.1% DMSO + 20 pM $E_2$) that was set to 100% and are expressed as mean ± standard deviation (N = 3), and adjusted for the background values.

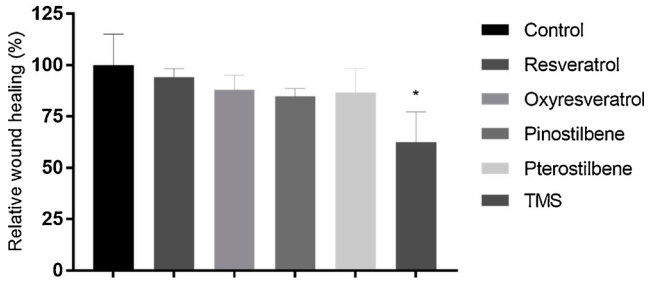


**Fig. 3.** Wound healing assay in MCF-7 cells. Cells were exposed to 3 μM resveratrol and its analogues for 48 h. Wound surface area is measured as marker for MCF-7 tumor cell migration. Data is expressed as mean wound surface area ± standard deviation (N = 3), relative to the vehicle-treated control (0.1% DMSO) cells. * Statistically significantly different from vehicle-treated control cells (P ≤ 0.05).

### *3.3. Effect of resveratrol and its analogues on AHR activation and CYP1A1 activity*

To assess the potency of resveratrol and its analogues to activate the AHR, a reporter gene assay was performed. HG2L7.5C1 cells, which are stably transfected human hepatoma HepG2 cells with an AHR responsive firefly luciferase reporter gene plasmid, were treated for 24 h with concentration ranges of the compounds (0–30 μM) with and without AHR antagonist CH-223191 (10 μM). Resveratrol did not cause a statistically significant effect on AHR activity in HG2L7.5C1 cells up to 30 μM (Fig. 4, left panel). In contrast, oxyresveratrol, pinostilbene, pterostilbene and TMS statistically significantly activated the AHR. AHR activation was maximal after exposure to 20 μM oxyresveratrol, 5 μM pterostilbene and 3 μM TMS, after which the activity declined (Fig. 4, left panels). This was not due to cytotoxicity as measured by an MTT assay (data not shown). No maximum AHR activation could be determined for pinostilbene. The EC50 value for AHR activation was 5 μM for oxyresveratrol, 2.6 μM for pterostilbene and 2.0 μM for TMS. Addition of AHR antagonist CH-223191 negated AHR activation by oxyresveratrol, pterostilbene and TMS, indicating an AHR-mediated effect of these resveratrol analogues. Co-exposure to CH-223191 had no significant effect on AHR activation by pinostilbene at the three highest exposure concentrations.

In addition to AHR activation, the potency of resveratrol and its analogues to induce CYP1A1 activity was assessed by means of an EROD assay. MCF-7 cells were treated for 24 h with concentration ranges of the compounds (0–30 μM) with and without AHR antagonist CH-223191 (10 μM). Pterostilbene and TMS concentration-dependently induced EROD activity in MCF-7 cells with EC50 values of 5 μM and 0.7 μM, respectively (Fig. 4, right panels). The induction of EROD activity by pterostilbene and TMS was inhibited by the AHR antagonist CH-223191, again confirming an AHR-mediated effect of these analogues. Resveratrol, oxyresveratrol and pinostilbene did not cause a statistically significant effect on EROD activity in MCF-7 cells after exposure to concentrations up to 30 μM (Fig. 4, right panels).

### *3.4. Effect of resveratrol and TMS on cell cycle status in MCF-7 and MCF-10A cells*

Overall, the viability, migration, AHR reporter gene assay and EROD assays revealed that resveratrol analogue TMS displays the strongest anti-tumorigenic properties and is the most potent AHR activator of all the analogues tested. Therefore, TMS was studied in more detail in MCF-7 cells and the non-tumorigenic breast epithelial cell line MCF-10A.

