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Perspective

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Chemical and Toxicological Characterization of Halobenzoquinones, an Emerging Class of Disinfection Byproducts

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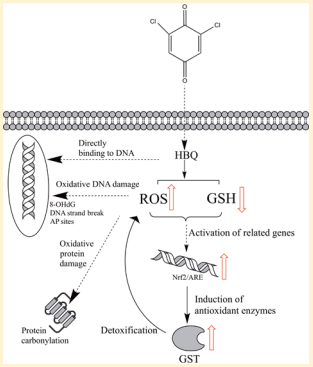
ABSTRACT: Halobenzoquinones (HBQs), a new class of disinfection byproducts (DBPs), occur widely in treated drinking water and recreational water. The main concern regarding human exposure to DBPs stems from epidemiological studies that have consistently linked the consumption of chlorinated drinking water with an increased risk of developing bladder cancer. The U.S. Environmental Protection Agency and Health Canada have set regulations on the amount of DBPs in drinking water to minimize the risk. However, these regulated DBPs do not account for the increased risk of bladder cancer because they have different target organs or lower magnitudes of risk based on animal carcinogenesis studies. Because of the pervasive exposure to DBPs, identification of DBPs relevant to human health has become one of the important research targets to address DBP-associated health concerns. Quantitative structure−toxicity relationship (QSTR) analysis has predicted HBQs to be potential bladder carcinogens. Therefore, this perspective focuses on the chemical and toxicological characterization of HBQs. *In vitro* cytotoxicity experiments have shown that HBQs induce greater cytotoxicity and/or greater developmental toxicity than most of the regulated DBPs. Cellular mechanistic studies indicate that HBQs are capable of producing reactive oxygen species (ROS) either within cells or in solution, depleting cellular glutathione levels, and influencing cellular antioxidant enzymes, which further induces oxidative stress and oxidative damage to cellular proteins and DNA. Oxidative damage to DNA was demonstrated in the form of significant increases in cellular levels of 8-hydroxydeoxyguanosine (8-OHdG), DNA strand breaks, and apurinic/apyrimidinic (AP) sites. HBQs can also form DNA adducts, affect genome-wide DNA methylation, and inhibit DNA repair enzymes. These findings demonstrate that HBQs are highly cytotoxic and potentially genotoxic and carcinogenic, although *in vivo* data corroborating this is not available. To fully understand the potential adverse health effects and cancer risk due to HBQ exposure, multidisciplinary research is required regarding human exposure, health risk assessment, and toxicological mechanisms of HBQs.



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1. INTRODUCTION

The chlorination of drinking water has been the most successful public health practice for preventing water-borne diseases, as evidence accumulated for over a century has shown. Disinfection kills pathogens, but, at the same time, it initiates reactions between disinfectants (e.g., chlorine or chloramine) and natural organic matter (NOM) present in the source water to generate a large number of disinfection byproducts (DBPs).[1]

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Since the first discovery of chloroform as a DBP in the 1970s, hundreds of DBPs have been identified. However, the identified DBPs only account for approximately 30% of the total predicted halo-DBPs based on total organic halogen (TOX) analysis. To date, the majority of DBPs remain unknown.[2] Why is it necessary to know what is unknown in our water? Epidemiological studies have consistently linked the consumption of chlorinated water with an increased risk of developing bladder cancer.[3] The regulated DBPs (including trihalomethanes, haloacetic acids, chlorite, bromate, and/or *N*-nitrosodimethylamine), whose concentrations in treated water are currently controlled by government guidelines, do not account for the cancer risk observed in epidemiological assessments based on animal toxicological studies.[4] Because everyone is exposed to DBPs through drinking water, showering, and swimming, DBP health issues challenge both the water industry and public health agencies. Therefore, identification of DBPs of health relevance has become one of the important research targets to address DBP-associated health concerns. A number of reviews have provided an excellent overview of DBP research.[1,4,5] This perspective focuses on one of the newly identified classes of DBPs, halobenzoquinones (HBQs), for several reasons: (1) quantitative structure−toxicity relationship (QSTR) analysis predicts that HBQs are up to 1000 times more toxic than the regulated DBPs,[6] (2) available toxicological results of 1,4-benzoquinone (1,4-BQ or *p*-BQ) indicate that HBQs are likely to be potent carcinogens, and (3) recent studies have provided evidence of the wide occurrence and toxicological importance of HBQs in drinking water.

1.1. QSTR Analysis. Epidemiological data has consistently indicated that an increased risk of bladder cancer is associated with the consumption of chlorinated water. However, animal studies have identified mainly the liver as the target organ for regulated DBPs, a significant difference from epidemiological studies that identified the bladder as the target organ. Moreover, the magnitude of risk from the carcinogenic activity found in animal studies for regulated DBPs is significantly lower than that which has been estimated from epidemiological studies. Therefore, recent efforts have been made to identify new DBPs that may have toxicological significance by contributing to the risk of cancer and being present in drinking water. QSTR analysis has been used to predict DBPs that are plausible carcinogens using TOPKAT software.[6] The first step of QSTR analysis is to predict the formation of novel DBPs from the reactions of substructures in NOM with commonly used disinfectants using principles of synthetic organic chemistry. The next step is to determine the potency of putative DBPs using several models in TOPKAT, mainly focusing on acute toxicity, chronic toxicity, and mutagenic and carcinogenic effects; then, assessing structure−activity relationships from the databases of the National Toxicology Program and the U.S. Food and Drug Administration. The results of QSTR analysis predicted five classes of putative DBPs as being high priority, including haloquinones (also known as halobenzoquinones), halocyclopentenoic acids, organic *N*-haloamines, nitrosamines and nitrosamides, and halonitriles and haloamides. The chronic lowest observed adverse effect levels (LOAELs) of HBQs were predicted to be 0.049 (2,6-dichloro-1,4-benzoquinone, DCBQ), 0.079 (2,6-dichloro-3-methyl-1,4-benzoquinone, DCMBQ), 0.033 (2,3,6-trichloro-1,4-benzoquinone, TriCBQ), and 0.159 (2,6-dibromo-1,4-benzoquinone, DBBQ) mg/kg body weight per day, with both DCMBQ and TriCBQ predicted to be bladder carcinogens.[6]

1.2. Benzoquinone Toxicity. QSTR analysis relies heavily on structure−activity relationships. For the HBQs, the analysis incorporated known toxicity information on 1,4-BQ, a structurally similar compound that is well-characterized toxicologically. The structure of 1,4-BQ in comparison to the HBQs can be found in Table 1. 1,4-BQ is a highly reactive metabolite of benzene, a recognized human carcinogen, whose toxicity is attributed to its highly reactive metabolites.[7] 1,4-BQ can cause a series of adverse effects either *in vivo* or *in vitro*, including sister chromatid exchange (SCE) and micronuclei (MN) formation in V79 cells[8] and induction of DNA single-strand breaks in human lymphocytes.[9] The mechanisms underlying 1,4-BQ-induced genotoxicity and carcinogenicity involve two major molecular pathways.[10,11] One pathway proposes that 1,4-BQ is capable of producing reactive oxygen species (ROS), causing oxidative damage to cellular DNA, proteins, and lipids. The covalent binding of 1,4-BQ to cellular macromolecules, such as DNA and protein, is the other major pathway.

