Published in final edited form as:

Chem Biol Interact. 2010 March 19; 184(1-2): 212–217. doi:10.1016/j.cbi.2009.12.016.

Role of Hydroquinone-Thiol Conjugates in Benzene-Mediated Toxicity

Serrine S. Lau¹, Christopher Kuhlman¹, Shawn B. Bratton^{2,3}, and Terrence J. Monks¹

¹ Southwest Environmental Health Sciences Center, Department of Pharmacology and Toxicology, The University of Arizona Health Sciences Center, Tucson, Arizona 85721

² Division of Pharmacology & Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, Texas 78712

³ Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712

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 hrs 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.

All correspondence should be addressed to: Dr. Serrine S. Lau, Southwest Environmental Health Sciences Center, Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, P.O. Box 210207, 1703 E. Mabel Street, Tucson, AZ 85721, (520) 626-0460 (TEL), (520) 626-6944 (FAX), lau@pharmacy.arizona.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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*. Coadministration 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 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 coadministration 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(i) 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 chemiluminescent reagent (ECL) and Hyperfilm ECL were purchased from Amersham Life Science (Arlington Heights, IL). Acivicin was obtained from Sigma.

2(ii) Animals

Male and Female SD rats (n=2–4@ 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(iii) 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 μ mol of p-nitroanilide formed/min at 37° C.

2(iv) 1D Western Analysis

Proteins (40 μ g) 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(v) 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% CO₂ 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(vi) 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 μ L) 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.

3. RESULTS

3(i) Effect of Blood Counts After Phenol/Hydroquinone Administration

Complete blood counts of rats 18 hr 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). All other leukocyte and erythrocyte counts were within their normal ranges following treatment.

3(ii) 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 minutes) neurological side-effect is observed. Interestingly the intensity of this side-effect correlates with the intensity of protein immuno-staining 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 Figure 1. Unique GS-HQ-immunopositive bands (26 kD, 43 kD, and 140 kD) were detected in bone marrow protein of rats co-administered HQ/PHE. GS-HQ-immunopositive bands, at 50 kD and 65 kD, were detected in bone marrow protein from both treated and untreated control male rats (Figure 1A). 4HNE immunopositive bands (25 kD, 27 kD, 43 kD and 59 kD) were detected in bone marrow protein of treated rats (Figure 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 (Figure 1B). The immuno-reactive bands from control rat represent endogenously modified protein(s), possibly derived from dietary sources.

3(iii) 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 (Figure 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(iv) Reactivity of HQ-thioethers: Effect on Plasma Erythropoietin

Erythrotoxicity of several of these conjugates was determined in rats using the erythrocyte ^{59}Fe incorporation assay [22]. Administration of 2,3,5-GS-HQ (17 $\mu\text{mol/kg}$, iv), 2,6-GS-HQ (50 $\mu\text{mol/kg}$, iv), and benzene (11 mmol/kg, sc) significantly decreased ^{59}Fe 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 ^{59}Fe incorporation to the same degree as benzene. These data suggest that HQ-GSH conjugates are erythrotoxic and may contribute to benzene-mediated hematotoxicity [22]. Because HQGSH 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 (Figure 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 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 erythrotoxicity 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 (Figure 3). We have previously shown that quinone-thioethers are “erythrotoxic” 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 (Figure 1), but may also exhibit overlap (eg the 50kD 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 exists 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 KKK] [24]. Although we have detected GS-HQ-adducted proteins in bone marrow of PHE/HQ treated rats (Figure 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 (Figure 2).

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 (Figure 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].

Acknowledgments

This work was supported by RO1 GM070890 (SSL). The authors also acknowledge the support of the P30 ES006694 Southwest Environmental Health Sciences Center, in particular the Arizona Proteomics Consortium (APC) and Dr. George Tsapralis, Director of the APC. The generous gift of anti-4HNE antibody from Dr. Dennis R. Petersen at the University of Colorado Health Sciences Center is greatly appreciated.

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- <i>bis</i> -(glutathion-S-yl)HQ
2,6-GS-HQ	2,6- <i>bis</i> -(glutathion-S-yl)HQ
2,3,5-GS-HQ	2,3,5- <i>tris</i> -(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

