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Role of hydroquinone–thiol conjugates in benzene-mediated toxicity

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ABSTRACT

Hydroquinone (HQ) is a metabolite of benzene, and in combination with phenol (PHE), reproduces benzene myelotoxicity. HQ readily oxidizes to 1,4-benzoquinone (1,4-BQ) followed by the reductive addition of glutathione (GSH). Subsequent cycles of oxidation and GSH addition give rise to a variety of mono-, and multi-GSH substituted conjugates. Following administration of PHE/HQ (1.1 mmol/kg/0.9 mmol/kg, ip) to male Sprague–Dawley (SD) rats, 2-(glutathion-S-yl)HQ [GS-HQ], 2,5-bis-(glutathion-S-yl)HQ [2,5-GS-HQ], 2,6-bis-(glutathion-S-yl)HQ [2,6-GS-HQ], and 2,3,5-tris-(glutathion-S-yl)HQ [2,3,5-GS-HQ] were all identified in bone marrow. 2-(Cystein-S-ylglycine)HQ [2-(CysGly)HQ], 2-(cystein-S-yl)HQ [2-(Cys)HQ], and 2-(N-acetylcystein-S-yl)HQ [2-(NACys)HQ] were also found in the bone marrow of PHE/HQ and benzene treated rats and mice, indicating the presence of an active mercapturic acid pathway within bone marrow. Moreover, 2,6-GS-HQ and 2,3,5-GS-HQ were hematotoxic when administered to rats. All of the HQ–GSH conjugates retain the ability to redox cycle and generate reactive oxygen species (ROS), and to arylate target proteins. Recent in vitro and in vivo studies in our laboratory revealed lysine and arginine residues as primary targets of 1,4-BQ, GS-HQ and 2-(NACys)HQ adduction. In contrast 1,4-BQ-adduction of cysteine residues may be a transient interaction, where physiological conditions dictate adduct stability. The generation of ROS and alkylation of proteins may both contribute to benzene-mediated myelotoxicity, and the two processes may be inter-dependent. However, the precise molecular mechanism by which benzene and HQ–GSH conjugates induce hematotoxicity remains to be determined. Within 18 h of administration of PHE/HQ to SD rats a significant decrease in blood lymphocyte count was observed. At this early time point, erythrocyte counts and hemoglobin concentrations remained within the normal range. Concomitant with the decrease in lymphocyte count, western blot analysis of bone marrow lysate, using HQ–GSH and 4-hydroxy-2-nonenal (4HNE) specific antibodies, revealed the presence of HQ–GSH- and 4HNE-derived protein adducts. Identification of these adducts is required before the functional significance of such protein modifications can be determined.

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1. Introduction

Benzene, a major industrial chemical and environmental pollutant, causes a variety of hematological disorders in man, including aplastic anemia, myelodysplastic syndrome, and acute myelogenous leukemia. While benzene must be metabolized to yield its hematotoxic and leukemogenic effects, no single metabolite of benzene reproduces these effects in vivo. Co-administration of PHE and HQ, however, does lead to myelotoxicity in rodents [1]. A pharmacokinetic interaction between these two metabolites results in increased concentrations of both metabolites in bone marrow [2]. Peroxidase and/or phenoxy-radical mediated oxidation of HQ then theoretically initiates redox cycling and formation of the reactive electrophile, 1,4-BQ, which is considered to be one of the ultimate hematotoxic metabolites of benzene [3–13]. 1,4-BQ is an electrophile, and covalent interactions of quinones with

Abbreviations: 2-BrHQ-NAC, 2-bromo-6-(N-acetylcystein-S-yl)hydroquinone; 1,4-BQ, 1,4-benzoquinone; ECL, enhanced chemiluminescence; EPO, erythropoietin; γ -GT, γ -glutamyl transpeptidase; GS-HQ, 2-(glutathion-S-yl)HQ; 2,5-GS-HQ, 2,5-bis-(glutathion-S-yl)HQ; 2,6-GS-HQ, 2,6-bis-(glutathion-S-yl)HQ; 2,3,5-GS-HQ, 2,3,5-tris-(glutathion-S-yl)HQ; 2-(CysGly)HQ, 2-(cystein-S-ylglycine)HQ; 2-(Cys)HQ, 2-(cystein-S-yl)HQ; 2-(NACys)HQ, 2-(N-acetylcystein-S-yl)hydroquinone; 4HNE, 4-hydroxy-2-nonenal; HQ, hydroquinone; PHE, phenol; QT, quinol–thioether; ROS, reactive oxygen species.

