Mechanisms of cytotoxicity induced by halobenzoquinone water disinfection byproducts

by

Jinhua Li

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Analytical and Environmental Toxicology
Department of Laboratory Medicine and Pathology
University of Alberta

Abstract

Drinking water disinfection effectively kills pathogens and prevents waterborne diseases, but it also generates a large number of water disinfection byproducts (DBPs). Epidemiological studies have consistently linked long-term consumption of chlorinated water with an increased risk of developing bladder cancer; however, the responsible DBP or DBPs have not been identified. Halobenzoquinones (HBQs) are an emerging class of DBPs that are predicted to be potent bladder carcinogens. However, little is known about the toxicological effects of HBQs. This thesis investigated the cytotoxicity and potential mechanism of toxicity induced by HBQs.

The cytotoxicity of HBQs was demonstrated in several cell lines: T24 human bladder carcinoma cells, CHO-K1 Chinese hamster ovary cells, and SV-HUC-1 human uroepithelial cells. At μM levels HBQs induced significantly higher toxicity than the regulated DBPs. HBQs generated significant intracellular reactive oxygen species (ROS), weakened the glutathione (GSH) antioxidant system, and caused severe oxidative DNA and protein damage (i.e., increased cellular level of 8-hydroxydeoxyguanosine (8-OHdG) and protein carbonyls). Additionally, toxicogenomic analysis showed that HBQs activate the nuclear factor E2-related factor 2 (Nrf2) antioxidant pathway; the major transcripts differentially up-regulated at both early and late exposure times were HMOX1, followed by NQO1, PTGS2 and TXNRD1. These results support oxidative stress as a key mechanism of HBQ-induced cytotoxicity.

Structure–toxicity relationship analysis demonstrated that formation of ROS, cytotoxicity, and genotoxicity were strongly affected by the structure of HBQs (i.e., position, type, and number of substitutions of halogens on the benzoquinone ring). Halogen substitution groups, isomeric structure, and electron distribution were shown to affect toxicity. The cytotoxicity of di-halogenated HBQs followed the trend: iodo- > bromo- > chloro-HBQs.

Additionally, 2,5-HBQs induced greater toxicity than their corresponding 2,6-HBQ isomers. Furthermore, HBQ toxicity was influenced by two major structural descriptors: the lowest unoccupied molecular orbital energy (E_{LUMO}) and dipole moment.

An established human embryonic kidney (HEK293) cell model, expressing specific multidrug resistance proteins (MRPs), was used to indirectly examine the role of MRPs on the cellular export of HBQs. Compared to the HEK-empty vector, HEK-MRP4 cells significantly decreased HBQ cytotoxicity and cellular ROS generation, supporting the role of MRP4 in the cellular detoxification of HBQs. The detoxification of HBQs by MRP4 was dependent on GSH.

This research provides several original contributions to the field. 1) HBQ DBPs can cause high toxicity in multiple human cell lines, and show significantly higher toxicity than regulated DBPs in CHO cells. 2) The structure–toxicity analysis of HBQs identified E_{LUMO} and dipole moment as a key parameter of HBQ toxicity. 3) Toxicogenomic analysis supports oxidative stress pathways as one of the key mechanisms of toxicity of HBQ DBPs. 4) The first study of MRPs and the toxicity of DBPs demonstrated the role of MRP4 in detoxifying HBQs. These results provide useful insight into the mechanisms of toxic effects of HBQs in *in vitro* systems.

Preface

Parts of Chapter 1 have been published as Jinhua Li, Wei Wang, Birget Moe, Hailin Wang, and Xing-Fang Li. Chemical and toxicological characterization of halobenzoquinones, an emerging class of disinfection byproducts. Chem. Res. Toxicol. 2015, 28(3): 306-318. Reprinted with permission. Copyright 2015 American Chemical Society.

Chapter 2 of this thesis has been published as Haiying Du, Jinhua Li (co-first author), Birget Moe, Claire F. McGuigan, Shengwen Shen, and Xing-Fang Li. Cytotoxicity and oxidative damage induced by halobenzoquinones to T24 bladder cancer cells. Environ. Sci. Technol., 2013, 47(6):2823-30. Dr. Du and I were responsible for the data collection and analysis as well as the manuscript composition. Dr. Moe, Dr. McGuigan, and Dr. Shen contributed to the manuscript editing. Dr. Li was the supervisory author and was involved with concept formation and manuscript composition. Reprinted with permission. Copyright 2013 American Chemical Society.

Chapter 3 of this thesis has been published as Jinhua Li, Birget Moe, Sai Vemula, Wei Wang, and Xing-Fang Li. Emerging disinfection by-products, halobenzoquinones: effects of isomeric structure and halogen substitution on cytotoxicity, formation of reactive oxygen species, and genotoxicity. Environ. Sci. Technol., 2016, 50 (13), 6744–6752. I was responsible for the experimental design, data collection and analysis, and manuscript composition. Mr. Vemula contributed to the preliminary experiments of this study. Dr. Moe and Dr. Wang contributed to the manuscript editing. Dr. Li was the supervisory author and was involved with concept formation and manuscript composition. Reprinted with permission. Copyright 2016 American Chemical Society.

Chapter 4 of this thesis has been published as Jinhua Li, Wei Wang, Hongquan Zhang, X. Chris Le, and Xing-Fang Li. Glutathione mediated detoxification of halobenzoquinone drinking water disinfection byproducts in T24 cells. Toxicol Sci, 2014, 141(2): 335-343. I was responsible for the experimental design, data collection and analysis, and manuscript composition. Dr. Wang contributed to the data collection and analysis of HBQ-GSH conjugates. Dr. Zhang and Dr. Le contributed to the manuscript editing. Dr. Li was the supervisory author and was involved with concept formation and manuscript composition. Reprinted with permission. Copyright 2014 Oxford University Press.

Acknowledgments

I would like to thank my supervisor, Dr. Xing-Fang Li, for her support, guidance, and patience over the last five years. She is an excellent example for me as a female professor. Thank you for your encouragement and training to help me transition from a student to a scholar in pursuit of my own academic career.

I'd like to thank my doctoral supervisory committee, Dr. X. Chris Le, Dr. Elaine Leslie, and Dr. Hongquan Zhang. Thank you for always providing excellent advice and suggestions during my program. Thanks also to my candidacy examiners, Dr. Jonathan Martin and Dr. Jason Acker. Thank you for all your help and support.

I am grateful for all the members of the AET Division, especially the Li and Le groups, who always gave me valuable suggestions on my research, and accompanied me in daily life like a big family. I'd like to thank Ms. Katerina Carastathis for her substantial help, especially editing my manuscripts and ordering reagents. Thank you to Ms. Dianne Sergy for all the help over the years. Thank you to Dr. Birget Moe, Dr. Haiying Du, Dr. Wei Wang, Dr. Yichao Qian, Ms. Lindsay Blackstock, and Ms. Katherine Fu for all your substantial collaboration with me. Thanks for the help and support from Ms. Xiufen Lu, Dr. Yanming Liu, Dr. Zhixin Wang, Dr. Feng Li, Dr. Hanyong Peng, Dr. Xiaowen Yan, Dr. Qi Zhang, Dr. Ping Jiang, Dr. Guang Huang, and Mr. Ian Vander Meulen.

My thanks and appreciation go out to Dr. Monika Keelan and Ms. Cheryl Titus for their constructive guidance and support on my professional development. Thanks for continual encouragement and support from Professor Juan Li. My thanks also to all the co-authors who have contributed to my publications.

Finally and most importantly, I'd like to thank my husband Zhigang Li, who gave me infinite love, support, help, encouragement, and company. Thanks to my little daughter Vivian, who always makes me happy and makes my life shining. Thanks to my beloved parents and younger sister, and parents-in-law for their boundless encouragement and understanding.