References

1. Eastmond DA, Smith MT, Irons RD. An interaction of benzene metabolites reproduces the myelotoxicity observed with benzene exposure. *Toxicol Appl Pharmacol* 1987;91(1):85–95. [PubMed: 2823417]
2. Legathe A, Hoener BA, Tozer TN. Pharmacokinetic interaction between benzene metabolites, phenol and hydroquinone, in B6C3F1 mice. *Toxicol Appl Pharmacol* 1994;124(1):131–138. [PubMed: 8291054]
3. Pirozzi SJ, Schlosser MJ, Kalf GF. Prevention of benzene-induced myelotoxicity and prostaglandin synthesis in bone marrow of mice by inhibitors of prostaglandin H synthase. *Immunopharmacology* 1989;18(1):39–55. [PubMed: 2504682]
4. Schattenberg DG, Stillman WS, Gruntmeir JJ, Helm KM, Irons RD, Ross D. Peroxidase activity in murine and human hematopoietic progenitor cells: potential relevance to benzene-induced toxicity. *Mol Pharmacol* 1994;46(2):346–351. [PubMed: 8078496]
5. Smart RC, Zannoni VG. DT-diaphorase and peroxidase influence the covalent binding of the metabolites of phenol, the major metabolite of benzene. *Mol Pharmacol* 1984;26(1):105–111. [PubMed: 6749127]
6. Smith CJ, Zhang Y, Koboldt CM, Muhammad J, Zweifel BS, Shaffer A, Talley JJ, Masferrer JL, Seibert K, Isakson PC. Pharmacological analysis of cyclooxygenase-1 in inflammation. *Proc Natl Acad Sci U S A* 1998;95(22):13313–13318. [PubMed: 9789085]
7. Thomas DJ, Sadler A, Subrahmanyam VV, Siegel D, Reasor MJ, Wierda D, Ross D. Bone marrow stromal cell bioactivation and detoxification of the benzene metabolite hydroquinone: comparison of macrophages and fibroblastoid cells. *Mol Pharmacol* 1990;37(2):255–262. [PubMed: 2154673]
8. Irons RD. Quinones as toxic metabolites of benzene. *J Toxicol Environ Health* 1985;16(5):673–678. [PubMed: 4093989]
9. Schlosser MJ, Shurina RD, Kalf GF. Metabolism of phenol and hydroquinone to reactive products by macrophage peroxidase or purified prostaglandin H synthase. *Environ Health Perspect* 1989;82:229–237. [PubMed: 2551664]
10. Subrahmanyam VV, Doane-Setzer P, Steinmetz KL, Ross D, Smith MT. Phenol-induced stimulation of hydroquinone bioactivation in mouse bone marrow in vivo: possible implications in benzene myelotoxicity. *Toxicology* 1990;62(1):107–116. [PubMed: 2343455]
11. Sipes, IG.; Maqueen, CA.; Gandolfi, AJ., editors. *Comprehensive Toxicology*. Elsevier Science Pub Co; 1997. Renal Toxicology.
12. Subrahmanyam VV, Kolachana P, Smith MT. Metabolism of hydroquinone by human myeloperoxidase: mechanisms of stimulation by other phenolic compounds. *Arch Biochem Biophys* 1991;286(1):76–84. [PubMed: 1654782]
13. Subrahmanyam VV, Ross D, Eastmond DA, Smith MT. Potential role of free radicals in benzene-induced myelotoxicity and leukemia. *Free Radic Biol Med* 1991;11(5):495–515. [PubMed: 1769608]
14. Greenlee WF, Sun JD, Bus JS. A proposed mechanism of benzene toxicity: formation of reactive intermediates from polyphenol metabolites. *Toxicol Appl Pharmacol* 1981;59(2):187–195. [PubMed: 7256763]
15. Smith MT, Yager JW, Steinmetz KL, Eastmond DA. Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. *Environ Health Perspect* 1989;82:23–29. [PubMed: 2551665]
16. Irons RD, Greenlee WF, Wierda D, Bus JS. Relationship between benzene metabolism and toxicity. A proposed mechanism for the formation of reactive intermediates from poly-phenol metabolites. *Adv Exp Med Biol* 1982;136A:229.
17. Yeowell-O'Connell K, Rothman N, Smith MT, Hayes RB, Li G, Waidyanatha S, Dosemeci M, Zhang L, Yin S, Titenko-Holland N, Rappaport SM. Hemoglobin and albumin adducts of benzene oxide among workers exposed to high levels of benzene. *Carcinogenesis* 1998;19(9):1565–1571. [PubMed: 9771926]