Cell cycle status was determined in MCF-7 and MCF-10A cells after a 20-h exposure to resveratrol and TMS to concentrations known to activate the AHR. 10 μM 2-methoxyestradiol (2-$MeOE_2$) was used as positive control for G2/M cell cycle arrest. Indeed, 2-MeOE2 reduced the cell cycle status of MCF-7 cells in the G1 phase with 30%, and increased the cell cycle status with 21% in the G2/M phase (Fig. 5, upper panel). The cell cycle status of the MCF-7 cells was not affected by resveratrol. Cell cycle status of MCF-7 cells shifted from the G1 phase to the G2/M phase upon exposure to 10 μM TMS. The fraction of cells in the G1 phase were statistically significantly reduced with 27% after exposure to TMS, while the fraction of cells in the G2/M phase increased with 18%.

In MCF10A cells, the number of cells in the G1 phase was reduced with 41% by 2-MeOE2, and increased in the G2/M phase with 33%. In contrast to MCF-7 cells, resveratrol, but not TMS, did affect cell cycle status in MCF-10A cells (Fig. 5, lower panel). Exposure to 30 μM resveratrol resulted in a shift in cell cycle status from the G1 to the S phase. The fraction of cells in the G1 phase reduced with 16% after the exposure to resveratrol, while the fraction of cells in the S phase increased with 12%. In contrast to MCF-7 cells, TMS did not affect cell cycle status in MCF-10A cells, even when the cells were exposed to a higher concentration of 30 μM TMS (Fig. 5, lower panel).

### *3.5. Effect of resveratrol and TMS on gene expression*

Gene expression patterns were analyzed in MCF-7 and MCF-10A cells after exposure to resveratrol or TMS to further elucidate the mechanism by which these compounds exert their effects.

In MCF-7 cells, CYP1A1 gene expression increased 289-fold and CYP1B1 expression increased 4.9-fold after a 48-h exposure to 3 μM TMS. CYP1A1 or CYP1B1 gene expression did not significantly change after exposure to 3 μM resveratrol. Nqo1 gene expression increased 2-fold in MCF-7 cells after a 48-h exposure to 3 μM TMS, while there was