Table of Contents

Chapter 1 Introduction **1**

1.1 Disinfection byproducts (DBPs) **1**

1.1.1 Importance of drinking water disinfection **1**

1.1.2 History and regulation of DBPs 1

1.1.3 Health concerns from epidemiological studies 2

1.1.4 Issues in DBP research 3

1.1.5 Toxicological evaluation of DBPs 3

1.1.5.1 *In vitro* studies 3

1.1.5.2 *In vivo* studies 5

1.1.5.3 Toxicological pathways 6

1.2 Halobenzoquinones (HBQs) **8**

1.2.1 Quantitative structure–toxicity relationship analysis 8

1.2.2 Occurrence of HBQs as DBPs 8

1.2.3 HBQ-induced cytotoxicity in CHO cells 9

1.3 Toxicological evaluation of quinone compounds **11**

1.3.1 Benzoquinone toxicity 11

1.3.2 Halogenated quinone (HQ)-induced oxidative stress 11

1.3.3 Effects of HQs on intracellular antioxidant systems 12

1.3.4 HQ-induced effects on DNA 14

1.3.4.1 Mutagenic effects 14

1.3.4.2 Oligonucleotide binding 14

1.3.4.3 Oxidative DNA damage 15

1.3.4.4 Other DNA effects 15

1.4. Rationale and objectives of thesis **16**

1.5 References **18**

Chapter 2 Cytotoxicity and oxidative damages induced by HBQs **39**

2.1 Introduction **39**

2.2 Materials and Methods **41**

2.2.1 Reagents 41

2.2.2 Cell culture 41

2.2.3 Cell viability 41

2.2.4 Detection of ROS 42

2.2.5 Determination of 8-OHdG 42

2.2.6 Determination of protein carbonylation 43
2.2.7 Determination of MDA 43
2.2.8 Data analysis 43
2.3 Results and Discussion 44
2.3.1 Effects of HBQs on the viability of T24 cells 44
2.3.2 HBQ-induced ROS in T24 cells 45
2.3.3 8-OHdG in genomic DNA extracted from HBQ-treated T24 cells 46
2.3.4 Protein carbonyl levels in HBQ-treated T24 cells 47
2.3.5 Malondialdehyde (MDA) protein adducts in HBQ-treated T24 cells 47
2.4 Conclusion 48
2.5 References 49
Chapter 3 Quantitative structure-toxicity relationship of HBQs 61
3.1 Introduction 61
3.2 Materials and Methods 62
3.2.1 Reagents 62
3.2.2 Cell culture 63
3.2.3 Cytotoxicity 63
3.2.4 Determination of cellular ROS 64
3.2.5 Determination of 8-hydroxy-2'-deoxyguanosine (8-OHdG) 65
3.2.6 Assessment of p53 protein 65
3.2.7 Physicochemical parameters 66
3.2.8 Statistical analysis 66
3.3 Results and Discussion 66
3.3.1 HBQ-induced cytotoxicity 66
3.3.2 HBQ-induced ROS production 68
3.3.3 HBQ-induced genotoxicity 70
3.3.4 HBQ structure–toxicity relationships 72
3.4 Conclusions 75
3.5 References 77
Chapter 4 The involvement of cellular glutathione defense system in HBQ-induced cytotoxicity 96
4.1 Introduction 96
4.2 Materials and Methods 97

4.2.1 Reagents 97
4.2.2 Cell culture 97
4.2.3 Effects of glutathione depletion on cell viability 97
4.2.4 Effects of exogenous glutathione on cell viability 98
4.2.5 Measurement of cellular glutathione levels 98
4.2.6 Measurement of cellular glutathione S-transferase (GST) activity 99
4.2.7 Measurement of cellular glutathione peroxidase (GPx) activity 99
4.2.8 Mass spectrometry analysis of the conjugation between HBQ and GSH 100
4.2.9 Data Analysis 100
4.3 Results 101
4.3.1 GSH Mediated detoxification of HBQs in T24 cells 101
4.3.2 Effects of HBQs on the intracellular levels of free reduced GSH 102
4.3.3 Effects of HBQs on cellular glutathione S-transferase (GST) activity 102
4.3.4 Effects of HBQs on cellular glutathione peroxidase (GPx) activity 103
4.3.5 Correlation between cellular GSH level and GST activity, GPx activity or ROS production 103
4.4 Discussion 103
4.5 References 106

Chapter 5 Multidrug resistance proteins (MRPs) protect cells from the toxic effects of HBQs 123
5.1 Introduction 123
5.2 Materials and Methods 124
5.2.1 Materials and reagents 124
5.2.2 Cell lines and cell culture 125
5.2.3 Cytotoxicity testing 125
5.2.4 Measurement of HBQ-induced cellular ROS levels 126
5.2.5 Data analysis 126
5.3 Results and Discussion 127
5.3.1 MRP-mediated protection of cells from the cytotoxicity of HBQs 127
5.3.2 MRP4 reduced ROS generation induced by HBQ 127
5.3.3 The detoxification of HBQs by MRP4 is GSH-dependent 128
5.3.4 The protection of MRP4 is HBQ compound-dependent 130
5.4 Conclusion 131
5.5 References 133

Chapter 6 Activation of Nrf2/ARE pathway by HBQs 147
6.1 Introduction 147
6.2 Materials and Methods 148
6.2.1 Reagents 148
6.2.2 Cell culture 148
6.2.3 Cell viability 149
6.2.4 Reactive oxygen species (ROS) detection 149
6.2.5 RNA extraction and reverse transcription PCR (RT-PCR) 150
6.2.6 Real-time PCR for time-course gene expression 150
6.2.7 Taqman® Array for pathway gene expression analysis 151
6.2.8 Data analysis 151
6.3 Results and Discussion 152
6.3.1 Optimization of experimental conditions for the PCR array study 152
6.3.2 Overview of transcriptome changes induced by HBQs 153
6.3.3 Common genes with altered expression at both early and late time points: the 4-gene set 154
6.3.4 Other common genes with altered expression at 2 h exposure 156
6.3.5 Other common genes with altered expression at 8 h exposure 156
6.3.6 Differences of transcriptome changes between HBQ isomers 158
6.3.7 Identification of potential pathways 159
6.4 Conclusion 159
6.5 References 161

Chapter 7 Conclusions and Future Work 185
7.1 Introduction 185
7.2 Advancements in Knowledge 186
7.2.1 Determination of the cytotoxicity and oxidative damage induced by HBQs (Chapter 2) 186
7.2.2 Investigation of the structure–toxicity relationship of HBQs (Chapter 3) 186
7.2.3 Investigation of the involvement of cellular glutathione defense system in HBQ-induced cytotoxicity (Chapter 4) 187
7.2.4 Investigation of multidrug resistance protein (MRP)-mediated export of HBQs (Chapter 5) 188
7.2.5 Identification of Nrf2/ARE pathway activated by HBQs (Chapter 6) 189
7.3 Conclusions 189
7.4 Perspectives 190
7.5 Future work 191

7.6 References..... 193

Bibliography 197

List of Tables

Table 1.1 USEPA Stage 2 DBP Rule [14] 33
Table 1.2 Relevant Health Canada Guidelines for DBPs [15] 34
Table 1.3 Relevant World Health Organization Guidelines for DBPs [16] 35
Table 1.4 Structure and occurrence of HBQs in drinking water 36
Table 1.5 Comparison of chronic cytotoxicity of regulated DBPs and HBQs in CHO cells 38
Table 2.1 Characteristics of the halobenzoquinones analyzed in this chapter 53
Table 3.1 Chemical structure, molecular formula and purity of HBQs 82
Table 3.2 Physicochemical parameters of HBQs 84
Table 3.3 Comparative IC_{50} Values at 6, 12, and 24 h Post-exposure Determined Using RTCA for Individual HBQs in CHO-K1 Cells 86
Table 3.4 Linear regression formula and R^2 of ROS production at 6, 12, and 24 h post-exposure for individual HBQs in CHO-K1 cells 91
Table 3.5 Percentage of p53 protein over control after CHO-K1 cells were treated with each HBQ (24 h IC_{50}) at 12 h and 24 h post exposure. 92
Table 3.6 Pearson correlation coefficient (r) of physicochemical and toxicological parameters of eight HBQs 93
Table 3.7 Pearson correlation coefficient (r) of physicochemical and toxicological parameters of isomeric HBQs or halogen-substituted HBQs after 24 h exposure 95
Table 4.1 Percentage of T24 viable cells after exposure to varying concentrations of BSO for 12 h of pretreatment 110
Table 4.2 IC_{50} values of HBQs in the absence and presence of pretreatment with BSO 112
Table 4.3 Pearson correlation analysis of cellular GSH level with cellular GST activity, GPx activity and ROS production after 24h in HBQ-treated T24 cells 120
Table 5.1 IC_{50} values and the relative resistance of human MRP-transfected HEK293 cells to each HBQ after 24 h exposure. 141
Table 5.2 IC_{50} values and the relative resistance of GSH-depleted human MRP4-transfected HEK293 cells to each HBQ after 24 h exposure. 146
Table 6.1 Primer sequences used for real-time PCR 167
Table 6.2 A list of genes used for the TaqMan PCR array 168
Table 6.3 24-h IC_{20} values (μM ± SEM) for each HBQ in SV-HUC-1 cells 172

Table 6.4 Significant gene expression changes vs concurrent negative controls after 2 h HBQ exposure in SV-HUC-1 cells 176
Table 6.5 Significant gene expression changes vs concurrent negative controls after 8 h HBQ exposure in SV-HUC-1 cells 178
Table 6.6 Canonical Pathways after HBQ exposure 183

List of Figures

Figure 1.1 Schematic of disinfection byproduct formation during water treatment 33
Figure 1.2 The principle of real-time cell analysis 37
Figure 2.1. Effects of HBQs on the viability of T24 cells after 24 h exposure 54
Figure 2.2 Effects of HBQs with or without 1 mM NAC on the viability of T24 cells after 24 h exposure 55
Figure 2.3 Effects of HBQs with or without 1 mM NAC on the viability of T24 cells after 24 h exposure 56
Figure 2.4 Levels of intracellular ROS in T24 cells after 24 h exposure to 25, 50, 75, 100, 125, 150 μM of BQ, 2,6-DCBQ, DCMBQ, TriCBQ, and 2,6-DBBQ 57
Figure 2.5 Relative increase of 8-hydroxydeoxyguanosine (8-OHdG) in genomic DNA of T24 cells after the cells were treated with one of the four HBQs (25–125 μM)..... 58
Figure 2.6 Concentrations of protein carbonyl in T24 cells after incubation with one of the four HBQs for 24 h 59
Figure 2.7 Malondialdehyde (MDA) adducts of proteins in T24 cells after incubation with one of the HBQs for 24 h..... 60
Figure 3.1 RTCA continuous profiles of the normalized cell index (NCI) over time for CHO-K1 cells exposed to HBQs 85
Figure 3.2 IC_{50} histograms of CHO-K1 cells after exposure to eight HBQs. 87
Figure 3.3 Cellular ROS production in CHO-K1 cells with linear regressions at (A) 6, (B) 12, and (C) 24 h post-exposure to HBQs. 88
Figure 3.4 Cellular ROS production in CHO-K1 cells at different time points..... 89
Figure 3.5 Percentage of 8-OHdG production over control after CHO-K1 cells were treated with each HBQ (24 h IC_{50}) at 12 h and 24 h post exposure..... 90
Figure 3.6 Calculated vs experimental logIC_{50} values for CHO-K1 cells treated with HBQs for 24 h..... 94
Figure 4.1 Effects of BSO and HBQs on viability of T24 cells..... 111
Figure 4.2 Effects of exogenous GSH on HBQ-cytotoxicity in T24 cells..... 113
Figure 4.3 Effect of solvent control, 0.4% methanol (v/v), on the cellular glutathione levels in T24 cells..... 114
Figure 4.4 Effect of HBQs on the cellular glutathione levels in T24 cells 115