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nucleophilic sites within cellular macromolecules may contribute to the toxic effects of benzene [7,14–16]. Indeed, the combined treatment of PHE and [^{14}C]-HQ increases myelotoxicity with concomitant increases in covalently bound radiolabel in blood and bone marrow [10]. Moreover, elevated levels of benzene oxide and HQ-derived (1,4-BQ?) adducts of hemoglobin and albumin have been observed in workers subjected to benzene exposure [17,18].

Focusing on cysteine-targeted protein adducts, McDonald et al. [19] reported that 1,4-BQ protein binding was favored over benzene oxide in mouse bone marrow. Although modification on selective target proteins occurs in bone marrow of mice following treatment with [^{14}C]-benzene [20], the exact nature of the adducted metabolite(s) and/or the specific site of adduction on target proteins are not known. Of particular relevance to the ability of benzene to induce aneuploidy, and other forms of chromosomal aberrations, histones were identified as potential targets of unknown reactive benzene metabolites [20]. 1,4-BQ readily conjugates with glutathione (GSH) to give 2-GS-HQ, 2,3-GS-HQ, 2,5-GS-HQ, 2,6-GS-HQ and 2,3,5-GS-HQ [21]. Moreover, HQ–GSH conjugates are present in the bone marrow of rats and mice following co-administration of PHE/HQ [22] and metabolized to more reactive cysteinylglycine and cysteine conjugates via the mercapturic acid pathway in bone marrow. Because HQ–thioether metabolites have an enhanced capacity to both redox cycle [Monks et al. (this issue)] and arylate tissue macromolecules [23,24], we suggest that they play an important role in benzene-mediated toxicity via a mechanism involving the production of ROS and/or macromolecular arylation. Interestingly, lysine residues appear to be a preferred target of quinone–thioether adduction [24,25]. ROS produced as a result of HQ–thioether redox cycling are also capable of oxidatively modifying both proteins and DNA thereby producing toxicity. Herein we report the presence of HQ–thioether and 4HNE-derived protein adducts following *in vivo* administration of PHE/HQ to rats. These two inter-dependent pathways of protein modification may contribute to benzene induced myelotoxicity.

2. Materials and methods

2.1. Materials

HQ and PHE were purchased from Sigma–Aldrich (St. Louis, MO). Cell Lysis Buffer (10 $\times$) was purchased from Cell Signaling Technology (Danvers, MA). Complete Protease Inhibitor Cocktail Tablets were purchased from Roche (Madison, WI). Antibody sources were as follows: rabbit anti-2-Br-6-(N-acetylcystein-S-yl)hydroquinone (anti-2-BrHQ–NAC) in-house [26]; anti-4HNE antibody was a generous gift from Dr. Dennis R. Petersen (University of Colorado Health Sciences Center) produced and characterized as previously described [27]; peroxidase labeled goat anti-rabbit IgG, Vector Laboratories (Burlingame, CA). Enhanced chemi-luminescent reagent (ECL) and Hyperfilm ECL were purchased from Amersham Life Science (Arlington Heights, IL). Acivicin was obtained from Sigma.

2.2. Animals

Male and female SD rats ($n=2\text{--}4$ @ 2–5 months) were purchased from Harlan Sprague–Dawley (Houston, TX) and used for all experiments. All animals were housed on a 12 h light/dark cycle and allowed food and water *ad libitum*. Blood samples were collected in EDTA coated micro-cuvettes via the retro-orbital sinus and cardiocentesis. Consistent with humane practices, animals were anesthetized via pentobarbital injection prior to blood collection. Blood samples were submitted to University Animal Care

Pathology Services (Tucson, AZ) for complete blood count analysis.

