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The fate of benzene-oxide

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ABSTRACT

Metabolism is a prerequisite for the development of benzene-mediated myelotoxicity. Benzene is initially metabolized via cytochromes P450 (primarily CYP2E1 in liver) to benzene-oxide, which subsequently gives rise to a number of secondary products. Benzene-oxide equilibrates spontaneously with the corresponding oxepine valence tautomer, which can ring open to yield a reactive α,β -unsaturated aldehyde, trans-trans-muconaldehyde (MCA). Further reduction or oxidation of MCA gives rise to either 6-hydroxy-trans-trans-2,4-hexadienal or 6-hydroxy-trans-trans-2,4-hexadienoic acid. Both MCA and the hexadienal metabolite are myelotoxic in animal models. Alternatively, benzene-oxide can undergo conjugation with glutathione (GSH), resulting in the eventual formation and urinary excretion of S-phenylmercapturic acid. Benzene-oxide is also a substrate for epoxide hydrolase, which catalyzes the formation of benzene dihydrodiol, itself a substrate for dihydrodiol dehydrogenase, producing catechol. Finally, benzene-oxide spontaneously rearranges to phenol, which subsequently undergoes either conjugation (glucuronic acid or sulfate) or oxidation. The latter reaction, catalyzed by cytochromes P450, gives rise to hydroquinone (HQ) and 1,2,4-benzene triol. Co-administration of phenol and HQ reproduces the myelotoxic effects of benzene in animal models. The two diphenolic metabolites of benzene, catechol and HQ undergo further oxidation to the corresponding *ortho*-(1,2-), or *para*-(1,4-)benzoquinones (BQ), respectively. Trapping of 1,4-BQ with GSH gives rise to a variety of HQ-GSH conjugates, several of which are hematotoxic when administered to rats. Thus, benzene-oxide gives rise to a cascade of metabolites that exhibit biological reactivity, and that provide a plausible metabolic basis for benzene-mediated myelotoxicity. Benzene-oxide itself is remarkably stable, and certainly capable of translocating from its primary site of formation in the liver to the bone marrow. However, therein lies the challenge, for although there exists a plethora of information on the metabolism of benzene, and the fate of benzene-oxide, there is a paucity of data on the presence, concentration, and persistence of benzene metabolites in bone marrow. The major metabolites in bone marrow of mice exposed to 50 ppm [^3H]benzene are muconic acid, and glucuronide and/or sulfate conjugates of phenol, HQ, and catechol. Studies with [$^{14}\text{C}/^{13}\text{C}$]benzene revealed the presence in bone marrow of protein adducts of benzene-oxide, 1,4-BQ, and 1,4-BQ, the relative abundance of which was both dose and species dependent. In particular, histones are bone marrow targets of [^{14}C]benzene, although the identity of the reactive metabolite(s) giving rise to these adducts remain unknown. Finally, hematotoxic HQ-GSH conjugates are present in the bone marrow of rats receiving the HQ/phenol combination. In summary, although the fate of benzene-oxide is known in remarkable detail, coupling this information to the site, and mechanism of action, remains to be established.

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1. One hundred and forty years, and counting

The metabolic conversion of benzene to phenol was demonstrated by Schultzen and Naunyn in 1867 [1], and the conjugation of phenol with sulfate was established in 1876 by Baumann [2]. At that

time, the mechanism of this metabolic conversion was not known, but following reports that anthracene [3], naphthalene [4], and phenanthrene [5] were all metabolized to dihydrodiols, Boyland [6] suggested that epoxide formation was the requisite intermediary in this metabolic reaction, simultaneously providing an explanation for the formation of other aromatic hydrocarbon metabolites (phenols, ring-opening). Boyland's suggestion occurred at about the same time (1949) that Dennis Parke joined R.T. Williams' group at St. Mary's Hospital Medical School, where he embarked on a study of all the known pathways of benzene metabolism, focusing particularly on whether benzene did indeed form an epoxide and/or a dihydrodiol, and on the isomers of muconic acid. The metabolism