Figure 4.5 Effect of HBQ compounds on cellular glutathione S-transferase (GST) activity 116
Figure 4.6 Effect of methanol control on cellular glutathione S-transferase (GST) activity..... 117
Figure 4.7 Effect of 4 HBQs on cellular glutathione peroxidase (GPx) activity 118
Figure 4.8 Effect of methanol control on glutathione peroxidase (GPx) activity..... 119
Figure 4.9 Isotope pattern of conjugation products of HBQs with GSH..... 121
Figure 4.10 Possible protection pathway involved GSH against HBQ-cytotoxicity..... 122
Figure 5.1 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cell viability of HEK-MRP1 and HEK-V cells after 24 h exposure..... 137
Figure 5.2 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cell viability of HEK-MRP3 and HEK-V cells after 24 h exposure..... 138
Figure 5.3 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cell viability of HEK-MRP4 and HEK-V cells after 24 h exposure..... 139
Figure 5.4 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cell viability of HEK-MRP5 and HEK-V cells after 24 h exposure..... 140
Figure 5.5 The fluorescence level of DCF in negative control cells of both HEK-MRP4 cells and HEK-V cells after 24 h of incubation 142
Figure 5.6 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cellular ROS levels of HEK-MRP4 and HEK-V cells after 24 h exposure..... 143
Figure 5.7 Cell viability of the negative control (NC) and control cells treated with 1 mM BSO after 24 h culture of (A) HEK-V and (B) HEK-MRP4 cells 144
Figure 5.8 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cell viability of GSH-depleted HEK-MRP4 and HEK-V cells after 24 h exposure..... 145
Figure 6.1 The 96-well format of the Taqman PCR Array..... 167
Figure 6.2 Cytotoxicity response profiles of the normalized cell index (CI) over time for SV-HUC-1 cells exposed to HBQs 171
Figure 6.3 Cytotoxicity response profiles of the normalized cell index (NCI) over time for SV-HUC-1 cells exposed to HBQ with the addition of 1 mM N-acetyl-L-cysteine (NAC)..... 172
Figure 6.4 IC_{50} values of HBQs with or without 1 mM NAC in SV-HUC-1 cells after 24 h, 48 h, and 72 h exposure 173
Figure 6.5 Time-course of ROS generation in SV-HUC-1 cells after each HBQ treatment at their respective IC_{20} values 174

Figure 6.6 Time-course of HBQ-induced (A) Nrf2 and (B) NQO1 gene response in SV-HUC-1 cells 175
Figure 6.7 Commonly shared genes by the 6 HBQs at 2 h and/or 8 h exposure times..... 180
Figure 6.8 Identification of 4 genes that were specifically altered by the 6 tested HBQs in SV-HUC-1 cells 181
Figure 6.9 The comparison of significantly altered genes between HBQ isomers in SV-HUC-1 cells 182
Figure 6.10 The typical Nrf2-mediated oxidative stress response pathway after HBQ treatment at 2 h or 8 h 184

List of Abbreviations

1,4-BQ	1,4-benzoquinone (also p-BQ)
2-CBQ	2-chloro-1,4-benzoquinone
2,3-DIBQ	2,3-diiodo-1,4-benzoquinone
2,5-DBBQ	2,5-dibromo-1,4-benzoquinone
2,5-DCBQ	2,5-dichloro-1,4-benzoquinone
2,6-DBBQ	2,6-dibromo-1,4-benzoquinone
2,6-DCBQ	2,6-dichloro-1,4-benzoquinone
4-HNE	4-hydroxyl-2-nonenal
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
8-OHdG	8-hydroxydeoxyguanosine
ABC	ATP-binding cassette
AP	apurinic/apyrimidinic
ARE	antioxidant response element
BAECs	bovine aortic endothelial cells
BER	base excision repair
BQ	benzoquinone
BSA	bovine serum albumin
BSO	buthionine sulfoximine
CAT	catalase
C-DBPs	only carbon-containing DBPs
CDKN1A	cyclin-dependent kinase inhibitor 1
CDNB	1-chloro-2,4-dinitrobenzene
CI	cell index
CHO	Chinese hamster ovary
COX-2	cyclooxygenase-2
Ct	cycle threshold
DBPs	disinfection byproducts
DCF	dichlorofluorescein
DCFH-DA	2′,7′-dichlorofluorescein diacetate

DCMBQ	2,6-dichloro-3-methyl-1,4-benzoquinone
dG	deoxyguanosine
DH-LA	dihydrolipoic acid
DMDBBQ	2,3-dibromo-5,6-dimethyl-1,4-benzoquinone
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
E_{HOMO}	energy of the highest occupied molecular orbital
E_{LUMO},	the lowest unoccupied molecular orbital energy
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCS	glutamate cysteine synthetase
GPx	glutathione peroxidases
GS	glutathione synthetase
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione S-transferase
GUSB	β-glucuronidase
HAA	haloacetic acid
HBQs	halobenzoquinones
HBTs	halobenzenetriols
HEK	human embryonic kidney
HMOX1	heme oxygenase-1 gene
HO-1	heme oxygenase 1
H_2O_2	hydrogen peroxide
HPRT1	hypoxanthine phosphoribosyltransferase 1
HQs	halogenated quinones
IAA	iodoacetic acid
IARC	International Agency for Research on Cancer
IC_{20}	the concentration of HBQs causing a 20% decrease in overall cell viability

IC_{50}	half-inhibitory concentrations, the concentration of HBQs causing a 50% decrease in overall cell viability
IPA	Ingenuity Pathway Analysis
LDH	lactate dehydrogenase
LOAELs	chronic lowest observed adverse effect levels
MDA	malondialdehyde
MN	micronuclei
MOA	mechanism of action
MPA	metaphosphoric acid
MRPs	multidrug resistance proteins
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium;
MX	3-chloro-4-(dichloromethyl)-5-hydroxy-5*H*-furan-2-one
MYH	MutY homologue
NAC	N-acetyl-L-cysteine
NCI	normalized cell index
N-DBPs	nitrogen-containing DBPs
NDMA	N-nitrosodimethylamine
NDPhA	Nnitrosodiphenylamine
NOM	natural organic matter
NOX	NADPH oxidases
NPip	N-nitrosopiperidine
NPyr	N-nitrosopyrrolidine
NQO	NADPH-quinone oxidoreductase
Nrf2	nuclear factor erythroid 2–related factor 2
NRU	neutral red uptake
OH-HBQs	halo-hydroxyl-benzoquinones
OGG1	8-oxoguanine glycosylase
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction

PGH2	prostaglandin H2
PGs	prostaglandins
PRDX	peroxiredoxin
PTGS2	prostaglandin-endoperoxide synthase 2
QSTR	quantitative structure–toxicity relationship
R	refractivity
ROS	reactive oxygen species
RR	relative resistance
RTCA	real-time cell analysis
S	molar surface area
SCE	sister chromatid exchanges
SCGE	single-cell gel electrophoresis
SD	standard deviation
SEM	standard error of means
SOD	superoxide dismutase
SRXN	sulfiredoxin
THM	trihalomethane
TCBQ	tetrachloro-l,4-benzoquinone
TCHQ	tetrachlorohydroquinone
TFBQ	tetrafluoro-1,4-benzoquinone
TOC	total organic carbon
ToF	time-of-flight
TOX	total organic halides
TriCBQ	2,3,6-trichloro-1,4-benzoquinone
Trx	thioredoxin
USEPA	U.S. Environmental Protection Agency
UV	ultralight
WHO	World Health Organization
μ	dipole moment

Chapter 1 Introduction[1]

1.1 Disinfection byproducts (DBPs)

1.1.1 Importance of drinking water disinfection

Drinking water disinfection is considered a significant milestone in public health history. Chlorine was first used to disinfect water in Europe in the late 1800s and has since provided an effective way to eliminate waterborne microorganisms.[1] Without disinfectants, contaminated drinking water can transport bacteria, viruses, parasites, and protozoa, which can lead to infection, resulting in disease, such as typhoid, hepatitis A, cholera,[2] and even death.[3-4] Additional benefits of disinfection include the removal of offensive taste and odor of water,[5] and prevention of algal growth in sedimentation basins and filters.[6] Today, adding disinfectants to drinking water is a basic but essential step in water treatment systems.[2] Despite the efficacy of disinfection, the 2015 estimate by the World Health Organization (WHO) showed over 600 million people still lack access to safe and clean drinking water.[7] Nearly one million deaths per year are attributable to unsafe drinking water and lack of appropriate sanitation.[7] Given the acute risk posed by pathogenic microorganisms, disinfection of drinking water is absolutely necessary to protect public health.

