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Position of an Active Thiosulfonate Group in New Phenolic Antioxidants is Critical for ARE-Mediated Induction of GSTP1 and NQO1

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Abstract :
Synthetic water-soluble phenolic antioxidants are known anti-inflammatory chemicals, some of them were shown to induce components of antioxidative defense enzymes NADPH:quinone oxidoreductase (NQO1) and glutathione-S-transferase (GST) via activation of Nrf2 pathway. Our experimental in vivo models have demonstrated an increase of both *gstp1* and *nqo1* expression in the liver of mice treated with novel organosulfur phenolic compounds TS-12, TS-13, TS-14. Both expression and activity of GSTP1 was dependent on the distance of active thiosulfonate group in para- position from the benzene ring of the above antioxidants. The increase of *nqo1* and *gstp1* gene expression was mediated by the antioxidant-binding response element (ARE). Our results revealed the role of Nrf2/ARE-dependent transcription regulation in response to the new phenolic antioxidants and suggested possible strategy to improve antioxidative properties of synthetic phenolic compounds.

Keywords: ARE – antioxidant-responsive element; GSTP; NQO1; phenolic antioxidants; ROS; tBHQ

Abbreviations ARE (antioxidant-responsive element), Nrf-2 (nuclear factor E2–related factor 2), Keap1 (Kelch like ECH-associated protein 1), tBHQ (tert-butylhydroquinone), GSTP1 (glutathione S-transferase P1), NQO1 (NAD(P)H: quinone oxidoreductase), mRNA (messenger RNA), qPCR (quantitative polymerase chain reaction), HPLC (high-performance liquid chromatography)

INTRODUCTION

A variety of natural antioxidant compounds including polyphenols have received a great deal of attention due to demonstrated therapeutic value in neurodegenerative disorders and resistance against a broad set of carcinogens [1-3]. Several groups of natural antioxidants have been described and categorized so far based on their chemical structures: (i) flavonoid polyphenols; (ii) non-flavonoid polyphenols; (iii) phenolic acids and diterpenes, and (iv) organosulfur compounds. All of them scavenge free radicals and increase the activity of endogenous cellular antioxidant defense system [4]. However, the direct evidence between antioxidative properties of these compounds and their biological effects is hard to validate as many of them can simultaneously trigger the hormone receptors, Red-Ox sensitive transcriptional factors, MAPK signaling and other universal pathways [5,6].

NADP(H)-quinone oxidoreductase 1 (NQO1) is a prototype phase 2 enzyme that is induced coordinately with other phase 2 proteins including glutathione S-transferases (GST) and has played a very useful role in the assessment of the potencies of phase 2 inducers, in the discovery and isolation of new inducers from natural sources, and in elucidating the chemistry of inducers [7]. These enzymes are regulated on transcriptional level by cis-acting antioxidant-responsive elements (ARE) via a nuclear factor (erythroid-derived 2)-like 2 (Nrf2) that controls both the inducible and constitutive gene expression [8,9]. Activation of the Nrf2-ARE is a major mechanism in the cellular defense against oxidative or electrophilic stress, which was shown to control the expression of more than 200 genes and encoded products involved in the detoxication and elimination of reactive oxygen species (ROS), xenobiotic metabolism and others [10]. Several binding sites including NF-κB, SP-1, AP-1-regulatory elements, three ARE and ARE-like elements are found in *gstp1* promoter region [11]. Similarly, ARE, XRE, TPA-responsive element (TRE) and AP-2 binding sites are found in *nqo1* promoter region [12], hence both *gstp1* and *nqo1* genes may serve as potential markers to detect ARE-mediated antioxidative capacity of chemical compounds in a living system. Moreover, a number of phenolic antioxidants, e.g. tBHQ, enhance ARE-mediated expression of genes responsible for metabolism of xenobiotics [13]. Therefore, one can suggest a common mechanism of antioxidative response for similar class of phenolic antioxidants.