2.3. Treatment protocols and isolation of bone marrow

Male and female SD rats were co-administered PHE (1.1 mmol/kg) and HQ (0.9 mmol/kg) and dissolved in a vehicle consisting of 0.85% phosphate-buffered saline (PBS):ethanol (60:40). Protective clothing was used and proper ventilation ensured to limit exposure to HQ, a potential carcinogen. After 18 h animals were euthanized by pentobarbital overdose, and each femur was quickly removed, cleansed of muscle, and placed on ice. The epiphyseal plates of each femur were then removed, and the marrow was flushed with 5 mL of ice cold 0.85% PBS, pelleted under centrifugation (Eppendorf, Model 5804R) for 10 min at 13,500 rpm, and suspended in Cell Lysis Buffer with protease inhibitor. The cell suspensions were quickly probe sonicated for 30 s on ice, freeze–thawed three times, and centrifuged (Eppendorf, Model 5415R) for 10 min at 13,500 rpm. The supernatant was then removed and stored at -80°C . γ -Glutamyl transpeptidase (γ -GT) activity and total protein levels were determined as previously described [28]. One unit of γ -GT activity is defined as 1 μmol of p-nitroanilide formed/min at 37°C .

2.4. 1D western analysis

Proteins (40 μg) and protein standards were loaded at 120 V (constant) through a 3% (w/v) acrylamide stacking gel and resolved at 140 V through the 10% (w/v) acrylamide resolving gel. Proteins were transferred to PVDF electrophoretically [29] and duplicate gels were stained with 0.05% (w/v) Imperial Blue (Pierce, Rockford, IL). Blots were incubated overnight at 4°C with either affinity-purified rabbit anti-BrHQ–NAC antibodies diluted 1:20 in TBST or affinity purified rabbit anti-4HNE antibodies diluted 1:5000 in TBST. We have demonstrated previously that anti-2-BrHQ–NAC antibodies detect *in vivo* covalent protein adducts of 2-BrHQ, HQ, and their corresponding GSH conjugates [23]. Additionally the anti-4HNE antibodies were characterized in Dr. Petersen's laboratory [27]. Blots were incubated with goat anti-rabbit IgG (HRP-labeled) diluted 1:3000 in TBST for 1 h at room temperature, washed and then incubated for 1 min in ECL solution. Finally blots were exposed to Hyperfilm ECL for 1–5 min, and the stained duplicate gels were aligned with the western blot. The immunopositive bands from the parallel Imperial Blue stained gel will be subjected to in-gel digestion followed by LC–MS/MS for protein identification using established protocol [24,25].

2.5. Cell lines and culture conditions

HL-60 cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY) containing 20% fetal bovine serum (FBS) in a 37°C , 5% CO_2 regulated incubator. Cells were routinely cultured at a density of 1.0×10^6 cells/mL. Immediately prior to all experiments, cells were washed and resuspended in RPMI 1640 containing 25 mM HEPES and 10% FBS.

2.6. Serum erythropoietin determination

The RIA kit for erythropoietin (EPO) determination (DSL Inc., Webster, TX) was a competitive binding assay using a radiolabeled ligand. Standards and samples (100 μL) were processed according to the manufacturer's suggested protocol and analyzed on a γ -counter (1282 CompuGamma, LKB Wallac). EPO concentrations were determined by comparing the DPM of a given sample with those of known standards.

Table 1

Effect of phenol/hydroquinone on blood lymphocyte counts.

	Lymphocyte count (K/ μ L) ^a	% decrease
Phenol/HQ treated		
Rat 1	0.51	86
Rat 2	1.57	58
Rat 3	0.42	89
Rat 4	1.53	59
Control		
Rat 1	4.18	
Rat 2	3.22	

^a Normal range of rat lymphocytes: 3.8–15.3 (K/ μ L).

3. Results

3.1. Effect of blood counts after phenol/hydroquinone administration

Complete blood counts of rats 18 h following co-administration of PHE/HQ (1.1/0.9 mmol/kg, ip) to rats revealed significant reduction (58–89%) of lymphocyte counts when compared to control values (Table 1). All other leukocyte and erythrocyte counts were within their normal ranges following treatment.

3.2. 1D western detection of HQ–GSH/4HNE-protein adducts in bone marrow

It should be noted that immediately following co-administration of phenol/hydroquinone, a transient (15–20 min) neurological side-effect is observed. Interestingly the intensity of this side-effect correlates with the intensity of protein immunostaining in the western blot analyses. Such correlations indicate that inter-individual differences in absorption/distribution (revealed in the neurological response “bioassay”) correlate with inter-individual differences in covalent binding and toxicity. It is therefore essential to present data from each individual animal in Fig. 1. Unique GS–HQ-immunopositive bands (26, 43, and 140 kD) were detected in bone marrow protein of rats co-administered HQ/PHE. GS–HQ-immunopositive bands, at 50 and 65 kD, were detected in bone marrow protein from both treated and untreated control male rats (Fig. 1A). 4HNE immunopositive bands (25, 27, 43 and 59 kD) were detected in bone marrow protein of treated rats (Fig. 1B). The 50 kD protein band that was immunopositive for HQ–thioether adduction in both control and treated rats also appeared to be immunopositive for 4HNE. Interestingly, the 50 kD 4HNE immunopositive band is of much stronger intensity in untreated bone marrow protein relative to the similar

protein from rats co-administered PHE/HQ (Fig. 1B). The immunoreactive bands from control rat represent endogenously modified protein(s), possibly derived from dietary sources.