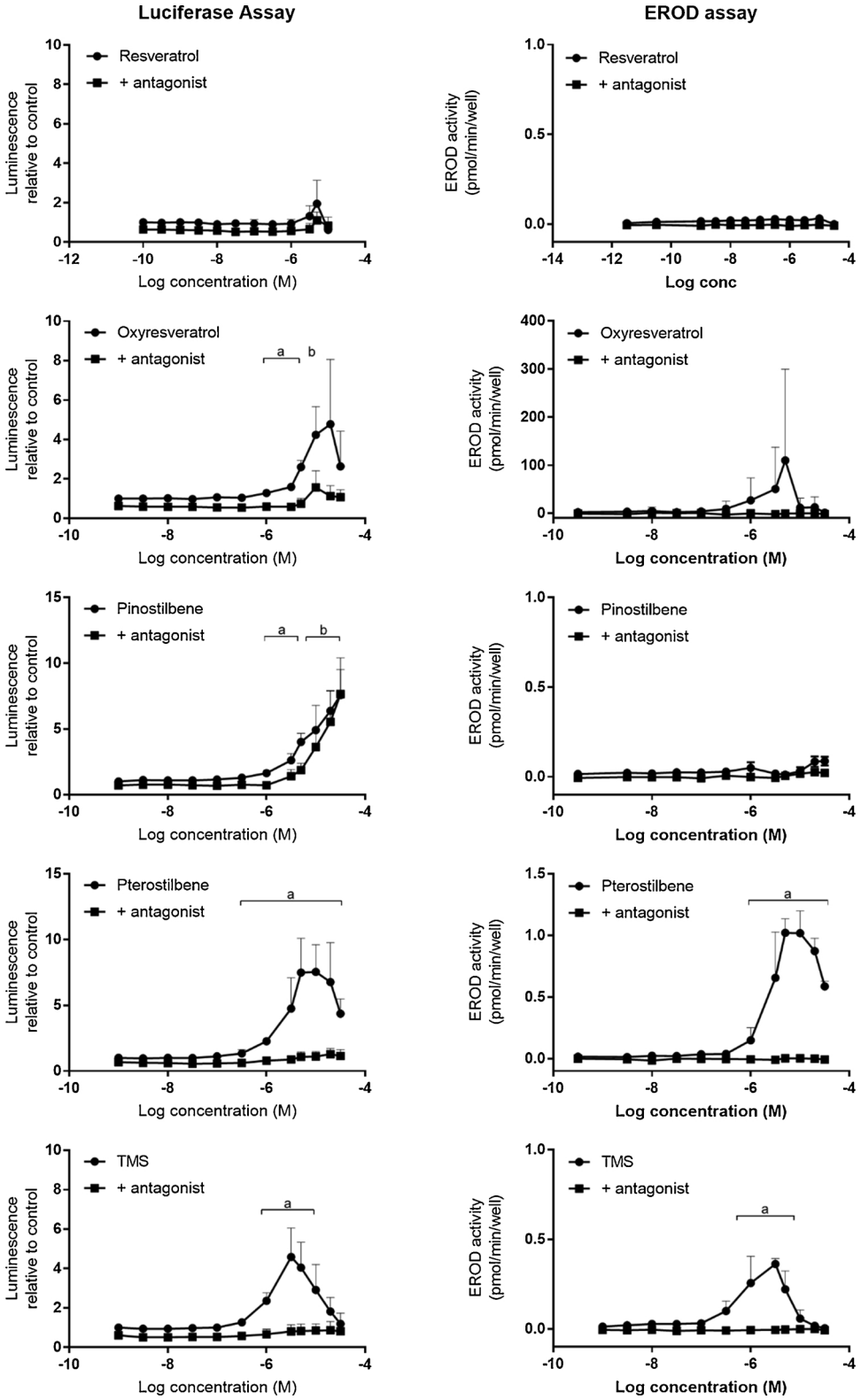


**Fig. 4.** AHR reporter gene assay in HG2L7.5C1 cells (left panels) and EROD activity in MCF-7 cells (right panels). Cells were exposed to resveratrol, oxyresveratrol, pinostilbene, pterostilbene, and tetramethoxystilbene (TMS) with concentration ranges up to 30 μM and in combination with 10 μM CH-223191 AHR antagonist. Data are expressed as means of three independent experiments with standard deviation (N = 3). [a] Statistically significantly different from vehicle-treated control cells and from treatment with inhibitor ($P \leq 0.05$); [b] Statistically significantly different from vehicle-treated control cells ($P \leq 0.05$).

no change in Nqo1 expression after exposure to 3 μM resveratrol. In addition, IL-8 gene expression decreased 3-fold in MCF-7 cells when exposed to 3 μM TMS, while no significant change in IL-8 gene expression was observed upon exposure to 3 μM resveratrol. AHR gene expression did not statistically significantly change in MCF-7 cells upon exposure to either 3 μM TMS or resveratrol (Fig. 6).

In the non-tumorigenic MCF-10A cells, CYP1A1 gene expression also increased, albeit only 5-fold, after a 48-h exposure to 10 μM TMS.

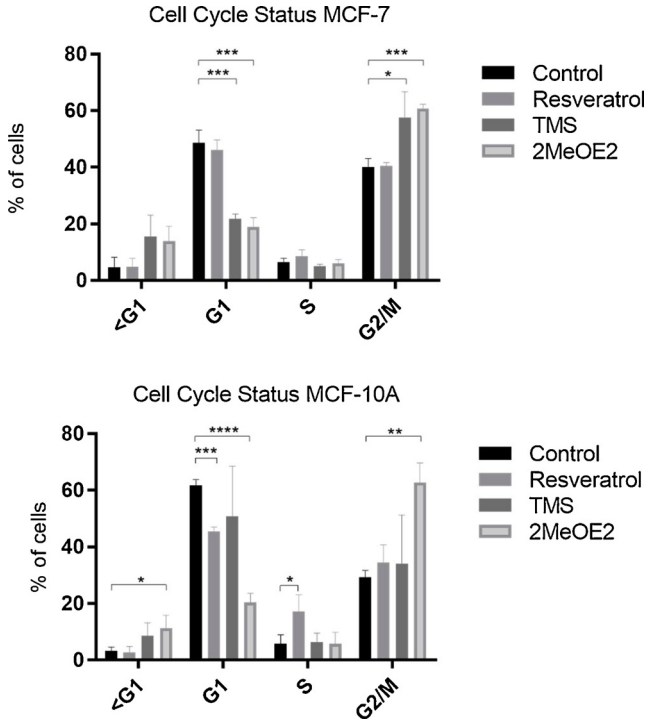


**Fig. 5.** Analysis of the cell cycle status of MCF-7 and MCF-10A cells. Cells were exposed to 10 μM (MCF-7) or 30 μM (MCF-10A) resveratrol or TMS, 0.1% DMSO (vehicle control) and 10 μM 2-MeOE2 (positive control) for 20 h. The bars represent the fraction of the cells distributed over each phase of the cell cycle and are expressed as mean ± standard deviation (N = 3). Asterisks indicate statistically significant differences from vehicle-treated control cells with * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, and **** P ≤ 0.0001.

