

Research article

THE EFFECT OF RESVERATROL AND ITS METHYLTHIO-DERIVATIVES ON THE Nrf2-ARE PATHWAY IN MOUSE EPIDERMIS AND HaCaT KERATINOCYTES

VIOLETTA KRAJKA-KUŹNIAK¹, HANNA SZAEFER¹,
 TOMASZ STEFAŃSKI², STANISŁAW SOBIĄK², MICHAŁ CICHOCKI¹
 and WANDA BAER-DUBOWSKA^{1,*}

¹Department of Pharmaceutical Biochemistry, Poznań University of Medical Sciences, Poznań, Poland, ²Department of Chemical Technology of Drugs, Poznań University of Medical Sciences, Poznań, Poland

Abstract: Resveratrol is the most extensively studied stilbene derivative. We previously showed that methylthiostilbenes were more effective inhibitors of CYP1A1 and 1B1 activity than resveratrol. In this study, we investigated whether resveratrol and its methylthio-substituted derivatives, i.e. 3-M-4'-MTS (S2), 3,5-DM-4'-MTS (S5) and 3,4,5-TM-4'-MTS (S7) could activate Nrf2 signaling in the mouse epidermis and in human keratinocytes. Western blot analysis showed translocation of Nrf2 from the cytosol to the nucleus in both models. All of the tested stilbenes increased GST activity, but resveratrol was the most effective inducer. Moreover, only resveratrol increased the protein level of GSTP in the mouse epidermis. GSTM was enhanced in HaCaT cells after the

* Author for correspondence. Email: baerw@ump.edu.pl, phone: +48 61 8546621, fax: +48 61 8546620

Abbreviations used: AhR – aryl hydrocarbon receptor; ARE – antioxidant response element; CDNB – 1-chloro-2,4-dinitrobenzene; CYPs – cytochromes P450; DMEM – Dulbecco's modified Eagle's medium; DMF – dimethylformamide; DMSO – dimethyl sulfoxide; DTT – dithiothreitol; FBS – fetal bovine serum; GSH – glutathione; GST – glutathione-S-transferase; GSTA – glutathione-S-transferase A; GSTM – glutathione-S-transferase M; GSTP – glutathione-S-transferase P; HaCaT – spontaneously immortalized human keratinocyte cell line; Keap1 – Kelch-like ECH-associated protein 1; MTT – 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; Nrf2 – nuclear factor erythroid 2-related factor 2; NQO – NAD(P)H: quinone oxidoreductase; PAHs – polycyclic aromatic hydrocarbons; Res – resveratrol; S2 – 3-methoxy-4'-methylthio-*trans*-stilbene (3-M-4'-MTS); S5 – 3,5-dimethoxy-4'-methylthio-*trans*-stilbene (3,5-DM-4'-MTS); S7 – 3,4,5-trimethoxy-4'-methylthio-*trans*-stilbene (3,4,5-TM-4'-MTS)

treatment with derivatives S2 and S5. The same effect was observed for GSTP in the case of compound S2. Resveratrol and its derivatives reduced the NQO2 protein level in HaCaT cells. Thus, it is possible that increased expression of GSTP or GSTM and GST activity was linked with NQO2 inhibition in these cells. The results of this study indicate that resveratrol and its methylthio-derivatives activate Nrf2 not only in the mouse epidermis, but also in human keratinocytes. Upregulating GST isozymes might be particularly important for deactivating chemical carcinogens, such as PAH.

Keywords: Nrf2, GST, NQO, Resveratrol, Methylthiostilbenes, HaCaT cells, Mouse epidermis

INTRODUCTION

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), a naturally occurring phytoalexin, is the most extensively studied stilbene derivative [1]. This compound has been shown to have several beneficial effects, including cancer chemopreventive activity. The latter was described for the first time in the pioneering work of the Pezzuto group, who used a two-stage mouse skin carcinogenesis model [2].

In this model, as in many others, the induction of carcinogenesis involved at least two well-defined steps: initiation and promotion [3]. During the initiation stage, which leads to DNA modifications, carcinogens usually undergo a phase I biotransformation, principally by cytochromes P450 (CYPs) to generate reactive intermediates that attack DNA and ultimately induce mutation in critical genes [4]. The elimination of reactive species, including electrophiles and reactive oxygen species, before they attack DNA is carried out by phase II detoxifying and antioxidant enzymes, such as glutathione-S-transferases (GST) and NAD(P)H:quinone oxidoreductases (NQO). NQOs are cytosolic enzymes that catalyze the metabolic reduction of quinones and their derivatives [5]. Therefore, inducers of cytoprotective proteins have been proposed as candidates for chemoprevention [6].

Several studies, including ours, showed that resveratrol is able to inhibit CYP1A1, 1A2, 1B1 and 2E1, which are responsible for the activation of most chemical carcinogens [7, 8]. Moreover, our studies showed that resveratrol, besides inhibiting CYPs, induced phase II enzymes in mouse skin treated with an initiating dose of benzo[a]pyrene or 7,12-dimethylbenz[a]anthracene [9, 10]. These polycyclic aromatic hydrocarbons (PAHs) are effective tumor initiators and complete carcinogens in the animal models. In order to exert their mutagenic and carcinogenic activity, PAHs require metabolic activation through the action of CYPs to yield chemically reactive species [11].

The key regulator of the inducible expression of phase II enzymes is transcription factor E2-related factor 2 (Nrf2) [12]. Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1). It undergoes proteasomal degradation following ubiquitination [13, 14]. Under oxidative or

electrophilic stress conditions, Keap1 cannot sequester and degrade Nrf2, but rather allows for a nuclear translocation of Nrf2 [15, 16]. In the nucleus, Nrf2 binds to specific DNA sequences known as antioxidant response elements (ARE), which are located in the promoter region of a variety of phase II detoxification/antioxidant enzymes [17]. The importance of phase II detoxifying enzymes for inactivating chemical carcinogens was highlighted in a study with Nrf2-deficient mice. These mice were found to be more susceptible to experimental carcinogenesis [18].