1.3. Occurrence of HBQs as DBPs in Treated Water. The occurrence of HBQs in treated drinking water was first identified in 2010.[12,13] In a follow-up study of nine treatment plants using different disinfection treatments, including coagulation with chlorination, chloramination, chlorination with chloramination, and ozonation with chloramination, DCBQ, DCMBQ, TriCBQ, and DBBQ (Table 1) were found to be at nanogram per liter levels in the treated water.[14] The concentration of HBQs ranged from 0.5 to 275 ng/L, but none of the HBQs was detected in raw water or in the blanks. DCBQ showed a 100% occurrence frequency and the highest median concentration of the tested HBQs (23.0 ng/L). The concentrations of DCBQ were above 100 ng/L in 5 of the 16 samples with the highest at 274.5 ng/L. DBBQ was detected in 11 samples (68.8%) at concentrations up to 37.9 ng/L. DCMBQ and TriCBQ were also detected in 6 (37.5%) and 3 (18.8%) samples, respectively, at concentrations lower than 10 ng/L. Zhang's group in Hong Kong has also identified brominated HBQs using their developed mass spectrometry methods in 2011[15] and 2014.[16] Both DBBQ and 2,6-dibromo-1,4-hydroquinone were identified as DBPs in simulated drinking water. These results confirm HBQs as DBPs with high occurrence frequency, highlighting the need for further research.

HBQs have also been identified in treated swimming pool water.[17] Analysis of HBQs in 10 swimming pools and their input tap water showed that DCBQ was widely present at concentrations from 19 to 299 ng/L. The concentration of DCBQ in the swimming pools was as much as 100 times higher than its concentration in the input tap water. TriCBQ and DBBQ were also detected at concentrations up to 11.3 and 3.9 ng/L, respectively. In addition, a new compound, 2,3-dibromo-5,6-dimethyl-1,4-benzoquinone, was identified as a DBP in water from two swimming pools at concentrations lower than 1 ng/L, which likely originated from personal care products such as sunscreen or lotion.[17]

2. TOXICITY ASSESSMENT OF HBQS

Research concerning HBQs over the past 5 years has demonstrated that HBQs are commonly occurring DBPs in disinfected waters. Hence, to understand the potential significance of this emerging class of DBPs to human health, a number of *in vitro* toxicological studies have been performed

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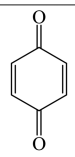
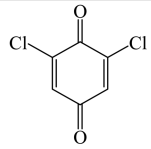
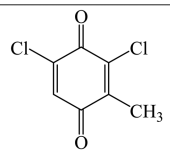
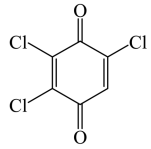
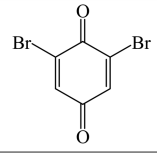
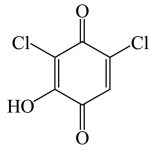
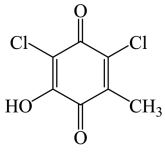
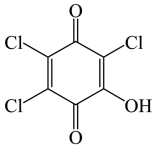
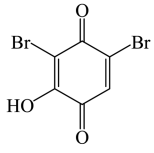
Table 1. Structure and Occurrence of HBQs in Drinking Water

HBQs	molecular formula	structure	occurrence frequency[14, 25]	concentration detected, ng/L[14, 25]
1,4-BQ	$C_6H_4O_2$		-	-
DCBQ	$C_6H_2Cl_2O_2$		16/16	4.5–274.5
DCMBQ	$C_7H_4Cl_2O_2$		6/16	< 0.9–6.5
TriCBQ	$C_6HCl_3O_2$		3/16	< 1.5–9.1
DBBQ	$C_6H_2Br_2O_2$		11/16	< 0.5–37.9
OH-DCBQ	$C_6H_2Cl_2O_3$		34/37	nd*–20
OH-DCMBQ	$C_7H_4Cl_2O_3$		12/37	nd–7
OH-TriCBQ	$C_6HCl_3O_3$		6/37	nd–20
OH-DBBQ	$C_6H_2Br_2O_3$		6/37	nd–10

*nd, not detected. The concentration is lower than the detection limit.

to evaluate the cytotoxicity of HBQs and to determine their potential mechanisms of toxicity.

2.1. Cytotoxicity of HBQs. The first evidence of HBQ-induced cytotoxicity was presented in a human urinary bladder epithelial carcinoma cell line, T24. Two traditional end-point cytotoxicity assays, the neutral red uptake (NRU) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assays, as well as a new impedance-based assay, real-time cell analysis (RTCA), were used to assess the cytotoxicity of DCBQ, DCMBQ, TriCBQ, and DBBQ, the first set of HBQs identified as DBPs in treated drinking water. The NRU assay determined 24 h IC_{50} values (the DBP concentration that results in a 50% decrease of the measured cellular response) of 11.4 μM for DCBQ, 148 μM for DCMBQ, 113 μM for TriCBQ, and 45.7 μM for DBBQ. Similarly, the MTS assay determined values of 94.5 μM for DCBQ, 110.1 μM for DCMBQ, 150.7 μM for TriCBQ, and 142.0 μM for DBBQ. Both traditional assays demonstrated

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IC_{50} values at micromolar levels for the tested HBQs in T24 cells.[18]

Conventional end-point cytotoxicity assays like the NRU and MTS assays have been extensively validated for use in cytotoxicity studies and are often fairly inexpensive. However, these end-point assays can require the preparation of multiple plates, limiting throughput, as well as require the use of labels or dyes, which can be invasive to cells. To improve throughput and to acquire multiple toxicological results using a single assay, the Li group has developed DBP cytotoxicity testing methods using a label-free technology, RTCA.[18−20] The principle and environmental application of RTCA have been described in a recent review.[21] Briefly, RTCA consists of a 96-microwell E-plate embedded with microelectrodes on the bottom of each well. RTCA measures the real-time changes in impedance resulting from changes in the population, morphology, and attachment of adherent cells on the surface of the microelectrodes. The measured impedance change is automatically converted to a unitless measurement, termed the cell index (CI) (Figure 1). Exposure of cells to DBPs may result in toxic