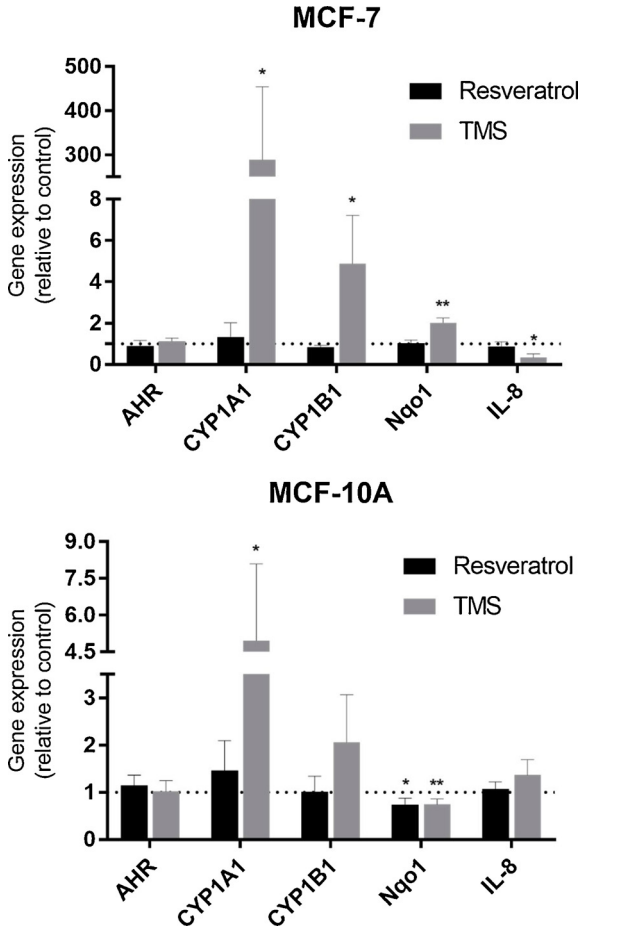


**Fig. 6.** Changes in gene expression of AHR, CYP1A1, CYP1B1, Nqo1 and IL-8 after a 48-h exposure in MCF-7 (upper panel) and MCF-10A cells (lower panel). Cells were exposed to resveratrol or TMS (3 μM in MCF-7 and 10 μM in MCF-10A). Data is expressed relative to DMSO treated controls that were set to 1 (represented by the dashed line) and expressed as mean and ± standard deviation with N = 3 for MCF-7 and N = 4 for MCF-10A. * Statistically significantly different from control treated cells (P ≤ 0.05) and ** significantly different from control (P ≤ 0.01).

CYP1B1 gene expression increased 2-fold after exposure to 10 μM TMS, yet this increase was not statistically significant. CYP1A1 and CYP1B1 gene expression in MCF-10A cells did not change upon exposure to 10 μM resveratrol. In contrast to MCF-7 cells, Nqo1 gene expression decreased 1.4-fold upon exposure to 10 μM resveratrol or TMS in MCF-10A cells. IL-8 gene expression increased 1.4-fold when MCF-10A cells were exposed to 10 μM TMS, although this increase was not statistically significant. Resveratrol did not affect IL-8 gene expression in MCF-10A cells. Similar to MCF-7 cells, AHR gene expression did not statistically significantly change by resveratrol or TMS exposure in MCF-10A cells (Fig. 6).