There is evidence that modification of the stilbene ring improves the efficacy of resveratrol and may overcome its limitations, such as poor bioavailability [19]. In this regard, our earlier studies showed that introducing the methylthio-group into the stilbene core may influence the efficacy and selectivity of the inhibitory potency of these compounds toward the P450 isozymes CYP1A1 and 1B1 [20]. Moreover, substitution of the 4' oxygen atom with a less electronegative sulfur atom reduced toxicity to HEK 293 cells and enhanced the ability to activate human SIRT1 [21]. Spontaneously immortalized through multiple passages, HaCaT cells are considered a suitable model of human skin keratinocytes, particularly to investigate xenobiotic metabolism pathways [22].

In this study, we investigated whether resveratrol and its three 4'-methylthio-*trans*-stilbene derivatives, which possess one (3-M-4'-MTS; S2), two (3,5-DM-4'-MTS; S5) or three (3,4,5-TM-4'-MTS; S7) additional methoxy groups, could activate Nrf2 signaling and its target proteins GSTA, GSTP, GSTM, GSTT and NQO2 in the mouse epidermis *in vivo* and in human keratinocytes *in culture*.

MATERIALS AND METHODS

Chemicals

Resveratrol (purity 99%), dithiothreitol (DTT), antibiotics solution (10^4 U penicillin, 10 mg streptomycin, 25 μ g amphotericin B), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB) and Tris were obtained from Sigma Chemicals. The spontaneously immortalized human keratinocyte cell line HaCaT was purchased from Cell Lines Service. Primary and secondary antibodies against GSTA, GSTM, GSTP, GSTT, NQO2, Nrf2, Keap1 and β -actin were obtained from Santa Cruz Biotechnology. Phospho-Nrf2 (Ser 40) was obtained from Abcam. Rainbow-colored protein molecular weight marker was purchased from Amersham Pharmacia Biotechnology. 4-methylthiobenzyl alcohol, thionyl chloride, triethyl phosphite, 3-methoxybenzaldehyde, 3,5-dimethoxybenzaldehyde, 3,4,5-trimethoxybenzaldehyde and sodium hydride (60% dispersion in mineral oil) were purchased from Aldrich Chemical Company. Anhydrous toluene and dimethylformamide (DMF) used in synthesis were obtained from Merck. All the other chemicals were commercial products of the highest purity available.

Synthesis of methylthio-stilbenes

Fig. 1 outlines the synthesis of 3-methoxy-4'-methylthio-*trans*-stilbene (S2), 3,5-dimethoxy-4'-methylthio-*trans*-stilbene (S5) and 3,4,5-trimethoxy-4'-methylthio-*trans*-stilbene (S7). The key step in stilbenoid synthesis is the generation of phosphonate ester (compound 2) diethyl 4-(methylthiobenzyl) phosphonate as an intermediate. This was prepared from commercially available 4-methylthiobenzyl alcohol in two steps. First, 4-methylthiobenzyl alcohol was converted to 4-methylthiobenzyl chloride (compound 1) using SOCl_2 in toluene at room temperature. Then, through the Michaelis-Arbuzov reaction [23, 24] of compound 1 with triethyl phosphite at 130°C , the corresponding phosphonate ester was obtained. Methylthio-*trans*-stilbene analogs were prepared by the Wittig-Horner (Horner-Wadsworth-Emmons) [24, 25] reaction of compound 2 with commercially available benzaldehydes in DMF using sodium hydride as a base. Under these reaction conditions only *trans*-isomers were obtained.

The geometries of these compounds were confirmed by their characteristic $^1\text{H-NMR}$ coupling constants for olefinic protons at about 16–16.5 Hz. Intermediates were characterized by NMR spectroscopy. The structure and purity of the final products were confirmed by NMR low- and high-resolution mass spectrometry (LRMS, HRMS). The high purity of the obtained methylthio-stilbenes enabled us to perform single-crystal X-ray structure analyses, which were in full accordance with the depicted structures.

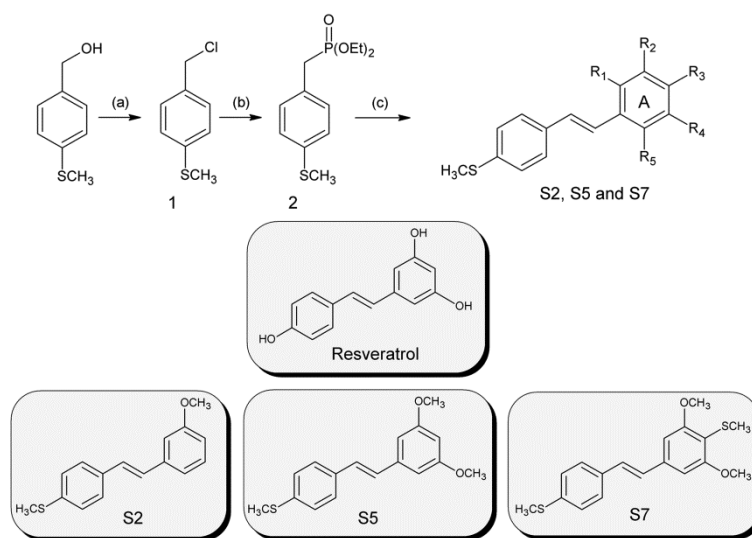


Fig. 1. General route for the synthesis of the methylthio-*trans*-stilbene analogs (S2, S5 and S7). Reagents and conditions: (a) SOCl_2 , toluene, room temp, 0.5 h, 84%; (b) $\text{P}(\text{OEt})_3$, 130°C , 2 h, 80%; (c) (R1-R5) PhCHO , NaH , DMF, 0°C to room temp, 2 h, 60-75%. 1: 4-methylthiobenzyl chloride; 2: diethyl 4-(methylthiobenzyl)phosphonate; S2: 3-methoxy-4'-methylthio-*trans*-stilbene; S5: 3,5-dimethoxy-4'-methylthio-*trans*-stilbene; S7: 3,4,5-trimethoxy-4'-methylthio-*trans*-stilbene.