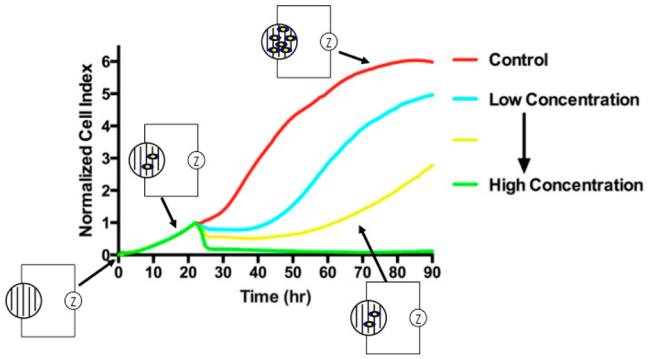

Figure 1. RTCA measures changes in impedance (Z) over time caused by cells adhering to microelectrodes on the bottom of specialized microwell plates. Z is presented as a unitless measure, termed the cell index (CI). Increases in CI correspond to the combined effects of cell proliferation, growth, and/or increased cell attachment to the microelectrodes; decreases in CI can represent inhibited cell proliferation, cell death, cell detachment, and/or decreased cell−electrode contact caused by an introduced cytotoxicant. In this cytotoxicity response profile, cells were seeded at time 0 h and were treated with three concentrations of a cytotoxicant after 24 h of growth. A dose response is clearly visible, as CI values decrease as the concentration of the cytotoxicant increases at any time point over the exposure period.

effects, resulting in lower CI values compared to those of untreated controls. Hence, this assay allows for continuous monitoring over a desired exposure period to provide dynamic cytotoxicity response profiles and temporal IC_{50} histograms. The RTCA method has been successfully used to assess the cytotoxicity of multiple emerging drinking water DBPs, including *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiphenylamine (NDPhA), *N*-nitrosopiperidine (NPip), *N*-nitrosopyrrolidine (NPyr), and phenazine, reporting 24 h IC_{50} values of 15, 0.59, 4.7, 14,[19] and 0.279 mM,[20] respectively, in T24 cells (Table 2). When T24 cells were exposed to varying concentrations (0−150 μM) of the individual HBQs and monitored for 80 h, the RTCA measurements generated real-time cytotoxicity response profiles of the CI over time and temporal IC_{50} histograms. For example, Figure 2 shows the RTCA analysis of T24 cells responding to DBBQ and DCMBQ

Table 2. Comparison of Cytotoxicity in T24 Cells of Some Classes of DBPs Using the RTCA Assay

DBPs	chemical class	24 h IC_{50}, mM	ref
DCBQ	HBQs	0.0019	18
DCMBQ		0.0587	
TriCBQ		0.0956	
DBBQ		0.0214	
N-nitrosodimethylamine (NDMA)	nitrosamines	15	19
N-nitrosopyrrolidine (NPyr)		14	
N-nitrosopiperidine (NPip)		4.7	
N-nitrosodiphenylamine (NDPhA)		0.59	
phenazine	–	0.279	20

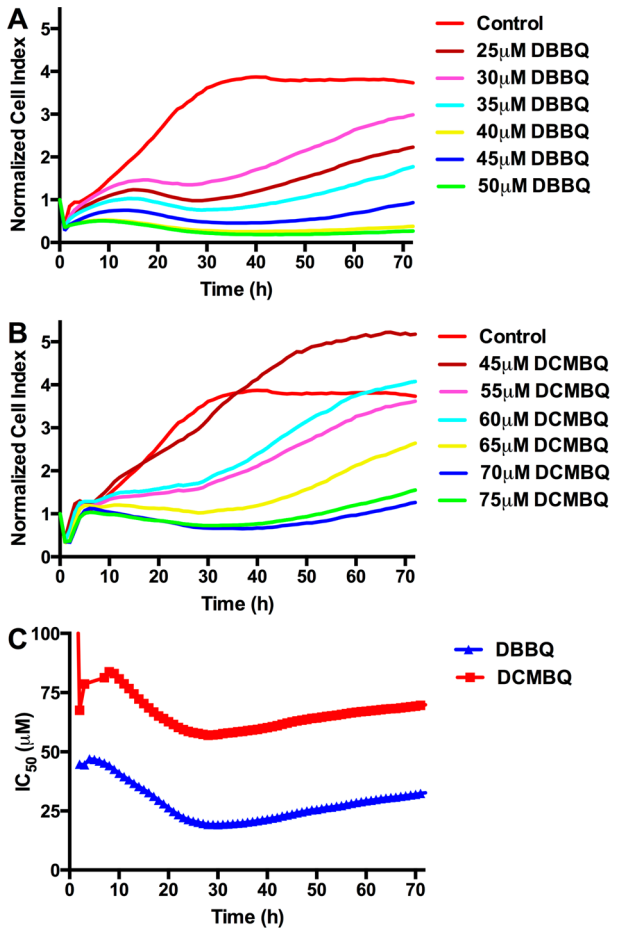

Figure 2. Cytotoxic response curves obtained from RTCA. (A, B) RTCA cytotoxicity response profiles (CI values over exposure time, normalized at time of cell treatment) showing the effect of DBBQ and DCMBQ on T24 cells. (C) IC_{50} histogram over 72 h post-treatment of DBBQ and DCMBQ. Reprinted with permission from ref 18. Copyright 2013 The Royal Society of Chemistry.

exposure. These response curves clearly demonstrate the dynamic nature of the cellular response over the exposure period, providing a clear advantage over conventional assays. The 24 h IC_{50} values were determined to be 1.9 μM for DCBQ, 58.7 μM for DCMBQ, 95.6 μM for TriCBQ, and 21.4 μM for DBBQ (Table 2). On the basis of these values, the cytotoxicity of the four HBQs in T24 was ranked in the order DCBQ > DBBQ > DCMBQ > TriCBQ. Furthermore, these results show HBQ-induced cytotoxicity to be the most potent of the DBPs tested by an order of magnitude or more.

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To better understand the relative cytotoxicity of HBQs in comparison to that of regulated DBPs, we compared the IC_{50} values of these DBPs in the immortalized Chinese hamster ovary (CHO) cell line. Table 3 shows the 72 h IC_{50} values

Table 3. Comparison of Cytotoxicity of Regulated DBPs and HBQs in CHO Cells

DBPs	chemical class	72 h IC_{50}, mM	ref
chloroform	trihalomethanes	9.62	22
bromodichloromethane		11.5	
dibromochloromethane		5.36	
bromoform		3.96	
monochloroacetic acid	haloacetic acids	0.81	23
dichloroacetic acid		7.3	
trichlorocetic acid		2.4	
monobromoacetic acid		0.01	
dibromoacetic acid		0.59	
bromate	–	0.963	31
chlorite	–	no data	
N-nitrosodimethylamine (NDMA)	nitrosamines	31	19
DCBQ	HBQs	0.0415	25
DCMBQ		0.0159	
TriCBQ		0.0729	
DBBQ		0.0355	

determined for the HBQs with those of regulated DBPs from the trihalomethane (THM) and haloacetic acid (HAA) classes in CHO cells. The IC_{50} values are between 3.96 and 11.5 mM for the THMs, between 0.01 mM (monobromoacetic acid) and 7.3 mM for the HAAs, 0.963 mM for bromate, 31 mM for NDMA, and between 0.01 and 0.07 mM for the HBQs.[19,22−24] The IC_{50} values show a wide variation in cytotoxicity of the DBPs, although it is important to note that different assays were used to generate these values. The IC_{50} values for the HBQs and NDMA were determined using the RTCA assay,[25] whereas the values for the remaining DBPs were determined using a dye-based assay developed by the Plewa group specifically for DBP testing.[24] This method is a microplate cytotoxicity assay that uses crystal violet dye to stain cell nuclei.[26] Nevertheless, on the basis of these findings, the HBQs are up to 1000 times more cytotoxic in CHO cells than some regulated DBPs (e.g., THMs) and are similarly cytotoxic to monobromoacetic acid at micromolar levels. This is consistent with a recent study from the Zhang group that evaluated the developmental toxicity of 20 halogenated DBPs in sewage effluent using the marine polychaete *Platynereis dumerilii*.[27] Here, it was demonstrated that 2,5-dibromohydroquinone induced the highest developmental toxicity among the tested halogenated DBPs. They reported an EC_{50} value (the DBP concentration at which 50% of the embryos developed normally) of 9.12 μM, which was hundreds to thousands of times lower than the values determined for the THMs and HAAs examined in the study.[27]