1.1.2 History and regulation of DBPs

Drinking water disinfection byproducts (DBPs) form unintentionally as a result of reactions between disinfectants (chlorine, chloramines, ozone, chlorine dioxide, ultraviolet or combination of these) and natural organic matter (NOM) (Figure 1.1), or other organic contaminants present in raw water.[8]

The trihalomethanes (THMs), chloroform, bromoform, dichlorobromomethane, and dibromochloromethane, were the first DBPs identified in chlorinated drinking water by Rook[9] and Bellar[10] in 1974. In 1976, the U.S. Environmental Protection Agency (USEPA) found that

[1] A version of Section 1.2 has been published as: Jinhua Li, Wei Wang, Birget Moe, Hailin Wang, and Xing-Fang Li. Chemical and toxicological characterization of halobenzoquinones, an emerging class of disinfection byproducts. Chem. Res. Toxicol. 2015, 28(3): 306-318.

THMs were widespread in chlorinated drinking water.[11] In the same year, the link between chloroform and cancer in laboratory animals was reported by the U.S. National Cancer Institute.[12] This led USEPA to set regulations for THMs in drinking water in 1979, which were extended by the Stage 1 Disinfectants/Disinfection By-Products Rule in 1998[13] and the Stage 2 Rule in 2006.[14] USEPA regulates two primary chemical classes of DBPs, THMs and haloacetic acids (HAAs), along with bromate and chlorite. The maximum contaminant levels set for these DBPs are shown in Table 1.1. Similarly, Health Canada has set maximum acceptable concentration guidelines for the same chemical classes of DBPs and N-nitrosodimethylamine (NDMA) (Table 1.2), [15] and WHO has established guideline values for 14 individual DBPs (Table 1.3).[16]

In addition to the regulated DBPs, emerging DBPs are continually discovered with the advancement of analytical techniques. To date, more than 600 (mostly halogenated) DBPs have been identified.[17-19] In recent years, new brominated, iodinated, and nitrogen-containing DBPs (N-DBPs) have been discovered.[8] However, based on the analysis of total organic carbon (TOC) and total organic halide (TOX), the reported DBPs only account for a small fraction (about 30%) of the total halogenated organic materials isolated after disinfection.[20] This suggests that the majority of DBPs in disinfected water remain to be identified.

1.1.3 Health concerns from epidemiological studies

The effects of long-term, low-level exposure to DBPs in disinfected water are unknown. Humans are exposed to DBPs through multiple routes: ingestion; inhalation of volatilized and aerosolized DBPs; and dermal absorption of DBPs during showering, bathing, and swimming.[21] Evaluating the effects of DBPs on humans is difficult due to these confounding exposure scenarios.

Concerns about the long-term health effects of DBPs have prompted a number of epidemiological studies to examine the association between chronic consumption of chlorinated drinking water and certain adverse health effects, including bladder cancer,[22-25] colorectal cancer,[26-27] and adverse reproductive outcomes.[28-31] Several review articles and book by Hrudey evaluated the strength of evidence and limitations for each health outcome.[32-34] To date, urinary bladder cancer risk with prolonged exposure to chlorinated water exhibited the greatest consistency.[14] Previous studies (1974–2004) have mostly focused on several case-control studies, and report the odds ratio above 1, indicating a strong association between consumption of

chlorinated drinking water and bladder cancer. Recent studies (2004–2014) have conducted meta-analyses to increase the power of the already published studies; the results support the association between consumption of chlorinated drinking water and bladder cancer. However, additional studies are required to improve the understanding of exposure response relationships.

1.1.4 Issues in DBP research

One of the major issues in DBP research is that the regulated DBPs (THMs and HAAs) cannot account for the observed bladder cancer risk based on the results of animal carcinogenicity tests.[35-36] First, animal studies for regulated DBPs identified the liver as the target organ, which contradicts epidemiological studies that identify the bladder. Moreover, the magnitude of risk from the carcinogenic activity found in animal studies for regulated DBPs is significantly lower than what has been estimated from epidemiological studies. Therefore, recent efforts have been made to identify new DBPs present in drinking water that may have the toxicological significance to contribute to the cancer risk. More toxicological data of DBPs are needed to elucidate inconsistencies between epidemiological studies and carcinogenicity studies.

Although more than 600 DBPs have been discovered in drinking water, only a small number (<90) have been assessed for toxicity.[36] The majority of DBPs remain unknown.[18, 37] It is an analytical challenge to devise advanced techniques to discover new species. Furthermore, the use of alternative disinfectants (chloramines, ozone, chlorine dioxide, UV) instead of chlorine will result in the formation of different classes of DBPs, even more toxic DBPs.[36] Determining the toxicity data of so many DBPs poses a big challenge, both money- and time-consuming. Therefore, developing a high-throughput assay to screen DBPs for high toxicity is needed.

1.1.5 Toxicological evaluation of DBPs

1.1.5.1 *In vitro* studies

Most of the identified DBPs have little to no toxicological data available. Due to the vast number of DBPs identified in water, it is not feasible to perform individual *in vivo* studies. *In vitro* toxicological studies have been conducted on several DBPs as a money- and time-saving alternative to *in vivo* analysis. These *in vitro* studies could provide insight and direction for future *in vivo* work.

A comprehensive review article regarding DBP toxicities was published in 2007.[36] It discussed the available toxicological data on the genotoxicity and carcinogenicity of 85 DBPs: 74 emerging DBPs and 11 DBPs regulated by USEPA. These DBPs have different cytotoxic, genotoxic, and mutagenic effects. In general, for both cytotoxicity and genotoxicity of halogenated DBPs, iodinated DBPs were the most toxic, followed by brominated DBPs, then chlorinated DBPs. Nitrogen-containing DBPs (N-DBPs) appear to be more cytotoxic and genotoxic than only carbon-containing DBPs (C-DBPs). However, there are gaps in the toxicological data of DBPs, and different methods have been used to evaluate the toxicity of different DBP species.

The most commonly used cell line for cytotoxicity analysis of DBPs are immortalized Chinese hamster ovary (CHO) cells. The Plewa team contributed significant effort to generate quantitative and comparative *in vitro* mammalian cytotoxicity data of DBPs, and provided a rank ordering of relative toxicities. The CHO cell chronic cytotoxicity assay measures the reduction in cell density as a function of DBP concentration over a period of approximately three cell divisions (72 h). The %C1⁄2 values generated are analogous to an IC_{50} value, and were used to rank the toxicity order.[38-39]

To date, six DBP chemical classes have been analyzed using this method: halomethanes, HAAs, haloacids, haloacetonitriles, haloacetamides, and haloacetaldehydes.[39-46] Additionally, this method has been used to examine disinfected water from swimming pools and hot tubs,[47] disinfected water containing iodinated X-ray contrast media,[48] X-ray contrast pharmaceutical iopamidol chlorination DBPs,[49] and organic fractions of water disinfected with or without chlorine and UV light.[50] In these studies, the DBPs or DBP mixture demonstrated varying cytotoxic effects to CHO cells. Since DBP cytotoxicity testing is routinely conducted with CHO cells, other emerging unregulated DBPs could be tested with this cell line to rank their relative toxicity. However, very few studies used human bladder cells for DBP cytotoxicity testing. Our research group found that selected nitrosamine DBPs were cytotoxic to CHO cells, as well as to human liver, bladder and lung cancer cells.[51] The human bladder cancer cell line T24 has only been used to profile the cytotoxicity of phenazine and nitrosamine DBPs.[51-53] As the target organ in epidemiological studies, there is a need to study cytotoxicity using bladder cells.

The mutagenicity of DBPs has been mostly examined using the *Salmonella typhimurium* histidine reverse mutation assay, known as the Ames assay. Another two mutagenicity

experiments used were the *E.coli* tryptophan reverse mutation assay and the SOS/umu test. [54-58] A standard methodology is needed to directly compare and rank-order the mutagenicity. In general, three of the four regulated THMs were mutagenic except for chloroform, and all the five regulated HAAs were mutagenic in *Salmonella*.[36] Brominated HAAs demonstrated more mutagenic potential compared to chlorinated HAAs.[59] For unregulated DBPs, some chemical classes have been shown to be mutagenic in *Salmonella*, including four nitrosamines, three dihalomethanes, halofuranones, and haloacetonitriles, among others. [36, 60-61] The mutagenic potential of DBP mixtures from disinfected drinking water has also been evaluated. The organic extracts or concentrates of water prepared from alternative disinfection methods (ozone, chloramine, chlorine dioxide) were less mutagenic in *Salmonella* than those from chlorinated water.[62-64] However, mutagenicity increased when these alternative disinfection methods underwent post-treatment with chlorine.[36] This indicated that future research could compare the toxicity before and after water treatment and attention should be paid to the methods used for disinfection.

Genotoxicity is another major research direction regarding the *in vitro* toxicity of DBPs. Of the several methods used to evaluate DBP genotoxicity, the comet assay (also called single-cell gel electrophoresis (SCGE) assay) is most commonly used followed by the micronucleus [65-68] and chromosome aberration[69-70] assays. The Plewa team also worked to generate quantitative and comparative genotoxicity data for DBPs. To date, Plewa's team has examined the genotoxicity of 47 individual DBPs from six classes using the SCGE assay.[36, 71] They evaluated the acute genotoxicity in CHO cells, and rank the relative order of genotoxicity for these DBPs. Most of the tested DBPs (75.8%) induced significantly increased levels of genomic DNA damage. Iodoacetic acid was the most genotoxic DBP investigated. N-DBPs were more genotoxic than C-DBPs. Iodinated DBPs were the most genotoxic, followed by their brominated and chlorinated analogues. While this data would be helpful to evaluate the genotoxicity of new DBPs, many known DBPs have not been tested. Additionally, the concentration used in these *in vitro* studies may not relevant to the DBP levels of environmental exposure.

