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The Toxicology of Hydroquinone — Relevance to Occupational and Environmental Exposure

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ABSTRACT: Hydroquinone (HQ) is a high-volume commodity chemical used as a reducing agent, antioxidant, polymerization inhibitor, and chemical intermediate. It is also used in over-the-counter (OTC) drugs as an ingredient in skin lighteners and is a natural ingredient in many plant-derived products, including vegetables, fruits, grains, coffee, tea, beer, and wine. While there are few reports of adverse health effects associated with the production and use of HQ, a great deal of research has been conducted with HQ because it is a metabolite of benzene. Physicochemical differences between HQ and benzene play a significant role in altering the pharmacokinetics of directly administered when compared with benzene-derived HQ. HQ is only weakly positive in *in vivo* chromosomal assays when expected human exposure routes are used. Chromosomal effects are increased significantly when parenteral or *in vitro* assays are used. In cancer bioassays, HQ has reproducibly produced renal adenomas in male F344 rats. The mechanism of tumorigenesis is unclear but probably involves a species-, strain-, and sex-specific interaction between renal tubule toxicity and an interaction with the chronic progressive nephropathy that is characteristic of aged male rats. Mouse liver tumors (adenomas) and mononuclear cell leukemia (female F344 rat) have also been reported following HQ exposure, but their significance is uncertain. Various tumor initiation/promotion assays with HQ have shown generally negative results. Epidemiological studies with HQ have demonstrated lower death rates and reduced cancer rates in production workers when compared with both general and employed referent populations. Parenteral administration of HQ is associated with changes in several hematopoietic and immunologic endpoints. This toxicity is more severe when combined with parenteral administration of phenol. It is likely that oxidation of HQ within the bone marrow compartment to the semiquinone or *p*-benzoquinone (BQ), followed by covalent macromolecular binding, is critical to these effects. Bone marrow and hematologic effects are generally not characteristic of HQ exposures in animal studies employing routes of exposure other than parenteral. Myelotoxicity is also not associated with human exposure to HQ. These differences are likely due to significant route-dependent toxicokinetic factors. Fetotoxicity (growth retardation) accompanies repeated administration of HQ at maternally toxic dose levels in animal studies. HQ exposure has not been associated with other reproductive and developmental effects using current USEPA test guidelines. The skin pigment lightening properties of HQ appear to be due to inhibition of melanocyte tyrosinase. Adverse effects associated with OTC use of HQ in FDA-regulated products have been limited to a small number of cases of exogenous ochronosis, although higher incidences of this syndrome have been reported with inappropriate use of unregulated OTC products containing higher HQ concentrations. The most serious human health effect related to HQ is pigmentation of the eye and, in a small number of cases, permanent corneal damage. This effect has been observed in HQ production workers, but the relative contributions of HQ and BQ to this process have not been delineated. Corneal pigmentation and damage has not been reported at current exposure levels of <2 mg/m^3. Current work with HQ is being focused on tissue-specific HQ-glutathione metabolites. These metabolites appear to play a critical role in the renal effects observed in F344 rats following HQ exposure and may also be responsible for bone marrow toxicity seen after parenteral exposure to HQ or benzene-derived HQ.

KEY WORDS: hydroquinone, *p*-benzoquinone, *p*-benzenediol, 1,4-benzenediol, quinol, quinone, benzene, myelotoxicity, carcinogenicity, developmental toxicity, reproductive toxicity, mutagenicity, genotoxicity, clastogenicity, aneuploidy, metabolism, pharmacokinetics, depigmentation, ocular toxicity, ocular pigmentation.

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I. INTRODUCTION

Hydroquinone (HQ; *p*-benzenediol; CAS# 123–31–9) is a high-volume commodity chemical with major uses as a reducing agent in black and white photographic developing solutions, as an antioxidant and polymerization inhibitor, and as an intermediate in the synthesis of other antioxidant derivatives.[1,2] Worldwide annual (1992) production capacity for HQ is estimated at 35,000 to 40,000 tonnes.[1,3] U.S. production is limited to three manufacturing sites, although National Institute of Occupational Safety and Health (NIOSH) data indicate that HQ may be employed in manufacturing and end use at 16,000 to 66,000 individual facilities.[4] A minor but important use of HQ is in topically applied, over-the-counter and prescription depigmenting skin creams and in other consumer personal care products. HQ is also present at significant levels in cigarette smoke and in certain fruits and vegetables (in the form of its glucose conjugate, arbutin), including pears and blueberries.[5,6]

HQ is a water-soluble, crystalline solid. When present in aqueous solution, HQ is susceptible to both redox and acid-base transformations (Figure 1). The products of these reactions, including *p*-benzoquinone (BQ), a semiquinone, and various activated oxygen species, are potentially important for the action of HQ in biological systems. The toxicology of HQ has been investigated in experimental animal studies by various routes of exposure and in *in vitro* model systems since the late 1800s. These studies have reported effects on a number of organ systems and cellular processes. The U.S. National Toxicology Program (NTP) classified HQ as demonstrating "some evidence" of carcinogenicity in animal studies, based primarily on kidney adenomas in male Fisher 344 rats.[7] The International Agency for Research on Cancer (IARC) includes HQ under its Group 3 category, that is, "not classifiable" as to its carcinogenicity in humans.[8] The American Conference of Governmental Industrial Hygienists (ACGIH) classifies HQ under its carcinogenicity designation "A3", that is, carcinogenic in animal studies, unknown relevance in humans.[9] Human clinical, occupational, and epidemiological studies have consistently demonstrated only dermal effects associated with excessive use of HQ-containing

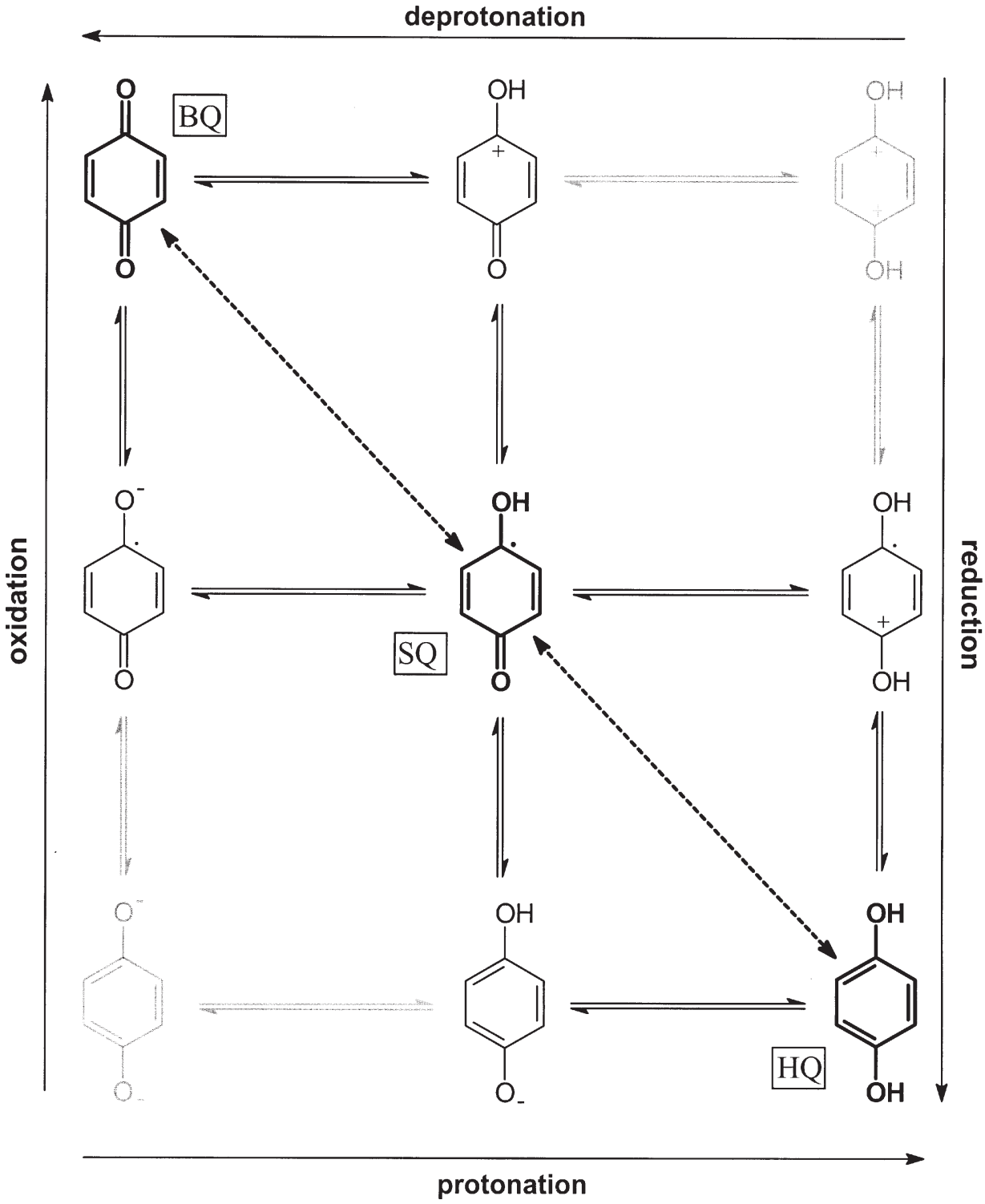

FIGURE 1. Acid-base and oxidation-reduction matrix for hydroquinone (HQ), semiquinone (SQ), and benzoquinone (BQ). Electron and proton transfer reactions are shown on vertical and horizontal axes, respectively. Most stable species under aqueous physiological conditions are indicated in bold; species shown in gray are highly unfavored. While all possible conversions are shown, *in vivo* interconversions between HQ, SQ, and BQ likely proceed via simultaneous electron-proton (i.e., hydrogen atom) transfers (diagonal dashed arrows).

skin lighteners and a unique ocular syndrome in heavily exposed HQ production workers. Several reviews of specific aspects of HQ toxicology are available.[10,11] In addition, more comprehensive reviews have been published by the World Health Organization, the Cosmetic Ingredient Review Expert Panel, and other authors.[1,12–15] The present article summarizes the available data on significant aspects of HQ toxicology, addresses newer information on nephrotoxicity, myelotoxicity, and macromolecular binding, and critically assesses the potential relevance of *in vitro* and experimental animal data on HQ to humans.

An important and controversial issue concerns the potential involvement of HQ in the molecular mechanism of benzene-induced myelotoxicity and leukemia in humans. HQ, along with phenol, BQ, and several other oxidized and ring-opened derivatives is a demonstrated metabolite of benzene in humans and experimental animals. Much of the basic research on HQ toxicology and its effects on cellular and molecular systems has been driven by the hypothesis that HQ, alone or in combination with other benzene metabolites, is involved in the mechanism of benzene-induced leukemia. Although full treatment of this issue is beyond the scope of the present article, relevant data from such studies are incorporated into this review where appropriate, and their potential significance for direct human exposure to HQ is evaluated.

II. HUMAN EXPOSURE TO HQ

Typical human occupational exposure scenarios for HQ consist primarily of inhalation of particulates and dermal contact with either solid HQ or aqueous solutions (as in photographic developers). Estimates of the number of workers with potential exposure to HQ vary, but range up to 80 to 100 for actual production, 560,000 for industrial uses, and 30,000 for photographic development.[1,4,15] The current Occupational Safety and Health Administration (OSHA) Permissible Exposure Limit (PEL) and ACGIH Threshold Limit Value (TLV) for HQ are both 2 mg/m^3 (8-h TWA), a value that is widely used in other countries as well.[1] In addition, an IDLH limit of 50 mg/m^3 has been recommended by NIOSH for HQ.

Airborne HQ dust levels are routinely monitored in occupational (i.e., HQ manufacturing) settings and generally average approximately 0.10 to 0.50 mg/m^3.[15] Prior to the institution of comprehensive dust control measures during HQ manufacture and use, air concentrations were substantially higher; approximately 2 to 5 mg/m^3 and up to 20 to 35 mg/m^3 in some cases.[16,17] In contrast, ambient air levels of HQ in the vicinity of photographic developing operations are much lower (<0.01 mg/m^3),[18] although levels during preparation of developing solutions (0.5 to 2.0% HQ) from dry HQ would probably be higher. The internalized dose of HQ is expected to be minimal following dermal exposure to HQ during photographic processing due to the low HQ concentrations present in developer solutions and the slow absorption of HQ through mammalian skin.[19] Estimates of dermal absorption following immersion of an adult hand in 5% HQ solution for 1 h indicated an uptake of only 200 μg (2.9 μg/kg for a 70-kg man).[5] Measurement of urinary levels of HQ in darkroom workers have revealed no increases compared with unexposed controls, indicating minimal uptake of HQ.[20] HQ may also be released directly to the environment as a fugitive emission during its production and use and in the effluent of photographic processes, although no specific data are available to judge the overall potential for human exposure via these releases.[2]

HQ occurs naturally in the leaves, bark, and fruit of a number of plants, some of which are used as food. In particular, HQ is present in various berries, pears, and other fruits, much of it in the form of the glucose conjugate 4-hydroxyphenyl-β-D-glucopyranoside (arbutin). Arbutin is expected to readily undergo acid hydrolysis to yield HQ, although whether this process actually occurs within the acidic environment of the human stomach has not been reported. Arbutin has been demonstrated to be stable to hydrolysis in human small intestine preparations *in vitro* and to be absorbed intact from the intestinal lumen by facilitated transport mechanisms.[21] However, direct studies in human volunteers did not reveal higher levels of free HQ in urine following glucronidase treatment, suggesting that arbutin

may not have been absorbed (and excreted) intact.[5]

In addition to arbutin, free HQ is also present in coffee beans and certain wheat-based products. It has been speculated that the low levels of HQ detected in the urine of unexposed persons[22] may be derived from HQ-containing foods, or, alternatively, from environmental exposure to benzene or phenol.[5,23] Other potential sources of such background HQ levels include cigarette smoke (110 to 300 μg per cigarette) and ingestion of acetaminophen (which is metabolized to HQ and other products).[5] One study revealed increases in plasma and urinary HQ shortly after consumption of a high-HQ meal and cigarette smoking, but not following a 1000 mg dose of acetaminophen in several human volunteers.[5] These authors also estimated that the typical exposure to HQ from food and other uncharacterized sources was approximately an order of magnitude greater than that predicted from occupational exposure during HQ production or use of photographic developers.

III. ACUTE EFFECTS OF HQ

Animal studies indicate that, in most species, HQ exhibits relatively low acute toxicity by oral and dermal routes of exposure. Acute oral LD_{50} values for HQ range from 70 mg/kg in the cat to 550 mg/kg in the guinea pig, with most species exhibiting values in the upper end of this range.[24–27] The higher sensitivity of the cat may be accounted for by relatively lower levels of glucuronide conjugation activity in the liver and intestinal tract.[28,29] The presence of food may decrease the rate and extent of absorption of HQ from the GI tract, as LD_{50} values of 310 and 1050 mg/kg have been reported for the fasted and unfasted rat, respectively.[26] A fairly steep dose-response curve for lethality is typically observed, and equivalent divided oral doses of HQ are substantially less toxic than large single doses.[25] The acute dermal LD_{50} of HQ (in an unidentified mammalian species) was estimated at 5970 mg/kg, suggesting low systemic absorption via this route.[30] The acute dermal toxicity in rats and mice is >3840 and >4800 mg/kg (respectively), as animals were able to survive these dose levels given repeatedly over 2 weeks.[7] LD_{50} values for HQ by parenteral administration have been reported as 160 mg/kg in the rat,[25] 115 mg/kg in the rat,[25] and 190 mg/kg in the mouse[31] by i.p., i.v., and s.c. injection, respectively.

The effects of acute high-level HQ exposure in animal studies are directed primarily toward the central nervous system (CNS). The CNS stimulatory effects of large acute doses of HQ were first shown in rabbits and frogs by Brieger in 1879[32] and since have been reproduced in a number of species and by various routes of administration. The signs associated with such acute exposure include tremor, salivation, hyperexcitability, incoordination, tonic and clonic convulsions, respiratory failure, coma, and death. The onset of CNS signs is quite rapid, and death generally occurs within 2 h of exposure of test animals. Rapid and complete recovery from the acute effects of large, sublethal doses of HQ in experimental animals is also characteristic. In contrast to the marked CNS effects of near-lethal doses, comprehensive functional-observational examination of Sprague-Dawley (SD) rats administered HQ at doses of 20, 64, or 200 mg/kg/day (5 days/week, p.o.) for 13 weeks revealed only transient tremors and decreased motor activity at the two higher dose levels.[33] No neuropathological changes were noted as a result of this dosing regimen.