2.1.1. Effects of Transformation on Cytotoxicity of HBQs. In the environment, quinones can undergo hydrolysis, redox, photodegradation, and nucleophilic reactions, resulting in transformation to semiquinones, hydroquinones, hydroxyl-quinones, and benzenetriols.[28−31] Transformation is an important facet of quinone toxicity, as the transformed products have been shown to have significantly different cytotoxic effects than the parent compound. For example, compared to 1,4-BQ, hydroquinone and 1,2,4-benzenetriols were less cytotoxic to CHO cells, and while 1,4-BQ induced DNA strand breaks, 1,2,4-benzenetriols were not able to do so.[32] Hence, HBQs, as a separate class of quinones, may also experience similar reactions. High-resolution mass spectrometry analysis of treated drinking water as it flowed through water distribution systems revealed that HBQs transformed to halo-hydroxyl-benzoquinones (OH-HBQs) over time.[25] The OH-HBQs (OH-DCBQ, OH-DCMBQ, OH-TriCBQ, and OH-DBBQ) with their corresponding HBQs (DCBQ, DCMBQ, TriCBQ, and DBBQ) were all identified as DBPs. With increasing distance from the water treatment plants, the concentrations of HBQs in the distribution system gradually decreased, whereas those of OH-HBQs increased, supporting the transformation of HBQs to OH-HBQs in drinking water distribution systems over time. The cytotoxicity of these identified OH-HBQs was assessed using RTCA, and 24 h IC_{50} values in CHO cells were determined to be 61 μM for OH-DCBQ, 20 μM for OH-DCMBQ, 64 μM for OH-TriCBQ, and 43 μM for OH-DBBQ, which are higher than the IC_{50} values for the HBQs in the same cell line (Table 4). Hence, OH-HBQs were shown to be less cytotoxic

Table 4. IC_{50} Values of the Four HBQs and Four OH-HBQs on CHO Cells[a]

	IC_{50} (μM)		
compd	24 h	48 h	72 h
DCBQ	27.3 ± 1.0	35.5 ± 1.0	41.5 ± 1.3
OH-DCBQ	61.0 ± 3.0	69.5 ± 5.6	90.6 ± 33.6
DCMBQ	11.4 ± 0.5	13.7 ± 0.5	15.9 ± 0.9
OH-DCMBQ	20.4 ± 0.6	21.5 ± 0.8	24.0 ± 1.1
TriCBQ	45.5 ± 2.5	63.7 ± 2.1	72.9 ± 3.6
OH-TriCBQ	64.4 ± 3.7	67.1 ± 4.7	69.8 ± 6.8
DBBQ	19.8 ± 1.5	29.2 ± 1.8	35.5 ± 0.7
OH-DBBQ	42.8 ± 6.2	51.8 ± 3.4	50.4 ± 7.6

[a]Reprinted from ref 25. Copyright 2014 American Chemical Society.

than HBQs in CHO cells.[25] HBQs can also undergo photodecay and transform to OH-HBQs through ultraviolet (UV) light irradiation.[33] This study also demonstrated HBQ transformation to halobenzenetriols (HBTs) and dechlorinated/debrominated OH-HBQs, although the cytotoxicity of these compounds has not been evaluated. It has also been shown that tetrachloro-l,4-benzoquinone (TCBQ or chloranil) can undergo hydrolysis and transform to less toxic species, including the OH-HBQ, 2,5-dichloro-3,6-dihydroxy-1,4-benzoquonine, which is accelerated in the presence of hydroxamic acids.[34]

The findings discussed above indicate that OH-HBQs are the major products of HBQs in the environment, whereas dehalogenated quinones and HBTs can also exist as transformation products. However, the transformation products of HBQs in biological systems are still poorly understood, although it has been postulated that 2,5-dibromohydroquinone can be metabolized to 2,5-dibromoquinone in the marine polychaete *Platynereis dumerilii*.[27] Nevertheless, transformation of HBQs has been shown to be an important facet of HBQ toxicity, as HBQ transformation products can result in significantly different cytotoxic effects. This was shown in the studies of HBQ and OH-HBQ cytotoxicity mentioned previously and also in the developmental toxicity study of 2,5-dibromohydroquinone, as it was postulated that the observed toxicity was a combination of the toxicities of both 2,5-dibromohydroquinone and 2,5-dibromoquinone.

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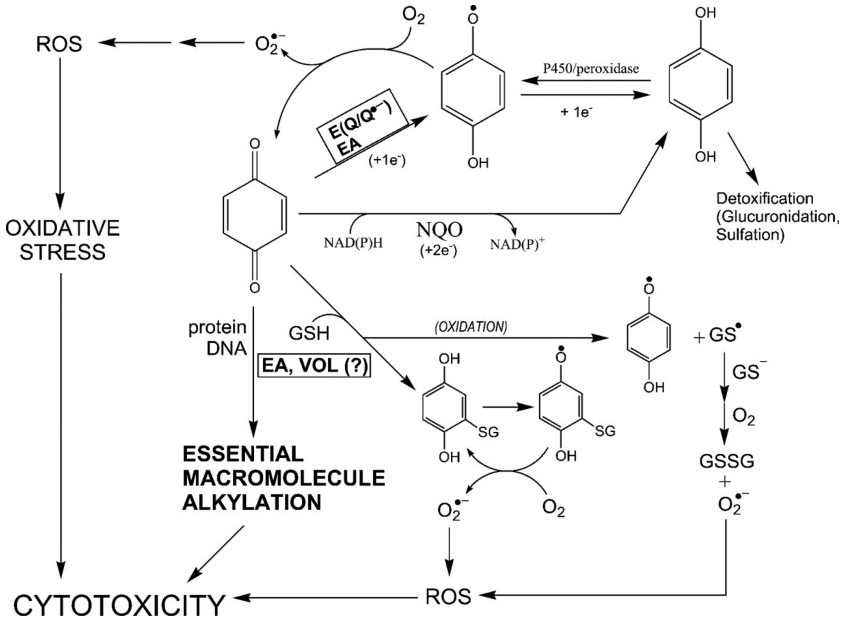
Scheme 1. Cellular Reaction Pathways for *p*-Benzoquinones and Proposed Involvement of Their Physicochemical Parameters[a]



[a]Reprinted with permission from ref 39. Copyright 2004 Oxford University Press. Note: E ($Q/Q^{\bullet-}$), one-electron redox potential; VOL, volume; EA, electron affinity.

