Reactive Ring-opened Aldehyde Metabolites in Benzene Hematotoxicity

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The hematotoxicity of benzene is mediated by reactive benzene metabolites and possibly by other intermediates including reactive oxygen species. We previously hypothesized that ring-opened metabolites may significantly contribute to benzene hematotoxicity. Consistent with this hypothesis, our studies initially demonstrated that benzene is metabolized *in vitro* to *trans-trans*-muconaldehyde (MUC), a reactive six-carbon diene dialdehyde, and that MUC is toxic to the bone marrow in a manner similar to benzene. Benzene toxicity most likely involves interactions among several metabolites that operate by different mechanisms to produce more than one biological effect. Our studies indicate that MUC coadministered with hydroquinone is a particularly potent metabolite combination that causes bone marrow damage, suggesting that the involvement of ring-opened metabolites in benzene toxicity may be related to their biological effects in combination with other benzene metabolites. Studies in our laboratory and by others indicate that MUC is metabolized to a variety of compounds by oxidation or reduction of the aldehyde groups. The aldehydic MUC metabolite 6-hydroxy-*trans-trans*-2,4-hexadienal (CHO-M-OH), similar to MUC but to a lesser extent, is reactive toward glutathione, mutagenic in V79 cells, and hematotoxic in mice. It is formed by monoreduction of MUC, a process that is reversible and could be of biological significance in benzene bone marrow toxicity. The MUC metabolite 6-hydroxy-*trans-trans*-2,4-hexadienoic (COOH–M–OH) is an end product of MUC metabolism *in vitro*. Our studies indicate that COOH–M–OH is a urinary metabolite of benzene in mice, a finding that provides further indirect evidence for the *in vivo* formation of MUC from benzene. Mechanistic studies showed the formation of *cis-trans*-muconaldehyde in addition to MUC from benzene incubated in a hydroxyl radical-generating Fenton system. These results suggest that the benzene ring is initially opened to *cis,cis*-muconaldehyde, an unstable isomer that rearranges to *cis-trans*-muconaldehyde, which further rearranges to *trans-trans*-muconaldehyde. The latter is not formed from benzene dihydrodiol by reactive oxygen species in a Fenton system that contains reactive oxygen species. — Environ Health Perspect 104(Suppl 6):1195–1199 (1996)

Key words: *trans-trans*-muconaldehyde, ring-opened benzene metabolites, benzene, metabolism

Introduction

Benzene toxicity is believed to involve biological interactions of multiple reactive intermediates with multiple cellular targets within the bone marrow. A major metabolic route consists of cytochrome P450-mediated oxidation of benzene to phenol and the polyhydroxylated metabolites hydroquinone, catechol, and 1,2,4-trihydroxybenzene. Reactive quinones generated through the oxidation of the polyhydroxylated metabolites have been postulated to be involved in benzene toxicity through alkylation of critical cellular components including DNA (*1,2*). The oxidation of polyhydroxylated benzene metabolites can generate reactive semiquinone free radicals as well as reactive oxygen species that have also been implicated in benzene hematotoxicity (*2*).

A quantitatively minor metabolic route consists of the ring-opening pathway. Definitive evidence for the existence of this pathway was provided by Parke and Williams (*3*), who identified *trans-trans*-muconic acid, a six-carbon diene diacid, as a urinary metabolite of benzene in rabbits. Our laboratory postulated that benzene toxicity is mediated, in part, by reactive ring-opened metabolites including *trans-trans*-muconaldehyde (muconaldehyde [MUC], CHO–M–CHO), the corresponding dialdehyde of *trans-trans*-muconic acid (*4*). Following is a short review of our studies on possible mechanisms of benzene ring-opening, the metabolism of muconaldehyde to other ring-opened compounds potentially important in benzene toxicity, and the toxic biological effects of reactive ring-opened compounds in relation to benzene toxicity.