3.3. Inhibition of γ -GT

A key enzyme in the GSH cycle, γ -GT is involved in salvaging cysteine. Since cysteine availability is rate limiting in the formation of GSH, inhibition of γ -GT enhances the susceptibility of cells to oxidative damage. 2,3,5-GS–HQ inhibits γ -GT in freshly isolated bone marrow homogenates and HL-60 cell lysates, in a concentration-dependent manner (Fig. 2). Acivicin was used as a positive control and Triton X-100 treated homogenates as a negative control. Acivicin, a potent inhibitor of γ -GT, also induces hematotoxicity in humans and rats [30–32]. Thus, inhibition of γ -GT in hematopoietic tissue dramatically reduces intracellular GSH levels [33,34], and TGHQ depletes GSH in HL-60 cells and induces apoptosis in an ROS-dependent manner [35]. HQ–thioethers might therefore adversely affect cellular GSH stores thereby exacerbating the effects of ROS generation in bone marrow.

3.4. Reactivity of HQ–thioethers: effect on plasma erythropoietin

Erythrototoxicity of several of these conjugates was determined in rats using the erythrocyte ⁵⁹Fe incorporation assay [22]. Administration of 2,3,5-GS–HQ (17 μ mol/kg, iv), 2,6-GS–HQ (50 μ mol/kg, iv), and benzene (11 mmol/kg, sc) significantly decreased ⁵⁹Fe incorporation into reticulocytes to 45 $\pm$ 6%, 28 $\pm$ 3%, and 20 $\pm$ 9% of control values, respectively. Although the doses of 2,3,5-GS–HQ and 2,6-GS–HQ represented only 0.2% and 0.4% of the dose of benzene, both conjugates reduced ⁵⁹Fe incorporation to the same degree as benzene. These data suggest that HQ–GSH conjugates are erythrototoxic and may contribute to benzene-mediated hematotoxicity [22]. Because HQ–GSH conjugates are capable of damaging cells within the proximal tubules of the kidney [21], the major site of EPO production, we questioned whether 2,3,5-GS–HQ and 2,6-GS–HQ might also be capable of indirectly inducing anemia by reducing serum EPO levels. Consistent with this reasoning, both 2,3,5-GS–HQ and 2,6-GS–HQ significantly reduced circulating EPO levels with levels returning to control values at 4 days in animals treated with 2,3,5-GS–HQ (Fig. 3). Two possibilities exist for the recovery of EPO levels with continued dosing. Firstly the % of reduction is modest (30% of control), and stimulation of the repair response in the kidney may be proceeding between days 2 and 4. Alternatively, the remaining healthy cells may well be compensating to the transient decrease by increasing EPO synthesis. The liver might also produce EPO if the kidneys are compromised. However when the damage to