The molecular mechanism of CNS stimulation by HQ is poorly understood, although aromatic phenols are well-known convulsant agents.[34] HQ is approximately one-third as potent as catechol (the most potent polyphenol) and equal to phenol in producing convulsions in rodent models.[35] CNS stimulation is believed to be a direct action of HQ, rather than metabolites such as BQ, due to the very rapid onset of effects and the lack of similar CNS signs with acute BQ exposure.[25] Early studies suggested that HQ and related phenols increase the size of end-plate potentials in peripheral motor nerve terminals without altering acetylcholine sensitivity,[36] and that HQ does not possess anticholinesterase activity.[37] A CNS activation mechanism involving both presynaptic and postsynaptic actions has been proposed.[38]

Acute exposure to HQ in humans by accidental or deliberate ingestion is also associated with CNS and other effects, although the attribution of specific signs and symptoms is frequently clouded

by the presence of other components and by the difficulty in accurately establishing exposure levels. Tremor, respiratory difficulty, convulsions, and unconsciousness have been reported in people ingesting multi-gram quantities of HQ.[39–41] For example, cyanosis, rapid pulse, and coma were reported after deliberate consumption of an estimated 12 g of HQ; the individual apparently recovered from this intoxication within 2 weeks.[40] Discolored (green) or dark urine has also been noted in these cases. No fatalities have been reported after ingestion of HQ itself. Acute gastroenteritis was reported in a large number of men consuming drinking water accidentally contaminated with an unknown amount of photographic developer containing HQ aboard a U.S. Navy ship.[42] Other toxic signs, including jaundice and hemolytic anemia, have also been reported, but only following ingestion of HQ in combination with other compounds, such as in photographic developers.[43–46] If not fatal, complete recovery from the effects of these combined exposures typically has been reported.

IV. TOXICOKINETICS OF HQ

The absorption, distribution, metabolism, and excretion of HQ has been studied extensively in both experimental animal models and human volunteers. These investigations have been prompted by the need for relevant data on HQ itself and by attempts to understand the potential involvement of HQ in the toxicity of benzene. Numerous reports indicate that HQ is well absorbed by the oral route. Single oral doses of up to 350 mg/kg were >90% absorbed in CD and F344 rats, with peak blood levels occurring within <1 h.[47–51] Similar findings were reported with oral exposure of rats to developer solution containing 3% HQ.[52] Rapid and substantial oral absorption in humans ingesting foods containing mg levels of HQ has been demonstrated.[5] HQ also appears to be well absorbed following intratracheal (i.t.) instillation in rats, suggesting that HQ from small (<5 μm) particles that reach the alveoli and dissolve would be rapidly taken up into the blood.[50,53,54]

Numerous studies have demonstrated that, in contrast to oral and i.t. administration, systemic absorption of HQ with dermal exposure occurs but is less efficient. *In vitro* studies with mouse and human skin exposed to aqueous solutions of HQ indicated permeability constants (K_p) of 28×10^{-6} and 4×10^{-6} cm/h, respectively.[55] Barber et al. reported K_p values of 23×10^{-6} with rat skin and 9.3×10^{-6} cm/h with human skin.[19] These human K_p values fall in the "very slow" and "slow" permeability ranges using the qualitative ranking system described by Marzulli et al.[56] Only minor systemic absorption of a dermal dose of HQ administered in aqueous solution to mice,[55] rats,[51] or dogs[57] was reported. More extensive penetration of HQ across human[58,59] and animal[7,59] skin when applied in an alcohol vehicle has been demonstrated. Most recently, dermal application of a 2% HQ cream formulation (dose of 2.5 mg spread over 25 cm^2 of skin) in human volunteers was associated with bioavailability (based on blood levels) of ~45% after 24 h, similar to that seen with *in vitro* human skin preparations.[60] Both HQ and BQ were detected in the *in vitro* human skin preparations. These workers reported an absorption rate fourfold higher than Barber et al., although K_p values calculated from these data still fall within the "slow" permeability range.[56]

Tissue to plasma partition coefficients for HQ were reported by Hill et al.[61] to range from 1.3 to 2.4, typical for a low-molecular-weight water- and lipid-soluble xenobiotic. Distribution of HQ is limited by extensive biotransformation following absorption, with differences observed between the oral and parenteral routes of administration. After oral administration of [^{14}C]-HQ to rats, <1% of radiolabel in blood was associated with parent compound, consistent with extensive first pass metabolism.[50] Greenlee et al.,[62,63] using whole body autoradiography and scintillation counting of tissue homogenates, reported concentrations of radiolabel in bone marrow and thymus 2 h following i.v. administration of 1.2 to 12 mg/kg [^{14}C]-HQ to rats. The latter study also demonstrated a time-dependent increase in covalently bound label in liver and bone marrow up to 24 h. In contrast, i.v. administration of [^{14}C]-HQ to dogs was associated with residual label in the skin, liver, and intestine after 24 h.[57] Wide distribution of radiolabel to various tissues, particularly liver and kidney, was demonstrated with both single and repeated oral [^{14}C]-HQ dosing regimens in the rat.[51]

Relative label in bone and spleen was approximately 10% of that seen in liver 48 h after a single 25 mg/kg oral dose. Administration of 75 mg/kg of [^{14}C]-HQ to mice by i.p. injection resulted in covalent binding to liver, kidney, blood, and bone marrow macromolecules, with liver having a 10-fold higher specific activity than bone marrow.[64]

While HQ is rapidly and extensively absorbed by the oral and i.t. routes, it is rapidly eliminated via urine, leaving little residual sequestration or binding to tissue (~1 to 3% of total dose). Elimination via expired air is negligible and elimination via feces is a generally minor pathway for HQ. Blood elimination half-lives (total radioactivity) of 18.7, 14.8 min, and 22.1 min have been reported for i.v., p.o., and i.t. administration of [^{14}C]-HQ, respectively, in F344 rats.[50] A parent compound half-life of 9 min was reported with i.p. injection in B6C3F$_1$ mice,[65] while 2.7 min was the mean HQ elimination half-life with i.t. instillation in SD rats.[54] Some evidence for saturation of elimination pathways at high doses of HQ is available, as a single oral dose of 350 mg/kg in the rat was associated with an average urinary excretion of 50% of total dose after 8 h, compared with >80% excretion at 25 mg/kg.[51] This trend was particularly evident in females.

The biotransformation of HQ (Figure 2) has been examined for over a century, with very early studies (cited by Garton and Williams[66]) identifying urinary sulfate and glucuronide conjugates as metabolites. Following single or multiple oral doses of up to 350 mg/kg HQ in experimental animals, approximately 90% of administered dose is recovered in the urine as one of these conjugates (2-to-1 glucuronide/sulfate ratio).[47–49,51] Similar values are seen after i.t. administration.[53] After i.t. instillation, all of the radiolabel in arterial blood represented parent compound at the initial (5 to 10 s) sample time, suggesting a lack of significant pulmonary metabolism of HQ.[54] At later time points (2 to 12 min), levels of glucuronide rapidly exceeded free HQ concentrations. Minor urinary products following oral administration include unchanged HQ, a mercapturic acid conjugate (*N*-acetyl-[*L*-cystein-*S*-yl]-HQ), and BQ.[51] The urinary BQ is likely formed *in situ* from autoxidation of HQ rather than biotransformation. Unchanged HQ, sulfate, glucuronide, and a putative glutathione (GSH) conjugate of HQ were also reported in plasma from rats 1 h after a 50 mg/kg oral dose of HQ.[50] Following dermal exposure to a 2% HQ cream formulation for 24 h in humans, urinary metabolites included only Phase II (glucuronide) conjugates.[60]

Parenteral administration of HQ to experimental animals is also associated with the presence of high levels of urinary sulfate and glucuronide conjugates. However, additional oxidized (Phase I) and conjugated (Phase II) products are observed. 1,2,4-Benzenetriol, presumably formed from cytochrome P450-mediated oxidation of HQ, was detected in the urine of rats and rabbits administered 50 mg/kg by i.p. injection.[22] *N*-Acetyl-(*L*-cystein-*S*-yl)-HQ was detected in the urine of rats 24 h after a 75 mg/kg i.p. dose and during the first 4 h following a 200 mg/kg i.p. dose of HQ.[67,68] 2-(*L*-Cystein-*S*-yl)-HQ and a number of GSH conjugates, including 2-glutathion-*S*-yl-HQ, 2,5- and 2,6-diglutathion-*S*-yl-HQ, and 2,3,5-triglutathion-*S*-yl-HQ, were detected in bile from rats in the latter study.[68] Together with urinary *N*-acetyl-(*L*-cystein-*S*-yl)-HQ, these biliary metabolites represented over 4% of the total HQ dose administered by i.p. injection. Additional data indicated that formation of these conjugates was in part NADPH- and P450-dependent. Major hepatic P450 isoforms responsible for HQ oxidation, based on *in vitro* studies with human microsomes, appear to be 1A1, 3A4, and 2E1.[69,70]

These toxicokinetic data clearly indicate that the relative proportion of oxidized and conjugated metabolites of HQ is both route of exposure and dose dependent. Following oral exposure, virtually all absorbed HQ is likely to be metabolized via hepatic portal and intestinal sulfate and glucuronide conjugation pathways, although saturation may occur with very high oral doses. In contrast, parenteral administration may bypass much of this conjugative capacity, with the resultant formation of higher levels of oxidized HQ metabolites and GSH conjugates. As discussed later, the GSH metabolites of HQ may be critical to the mechanism of nephrotoxicity observed in animal studies, in particular those utilizing the F344 rat. A PBPK model of HQ disposition, which described a wide variety of experimental data, predicted that male F344 rats form more glutathione conjugates than male SD rats at equivalent dose levels.[71] The model also predicted that

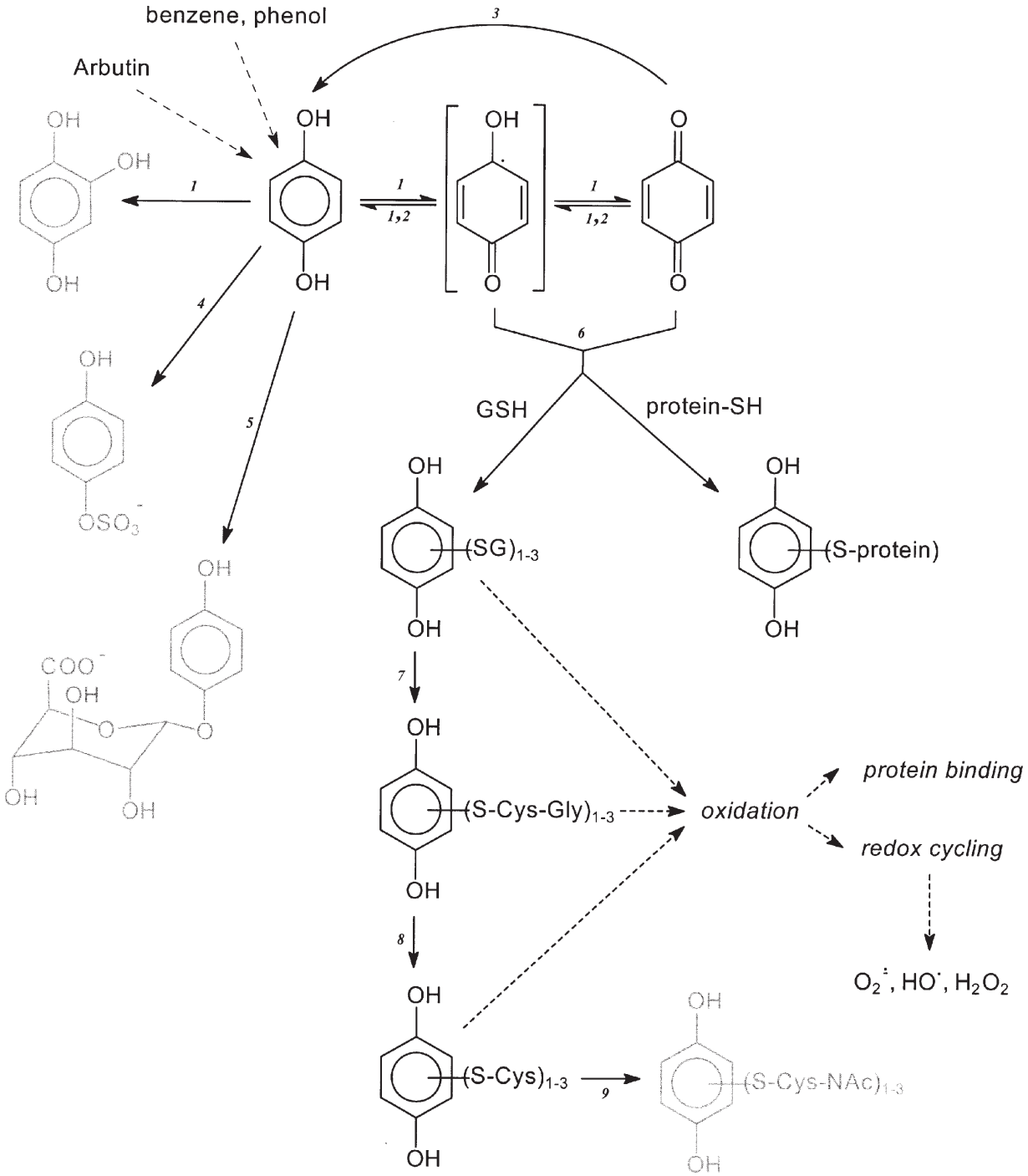

FIGURE 2. Comprehensive metabolic scheme for HQ. Possible input from arbutin (naturally occurring glucose conjugate of HQ), benzene, and phenol is also indicated (long dashed line arrows). Derivatives shown in gray are considered detoxified metabolites. Proposed mechanism of covalent binding and activated oxygen species production from HQ-SG-derived conjugates also shown (short dashed line arrows). Enzyme (or process) associated with each conversion indicated by numbers: (1) spontaneous reaction (slow), cytochrome P450, or various peroxidases; (2) cytochrome P450 or b_5 reductase; (3) NQO1 or carbonyl reductase; (4) sulfotransferase; (5) glucuronyl transferase; (6) spontaneous reaction or glutathione-*S*-transferase; (7) γ-GT; (8) dipeptidase; (9) *N*-acetyltransferase. (GSH = glutathione.)

i.p. administration, which bypasses much of the glucuronide and sulfate conjugation capability of the GI tract, should result in greater amounts of glutathione conjugates than comparable oral doses. With parenteral exposure, increased levels of unchanged HQ are likely to be available to extrahepatic tissues, where non-P450-mediated oxidation of HQ to BQ may occur (particularly in bone marrow).

Finally, while recent evidence suggests that interindividual variation in glucuronyl and sulfotransferase enzymes in liver and other relevant tissues may impact the overall pattern and extent of HQ metabolism from benzene,[72] these differences may not be as important with direct HQ exposure. This is because intestinal pathways provide significant additional glucuronidation capacity for the oral route,[73,74] the most common route of HQ exposure in man. For dermal and inhalation routes, hepatic metabolism of HQ is unlikely to be a limiting factor for detoxication, as lower net absorption of HQ is likely, either because of slow dermal penetration or because the physical characteristics of HQ (i.e., particle size of commercially produced material >100 μm; low volatility) result in lesser amounts absorbed via inhalation for ultimate presentation to the liver. These considerations are important in assessing the potential risk to humans exposed to HQ under typical exposure scenarios (see under Conclusions).

V. MACROMOLECULAR BINDING AND REDOX REACTIONS

Covalent reactivity may underlie certain aspects of HQ toxicity, and the macromolecular binding potential of HQ has been investigated in a number of *in vitro* and *in vivo* models. Because HQ itself lacks a strong electrophilic center, such binding must be preceded by oxidation to either the semiquinone or to BQ, both of which are substantially more reactive toward protein and DNA nucleophiles. The redox chemistry of quinones has been reviewed comprehensively in a number of articles.[75–78] The acid-base and redox conversions of the quinones are closely interrelated, and a total of nine putative ionic and/or radical quinone species combinations are possible (Figure 1). Most of these require relatively extreme conditions of pH or redox potential to form and thus are unlikely to be present under aqueous physiological conditions. Others, however, may be formed and exist as either reactive intermediates or relatively stable species that can mediate toxic effects. Transformations between these species may occur by either one- or two-electron transfers. One-electron redox reactions leading to semiquinones can result in the phenomenon of "redox cycling", where electrons are transferred to molecular oxygen to form superoxide anion. This process has been proposed to result in an increased oxidative stress that may be significant for quinone toxicity,[79,80] although evidence is available that this process is slow at physiological pH.[81] Semiquinone radicals can also combine to regenerate HQ and BQ by means of disproportionation. Brunmark and Cadenas proposed[48] an alternative mechanism of redox cycling, in which P450-mediated oxidation of HQ accompanied by GSH addition would form benzenetriol-SG, a derivative more likely to participate in cycling than HQ itself.