Formation of Ring-opened Metabolites of Benzene

Drumond and Finar (*5*) showed that benzene metabolism involves ring opening, as indicated by the urinary excretion of *trans-trans*-muconic acid (muconic acid [MA]), a ring-opened six-carbon diene dicarboxylic acid (COOH–M–COOH). The formation of *trans-trans*-muconic acid from benzene was definitively established by Parke and Williams (*3*) who observed the excretion of ^{14}C-muconic acid in the urine of rabbits administered ^{14}C-benzene. We postulated that muconaldehyde, the aldehydic analog of MA, was a potential reactive toxic intermediate of benzene (*4*). This hypothesis was based in part on the known toxicity of α,β-unsaturated aldehydes as well the inability of hydroxylated metabolites to produce bone marrow toxicity in animal models. Muconaldehyde was identified as a compound formed during the microsomal metabolism of benzene (*6*). The identification of MUC derived from ^{14}C-benzene incubated with microsomes, isolated from the livers of male CD-1 mice induced with benzene, was based on molecular weight determined by mass spectrometry, coelution with authentic standard during high performance liquid chromatography (HPLC), and the trapping of MUC as the thiobarbituric acid adduct.

The metabolism of muconaldehyde is of interest since it could be the source of other reactive ring-opened compounds potentially

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Abbreviations used: CHO-M-COO, 6-oxo-*trans-trans*-2,4-hexadienoic acid; COOH-M-OH, 6-hydroxy-*trans-trans*-2,4-hexadienoic acid; GSH, glutathione; HO-M-OH, 1,6-dihydroy-*trans-trans*-2,4-hexadiene; muconaldehyde/MUC/CHO-M-CHO, *trans-trans*-muconaldehyde; muconic acid/MA/COOH-M-COOH, *trans-trans*-muconic acid; NAD+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced; TBA, thiobarbituric acid.

involved in benzene hematotoxicity. Like other aldehydes, there are two major pathways involved in the metabolism of muconaldehyde: the reduction pathway to form alcohols by alcohol dehydrogenase and the oxidation pathway to form carboxylic acids by aldehyde dehydrogenase. Initial studies of muconaldehyde metabolism by Kirley et al. (*7*) showed that muconaldehyde is oxidized by mouse liver cytosol to 6-oxo-*trans-trans*-2,4-hexadienoic acid (CHO–M–COOH). The latter is further metabolized to muconic acid by purified yeast aldehyde dehydrogenase and mouse liver cytosol supplemented with nicotinamide adenine dinucleotide (NAD^+). In addition, Goon et al. (*8*) identified 6-hydroxy-*trans-trans*-2,4-hexadienal (CHO–M–OH), 6-hydroxy-*trans-trans*-2,4-hexadienoic acid (COOH–M–OH) and 1,6-dihydroxy-*trans-trans*-2,4-hexadiene (HO–M–OH) as products derived from muconaldehyde incubated with purified yeast aldehyde dehydrogenase and yeast alcohol dehydrogenase supplemented with NAD^+ and nicotinamide adenine dinucleotide, reduced (NADH). Using mouse liver cytosol supplemented with NAD^+, we demonstrated that COOH–M–OH and MA are the end products of muconaldehyde metabolism (*9*). The same compounds were also reported to be end products of muconaldehyde metabolism in rat liver hepatocytes (*8*). Studies with mouse liver cytosol showed that the formation of CHO–M–OH from MUC is reversible (*9*). The formation of COOH–M–OH occurs by two pathways, the major one involving an initial reduction of muconaldehyde to CHO–M–OH followed by oxidation to COOH–M–COOH, and a minor pathway in which the monooxidation product CHO–M–COOH is reduced to COOH–M–OH (*9*). The metabolism of muconaldehyde is shown in Figure 1.