Here we studied derivatives of orto-tert-butyl-phenols TS-12, TS-13, TS-14 which were previously shown to increase gene expression of *gstp1* in hepatoma cell lines [14]. Using mice models we have demonstrated an increase expression and activity of GSTP1 in the liver of animals treated with the above derivatives. We also showed an accumulation of the nuclear ARE-protein complexes in the liver homogenates of animals exposed to tBHQ and TS-13. The increase of *nqo1* and *gstp1* expression was dependent on the distance of thiosulfonate ligand from the benzene ring. Our results suggest the role of Nrf2/ARE-dependent transcription regulation in response to the phenolic antioxidants.

MATERIALS AND METHODS

Reagents and chemicals The following chemicals have been used in the current study: antioxidants – tBHQ, 3-(3'-*tert*-butyl-4'-hydroxyphenyl)ethylthiosulfonate Na (TS-12),

3-(3'-*tert*-butyl-4'-hydroxyphenyl)propylthiosulfonate Na (TS-13), 4-(3'-*tert*-butyl-4'-hydroxyphenyl) butylthiosulfonate Na (TS-14). Synthesis and chemical properties of these compounds have been described previously [15-17].

Animal treatment BALB male mice were provided with food containing corresponding phenolic derivatives (0.6% of the food mass). Mice have been sacrificed by decapitation. All protocols used in this study were approved by the Institutional Animal Care and Use Committee.

RNA Isolation and Reverse Transcription Pieces of animal livers (25-35 mg) were frozen in liquid nitrogen and total RNA was isolated with RNeasy mini kit (Qiagen). The quality of RNA was tested in 2% agarose gel and by measuring absorbance at 260/320 nm (netAbs 260), and compared it with absorbance for proteins at 280/320 nm (netAbs 280) and for polysaccharides at 230/320 nm (netAbs 230). 40 μg RNA was considered to give OD=1 and the probes with netAbs260/netAbs280=1.4-1.7 were utilized for further experiments. 2 μg of RNA were used for the generation of cDNA in reverse transcriptase reaction with a High-capacity cDNA archive kit (Applied Biosystems), according to the manufacturer's instructions.

Real-time qPCR Real-time qPCR was performed in a two-step reaction. First cDNA was prepared from 2 μg of total RNA. Next, qPCR was performed using 5μL of cDNA described above on ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using pre-developed TaqMan-probe (Applied Biosystems) for *gstp1* and *nqo1,* labeled with FAM. VIC labeled 18S RNA pre-made TaqMan probes (Applied Biosystems) were used for internal control. The efficiency of PCR was evaluated by performing a 10-fold dilution series experiment using the target assay. The standard curves were translated into efficiency values which were equal 99-100%. Results were analyzed by $2^{-\Delta\Delta Ct}$ methods with "Applied Biosystems" software. A statistical analysis was performed using one-factor Dispersion analyses (ANOVA) following t-test and $p<0.05$ was considered significant.

Preparation of cytosolic fraction and Western blotting Mice livers were placed on ice in the buffer containing 20 mM tris-HCl, pH 7.4, 1.15 % KCl. After homogenization, samples were fractionated by centrifugation at 12100 g for 20 min; supernatant was processed for further centrifugation at 105000g for 1h. Equal loading was achieved by normalization of total protein concentrations as measured by Quick Start Bradford Protein Assay kit (Bio-Rad). For Western blotting protein extracts from cytozolic fractions were resolved in 15% SDS–PAGE, transferred to PVDF membranes, and blocked for 1 h in Tris-buffered saline (TBS), 0.1% (v/v) Tween-20, and 3% (w/v) bovine serum albumin (BSA). The PVDF membranes were incubated for 2 h with primary monoclonal antibody against GSTP1 (BD, clone 3, G-59720) or beta-actin (Sigma, AC-74). After washing with 0.1% (v/v) Tween 20 in TBS, the membranes were incubated with mouse anti-goat IgG coupled with alkaline phosphatase. Images from several blots were digitized and processed with Versa doc software Image Quantity 1D 4.6.1 (Bio-Rad).