Under physiological conditions, one- and two-electron redox reactions of HQ and BQ can occur either spontaneously or via enzyme-mediated processes. Slow autoxidation of HQ to BQ in phosphate buffer at physiological pH was reported by Greenlee et al.,[63] a reaction that was substantially accelerated by superoxide dismutase. As discussed above, enzymatic conversion of HQ to BQ primarily involves cytochrome P450-mediated oxidation in the liver. However, peroxidases (including myeloperoxidase and related enzyme activities) can mediate this conversion in extrahepatic organs. One-electron reduction of BQ to the semiquinone may occur via cytochrome P450 or b_5 reductase, while two-electron reduction can be catalyzed by NADPH:quinone acceptor oxidoreductase (NQO1; also known as DT-diaphorase or quinone reductase) or carbonyl reductase.[78,79] Depending on the particular organ or tissue in which they occur and on local redox status and metabolite flux, these various metabolic conversions can result in either net activation or detoxification. This is because factors such as intracellular pH, O_2 tension, and thiol balance will influence the direction and extent of these reactions.[78] It should also be noted that the *in vitro*

oxidation and reduction activity of DT-diaphorase on hydroquinones may be modulated in unpredictable ways by ancillary materials such as Triton X-100, Tween 20, and bovine serum albumin.[82] Consequently, *in vitro* studies involving the redox potential of HQ need to be conducted under very carefully controlled conditions to avoid false-positive and false-negative results.[82] As many such studies have not controlled for the impact of these ancillary agents, the significance of certain *in vitro* results is unclear.

As discussed previously, administration of radiolabeled HQ to experimental animals results in covalently bound protein label in a variety of tissues. Peroxidase-mediated binding of [^{14}C]-HQ to albumin was observed with mouse peritoneal macrophage lysates and with purified prostaglandin-H synthase in the presence of H_2O_2; binding was inhibited by cysteine, suggesting the formation of thiol adducts.[81,83] Covalent adducts of HQ to protein sulfur nucleophiles were measured in tissues of rats after administration of HQ.[84] Total adducts were determined as well as individual bound forms of HQ in which the HQ nucleus was substituted with mono-, di-, tri-, or tetra-substituents. The concentration of total adducts was greatest in the liver, followed by kidney and blood. However, the pattern of adduct type was highly tissue specific, with mono-adducts predominating in the liver and tri- and tetra-adducts predominating in the kidneys. These results are consistent with the nephrotoxicity of di- and tri-glutathione conjugates of HQ acting through adduction of protein sulfhydryl groups (yielding tri- and tetra-adducts, respectively). Levels of adducts in kidney correlated with acute nephrotoxicity of HQ as a function of route of administration (i.p. > p.o.) and sex (female F344 rat > male F344 rat) and strain (F344 rat > SD rat).[84] The thiol reactivity of bioactivated HQ is reflected in the rapid depletion of hepatic GSH in rats following p.o. administration of 100 or 200 mg/kg HQ and in isolated hepatocytes exposed to HQ.[85–87] In contrast, only a modest decrease (0 to 20%) in renal GSH in rats was observed after p.o. administration of 400 mg/kg HQ.[88] *In vivo* HQ treatment also resulted in enhanced lipid peroxidation based on recovery of malondialdehyde in urine,[85] although no change in peroxidized lipid levels in the kidneys was found.[89]

Protein binding of HQ/BQ has been reported in other model and *in vivo* systems as well. Covalent labeling of protein with [^{14}C]-HQ was observed in mouse bone marrow macrophages in the presence of H_2O_2 but not arachidonic acid.[90] *In vitro* incubation of HQ with tubulin in the presence of horseradish peroxidase and H_2O_2 resulted in dose-related binding to both α- and β- subunits.[91] Irreversible binding of [^{14}C]-HQ to plasma proteins from F-344 and SD rats and from humans decreased dramatically when incubation temperature was lowered from 37°C to 0°C, suggesting temperature-dependent biotransformation to a reactive species.[92] Assays have been developed to quantitate BQ-derived adducts in hemoglobin and albumin from humans and rodents.[93,94] Human studies have demonstrated high background levels of such adducts, possibly reflecting dietary or other environmental sources.[93] Species- and tissue-dependent, BQ-derived protein adduction in blood and bone marrow has been reported in rats and mice treated with single p.o. doses of benzene.[95] Evidence for covalent binding to DNA polymerase and topoisomerase II has also been obtained for HQ and BQ.[96,97] Finally, *in vitro* inactivation of cytochrome P450, presumably via covalent binding of BQ and/or the semiquinone, has been reported with liver microsomes treated with HQ.[98,99]

Additional studies have shown DNA adduct formation following HQ exposure in tissue culture and cell-free systems, but not *in vivo*. Rushmore demonstrated that *in vitro* incubation of rat bone marrow mitochondria with HQ resulted in formation of guanine and possibly adenine adducts to mitochondrial DNA.[100,101] Two guanine adducts were characterized when purified calf thymus DNA was reacted with HQ,[102] while three adducts were noted following *in vitro* HQ treatment of bone marrow from B6C3F$_1$ mice.[103] Rat Zymbal glands incubated with 750 or 1000 μg HQ/ml for 48 h displayed a characteristic DNA adduct pattern and adduct levels of 1080 and 1250 adducts/10^9 nucleotides, respectively, as measured by ^{32}P-postlabeling assay.[104] Dose- and time-dependent formation of DNA adducts detected using ^{32}P-postlabeling was also observed in HL-60 cells treated with HQ.[105–108] The same adduct was produced with BQ, although BQ was a more potent adduction agent than HQ in this

system, suggesting that BQ is the actual reactive species in this process.[105] Addition of H_2O_2 or cumene hydroperoxide potentiated DNA adduct formation by HQ in HL-60 cells.[109] Chemical studies[110] have indicated that the major DNA adduct formed in HL-60 cells by both HQ and BQ is likely to be N^2-(4-hydroxyphenyl)-dG, which differs from the primary guanine adduct formed in cell-free systems, that is, 3′-hydroxy-1,N^2-benztheno-2′-dG.[102]

Formation of modified DNA bases due to activated oxygen species as opposed to direct adduction by HQ metabolites has also been reported. Levels of 8-hydroxydeoxyguanosine (8-OHdG), considered indicative of damage from superoxide and/or hydroxyl radicals, were increased during incubation of HQ and purified calf thymus DNA.[6] This effect was inhibited in the presence of tyrosinase or catalase. 8-OHdG formation was also demonstrated in DNA from HL-60 cells treated with HQ,[111] and time-dependent covalent DNA adduction has been reported in mixtures of calf thymus DNA and HQ in the presence of prostaglandin-H synthase and either arachidonic acid or H_2O_2.[112]

In contrast to the clearly demonstrated formation of DNA adducts by HQ exposure *in vitro*, evidence for similar adduction in whole animal experiments is absent. Using two variations of the ^{32}P-postlabeling assay, Reddy et al.[113] failed to demonstrate adducts in bone marrow, Zymbal gland, liver, or spleen DNA from female SD rats after HQ exposure (75 or 150 mg/kg/day, 4 days, p.o.). No increase in adducts by postlabeling was seen in kidney DNA from male or female F344 rats exposed to 50 mg/kg/day HQ, p.o., for 6 weeks.[114] The treatment did, however, decrease levels of "I-compounds", which are believed to represent adducts formed by endogenous compounds or oxidative metabolism. The same 6-week treatment regimen did not result in increased 8-OHdG levels in kidney nuclear DNA of male and female F344 rats.[115] Treatment of male $B6C3F_1$ mice with 75 mg/kg HQ, i.p., and of male SD rats with 250 mg/kg HQ, i.p., also did not result in increased 8-OHdG levels in bone marrow or in liver or kidney DNA, respectively.[111,116]

These studies indicate that HQ conversion to the semiquinone and BQ can potentially result in covalent adduction to both protein and DNA *in vitro*. In addition, formation of activated oxygen species via HQ oxidation can lead to increased 8-OHdG adduction in DNA *in vitro*. However, the demonstrated lack of DNA adduct or 8-OHdG formation by HQ *in vivo*, coupled with the generally negative results for HQ mutagenicity in short-term tests (discussed below), argue against a significant role for these phenomena in HQ toxicity. In contrast, thiol adduction (either with GSH or with protein sulfhydryl groups) clearly occurs following *in vivo* HQ exposure. This binding potential has implications for the mechanisms of HQ-induced clastogenicity, nephrotoxicity, and myelotoxicity.

VI. GENOTOXICITY

A voluminous literature exists examining the potential genotoxic effects of HQ in a variety of *in vitro* and *in vivo* systems. The results of these studies have been tabulated in several previous reviews.[1,11–13] While the genotoxicity data base for HQ is extensive, the results are highly dependent on exposure route. *In vitro* studies are frequently, but not always, positive, while *in vivo* studies are typically negative unless detoxication pathways are overcome by parenteral administration. With few exceptions, HQ has been shown to be inactive as a direct mutagen in various short-term assays. Negative results have been reported with various strains of *S. typhimurium* and *E. coli*, with or without metabolic activation,[117–124] in yeast (*S. cerevisiae*, strain D4),[117] and in *Drosophila*.[7,125,126] In addition, no evidence for mutagenicity was demonstrated in an *in vivo* mouse spot test[127] and in a dominant lethal assay in male rats following p.o. doses of 30, 100, or 300 mg/kg/day HQ for 10 weeks.[128]

Occasional positive results for HQ in reverse mutation assays have been reported, that is, in a single strain (TA1535A) of *S. typhimurium* using a nonstandard incubation medium (ZLM medium) without metabolic activation,[125] in several strains of *S. typhimurium* without activation,[129,130] in a "fluctuation test" using one strain of *S. typhimurium* only in the presence of S9[131] and in *S. cerevisiae* strain D3.[118] A high potency for the induction of mutations leading to 6-thioguanine resistance in Chinese hamster V79 cells by

HQ has been demonstrated also.[124] A recent paper reported increases in mutation to both ouabain and 6-thioguanine resistance in Syrian hamster embryo (SHE) cells exposed to HQ, although the increases were not dose related.[132] In addition, positive results were obtained in forward mutation assays using mouse lymphoma L5178Y cells exposed *in vitro* to HQ, with and without metabolic activation.[133]

In contrast to the largely negative data for direct mutagenicity, numerous studies have shown the effects of HQ on clastogenic, mitotic, and aneuploidigenic endpoints. These effects are manifested as chromosomal aberrations, abnormal mitoses, formation of micronuclei (MN), aneuploidy, DNA strand breakage, and sister chromatid exchange (SCE). Many of these studies were conducted as part of a large interlaboratory aneuploidy screening program conducted by the European Communities Directorate General Environmental Research Programme, which included HQ as one of the core chemicals selected for study.[134,135] Early reports indicated that HQ disrupts mitosis in a variety of cell types.[136,137] These were followed by demonstration of the induction of SCE in Chinese hamster ovary (CHO) cells,[138] Chinese hamster V79 cells,[124] SHE cells,[132] and human lymphocytes.[139–144] Generally positive results were found with or without S9 metabolic activation in these studies. Negative SCE findings have been reported in bone marrow cells from mice treated with HQ at doses up to 120 mg/kg, i.p.,[145] and in one *in vitro* study with V79 cells.[129]

A number of *in vitro* studies have demonstrated MN induction in HQ-treated human lymphocytes.[146–149] Induction of MN in human lymphocytes has not been reported in whole blood cultures except at toxic dose levels.[150] MN induction *in vitro* has also been reported for fibroblasts,[151] and embryonal liver cells,[152] Chinese hamster V79, XEM2, LUC2, and/or SD1 cells,[124,153–156] and rat intestinal cells.[152] Using oral gavage, s.c., or i.p. dosing regimens, largely positive results have been obtained with HQ in the mouse bone marrow micronucleus assay.[125,145,157–170] Transplacental induction of MN in blood cells from fetal mouse liver following exposure of pregnant dams to HQ has been reported.[171] The lowest positive dose of HQ in these experiments was approximately 40 to 50 mg/kg, and i.p. dosing was found to be substantially more effective than oral exposure, which was associated with weak induction of MN.[160] Some of these studies also examined the proportion of kinetochore-positive and -negative MN, with general indications that HQ can produce MN by mechanisms involving both whole chromosome loss and chromosome fragmentation.[146,148,151,156,164–166,169,170,172]

Additional experiments have assessed induction of aneuploidy (abnormal chromosome number) by HQ *in vitro* and *in vivo*. Doses of 80 to 120 mg/kg, i.p., produced hyperploidy in mouse spermatocytes,[173,174] while aneuploidy was detected in mouse bone marrow cultures[175] and human lymphocytes[176] treated with HQ. In contrast, HQ exposure to SHE and yeast cells did not result in aneuploidy.[132,177] Chromosomal aberrations, including gaps, breaks, exchanges, dicentrics, and complete fragmentation, resulting in chromosome malsegregation and aberrant cells, have been reported for HQ *in vivo* in mouse bone marrow cells[178] and spermatocytes (following i.p. injection),[179] and *in vitro* in *Aspergillus nidulans*,[180,181] CHO,[138] and SHE cells.[132] DNA strand breaks and other damage has been noted in a variety of *in vitro* systems, including isolated DNA,[112,182–186] rat liver cell nuclei and hepatocytes,[187,188] mouse lymphoma cells,[189] Chinese hamster bone marrow[190] and ovary[191] cells, human lymphocytes,[192] and human lung carcinoma cells.[193] Induction of DNA damage *in vitro* in human peripheral lymphocytes was found to require much higher concentrations of HQ in mitogen-stimulated as compared to resting cells.[194] Addition of cytosine arabinoside (a DNA repair inhibitor) increased the sensitivity of mitogen-stimulated cells to HQ, suggesting protection by DNA repair mechanisms from DNA-damaging activated oxygen species. Recent studies have indicated induction of monosomy and long arm deletion of chromosomes 5 and 7 in human whole blood lymphocyte cultures incubated with HQ.[195] These changes are commonly observed in benzene-induced myelodysplastic syndrome (MDS) and leukemia in humans.[196] Additional positive (and occasional negative) responses for HQ have been reported in various other types of assays, including mitotic disruption and arrest,[136,181,197–200] inhibition of DNA and RNA synthesis,[97,201–205] and induction of unscheduled DNA synthesis.[132]

HQ exposure appears to affect the mitotic apparatus and/or protein components of the chro-

mosome. Irons and co-workers first demonstrated that HQ could inhibit rat tubulin polymerization *in vitro*, that this process required the presence of O_2, and that specific covalent modification of one or two sites within the high-molecular-weight subunit of tubulin was involved.[206,207] These sites were characterized as reactive protein thiols distinct from the colchicine binding site and possibly at or near the GTP binding site. Weak inhibition of tubulin assembly *in vitro* by HQ has also been demonstrated for bovine, but not porcine, tubulin.[208,209] Based on these data, Irons proposed that the mitotic effects of HQ were mediated by spontaneous or enzyme-catalyzed oxidation to a reactive species, probably BQ, followed by covalent modification of tubulin, inhibition of tubulin polymerization, and disrupted spindle formation. This mechanism is supported by evidence indicating the enhancement of HQ-mediated inhibition of microtubule formation during peroxidative metabolism.[91] More recently, Dobo and Eastmond[156] demonstrated that prostaglandin-H synthase (PHS) mediated oxidation of HQ increased the incidence of kinetochore-positive (i.e., whole chromosome-containing) MN in Chinese hamster V79 cell cultures, an effect blocked by GSH. While this proposed mechanism is reasonable, direct experimental evidence that inhibition of tubulin polymerization leads to aneuploidy and formation of kinetochore-positive MN in susceptible cells following *in vivo* HQ exposure is lacking.