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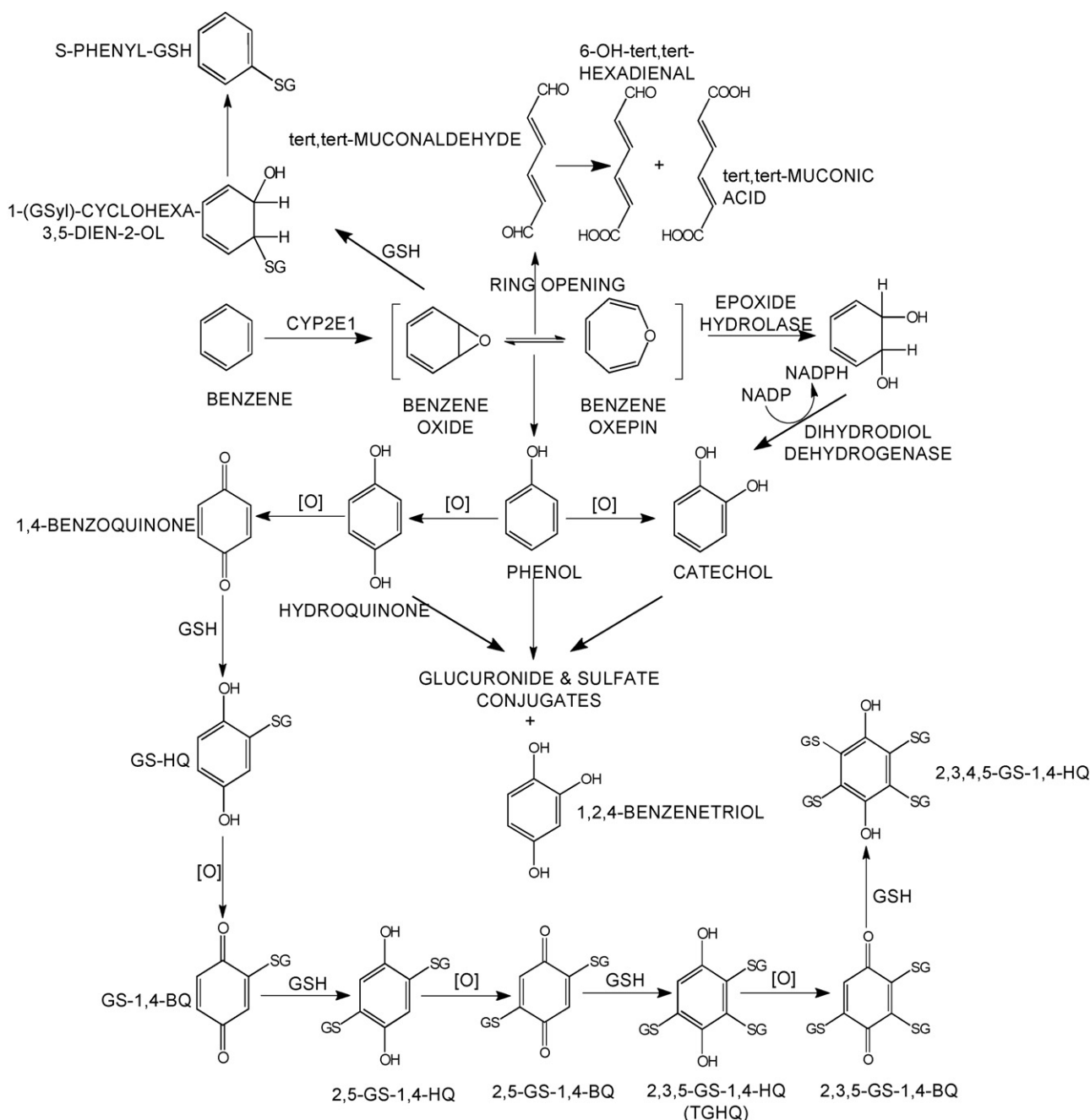


Fig. 1. The disposition of benzene-oxide. See text for detailed description of the various metabolic pathways.

of the simplest of aromatic hydrocarbons has subsequently been revealed to be comparatively complex, giving rise to a myriad of metabolites.