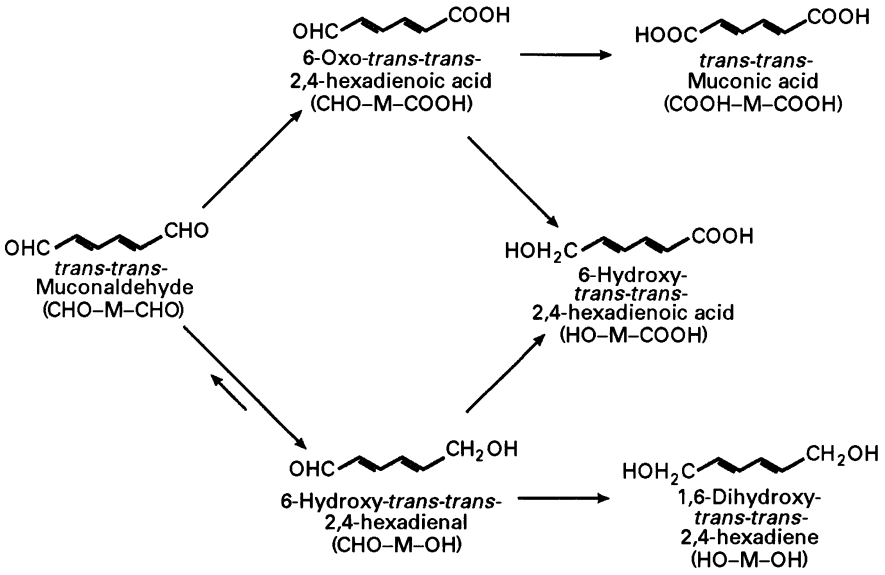

Figure 1. Metabolism of muconaldehyde.

At present there is no direct evidence for the formation of muconaldehyde from benzene *in vivo*. The studies described above and *in vivo* studies with mice administered muconaldehyde (*10*) indicate that muconic acid is a product of muconaldehyde metabolism, suggesting that urinary muconic acid in animals administered benzene is derived from muconaldehyde. Other studies showed that COOH–M–OH is present in the urine of mice administered benzene or muconaldehyde (*11*), thus providing additional indirect evidence for ring-opening of benzene to muconaldehyde *in vivo*. These studies also indicate that the reduction pathway of MUC occurs *in vivo* since COOH–M–OH is mainly formed from CHO–M–OH, an initial monoreduction product of muconaldehyde (*9*).

The mechanism(s) and pathway(s) involved in the formation of muconaldehyde are not well understood. Loeff and Stein (*12*) detected muconaldehyde in aqueous solutions of benzene irradiated with γ-irradiation, a process known to generate hydroxyl radicals. Wei et al. (*13*) showed that muconaldehyde is formed from benzene during photooxidation, presumably via singlet oxygen attack. Latriano et al. (*14*) showed that benzene incubated in a hydroxyl radical-generating system forms products that react with thiobarbituric acid (TBA) to form chromophores absorbing at 495 and 532 nm. These results suggested ring-opening of benzene to unsaturated aldehydes, which are known to react with TBA to form 495 and 532 nm absorbing chromophores (*15*). The finding that muconaldehyde reacts with TBA and forms a 495-nm absorbing chromophore provided further evidence in support of benzene ring-opening to muconaldehyde in a Fenton system. Using ^{14}C-benzene, muconaldehyde was identified as the TBA adduct by HPLC analysis of the TBA-reacted Fenton system incubated with benzene (*16*).

The actual oxidant involved in benzene ring-opening in the Fenton system is not known. The hydroxyl radical scavengers, dimethyl sulfoxide, mannitol, and ethanol were found to cause a dose-dependent decrease in the formation of muconaldehyde, suggesting the involvement of hydroxyl radicals in the ring-opening process in the Fenton system (*16*). However, in a system in which hydroxyl radicals were generated radiolytically in N_2O/O_2 saturated aqueous solutions, benzene was not reported to form muconaldehyde (*17*). The Fenton system is complex and, in addition to hydroxyl radicals, other reactive oxygen species such as the ferryl species $[FeO]^{2+}$ may be produced (*18*). In anhydrous acetonitrile, iron and H_2O_2 were reported to produce ferryl species as well as singlet oxygen (*19*). Benzene ring-opening could involve more than one reactive oxygen species, and thus more than one mechanism could lead to the formation of ring-opened products.

In the studies described above, the TBA–MUC adduct was semipurified by solid phase extraction using reverse phase C-18 packing material prior to HPLC analysis. In our recent studies, Fenton reaction mixtures were directly injected onto the HPLC for product analysis. The results of these studies (*20*) indicate that *cis-trans*-muconaldehyde and MUC are present in Fenton reaction mixtures of benzene, suggesting that the benzene ring is initially opened to *cis-cis*-muconaldehyde, which then rearranges to form the more stable *cis-trans*-muconaldehyde. This, in turn, rearranges to *trans-trans*-muconaldehyde, the most stable form of the three muconaldehyde isomers.