2.2. HBQ-Induced Oxidative Stress. To investigate the mechanisms of HBQ toxicity, it is important to determine the similarities and differences compared to the known mechanisms of 1,4-BQ toxicity, as 1,4-BQ is the basic structure of HBQs (Table 1). There are two well-accepted mechanisms of action for quinone toxicity: (1) alkylation of cellular essential proteins and/or DNA and/or (2) oxidative stress due to formation of ROS due to redox cycling.[10,11] In biological systems, quinones can undergo either one-electron reduction or two-electron reduction (Scheme 1).[35] NADPH-quinone oxidoreductase (NQO) catalyzes the two-electron reduction of quinones to hydroquinones, which is often believed to be a detoxification process. In contrast, NADPH-cytochrome P450 reductase catalyzes the one-electron reduction of quinones to semiquinones. Semiquinone free radicals can directly damage cellular macromolecules, such as proteins and DNA. Semiquinones can also react with oxygen to form ROS, including superoxide anions, perhydroxyl radicals, hydrogen peroxide (H_2O_2), and hydroxyl radicals. These ROS can further induce oxidative damage to cellular macromolecules. Unrepaired molecular and cellular damage may eventually lead to carcinogenesis or cell death.

To demonstrate the involvement of oxidative stress in the observed cytotoxicity of HBQs to T24 cells, the effects of HBQs on T24 cell viability were examined with and without the addition of *N*-acetyl-L-cysteine (NAC),[36] as NAC functions either as a reservoir of cysteine in cells or as a scavenger of free radicals.[37,38] The presence of NAC significantly reduced the cytotoxic effects induced by HBQs, indicating that ROS are a significant contributor to HBQ cytotoxicity. Analysis of ROS in HBQ-treated (25−150 μM) T24 cells demonstrated that the four HBQs, DCBQ, DCMBQ, TriCBQ, and DBBQ, can generate intracellular ROS in T24 cells in a concentration-dependent manner. When compared to 1,4-BQ, the four HBQs generated significantly higher ROS at the same concentration (Figure 3). This is consistent with a previous study of 1,4-BQ congeners by Siraki and colleagues reporting that chlorine-

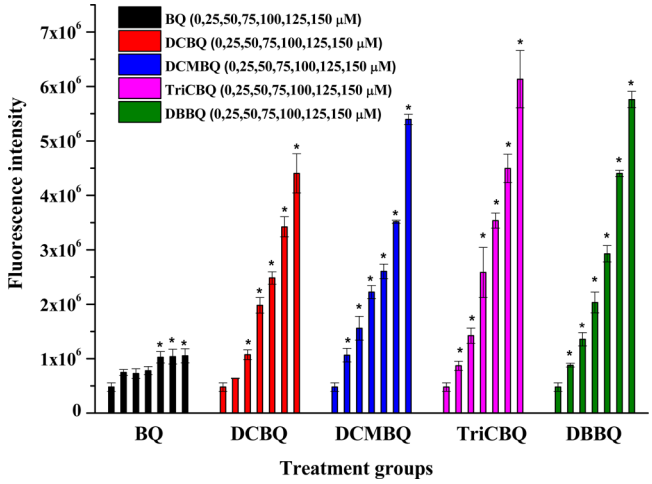


Figure 3. Levels of intracellular ROS in T24 cells after 24 h exposure to 25, 50, 75, 100, 125, or 150 μM of BQ (1,4-BQ), DCBQ, DCMBQ, TriCBQ, and DBBQ. ROS production was determined using 2′,7′-dichlorofluoroscein diacetate (DCFH-DA).[85] All values are expressed as the mean ± SD. Significant differences were observed in all treatment groups of HBQs compared to their respective control group (*P < 0.05). Reprinted from ref 36. Copyright 2013 American Chemical Society.

substituted 1,4-BQs (2,5-dichloro-l,4-benzoquinone and TCBQ) were capable of producing a significantly higher amount of ROS than 1,4-BQ and nonhalogenated quinones in primary cultured rat hepatocytes and PC12 cells.[39] Furthermore, these two HBQs were more cytotoxic in both cell types than the nonhalogenated quinones, indicating that chlorine substitution of 1,4-BQ may increase its toxicity. This is consistent with the fact that halogen substitutions are electron-withdrawing groups, which are more electrophilic than their parent compound quinones.[39,40] Another class of halogenated quinones, polychlorinated biphenyl (PCB) quinones, also

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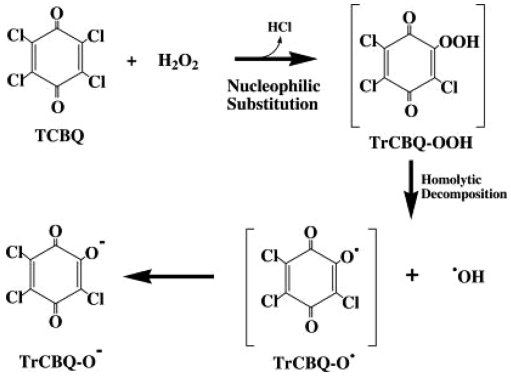
demonstrates the ability to yield the corresponding semiquinone free radicals in solution, which is correlated with the number of chlorines on the quinone ring.[41]

The ability of HBQs to produce ROS in solution has been investigated by Zhu and colleagues.[42−44] Using an electron spin resonance secondary spin-trapping method,[42−44] the Zhu group demonstrated that HBQs can generate free radicals with H_2O_2 via metal-independent reactions. When TCBQ or other HBQs were mixed with H_2O_2 in the absence of transition metal ions, hydroxyl radicals were generated via a nucleophilic substitution of H_2O_2 to TCBQ followed by a hemolytic decomposition reaction (Scheme 2).[44,45] In addition to hydroxyl radicals,

Scheme 2. Proposed Mechanism for Hydroxyl Radical Production by TCBQ and H_2O_2 in Solution[a]



[a]Reprinted with permission from ref 44. Copyright 2007 National Academy of Sciences.