2. Benzene-oxide

The first step in the metabolism of benzene is the cytochrome P450 catalyzed formation of benzene-oxide (Fig. 1). Epoxides such as benzene-oxide can be somewhat ambiguous in their nature. On the one hand, they are sufficiently electrophilic that they can react and covalently bind to nucleophilic sites within proteins and DNA. In contrast, epoxides can be relatively stable, in chemical terms, with half-lives in biological milieu ranging from seconds to minutes. For example, bromobenzene-3,4-oxide is capable of diffusing out of hepatocytes and being trapped extracellularly by

glutathione (GSH) and the requisite GSH transferase (GST) [7]. Indeed, bromobenzene-3,4-oxide is sufficiently stable that it can be detected in venous blood of rats receiving bromobenzene via intraperitoneal injections, with a half-life of ~13.5 s [8], which is more than sufficient time to reach extrahepatic targets. Benzene-oxide has also been directly identified in the blood of rats administered benzene (400 mg/kg) and has a half-life of ~8 min when added to rat blood *ex vivo* [9]. Since the mean circuit time of blood in rats (blood volume/cardiac output) is about 5–10 s, the concentration of benzene-oxide will not decrease greatly in a single pass between organs. Thus, nearly all of the benzene-oxide leaving the liver (and any other organ in which it is formed) will enter the lung, and the concentration of the epoxide leaving the lung will be essentially identical to its concentration in arterial blood entering the other organs/tissues of the body, including the bone

marrow. The extent to which benzene-oxide in arterial blood contributes to its covalent binding in extrahepatic tissue, in particular bone marrow, will depend on the ability of the various tissues to convert the epoxide into downstream metabolites, and on its non-enzymatic rearrangement to phenol and ring-opened products. However, each particular non-enzymatic rearrangement reaction should occur at the same rate in all tissues. It will therefore be the relative distribution of the enzymes that catalyze the conversion of benzene-oxide into the dihydrodiol (epoxide hydrolase) and *S*-phenylpremercaptopuric acids (GST) that limit the exposure of extrahepatic tissues to blood-borne benzene-oxide.

Although liver-derived benzene-oxide is clearly capable of reaching bone marrow, is it sufficiently biologically reactive to play a major direct role in benzene-mediated hematotoxicity? The half-life of benzene-oxide in aqueous medium (95:5 [v/v] phosphate buffer in D₂O with [CD₃]₂SO) at 25 °C (pD/pH 7) was ~34 min [10]. Moreover, the half-life did not change in the presence of GSH (2–15 mM) or with a combination of GSH (2 mM) and GST [10]. In fact, the major product observed under these experimental conditions was phenol. These findings are consistent with the fact that *S*-phenyl-mercaptopuric acid is a relatively minor urinary metabolite of benzene. Perhaps the role of benzene-oxide in benzene hematotoxicity is to assist in the delivery of phenol and/or the ring-opened products (see below) to the bone marrow, where they may undergo further metabolic transformations into more reactive metabolites.

In contrast to the apparent stability of benzene-oxide, and it is inefficient reaction with GSH, *S*-phenyl-L-cysteine, presumed to arise from the interaction of benzene-oxide with cysteine residues within proteins, has been isolated from hemoglobin and bone marrow proteins [11]. It would seem likely that the protein microenvironment increases the efficiency with which these cysteinyl thiols react with benzene-oxide. Consistent with this view, increasing the pH from 7.0 to 8.5 dramatically increases (20-fold) the reaction of GSH with benzene-oxide [10]. Nonetheless, the major protein adducts identified in mouse marrow after benzene administration were derived from 1,4-benzoquinone (see Section 4) rather than benzene-oxide (<2%) [11]. Interestingly, the metabolites representing the major source of benzene-derived protein adducts in bone marrow remain to be identified. A more in-depth analysis of this issue can be found in the following presentations.

3. trans-trans-Muconaldehyde (MCA)

Benzene-oxide can equilibrate spontaneously with the corresponding oxepin valence tautomer, which can ring open to initially yield a reactive α,β -unsaturated aldehyde, cis-cis-muconaldehyde, which subsequently isomerizes to the cis-trans, and ultimately to the trans-trans-muconaldehyde (MCA) isomer. The mechanism of ring-opening remains debatable, but has been proposed to occur *via* cytochrome P450-mediated metabolism of the oxepin to the oxepin oxide [12]. Further reduction or oxidation of MCA gives rise to a variety of products. *In vitro* model systems indicate that cytosolic aldehyde dehydrogenases may oxidize MCA to 6-oxo-trans-trans-hexadienoic acid, a mixed-aldehyde acid whose aldehyde functional group can undergo (i) oxidation to form trans-trans-muconic acid or (ii) reduction to 6-hydroxy-2,4-trans-trans-hexadienoic acid. Muconic acid was the first ring-opened urinary metabolite to be identified from benzene-treated animals, by Jaffe in 1909 [13]. However, at that time the prevailing view was that the cis-cis isomer was the primary product, and it was not until 1952 that Parke and Williams unequivocally confirmed the structure as trans-trans-muconic acid, following [¹⁴C]-benzene administration to rabbits [14]. Alternatively, cytosolic alcohol dehydrogenases can reduce (reversible reaction) MCA to 6-hydroxy-2,4-trans-trans-hexadienal, a mixed-aldehyde