In contrast to mitotic disruption, the occurrence of chromosomal fragmentation (indicated by the presence of kinetochore-negative MN) and DNA strand breaks cannot be accounted for by inhibition of tubulin polymerization. These effects have instead been attributed to either direct damage to DNA from reactive oxygen species, covalent DNA adduct formation via BQ or the semiquinone, or inhibition of enzymes involved in nucleic acid metabolism. The presence of catalase resulted in partial inhibition of kinetochore-negative MN formation by HQ in Chinese hamster V79 cell cultures, suggesting the involvement of H_2O_2 and other activated oxygen species.[156] In addition, Cu^{+2}-mediated autoxidation of HQ with consequent reactive oxygen species formation resulted in enhancement of DNA strand breaks.[210–212] GSH was found to enhance the HQ-mediated induction of double-strand DNA breaks *in vitro,* an effect that was reduced in the presence of superoxide dismutase.[213] However, other results indicated that the presence of oxygen radical scavengers did not reduce HQ-induced phage DNA single strand break*s in vitro*,[184] while mouse bone marrow cell DNA breakage was induced by BQ despite its low oxygen radical-generating potential.[214] Finally, *in vitro* inhibition of DNA and RNA polymerases and of DNA topoisomerases by oxidized HQ metabolites has been reported.[96,97,100,202,205,215]

The genotoxicity database for HQ supports a low potential for direct mutagenicity (i.e., induction of frame-shift and point mutations) for this compound. A similar conclusion can be made for BQ. This suggests that the observed *in vitro* formation of DNA adducts by HQ and BQ may be of questionable toxicological significance. In contrast, both agents have clastogenic and aneugenic potential and can interfere with the mitotic process. These effects are likely to be mediated through the protein thiol binding capability of BQ, although the involvement of activated oxygen species in certain of these processes may be important. Other than tubulin, it is not known which specific proteins may represent the critical targets for this adduction. Alternatively, adduction/inactivation of DNA polymerase, topoisomerase II, or other relevant enzymes may be significant in the mechanism of HQ clastogenicity. In any case, these epigenetic effects appear to depend heavily on toxicokinetic factors, particularly route of exposure, suggesting that exposure thresholds may exist. Despite gaps in knowledge concerning specific mechanisms of clastogenicity and aneuploidy for HQ, modeling of dose response data from mice given HQ i.p. indicate a very good linear fit between 25 to 75 mg/kg using a linear quadratic model with a no-observed effect level at 12.5 mg/kg.[169] The model suggests a no-effect threshold between 12.5 and 25 mg/kg i.p. Because oral exposure is much less effective at inducing such effects, the threshold for an oral no-effect level would be expected to be higher than the i.p. no-effect level.

VII. NEPHROTOXICITY AND RENAL CARCINOGENESIS

Early studies examining single or repeated dose toxicity of HQ in experimental animals did

not reveal marked evidence of nephrotoxicity, although comprehensive evaluations of renal function were generally not performed in these studies. For example, feeding of SD rats with up to 1% HQ in the diet for 2 years did not result in histopathological alterations in the kidney, while administration of 100 mg/kg/day, p.o., to dogs for 6 months produced neither histopathology nor changes in urine chemistry values.[26] These same investigators reported no urinary alterations in male human volunteers ingesting 300 to 500 mg/day (4.3 and 7.1 mg/kg/day, respectively, for a 70-kg man) for 3 to 5 months. Christian reported only a mild increase in relative (to body) kidney weights, with no accompanying pathological changes, in rats administered 1% or 0.4% HQ in the drinking water for 8 or 15 weeks, respectively.[27] Woodard exposed several dogs to daily oral doses of HQ of 25 or 50 mg/kg for more than 2 years, but did not report any clinical or gross pathological findings consistent with renal impairment.[25] In one study, chronic exposure (length unspecified) of rats and mice to 50 to 100 mg HQ/kg/day was reported to cause "dystrophic changes" in kidney and other organs.[216] In addition, daily i.p. injection of BQ (2 mg/kg/day) or benzenetriol (6.25 mg/kg/day) but not HQ (10 mg/kg/day) for 6 weeks produced histologically demonstrable kidney damage in mice.[217]

In 1979, the NTP initiated testing on HQ that included subchronic (90-day) and chronic (2-year, with a 15-month interim sacrifice) toxicity studies in F344/N rats and B6C3F_1 mice. The results of these studies have been published as a monograph and summarized in a peer-reviewed literature report.[7,218] In the subchronic study, animals were administered HQ in corn oil by gavage at dose levels of 0, 25, 50, 100, 200, or 400 mg/kg/day (5 days/week). Mortality in the highest dose group was 100 and 80% for rats and mice, respectively. Moderate to marked nephrotoxicity, characterized by tubular cell degeneration and regeneration in kidney cortex, was noted in the majority of male and female rats at 200 mg/kg/day and in 1 of 10 female rats at 100 mg/kg/day. The lesions present in male rats were graded as more severe than those in females. No renal lesions were observed in rats at 50 mg/kg/day or lower or in mice at any dose level. Relative kidney weights were not reported in this study. These effects were considered to represent dose-related primary kidney toxicity due to HQ exposure.

Sex-, strain-, and species-related differences in HQ-induced nephrotoxicity have also been examined in numerous studies. A single oral dose of 400 mg/kg HQ to F344 rats resulted in increased urinary excretion of alanine aminopeptidase, *N*-acetyl glucosaminidase, alkaline phosphatase, γ-GT, and glucose, all indicative of proximal tubule damage.[219] Increases in creatinine and BUN were also seen in female rats. Interestingly, the increases were more pronounced for female rats, in contrast to the higher susceptibility of male rats to histologically demonstrable nephrotoxicity with subchronic HQ administration.[7,220] Similar changes were not seen in SD rats, or in B6C3F_1 mice, except for increased BUN in both sexes of mice administered 350 mg/kg HQ.[219] Urine osmolality tended to be lower and urine volume higher than in controls for both F344 and SD rats. Microscopic analysis of urine demonstrated increased epithelial cell counts for F344 rats. These studies provide further confirmation of the high susceptibility of this rat strain to HQ-induced nephrotoxicity.

In addition to the sex- and strain-specificity of kidney effects, the expression of kidney toxicity depends upon route of administration and age of the animal. In a 13-week dermal toxicity and cell proliferation study, the male F344 rat-specific kidney effects observed in earlier oral studies were absent.[221] HQ was applied as an oil-in-water emulsion at a maximum level of 5%, corresponding to a dose of approximately 75 mg/kg/day. The lack of observed effect is most readily explained by low dermal absorption of HQ.[19] However, other toxicokinetic factors may be involved, as applied dermal doses of HQ (in ethanol) of up to 3840 mg/kg/day for 14 days in F344 rats was also not associated with gross indicators of nephrotoxicity (i.e., body weight changes and gross kidney pathology).[7] At this high-dose level and with this dosing vehicle, HQ was detected in urine in this study, suggesting substantial dermal absorption. Similarly, the histopathology and cell proliferation seen after 6-week gavage administration to young adult male F344 rats was not present in 1-year-old rats on the same treatment regimen, consistent with a possible age-related difference in susceptibility.[222]

The evidence for nephrotoxicity following oral exposure to HQ has stimulated much research on mechanism of action. These efforts have primarily focused on the sex- and species-dependence of the phenomenon and on the possible involvement of GSH conjugates of HQ as initiators of nephrotoxicity (Figure 3). Mono-, di-, and tri-GSH conjugated HQ metabolites have been detected in rat bile following i.p. injection of HQ.[68] Direct parenteral administration of di- and tri-GSH conjugates to rats resulted in proximal tubule necrosis and nephropathy, with the 2,3,5-triglutathion-*S*-yl-HQ (tri-G-HQ) derivative being the most potent, and the fully substituted 2,3,5,6-tetraglutathion-*S*-yl-HQ exhibiting no activity.[223] Maximum damage was localized to the P3 segment of the proximal tubule. Nephrotoxicity due to GSH conjugates of HQ was blocked by pretreatment of animals with AT-125 (Acivicin, a potent inhibitor of γ-GT).[223] This enzyme catalyzes the cleavage of glutamic acid from GSH conjugates to yield a cysteinyl-glycine conjugate and is present at high activity in proximal tubule epithelium.

Hill et al.[224] demonstrated a decrease in state 3 mitochondrial respiration in rats administered

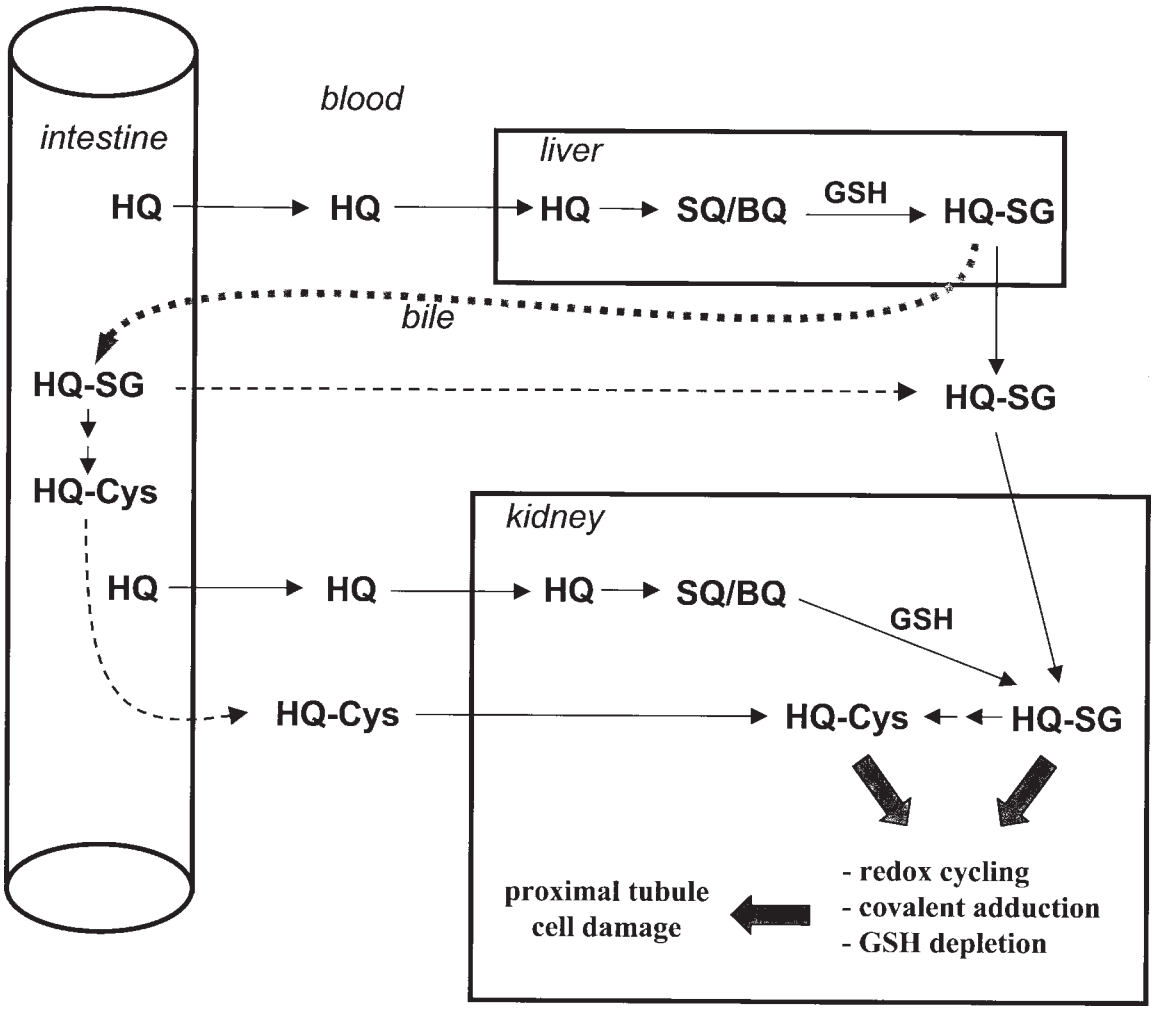

FIGURE 3. Summary of proposed mechanisms of hydroquinone (HQ) nephrotoxicity following p.o. administration and oxidation to semiquinone (SQ) and/or benzoquinone (BQ) in liver and kidney. Note dual pathways of formation of putative nephrotoxic HQ-glutathione (HQ-SG) and HQ-cysteine (HQ-Cys) conjugates. HQ-SQ and HQ-Cys can be formed in the liver and intestine, respectively, and then transported in blood to the kidney. Alternatively, each derivative can be formed directly in the kidney from HQ via SQ/BQ.

tri-G-HQ, although it was unclear whether this change represented a cause or effect of subsequent nephrotoxicity. Using the *in situ* perfused rat kidney (ISPRK) model, Hill et al.[225] showed a time-dependent release of γ-GT into the urine of rats infused with 2-glutathion-*S*-yl-HQ or tri-G-HQ. A greater retention of tri-G-HQ within the kidney was found, leading to speculation that the oxidative metabolism of the conjugate resulted in formation of reactive derivatives that subsequently bound to kidney macromolecules. Additional studies have shown that mono- and di-G-substituted HQ derivatives readily undergo autoxidation to tri-G-HQ under physiological conditions.[226]

In vitro studies have revealed that *N*-acetylation of 2-(*L*-cystein-*S*-yl)-HQ to form the mercapturate was less active in the male than in the female F344 rat or in the male SD rat, a nephrotoxicity-resistant strain.[227] Formation of the mercapturate is considered a detoxication reaction, as it oxidizes less readily to putative reactive products than does the cysteine conjugate. Male F344 rats exhibited a moderate decrease in GSH and cysteine concentrations in kidney following 400 mg/kg HQ p.o., while male SD rats did not.[88] Studies of differences in species susceptibility to nephrotoxicity induced by i.v. injection of tri-G-HQ indicated that only male F344 rats and guinea pigs developed proximal tubule dysfunction, while females of these species and both sexes of BALB/c and $B6C3F_1$ mice and of hamsters were resistant.[228] Some correlations between the activities of γ-GT, *N*-acetylase, and *N*-deacetylase and species susceptibilities were noted.

The overall mechanistic database for HQ points to the involvement of GSH-HQ conjugates and a critical influence of sex-, strain-, species-, dose-, and route-specific factors in the pathogenesis of nephrotoxicity. The current paradigm[1,10,11,114,225,229] suggests that oral HQ exposure is followed by rapid and extensive sulfate and glucuronide conjugation in liver and intestine and elimination of these detoxified metabolites via urine. Saturation of this process by high oral doses or partial bypass via parenteral administration leads to the formation of various GSH conjugates in the liver, which are either excreted via bile or enter the circulation. These conjugates enter proximal tubule cells either directly from the blood or following reabsorption from the urine. Within proximal tubule cells, HQ-GSH conjugates or their γ-GT/dipeptidase cleavage products can be oxidized either spontaneously or (more likely) via enzymatic processes to yield reactive species such as BQ- and/or semiquinone-conjugates. These derivatives may either directly adduct renal cell macromolecules or undergo redox cycling to produce reactive oxygen species that can also cause macromolecular damage. Alternatively, HQ-GSH conjugates may be excreted as detoxified urinary mercapturates following sequential processing by γ-GT, dipeptidase, and *N*-acetylase. Thus, the sex-, strain-, and species-specific characteristics of HQ nephrotoxicity may be due to differences in the relative balance between toxifying and detoxifying metabolic pathways in the proximal tubule cell, which in turn may be governed by genetic factors.

Some uncertainty still remains concerning the mechanism of nephrotoxicity following higher-dose HQ exposure in experimental studies. For example, although HQ thiol adducts have been demonstrated following oral dosing, HQ-GSH conjugate formation has not been conclusively demonstrated in orally dosed rats. Covalent protein adducts in the kidney derived from HQ have been detected and quantified but not characterized, and their precise role in kidney toxicity remains to be determined. In addition, macromolecular changes consistent with oxidative damage occurring within proximal tubule cells have not been reported.

The NTP also conducted a chronic study, in which rats were administered 0, 25, or 50, and mice 0, 50, or 100 mg/kg/day (5 days/week) of HQ by gavage in deionized water for 103 weeks.[7] An interim sacrifice of 10 animals per group was performed at 65 weeks. Unlike the subchronic studies, no clear evidence of chemically induced nephrotoxicity was apparent in HQ-treated rats or mice. In contrast, evidence of chronic nephropathy was present in nearly all control and dosed rats at 103 weeks. While this syndrome (also termed "spontaneous nephropathy" or "chronic progressive nephropathy"; CPN) is common in aged male rats, the severity of the lesion was greatest in males from the 50 mg/kg/day HQ-treated group. This trend was also apparent in male rats at the 65-week interim sacrifice, although no alterations in urinalysis parameters

consistent with nephropathy were apparent at this time point. Histopathological findings associated with this nephropathy included degeneration and regeneration of tubular epithelium, tubular atrophy and dilation, the presence of hyaline casts in tubule lumen, glomerulosis, interstitial fibrosis, and inflammation. High-dose male rats with marked levels of nephropathy also exhibited papillary hyperplasia of the transitional epithelium covering renal papillae and cysts. Due to the lack of hyaline droplets, cast formation in the loop of Henle, and mineralization, the kidney lesions were not considered related to α_{2u}-globulin nephropathy. Elevated relative (to body) kidney weights were also observed for high-dose male rats at the 65- and 103-week sacrifices. Dose-related increases in the severity of chronic nephropathy were not seen in female rats and except for a high incidence of chronic renal inflammation in all groups mice did not exhibit kidney lesions.