1.1.5.2 *In vivo* studies

In vivo animal carcinogenicity studies could provide the most powerful and direct evidence of the carcinogenic potential of DBPs. Ten of the 11 USEPA regulated DBPs (except for

bromoacetic acid) have been tested using two-year rodent carcinogenicity assays through gavage of drinking water or in combination with inhalation.[36, 72-76] Nine of them (excluding chloroacetic acid) demonstrated evidence of carcinogenicity. However, as mentioned above, the major tumor sites from these assays are liver, kidney, and intestine, instead of the bladder, identified from epidemiological findings. Only a few unregulated DBPs have been evaluated using two-year rodent carcinogenicity studies.[36] Among them, just four unregulated DBPs demonstrated some evidence of the level of carcinogenicity, including formaldehyde, acetaldehyde, 3-chloro-4-(dichloromethyl)-5-hydroxy-5*H*-furan-2-one (MX), and the nitrosamines. Again, the liver was found to be the major tumor site for these tested unregulated DBPs.

Taken together, the International Agency for Research on Cancer (IARC) has only identified a few DBPs as carcinogens.[77] Formaldehyde is considered as a class 1 carcinogen, carcinogenic to humans; NDMA is a class 2A carcinogen, probably carcinogenic to humans; bromodichloromethane, bromate, dichloroacetic acid, acetaldehyde, dibromoacetonitrile and MX are class 2B carcinogens, possibly carcinogenic to humans. More carcinogenicity studies for either single DBPs or water samples are required to better understand the bladder cancer risk from epidemiological studies. Evaluating the carcinogenic potential of those DBPs that have already demonstrated highly toxic effects in the *in vitro* studies is a high priority.

1.1.5.3 Toxicological pathways

Although some of the DBPs have been evaluated with either *in vitro*, *in vivo*, or both toxicity studies, there is still a need for high-throughput toxicity tests that may predict the potential toxic mechanism of action (MOA). The mechanisms through which DBPs may cause cancer have not been clarified, although genotoxicity and oxidative stress have been suggested.[78]

Identify critical toxicity pathway perturbations using new toxicity testing tools and technologies for the next generation of risk assessment is recommended.[79] Toxicogenomics is a new tool that offers insight into discovering such molecular mechanisms and toxicity pathways. Toxicogenomics is defined as "the study of the relationship between the structure and activity of the genome and the adverse biological effects of exogenous agents".[80] With the development of microarray technology, it is possible to study multiple pathways and mechanisms simultaneously, although quantitative studies are needed to validate data. Toxicogenomics has been applied to some studies to elucidate mechanisms of toxicity and identify gene expression patterns of DBPs.

Three earlier toxicogenomics studies used high levels of DBP exposure in rodent models by microarray. The evaluated DBPs were dichloroacetic acid,[81] bromochloroacetic acid,[82] and potassium bromate.[83] Three major pathways were involved: cancer induction, cell death, and oxidative stress. However, most of the identified genes were not quantitatively confirmed by real-time polymerase chain reaction (real-time PCR) analysis.

More recently, additional cell lines have been used to evaluate the toxicogenomics of DBP exposure. A human tumor cell line, lymphoblastoid TK6 cells, was used to profile gene expression after potassium bromate exposure. The results demonstrated that the genes altered by bromate were mainly related to DNA repair and cell cycle.[84] The toxicogenomic analysis of MX-treated *Salmonella* revealed that MX induced changes in the expression of genes involved in DNA-damage response, membrane transport, and porphyrin metabolism.[85] Using the ARE-luciferase reporter and real-time PCR, organic contaminant (OC) extracts from drinking water demonstrated the ability to activate nuclear factor E2-related factor 2 (Nrf2)-mediated antioxidant response in HepG2 cells, a human liver cancer cell line.[86]

The toxicogenomics of monoacetic acids (iodoacetic, bromoacetic, and chloroacetic acids) was evaluated using a normal non-transformed human cell line, human small intestine epithelial cells FHs 74 Int. Quantitative PCR array results demonstrated that the transcriptome profiles altered were mainly associated with genes responding to DNA damage/repair, cell cycle regulation, apoptosis, oxidative stress, inflammation, and cancer. [87-89] Using two beta-lactamase-based reporter gene assays, monoacetic acids were found to activate two adaptive stress response pathways, Nrf2 and p53.[90] Similarity, iodoacetic acid was able to activate Nrf2-mediated antioxidant response both in HepG2 cells and in rats using the ARE-luciferase reporter and qRT-PCR.[91]

A comprehensive study was performed to explore the reactive toxicity pathway fingerprint of 50 DBPs. Reporter gene assays were used to indicate the adaptive stress responses to genotoxicity, oxidative stress, and inflammation. The results demonstrated that 98% of tested DBPs could activate adaptive stress responses to oxidative stress and 68% could activate adaptive stress responses to genotoxicity.[92]

Since only a few DBPs were studied with toxicogenomics, further research is required to examine more DBPs. These works could lead to the discovery of biomarkers, specific toxicity pathways, and potential molecular mechanisms, related to DBP exposure. In addition, lower

concentrations should be applied in these molecular experiments, more comparable to concentration in real waters. More confirmatory experiments are needed to validate the findings in these microarray experiments.

1.2 Halobenzoquinones (HBQs)

1.2.1 Quantitative structure–toxicity relationship analysis

The interest to confirm halobenzoquinones (HBQs) as DBPs resulted from a quantitative structure–toxicity relationship (QSTR) analysis. Since the inconsistent results from epidemiological studies and animal carcinogenicity studies, recent efforts have been made to identify new DBPs that may have the toxicological significance to contribute to the cancer risk and be present in drinking water. Therefore, QSTR analysis has been used to predict DBPs that are plausible carcinogens using TOPKAT software.[93] The first step 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, mutagenic and carcinogenic effects, and assessing structure–activity relationships from the databases of the U.S. National Toxicology Program and the U.S. Food and Drug Administration. The results of QSTR analysis predicted five classes of putative DBPs as 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, 2,6-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, 2,6-DBBQ) mg/kg body weight per day, with both DCMBQ and TriCBQ predicted to be bladder carcinogens.[93]

1.2.2 Occurrence of HBQs as DBPs

The occurrence of HBQs in treated drinking water was first identified in 2010.[94-95] 2,6-DCBQ, DCMBQ, TriCBQ, and 2,6-DBBQ (Table 1.4) were determined at ng/L levels in treated water collected from nine water treatment plants and distribution systems. In the drinking water

treatment plants and distribution systems studied, 2,6-DCBQ occurred with 100% frequency, followed by 2,6-DBBQ with 72%, and TriCBQ and DCMBQ around 10%. The concentration of HBQs in the treated water ranged from 0.5–275 ng/L, but none of the HBQs were detected in raw water or in the blanks. These results confirmed HBQs as DBPs with high occurrence frequency, highlighting the need for further research.

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, 2,6-DCBQ again showed a 100% occurrence frequency and the highest median concentration (23.0 ng/L) of the tested HBQs. The concentrations of 2,6-DCBQ were above 100 ng/L in five of the 16 samples with the highest at 274.5 ng/L. 2,6-DBBQ was detected in 11 samples at concentrations up to 37.9 ng/L. DCMBQ and TriCBQ were also detected in six and three samples, respectively, at concentrations lower than 10 ng/L. Brominated HBQs, 2,6-DBBQ and 2,6-dibromo-1,4-hydroquinone, were also identified as DBPs in simulated drinking water by Zhang's group in Hong Kong using their developed mass spectrometry methods in 2011[96] and 2014.[97]

HBQs have also been identified in treated swimming pool waters.[98] Analysis of HBQs in 10 swimming pools and their input tap water showed that 2,6-DCBQ was widely present at concentrations of 19–299 ng/L. The concentration of 2,6-DCBQ in the swimming pools was as much as 100 times higher than its concentration in the input tap water. TriCBQ and 2,6-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 (DMDBBQ), was identified as a DBP in two swimming pool waters at concentrations lower than 1 ng/L, which likely originated from personal care products such as sunscreen or lotion.[98]

1.2.3 HBQ-induced cytotoxicity in CHO cells

Research concerning HBQs in the past five years has demonstrated that they are commonly occurring DBPs in disinfected water. However, there is little experimental data available on the toxicity of HBQs as DBPs.

The Li group has developed DBP cytotoxicity testing methods using the label-free technology, real-time cell analysis (RTCA).[51-52, 99] The principle and environmental application of RTCA are described in a recent review.[100] 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 cell index (CI) (Figure 1.2). Exposure of cells to DBPs may result in toxic effects, resulting in lower CI values compared to 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 mM, 0.59 mM, 4.7 mM, 14 mM,[51] and 0.279 mM,[52] respectively, in T24 cells.