Animals and treatment

Female CD-1 mice, aged 7–9 weeks old (18–20g) were provided by Charles River Laboratories International, Inc. They were housed in polycarbonate cages containing hardwood chip bedding. A standard pellet diet and distilled water were available without restriction. The mice were randomly divided into five experimental groups of three mice each. They were shaved on their dorsal side using surgical clippers 2 days before treatment. The mice were treated topically with resveratrol or the methylthio-derivatives (S2, S5 and S7) in doses of 16 μmol in 0.2 ml of acetone per mouse. The dosage of resveratrol was established on the basis of our previous studies [9]. A control group of mice was treated with acetone alone. The mice were killed 4 h after treatment with the tested compounds. The dorsal skin was excised, washed in ice-cold PBS, placed on a glass plate and cooled with ice. The epidermis was scraped into ice-chilled tubes filled with a buffer appropriate for each assay. All of the experiments were conducted according to European guidelines on the care and use of laboratory animals and were approved by a Regional Ethics Committee (No. 33/2007). All efforts were made to minimize animal pain and suffering.

Cell culture and treatment

The spontaneously immortalized human keratinocyte cell line HaCaT was grown in DMEM containing 10% FBS supplemented with antibiotics (penicillin, streptomycin and amphotericin B). The cells were incubated at 37°C in an atmosphere consisting of 95% air and 5% CO₂ in a humidified incubator until 80% confluence. One million cells were seeded in 100 mm diameter culture dishes. After 24 h, the cells were treated with either 20 or 60 μM resveratrol, 20 or 60 μM S2 derivative, 5 or 20 μM S5 compound, or 0.1% DMSO (control cells). The applied compounds were solved in DMSO. Incubation was continued for a subsequent 24 h, then the cells were harvested. The applied concentrations of the compounds were established on the basis of a cell viability assay called the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide cleavage assay (MTT test) [26]. The S7 derivative was excluded from the cell culture experiment because of its high cytotoxicity (9.87 μM).

Preparation of nuclear and cytosolic extracts

The nuclear and cytosolic extracts were prepared using a Nuclear/Cytosol Fractionation Kit (BioVision Research) according to manufacturer's instructions. Briefly, HaCaT cells or the scraped epidermis were collected by centrifugation at 600 g for 5 min at 4°C. Pellets were resuspended in ice-cold cytosol extraction buffer containing DTT and protease inhibitors. After incubation in an ice bath for 10 min, the samples were centrifuged for 5 min at 16000 g at 4°C to collect the cytosolic fraction. The supernatants (cytosolic fractions) were transferred to clean tubes. The pellets were resuspended in ice-cold nuclear extraction buffer containing DTT and protease inhibitors and incubated again in an ice bath for 40 min with vortex mixing for 15 sec every

10 min. The lysed suspensions of nuclei were then centrifuged at 16000 g at 4°C for 10 min, and the collected nuclear extracts were stored at -70°C.

Western blot analysis

We used the immunoblot assay to determine the levels of GSTA, GSTP, GSTM, GSTT, NQO2, Nrf2, p-Nrf2 (Ser 40) and Keap1 proteins [27, 28]. The protein content in the samples was determined using the Lowry method [29]. All of the experiments were repeated three times. Nuclear extracts (Nrf2, p-Nrf2 Ser40) or cytosolic proteins (GSTA, GSTP, GSTM, GSTT, NQO2, Nrf2, Keap1; 100 µg of protein) were resolved on 12% or 10% SDS-PAGE slab gels, depending on the molecular weight. Subsequently separated proteins were transferred to nitrocellulose membranes. After blocking with 10% skimmed milk, the proteins were probed with rabbit anti-human GSTA, goat anti-rat GSTM, rabbit anti-human GSTP, human anti-mouse GSTT, goat polyclonal NQO2, goat anti-rat Keap1, rabbit anti-mouse Nrf2, rabbit monoclonal p-Nrf2 and rabbit anti-human β-actin antibodies. The β-actin protein was used as an internal control. Alkaline phosphatase-labeled anti-goat IgG, anti-human IgG or anti-rabbit IgG were used as the secondary antibodies in the staining reaction. Bands were visualized with a BioRad AP Conjugate Substrate Kit NBT/BCIP. The amount of the immunoreactive product in each lane was determined using the Quantity One software (BioRad Laboratories). Values were calculated as relative absorbance units (RQ) per mg of protein.

GST activity assay

The activity of GST was measured using the method of Habig et al. [30] with CDNB as the substrate. The assay mixture contained a cytosol fraction (0.3 mg protein), 1 mM GSH, 1 mM CDNB, and 0.2 mM phosphate buffer (pH 6.5). The reaction was monitored for 1 min at 30°C in a Hitachi spectrophotometer at 340 nm. The results were calculated using a molar absorption coefficient of $9.6 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. The specific activity of GST was expressed as nanomoles of the GSH-CDNB conjugate formed per milligram of protein per minute.

Statistical analysis

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

RESULTS

Mouse epidermis

The effect of resveratrol and methylthio-stilbenes on Nrf2 activation

To investigate the translocation of Nrf2 due to resveratrol and methylthio-stilbene treatment, the level of Nrf2 in the cytoplasm and nuclear fractions was evaluated by western blot analysis. As is shown in Fig. 2A, resveratrol at a dose of 16 µmol increased the nuclear level of Nrf2 in the mouse epidermis in

comparison with the result from the control group. An enhanced nuclear/cytosol Nrf2 ratio was also observed after treatment with compounds S5 and S7, although at a lower level than for resveratrol (1.63 vs 1.28 and 1.25, respectively; Fig. 2B). Phosphorylation of Nrf2 at Ser40 was detected as a result of treatment with all of the tested compounds, but its level did not differ significantly from that found in the control group of animals (Fig. 2C and D). Since the activation of Nrf2 is generally accompanied by degradation of Keap1, we also measured the amount of the Keap1 cytosolic protein. The amount of the Keap1 protein was not significantly changed by either the methylthio-stilbenes or resveratrol (Fig. 2E and F).