Muconaldehyde was initially demonstrated to be formed in a mouse liver microsomal system supplemented with reduced nicotinamide adenine dinucleotide phosphate (NADPH) (*6*). A number of pathways can be postulated to lead to ring-opening and the formation of muconaldehyde, as shown in Figure 2. Ring-opening could occur via a pathway involving two successive oxidations by cytochrome P450, followed by rearrangement of the oxidized benzene oxepin to muconaldehyde. Other pathways could consist of ring-opening mediated solely by reactive oxygen species, while yet other pathways might consist of a combination of enzymatic steps and reactions involving reactive oxygen species. The formation of muconaldehyde via benzene dihydrodiol is an example of the latter pathway. This pathway is unlikely, though, as benzene dihydrodiol did not form muconaldehyde upon incubation in a Fenton system (*20*). Studies in mouse liver microsomes indicate that benzene ring-opening is enhanced by addition of iron and that Fenton chemistry may be involved in the ring-opening of benzene (*21*). The results from hydroxyl radical and singlet oxygen scavenger studies suggest that reactive oxygen species may participate in the ring-opening of benzene in the microsomal system. The formation of muconaldehyde from benzene in liver microsomes (*6,21*) and the finding of muconic acid in perfusate of rat liver perfused with benzene (*22*) suggest that the liver is a site of ring opening *in vivo*.

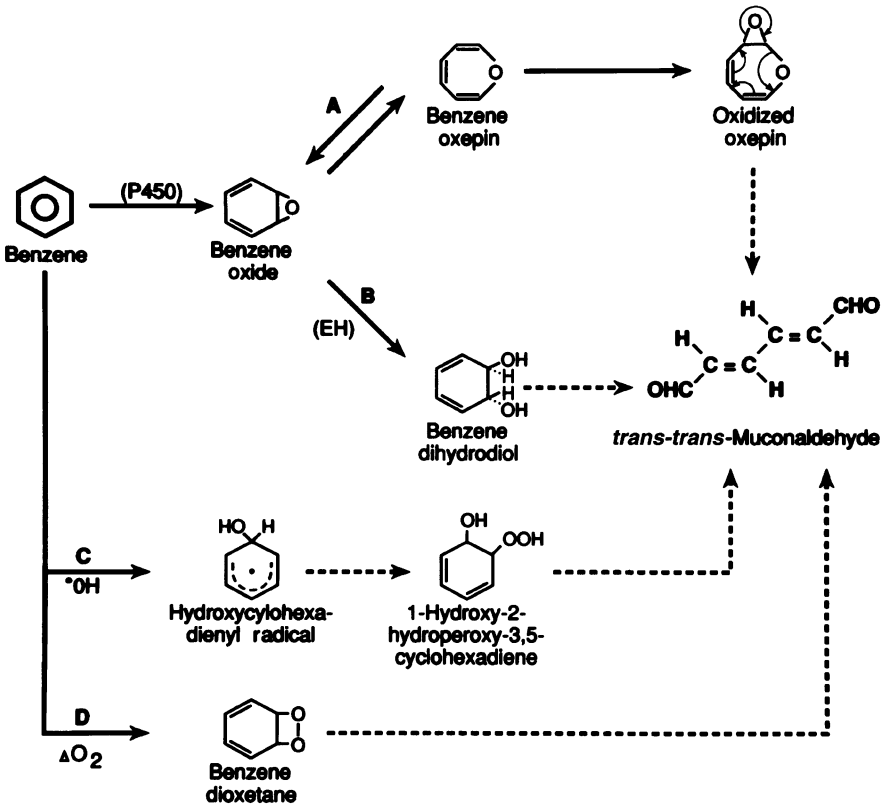

Figure 2. Potential pathways for the formation of muconaldehyde. Letters A–D refer to specific pathways; broken arrows indicate that several steps are required to go from one intermediate to the next. EH, epoxide hydrolase.