To better understand the relative cytotoxicity of HBQs in comparison to regulated DBPs, the IC_{50} values of these DBPs were compared in the immortalized Chinese hamster ovary (CHO) cell line. Table 1.5 shows the 72 h-IC_{50} values 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 3.96–11.5 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 0.01–0.07 mM for the HBQs.[39, 43, 51, 71] The IC_{50} values show a wide variation in cytotoxicity of 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,[101] while the values for the remaining DBPs were determined using a dye-based assay developed by the Plewa group specifically for DBP testing.[39] This method is a microplate cytotoxicity assay which uses crystal violet dye to stain cell nuclei.[102] Nevertheless, based on 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 μM levels. This is consistent with a recent study from the Zhang group, which evaluated the developmental toxicity of 20 halogenated DBPs in sewage effluent using the marine polychaete *Platynereis dumerilii*. Here, it was demonstrated that 2,5-dibromohydroquinone induced the highest developmental toxicity amongst 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.[103]

1.3 Toxicological evaluation of quinone compounds

1.3.1 Benzoquinone toxicity

QSTR analysis relies heavily on structure-activity relationships. For the HBQs, the analysis incorporated toxicity information known about 1,4-benzoquinone (1,4-BQ or *p*-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 1.4. 1,4-BQ is a highly reactive metabolite of benzene, which is a recognized human carcinogen. The toxicity of benzene is attributed to its highly reactive metabolites, 1,4-BQ.[104] 1,4-BQ can cause a series of adverse effects either *in vivo* or *in vitro*, including sister chromatid exchanges (SCE) and micronuclei (MN) formation in V79 cells,[105] and induction of DNA single-strand breaks in human lymphocytes.[106] The mechanisms underlying 1,4-BQ-induced genotoxicity and carcinogenicity involve two major molecular pathways.[107-108] 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 proposed major pathway.

1.3.2 Halogenated quinone (HQ)-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 1.4). 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 because of redox cycling.[107-108] In biological systems, quinones can undergo either one-electron reduction or two-electron reduction.[109] 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.

Halogenated quinones (HQs) can also produce ROS and induce oxidative damage at cellular and molecular levels. The halogen-substitutions of quinones increase their ability to generate free radicals and likely induce more oxidative damage, resulting in higher toxicity. The ability of HQs to produce ROS in solution has been investigated by Zhu and colleagues. Using an electron spin resonance secondary spin-trapping method,[110-112] the Zhu group has demonstrated that these HQs can generate free radicals with H_2O_2 via metal independent reactions. When (terachloro-1,4-benzoquinone) TCBQ or other HQs 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.[112-113] In addition to hydroxyl radicals, HQs also produced alkoxyl radicals and quinone ketoxyl radicals in solution through decomposition of hydroperoxides, independent of transition metal ions.[114-115] The Zhu group also observed potential oxidative damage to lipids, as HQs could form the reactive lipid radicals pentyl and 7-carboxyheptyl, and reactive product 4-hydroxyl-2-nonenal (4-HNE) in solutions through the decomposition of lipid hydroperoxide 13-HPODE without the catalysis of transition metal ions.[116]

1.3.3 Effects of HQs 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 non-enzyme antioxidant that is present in cells at concentrations as high as millimolar levels.[117] The radical scavenger GSH and the antioxidant enzymes together constitute the first frontier 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.

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 to become more water-soluble and more easily excreted. The decreases in cellular GSH levels were observed in studies where rat hepatocytes and PC12 cells were exposed to TCBQ and 2,5-

dichloro-1,4-benzoquinone, and where HepG2 cells were exposed to PCB quinones.[118-119] When the PCB quinones were co-treated 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 form in cells.[118, 120] The decreased levels of cellular GSH may be due to conjugation of HBQs with GSH. This mechanism of conjugation has been previously reported in a study by Song and colleagues.[121] Chlorinated quinones can undergo non-enzymatic dechlorination upon reaction with GSH; for example, incubation of TCBQ with excess GSH formed its $(GS)_4$-Q adduct.[121] 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.

Since GSH likely plays an important role in the protection of cells from HQs, it is also important to clarify whether HQs induce some concomitant changes in GSH-related antioxidant enzymes. Glutathione S-transferases (GST) are a family of enzymes which catalyze the conjugation of GSH with electrophilic substrates,[122] while glutathione peroxidases (GPx) are responsible for the metabolism of hydrogen peroxide or hydroperoxides.[123] The results in 1,4-BQ-treated MCF7 cells showed the mRNA gene expression level of GST was enhanced while the mRNA gene expression level of GPx did not show modulation.[124] 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 the nuclear factor erythroid 2–related factor 2 (Nrf2), which is the principal regulator of the antioxidant response element (ARE) located in the genes of antioxidant enzymes.[125-127] In contrast, TCBQ is a very effective inhibitor of rat and human GST, as observed in incubation studies of purified GST and TCBQ.[128-129] 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. [118] This was accompanied by a significant up-regulation of 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.[130]

1.3.4 HQ-induced effects on DNA

According to the results of QSTR analysis, none of the HBQs were predicted as potential mutagens, although DCMBQ and TriCBQ were predicted as potential carcinogens.[93, 131] There is very limited data on HBQ mutagenic, genotoxic, and carcinogenic effects. 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.[107-108, 132] Halogen substitution on quinones may also have effects 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.[109, 131] Some evidence has suggested that halogenated quinones are also capable of inducing mutagenic and genotoxic effects.

1.3.4.1 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.[133] 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 (> 85%) being single base mutations.[134] The Ames assay (or the bacterial reverse mutation assay) is a standard method used for detecting the mutagenic potential of testing compounds.[135] Based on the Ames tests, 2,6-DCBQ, DCMBQ, TriCBQ, and 2,6-DBBQ appear to be mutagenic.[136] However, further studies are required to understand the underlying mechanisms of HBQ-induced mutagenic effects.

1.3.4.2 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 < 2,6-DCBQ << 2,6-DBBQ. Binding was thought to occur through a non-covalent mechanism characterized by H-bonding and partial intercalation.[137] Bromobenzoquinone interacted with oligonucleotides in the formation of bromide oligonucleotides, and the interaction depended on the extent of bromination of 1,4-BQ.[138]

The adduct of deoxyguanosine (dG) and TCBQ was found to be a dichlorobenzoquinone nucleoside in solution using LC-MS.[139] When exposed to nucleosides and calf thymus DNA, TCBQ preferentially formed adducts with dG, but would also form adducts with deoxycytidine and deoxythymidine.[140] 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), while co-exposure of the HBQs with H_2O_2 revealed that TFBQ also generates hydroxyl radicals that damage DNA bases.[141] The formation of direct adducts has been observed in TCBQ- or TCHQ- treated calf thymus DNA, human Hela S3 cells, and rats.[142-144]

1.3.4.3 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.[145] Among them, 8-OHdG is a well-accepted index of oxidative DNA damage when biological systems are exposed to xenobiotics.[146] 8-OHdG, as a DNA adduct, is formed due to the hydroxylation of the guanine base residues in DNA. The formation of 8-OHdG was also observed after V79 Chinese hamster lung cell exposure to TCBQ,[147] and after human HeLa S3 tumor cell exposure to TCBQ. [144]

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,[147] in 1,4-BQ- and hydroquinone-treated CHO cells,[148] and in HepG2 cells upon exposure to PCB quinones.[149] The underlying pathway of 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 co-incubated with H_2O_2 in solution.[150]

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.[144] 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.[151-152]

1.3.4.4 Other DNA effects

HQs are able to induce the formation of 5-hydroxymethylcytosine (5hmC) from 5-methylcytosine (5mC) genome-wide in cultured human cells by stimulating the Tet-dependent

oxidative conversion of 5mC to 5hmC.[153] 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.[154] These findings have led to a newly 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 micronucleus and chromosome aberrations in CHO cells.[155] 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.

1.4. Rationale and objectives of thesis

Evidence accumulated over a century has proven chlorination of drinking water is the most successful public health practice for prevention of waterborne diseases. Disinfection kills pathogens, but at the same time it generates a large number of DBPs. Epidemiological studies have consistently linked the consumption of chlorinated water with an increased risk of developing bladder cancer. The regulated DBPs, 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. 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, to address DBP health concerns, identification of DBPs of health relevance becomes an important research target.

My thesis 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; (2) available toxicological results of benzoquinones (BQs) indicate that HBQs are likely to be potent carcinogens; (3) recent studies provide evidence of wide occurrence and toxicological importance of HBQs in drinking water; and (4) there is little existing toxicological data on HBQs. Therefore, I propose to determine the cytotoxicity of HBQ DBPs and to investigate the possible mechanisms of toxicity of HBQs in human cells. Because CHO cells have been commonly used to test the cytotoxicity of the regulated DBPs, I will test the cytotoxicity of HBQs in CHO cells to compare the cytotoxicity of HBQs with other DBPs. In addition, T24 (a human urinary

bladder epithelial carcinoma cell line) and SV-HUC-1 (a normal human uroepithelial cell line), are chosen as a model to represent potential target organs of DBPs and demonstrate cell-specific effects. Specifically, I will aim:

1) To investigate the cytotoxicity and oxidative damage induced by HBQs in T24 cells;

2) To investigate the structure–toxicity relationship of HBQs in CHO-K1 cells;

3) To investigate the involvement of the cellular glutathione defense system in HBQ cytotoxicity;

4) To investigate the roles of multidrug resistance proteins (MRPs) in protecting cells from HBQ cytotoxicity;

5) To identify toxicity response genes and toxicity pathways influenced by HBQs in SV-HUC-1 cells.

The research outcomes will provide new *in vitro* assays and new toxicological results that will contribute to the knowledge of cytotoxic effects and the mechanisms of potential toxic effects of HBQs in *in vitro* systems. These results are relevant to better understanding of the potential health effects of human exposure to HBQ DBPs. Protection of public health is the ultimate goal, to which this research will contribute.