HBQs also produced alkoxyl radicals and quinone ketoxyl radicals in solution through decomposition of hydroperoxides, independent of transition metal ions.[46,47]

To evaluate the ability of HBQs to induce oxidative molecular damage, the Li group measured (1) 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative DNA damage, and (2) the formation of carbonyl proteins, a marker of oxidative protein damage, in HBQ-treated T24 cells.[36] It was found that DCBQ, DCMBQ, TriCBQ, and DBBQ produce elevated levels of 8-OHdG in genomic DNA and elevated carbonyl levels in cellular proteins of T24 cells. These results support the idea that ROS-induced oxidative damage to DNA and proteins is involved in the observed cytotoxicity of these four HBQs in T24 cells. These results are consistent with a previous study in HepG2 cells that found that PCB quinones induced ROS production and resulting oxidative damage to lipids after a 3 h exposure.[48] The Zhu group also observed potential oxidative damage to lipids, as HBQs could form reactive lipid radicals pentyl and 7-carboxyheptyl and reactive product 4-hydroxyl-2-nonenal (4-HNE) in solution through the decomposition of lipid hydroperoxide 13-HPODE without the catalysis of transition metal ions.[49]

HBQs and related halogenated quinones can produce ROS and induce oxidative damage at the cellular and molecular levels. The halogen substitutions of quinones increases their ability to generate free radicals and likely induces more oxidative damage, resulting in higher toxicity. This supports the previous QSTR prediction of HBQs.[4,6]

2.3. Effects of HBQs on Intracellular Antioxidant Systems. In normal cells, there is equilibrium between free radicals and the antioxidant defense systems. A primary defense against oxidative damage is provided by glutathione (GSH), the major nonenzyme antioxidant which is present in cells at concentrations as high as millimolar levels.[50] The radical scavenger GSH and the antioxidant enzymes together constitute the first line of defense against cellular oxidative stress. Hence, these antioxidant defense systems change rapidly in response to oxidative stress induced by a xenobiotic. Additionally, many xenobiotics can induce or inhibit these enzymes causing their toxicity.

2.3.1. Glutathione. It has been observed that cellular GSH depletion by quinones is either by conjugation between GSH and the quinone or oxidation of GSH to glutathione disulfide (GSSG). In biological systems, xenobiotics undergo phase II metabolism, forming GSH conjugates that are more water-soluble and more easily excreted.

The protective role of GSH against HBQ toxicity has been demonstrated through several pieces of evidence.[51] It was found that treatment of T24 cells with DCBQ, DCMBQ, TriCBQ, and DBBQ significantly reduced cellular GSH levels compared to those in untreated control cells, and the reduction of GSH levels was correlated with the concentration of the HBQs used for treatment (Figure 4). These results are

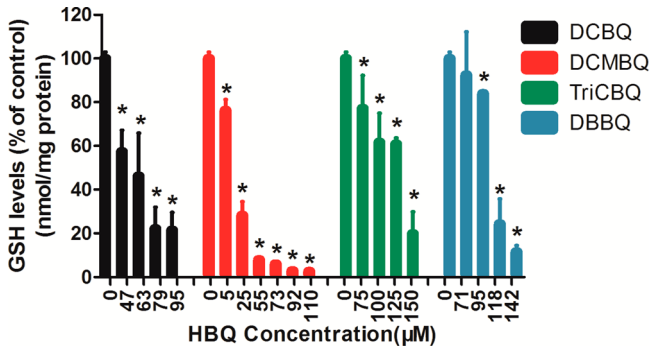


Figure 4. Effect of HBQs on cellular glutathione levels in T24 cells. GSH levels were determined after 24 h of exposure to HBQs and compared to the levels of the negative control. Error bar represents SD. *P < 0.05, HBQ treatment groups compared with negative control. Reprinted with permission from ref 51. Copyright 2014 Oxford University Press.

consistent with decreases in cellular GSH levels that were also observed in studies in which rat hepatocytes and PC12 cells were exposed to TCBQ and 2,5-dichloro-1,4-benzoquinone and HepG2 cells were exposed to PCB quinones.[39,48] When the PCB quinones were cotreated with dihydrolipoic acid (DH-LA) in HepG2 cells, DH-LA significantly reduced PCB quinone-induced oxidative stress, further supporting the protective role of GSH toward HBQ-induced toxicity, as DH-LA is a sulfur-containing fatty acid that is able to regenerate both vitamin C and GSH in their reduced forms in cells.[48,52]

To further demonstrate the involvement of GSH in the mechanisms of HBQ toxicity, the cytotoxicity of DCBQ, DCMBQ, TriCBQ, and DBBQ to T24 cells was determined when cellular GSH was depleted by buthionine sulfoximine (BSO), an irreversible inhibitor of GSH synthesis. It was found that IC_{50} values of HBQs significantly decreased by 1.5−5-fold compared to when there was no depletion of cellular GSH.[51] Furthermore, when cellular GSH was depleted and the culture media was supplemented with 10 mM GSH prior to HBQ treatment, the results indicated that supplementing 10 mM exogenous GSH can significantly reduce HBQ-induced cytotoxicity in T24 cells.[51] Hence, supplementation of extracellular

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GSH was found to further support the finding that GSH likely plays an important role in preventing HBQ-induced cytotoxicity.

The decreased levels of cellular GSH may be due to conjugation of HBQs with GSH. Conjugation products of GSH and HBQs could be formed after incubation of HBQs with GSH in solution,[51] which was confirmed by mass spectrometry analysis. This mechanism of conjugation has been previously reported in a study by Song and colleagues.[53] Chlorinated quinones can undergo nonenzymatic dechlorination upon reaction with GSH; for example, incubation of TCBQ with excess GSH formed its $(GS)_4$-Q adduct.[53] However, it is unknown whether oxidation of GSH or conjugation of GSH plays the major role in depletion of cellular GSH, prompting the need for future studies.

2.3.2. Antioxidant Enzymes. GSH likely plays an important role in the protection of cells from HBQs. It is also important to clarify whether HBQs induce some concomitant changes in GSH-related antioxidant enzymes. Glutathione S-transferases (GST) are a family of enzymes that catalyze the conjugation of GSH with electrophilic substrates,[54] whereas glutathione peroxidases (GPx) are responsible for the metabolism of hydrogen peroxide or hydroperoxides.[55]

To assess the effects of HBQs on the activity of these enzymes, T24 cells were incubated with DCBQ, DCMBQ, TriCBQ, and DBBQ at concentrations of equivalent biological response (from 1/2 of IC_{50} to 1 of IC_{50}). It was found that HBQ exposure significantly increased GST activity and slightly decreased or did not change GPx activity in T24 cells after 24 h of HBQ exposure.[51] Similar results were observed in 1,4-BQ-treated MCF7 cells, where the mRNA gene expression level of GST was enhanced, whereas the mRNA gene expression level of GPx did not show modulation.[56] The induction of antioxidant enzymes, such as GST, is thought to be critical for protection of cells from quinone toxicity. Their induction is regulated by nuclear factor erythroid 2-related factor 2 (Nrf2), which is the principal regulator of the antioxidant response element (ARE) located in the genes encoding antioxidant enzymes.[57−59] In contrast, TCBQ is a very effective inhibitor of rat and human GST, as observed in incubation studies of purified GST and TCBQ.[60,61] The contrasting results on GST may be due to the lack of information on the specific GST isoforms, the use of different exposure times and different concentrations of quinones, or the different halogen substitutions of the quinones.