## 4. Discussion

In this study, the anti-tumor properties of resveratrol and several structural analogues on MCF-7 tumor cell growth and migration were assessed. The potency of these compounds to initiate anti-tumorigenic effects seemingly corresponds with their potency to activate the AHR. TMS was the most potent anti-tumorigenic resveratrol analogue. Noticeably, TMS caused a cell cycle arrest in the malignant MCF-7 cells, but not in non-tumorigenic MCF-10A breast epithelial cells. Although TMS activates the AHR in both MCF-7 and MCF-10A cells, this appears to result in cell-type specific actions possibly related to tumorigenic status.

### *4.1. Anti-tumor properties of resveratrol and its analogues*

Resveratrol and its analogues have previously been described to possess anti-tumor properties, such as inhibition of tumor cell viability, proliferation and migration. In present study, resveratrol did not affect tumor cell growth, not even at the highest exposure concentration of 30 μM. Earlier reports mention inhibitory effects by resveratrol on MCF-7 cell proliferation, yet higher exposure concentrations were used (up to 300 μM) (Joe et al., 2002; Mgbonyebi et al., 1998). The anti-tumor properties of the compounds tested appear to increase with the number of methoxy substitutions to the stilbene backbone of resveratrol. An additional hydroxy group in the stilbene backbone of resveratrol, like in oxyresveratrol, did not significantly affect tumor cell viability nor migration in our study. However, if the hydroxy group was replaced by a methoxy group on the stilbene backbone, an increase in anti-tumor properties, as measured by a viability and migration assay, was observed. Pinostilbene, pterostilbene and TMS with respectively one, two and four methoxy groups, all reduced tumor cell viability and migration in MCF-7 cells with TMS being the most potent compound. Others have also reported an inhibition of cell proliferation by methoxylated resveratrol derivatives, e.g. pterostilbene in MCF-7 cells (Daniel and Tollefsbol, 2018), trimethoxyl stilbene in a A549 lung cancer cells (Liu et al., 2011) and tetramethoxystilbene in MCF-7 cells (Androutsopoulos et al., 2011). The observed concentration at which TMS inhibited MCF-7 cell migration (3 μM) is in the same order of magnitude as the 50% decrease of MCF-7 cell viability (3.6 μM). In our experimental set-up, the 50% decrease in viability assay was obtained after 8 days of exposure to TMS, hence involving multiple cell cycles and cell divisions. The average doubling time of MCF-7 cells is 30–40 h in regular culture medium. Therefore, after a period of only 48 h, the effect of TMS on cell

proliferation in the migration assay in present study was considered to be relatively minor.

Analysis of the cell cycle distribution showed that resveratrol did not affect the cell cycle progression of malignant MCF-7 cells at a concentration of 10 μM, while the non-tumorigenic MCF-10A cells accumulated in the S-phase of the cell cycle at 30 μM resveratrol. This cell phase specific accumulation for resveratrol has been reported previously for MCF-7 and MCF-10A-Tr cells, at slightly higher or comparable concentrations as in our experiments (Mohapatra et al., 2014; Pozo-Guisado et al., 2002). Joe et al. also reported an S-phase arrest by resveratrol in MCF-7 cells at 300 μM as well as in the HCE7 human esophageal squamous carcinoma and HL60 human promyelocytic leukemia cell lines (Joe et al., 2002). The concentration resveratrol in present study was an order of magnitude lower than the concentration used by Joe et al. (2002), which may explain the absence of an S-phase arrest by resveratrol in the MCF-7 cells. TMS induced a shift from the G1 phase to the G2/M phase causing a cell cycle arrest in the MCF-7 cells, which was not observed for resveratrol. In line with our findings, other resveratrol analogues with four methoxy-groups (3,4,4',5-tetramethoxy-*trans*-stilbene) induced the same G2/M phase cycle arrest in a prostate cancer cell line (Horvath et al., 2007), ovarian cancer cell line (Piotrowska et al., 2012) and in MCF-7 and HepG2 cell lines at similar concentrations as our experiments (Androutsopoulos et al., 2011). In addition, we noticed a slight, yet not statistically significant, increase in cells in the sub-G1 phase upon TMS exposure. This suggests an increase in apoptotic cells, which corroborates with the observed decrease in cell viability by TMS in our study.