GSTP1 activity assay by HPLC The GSTP1-specific activity was determined according to the method described in [18]. Briefly, 50 μg of cytoplasmic proteins were dissolved in 0.5ml of reaction mixture containing 25 mM potassium phosphate, 1 mM EDTA, 0.25 mM glutathione, 300 μM ethacrynic acid (pH 6.5). Reaction mixture was preincubated for 4 min at 37ºC and 5 mM glutathione was added. Reaction was stopped by vortexing with 17 μL 15% TCA and 50μL of ethanol, 5 μg of ethyl para-hydroxybenzoate (Ethylparaben, internal standard) were added. After spinning at 4500 g for 5 min, 400μl aliquots were taken from the water phase and the pH was adjusted to 3.5. The amount of ethacrynate-GSH conjugates (EA-SG) was measured by HPLC method [19], with our modification. 5μl of probe was loaded onto HPLC C18 Nucleosil column (250x2 mm) following separation in acetonitrile (40% v/v) and acetic acid (1%) at the flow rate of 150 μl/min. EA-SG was detected at 270 nm. The activity was calculated by the EA-SG concentration subtracted by the non-enzymatic EA-SG products. For multiple comparison analyses, Kruskal – Wallis ANOVA with post hoc comparison was applied. Mann-Whitney U test was applied for paired comparison, analyses. In both cases, $p < 0.05$ was considered to be significant.

Nuclear cell extracts and chromatin isolation Nuclear cell extracts from liver homogenates were prepared as described earlier [20] with some modifications [21]. All procedures were performed at 4°C. Livers from decapitated mice were extracted, washed with 20ml perfusion buffer (10мM HEPES pH=7.8; 25mM KCl; 1mM EDTA), sliced by small pieces and homogenized in x2V buffer (10mM HEPES pH 7.8; 25mM KCl; 0.15mM spermin, 0.5mM spermidine, 1mM EDTA; 2M sucrose; 10% glycerol). Homogenates were overlaid onto the same buffer and centrifuged at 100.000 g for 40 min at 0°C. Nuclear pellets were resuspended in 4 ml lyses buffer (10mM HEPES pH 7.8, 100mM KCl, 2mM $MgCl_2$; 0.1mM EDTA, 1mM DTT, 0,1mM PMSF and 10% glycerol). Precipitation was performed by adding 0.1V of saturated (4°C) $(NH_4)_2SO_4$. Chromatin fraction was centrifuged at 120.000 g for 90 min at 0°C. Proteins were precipitated by adding dry $(NH_4)_2SO_4$ to the supernatants (0.252 g/ml) followed by centrifugation at 120.000 g for 20 at 0°C. Precipitates were dissolved in 0,2 ml of dialyses buffer (25mM HEPES pH 7.8, 80mM KCl, 0.2mM EDTA, 1mM DTT, 0.2mM EGTA and 10% glycerol) containing protease cocktail inhibitors (Pierce). Dialyses against 150 ml of dialyses buffer for 40 min was performed three times. Protein concentrations in the samples were measured by the method of Bradford [22].

Electrophoretic mobility shift assay (EMSA) For determining the Nrf2/ARE-binding activity

Nuclear extracts containing salmon sperm (1μg DNA per 7μg total protein) were sonicated for 10 min on ice; with 4μg of sonicated complexes were incubated with 0.05 pmol [^{32}P]-labelled oligonucleotides in 15 μl of dialyses buffer for 15 min at room temperature followed by separation by 4.5% PAGE in 0.5 × TBE; gels were dried and exposed to X-ray film. The following oligonucleotides containing