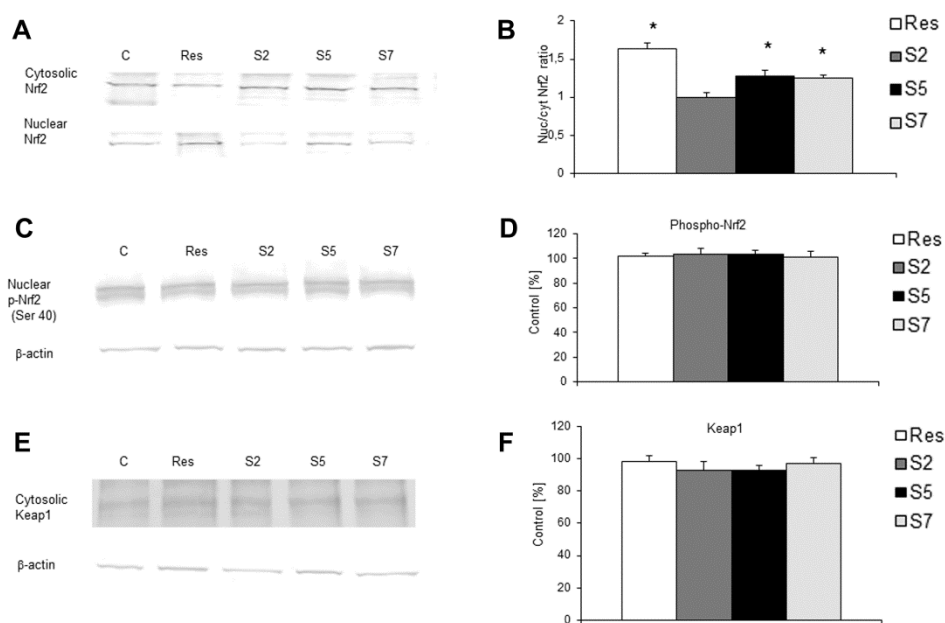


Fig. 2. The effect of resveratrol and methylthio-derivatives on Nrf2 activation in the mouse epidermis. A – Representative immunoblots for the analysis of the cytosolic and nuclear level of the Nrf2 protein. B – The ratio of the content of Nrf2 in the nuclear and cytosolic fractions relative to the control level, which equals 1. C – Representative immunoblots for the analysis of the nuclear level of the p-Nrf2 protein. D – Results of western blot analysis of the nuclear content of the p-Nrf2 protein compared with the control level, which equals 100%). E – Representative immunoblots for the analysis of the cytosolic level of Keap1 protein. F – Results of western blot analysis of the cytosolic content of the Keap1 protein compared with the control level, which equals 100%. The data are the means \pm SEM from three separate experiments. The asterisk (*) above the bar denotes statistically significant differences from the control group, $p < 0.05$. C: vehicle control; Res: resveratrol; S2: 3-methoxy-4'-methylthio-*trans*-stilbene, S5: 3,5-dimethoxy-4'-methylthio-*trans*-stilbene; S7: 3,4,5-trimethoxy-4'-methylthio-*trans*-stilbene.

The effect of resveratrol and methylthio-stilbenes on the activity of GST

Data illustrating the influence of resveratrol and methylthio-stilbenes on the activity of GST in the mouse epidermis are presented in Table 1. All of the tested compounds significantly enhanced the activity of GST (by about 28–63%) in comparison with the value obtained for the control group. The most effective inducer of GST activity was resveratrol.

Table 1. The enzymatic activity of GST in mouse epidermis treated with resveratrol (Res), methylthio-stilbenes (S2, S5, S7) or acetone (vehicle control).

Compounds	Dose ($\mu\text{mol}/\text{mouse}$)	GST	
		nmol/min/mg protein	% of control
Control	0.2 ml of acetone	33.43 ± 3.00^a	100
Res	16	54.46 ± 6.17^b	163 ^b
S2	16	52.33 ± 5.18^b	157 ^b
S5	16	51.03 ± 6.20^b	153 ^b
S7	16	42.81 ± 2.98^b	128 ^b

^aValues are the means \pm SEM of three separate experiments run in triplicate. ^bSignificantly different from the control value, $p < 0.05$

The effect of resveratrol and methylthio-stilbenes on the protein levels of GSTA, GSTM, GSTP, GSTT and NQO2

The levels of the GSTA, GSTP, GSTM, GSTT and NQO2 proteins in the mouse epidermis were evaluated using the western blot assay with specific antibodies against these proteins (Fig. 3A). The cytosolic content of GSTP (Fig. 3B) increased by about 22% after treatment with 16 μmol resveratrol. By contrast, no significant increase in the GSTM, GSTA, GSTT and NQO2 protein levels was found in the mouse epidermis as a result of treatment with stilbenes (Fig. 3B).

HaCaT cells***The effect of resveratrol and methylthio-stilbenes on Nrf2 activation***

Just as in the mouse epidermis, the translocation of Nrf2 after treatment with methylthio-stilbenes or resveratrol was found in human keratinocytes (Fig. 4A). The Nrf2 protein was accumulated and phosphorylated at Ser 40 in the nucleus after exposure to 60 μM of resveratrol, 20 μM and 60 μM of S2 and 5 μM and 20 μM of S5. Simultaneously, a decrease in its level in the cytosolic fractions in HaCaT cells was observed (Fig. 4A–D). The enhanced Nrf2 content confirmed that the nuclear/cytosol ratio significantly increased as a result of HaCaT cell treatment with stilbenes (Fig. 4B). Similarly to mouse epidermis, the level of Keap1 protein was not significantly changed by any of the tested compounds (Fig. 4E and F).