alcohol whose aldehyde functional group can undergo oxidation to form 6-hydroxy-2,4-trans-trans-hexadienoic acid [15]. It is important to note, however, that while [³H]-trans-trans-muconic acid and [¹⁴C]-6-hydroxy-2,4-trans-trans-hexadienoic acid have been isolated from the urine, blood, and/or bone marrow of rodents treated with [³H] or [¹⁴C]benzene, the reactive aldehydes, MCA and 6-hydroxy-2,4-trans-trans-hexadienal, have yet to be isolated *in vivo*. Thus, while MCA is produced in liver microsomal incubations containing benzene [16], and is undoubtedly an *in vivo* metabolite of benzene, albeit transient, the reactivity of this metabolite (6 s in the presence of GSH) suggests that hepatic-derived MCA is unlikely to reach the bone marrow in sufficient quantities to cause toxicity [17]. This again emphasizes the critical balance between the chemical stability and the biological reactivity of benzene metabolites with respect to their potential role in benzene-mediated hematotoxicity. Both MCA and the hexadienal metabolite are myelotoxic in animal models [18]. Of current interest is the ability of benzene metabolites to block gap junction intercellular communication, in particular hydroquinone, MCA, and 6-hydroxy-2,4-trans-trans-hexadienal [19]. MCA decreases connexin 43 levels, inhibition of which has been linked to disruptions in hematopoiesis. More recently, MCA-induced cross-linking of connexin 43 likely underlies the inhibition of gap junction intercellular communication [20], further elaboration of which will be provided later in this session. However, information on the presence of ring-opened metabolites in bone marrow following exposure to benzene remains limited (see Section 5).

4. Phenol and hydroquinone

Benzene-oxide spontaneously rearranges to phenol, the majority of which undergoes conjugation with either glucuronic acid or sulfate. That fraction that escapes conjugation can be further oxidized, with hydroquinone (HQ) as one of the products. Phenol itself is not hematotoxic, but synergistic effects of phenol and HQ, both of which accumulate in bone marrow, has become an established model of benzene hematotoxicity [21]. It should be emphasized that although animal models can be an appropriate approximation for human benzene metabolism, they are a less than ideal model for the human myelotoxic effects of benzene; perhaps highlighting the fact that metabolism may be necessary, but not sufficient for benzene hematotoxicity. The effectiveness of the HQ/phenol combination appears to be related, in part, to a pharmacokinetic interaction between HQ and phenol [22]. Phenol likely competes with HQ for conjugative enzymes and depletes the liver of UDPGA and PAPS, resulting in greater fractions of HQ and phenol available for delivery to bone marrow [23]. Bone marrow suppression may then result from phenol-stimulated peroxidase and/or phenoxyl radical-mediated oxidation of HQ, which (in theory) initiates redox cycling and leads to the formation of the reactive intermediates, 1,4-benzo-semiquinone and 1,4-BQ. However, the current model of benzene hematotoxicity may need to be extended to include a role for thioether metabolites of HQ (see below). While depletion of cofactors for glucuronidation and sulfation increases the amount of “free” HQ and phenol in bone marrow, it will also increase the fraction available for oxidation and GSH conjugation. Such increases could be critical since both 2,6-(GS)-HQ and 2,3,5-(GS)-HQ are very potent hematotoxicants *in vivo*, inhibiting [⁵⁹Fe] incorporation to the same degree as benzene at significantly lower doses (see Lau et al., *in press*). Based on the (re)activity of HQ-GSH conjugates, it is possible that many of the hematotoxic effects attributed to HQ (or 1,4-BQ) may, in fact, be mediated by their thiol conjugates. Indeed, although semiquinone formation from a variety of quinones leads to O₂ consumption, no O₂ consumption occurs in reactions in which the 1,4-benzosemiquinone free radical