specific transcriptional binding sites were used as DNA probes: Ets 5'-cagt TCG AAC TTC CTG CTC GA -3'; 5'-cagt TCG AGC AGG AAG TTC GA -3' [23] and NF-E2 5'- cagt TGG GGA ACC TGT GCT GAG TCA CTG GAG -3'; 5'-cagt CTC CAG TGA CTC AGC ACA GGT TCC CCA -3', mutant form NF-E2mut 5'-cagt TGG GGA ACC TGT GCT AGG TCA CTG GAG -3'; 5'-cagt CTC CAG TGA CCT AGC ACA GGT TCC CCA -3'. Sequences for NF-E2 и NF-E2mut were taken from Santa Cruz database for gel-shift (Santa Cruz Biotechnology, Inc.). Probes were P32-labeled by incubation of 0.01mM oligonucleotides in 1 µl of 10x labeling buffer (500 mM Tris-HCl pH 8.0, 100mM NaCl, 100mM $MgCl_2$, 1mM DTT, 2mM dGTP, 2mM dTTP, 2mM dCTP) with 2U of Klenow fragment and 10 uCi [α-^{32}P]dATP. Labeled oligonucleotides were purified on DEAE-81 resin (paper) followed by washing with 0.25M KH_2PO_4. NF-E2 specific DNA sequence was used as ARE element. Ets transcription factor was used as internal control and DNA-binding activity of Ets with cognate binding site was used as a baseline control. Densitometry of scanned films was performed using Quantity One computer software (Bio-Rad).

RESULTS

Increased *nqo1* and *gstp1* gene expression in the mice liver after treatment with antioxidants To determine the role of phenolic derivatives in *nqo1* and *gstp1* gene expression in the liver of BALB mice the following antioxidants have been tested: TS-12, TS-13, TS-14 (Figure 1). The choice for these derivatives was stipulated by the earlier experiments in vitro, where variations in antioxidative and antiradical properties for these chemicals were correlated with the arrangement of OH-group as well as the antioxidative property of divalent sulfur in the thiosulfonate ligand [16]. tBHQ was used as a validated ARE-mediated antioxidant [24,25]. As revealed by real-time PCR, *gstp1* mRNA expression was increased in the liver homogenates of mice receiving TS-12 (2.10 times), TS-13 (1.96 times) and TS-14 (2.46 times) but not with the t-BHQ compound as compared to homogenates from control group (Table 1). However, *nqo1* mRNA was increased by treatment with t-BHQ (1.8 times), TS-12 (3.79), TS-13 (6.69) and TS-14 (8.21) (Table 1). These data demonstrate that animals treated with novel phenolic antioxidants show an increase in both *gstp1* and *nqo1* gene expression. Importantly, *gstp1* and *nqo1* gene expression level demonstrate direct dependence on the distance of active group in para-position from the benzene ring.

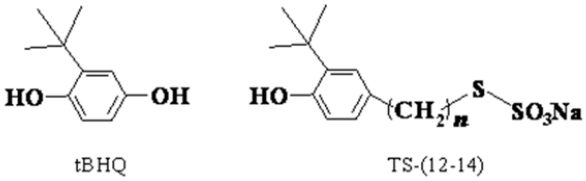

Fig. 1. Structures of phenolic antioxidants used in current study. tBHQ, TS-12 (n=2), TS-13 (n=3), TS-14 (n=4).

Induction of GSTP1 protein in the liver of mice in response to phenolic antioxidants In order to estimate the induction of GSTP1 on a protein level, we performed Western blot analyses. (Figure 2A). As revealed by measurement of GSTP1/β-actin signals, the GSTP1 protein expression levels were increased in the groups of animals treated with tBHQ (1.47), TS-12 (1.46), TS-13 (1.57), and TS-14 (1.41) (Figure 2B). In line with previous findings, GSTP1 expression was proportional to the length of spacer separating active group from the benzene ring.

GSTP1 activity in the mice liver is increased upon treatment with antioxidants GSTP1 is an important enzyme of the intracellular antioxidative defense system and possess high substrate specificity when metabolizing electrophilic compounds by conjugating them with glutathione and increasing their solubility and excretion from a cell [26]. In order to assess activities of GSTP1 in response to antioxidants we measured the velocity of ethacrynic acid conjugation with glutathione in the cytosoles of liver homogenates isolated from BALB mice treated with indicated phenolic compounds. Our results demonstrate that GSTP1 activity in the liver of animals treated with tBHQ, TS-13, and TS-14 is increased comparatively to control group (Table 2). This increase of activities corroborates with the previously shown dependence on the distance of thiosulfonate group from the benzene ring.