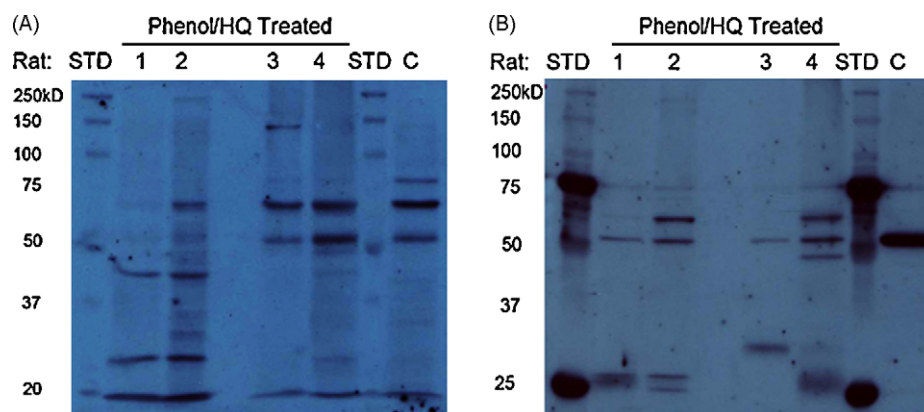


Fig. 1. 1D Western blotting of protein adducts in bone marrow. (A) HQ–GSH adducts and (B) 4–HNE adducts in rat bone marrow following co-administration of phenol (1.1 mmol/kg) and hydroquinone (0.9 mmol/kg). Lane 1,2 and 3,4 are phenol/HQ treated female and male SD rats, respectively; STD, ladder; C, vehicle treated control male rat.

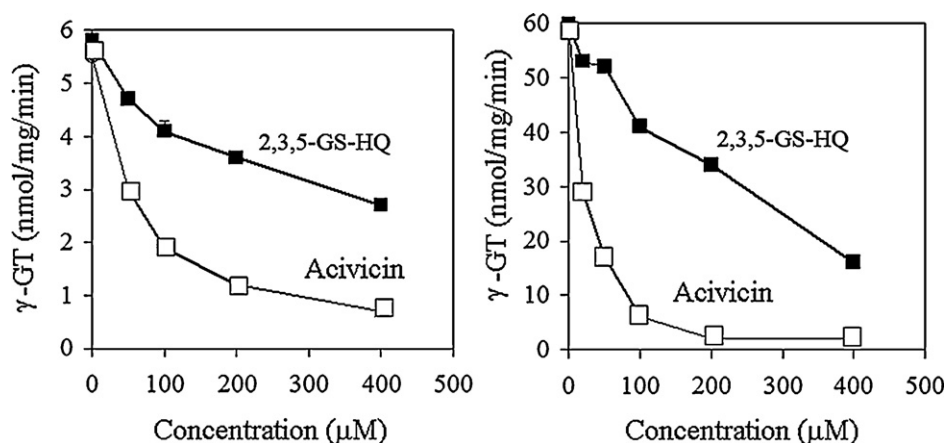


Fig. 2. The effect of 2,3,5-GS-HQ on γ -GT activity in bone marrow (left) and HL-60 cell (right) lysates. Bone marrow was flushed from the femurs of male Sprague–Dawley rats with 1 ml of Tris/KCl (20 mM/1.15%) containing 1% Triton X-100. The marrow was homogenized, incubated on ice for 30 min, and centrifuged at $800 \times g$ for 10 min. The supernatant was used as the enzyme source. (■) 2,3,5-GS-HQ; (□) acivicin.

kidney is too severe, as in the case following 2,6-GS-HQ administration the recovery in EPO was minimal, indicating that the kidneys are the primary organ for EPO production.

4. Discussion

HQ–thioethers are present in the bone marrow of rats following co-administration of PHE/HQ, the majority of which appear to be generated *in situ* and further metabolized via the mercapturic acid pathway [22]. This pathway is important in modulating the reactivity of HQ–GSH conjugate. Based on the (re)activity of HQ–GSH conjugates, we speculated that some of the hematotoxic effects attributed to HQ (or 1,4-BQ) may, in fact, be mediated by their thiol conjugates. Indeed, as shown by Monks et al (this issue), HQ–GSH conjugates are far more efficient generators of superoxide anion than the HQ/1,4-BQ redox couple. Moreover, HQ–GSH conjugates are toxic to developing erythrocytes *in vivo* [22]. Whether this is a direct effect of these conjugates in bone marrow, or whether additional tissues/organs contribute to the observed erythroid toxicity is not known. The hematopoietic microenvironment is regulated by stromal cells which secrete a variety of cytokines, including interleukin-1 (IL-1), tumor necrosis factor- α , granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and macrophage

colony-stimulating factor (M-CSF). In addition, kidney peritubular cells produce and secrete the hormone, erythropoietin (EPO) [36,37]. These growth factors interact with various hematopoietic stem/progenitor cells to control cellular proliferation and differentiation. Because HQ–GSH conjugates are capable of damaging cells within the proximal tubules of the kidney [21], the major site of EPO production, we hypothesized that 2,3,5-GS-HQ and 2,6-GS-HQ might also be capable of indirectly inducing anemia by reducing serum EPO levels. Consistent with this reasoning, both 2,3,5-GS-HQ and 2,6-GS-HQ caused reductions in circulating EPO levels (Fig. 3). We have previously shown that quinone–thioethers are “erythroid toxic” based on a reduction in iron uptake [22]. However, EPO drives RBC production and consequently the incorporation of iron. It is therefore possible that the reduced iron uptake involved both decreased production of EPO as well as direct toxicity to existing RBCs. In this report we have shown that quinone–thioethers induced a reduction in circulating lymphocytes, consistent with bone marrow toxicity, as previously reported [22]. However a possible redistribution of lymphocytes from the peripheral blood into tissues cannot be ruled out.