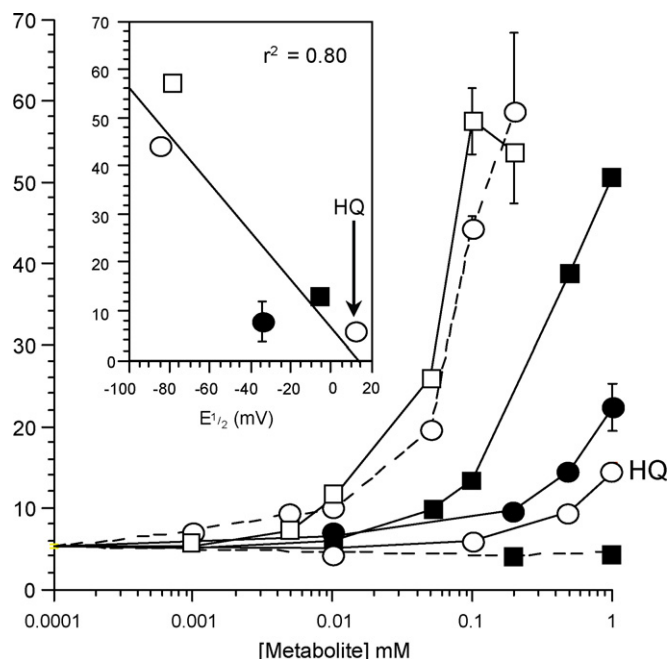


Fig. 2. Microsomes (0.5 mg/mL protein) were preincubated with acivicin (10 mM) for 15 min and then incubated with various concentrations of either phenol (■, dashed line), HQ (○), GS-HQ (●), 2,5-(GS)-HQ (○, dashed line), 2,6-(GS)-HQ (□), or 2,3,5-(GS)-HQ (■), in the presence of succinylated cytochrome C (12.5 μM) and an NADPH generating system. Superoxide anion formation is expressed as nmol/(mg protein min). The inset shows the correlation between the oxidation potentials [$E_{1/2}$ (mV)] for the HQ and its GSH conjugates, and their ability to catalyze superoxide anion formation. Each data point represents the mean $\pm$ SEM ($n = 3$).

is formed enzymatically [24], because 1,4-benzoquinone is so electron affinic that its rate of reduction by $O_2^{\bullet-}$ [25] is over four orders of magnitude faster than the reverse reaction (reduction of O_2 to $O_2^{\bullet-}$) [26,27], which is usually responsible for O_2 consumption via redox cycling. Thus, although redox cycling of semiquinone radicals resulting in the generation of reactive oxygen species is proposed to be of major importance in the toxicity of many quinones, this mechanism is clearly excluded in the case of 1,4-BQ [28] (see also Fig. 2). Indeed, although 1,4-BQ is cytotoxic to hepatocytes, it causes rapid depletion of cellular thiols without oxidative stress [29].

5. Hydroquinone-thiol conjugates

HQ is readily oxidized to 1,4-BQ, which is then efficiently scavenged by GSH. However, this reaction does not represent a true detoxication reaction, since the initial conjugate, 2-(Glutathion-S-yl)hydroquinone (GS-HQ), is also readily oxidized to the corresponding GS-1,4-BQ. This cycle of the reductive addition of GSH to the quinone and subsequent cross-oxidation continues, resulting in the eventual formation of all possible addition products, GS-HQ, 2,3-(GS)-HQ, 2,5-(GS)-HQ, 2,6-(GS)-HQ, 2,3,5-(GS)-HQ, and 2,3,4,5-(GS)-HQ [30]. The *in vivo* relevance of these reactions was demonstrated following the administration of HQ (1.8 mmol/kg, i.p.) to AT-125 (an inhibitor of γ -glutamyl transpeptidase [γ -GT]) pretreated male Sprague–Dawley rats. Five S-conjugates of HQ were identified in bile and one S-conjugate in urine [31]. The major biliary S-conjugate identified was GS-HQ. Additional biliary metabolites were 2,5-(GS)-HQ, 2,6-(GS)-HQ, and 2,3,5-(GS)-HQ. 2-(N-Acetylcystein-S-yl)HQ was the only urinary thioether metabolite identified. The quantity of S-conjugates excreted in urine and bile within 4 h of HQ administration was sufficient to propose a role for such metabolites in HQ mediated nephrotoxic-

ity and nephrocarcinogenicity. Whether the conjugates are formed in sufficient amounts following either HQ/phenol or benzene administration to contribute to benzene-mediated hematotoxicity remains to be determined, but they can be identified in the bone marrow of HQ/phenol or benzene-treated animals (see Section 6).