Table 1. Increase of *nqo1* and *gstp1* mRNA in the liver of BALB mice treated with phenolic antioxidants. Results are $2^{-\Delta\Delta Ct}$ – copy ratio of *nqo1* and *gsp1* mRNA in control and experiments (ΔΔCt + SD; ΔΔCt – SD), SD – standard deviation of ΔΔCt. Group size consisted of 5-6 animals. *p<0.05 vs control, #p<0.05 vs. tBHQ.

Gene	*Control*	*t-BHQ*	*TS-12*	*TS-13*	*TS-14*
gstp1	1.00 (0.78-1.28)	0.86 (0.64-1.17)	2.10*, # (1.89-2.33)	1.96*, # (1.56-2.46)	2.46*, # (2.03-2.99)
nqo1	1.00 (0.64-1.54)	1.82* (1.78-2.86)	3.79*, # (1.93-4.49)	6.69*, #(4.25-8.18)	8.21*, #(4.53-9.55)

Table 2. Velocity of ethacrynic acid and glutathione conjugation in the cytosol of liver homogenates isolated from BALB mice treated with indicated phenolic compounds. n- number of animals in the group, p-values represent differences with control group.

Groups	Median	Min	Max	Lower Quartile	Upper Quartile	p value
Control (n = 11)	244.43	127.06	370.44	184.93	336.9	
tBHQ (n = 17)	357.19	130.31	596.15	297.43	418.28	0.0287
TC-12 (n = 5)	286.0	162.98	323.07	285.71	293.58	0.913
TC-13 (n = 9)	349.57	264.78	426.73	340.05	417.68	0.0097
TC-14 (n = 6)	449.23	301.80	781.05	318.62	675.98	0.0103

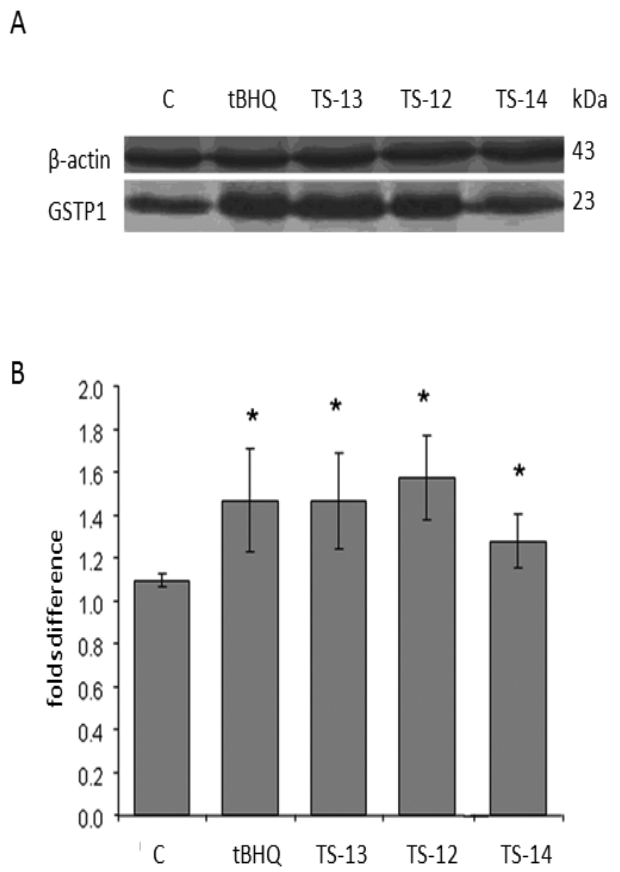

Fig. 2. The effect of antioxidants on GSTP1 protein expression in the liver cytosoles isolated from BALB mice. (A) BALB mice have been treated with vehicle (control), TS-12, TS-13, TS-14 or tBHQ for two weeks and corresponding fractions were assayed by Western blot followed by probing with anti-GSTP1 or b-actin antibodies. (B) For each band, the amount of GSTP proteins was determined by densitometric analysis and normalized to that of β-actin. *p<0.05 vs. vehicle.