1.5 References

1. White, G. C., *Handbook of Chlorination; for Potable Water, Wastewater, Cooling Water, Industrial Processes, and Swimming Pools*. Van Nostrand Reinhold Co.: New York,, 1972.
2. Akin, E. W.; Hoff, J. C.; Lippy, E. C., Waterborne outbreak control - which disinfectant. *Environ. Health. Perspect.* **1982,** *46*, 7-12.
3. Bull, R. J.; Birnbaum, L. S.; Cantor, K. P.; Rose, J. B.; Butterworth, B. E.; Pegram, R.; Tuomisto, J., Water chlorination: essential process or cancer hazard? *Fund. Appl. Toxicol.* **1995,** *28* (2), 155-166.
4. Centers for Disease Control and Prevention (CDC). Morbidity and Mortality Weekly Reports. **1999**, 48(29): 621-629.
5. Lalezary, S.; Pirbazari, M.; Mcguire, M. J., Oxidation of 5 earthy musty taste and odor compounds. *J. Am. Water Works Assoc.* **1986,** *78* (3), 62-69.
6. American Water Works Association (AWWA), *Water Quality and Treatment: a handbook of community water supplies*. 4th ed.; McGraw-Hill: New York, 1990.
7. World Health Organization (WHO), UNICEF., *Progress on Sanitation and Drinking Water: 2015 update and MDG assessment*. New York, Geneva, 2015.
8. Richardson, S. D.; Ternes, T. A., Water analysis: emerging contaminants and current issues. *Anal. Chem.* **2014**, 86 (6), 2813-48.
9. Rook, J. J., Formation of haloforms during chlorination of natural waters. *Water Treat. Exam.* **1974,** *23*, 234-243.
10. Bellar, T. A.; Lichtenberg, J. J.; Kroner, R. C., Occurrence of organohalides in chlorinated drinking waters. *J. Am. Water Works Assoc.* **1974,** *66* (12), 703-706.
11. Kopfler, F. C.; Melton, R. G.; Lingg, R. D.; Coleman, W. E., GC/MS determination of volatiles for the national organics reconnaissance survey (NORS) of drinking water, In: *Identification and Analysis of Organic Pollutants in Water*, Keith, L.H., (Ed.), Ann Arbor Science, Ann Arbor, MI. **1976**, 87-104.
12. *Report on Carcinogenesis Bioassay of Chloroform*; Carcinogenesis Program, Division of Cancer Cause and Prevention: Bethesda, MD, 1976.
13. U. S. Environmental Protection Agency, National primary drinking water regulations: Stage 1 Disinfectant and Disinfection Byproducts Rule. http://water.epa.gov/lawsregs/rulesregs/sdwa/stage1/index.cfm.

14. U. S. Environmental Protection Agency, National primary drinking water regulations: Stage 2 Disinfectants and Disinfection Byproducts Rule. http://water.epa.gov/lawsregs/rulesregs/sdwa/stage2/index.cfm.

15. Health Canada (2014). Guidelines for Canadian Drinking Water Quality—Summary Table. Water and Air Quality Bureau, Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa, Ontario.

16. World Health Organization., *Guidelines for Drinking-Water Quality*. 4th ed.; World Health Organization: Geneva, 2011.

17. Krasner, S. W.; Mcguire, M. J.; Jacangelo, J. G.; Patania, N. L.; Reagan, K. M.; Aieta, E. M., The Occurrence of disinfection by-products in United States drinking water. *J. Am. Water Works Assoc.* **1989,** *81* (8), 41-53.

18. Krasner, S. W.; Weinberg, H. S.; Richardson, S. D.; Pastor, S. J.; Chinn, R.; Sclimenti, M. J.; Onstad, G. D.; Thruston, A. D., Occurrence of a new generation of disinfection byproducts. *Environ. Sci. Technol.* **2006,** *40* (23), 7175-7185.

19. Richardson, S. D., *Disinfection By-Products: Formation and Occurrence in Drinking Water.* Elsevier: Burlington, MA, 2011; Vol. 2.

20. Richardson, S. D.; Ternes, T. A., Water analysis: emerging contaminants and current issues. *Anal. Chem.* **2011,** *83* (12), 4614-4648.

21. Nieuwenhuijsen, M. J.; Toledano, M. B.; Elliott, P., Uptake of chlorination disinfection by-products; a review and a discussion of its implications for exposure assessment in epidemiological studies. *J. Expo. Anal. Environ. Epidemiol.* **2000,** *10* (6), 586-599.

22. Mcgeehin, M. A.; Reif, J. S.; Becher, J. C.; Mangione, E. J., Case-control study of bladder cancer and water disinfection methods in Colorado. *Am. J. Epidemiol.* **1993,** *138* (7), 492-501.

23. Cantor, K. P.; Lynch, C. F.; Hildesheim, M. E.; Dosemeci, M.; Lubin, J.; Alavanja, M.; Craun, G., Drinking water source and chlorination byproducts I. Risk of bladder cancer. *Epidemiology* **1998,** *9* (1), 21-28.

24. King, W. D.; Marrett, L. D., Case-control study of bladder cancer and chlorination by-products in treated water (Ontario, Canada). *Cancer Cause Control* **1996,** *7* (6), 596-604.

25. Villanueva, C. M.; Cantor, K. P.; Grimalt, J. O.; Malats, N.; Silverman, D.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Marcos, R.; Rothman, N.; Real, F.

X.; Dosemeci, M.; Kogevinas, M., Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am. J. Epidemiol.* **2007,** *165* (2), 148-156.

26. Hildesheim, M. E.; Cantor, K. P.; Lynch, C. F.; Dosemeci, M.; Lubin, J.; Alavanja, M.; Craun, G., Drinking water source and chlorination byproducts II. Risk of colon and rectal cancers. *Epidemiology* **1998,** *9* (1), 29-35.

27. Rahman, M. B.; Driscoll, T.; Cowie, C.; Armstrong, B. K., Disinfection by-products in drinking water and colorectal cancer: a meta-analysis. *Int. J. Epidemiol.* **2010,** *39* (3), 733-745.

28. Tardiff, R. G.; Carson, M. L.; Ginevan, M. E., Updated weight of evidence for an association between adverse reproductive and developmental effects and exposure to disinfection by-products. *Regul. Toxicol. Pharm.* **2006,** *45* (2), 185-205.

29. Yang, C. Y.; Xiao, Z. P.; Ho, S. C.; Wu, T. N.; Tsai, S. S., Association between trihalomethane concentrations in drinking water and adverse pregnancy outcome in Taiwan. *Environ.l Research* **2007,** *104* (3), 390-395.

30. Grellier, J.; Bennett, J.; Patelarou, E.; Smith, R. B.; Toledano, M. B.; Rushton, L.; Briggs, D. J.; Nieuwenhuijsen, M. J., Exposure to disinfection by-products, fetal growth, and prematurity a systematic review and meta-analysis. *Epidemiology* **2010,** *21* (3), 300-313.

31. Jeong, C. H.; Wagner, E. D.; Siebert, V. R.; Anduri, S.; Richardson, S. D.; Daiber, E. J.; McKague, A. B.; Kogevinas, M.; Villanueva, C. M.; Goslan, E. H.; Luo, W. T.; Isabelle, L. M.; Pankow, J. F.; Grazuleviciene, R.; Cordier, S.; Edwards, S. C.; Righi, E.; Nieuwenhuijsen, M. J.; Plewa, M. J., Occurrence and toxicity of disinfection byproducts in European drinking waters in relation with the HIWATE epidemiology study. *Environ. Sci. Technol.* **2012,** *46* (21), 12120-12128.

32. Hrudey, S. E., Chlorination disinfection by-products, public health risk tradeoffs and me. *Water. Res.* **2009,** *43* (8), 2057-2092.

33. Hrudey, S. E., *Epidemiological Inference and Evidence on DBPs and Human Health*. IWA Publishing: London, U.K., 2012.

34. Hrudey, S. E.; Backer, L. C.; Humpage, A. R.; Krasner, S. W.; Michaud, D. S.; Moore, L. E.; Singer, P. C.; Stanford, B. D., Evaluating evidence for association of human bladder cancer with drinking-water chlorination disinfection by-products. *Journal Toxicol. Environ. Health. Part B, Crit. Rev.* **2015,** *18* (5), 213-241.

35. Bull, R. J.; Reckhow, D. A.; Rotello, V.; Bull, O. M.; Kim, J., *Use of Toxicological and Chemical Models to Prioritize DBP Research. Project 2867. Report to the Awwa Research Foundation*. Denver, CO., 2006.

36. Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; Demarini, D. M., Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutat. Res.* **2007,** *636* (1-3), 178-242.

37. Boorman, G. A.; Dellarco, V.; Dunnick, J. K.; Chapin, R. E.; Hunter, S.; Hauchman, F.; Gardner, H.; Cox, M.; Sills, R. C., Drinking water disinfection byproducts: review and approach to toxicity evaluation. *Environ. Health. Perspect.* **1999,** *107*, 207-217.

38. Plewa, M. J.; Kargalioglu, Y.; Vankerk, D.; Minear, R. A.; Wagner, E. D., Development of a quantitative comparative cytotoxicity and genotoxicity assays for environmental hazardous chemicals. *Water Sci. Technol.* **2000,** *42* (7-8), 109-116.