In another chronic study conducted by different investigators (Shibata et al.), an increase in the severity of chronic nephropathy, in addition to papillary and tubular hyperplasia, was noted in male F344 rats fed 0.8% HQ in the diet for 104 weeks.[220] This dietary level corresponded to an average dose of 351 mg/kg/day based on measured food consumption, substantially higher than that used in the NTP study. An increased incidence of mild nephropathy was seen in female F344 rats, with an average HQ intake of 386 mg/kg/day. Renal changes in male and female $B6C3F_1$ mice fed 0.8% HQ, with average doses of 1046 and 1486 mg/kg/day, respectively, consisted of a 30% incidence of tubular hyperplasia without nephropathy in males; interim tissue examinations were not conducted. Relative (to body) kidney weights were increased in both sexes of rats and in female mice. The results of urinalysis were not reported. The overall findings regarding CPN were generally consistent with those of the NTP study.

Both the NTP and Shibata studies provided evidence for renal carcinogenesis in male rats chronically exposed to HQ. In addition to increased severity of CPN, male F344 rats in the NTP study exhibited 7% (4/55) and 15% (8/55) incidences of tubular adenomas in the low- and high-dose groups, respectively, although the incidence in low-dose rats was not significantly different from control. These lesions were described[7] as "discrete masses of epithelial cells arranged in solid clusters or nests separated by a scant stroma." Hyperplasia of tubular epithelium, a potential preneoplastic lesion, was also noted in two male high-dose rats. None of the tumors were reported to be grossly observable. Adenomas were not observed in female F344 rats or in either sex of $B6C3F_1$ mice. Based on these findings, NTP concluded that there was "some evidence" of carcinogenic activity for HQ in the male rat. Tumor formation was suggested to be chemically induced but independent of the nephrotoxicity observed in the subchronic studies.[218]

Renal adenomas were also reported in male F344 rats fed 0.8% HQ in the diet for 2 years, with an incidence rate of 47% (14/30).[220] The higher incidence in this study was attributed to the larger average daily dose (50 vs. 351 mg/kg/day) in these animals when compared with the NTP study. Renal papillary hyperplasia, considered indicative of advanced CPN, was seen in 37% of male rats, while all male rats exhibited renal tubular hyperplasia. Adenomas were also reported in 3 of 30 male $B6C3F_1$ mice, although this incidence rate was not significantly different from control. Increased relative (to body) kidney weights were noted in both sexes of rats and in female mice, while absolute kidney weights were increased only in male rats. Based on these data, Shibata and co-workers concluded that HQ was clearly carcinogenic in male rats and that this effect was probably linked to CPN. However, unlike the NTP investigators, they suggested an involvement of free radicals and/or quinone metabolites in the carcinogenic mechanism.

The NTP and Shibata et al. conclusions regarding HQ-induced renal toxicity and tumorigenesis have been criticized based on both methodological concerns (e.g., the potential renal effects of a concurrent sialodacryoadenitis infection within the test colony in the NTP study) and overall interpretation of the database.[10,11,229,230] The possible relationships between proximal tubule cell degeneration and regeneration, CPN, and renal adenomas in F344 rats exposed to HQ have been examined recently by means of a reevaluation[10] of the pathology data from the NTP chronic study and additional experimentation. The reevaluation indicated that 36 of 51 high-dose (50 mg/kg/

day) male rats had pathological alterations consistent with either severe or end-stage CPN. In addition, 11/51 high-dose rats exhibited foci of atypical tubular hyperplasia, considered to be a preneoplastic lesion, compared with the originally reported NTP incidence of 1/55. The reassessment of tumor data also resulted in revised renal tubule adenoma incidences of 3/49 and 7/51 in low- and high-dose male F344 rats, respectively, when compared with 4/55 and 8/55 in the original report. The key finding of this reevaluation was the high correlation between the presence (occurrence and location) of hyperplasia or adenomas and that of severe to end-stage grade CPN, suggesting that HQ acts in an epigenetic manner to accelerate the spontaneous CPN process.

It is widely accepted that compensatory cell proliferation in general can increase the risk of tumorigenesis. To further clarify this issue for HQ and renal carcinogenesis, the time- and dose-dependence and anatomic localization of cell proliferation in the proximal and distal tubules of F344 rats were examined in animals administered oral doses of HQ at 0, 2.5, 25, or 50 mg/kg/day for up to 6 weeks.[229] Increased cell proliferation is characteristic of CPN,[231] and the selected dose levels and dosing protocol were designed to mimic those employed during the initial period of the NTP chronic study. As measured by BrdU incorporation, cell proliferation in the P1 and P2 segments of kidney tubules was increased over control values for male F344 rats after 6, but not 3, weeks of HQ exposure at the 50 mg/kg/day level. Interestingly, this finding contrasts with the extensive damage seen in the P3 segment of rats exposed via parenteral injection to tri-G-HQ and other GSH-HQ conjugates,[223] suggesting that HQ-induced nephrotoxicity and accelerated CPN may proceed by different mechanisms. Degenerative and regenerative foci and interstitial inflammation were also observed in these animals by histopathologic examination. A nonsignificant trend toward increased proliferation was seen in male rats at 25 mg/kg/day. In contrast, no significant changes in these parameters were observed at lower dose levels in male rats or at any dose level in female F344 or either sex of SD rats. The delay in appearance of cell proliferation is indicative of a compensatory response to slowly developing nephropathy characterized by proximal tubule cytotoxicity and cell replacement, consistent with an epigenetic mechanism.

The nature of the causal relationship, whether direct or indirect, between HQ exposure, CPN, and induction of renal tubule adenomas in F344 rats is currently unclear. In one recent model, HQ is proposed to act at two stages; to accelerate the normal evolution of CPN in the rat from mild to end-stage, and to increase tubule cell proliferation, particularly that associated with progression from CPN to tubular hyperplasia and renal adenoma formation.[10,11] This model is based on the better correlation of incidence of hyperplasia with severity of CPN than with dose of HQ, and on data showing the presence of atypical hyperplasia and adenomas in low-dose male rats in the absence of enhanced CPN. Clearly, questions remain to be answered concerning the molecular mechanism(s) by which HQ might promote, accelerate, or synergize spontaneous CPN and cause increased cell proliferation. Despite these uncertainties, the overall genotoxicity, nephrotoxicity, and mechanistic database supports a species-dependent, epigenetic mechanism for HQ-induced renal carcinogenesis. The possible implications of these findings for human renal carcinogenesis due to direct HQ exposure are discussed later in this article.

VIII. OTHER DATA RELATED TO CARCINOGENESIS

In addition to the renal tumorigenic effects discussed above, long-term bioassays with HQ have provided some indication for liver and forestomach neoplasia and mononuclear cell leukemia in rodents. In the 2-year NTP bioassay, male and female mice exhibited significantly increased relative liver weights at 15 months, while hepatic fatty change and cytomegaly were present in male mice in the 100 mg/kg/day group at this time point.[7,218] After 2 years, the incidence of liver adenomas was increased in both sexes of mice at the 50 and 100 mg/kg/day dose levels. However, the incidences of these lesions were highest in the 50 mg/kg/day group, suggesting a lack of clear dose response. Hepatic carcinomas occurred at a lower incidence in male mice given HQ compared with the control, while there was no differ-

ence in the incidence of carcinomas between female mice given HQ and their control group. Thus, when adenomas and carcinomas were combined, only the female mice were considered to have an increased incidence of liver tumors. Based on these data, NTP concluded that there was "some evidence" for carcinogenic activity in the female mouse.[7]

Shibata et al.[220] reported increased relative liver weight and incidence of hepatocellular hypertrophy, altered foci, and adenomas in male but not female mice fed 0.8% HQ in the diet (average dose 1486 mg/kg/day). The basis for the inconsistency in the sex-specific incidence rates for hepatic adenomas between the two studies is unclear. As in the NTP study, hepatocellular carcinomas were not increased for male or female mice in the bioassays reported by Shibata et al.[220] In addition, hepatic neoplastic lesions were not observed in rats in either study, and HQ exposure was found to decrease the number of foci of cellular alteration in livers of both male and female rats. These inconsistencies and negative findings have prompted other investigators to conclude that the evidence for HQ-induced hepatic neoplasia is only equivocal.[11,230]

The study of Shibata et al.[220] also reported a significant increase in squamous cell hyperplasia (but not carcinoma) of the forestomach in male and female mice. This effect was not observed in the NTP 2-year bioassay,[7] although inflammation and epithelial hyperplasia were noted in rats and mice at 200 and 400 mg/kg/day, respectively, in the NTP preliminary subchronic studies. In contrast, administration of 0.5% HQ in the diet of Syrian golden hamsters for 20 weeks did not result in histopathological forestomach changes or an increase in mitotic labeling index of forestomach epithelial cells.[232] HQ (0.8% in the diet for 51 weeks) also did not enhance forestomach or glandular stomach neoplasia in F344 rats given a single initiating dose of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine,[233] but did slightly increase the incidence of esophageal carcinomas following initiation by methyl-*N*-amylnitrosamine.[234] No histopathological changes or increases in forestomach epithelial cell DNA synthesis were observed in male F344 rats fed 0.8% HQ in the diet for 8 weeks.[235] Only mild forestomach hyperplastic responses were present in rats fed 2% HQ for 4 weeks.[236] Taken together, these results do not support a significant potential for HQ to induce gastrointestinal neoplasia in rodent bioassays.

An increased incidence of mononuclear cell leukemia was noted in the NTP bioassay for HQ in female F344/N rats after administration of 50 mg/kg, p.o., for 2 years.[7,218] Staging of the severity of the leukemia in the 22 affected animals, based on histopathological alterations in the spleen, indicated 1, 7, and 14 rats with Stage 1, 2, or 3 leukemia, respectively. These data led NTP to classify HQ as having "some evidence" of carcinogenicity in the female rat. Concern has been raised over the leukemogenic effect of HQ as reported in the NTP study because of the well-known phenomenon of benzene-induced leukemia in humans. However, benzene itself does not induce mononuclear cell leukemia in rats,[237] and no similar effects were observed in the Shibata HQ bioassay,[10,230] despite much higher dose levels. Mononuclear cell leukemia in rats is considered to originate in the spleen[238] and not the bone marrow, which is considered to be the origin of benzene-induced leukemia. No specific evidence for bone marrow alterations consistent with a leukemogenic process was reported in either study. It should be noted that the reported historical control incidence of mononuclear cell leukemia observed in NTP bioassays using female F344 rats increased progressively during the 1980s.[239] This trend, in addition to the isolated nature of the NTP finding, weakens the strength of evidence for a significant leukemogenic potential for HQ.

Various tumor initiation and/or promotion assays have also been conducted with HQ, with generally negative results. An early study involving a single application of 20 mg HQ (in acetone) to mouse skin, followed by promotion with croton oil, did not reveal initiating activity.[240] HQ was also negative as a promoter in a mouse skin carcinogenesis model following initiation with dimethylbenzanthracene.[241] Both a lack of promoting activity and an ability to partially inhibit benzo[*a*]pyrene-initiated skin carcinogenesis have been demonstrated for HQ.[242] Dietary exposure to HQ (1.5%) also inhibited pancreatic lesions (combined preneoplastic and neoplastic) following administration of *N*-nitrosobis(2-oxopropyl) amine.[243] Administration to F344 rats of a nitrosamine initiator, followed by 0.8% HQ in the diet

for 32 weeks did not result in the development of bladder hyperplasia or neoplasia.[244] HQ neither initiated nor promoted *N*-butyl-*N*-(hydroxybutyl)nitrosamine initiated bladder carcinogenesis when given at 0.2% for 22 weeks to male F344 rats that had one ureter ligated.[245] Male F344/Du Crj rats were fed 0.8% HQ diets for 30 weeks following exposure to *N-bis*(2-hydroxypropyl) nitrosamine as an inducer of lung tumors.[246] HQ did not promote lung tumors or enhance the incidences of thyroid, kidney, or urinary bladder tumors that were observed following exposure to the initiator alone.

In contrast to these negative findings, rats treated with *N*-ethyl-*N*-hydroxyethylnitrosamine followed by 0.8% HQ in the diet for 32 weeks did display a significant increase in renal microadenomas and renal cell tumors when compared with the nitrosamine alone.[89] However, similar HQ treatment without initiator did not result in renal changes. Boyland et al.[247] reported an increased incidence of bladder carcinomas in mice implanted with HQ/cholesterol pellets in the urinary bladder for 25 weeks, although the relevance of this model for determination of overall carcinogenic potential is questionable. Bladder tumorigenesis has not been reported in any long-term bioassay with HQ.

Several studies have employed "medium-term" liver carcinogenesis bioassays or related protocols to examine initiation and promotion effects of HQ. Stenius reported an increase in *N*,*N*′-diethylnitrosamine (DEN)-induced γ-GT-positive hepatic foci in partially hepatectomized rats following 7 weeks exposure to 100, but not 200, mg/kg/day HQ p.o.[187] In contrast, DEN initiation, followed by partial hepatectomy and dietary administration of 2% HQ to rats for 6 weeks, resulted in a significant decrease in glutathione-*S*-transferase (GST)-positive hepatic foci as compared with DEN treatment alone.[248] Other data indicate that HQ does not selectively damage GST-positive hepatocytes.[249] Finally, a recent study[250] examining hepatic tissue from a previous HQ carcinogenicity bioassay[220] indicated that prolonged (2-year) dietary exposure of F344 rats to 2% HQ resulted in decreased numbers and area of GST-positive hepatic foci. These findings generally support the conclusion that HQ lacks significant hepatocarcinogenic activity in rodents.[11]

IX. MYELOTOXICITY AND IMMUNOTOXICITY

Early animal studies provided limited evidence that prolonged, high-dose HQ administration might affect blood cells and/or bone marrow. Hematological changes were reported in cats following parenteral administration of single doses of 60 to 100 mg/kg HQ (cited by Von Oettingen[341]), and after p.o. exposure to 15 mg/kg/day for 40 days in rats.[251] Woodard reported histopathological changes in bone marrow and spleen from dogs fed 25 or 50 mg/kg/day for 819 days.[25] Severe toxicity and decreased bone marrow cellularity was observed in rats fed 5% HQ in the diet for 9 weeks;[26] however, results at this dose level are confounded by severe body weight loss. Decreased red blood cell (RBC) counts, but not hematocrit or colony forming cells in bone marrow, were seen in mice given HQ in the drinking water (average dose 16.9 mg/kg/day) for 28 days.[252] Administration of HQ (10 mg/kg/day, i.p., 6 weeks) to rats produced decreased RBC counts and bone marrow cellularity.[217] In the single available experimental animal inhalation study reported in the literature, rats exposed to HQ at an air level of 10 mg/m^3, 4 h/day for 17 weeks exhibited normocytic anemia and RBC count changes (additional details not reported).[30] In contrast, other studies, including longer-term bioassays employing standard protocols, have not indicated major effects of HQ on bone marrow or hematologic parameters.[7,220,253]

While some evidence for HQ myelotoxicity is available from animal bioassays, as discussed above, the majority of data on this issue are derived from *in vivo* mechanistic studies on benzene myelotoxicity and leukemogenesis and the postulated involvement of benzene metabolites (including HQ) in this phenomenon.[254,255] Some investigations have employed overall measures of bone marrow cellularity or various versions of "colony-forming unit" (CFU) assays to detect toxic effects directed toward specific subpopulations of hematopoietic cells. Tunek et al.[256] reported a decrease in bone marrow granulopoietic cellularity, as measured by total cell or by CFU-culture (CFU-C) assay results, in male NMRI mice given 6 daily s.c. injections of 80, but not 50 mg/kg HQ. Wierda and Irons[257] found that two daily s.c. or

i.v. injections of 100 mg/kg HQ in C57BL/6 mice also reduced spleen and bone marrow cellularity when cells were harvested 24 h later. In contrast, only mild, transient suppression of bone marrow cellularity was observed in male $B6C3F_1$ mice administered repeated (two times/day) i.p. doses of 100 mg/kg HQ for up to 36 days.[258] One *in vivo* study indicated increased levels of granulocyte-macrophage colony-forming cells (GM-CFC) in bone marrow from mice given twice-daily i.p. injections of 75 mg/kg HQ for 11 days, without an accompanying decrease in marrow cellularity.[259] HQ administered to C57BL/6J mice was reported to induce differentiation of myeloblasts but to block maturation at the myelocyte stage.[260]