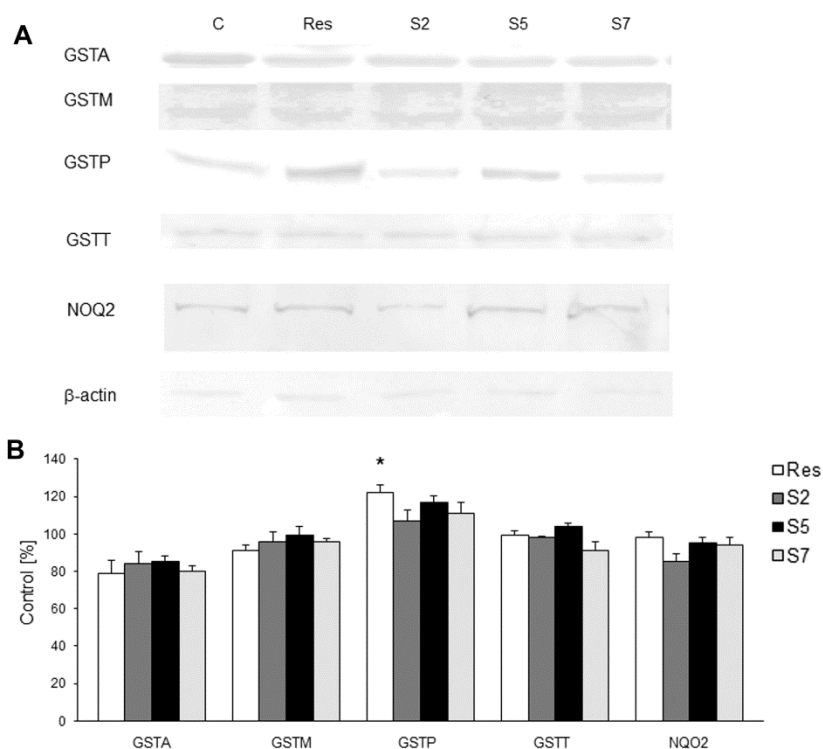


Fig. 3. The effect of resveratrol and methylthio-derivatives on the expression of Nrf2 target genes at the protein level in the mouse epidermis. A – Representative immunoblots for the analysis of GSTA, GSTM, GSTP, GSTT and NQO2. B – The results of western blot analysis of the content of GSTA, GSTM, GSTP, GSTT and NQO2 proteins compared with the control level, which equals 100%. The data are the means \pm SEM from three separate experiments. The asterisk (*) above the bar denotes statistically significant differences from the control group, $p < 0.05$.

Table 2. Enzymatic activity of GST in HaCaT cells treated with resveratrol (Res), methylthio-stilbenes (S2, S5) or DMSO (vehicle control).

Compounds	Concentration (μ M)	GST	
		nmol/min/mg protein	% of control
Control	0.1% of DMSO	151.96 \pm 9.21 ^a	100
Res	20	182.35 \pm 8.23 ^b	120 ^b
Res	60	197.72 \pm 10.34 ^b	130 ^b
S2	20	195.89 \pm 9.73 ^b	129 ^b
S2	60	185.91 \pm 8.11 ^b	122 ^b
S5	5	186.21 \pm 6.74 ^b	123 ^b
S5	20	213.91 \pm 10.45 ^b	141 ^b

^aValues are the means \pm SEM of three separate experiments run in triplicate. ^bSignificantly different from the control, $p < 0.05$.

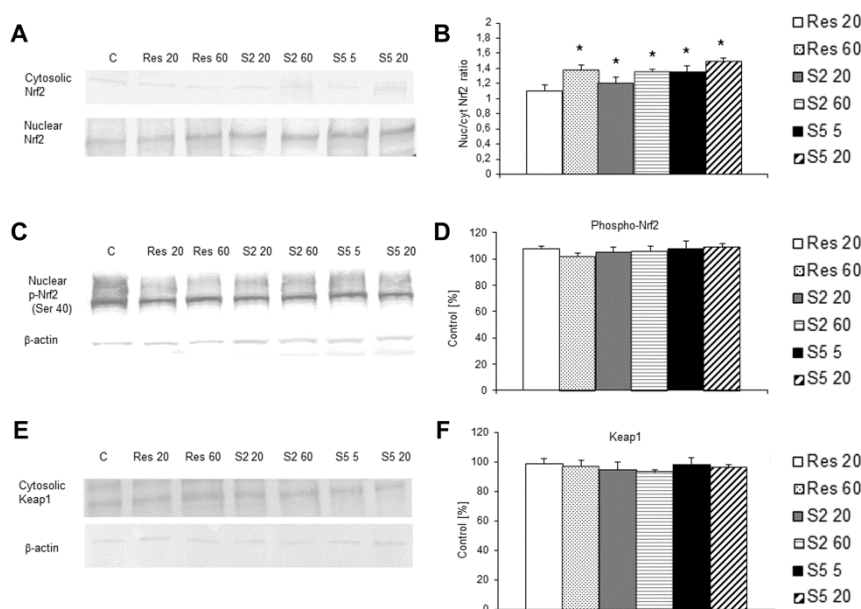


Fig. 4. The effect of resveratrol and methylthio-derivatives on Nrf2 activation in HaCaT cells. A – Representative immunoblots for the analysis of the cytosolic and nuclear level of the Nrf2 protein. B – The ratio of the content of Nrf2 in the nuclear and cytosolic fractions is relative to the control level, which equals 1. C – Representative immunoblots for the analysis of the nuclear level of the p-Nrf2 protein. D – Results of western blot analysis of the nuclear content of the p-Nrf2 protein compared with the control level, which equals 100%. E – Representative immunoblots for the analysis of the cytosolic level of the Keap1 protein. F – Results of western blot analysis of the cytosolic content of the Keap1 protein compared with the control level, which equals 100%. The data are the means \pm SEM from three separate experiments. The asterisk (*) above the bar denotes statistically significant differences from the control group, $p < 0.05$. C: vehicle control; Res: resveratrol (20 μ M and 60 μ M); S2: 3-methoxy-4'-methylthio-*trans*-stilbene (20 μ M and 60 μ M); S5: 3,5-dimethoxy-4'-methylthio-*trans*-stilbene (5 μ M and 20 μ M).

The effect of resveratrol and methylthio-stilbenes on the activity of GST

Data illustrating the effects of methylthio-stilbenes and resveratrol on GST activity in HaCaT cells are presented in Table 2. Treating the cells with both doses of resveratrol increased the activity of GST by about 20–30% in comparison with results obtained in the control cells. A similar effect was observed after incubation with both methylthio-stilbenes. These compounds enhanced the activity of GST by about 22–41%.

The effect of resveratrol and methylthio-stilbenes on the protein level of GSTA, GSTM, GSTP, GSTT and NQO2

Fig. 5A and B present the levels of GSTs and NQO2 proteins in HaCaT cells. The results of the western blot assay showed that the GSTM level increased by

about 18–28% after treatment with both methylthio-stilbenes. The same effect was observed for GSTP for compound S2. No significant effects of stilbene on GSTA and GSTT were observed. Resveratrol did not change the protein level of any of these GST isoforms. By contrast, the NQO2 level was significantly decreased in the HaCaT cells as a result of resveratrol and S5 compound treatment (Fig. 5B).