PCB quinones have also been shown to modulate antioxidant enzymes. After treatment of HepG2 cells with PCB quinones, there was not only an increase in superoxide dismutase (SOD) activity but also a decrease in catalase (CAT) and GST activity.[48] This was accompanied by a significant upregulation in the mRNA levels of NQO1 and heme oxygenase 1 (HO-1) and significant increases in protein expression of NQO1 and HO-1, which is associated with the activation of the Nrf2/ARE pathway in HepG2 cells.[62]

2.4. HBQ-Induced Effects on DNA. According to the results of QSTR analysis, none of the HBQs was predicted to be a potential mutagen, although DCMBQ and TriCBQ were predicted to be potential carcinogens.[4,6] There is very limited data on the mutagenic, genotoxic, and carcinogenic effects of HBQs. However, the potential mechanisms of quinone genotoxicity are more well-known, which includes the generation of ROS with subsequent oxidative damage to DNA, the direct alkylation of cellular DNA, and the direct intercalation of DNA.[10,11,31] Halogen substitution on quinones may also have an effect on the mechanism of toxicity, as halogenation increases the octanol−water partition coefficient, which is an important parameter related to absorption, distribution, metabolism, and excretion in the body.[4,35] Some evidence has suggested that halogenated quinones are also capable of inducing mutagenic and genotoxic effects. HBQ-induced DNA damage is summarized in Table 5.

2.4.1. Mutagenic Effects. 1,4-BQ has been shown to produce single-base substitution mutations in human fibroblast cell lines, as measured by the *supF* forward mutation assay.[63] A recent study investigated the mutagenicity of tetrachlorohydroquinone (TCHQ) using the *supF* shuttle vector system. The results indicated that TCHQ is a potent mutagen, with the majority (above 85%) being single-base mutations.[64] The Ames assay (or the bacterial reverse mutation assay) is a standard method used for detecting the mutagenic potential of testing compounds.[65] On the basis of Ames tests, DCBQ, DCMBQ, TriCBQ, and DBBQ appear to be mutagenic (unpublished work).[66] However, further studies are required to understand the underlying mechanisms of HBQ-induced mutagenic effects.

2.4.2. Oligonucleotide Binding. Previous experiments have shown that HBQs are capable of binding to single- or double-stranded oligodeoxynucleotides, as measured by electrospray ionization mass spectrometry. The ranking of binding affinities was found to be TriCBQ ≈ DCMBQ < DCBQ ≪ DBBQ, and binding was thought to occur through a noncovalent mechanism characterized by H-bonding and partial intercalation.[67] Bromobenzoquinone interacted with oligonucleotides in the formation of bromide oligonucleotides, and the interaction depended on the extent of bromination of 1,4-BQ.[68]

The adduct of deoxyguanosine (dG) and TCBQ was found to be a dichlorobenzoquinone nucleoside in solution using LC-MS.[69] When exposed to nucleosides and calf thymus DNA, TCBQ preferentially formed adducts with dG, but it also formed adducts with deoxycytidine and deoxythymidine.[70] Furthermore, exposure of immobilized double-stranded DNA on a biosensor to TCBQ and tetrafluoro-1,4-benzoquinone (TFBQ) revealed that both TCBQ and TFBQ covalently bind DNA to form DNA adducts (TCBQ > TFBQ), whereas coexposure of the HBQs with H_2O_2 revealed that TFBQ also generates hydroxyl radicals that damage DNA bases.[71] The formation of direct adducts has been observed in TCBQ- or TCHQ-treated calf thymus DNA, human HeLa S3 cells, and rats.[72−74]

2.4.3. Oxidative DNA Damage. ROS-induced DNA damage can include base modification, single- or double-stranded DNA breaks, modification of deoxyribose, apurinic/apyrimidinic (AP) sites, and DNA cross-links.[75] Among them, 8-OHdG is a well-accepted indicator of oxidative DNA damage when biological systems are exposed to xenobiotics.[76] 8-OHdG, as a DNA adduct, is formed due to the hydroxylation of the guanine base residues in DNA. Incubation of T24 cells with DCBQ, DCMBQ, TriCBQ, and DBBQ significantly induced generation of 8-OHdG, with the highest levels of 8-OHdG (an approximately 10-fold increase) induced by DCMBQ.[36] The formation of 8-OHdG was also observed after V79 Chinese hamster lung cell exposure to TCBQ[77] and after human HeLa S3 tumor cell exposure to TCBQ.[74]

DNA strand breaks have also been commonly reported when cells are exposed to quinone treatments. They have been observed in V79 cells upon exposure to TCBQ,[77] in 1,4-BQ- and hydroquinone-treated CHO cells,[32] and in HepG2 cells upon exposure to PCB quinones.[78] The underlying pathway of

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Table 5. HBQ-Induced DNA Damage[a]

	HBQs	results	method	exposure	ref
Oligonucleotide binding	DCBQ, DCMBQ, TriCBQ, DBBQ	Bind to single- or double-stranded oligodeoxynucleotides	ESI-MS/MS	In solution	67
	TCBQ	Bind to deoxyguanosine	LC-ESI-MS/MS, LC-ESI-MS, NMR, IR, UV, cydic voltammetry	In solution	69
	TCBQ, TFBQ	Mainly bind to deoxyguanosine, also bind to deoxycytidine and deoxythymidine	LC-ESI-MS/MS, LC-ESI-MS, HPLC, UV, NMR	Nucleosides and calf thymus DNA in solution	70
	TCBQ	Bind to double-stranded DNA	Photoelectrochemical measurement	Immobilized double-stranded DNA on a biosensor	71
	TCBQ	0.78×10^7 DNA adducts per total nucleotides	^{32}P-postlabeling following nuclease P1 adduct enrichment	F344 rats, dietary exposure to 60 mg/kg/day of PCP for 27 weeks	72
	TCBQ (50−300 μM)	3−6 adducts per 10^8 total nucleotides	^{32}P-postlabeling assay	Human HeLa S3 tumor cells, 0.5−2 h	74
	TCBQ (5 mM)	3.5 adducts per 10^5 total nucleotides	^{32}P-postlabeling assay	Calf thymus DNA	73
Oxidative DNA Damage					
8-OHdG	DCBQ, DCMBQ, TriCBQ, DBBQ (25−150 μM)	Increased 10-fold above background levels	ELISA	Human T24 bladder cancer cells, 24 h	36
	TCBQ (25 μM)	Increased 2−2.5 fold above background levels	Hewlett-Packard 1049A electrochemical detector	V79 Chinese hamster lung cells, 1 h	77
	TCBQ (50−300 μM)	Increased 2−5 fold above background levels	HPLC/electrochemical detection approach	Human HeLa S3 tumor cells, 0.5−2 h	74
DNA strand breaks	TCBQ (25 μM)	Significantly increased DNA single-strand breaks	Alkaline elution assay	V79 Chinese hamster lung cells, 1 h	77
	1,4-BQ (25−100 μM)	Significantly increased DNA strand breaks	Fluorimetric analysis of DNA unwinding	CHO cells, 45 min	32
	PCB quinones (12.5 and 25 μM)	Significantly increased DNA strand breaks	Single cell gel electrophoresis assay	Human HepG2 liver cells, 1.5, 3, and 6 h	78
AP sites	TCHQ (300 μM)	Increased 2 fold	ASB assay	Human HeLa S3 tumor cells, 0.5−2 h	74
Other DNA Damage					
DNA methylation change	TCBQ or TCHQ, (1−50 μM)	Induced the formation of 5-hydroxymethylcytosine (5hmC) from 5-methylcytosine (5mC)	UHPLC-MRM MS/MS analysis, and antibody-based dot-blot analysis	Human MRC-5 fetal lung fibroblast cells, human A549 lung adenocarcinoma cells, and human HepG2 liver cells, 6−72 h	82
Micronucleus and chromosome aberrations	1,4-BQ (1−50 μM)	Increased cellular levels of micronucleus and chromosome aberrations	Flow cytometry for micronuclei, and the OECD guidelines method for chromosomal aberration test	CHO cells	84

[a]ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; IR, infrared; UV, ultraviolet; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; ASB assay, aldehydic slot blot assay; UHPLC, ultra high-performance liquid chromatography; MRM, multiple reaction monitoring.