In vitro work has also shown effects of HQ on certain parameters associated with myelopoeisis and myelocytic colony growth. DNA synthesis in mouse bone marrow cells, but not in an isolated cell-free system, was significantly decreased by incubation with 24 μ*M* HQ.[205] Boyd et al.[261] exposed mouse CFU-GM cultures to micromolar concentrations of HQ and observed suppression of colony growth. Dose-related inhibition of granulocyte/macrophage colony-stimulating factor (GM-CSF) induced colony formation in both $B6C3F_1$ mouse and human bone marrow cell cultures, and in purified human hematopoietic progenitor cells, was produced by co-incubation with up to 50 μ*M* HQ.[262] These studies also demonstrated an increased effect of HQ on bone marrow cultures with ambient (19%) as opposed to physiologic (5%) O_2 concentrations, suggesting the importance of oxidative reactions. Granulocytic progenitor cells were more sensitive to HQ inhibition than were myeloid progenitors in mouse cultures, while a similar differential susceptibility was not observed in human cell cultures. In contrast, preincubation of C57BL/6 mouse and of human $CD34^+$ bone marrow cells with HQ generally results in stimulation of CFU-GM colony formation.[263,264]

Some studies have explored the possible effects of HQ on differentiation and/or apoptosis of bone marrow cells. HQ-induced alterations in myelocytic differentiation have been examined in human HL-60 promyelocytic leukemia cell cultures. Treatment with HQ (1 to 5 μ*M*) produced a dose-related suppression of monocytic, but not granulocytic differentiation.[265] Retinoic acid-induced maturation of human HL-60 cells to granulocytes was blocked by HQ pretreatment.[108] Exposure of the mouse myeloblastic cell line 32D to low micromolar concentrations of HQ resulted in stimulation of granulopoiesis to the myelocyte stage, but not beyond.[260,266] Recent work has also demonstrated increased apoptosis in HL60 cell cultures and human $CD34^+$ cells from bone marrow following *in vitro* treatment with 50 μ*M* HQ,[212,267] in addition to inhibition of apoptosis in mouse 32D cell cultures at HQ concentrations of 1 to 6 μ*M*.[268] Recently, it has been proposed that HQ acts synergistically with GM-CSF to induce proliferation of progenitor cells that would not normally be recruited by the cytokine, possibly via activation of secondary gene expression signals.[264]

In vitro work has also indicated suppression of erythropoiesis by HQ. Decreased incorporation of iron into erythrocytes has been reported following s.c. or i.p. injection of 100 mg/kg HQ in mice.[269–272] The effect of HQ on growth of burst-forming unit, erythroid (BFU-E), colony-forming unit, erythroid (CFU-E), and CFU-C colonies from mouse bone marrow revealed CFU-E activity to be most sensitive, with suppression observed at low micromolar concentrations.[273] Dose-dependent inhibition of CFU-E and BFU-E was observed in HQ-treated SW and C57B1/6J mouse and human bone marrow cells, respectively.[274,275] A decrease in the ratio of bone marrow cell surface antigens associated with the erythroid line to those associated with granulocytic cells, consistent with a shift in the differentiation pattern, was reported in human CD34 hematopoietic precursor cells treated with 10 μ*M* HQ.[276]

In addition to potential direct effects on erythropoietic and granulopoietic progenitor cells, numerous studies have examined HQ action on bone marrow stromal cells. These cells, which include macrophages and supporting fibroblastic cells, influence hematopoiesis by providing both a supporting physical framework and a variety of cell-specific cytokines and growth factors (e.g., colony-stimulating factors, interleukins).[277,278] Such studies typically have employed co-culture models of adherent stromal and nonadherent progenitor cells. Gaido and Wierda[279] first demonstrated the ability of low micromolar levels of HQ to inhibit both the formation of stromal cell colonies

and the ability of stromal cells to support CFU-GM colony growth. The latter effect was partially reversed by pretreatment with indomethacin, suggesting the involvement of prostaglandin synthetase in this process.[280] Reconstituted culture experiments indicated that the macrophage was the more sensitive component of the bone marrow stroma, while fibroblastoid cells were unaffected by HQ exposure.[281] This study also showed a reduction of interleukin-1 (IL-1) activity in HQ-treated stromal cultures. This effect was later shown to be due to inhibition of processing of pre-IL-1α to the mature cytokine,[282] possibly via inhibition of the enzyme calpain II.[283] Inhibition of processing of pre-IL-1β, and of the required converting enzyme, in a human myeloid tumor cell line has been reported.[284,285] Incubation of human peripheral monocytes with 5 μ*M* HQ resulted in substantially decreased IL-1α and -1β secretion and RNA and protein synthesis.[286] An IC_{50} of 25 μ*M* for inhibition of RNA synthesis was demonstrated for HQ in mouse peritoneal macrophages.[287]

The oxidation of HQ to reactive products (i.e., semiquinone and/or BQ) either spontaneously or via metabolism within the bone marrow compartment has been postulated to represent a critical step in the mechanism of HQ (and benzene) myelotoxicity.[288] Macrophage myeloperoxidase activity, which is also present in early bone marrow progenitor cells,[289] has been implicated in the metabolic sequence leading to such reactive products. Alternatively, the enzyme(s) responsible for this tissue-specific biotransformation may be eosinophil peroxidase or the peroxidase function of prostaglandin H-synthetase (PHS).[280,290] Pirozzi et al.[291] observed indomethacin inhibition of arachidonic acid-mediated oxidation of HQ to BQ by purified PHS. In contrast, no similar inhibition was found for H_2O_2-mediated oxidation by either PHS or myeloperoxidase. Conversion of HQ to BQ by purified human myeloperoxidase in the presence of H_2O_2 has been demonstrated.[292] Recent data have demonstrated the presence of polymorphisms in the lung myeloperoxidase enzyme.[293] Although the occurrence of similar genotypic variations in bone marrow myeloperoxidase have not been reported, such polymorphisms could influence the expression of myelotoxicity following parenteral HQ exposure in animal models.

Formation of HQ-derived oxidation products can lead to covalent protein binding in hematopoietic cells and bone marrow cultures. Schlosser et al.[81] incubated ^{14}C-labeled HQ with a mouse peritoneal macrophage lysate and observed an H_2O_2-dependent protein binding of HQ-derived radiolabel; binding was inhibited by cysteine and by the peroxidase inhibitor aminotriazole but not by hydroxyl radical scavengers. These workers also reported HQ-derived covalent binding to both protein and DNA with purified PHS in the presence of either arachidonic acid or H_2O_2, a reaction inhibited by indomethacin.[81,83,112] Concomitant exposure of human HL-60 cells to HQ and H_2O_2 resulted in cytotoxicity and covalent protein binding.[294] While the thiol reactivity of BQ and/or the semiquinone likely underlies HQ-derived covalent binding in bone marrow, the specific targets of this binding, whether small molecule or protein based, are unidentified. A recent report indicated that i.v. administration of HQ-GSH conjugates (postulated to form via reaction of BQ and GSH in bone marrow) to rats resulted in decreased ^{59}Fe incorporation into erythrocytes.[295]

Studies using mouse bone marrow stromal cell cultures have indicated a 16-fold greater binding of ^{14}C-HQ to macrophage than to fibroblast protein, consistent with the higher sensitivity of macrophages to HQ.[292] The relatively high level of HQ-derived covalent binding in macrophages was attributed to a higher level of peroxidase activity and/or a lower level of NQO1 activity in these cells when compared with fibroblasts. As discussed previously, NQO1 is a detoxifying enzyme that reduces BQ to HQ. Additional studies have confirmed these relative enzyme activity differences and have demonstrated that, in contrast, GSH levels between these two cell types are not significantly different.[90] The potential significance of NQO1 activity in HQ myelotoxicity was further assessed in studies showing that bone marrow stromal cells from DBA/2 mice have a lower basal NQO1 activity and are more susceptible to HQ cytotoxicity than cells derived from the more resistant C57BL/6 strain.[296] Correlations between relative susceptibility to HQ and NQO1 content of bone marrow cells have also been demonstrated for rats and mice.[297] In addition, induction of both NQO1 and GSH in mouse bone marrow cells by 1,2-dithiole-3-thione (D3T) was found to protect against HQ cytotoxicity, while

addition of dicoumarol, a NQO1 inhibitor, potentiated these effects.[298] Protective effects were reported in human ML-1 and HL-60 myeloid cell lines treated with D3T and HQ, and in DBA/2 mice fed D3T in the diet prior to isolation of the bone marrow and *in vitro* challenge with HQ.[299,300] Some direct human data on the importance of NQO1 as a protective factor are also available. Recent studies have demonstrated that individuals with an *NQO1* $^{609}C \rightarrow T$ mutation, which results in a "null" phenotype, may be more susceptible to benzene-induced hematotoxicity.[301]

Finally, other aspects of oxidative metabolism in bone marrow have been suggested to be altered by HQ treatment. Laskin et al.[302] demonstrated that marrow cells from BALB/c mice exposed to HQ (100 mg/kg, i.p., three daily doses) released significantly more nitric oxide on stimulation by various inflammatory mediators than cells from control mice, an effect proposed to be significant to HQ myelotoxicity. Studies in human HL-60 cells indicated that H_2O_2, superoxide anion, and nitric oxide levels were all increased by HQ treatment in the presence of phorbol ester, suggesting an enhancement of oxidative stress by HQ.[303]

A number of studies have also explored the effects of *in vitro* HQ exposure on immune cells and immunological parameters in various model systems. Early studies demonstrated inhibition of lectin-stimulated lymphocyte blastogenesis and agglutination following pretreatment with micromolar levels of HQ.[207,304] Based on the lack of a concurrent decrease in energy production and the protection from inhibition by thiol reagents, this effect was attributed to cytoskeletal disruption in lymphocytes due to the microtubule sulfhydryl reactivity of HQ.[305] Parenteral treatment of C57BL/6 mice with HQ (100 mg/kg, two daily doses for 3 days) resulted in decreased spleen and bone marrow cellularity, in addition to decreased development of viable, plaque-forming cells derived from these tissues.[257] These results suggested a reduction in the level of B-lymphocyte progenitor cells in bone marrow by HQ. A 50% decrease in RNA synthesis was reported in mouse splenic lymphocytes treated with 10 to 20 μ*M* HQ.[203] Inhibition of maturation of pre-B cells was demonstrated in $B6C3F_1$ mouse bone marrow cultures pretreated with HQ (0.1 μ*M*, 1 h).[306] This effect may be mediated by decreased IL-1 release from stromal macrophages, leading to inhibition of IL-4 production by stromal fibroblasts.[307]

In vitro mitogen-induced proliferation of mouse, rat, and human B- and T-lymphocytes was inhibited in a dose-related manner by HQ.[308] HQ was found to block IL-2-dependent proliferation of human T-lymphoblasts *in vitro* without affecting either IL-2 production, IL-2 receptor binding, cell viability, or GSH levels,[309] possibly via interference with iron metabolism and ribonucleotide reductase activity.[310,311] Expression of transferrin receptors, an early cellular event mediated by IL-2, was inhibited by HQ in human T-lymphoblasts.[312] Thiol reagents were not effective in reversing the effect of HQ on T-lymphoblast proliferation, while transfection of the M2 subunit of ribonucleotide reductase did provide protection.[313] Mild inhibition of pre-B cell proliferation when co-cultured with bone marrow stromal cells was observed with 10 μ*M* HQ, probably due to inhibition of IL-7 production by the stromal cells.[314] A 55% decrease in interferon gamma (IFNγ) production, without concomitant reduction in cell viability, was observed in mouse spleen cell cultures exposed to 50 μ*M* HQ for 1 h.[315] Exposure of the mouse fibroblast L-929 cell line to HQ also resulted in inhibition of IFNα/β production.[316] Recent work has shown reversible inhibition of the NF-κB transcription factor by 1 μ*M* HQ in human $CD4^+$ T-cell cultures.[317]

The effects of *in vitro* HQ exposure on phagocytic and cytotoxic immune cell function have also been explored. Dose-dependent inhibition (1 to 10 μ*M* range) of mouse spleen natural killer (NK) cell activity *in vitro* by HQ has been demonstrated.[318] Lewis et al.[185] reported inhibition of H_2O_2 production and cytolytic activity in mouse peritoneal macrophages treated with HQ, without concomitant disruption of general cellular functions. Additional experiments revealed concentration-dependent inhibition of Fc receptor-mediated phagocytosis *in vitro* in peritoneal macrophages.[319] The ability of human HL-60-cell-derived granulocytes to phagocytize sheep RBCs and to reduce nitroblue tetrazolium (indicators of immune functionality) was also diminished by HQ pretreatment.[108]

It is clear from the extensive database discussed above that HQ exposure *in vitro* or in

whole animal systems using parenteral routes can affect numerous hematopoietic and immunologic parameters. It is also likely that oxidation of HQ within the bone marrow compartment to the semiquinone or BQ, followed by covalent macromolecular binding, is critical to these effects (Figure 4). Whether myelotoxicity is mediated by effects of HQ on mitosis or other cellular processes (or both) is not known. While specific mechanisms have still not been elucidated, the molecular effects of HQ metabolites appear to involve a combination of inhibition and enhancement of different metabolic and cellular differentiation pathways in bone marrow, leading to an overall imbalance in the hematopoietic process. Primary among these specific effects are stimulation of progenitor cell proliferation to the promyelocyte stage, inhibition of progenitor cell proliferation to the erythroblast stage, and disruption of cytokine production by marrow stromal cells. Thus, an imbalance, manifested as a relative increase in granulocytic ontogenesis at the expense of erythropoietic (and, possibly, monocytic and lymphocytic) development evolves. In addition, functional effects on certain immune cells may occur.

Mechanistic studies with HQ and its metabolites have provided much information regarding the myelotoxicity in these experimental systems. However, many of these studies have been designed to examine the potential involvement of HQ in benzene myelotoxicity and/or leukemia, making it difficult to judge the relevance of these data for HQ toxicity per se. As discussed previously, toxicokinetic and species-specific factors are critical to assessing the pattern of HQ toxicity in mammalian systems, and thresholds for certain effects are likely. Several authors have addressed this issue in detail.[11,320] It must also be emphasized that bone marrow and hematologic effects are generally not characteristic of HQ exposure in animal bioassays employing routes of exposure other than parenteral. In addition, myelotoxic changes have not been reported in humans as a result of long-term occupational HQ exposure.

XI. REPRODUCTIVE AND DEVELOPMENTAL EFFECTS

The overall *in vitro* and animal toxicity database indicates that HQ may cause maternal toxicity, which is essentially the same as that seen in non-pregnant animals given similar exposures to acutely toxic dose levels of HQ. Fetotoxicity, primarily manifested as growth retardation, may be seen at high-dose levels that also induce maternal toxicity. However, reproductive and/or teratogenic effects are not prominent, even at high-exposure levels.

In vitro effects of HQ on various developmental parameters have been examined. HQ produced embryotoxicity but no statistically significant increase in malformations in a chick embryo assay at doses of up to 40 μg/egg.[321] Treatment of whole-rat embryos in culture with HQ at 10, 50, 100, or 200 μ*M* concentrations resulted in 0% viability at the two higher levels, but no effects at 10 or 50 μ*M*.[322] Similar exposure of rat embryo cultures to 45 or 68 μ*M* HQ produced growth retardation, while the higher concentration also resulted in certain structural defects (hind limb absence and tail abnormalities).[323]

Certain early studies, using nonstandard protocols, did suggest a potential for reproductive effects of HQ at high-dose levels. Increased fetal resorption rates were reported in pregnant female rats fed a total dose of 0.5 g HQ in the diet over an unspecified length of time.[324] Administration of 200 mg/kg/day HQ, p.o., to female rats for 14 days resulted in inhibition of estrus but also significant toxicity (clonic seizures, respiratory effects, 30% mortality).[325] Interruption of estrus was reported in female rats following s.c. injection of 10 mg/kg/day HQ for 11 days.[326] Male rats injected s.c. with 100 mg/kg/day HQ for 51 days exhibited inhibition of spermatogenesis, testicular changes, and decreased fertility.[327] In contrast, Ames et al.[328] reported no effects on reproductive parameters in female rats fed 0.3% HQ in the diet for 10 days prior to mating. Kavlock et al.[329] reported reproductive effects in pregnant female SD rats treated with 100, 333, 667, or 1000 mg/kg of HQ, p.o., on gestational day (gd) 11. Except for the low dose level, the other dose levels approached or exceeded the reported oral LD_{50} levels for rats. Decreased maternal weight and total litter mass, and increased perinatal loss, were observed with the two highest dose levels, while decreased litter size was noted at 1000 mg/kg.