Hematotoxicity of Muconaldehyde and 6-Hydroxy-*trans-trans*-2,4-hexadienal

Muconaldehyde induces hematotoxic effects in mice similar to those induced by benzene. Intraperitoneal administration of 2 mg/kg/day MUC to CD-1 mice for 16 days produced significant decreases in bone marrow cellularity, lymphocytes, red blood cell count, hematocrit, and hemoglobin; and significant increases in white blood cell count, mainly due to neutrophils, and spleen weight (*23*). Significant decreases in nucleated bone marrow cells were also observed after ip muconaldehyde administration of 2 mg/kg/day for 10 days. When the mice were given the same total dose of 2 mg/kg divided into three daily ip injections of 0.67 mg/kg MUC for 10 and 16 days, similar effects, but of much lesser magnitude, were observed. Bone marrow toxicity of MUC was also demonstrated by Snyder et al. (*24*) using inhibition of incorporation of radioactive iron into cell hemoglobin as a toxic end point indicative of inhibitory effects on proliferation and differentiation of bone marrow erythroid cells (*25*). In their studies, dose-dependent decreases in ^{59}Fe incorporation were observed in Swiss albino mice administered 1, 2, or 4 mg/kg MUC in an experimental regimen that involved three treatments per dose during a 24-hr period. Benzene (150 and 300 mg/kg) as well as hydroquinone (75 and 100 mg/kg) also significantly inhibited ^{59}Fe incorporation, compared with phenol (25–100 mg/kg) or catechol (25–100 mg/kg), which had little or no effect on ^{59}Fe uptake. Similar to Eastmond et al. (*26*), who reported bone marrow depression upon coadministration of phenol and hydroquinone at doses that were nonhematotoxic when administered alone, Snyder et al. (*24*) found synergistic effects between benzene metabolites on the inhibition of ^{59}Fe incorporation. Their results indicate that the hydroquinone/MUC combination is the most potent combination of two benzene metabolites tested in producing suppression of erythropoiesis.

6-Hydroxy-*trans-trans*-2,4-hexadienal (CHO–M–OH), a reactive metabolite of MUC, is also hematotoxic in mice (*27*). It significantly inhibited the uptake of ^{59}Fe by bone marrow erythroid cells at 20 mg/kg administered using the same dosing schedule as that used for the muconaldehyde studies described above. The compound CHO–M–OH also decreased bone marrow cellularity and lymphocytes in the peripheral blood after ip administration of 25 mg/kg/day for 3 days followed by 20 mg/kg for 1 day. The latter dose regimen produced significant increases in peripheral blood neutrophils, as did administration of 5 mg/kg/day for 16 days, compared with administration of 10 mg/kg/day for 16 days, which had no effect on this parameter. This lack of dose-related effects in mouse neutrophils is not unexpected (*28*). On a molecular level, the reactivity of CHO–M–OH toward cellular targets such as cell membranes, sulfhydryl (SH)-dependent regulatory enzymes, or DNA may vary, and consequently different cellular processes are expected to be affected at different doses. Furthermore, the bone marrow is extremely complex in terms of

Table 1. Toxic effects of *trans-trans*-muconaldehyde and 6-hydroxy-*trans-trans*-2,4-hexadienal.

Compound	Toxicological effects	Reference
Muconaldehyde (CHO–M–CHO)	Hematotoxic in mice, decrease in bone marrow cellularity, suppression of erythropoiesis, increase in white blood cell count, segmented neutrophils, and decrease in peripheral blood lymphocytes and red blood cells	Witz et al. (*23*), Snyder et al. (*24*)
	Synergistic interaction with hydroquinone in suppression of erythropoiesis in mice	Snyder et al. (*24*)
	Increase of spleen weight and decrease of hepatic total and free sulfhydryls in CD-1 mice	Witz et al. (*23*)
	Increase of sister chromatid exchange in bone marrow cells in B6C3F1 mice and the percentage of cells with micronuclei in CHO cells	Witz et al. (*31*)
	Increase of mutation frequency in Chinese hamster V79 cells	Glatt and Witz (*32*), Chang et al. (*33*)
	Weakly mutagenic in *Salmonella typhimurium* TA97, TA100, TA102, TA104	Witz et al. (*31*), Glatt and Witz (*32*)
	Causes SOS response, cell transformation, and increases micronuclei in SHE system	Henschler et al. (*34*)
	Highly cytotoxic to erythroid stem cells in human peripheral blood cultures, Chinese hamster V79 cells, CHO cells, and primary hepatocytes	Goldstein et al. (*4*), Witz et al. (*31*), Glatt and Witz (*32*)
	Decrease of stimulted superoxide anion radical production by NADPH-oxidase in human neutrophils and rat lung macrophages	Witz et al. (*35,36*)
	Decrease of cell membrane lipid fluidity, plasma membrane sulfhydryls, and GSH in rat lung macrophages	Witz et al. (*36*), Witz (*37*)
6-Hydroxy-*trans-trans*-2,4-hexadienal (CHO–M–OH)	Hematotoxic in mice, decrease in bone marrow cellularity, suppression of erythropoiesis, changes in peripheral blood lymphocytes, and increase in neutrophils	Zhang et al. (*27*)
	Decrease of sulfhydryls in bone marrow cells in CD-1 mice	Zhang et al. (*27*)
	Increase of mutation frequency and cytotoxicity in Chinese hamster V79 cells	Chang et al. (*33*)

interacting cell types that may not be equally sensitive to CHO–M–OH.