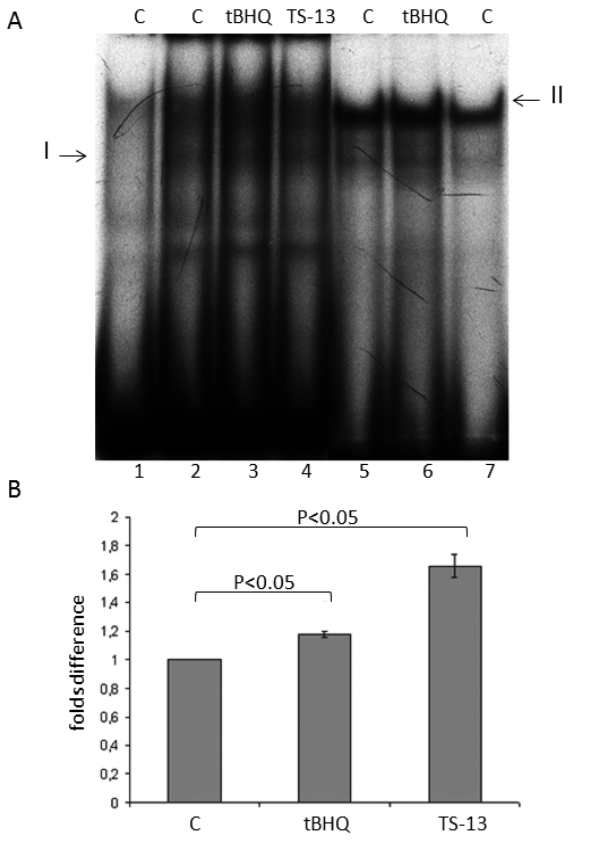

Fig. 3. The effect of antioxidants on ARE-binding complexes from nuclear extracts of liver homogenates. (A) NF-E2mut (lane 1), NF-E2 (lanes 2,3,4) radiolabelled probes specific to NF-E2 protein were incubated with nuclear cell extracts isolated from liver homogenates of BALB mice treated with control, t-BHQ, or TS-13 antioxidants. DNA-binding complexes I and II (depicted in arrows) were visualized using radioautography. (B) Quantification of DNA binding as measured by densitometry of corresponding radioautographs.

Interaction of ARE with Nrf2 transcription factor is enhanced in response to phenolic antioxydants The transcription factor Nrf2 appears to represent a key regulator in oxidative stress response and recognizes the ARE in the promoter region of its target genes [27]. Previous work suggests that tBHQ may activate ARE-dependent transcription of Ox-RedOx genes [28]. Nrf and other transcriptional activators bind to 5'–A/GTGAC/TnnnGCA/G–3'sequence of cis-acting ARE regulatory element [29]. In normal physiological conditions Nrf2 level is low and predominantly associated with Keap1 regulatory protein on the nuclear envelop or on cytoskeleton [30]. However, in many pathological states Nrf2/Keap1 signaling is activated and binding of Nrf2 to ARE confers increased tolerance to oxidative stress [31].

To gain further insights into the regulatory mechanisms of the Nrf2-ARE signaling pathway, we performed mobility-shift assay to measure ARE-bond complexes in the nuclear extracts of liver homogenates. Results from EMSA assay demonstrate accumulation of ARE-protein complexes in the probes obtained from the liver homogenates of mice treated with tBHQ and TS-13 antioxidants (Figure 3). Since NF-E2 specific DNA sequence was utilized as ARE element in EMSA, disappearance of complex I (lane 1) and appearance of complex II (lane 2) suggests involvement of NF-E2 factor in response to the above antioxidant treatment.

DISCUSSION

Activation of Nrf2-ARE signaling pathway is initiated by covalent modification of cysteine thio-disulfide bonds of Keap1 by electrophilic antioxidants, radicals and heavy metals [31]. OxRedox properties of synthetic chemicals are crucial for ARE-mediated activators [32,7]. SH-containing derivatives (isocyanates dithiol tions, mercaptans etc.) are able to enhance transcription of ARE-regulated genes [33, 34]. Despite structural differences of ARE activators, oll of them are electrophiles which are capable of inactivating Keap1 by alkylation or oxidation of its cysteine SH-groups [35]. Interestingly, both para- and orto-position of active groups in benzene ring of phenolic compounds are

important for Nrf2/Keap1-mediated signaling, whereas neither benzene nor phenol reveals ARE-activating properties. While OH-group in meta position of di- and tri-phenolic compounds (resorcinol or 1,3,5-trihydroxybenzene) does not evoke any ARE-regulated gene expression, presence of OH-group in orto or para position defines transcriptional activity of targeted genes [36]. Other phenolic compounds reveal ARE-mediated induction of phase II biotransformation of xenobiotics only when both Michael acceptors and hydroxyl are present together [37,38,34].