In contrast to these studies, the results of standardized teratogenicity and reproduction bioasssays for HQ have generally revealed a lack

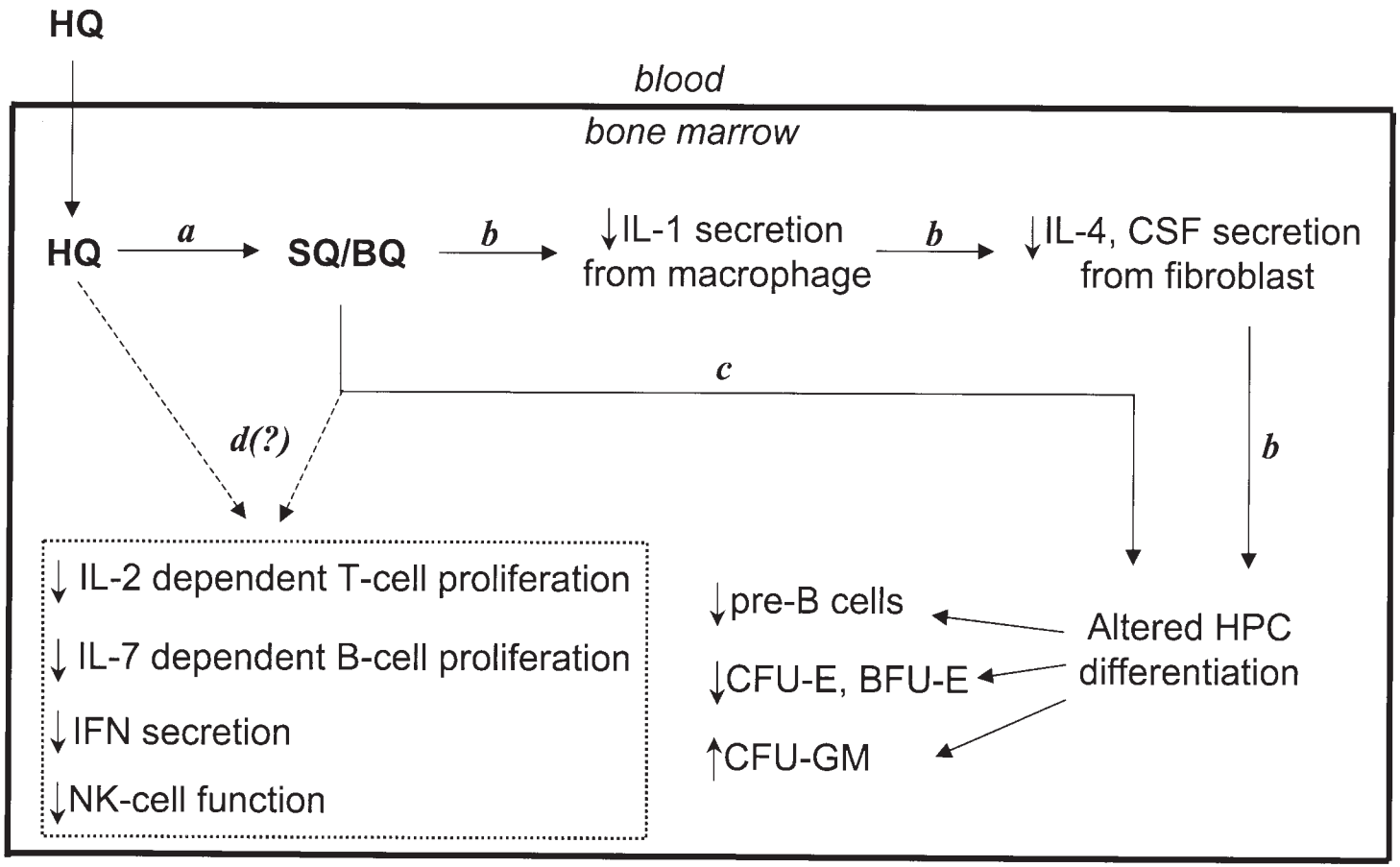

FIGURE 4. Summary of proposed mechanisms of hydroquinone (HQ) myelotoxicity following i.p. administration. HQ is taken up by bone marrow from blood, followed by one-electron oxidation by myeloperoxidase (or other peroxidases) to semiquinone (SQ) and benzoquinone (BQ) (path "*a*"). These metabolites are proposed to interfere with stromal macrophage and fibroblast secretion of growth and differentiation factors to result in modified hematopoietic progenitor cell (HPC) development (path "*b*"). Alternatively, these metabolites may directly influence mitotic or other cellular processes in HPCs (path "*c*"). Reported effects on immune cell physiology and function may be due to either HQ or oxidized metabolites (path "*d*") and may occur in compartments other than bone marrow (dashed box). ↑ = relative increase in level compared to unexposed; ↓ = relative decrease in level compared with unexposed.

of effect, even at high-dose levels. Teratogenic effects were not observed in offspring of pregnant female SD rats following dermal HQ exposure of up to 810 mg/kg/day on gd 6 to 19.[330] In a dominant lethal assay in mice, males were exposed to HQ at doses of up to 300 mg/kg/day (5 d/week, 10 weeks, p.o.) and then mated to untreated females. No treatment-related effects on fertility rates, implantations, fetal survival, or other reproductive parameters on offspring were noted.[128] Developmental toxicity of HQ was investigated in female New Zealand White rabbits dose with 25, 75, or 150 mg/kg HQ, p.o., on gd 6 to 18.[331] Slightly decreased maternal body weights and food consumption were noted at the two higher dose levels. No statistically significant teratogenic alterations were observed in offspring from treated rabbits when compared with control. A similar decrease in maternal body weight and lack of teratogenic changes were obtained in female SD rats treated with 30, 100, or 300 mg/kg HQ, p.o., on gd 6 to 15.[310] A two-generation study in SD rats did not demonstrate selective reproductive effects at HQ dose levels of up to 150 mg/kg/day.[332]

XII. EFFECTS OF HQ EXPOSURE IN MAN

A. Systemic Effects

With the exception of ocular and dermal effects (described below), there are no clearly demonstrated impacts of prolonged HQ exposure in humans. No adverse hematological or urinary changes were observed in two male volunteers ingesting daily doses of 500 mg/day HQ for 5 months, or in 17 male and female volunteers ingesting 300 mg/day for 3 to 5 months.[26] Isolated case reports have postulated the occurrence of health effects due to HQ exposure without any specific causal evidence. For example, a recent report described hepatotoxicity in a darkroom worker and attributed this finding to inhalation to HQ "fumes" or dust.[333] Little-to-no HQ is present, however, in ambient air during such uses, and no data were reported to implicate HQ (other than its presence in developer fluid) or to discount other more likely causes.[16,334]

A number of occupational epidemiologic studies have been conducted on worker cohorts with potential exposure to HQ, primarily via inhalation of dust and direct dermal contact. No evidence for systemic toxic effects was reported in a cohort of HQ production workers with potentially significant airborne HQ dust (up to 30 mg/m^3) and BQ vapor (up to 1 mg/m^3) exposure.[335] No increase over control in the measured outcomes of mortality, cancer incidence, or sickness/absence were observed in epidemiological studies of laboratory film processors with potential exposure to numerous chemicals, including HQ.[18] Airborne HQ levels for these workers were much lower than for production workers; typically <0.01 mg/m^3 during the period 1940 to 1964. Over 400 workers were followed for up to 16 years in this study. A study of over 9000 plant workers at a major U.S. producer of HQ revealed significantly lower incidences of mortality due to a number of diseases, including cancer, when compared with general population controls.[336] A similar result was reported for the subset of workers with primary exposure to HQ, although actual HQ air levels were not reported. Finally, a recent comprehensive mortality and exposure study of 879 male and female workers involved in HQ production and use over a 50-year period did not reveal statistically significant increases in deaths from a number of malignant and nonmalignant disease categories.[337] No cumulative exposure-related trends in selected causes of mortality were found. Overall death rates and cancer incidence were significantly decreased in HQ-exposed workers when compared with an employed referent population, demonstrating that the improved outcome in HQ workers was not related to the healthy worker effect. These studies provide substantial evidence for a lack of systemic toxic (including carcinogenic) effects from long-term occupational exposure to HQ.

A few studies have reported systemic effects in workers with potential exposure to HQ. Choudat and co-workers[338] reported an increase in respiratory symptoms (i.e., cough in a smoky atmosphere and hay fever) in workers exposed by inhalation to a combination of HQ, trimethyl-HQ, and retinene-HQ (relative and absolute concentrations of each compound not specified). Decreased pulmonary function test results (i.e.,

decreased forced expiratory volume and vital capacity) and elevated serum IgG were reported. Based on these data, the authors concluded that HQ may induce respiratory and immunological changes in exposed workers, although the mixed exposure scenario complicates interpretation of their findings. Nielsen et al.[339] reported a relative risk of 3.4 for malignant melanoma in a cohort of 836 Danish lithographers, about 200 of whom reported regular use of photographic chemicals, including HQ. Exposure to numerous other industrial chemicals, including dyes, solvents, metals, and acids, was also reported by these investigators. No quantitative exposure data for HQ or any other agent were provided. The authors suggested HQ as a possible causative agent in melanoma, based on its "biological effect upon the melanocyte." However, malignant melanoma has not been reported in other, more carefully controlled, HQ occupational epidemiological studies or as a consequence of excessive use of HQ-containing skin preparations. In addition, animal studies (discussed below) generally demonstrate melanocyte cytotoxicity with HQ treatment, including an inhibitory effect on growth of implanted melanomas in mice. These results are difficult to reconcile with the authors' proposal. Excess melanomas were reported among laboratory employees, associated with risk factors, including chemist duties and working with high explosives, sources of ionizing radiation, or "volatile photographic chemicals".[340] However, no specific exposure data were given in this report and, based on its physical characteristics, it would not be appropriate to include HQ in the latter risk category.

B. Ocular Effects

While isolated reports of the human ocular effects of HQ in occupational settings[341,342] had appeared earlier, a comprehensive description and study of this phenomenon did not occur until the 1940s. At that time, a number of cases of conjunctival and corneal pigmentation appeared among workers at a large production facility, where HQ manufacture had begun in 1930.[343] These cases were described in a series of publications by Sterner, Oglesby, and Anderson.[17,335,344] The conjunctival lesions have been described as proceeding from slight, diffuse light brownish staining to marked globular, dark brown staining of the interpalpebral zone with increasing time and intensity of HQ exposure. The corneal lesions were noted to include horizontal brown pigmented lines (Stahli's lines), vertical striations in Descemet's membrane, and diffuse brown corneal staining. In severe cases, a grayish-white scarlike tissue was found to replace some or all of the cornea. A histopathological study has described degenerative corneal changes in several cases of HQ-induced ocular changes that occurred in a German plant manufacturing HQ.[345] While the adverse effects associated with the conjunctival lesions were largely cosmetic in nature, changes in visual acuity as a result of corneal damage were noted. These were found to range from mild deficiencies in night vision, to excessive internal scattering and reflection of light within the cornea, to near blindness in the most extreme cases. Reversibility of the conjunctival, and to a lesser extent, the corneal pigmentation was noted after reduction in HQ exposure and during long-term follow up.[335] In some cases, however, corneal changes were seen to persist and even increase after cessation of exposure.[346]

Ogelsby et al.[17] studied the characteristics of plant exposures that were associated with development of ocular effects in workers. Because the synthetic process involved sequential oxidation of aniline to BQ followed by reduction to HQ, it was assumed that exposure to both BQ vapor (known to be a potent eye irritant) and HQ dust may have been relevant. Development of the syndrome of ocular effects appeared to require prolonged exposure, with initial pigmentation typically appearing no earlier than 2 to 3 years after initiation of heavy HQ and BQ exposure. Severe corneal damage was generally associated only with continuous exposure of >5 years. Despite long-term exposure, certain individuals were found to be relatively free of ocular pigmentation, suggesting the influence of additional environmental and/or individual susceptibility factors in the development of these effects. Correlation of ambient airborne levels of BQ vapor and HQ dust for various plant operations with the severity of ocular changes eventually led to establishment of 0.1 ppm and 2 mg/m^3 as exposure limits for these contaminants, respectively.[343] In addition, other

industrial hygiene measures and engineering controls were instituted in the HQ production process. Similar ocular lesions have not been described among end users or consumers of HQ or BQ or products containing these materials.

Although both HQ and BQ have been implicated in causing ocular effects, the mechanism of this phenomenon remains unclear. Acute ocular toxicity studies with HQ in rabbits and dogs have generally shown only mild irritant and reversible corneal impacts.[1,347] With the exception of a single report,[348] attempts to reproduce the conjunctival and corneal pigmentation in animal models have been unsuccessful. It is generally accepted that these effects are due to a direct action of HQ and/or BQ on the eye rather than an indirect effect of systemic intoxication.[344] The most likely possibility is that the corneal and conjunctival pigmentation represents formation and deposition of oxidized HQ- and BQ-derived polymers, quinhydrone (a molecular dimer of BQ and HQ), and/or modified ocular proteins.[335,342] In any case, the effectiveness of industrial hygiene measures and medical surveillance efforts in HQ production is indicated by the lack of literature reports of new cases of HQ-induced ocular effects in humans over the last several decades.

C. Dermal Effects

HQ is only a mild skin irritant when tested using classic acute dermal irritation bioassays.[1] However, subchronic exposure to HQ may lead to reduced pigmentation on both animal and human skin. The first indication of this potential came from early animal toxicology studies, where oral administration of HQ and other phenolics to black-haired cats or mice resulted in reduced pigmentation of fur after 6 to 8 and 4 to 20 weeks, respectively.[349,350] Similar results were reported with s.c. injections of HQ in black goldfish.[351,352] Subsequent work demonstrated skin pigmentation reduction in black guinea pigs following daily topical application of 1 to 10% HQ in an ointment formulation for up to 1 month.[353,354] The higher concentration preparations were also associated with skin irritation and inflammatory reactions. The latter study showed that HQ application caused a reduction in melanin content of melanosomes and eventually produced a degeneration of melanocytes.

These findings are consistent with a hypothesis that HQ is an inhibitor of tyrosinase, the enzyme involved in conversion of tyrosine to L-dopa and then to dopaquinone, the initial steps in melanin formation.[355–357] A 90% inhibition of tyrosinase activity in black goldfish skin homogenates was observed following incubation with 0.9 m*M* HQ, although higher concentrations resulted in moderate activation of the enzyme.[358] HQ was also shown to directly inhibit purified tyrosinase from human melanoma cell lines.[359] More recent work with purified tyrosinase has demonstrated that, in the presence of catalytic amounts of L-dopa, HQ actually competes with tyrosine as a substrate for the enzyme in a reaction that can result in formation of trihydroxybenzene and hydroxybenzoquinone.[360] These products may undergo further metabolism and/or spontaneous conversion to toxic and/or reactive species; however, such additional metabolites have not been identified following *in vivo* exposures to HQ. The effectiveness of tyrosinase substrates as depigmenting agents has been suggested to be related to the extent of their further conversion to such metabolites.[361] Recent work indicates that inhibition of GSH synthesis results in potentiation of the depigmenting action of HQ on black guinea pig skin, presumably by increasing the level of oxidative species that would normally be trapped by the intracellular thiol.[362]

A number of studies have examined the effects of HQ on melanomas *in vivo* and on melanotic cell lines *in vivo*, both as mechanistic investigations and as a means of testing the utility of HQ as a possible chemotherapeutic agent. Cytotoxicity in mouse melanoma cell cultures treated with HQ was reported by Hu.[363] Abramowitz and Chavin[364] found complex changes in cyclic nucleotide levels and tyrosinase activities of mouse B-16, S-91, and HP tumors exposed *in vitro* to HQ. A 30-fold lower ED_{50} for inhibition of tritiated thymidine incorporation was found for HQ in melanotic when compared with nonmelanotic cell lines, suggesting the participation of tyrosinase inhibition in cytotoxicity.[365] These investigators postulated active oxygen species generated externally to the cell as the actual cytotoxic agents. Another study using melanotic, amelanotic, and

nonmelanotic cell lines demonstrated that HQ cytotoxicity is dependent on tyrosinase, but not melanin, content of the cell.[366] In contrast, a series of phenols (including HQ) that are substrates for tyrosinase were found to be cytotoxic to both melanotic and nonmelanotic cell lines, suggesting that tyrosinase may not be involved in the cytotoxic mechanism.[367,368] The reasons for these conflicting results have not been determined. Finally, treatment with nine daily s.c. injections of 80 mg/kg HQ significantly decreased tumor formation and increased survival of BALB/c mice given melanoma implants, consistent with a specific cytotoxic effect on melanocytes *in vivo*.[369]

Intentional use of HQ formulations for skin lightening in humans began in the 1950s, following anecdotal reports from the southern U.S. of depigmentation occurring as a side effect of an HQ-containing preparation designed as a sunscreen.[370] Early controlled clinical studies suggested that daily dermal application of creams containing 1.5% or greater concentrations of HQ were effective in producing at least mild cosmetic skin depigmentation after approximately 1 month of use,[371–374] although some clinicians reported negative results for HQ in the absence of additional ancillary components.[375] The incidence of skin irritation and inflammation associated with this treatment appeared to increase substantially with HQ concentrations above 2 to 3%. Occasional hypersensitivity reactions have also been noted, although HQ is not a consistent sensitizer in human or animal studies.[1,376]

Since the 1950s, HQ-containing creams have been employed in clinical dermatology for treatment of hypermelanosis, senile lentigo, vitiligo, and melasma.[377] Prolonged use of over-the-counter preparations containing higher concentrations of HQ or continued use of HQ-based skin lighteners in the presence of skin inflammation or dermatitis has resulted in a small number of reports of adverse skin reactions in the U.S. Reports of reactive skin hyperpigmentation following excessive use of these products appeared in South Africa during the 1970s.[374,378] This phenomenon was first reported by Findlay and co-workers following the extensive use of skin creams with high HQ contents (6 to 8%).[379,380] In some cases hyperpigmentation can appear as colloid milium or exogenous ochronosis. Clinically, these conditions are characterized by collections of dark papules or macules against lighter colored skin (colloid milium), with histopathological analysis of the affected skin revealing parallel bundles of ochre-colored elastoid fibers (ochronosis). Development of the condition is accelerated and aggravated by sunlight, by the presence of other active derivatives (e.g., resorcinol and phenol), and by the use of penetrating vehicles.[381] After cessation of exposure to HQ and sunlight, the effects are at least partially reversible.