The reversible formation of MUC from CHO–M–OH may have biological significance. If MUC, a reactive direct-acting alkylating agent, is formed in the liver, it may immediately react with glutathione (GSH) and other thiol groups in the liver or the blood and therefore not survive the transport from liver to bone marrow, the target tissue. The compound CHO–M–OH is less reactive than MUC, has a longer half-life in the presence of GSH (*29*), and therefore has a better chance to survive transport from liver to bone marrow, where conceivably it could be oxidized to MUC. In this scenario, CHO–M–OH functions as a transport form of muconaldehyde. In light of this, the question might be asked whether the hematotoxicity of CHO–M–OH in mice is due to muconaldehyde. Our studies indicate that CHO–M–OH is about one-tenth as potent as MUC in producing bone marrow toxicity. Oxidation of 10% of CHO–M–OH administered at 25 mg/kg would yield about 2 mg MUC, a hematotoxic dose. Regeneration of MUC from CHO–M–OH in the bone marrow would produce the reactive intermediate directly at the target tissue without loss to the liver or intervening targets prior to the bone marrow. At present, it is not known whether muconaldehyde or CHO–M–OH reaches the bone marrow when administered to mice.

Distribution studies in our laboratory (unpublished results) show that a substantial amount of radioactivity is found in the bone marrow of the mice administered ^{14}C-muconaldehyde ip, suggesting that either muconaldehyde or its metabolites or conjugates could reach the bone marrow from the liver.

The observation of a narrow window of toxicity in both the hematotoxicity and the ^{59}Fe incorporation studies for CHO–M–OH in mice (*27*) suggests that reversibility of CHO–M–OH to MUC could occur *in vivo*. This narrow window of toxicity may be caused by saturation of the detoxification metabolism of CHO–M–OH. At a low dose, most of CHO–M–OH may be metabolized to COOH-M-OH, a stable MUC metabolite (*8,9*). At a high dose, this pathway may be saturated; therefore, more CHO–M–OH is oxidized back to MUC (*9*), which is hematotoxic in mice at 2 mg/kg/day (*23*).

Conclusions

Benzene hematotoxicity is a complex process that most likely involves interaction among several metabolites (*30*). Possible intermediates considered at present important in benzene hematotoxicity are polyhydroxylated benzene metabolites, e.g., hydroquinone and 1,2,4-benzenetriol; their quinone oxidation products, e.g., *p*-benzoquinone, formed via oxidation of hydroquinone; semiquinone free radical intermediates formed during the oxidation of polyhydroxylated metabolites to quinones; reactive oxygen species formed during the oxidation of the polyhydroxylated metabolites and ring-opened aldehydic benzene metabolites, e.g., muconaldehyde and 6-hydroxy-*trans-trans*-2,4-hexadienal. This array of intermediates includes directly acting alkylating agents, radical metabolites, and reactive oxygen species including the reactive hydroxyl radical. A multitude of cellular effects could ensue in response to alkylation and oxidative processes involving these intermediates.

Muconaldehyde and its metabolite 6-hydroxy-*trans-trans*-2,4-hexadienal are reactive ring-opened hematotoxic compounds. They exhibit a host of biological activities that could potentially be important in their mechanisms of toxicity in relation to benzene (Table 1). Muconaldehyde and 6-hydroxy-*trans-trans*-2,4-hexadienal are multifunctional alkylating agents that have the potential to cross-link cellular DNA and protein. They also react with GSH and have the ability to deplete cellular GSH, thus decreasing the concentration of this important cellular antioxidant. The chemical reactivity of muconaldehyde, and perhaps of 6-hydroxy-*trans-trans*-2,4-hexadienal, as an alkylating agent, cross-linking agent, and thiol depleter may provide the basis for molecular interactions that lead to bone marrow toxicity. Future studies will include mechanistic approaches designed to probe the muconaldehyde–hydroquinone interaction and analytical studies on the *in vivo* identification of muconaldehyde in the form of adducts.

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