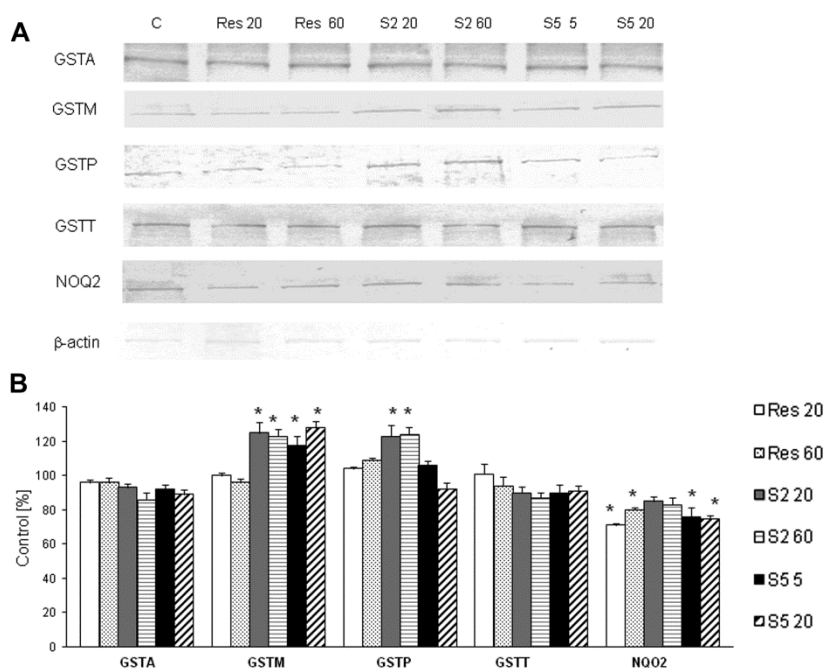


Fig. 5. The effect of resveratrol and methylthio-derivatives on the expression of Nrf2 target genes at the protein level in HaCaT cells. A – Representative immunoblots for the analysis of GSTA, GSTM, GSTP, GSTT and NQO2. B – Results of western blot analysis of the content of GSTA, GSTM, GSTP, GSTT and NQO2 proteins compared with the control level, which equals 100%. The data are the means \pm SEM from three separate experiments. The asterisk (*) above the bar denotes statistically significant differences from the control group, $p < 0.05$.

DISCUSSION

Inducing enzymes that enhance the detoxification of chemical carcinogens has been found to be a broadly effective strategy for chemoprevention in experimental carcinogenesis with rodent models and in some clinical intervention studies. Since the mid-1990s, it has been well accepted that the Nrf2 transcription factor plays a key role in the regulation of the inducible expression of enzymes such as GST and NQO. These catalyze the detoxification of reactive electrophiles and oxidants that contribute to the formation of mutations and ultimately cancers [31].

Derivatives of stilbene, such as resveratrol, are thought to affect the initiation of tumorigenesis principally through their interaction with the Ah receptor, and thus through inhibition of CYPs, which are involved in carcinogen activation [7]. Data on their effect on phase II enzymes are conflicting. For example, in mice treated with resveratrol, GST significantly decreased, particularly in the lung, after a single administration of 25 mg/kg body weight. An adverse response was observed for UDP-glucuronosyltransferase, where significant induction was observed (approximately 83%) in the liver, but a significant reduction was found in the lung (up to approximately 83% loss) following treatment with 25 mg/kg body weight for seven days [32].

In this study, we compared the effect of resveratrol and methylthio-stilbenes on Nrf2 activation in mouse epidermis and human HaCaT cells. Western blot analysis revealed the translocation of Nrf2 from the cytosol to the nucleus as an effect of stilbene treatment in both models. In the mouse epidermis, the most effective inducer of Nrf2 translocation was the parent compound, indicating that the number of methoxy-groups and the substitution of the 4' oxygen atom in the stilbene molecule does not affect Nrf2 activation/translocation. Since the regulation of Nrf2 function relies on changes in subcellular distribution rather than induction of this transcription factor through *de novo* synthesis [33], the results of our study indicate that the stilbene derivatives, particularly resveratrol, may react with redox reactive cysteines in Keap1 and disrupt the interaction between Nrf2 and Keap1, allowing its translocation, as was suggested by Dinkova-Kostova [34]. Such a mechanism further supports the unchanged Keap1 protein level and induction of GST expression and activity observed in the mouse epidermis. However, further studies are required to confirm this mechanism and to identify the cysteine residue(s) of Keap1 that are directly or indirectly modified by stilbenes.

While all of the tested stilbenes increased GST activity, resveratrol was the most effective inducer. Moreover, only resveratrol increased the GSTP protein level. Thus, increased expression of this isoform may be responsible for the enhanced activity of GST in the mouse epidermis as a result of resveratrol treatment. The other GST isozymes evaluated in this study were not affected in the mouse epidermis. This observation is consistent with that found in a human intervention study where an induction of the GST-pi level by resveratrol was found [35]. Moreover, GSTP1 was detected as a new resveratrol-targeting protein in cultured prostate cancer cells [36]. This isoform is involved in the detoxification of reactive polycyclic aromatic hydrocarbon metabolites, and thus its induction may protect against carcinogen initiation by these compounds.

The lack of effect of the other stilbenes on the GST isoforms may suggest that either the other untested isoforms were induced or methylthio-substitution led to direct interaction with GST protein to affect the enzyme activity in this way. It was suggested that allosteric regulation of GST is possible since a binding site distinct from the catalytic center was found on a GSTP molecule [37].

Moreover, in contrast to the mouse epidermis, in human HaCaT keratinocytes, resveratrol did not affect the expression of any of the investigated GST isoforms, while GSTP expression was increased by S2 and GSTM expression by both S2 and S5. S5 was also the most potent inducer of GST activity. GSTM1 shows catalytic activity toward phospholipid hydroperoxides, so the induction of this isoform may protect against DNA damage from lipid peroxides formed endogenously as a result of oxidative stress [38]. Spontaneously immortalized keratinocytes, HaCaT cells differ in the expression level of several proteins, including GST. Zhang et al. [39] reported that normal human keratinocytes and melanocytes expressed GST activity for which GSTP1-1 was mainly responsible. However, they did not observe the increase in GSTP1-1 expression in transformed keratinocytes (HaCaT) after treatment with inducers of the antioxidant response element (ARE).