Sangjun et al. studied the effects of several methoxy additives to *cis*-stilbenes derivatives in MCF-7 cells and found that methoxy groups at the *para* position of the left aromatic ring to be a key position to induce cell cycle arrest (Sangjun et al., 2009). There was indeed a methoxy group positioned at that site of 3,4,4',5-TMS used by Horvath et al. (2007); Piotrowska et al. (2012) and Androutsopoulos et al. (2011) who observed a cell cycle arrest by TMS. However, while 2,3',4,5'-TMS in our study did induce a cell cycle arrest, it did not hold a methoxy group at this particular site. Possibly, that specific methoxy position may be of more importance in the *cis*-stilbenes than for the *trans*-stilbenes, as was used in this study.

Strikingly, TMS selectively induced a cell cycle arrest in the malignant MCF-7 cells and not in the non-tumorigenic MCF-10A cells, while resveratrol only induced an arrest in the MCF-10A cells and the positive control 2-MeOE2 induced a cell cycle arrest both cell lines. The endogenous estrogen metabolite 2-MeOE2 has been shown to cause a cell cycle arrest and induce apoptosis via inhibition of kinase pathways and Bcl2 downregulation (Lee et al., 2008). To the best of our knowledge, no other studies have described the effects of TMS on the cell cycle of MCF-10A cells. The differences in anti-migration and cell cycle arrest properties of TMS in the two cell lines may be explained by differences in AHR activation. Cell type specific differences may arise from different AHR ligand binding pockets, different recruitment of and/or cell-type specific expression of co-activators/repressors and different availability of ligand DNA recognition sites between the cell lines (Denison and Faber, 2017). The involvement of differential AHR activation between the cell lines is supported by clear different gene expression patterns in MCF-7 and MCF-10A cells that was inflicted by TMS exposure (further discussed below).

### 4.2. AHR activation by resveratrol and its analogues

Several studies have previously described that resveratrol can activate the AHR and induce CYP1A1 activity (Aggarwal et al., 2004; Beedanagari et al., 2009). In our study using the AHR reporter cell line HG2L7.5C1, resveratrol did not activate the AHR at concentrations up to 30 μM. However, adding methoxy groups to the stilbene backbone significantly increased the potency of the resveratrol analogues to activate the AHR with TMS being the most potent analogue followed by pterostilbene and oxyresveratrol. The increased AHR activity was corroborated with an increase in CYP1A1/1B1-mediated EROD activity in MCF-7 cells. Interestingly, while pinostilbene did induce AHR activity in the reporter gene assay, it could not be negated by adding the AHR antagonist CH-223191. In addition, pinostilbene did not induce EROD activity in MCF-7 cells. Possibly, pinostilbene binds to a different activation site of the AHR than the antagonist CH-223191 and the other resveratrol analogues tested. Another interesting finding was the fact that all resveratrol analogues caused a decrease in AHR activation as well as EROD activity at higher concentrations. It has been reported that resveratrol can inhibit CYP1A1 and CYP1B1 activity in human recombinant supersomes with a reported IC50 of 26 μM and 11 μM, respectively (Mikstacka et al., 2008). TMS exhibited stronger CYP1A1 and CYP1B1 inhibitory properties with previously reported IC50 values of 300 nM and 6 nM respectively, which also indicates that TMS exhibits a selective catalytic inhibition of CYP1B1 activity (Chun et al., 2001). This may explain the decrease in EROD activity at higher concentrations of the resveratrol analogues that we observed. In present study, CYP1A1 and CYP1B1 gene expression was induced by TMS in both MCF-7 and MCF-10A cell lines. CYP1A1 and CYP1B1 gene expression was more strongly induced in the MCF-7 cells compared to MCF-10A cells, which may relate to observations that the AHR is overexpressed in many tumor cells (Go et al., 2015; Safe et al., 2017). Piotrowska et al. (2012) studied the effect of 3,4,4',5-tetramethoxy stilbene in two ovarian cancer cell lines. Although they also observed an inhibition of proliferation and induction of cell cycle arrest by TMS, they found that TMS decreased CYP1A1 and CYP1B1 mRNA and protein expression after 24 h of exposure (Piotrowska et al., 2012). Possibly, this inhibitory effect is attributed to the different structural arrangement of the TMS that was used in that study. Additionally, the formation of a TMS metabolite is suggested to be responsible for the modulation of CYP1A1 and CYP1B1 expression (Piotrowska-Kempisty et al., 2017). Similar to our study, Einem Lindeman et al., 2011 also used 2,3',4,5'-tetramethoxystilbene and did not observe an inhibitory effect on CYP1A1 and CYP1B1 expression in MCF-7 cells (Einem Lindeman et al., 2011).