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DNA strand breaks caused by TCBQ has been attributed to the likely intercalation of TCBQ in double-stranded DNA, which could lead to the generation of hydroxyl radicals when coincubated with H_2O_2 in solution.[79]

AP sites are another byproduct of oxidative DNA damage reportedly induced by HBQ exposure. Formation of AP sites has been observed in HeLa S3 cells after treatment with TCHQ.[74] A possible pathway for AP site formation is the direct abstraction of hydrogen from the deoxyribose of DNA, which will further form 5′-nicked oxidized AP sites.[80,81]

2.4.4. Other DNA Effects. Halogenated quinones are able to induce the formation of 5-hydroxymethylcytosine (5hmC) from 5-methylcytosine (5mC) genome-wide in several cultured human cell lines by stimulating the Tet-dependent oxidative conversion of 5mC to 5hmC.[82] A change in genome-wide DNA methylation can affect gene expression, which is involved in a broad range of cellular functions. Thus, it has been proposed to potentially mediate oncogene activation, further inducing carcinogenesis.[83] These findings have led to a new proposed mechanism for HBQ-induced genotoxic and carcinogenic effects. 1,4-BQ and hydroquinone have been demonstrated *in silico* to induce DNA strand breaks and the formation of MN and chromosome aberrations in CHO cells.[84] These genotoxic effects were mainly due to the inhibition of topoisomerase, a major DNA replication and repair enzyme. The inhibition of DNA replication and repair enzymes is therefore another possible direction for the future study of HBQ-induced DNA damage.

3. SUMMARY AND FUTURE RESEARCH NEEDS

Both QSTR analysis and the *in vitro* experiments discussed in this perspective have shown that HBQs are highly cytotoxic. In comparison to some regulated DBPs, HBQs were over 1000 times more cytotoxic toward an immortalized mammalian cell line. Furthermore, evidence has shown that there are similarities between the cellular mechanisms of HBQ-induced toxicity and those of 1,4-BQ, which is a highly reactive metabolite of a known human carcinogen, with the results of many *in vitro* studies supporting oxidative stress pathways playing a key role in HBQ-induced toxicity. HBQs can undergo redox cycling to produce ROS, causing oxidative damage to cellular macromolecules, including proteins and DNA. This oxidative damage is exacerbated by direct interactions between HBQs and the cells' innate antioxidant defense system, including GSH and the antioxidant enzymes, GST and GPx. Several studies have shown that the DNA damage induced by HBQs is extensive due to both the direct and indirect (via ROS) interactions with DNA. This includes the formation of DNA adducts, DNA strand breaks, and AP sites. These results are consistent with reports of mutagenic and genotoxic effects of HBQs observed *in vitro*.

The *in vitro* evidence supporting HBQ-induced DNA damage strongly suggests that HBQs are potentially genotoxic and carcinogenic, although *in vivo* data is missing. Future *in vivo* studies are needed to evaluate the carcinogenic potential of this emerging class of DBPs to better understand the potential human health hazards presented by the presence of these compounds in disinfected water. This is particularly important because recent studies have shown that some HBQs occur widely in both treated drinking water and treated recreational water, such as swimming pools. Hence, more toxicological studies are warranted as well as studies of HBQ formation, transformation, and global occurrence.

HBQ formation studies can identify how current water disinfection processes affect HBQ formation in treated water, promoting the development of alternative disinfection techniques that either avoid HBQ formation or can control their formation and/or removal from treated water to prevent human exposure. More analytical research is also needed to identify HBQ transformation products in biological systems, as current evidence indicates that the cytotoxicity of HBQ transformation products can be significantly altered from that of their parent compound. The global occurrence of HBQs is another important avenue of research, as global surveys of HBQs are lacking. Because HBQ formation is highly dependent on the quality of source water, it is necessary to examine HBQs in drinking water in underdeveloped countries where source water quality may be low. In conclusion, multidisciplinary studies will be required to determine both the toxicological mechanisms of HBQs *in vivo* and the pathways of human exposure. Together, these future studies will provide a better understanding of the potential health risks associated with HBQ exposure and will allow for the establishment of drinking water regulations, with the ultimate goal being the protection of human health.

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ABBREVIATIONS

1,4-BQ or *p*-BQ, 1,4-benzoquinone; 4-HNE, 4-hydroxyl-2-nonenal; 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; 8-OHdG, 8-hydroxydeoxyguanosine; AP, apurinic/apyrimidinic; ARE, antioxidant response element; BSO, buthionine sulfoximine; CAT, catalase; CI, cell index; CHO, Chinese hamster ovary; DBBQ, 2,6-dibromo-1,4-benzoquinone; DBPs, disinfection byproducts; DCBQ, 2,6-dichloro-1,4-benzoquinone; DCMBQ, 2,6-dichloro-3-methyl-1,4-benzoquinone; dG, deoxyguanosine; DH-LA, dihydrolipoic acid; GPx, glutathione peroxidases; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferases; HAA, haloacetic acid; HBQs, halobenzoquinones; HBTs, halobenzenetriols; HO-1, heme oxygenase 1; H_2O_2, hydrogen peroxide; LOAELs, chronic lowest observed adverse effect levels; MN, micronuclei; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; NAC, *N*-acetyl-L-cysteine; NDMA, *N*-nitrosodimethylamine; NDPhA, *N*-nitrosodiphenylamine; NOM, natural organic matter; NPip, *N*-nitrosopiperidine; NPyr, *N*-nitrosopyrrolidine; NQO, NADPH-quinone oxidoreductase; Nrf2, nuclear factor erythroid 2-related factor 2; NRU, neutral red uptake; OH-HBQs, halohydroxyl-benzoquinones;

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PCB, polychlorinated biphenyl; QSTR, quantitative structure−toxicity relationship; ROS, reactive oxygen species; RTCA, real-time cell analysis; SCE, sister chromatid exchanges; SOD, superoxide dismutase; THM, trihalomethane; TOX, total organic halogen; TCBQ, tetrachloro-1,4-benzoquinone; TCHQ, tetrachlorohydroquinone; TFBQ, tetrafluoro-1,4-benzoquinone; TriCBQ, 2,3,6-trichloro-1,4-benzoquinone; UV, ultraviolet

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