Thus, the induction of GSTM by methylthio-derivatives of resveratrol in these cells is an interesting observation. Further studies are required to explain the mechanism of stilbene derivative interaction with GST in both systems. Collectively, these results indicate that an alteration in the GST profile and activity depends not only on the resveratrol dose, duration and means of administration as suggested by Canistro et al. [32], but also on the experimental system.

Cytosolic NQOs have two isoforms, NQO1 and NQO2, which were purified and cloned from mouse and human liver [40]. NQO2 is the only enzyme known to accept electrons from dihydronicotinamide riboside (NRH). Experiments with NQO2^{-/-} mice provided evidence that NQO2 protects against chemical carcinogenesis. However, protection against promotion rather than initiation of carcinogenesis was suggested [5].

Some studies also showed that the inhibition of NQO2 increased the concentration of endogenous electrophiles and consequently upregulated the expression of some antioxidant enzymes [41]. In our study, resveratrol and its methylthio-derivatives did not affect the NQO2 protein level in the mouse epidermis. However, in HaCaT cells, reduced expression was found as a result of treatment with all of the stilbenes, although a statistically significant reduction was found only in the case of resveratrol and compound S5. Thus, we can speculate that increased GSTP or GSTM expression and GST activity may be linked with NQO2 inhibition in HaCaT cells.

NQO2 possesses a unique active-site cleft that can accommodate a *trans*-stilbene. Such a mechanism was proposed for some dietary polyphenols, including flavonoids and chalcone derivatives [42]. The results of our study indicate that methylthio-stilbenes also could bind to the same site in NQO2. Thus, the results of our study confirm an earlier suggestion [43] that NQO2 may be a common target of the chemopreventive action of not only naturally occurring but also synthetic polyphenols.

This study showed that resveratrol and its methylthio-derivatives may activate the Nrf2 transcription factor in the mouse epidermis and in human keratinocytes.

It may also upregulate GST isozymes involved in carcinogen deactivation. Moreover, these compounds may inhibit NQO2. The overall effect is moderate and suggests that resveratrol and its synthetic methylthio-substituted derivatives may interfere with carcinogenesis, mostly through inhibition of phase I enzymes, namely CYP1A1/1A2 and 1B1, as was shown earlier [20, 21]. However, the activity of methylthio-stilbenes toward cytochromes P450 has to be confirmed in vivo.

Declaration of interest. The authors report no declarations of interests.

Acknowledgements. This study was supported by the Ministry of Science and Higher Education of Poland, grant N N 405 209737.

REFERENCES

1. Gescher, A., Steward, W.P., Brown, K. Resveratrol in the management of human cancer: how strong is the clinical evidence? *Ann. NY Acad. Sci.* **1290** (2013) 12–20.
2. Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W., Fong, H.H., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G., Moon, R.C. and Pezzuto, J.M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275** (1997) 218–220.
3. DiGiovanni, J. Multistage carcinogenesis in mouse skin. *Pharmacol. Ther.* **54** (1992) 63–128.
4. Anzenbacher, P. and Anzenbacherová, E. Cytochromes P450 and metabolism of xenobiotics. *Cell. Mol. Life Sci.* **58** (2001) 737–747.
5. Iskander, K., Paquet, M., Brayton, C. and Jaiswal, A.K. Deficiency of NRH:quinone oxidoreductase 2 increases susceptibility to 7,12-dimethylbenz(a)anthracene and benzo(a)pyrene-induced skin carcinogenesis. *Cancer Res.* **64** (2004) 5925–5928.
6. Dinkova-Kostova, A.T. and Talalay, P. Direct and indirect antioxidant properties of inducers of cytoprotective proteins. *Mol. Nutr. Food Res.* **52** (2008) S128–138.
7. Baer-Dubowska, W. and Szaefer, H. Modulation of carcinogen-metabolizing cytochromes P450 by phytochemicals in humans. *Expert Opin. Drug Metab. Toxicol.* **9** (2013) 927–941.
8. Mikstacka, R., Gnojowski, J. and Baer-Dubowska W. Effect of natural phenols on the catalytic activity of cytochrome P450 2E1. *Acta Biochim. Pol.* **49** (2002) 917–925.
9. Szaefer, H., Cichocki, M., Brauze, D. and Baer-Dubowska W. Alteration in phase I and II enzyme activities and polycyclic aromatic hydrocarbons-DNA adduct formation by plant phenolics in mouse epidermis. *Nutr. Cancer* **48** (2004) 70–77.
10. Szaefer, H., Krajka-Kuźniak, V. and Baer-Dubowska, W. The effect of initiating doses of benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene on