Despite governmental controls on the maximum level of HQ permitted in these products, an epidemiological study conducted in South Africa 15 years after these initial reports indicated a prevalence rate of 69% for exogenous ochronosis among users of skin lighteners.[382] These data have resulted in calls for the banning of HQ-based skin creams as over-the-counter cosmetic preparations.[383–385] A small number of cases of exogenous ochronosis attributed to the use of HQ-based products have also been reported in the U.S. and countries other than South Africa.[381,386–392] Paradoxical pigmentation of the nails has also been reported as a side effect of the chronic use of HQ.[393,394] In contrast, ochronosis or hyperpigmentation has not been reported during the manufacture or the use of HQ in occupational settings. Occasional reports of leukoderma and/or contact dermatoses are also encountered, primarily associated with use of photographic developers.[395–400]

The mechanism of ochronotic skin changes following prolonged dermal HQ exposure is unclear. The presence of melanocytes appears to be required for ochronosis to occur.[401] Findlay initially proposed that, with constant exposure, melanocytes might eventually become resistant to the uptake and inhibitory effects of HQ (termed "melanocyte recovery"), leading to increased production of melanin.[379] This would be accompanied by increased transfer of HQ into the dermis, with deposition of oxidized pigments and/or damage to connective tissue fibers. Penneys[388] proposed that, in analogy to the mechanism of endogenous ochronosis in alkaptonuria, HQ may inhibit homogentisic acid oxidase in the skin, leading to deposition of homogentisic acid-based pigments. Other hypotheses include activation of tyrosinase at high HQ concentrations, leading to increased melanin production,[402] formation of oxidized,

colored hydroxylated indoles,[386] uptake of melanin by macrophages in the dermis,[379] or degeneration of dermal collagen leading to the deposition of pigmented fibers.[403]

Despite the occasional appearance of clinical case reports in the published literature, significant side effects from the use of skin preparations containing HQ are uncommon and generally associated with improper or excessive use. In the U.S., dermatological and cosmetic creams containing 2% or less of HQ are considered safe for use in skin depigmentation (with avoidance of sunlight and discontinuance after 2 months).[404,405] Extensive reviews of HQ toxicity as related to cosmetic and dermatologic applications have been published.[12,13,371,379,380]

XIII. CONCLUSIONS: IMPLICATIONS FOR HUMAN HEALTH EFFECTS

As with all xenobiotics, the potential risk to humans from occupational, environmental, and consumer exposure to HQ is a function of both the extent of exposure and the inherent toxicity of the chemical. Thus, risk characterization relies on scientific judgment based on a consideration of both exposure assessment and dose-response analysis. This can be a highly uncertain undertaking with major areas of uncertainty rising from animal-to-human extrapolation, and extrapolation with respect to dose level, route, and rate of dose administration. In the case of HQ, much information is available on mode of action and route of exposure- and species-dependent differences in toxicokinetics and toxic effects; these address some of the uncertainties and provide insight into the overall risks associated with HQ exposure in humans. It is clear that the potential human effects of direct HQ exposure must be distinguished from those of indirect exposure via metabolism of benzene. These issues are considered in the following discussion, in light of the current extensive knowledge base for HQ. Other published articles and reviews have also explored these issues to various extents.[1,11,254,337,406]

HQ appears to more or less specifically affect the kidney and hematopoietic systems in experimental animals and the skin and eye in humans (Table 1). In view of the apparent diversity of these effects, it is valuable to discuss the evidence for and against common underlying mechanisms of action for HQ. Such an assessment is critical to extrapolation of animal (and *in vitro*) results to man and for predicting risk of HQ exposure in humans. One critical question involves the nature of the chemical species responsible for the effects observed in each organ system. Is HQ both necessary and sufficient to produce an effect or are other metabolites or reaction products required? The database available to evaluate this issue is extensive and generally points to BQ and the semiquinone as the primary mediators of HQ toxicity, while evidence for the involvement of activated oxygen species in HQ toxicity is less compelling. More specifically, the covalent reactivity of BQ and the semiquinone with cellular nucleophiles (particularly thiol groups) is likely to mediate the majority of HQ's effects. Despite this generalization, the actual molecular targets for each tissue- and organ-specific effect of HQ appear to be quite unique. Thus, formation of toxic HQ-GSH conjugates that impair mitochondrial function may underlie kidney toxicity, while covalent modification of proteins and thiol-containing biomolecules involved in mitosis and hematopoiesis may be important in myelotoxicity. A exception to this model concerns the acute CNS effects of HQ, which are probably dependent on the parent molecule itself.

Because HQ does not appear to be a direct genotoxicant or mutagen, epigenetic factors, such as toxicokinetic and other species-, strain-, and sex-related differences will determine the specificity of toxic effects and will impact the prediction of human risk. In the case of renal toxicity following high-level HQ exposure, the rate of oxidative metabolism of HQ to BQ in the liver and kidney, the rate of formation and release of HQ-GSH conjugates from the liver and subsequent uptake by the kidney, tissue-specific GSH contents, and relative levels of detoxifying enzymes (e.g., glucuronyltransferases, sulfotransferases, and NQO1) will all determine the species-specific exposure threshold for this effect.

As discussed previously, the male F344 rats appears to be most susceptible to HQ-induced nephrotoxicity and renal adenoma induction. This strain exhibits high background rates of CPN and of cell proliferation in kidney tubule cells, condi-

TABLE 1
Summary of Toxicity Endpoints for HQ in Experimental Animals and Man

Target organ or target effect	Proposed critical toxicant(s)	Effect present in:		Reported effect(s)
		Animals	Humans[a]	
Acute CNS	unchanged HQ	+	+	Acute excitatory action; tremor, incoordination, seizures, respiratory difficulty, coma
Bone marrow and blood	SQ/BQ; reactive oxygen species (?)[b]	+	–	Anemia, decreased cellularity, altered hematopoietic precursor cell differentiation, altered immune cell function
Kidney	SQ/BQ; HQ-SG; HQ-Cys; reactive oxygen species (?)	+	–	Nephrotoxicity, exacerbation of chronic progressive nephropathy, tubular cell adenomas
Eye	SQ/BQ	–	+	Pigment deposition in conjunctiva and cornea
Skin	HQ (?); SQ/BQ	+	+	Depigmentation; exogenous ochronosis
Genotoxicity	SQ/BQ; reactive oxygen species (?)	+	–	Clastogenicity; mitotic disruption

Note: **Abbreviations:** BQ = benzoquinone; HQ = hydroquinone; HQ-SG = glutathione conjugates of HQ; HQ-Cys = cysteine conjugates of HQ; SQ = semiquinone.

a With the exception of some reported acute poisoning incidents and, possibly, early occupational exposures, human data are based on dose levels likely to be substantially lower than for the animal data.

b Indicates weak and/or inconsistent data to support involvement of particular metabolite.

tions that are expected to enhance the renal effects of compounds such as HQ. The evidence discussed earlier indicates that the nephrotoxicity induced by short-term HQ exposure in experimental animals is mechanistically distinct from the enhancement of CPN and cell proliferation seen after chronic exposure. These data indicate likely epigenetic mechanisms for these effects, with acute effects dependent on toxicokinetic factors and chronic changes on species-specific differences in cellular responses to continuing proliferative stimulation. Based on the absence of evidence for similar predisposing factors in other species, the lack of demonstrated renal effects in humans exposed to significant levels of HQ, and the minimal direct mutagenic potential of HQ, it is unlikely that nephrotoxicity or renal carcinogenesis represent relevant risk extrapolation endpoints for HQ in man.

Similar arguments can be made for the observed myelotoxic effects of HQ in experimental systems, where the mouse (and mouse bone marrow cells) appears to be most susceptible. The basis for this sensitivity is not known, but probably involves relative differences in activating and detoxifying enzymes within specific bone marrow cell types.[407] In addition, for myelotoxicity, toxicokinetic considerations become critical in determining the likelihood of effects in man. The majority of data on this phenomenon have been derived from animal studies employing parenteral routes of exposure. As has been demonstrated, this technical approach, while experimentally convenient, results in lower overall HQ detoxification (via conjugation) by the liver and increased levels of unchanged HQ available to the bone marrow for activation by myeloperoxidase and other oxidative enzymes. This would not be expected to occur under reasonably anticipated conditions of human exposure, where respiratory, dermal, and oral routes would predominate.

One issue that deserves further scrutiny in predicting the possible effects of direct HQ exposure in man is that of individual susceptibility. Many of the enzymes involved in HQ metabolism are polymorphic in animals and/or humans. For example, phenol sulfotransferase, the enzyme likely involved in HQ sulfation and detoxication, exists in two forms, a high activity themostable and a lower activity thermolabile form.[72] Variations in the activity of UDP-glucuronosyl transferase (UGT), which catalyzes formation of HQ-glucuronide, have also been reported in human liver.[72] The presence of the low-activity forms of these enzymes, particularly UGT, in subgroups of individuals could hypothetically result in decreased conjugation and urinary excretion, and the higher distribution of parent molecules to peripheral tissues such as kidney and bone marrow. This could in turn result in enhanced toxicity. However, because of the demonstrated high metabolic capacity of these systems (especially in the intestine),[72,74] there is likely to be adequate detoxication activity present for all except "null" genotypes for these loci. It is unclear whether such genotypes exist for these enzymes in humans. Similar arguments for increased susceptibility could be made in the case of genetic variations resulting in increased activity of bone marrow myeloperoxidase or kidney γ-GT, or decreased activity of bone marrow NQO1. In the absence of molecular epidemiological data on the existence, tissue specificity, and metabolic capacity of these possible variants, and on tissue levels of HQ and metabolites in individuals exposed directly to HQ, such arguments are speculative. However, this is clearly an area that deserves further investigation.

In discussing the potential for HQ-induced myelotoxicity in humans, comparisons with benzene are inevitable. As stated earlier, much of the available data on mechanisms of HQ-induced effects in bone marrow are derived from studies examining mechanisms of benzene toxicity, rather than as a result of concern over direct HQ exposure. Benzene is a potent myelotoxin in experimental animals and causes pancytopenia, MDS, aplastic anemia, and acute myelogenous leukemia in man.[255] Early work demonstrated that benzene myelotoxicity is probably due to the presence of a metabolite, rather than the parent compound, in bone marrow. HQ has long been known to be a metabolite of benzene, formed following oxidation of phenol produced from the initial P450-mediated oxidation of benzene. Significant levels of HQ are present in bone marrow following acute inhalation exposure of rats to benzene.[408] In addition, structurally similar DNA adducts are formed in bone marrow following either *in vitro* HQ or *in vivo* benzene exposure in mice.[103]

While metabolism is clearly important in benzene myelotoxicity, no single benzene metabolite, including phenol, HQ, or BQ, has exhibited the potency and level of myelotoxic effect of benzene itself in animal studies. Eastmond et al.[258] first demonstrated that coadministration of phenol and HQ by i.p. injection in B6C3F$_1$ mice resulted in dose-related decreases in bone marrow cellularity comparable to those noted with benzene exposure. Phenol was also found to enhance peroxidase-dependent HQ metabolism to BQ and subsequent covalent protein binding. These authors proposed that phenol-mediated stimulation of HQ metabolism by myeloperoxidase in bone marrow was responsible for this phenomenon and that such a mechanism might underlie benzene myelotoxicity. Since that report, other studies have generally corroborated these findings. Coadministration with phenol has been found to decrease the metabolic clearance of HQ[65] and to exacerbate the inhibition of erythrocyte iron uptake,[271,272] formation of macromolecular adducts,[64,106,111,409] generation of activated oxygen species,[410] and MN induction[162] encountered with HQ exposure alone. Stimulation of HQ metabolism by myeloperoxidase has also been demonstrated for BQ[411] and additional phenolic compounds.[412]

Becuase phenol is metabolized to HQ in the liver, the lack of myelotoxicity associated with p.o. administration of phenol alone in experimental animal studies is surprising. Toxicokinetic models have been developed to account for this finding.[320,413] These models predict that route of exposure differences and competitive hepatic oxidative and conjugative metabolism between benzene and phenol result in a higher net HQ delivery to bone marrow following benzene as opposed to phenol administration. Assuming that myeloperoxidase action on HQ is critical to myelotoxicity, that phenol stimulates HQ oxidation, and that relatively similar phenol levels are present in bone marrow following either phenol or benzene administration, then this explanation is tenable. This paradigm also has bearing on the myelotoxicity associated with direct HQ exposure. Because phenol is not a metabolite of HQ, the degree of myelotoxicity will depend only on the level of unchanged HQ present in bone marrow. As indicated earlier, this parameter is route of exposure dependent, and is typically low after nonparenteral HQ administration.

If the level of HQ in the bone marrow is a critical determinant of bone marrow toxicity, then plausible explanations for the distinct spectrum of myelotoxic effects of benzene follow. One could predict that, with low benzene exposure, little overall metabolism to HQ occurs. The HQ that is formed would be readily removed by conjugation and urinary excretion, leaving little to ultimately reach the bone marrow. In contrast, high-level benzene exposure would lead to very elevated levels of HQ in bone marrow and significant hematopoietic cell death, potentially leading to aplastic anemia. At intermediate benzene exposure levels, a complex series of myelodysplastic effects (e.g., clastogenicity, clonal selection, altered cell physiology) would result, some or all of which might represent a preleukemic condition. The specific dose ranges associated with each level of effect might be influenced by an individual's overall genotype for the relevant enzymes (including CYP450) involved in benzene metabolism.

In conclusion, review of the overall *in vitro*, experimental animal, and human toxicological and epidemiological databases strongly suggests that direct HQ exposure under current occupational and environmental conditions poses little risk. Several recent quantitative risk assessments for HQ, using worst case scenarios, have concluded that such exposure is well below levels conservatively estimated to be associated with health effects in humans.[11,414] This conclusion is further supported by the lack of evidence for HQ toxicity in occupational cohorts in a number of well-conducted epidemiological and clinical studies and as a result of consumption of foods containing significant levels of HQ. In addition, the major effects of HQ reported in animal studies (i.e., renal toxicity and myelotoxicity) are of uncertain relevance to humans, because of the dependence of these endpoints on toxicokinetic and species-specific factors. While hypothetical genetic polymorphisms in relevant metabolic enzymes could heighten the risk of renal toxicity or myelotoxicity in specific exposed individuals, there are no data available to indicate that this actually occurs. Finally, concerns related to the possibility that effects similar to those reported in *in vitro* and

animal studies with benzene may occur following direct HQ exposure are likely to be exaggerated due to the major toxicokinetic and toxicodynamic differences between these substances. Only in the improbable case of simultaneous parenteral or very high-level oral exposure to HQ and phenol (or related compounds) might the effects of co-exposure reported in experimental studies be potentially significant to man.

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