6. Benzene metabolites in bone marrow

The gap in our knowledge with respect to benzene metabolism is the extent to which each of the metabolites exist/persist in bone marrow. The glucuronide conjugates of both phenol and HQ were identified in the bone marrow of B6C3F1 mice, but not in the bone marrow of F344 rats exposed for 6 h to 50 ppm benzene [32] an intriguing finding especially given the fact that such conjugates are readily recoverable in the urine of these animals. More intriguing is the observation that HQ conjugates could not be detected in blood, liver, or lung of F344 rats, whereas they are readily detected in these tissues in B6C3F1 mice. Whether phenol is generated within bone marrow, or delivered there either as the conjugate or in the free form is not known. In addition to HQ and phenol conjugates, trans-trans-muconic acid and phenyl or catechol sulfate were also identified in the bone marrow of B6C3F1 mice and F344 rats, with the latter representing the highest fraction of benzene metabolites in both species. The presence of benzene metabolites in target tissue in humans is complicated by the potential contribution of dietary, over-the-counter medicinals, and perhaps other environmental sources (cigarette smoke) of such metabolites. For example, phenol, HQ, catechol, and trans-trans-muconic acid may all be derived from dietary sources [33,34], in addition to environmental/occupational exposures to benzene.

More recently, following co-administration of HQ/phenol (2.0 mmol/kg, i.p.) to rats, GS-HQ, 2,5-(GS)-HQ, 2,6-(GS)-HQ, and 2,3,5-(GS)-HQ were all detected in bone marrow [35]. The γ -GT catalyzed metabolite of GS-HQ, 2-(Cys-Gly)HQ, the dipeptidase metabolite, 2-(Cys)HQ, and the mercapturic acid metabolite, 2-(NAC)HQ were also all identified in bone marrow. Moreover, 2-(Cys)HQ and 2-(NAC)HQ appeared to persist in bone marrow. A similar metabolic profile was observed in mice, with the exception that 2,5-(GS)-HQ, 2,6-(GS)-HQ, and 2,3,5-(GS)-HQ were only sporadically detected. The latter conjugates were only observed in mice exhibiting high concentrations of GS-HQ in marrow. Nonetheless, concentrations of quinol thioethers in bone marrow were higher in mice than in rats of HQ/phenol-treated animals, which correlates with the relative sensitivity of these two species to benzene-induced hematotoxicity [36]. Similar results were obtained in benzene-treated animals. Following twice daily administration of benzene (11.2 mmol/kg) for 2 days, GS-HQ, (Cys-Gly)-HQ, (Cys)-HQ, and (NAC)-HQ were all detected in the bone marrow of rats and mice. The presence of a functional mercapturic acid pathway in bone marrow of both species was confirmed by *in vitro* studies. Concentrations of (Cys)-HQ were relatively high and persisted in bone marrow. The availability of bone marrow γ -GT and dipeptidases for processing of GSH conjugates is important, because Cys-Gly and Cys conjugates of HQ are more chemically (re)active than their corresponding GSH conjugates [37]. Consequently, such metabolites will be more potent arylators and redox cyclers. The half-wave oxidation potentials of the GSH conjugates of hydroquinone are also considerably lower than the half-wave oxidation potential of HQ [31]. In addition, the conjugates are also more readily oxidized by cytochrome(s) P450 to the corresponding quinones than HQ and generate more superoxide anions (Fig. 2).