- the expression of PAH activating enzymes and its modulation by plant phenols. **Toxicology** 251 (2008) 28–34.
11. Shimada T., Sugie, A., Yamada, H., Kawazoe, H., Hashimoto, M., Azuma, E., Nakajima, T., Inoue, K., Oda, Y. Dose – response studies on the induction of liver cytochrome P4501A1 and 1B1 by polycyclic aromatic hydrocarbons in arylhydrocarbon-responsive C57BL/6J mice. **Xenobiotica** 33 (2003) 957–971.
 12. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D. and Yamamoto, M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. **Genes Dev.** 13 (1999) 76–86.
 13. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'Connor, T. and Yamamoto, M. Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. **Genes Cells** 8 (2003) 379–391.
 14. McMahon, M., Itoh, K., Yamamoto, M. and Hayes, J.D. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. **J. Biol. Chem.** 28 (2003) 21592–21600.
 15. Itoh, K., Tong, K.I. and Yamamoto, M. Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. **Free Radic. Biol. Med.** 36 (2004) 1208–1213.
 16. Nguyen, T., Yang, C.S. and Pickett, C.B. The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. **Free Radic. Biol. Med.** 37 (2004) 433–441.
 17. Surh, YJ. Cancer chemoprevention with dietary phytochemicals. **Nat. Rev. Cancer** 3 (2003) 768–780.
 18. Ramos-Gomez, M., Kwak, M.K., Dolan, P.M., Itoh, K., Yamamoto, M., Talalay, P. and Kensler, T.W. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. **Proc. Natl. Acad. Sci. USA** 98 (2001) 3410–3415.
 19. Walle, T., Hsieh, F., DeLegge, M.H., Oatis, J.E.J., Walle, U.K. High absorption but very low bioavailability of oral resveratrol in humans. **Drug Metab. Dispos.** 32 (2004) 1377–1382.
 20. Mikstacka, R., Baer-Dubowska, W., Wieczorek, M. and Sobiak, S. Thiomethylstilbenes as inhibitors of CYP1A1, CYP1A2 and CYP1B1 activities. **Mol. Nutr. Food Res.** 52 (2008) S77–83.
 21. Mikstacka, R., Rimando, A.M., Dutkiewicz, Z., Stefański, T. and Sobiak, S. Design, synthesis and evaluation of the inhibitory selectivity of novel trans-resveratrol analogues on human recombinant CYP1A1, CYP1A2 and CYP1B1. **Bioorg. Med. Chem.** 20 (2012) 5117–5126.
 22. van Eijl, S., Zhu, Z., Cupitt, J., Gierula, M., Götz, C., Fritsche, E. and Edwards, R.J. Elucidation of xenobiotic metabolism pathways in human skin and human skin models by proteomic profiling. **PLoS One** 7 (2012) e41721.
 23. Rajeshwaran, G.G., Nandakumar, M., Sureshbabu, R. and Mohanakrishnan, A.K. Lewis acid-mediated Michaelis-Arbuzov reaction at room temperature:

- A facile preparation of arylmethyl/heteroarylmethyl phosphonates. **Org. Lett.** 13 (2011) 1270–1273.
24. Ianni, A. and Waldvogel, S.R. Reliable and versatile synthesis of 2-Aryl-substituted cinnamic acid esters. **Synthesis** 13 (2006) 2103–2112.
 25. Claridge, T.D.W., Davies, S.G., Lee, J. A., Nicholson, R.L., Roberts, P.M., Russel, A.J., Smith, A.D. and Toms, S.M. Highly (*E*)-selective wadsworth-emmons reactions promoted by methylmagnesium bromide. **Org. Lett.** 10 (2008) 5437–5440.
 26. Mosman, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. **J. Immunol. Methods** 65 (1983) 55–63.
 27. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature** 227 (1970) 680–685.
 28. Towbin, H., Staehelin, T. and Gordon, J. Electrophoretic transfer of proteins from polyarylamide gels to nitrocellulose sheets: procedure and some applications. **Proc. Natl. Acad. Sci. USA** 76 (1979) 4350–4354.
 29. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with the Folin phenol reagent. **J. Biol. Chem.** 193 (1951) 265–275.
 30. Habig, W.H., Pabst, M.J. and Jakoby, W.B. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. **J. Biol. Chem.** 249 (1974) 7130–7139.
 31. Slocum, S.L. and Kensler, T.W. Nrf2: control of sensitivity to carcinogens. **Arch. Toxicol.** 85 (2011) 273–284.
 32. Canistro, D., Bonamassa, B., Pozzetti, L., Sapone, A., Abdel-Rahman, S.Z., Biagi, G.L. and Paolini, M. Alteration of xenobiotic metabolizing enzymes by resveratrol in liver and lung of CD1 mice. **Food Chem. Toxicol.** 47 (2009) 454–461.
 33. Numazawa, S. and Yoshida, T. Nrf2-dependent gene expressions: a molecular toxicological aspect. **J. Toxicol. Sci.** 29 (2004) 81–89.
 34. Dinkova-Kostova, A.T., Holtzclaw, W.D., Cole, R.N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., and Talalay, P. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. **Proc. Natl. Acad. Sci. USA** 99 (2002) 11908–11913.
 35. Chow, H.H., Garland, L.L., Hsu, C.H., Vining, D.R., Chew, W.M., Miller, J.A., Perloff, M., Crowell, J.A. and Alberts, D.S. Resveratrol modulates drug- and carcinogen-metabolizing enzymes in a healthy volunteer study. **Cancer Prev. Res. (Phila)** 3 (2010) 1168–1175.
 36. Hsieh, T.C., Wang, Z., Deng, H. and Wu, J.M. Identification of glutathione sulfotransferase-pi (GSTP1) as a new resveratrol targeting protein (RTP) and studies of resveratrol-responsive protein changes by resveratrol affinity chromatography. **Anticancer Res.** 28 (2008) 29–36.

37. Phillips, M.F. and Mantle, T.J. The initial-rate kinetics of mouse glutathione S-transferase YfYf. Evidence for an allosteric site for ethacrynic acid. **Biochem. J.** 275 (1991) 703–709.
38. Hayes, J.D. and Pulford, D.J. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. **Crit. Rev. Biochem. Mol. Biol.** 30 (1995) 445–600.
39. Zhang, Y., Gonzalez, V. and Xu, M.J. Expressions and regulation of glutathione S-transferase P1-1 in cultured human epidermal cells. **J. Dermatol. Sci.** 30 (2002) 205–214.
40. Radjendirane, V., Joseph, P. and Jaiswal, A.K. Gene expression of DT-diaphorase (NQO1) in cancer cells. in: **Oxidative stress and signal transduction.** (Forman, H.J., Cadenas, E., Eds.), Chapman & Hall, New York, 1997, 441–475.
41. Shen, G., Kong A.N. Nrf2 plays an important role in coordinated regulation of Phase II drug metabolism enzymes and Phase III drug transporters. **Biopharm. Drug Dispos.** 30 (2009) 345–355.
42. Buryanovskyy, L., Fu, Y., Boyd, M., Ma, Y., Hsieh, T.C., Wu, J.M. and Zhang, Z. Crystal structure of quinone reductase 2 in complex with resveratrol. **Biochemistry** 43 (2004) 11417–11426.
43. Zhang, X., Wang, Y., Yang, W., Hou, X., Zou, J. and Cao, K. Resveratrol inhibits angiotensin II-induced ERK1/2 activation by downregulating quinone reductase 2 in rat vascular smooth muscle cells. **J. Biomed. Res.** 26 (2012) 103–109.