Adduction of proteins by reactive electrophiles is not a random event, but rather specific proteins appear to be targeted. These protein targets may differ between different electrophiles, as illustrated with HQ–GSH and 4HNE modified proteins (Fig. 1), but may also exhibit overlap (e.g. the 50 kD proteins). Chemical structure, reactivity, and ability to localize within the various intracellular compartments are several characteristics that likely govern which proteins are targeted by a given electrophile. We have provided evidence in support of the existence of “electrophile binding motifs” (EBMs) within proteins in the kidney that are selectively adducted by reactive electrophiles following GS–HQ administration [24]. Whether similar EBMs exist within the bone marrow adductome deserves attention. With respect to the reactive metabolites derived from GS–HQ, they appear to selectively target (i) proteins with a high lysine (basic amino acid) content, and specifically (ii) proteins that contain lysine residues either flanking a potentially nucleophilic amino acid [KXX], or containing two lysine residues preceded or followed by a nucleophilic amino acid [XKK or KXX] [24]. Although we have detected GS–HQ-adducted proteins in bone marrow of PHE/HQ treated rats (Fig. 1) the identity of these proteins, and the site(s) of adduction, remain to be determined. One such target may be γ -GT. Indeed, HQ–GSH conjugates are substrates for, and inhibit, renal γ -GT [38]. Consistent with this view, 2,3,5-GS-HQ inhibits γ -GT in bone marrow cell lysates and in HL-60 cells (Fig. 2).

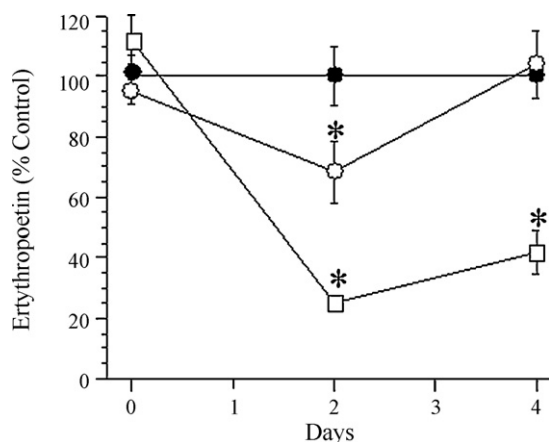


Fig. 3. Effect of HQ–GSH conjugates on rat serum erythropoietin levels. Rats were given daily doses of either (●) saline; (○) 2,3,5-GS-HQ (10 μ mol/kg, i.v.) or; (□) 2,6-GS-HQ (50 μ mol/kg, i.v.). Each point represents the mean $\pm$ SEM ($n=5$). One-way ANOVA; SNK.

Both “free” and protein-bound HQ–GSH conjugates (and metabolites thereof) retain the ability to redox cycle and generate ROS. One consequence of which is the initiation of lipid peroxidation. Cysteine thiols are common targets of reactive electrophilic metabolites, and of endogenous electrophiles, including lipid-derived α,β -unsaturated aldehydes, such as 4-hydroxynonenal (4HNE) and 4-oxononenal (4ONE), and ROS. In addition to the formation of HQ–GSH-derived protein adducts, PHE/HQ administration to rats also results in the formation of 4HNE–protein adducts (Fig. 1). Although in most cases, the toxicological significance of protein adducts remains uncertain, physiological concentrations of either 4HNE or 4ONE cause the cross-linking of bovine brain tubulin, and an inability of tubulin to polymerize [39]. 4HNE also reduces ERK-1/2 phosphorylation causing a loss of activity and of nuclear localization. Interestingly, the loss of ERK activity is caused by a single 4HNE modification, on histidine 178. While the precise toxicological implication of proteins modified by 4HNE is not known, recent evidence suggests a role in the pathogenesis of several diseases [40].

Conflict of interest

None.

Acknowledgements

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