The above structural differences in position of oxidative groups suggest that ARE-mediated activation occurs by 2-electron oxidation-reduction of Nrf2–Keap1 complex, where orto- and para- but not meta-hydroxyl groups of phenolic compounds are involved. Therefore, in this study we investigated novel phenolic antioxidants TS-(12-14) with various combinations of tret-butyl fragments in orto- or alkyl groups in para- position, all of them contained thiosulphonate group linked to a benzene ring via methyl-chained spacers (n=2,3,4). We have showed that all chemicals were able to increase gene expression of two major enzymes of antioxidative defence system *gstp1* and *nqo1* in the liver of BALB mice. Importantly, this increase was dependent on the distance of thiosulphonate groups in para-position from the benzene ring of phenolic compounds. This may suggest possible strategy to enhance antioxidative response by varying the length of linker to which specific groups are separated from the benzene ring. This observation corroborates well with the previous findings describing dependence of GST induction mediated by anticancer compound sulforaphane on the number of methyl groups of electrophiles CH_3–S–$(CH_2)n$–N=C=S, CH_3—SO–$(CH_2)n$–N=C=S и CH_3–SO_2–$(CH_2)n$–N=C=S [39]. In all of our experiments with phenolic compounds performed at equal concentrations, the control antioxidant tBHQ revealed lower induction levels of *nqo1* и *gstp1* than the newly synthesized TS-12-14. Our results from gel retardation assay suggest ARE-mediated transcriptional activation of these genes.

In light of the conventional model for ARE-dependent gene induction of, one may suggest that thiosulphonate groups of TS compounds interact with nucleophilic thiols from the Keap1 Cys residues resulting in dissociation of Nrf2–Keap1 complex. Interestingly, 624 amino acid mouse Keap 1 has 25 cysteine residues [40] and 102 amino acid Nrf-fixing IVR domain of Keap1 has eight Cys residues, five of which (Cys257, Cys273, Cys288, Cys297 and Cys613) are thought to form disulfide bonds during oxidative modifications of phenolic compounds [35]. In turn, seven Cys residues of 597 amino acid mouse Nrf2 protein are much more tolerant to oxidation and seem to have no effect on dissociation of Nrf2–Keap1 [41]. Based on the published literature, we may propose that antioxidative properties of TS-12, TS-13, TS-14 are defined by the presence of sulpho-containing ionogenic group which interacts with nucleophilic Cys-residues of Keap1. In some antioxidants, inactivating of electrophilic group was shown to the loss of ARE-mediated binding capacity with cognate factors, although other factors including intracellular concentration, non-covalent protein-target recognition, simultaneous influence of metabolites of oxidants should be considered [42].

ARE-mediated gene induction depends on the chemical nature of inducer, sequence composition of ARE and cell specificity [33]. Our findings on different levels of *nqo1* and *gstp1* gene induction may reflect the variations in the architecture of ARE and targeted complexes. Indeed, dimerization of nuclear Nrf2 with Maf- or Jun proteins following ARE binding and transcriptional activation [9] is dependent on the ARE sequence that differentially regulates accommodation of Nrf2-Maf dimers and thus may affect induction level of certain genes [43].

In general, Keap1/Nrf2 signaling confers antioxidative property of cellular system and is pivotal to defend organisms against oxidative stress, a common hallmark of cancer, chronic inflammation, or aging-associated diseases. Both Nrf2 and Keap1 are prominent targets for drug discovery hence, finding and characterization of novel molecules with controlled antioxidant activities is crucial for novel pharmacological approaches.

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