18. Yeowell-O'Connell K, Rothman N, Waidyanatha S, Smith MT, Hayes RB, Li G, Bechtold WE, Dosemeci M, Zhang L, Yin S, Rappaport SM. Protein adducts of 1,4-benzoquinone and benzene oxide among smokers and nonsmokers exposed to benzene in China. *Cancer Epidemiol Biomarkers Prev* 2001;10(8):831–838. [PubMed: 11489749]
19. McDonald TA, Yeowell-O'Connell K, Rappaport SM. Comparison of protein adducts of benzene oxide and benzoquinone in the blood and bone marrow of rats and mice exposed to [¹⁴C/¹³C₆] benzene. *Cancer Res* 1994;54(18):4907–4914. [PubMed: 8069856]
20. Williams KE, Carver TA, Miranda JJ, Kautiainen A, Vogel JS, Dingley K, Baldwin MA, Turteltaub KW, Burlingame AL. Attomole detection of in vivo protein targets of benzene in mice: evidence for a highly reactive metabolite. *Mol Cell Proteomics* 2002;1(11):885–895. [PubMed: 12488464]
21. Lau SS, Hill BA, Highet RJ, Monks TJ. Sequential oxidation and glutathione addition to 1,4-benzoquinone: correlation of toxicity with increased glutathione substitution. *Mol Pharmacol* 1988;34(6):829–836. [PubMed: 3200250]
22. Bratton SB, Lau SS, Monks TJ. Identification of quinol thioethers in bone marrow of hydroquinone/phenol-treated rats and mice and their potential role in benzene-mediated hematotoxicity. *Chem Res Toxicol* 1997;10(8):859–865. [PubMed: 9282834]
23. Kleiner HE, Jones TW, Monks TJ, Lau SS. Immunochemical analysis of quinol-thioether-derived covalent protein adducts in rodent species sensitive and resistant to quinol-thioether-mediated nephrotoxicity. *Chem Res Toxicol* 1998;11(11):1291–1300. [PubMed: 9815189]
24. Labenski MT, Fisher AA, Lo HH, Monks TJ, Lau SS. Protein electrophile-binding motifs: lysine-rich proteins are preferential targets of quinones. *Drug Metab Dispos* 2009;37(6):1211–1218. [PubMed: 19251821]
25. Fisher AA, Labenski MT, Malladi S, Gokhale V, Bowen ME, Milleron RS, Bratton SB, Monks TJ, Lau SS. Quinone electrophiles selectively adduct “electrophile binding motifs” within cytochrome c. *Biochemistry* 2007;46(39):11090–11100. [PubMed: 17824617]
26. Kleiner HE, Rivera MI, Pumford NR, Monks TJ, Lau SS. Immunochemical detection of quinol--thioether-derived protein adducts. *Chem Res Toxicol* 1998;11(11):1283–1290. [PubMed: 9815188]
27. Roede JR, Stewart BJ, Petersen DR. Decreased expression of peroxiredoxin 6 in a mouse model of ethanol consumption. *Free Radic Biol Med* 2008;45(11):1551–1558. [PubMed: 18852041]
28. Lau SS, Monks TJ, Everitt JI, Kleyменова E, Walker CL. Carcinogenicity of a nephrotoxic metabolite of the “nongenotoxic” carcinogen hydroquinone. *Chem Res Toxicol* 2001;14(1):25–33. [PubMed: 11170505]
29. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979;76(9):4350–4354. [PubMed: 388439]
30. Baruchel S, Bernstein M, Whitehead VM, Devine S, Bell B, Dubowy R, Grier H, Kretschmar C, Langevin AM, Vietti T. A phase I study of acivicin in refractory pediatric solid tumors. A Pediatric Oncology Group study. *Invest New Drugs* 1995;13(3):211–216. [PubMed: 8729948]
31. Earhart RH, Khandekar JD, Faraggi D, Schinella RA, Davis TE. Phase II trial of continuous drug infusions in advanced ovarian carcinoma: acivicin versus vinblastine. *Invest New Drugs* 1989;7(2–3):255–260. [PubMed: 2793383]
32. Maroun JA, Stewart DJ, Verma S, Evans WK, Eisenhauer E. Phase I study of acivicin and cisplatin in non-small-cell lung cancer. A National Cancer Institute of Canada study. *Am J Clin Oncol* 1990;13(5):401–404. [PubMed: 2171318]
33. Dethmers JK, Meister A. Glutathione export by human lymphoid cells: depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation. *Proc Natl Acad Sci U S A* 1981;78(12):7492–7496. [PubMed: 6950392]
34. van den Dobbelen DJ, Nobel CSI, Slater AF, Orrenius S. Regulation and mechanisms of apoptosis in T lymphocytes. *Arch Toxicol* 1996;19(Suppl):77–85.
35. Bratton SB, Lau SS, Monks TJ. The putative benzene metabolite 2,3, 5-tris(glutathion-S-yl) hydroquinone depletes glutathione, stimulates sphingomyelin turnover, and induces apoptosis in HL-60 cells. *Chem Res Toxicol* 2000;13(7):550–556. [PubMed: 10898586]

36. Blanchard KL, Acquaviva AM, Galson DL, Bunn HF. Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. *Mol Cell Biol* 1992;12(12):5373–5385. [PubMed: 1448072]
37. Groopman JE, Molina JM, Scadden DT. Hematopoietic growth factors. Biology and clinical applications. *N Engl J Med* 1989;321(21):1449–1459. [PubMed: 2682244]
38. Hill BA, Lo HH, Monks TJ, Lau SS. The role of gamma-glutamyl transpeptidase in hydroquinone-glutathione conjugate mediated nephrotoxicity. *Adv Exp Med Biol* 1991;283:749–751. [PubMed: 1676872]
39. Stewart BJ, Doorn JA, Petersen DR. Residue-specific adduction of tubulin by 4-hydroxynonenal and 4-oxononenal causes cross-linking and inhibits polymerization. *Chem Res Toxicol* 2007;20(8):1111–1119. [PubMed: 17630713]
40. Petersen DR, Doorn JA. Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radic Biol Med* 2004;37(7):937–945. [PubMed: 15336309]

Figure 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.

Figure 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 X g for 10 min. The supernatant was used as the enzyme source. (●) 2,3,5-GS-HQ; (○), Acivicin.

Figure 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.

Table

Effect of Phenol/Hydroquinone on Blood Lymphocyte Counts

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

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