Because the liver produces significant amounts of the multi-substituted GSH conjugates following administration of HQ (1.8 mmol/kg) [31], it seems plausible that the conjugates identified in the bone marrow might be transported there

via the circulation. However, an intravenous dose of GS-HQ (100 $\mu\text{mol/kg}$) yields quinol thioether concentrations in bone marrow far below those detected following co-administration of HQ/phenol (0.9/1.1 mmol/kg, i.p.), suggesting that the major fraction of the HQ-GSH conjugates present in bone marrow is formed *in situ* [35]. Myeloperoxidase- and prostaglandin H synthase-mediated oxidation of HQ to 1,4-BQ, in the presence of GSH, has been shown to yield GS-HQ *in vitro* [38]. This reaction should therefore occur in bone marrow which contains both enzymes [39]. If concentrations of GS-HQ saturate bone marrow γ -GT ($k_m = 68 \mu\text{M}$), subsequent oxidation and GSH substitution will lead to the formation of multi-substituted GSH conjugates of HQ. Since estimates of quinol thioether concentrations in bone marrow exceed 180 μM , this metabolic pathway should occur readily *in vivo*. This view is supported by the identification of 2,5-(GS)-HQ, 2,6-(GS)-HQ, and 2,3,5-(GS)-HQ in the bone marrow of rats following co-administration of HQ/phenol.

7. Some speculation on the potential involvement of HQ-GSH conjugates in benzene-mediated hematotoxicity

GSH conjugates of HQ possess unique structural features that confer upon them the ability to interact with proteins that utilize GSH/cysteine or GSH/cysteine containing molecules as substrates or cofactors. The fact that such proteins play important roles in hematopoiesis provides a basis for these specific benzene metabolites to interfere with this process, and to mimic the actions of benzene. For example; (i) HQ-GSH conjugates inhibit γ -GT [40], and acivicin, a potent inhibitor of γ -GT, is hematotoxic [41]. γ -GT possesses a unique thiol that is not required for catalysis, but is present in the active site of the enzyme on the light subunit [42]. This thiol may be a target for HQ-GSH conjugates. The major function of γ -GT is to regulate the transport of amino acids into cells, but its relative role in maintaining intracellular GSH concentrations is cell-type specific. γ -GT expression in the kidney is so high that even when 95% of the enzyme is inhibited, there remains more total activity than in most other tissues [43]. Consequently, renal GSH levels are only minimally decreased following treatment with acivicin. Tissues expressing very low levels of γ -GT usually possess a very active cystathionase pathway, in which cystathionine is deaminated and cleaved to form free cysteine and α -ketobutyrate. Therefore, even in the presence of acivicin, these tissues maintain high levels of GSH [44]. However, γ -GT activity in bone marrow is relatively low [43], and the more immature, undifferentiated cells within the marrow (targets of benzene) express almost no cystathionase [45]. Thus, inhibition of γ -GT in hematopoietic tissue dramatically reduces intracellular GSH levels [46]. (ii) Benzene- and HQ-treated mice exhibit increased granulopoiesis in bone marrow [47,48]. Increases in G-CSF and GM-CSF can stimulate granulocytic differentiation [49] and HQ synergizes with GM-CSF to increase the number of myeloid progenitor cells in isolated mouse bone marrow [50]. HQ also mimicks the action of leukotriene D₄ (LTD₄) a downstream mediator of G-CSF, to initiate terminal differentiation in IL-3-dependent murine myeloblasts [51]. Interestingly, the ability of 1,4-BQ to induce granulocytic differentiation is prevented by inhibitors of γ -GT and by LTD₄ receptor antagonists [52]. Subsequently, 1,4-BQ was shown to induce granulocytic differentiation by activating the LTD₄ receptor, mimicking LTD₄ [53]. How does the inhibition of γ -GT prevent 1,4-BQ-induced granulopoiesis? If the effects of 1,4-BQ are mediated by the corresponding GSH conjugates, then inhibition of γ -GT will prevent processing of the conjugates to the cysteinylglycine conjugate and interaction with the LTD₄ receptor. LTD₄ is a cysteinylglycine conjugate, and we predict that HQ-thioether conjugates will possess greater selectivity for the LTD₄ receptor than HQ/1,4-BQ. (iii) Finally, functional roles

for ATP-binding cassette (ABC) transporter proteins in hematopoietic stem cell function have recently been described [54,55]. Structurally related catechol-GSH conjugates are potent inhibitors of MRP-1 mediated LTC₄ transport [56] suggesting that GSH-HQ conjugates have the potential to interfere with ABC transporter function within hematopoietic stem cells. Moreover, ABC transporter expression/conformation/function are modulated by ROS, which induce defects in hematopoietic stem cell homeostasis [55].

Conflict of interest

None.

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