While AHR activation is often associated with dioxin-like toxicity, such as chloracne in humans, it should be recognized that the activation of the AHR and concurrent induction of CYP1A1 and CYP1B1 by itself do not necessarily indicate toxicity. Nowadays, the induction of CYP450 isoforms is usually considered as the earliest and most sensitive signal of AHR activation. A wide range of non-dioxin-like compounds, such as polycyclic aromatic hydrocarbons (PAHs) but also other natural compounds like indole-3-carbinol, are causing CYP1A1 and CYP1B1 induction without expressing the classical toxic endpoints associated with halogenated dioxins, dibenzofurans or PCBs. Investigating the effects on CYP1A1/1B1 gene expression and activity of potential anticancer agents are especially important with respect to the role of CYPs in formation of genotoxic estrogen metabolites. CYP1A1-mediated estrogen metabolism yields predominantly 2-hydroxylated estrogens, which are considered to protect against tumor formation. In contrast, CYP1B1 catalyzes estrogen 4-hydroxylation, which may lead to quinones that react with DNA and as a result may initiate cancer (Cavalieri and Rogan, 2011). Further studies should be performed to elucidate the effect of TMS on estrogen metabolism.

While gene expression of CYP1A1 and CYP1B1 was increased in both MCF-7 and MCF-10A cells, gene expression of Nqo1, a hallmark for Nrf2 pathway activation, was increased by TMS in MCF-7 cells but decreased in the MCF-10A cells. This indicates that TMS activates a pathway that is induced in response to cellular oxidative stress, but only in the malignant breast tumor cells. Resveratrol has also been reported to induce Nqo1 expression in the human K562 leukemia cell line (Hsieh et al., 2006). However, concentrations used in that study were 10-fold higher than in our experiments, which may explain the observed absence of Nqo1 induction by resveratrol in our study. Interesting is the finding of Lu et al. (Lu et al., 2008) who describe a slight increase in

Nqo1 protein expression and enzymatic capacity 48 h after exposure to resveratrol (50 μM) in MCF-10F cells, which are similar to MCF-10A regarding for example hormone receptor status (Subik et al., 2010). Our results show a decrease in Nqo1 gene expression after 48 h in MCF-10A. This does not exclude that resveratrol and TMS do not induce an oxidative stress response in these cells, as gene expression changes often do not corroborate with protein expression changes when determined at the same time point. An increase in Nqo1 capacity may protect the cell against formation of quinones and potential genotoxic events by estrogen metabolites.

Gene expression of IL-8 was reduced by TMS in MCF-7, but no clear effect on IL-8 gene expression was observed in the MCF-10A cells. Others have associated AHR activation with IL-8 downregulation which can be linked to cell cycle arrest via PTEN/Akt signaling pathways (Freund et al., 2004; Shen et al., 2003; Todorović-Raković and Milovanović, 2013). These studies are in line with our findings that TMS exposure reduces IL-8 gene expression and induces a cell cycle arrest in MCF-7 cells. The lack of IL-8 downregulation in MCF-10A cells may also explain the absence of a cell cycle arrest by TMS in that cell line.

## 5. Conclusions

Taken together, our data suggest that the methoxylated analogues of resveratrol hold more potent anti-tumor properties than resveratrol itself, which may be mediated by the AHR. TMS, but not resveratrol, selectively inhibited the cell cycle of the malignant MCF-7 cells, but not the non-tumorigenic MCF-10A cells. The methoxylated analogues of resveratrol could therefore be potentially more useful as anti-cancer agents than resveratrol itself. The findings from our study and others suggest that the anti-cancer properties of TMS may be dual: it may act as anti-tumor agent through inhibition of cell viability, migration and cell cycle arrest in tumor cells, but also as cancer chemo-preventive agent in non-malignant breast cells via inhibition of CYP1B1-mediated estrogen metabolism and possibly via Nqo1 and IL-8-mediated pathways.

## Conflict of interest

None of the authors have competing interests to declare. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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