39. Plewa, M. J.; Kargalioglu, Y.; Vankerk, D.; Minear, R. A.; Wagner, E. D., Mammalian cell cytotoxicity and genotoxicity analysis of drinking water disinfection by-products. *Environ. Mol. Mutagen* **2002,** *40* (2), 134-142.

40. Plewa, M. J.; Wagner, E. D.; Jazwierska, P.; Richardson, S. D.; Chen, P. H.; McKague, A. B., Halonitromethane drinking water disinfection byproducts: chemical characterization and mammalian cell cytotoxicity and genotoxicity. *Environ. Sci. Technol.* **2004,** *38* (1), 62-68.

41. Plewa, M. J.; Muellner, M. G.; Richardson, S. D.; Fasanot, F.; Buettner, K. M.; Woo, Y. T.; Mckague, A. B.; Wagner, E. D., Occurrence, synthesis, and mammalian cell cytotoxicity and genotoxicity of haloacetamides: an emerging class of nitrogenous drinking water disinfection byproducts. *Environ. Sci. Technol.* **2008,** *42* (3), 955-961.

42. Jeong, C. H.; Richardson, S. D.; Marinas, B. J.; Wagner, E. D.; Plewa, M. J., Mammalian cell cytotoxicity and genotoxicity of haloacetaldehyde drinking water disinfection byproducts. *Environ. Mol. Mutagen* **2010,** *51* (7), 726.

43. Plewa, M. J.; Simmons, J. E.; Richardson, S. D.; Wagner, E. D., Mammalian cell cytotoxicity and genotoxicity of the haloacetic acids, a major class of drinking water disinfection by-products. *Environ. Mol. Mutagen* **2010,** *51* (8-9), 871-878.

44. Jeong, C. H.; Postigo, C.; Richardson, S. D.; Simmons, J. E.; Kimura, S. Y.; Marinas, B. J.; Barcelo, D.; Liang, P.; Wagner, E. D.; Plewa, M. J., Occurrence and comparative toxicity of

haloacetaldehyde disinfection byproducts in drinking water. *Environ Sci Technol* **2015**, *49*, 13749−13759.

45. Postigo, C.; Jeong, C. H.; Richardson, S. D.; Wagner, E. D.; Plewa, M. J.; Simmons, J. E.; Barcelo, D., Analysis, Occurrence, and Toxicity of Haloacetaldehydes in Drinking Waters: Iodoacetaldehyde as an Emerging Disinfection By-Product. *Recent Advances in Disinfection by-Products* **2015,** *1190*, 25-43.

46. Kimura, S. Y.; Vu, T. N.; Komaki, Y.; Plewa, M. J.; Marinas, B. J., Acetonitrile and N-chloroacetamide formation from the reaction of acetaldehyde and monochloramine. *Environ. Sci. Technol.* **2015,** *49* (16), 9954-63.

47. Plewa, M. J.; Wagner, E. D.; Mitch, W. A., Comparative mammalian cell cytotoxicity of water concentrates from disinfected recreational pools. *Environ. Sci. Technol.* **2011,** *45* (9), 4159-4165.

48. Duirk, S. E.; Lindell, C.; Cornelison, C. C.; Kormos, J.; Ternes, T. A.; Attene-Ramos, M.; Osiol, J.; Wagner, E. D.; Plewa, M. J.; Richardson, S. D., Formation of toxic iodinated disinfection by-products from compounds used in medical imaging. *Environ. Sci. Technol.* **2011,** *45* (16), 6845-6854.

49. Wendel, F. M.; Ternes, T. A.; Richardson, S. D.; Duirk, S. E.; Pals, J. A.; Wagner, E. D.; Plewa, M. J., Comparative toxicity of high-molecular weight iopamidol disinfection byproducts. *Environ. Sci. Technol. Lett.* **2016,** *3* (3), 81-84.

50. Plewa, M. J.; Wagner, E. D.; Metz, D. H.; Kashinkunti, R.; Jamriska, K. J.; Meyer, M., Differential toxicity of drinking water disinfected with combinations of ultraviolet radiation and chlorine. *Environ. Sci. Technol.* **2012,** *46* (14), 7811-7817.

51. Boyd, J. M.; Huang, L.; Xie, L.; Moe, B.; Gabos, S.; Li, X.-F., A cell-microelectronic sensing technique for profiling cytotoxicity of chemicals. *Anal. Chim. Acta.* **2008,** *615* (1), 80-87.

52. McGuigan, C. F.; Li, X.-F., Cytotoxicity and genotoxicity of phenazine in two human cell lines. *Toxicol. In Vitro* **2014,** *28* (4), 607-615.

53. Zhou, W. J.; Lou, L. J.; Zhu, L. F.; Li, Z. M.; Zhu, L. Z., Formation and cytotoxicity of a new disinfection by-product (DBP) phenazine by chloramination of water containing diphenylamine. *J. Environ. Sci.* **2012,** *24* (7), 1217-1224.

54. Harrington-Brock, K.; Doerr, C. L.; Moore, M. M., Mutagenicity of three disinfection by-products: di- and trichloroacetic acid and chloral hydrate in L5178Y/TK+/- -3.7.2C mouse lymphoma cells. *Mutat Res.* **1998,** *413* (3), 265-276.

55. Giller, S.; LeCurieux, F.; Gauthier, L.; Erb, F.; Marzin, D., Genotoxicity assay of chloral hydrate and chloropicrine. *Mutat. Res. Lett.* **1995,** *348* (4), 147-152.

56. Wu, Q. Y.; Li, Y.; Hu, H. Y.; Ding, Y. N.; Huang, H.; Zhao, F. Y., Removal of genotoxicity in chlorinated secondary effluent of a domestic wastewater treatment plant during dechlorination. *Environ. Sci. Pollut. Res. Int.* **2012,** *19* (1), 1-7.

57. Wu, Q. Y.; Li, Y.; Hu, H. Y.; Sun, Y. X.; Zhao, F. Y., Reduced effect of bromide on the genotoxicity in secondary effluent of a municipal wastewater treatment plant during chlorination. *Environ. Sci. Technol.* **2010,** *44* (13), 4924-9.

58. Cao, N.; Miao, T.; Li, K.; Zhang, Y.; Yang, M., Formation potentials of typical disinfection byproducts and changes of genotoxicity for chlorinated tertiary effluent pretreated by ozone. *J. Environ. Sci.* **2009,** *21* (4), 409-13.

59. Kargalioglu, Y.; McMillan, B. J.; Minear, R. A.; Plewa, M. J., Analysis of the cytotoxicity and mutagenicity of drinking water disinfection by-products in Salmonella typhimurium. *Teratogen Carcinogen Mutagen* **2002,** *22* (2), 113-128.

60. Wagner, E. D.; Hsu, K. M.; Lagunas, A.; Mitch, W. A.; Plewa, M. J., Comparative genotoxicity of nitrosamine drinking water disinfection byproducts in *Salmonella* and mammalian cells. *Mutat Res* **2012,** *741* (1-2), 109-15.

61. Kundu, B.; Richardson, S. D.; Granville, C. A.; Shaughnessy, D. T.; Hanley, N. M.; Swartz, P. D.; Richard, A. M.; DeMarini, D. M., Comparative mutagenicity of halomethanes and halonitromethanes in Salmonella TA100: structure-activity analysis and mutation spectra. *Mutat. Res. Fund. Mol Mech. Mutagen.* **2004,** *554* (1-2), 335-350.

62. Noot, D. K.; Anderson, W. B.; Daignault, S. A.; Williams, D. T.; Huck, P. M., Evaluating treatment processes with the Ames mutagenicity assay. *J. Am. Water Works Assoc.* **1989,** *81* (9), 87-102.

63. DeMarini, D. M.; Abu-Shakra, A.; Felton, C. F.; Patterson, K. S.; Shelton, M. L., Mutation spectra in salmonella of chlorinated, chloraminated, or ozonated drinking water extracts: comparison to MX. *Environ. Mol. Mutagen.* **1995,** *26* (4), 270-85.

64. Patterson, K. S.; Lykins, B. W.; Richardson, S. D., Mutagenicity of drinking-water following disinfection. *.J Water Supply Res. T.* **1995,** *44* (1), 1-9.

65. Liviac, D.; Creus, A.; Marcos, R., Genotoxicity analysis of two halonitromethanes, a novel group of disinfection by-products (DBPs), in human cells treated in vitro. *Environ. Res.* **2009,** *109* (3), 232-8.

66. Laingam, S.; Froscio, S. M.; Humpage, A. R., Flow-cytometric analysis of in vitro micronucleus formation: comparative studies with WIL2-NS human lymphoblastoid and L5178Y mouse lymphoma cell lines. *Mutat. Res.* **2008,** *656* (1-2), 19-26.

67. Liviac, D.; Creus, A.; Marcos, R., Genotoxicity testing of three monohaloacetic acids in TK6 cells using the cytokinesis-block micronucleus assay. *Mutagenesis* **2010,** *25* (5), 505-9.

68. Laingam, S.; Froscio, S. M.; Bull, R. J.; Humpage, A. R., In vitro toxicity and genotoxicity assessment of disinfection by-products, organic N-chloramines. *Environ. Mol. Mutagen.* **2012,** *53* (2), 83-93.

69. Gunter, S.; Haupter, S.; Schutz, P.; Kreis, P., Comparative evaluation of the genotoxic properties of potassium bromate and potassium superoxide in V79 Chinese hamster cells. *Mutat. Res.-Gen. Tox. En.* **1999,** *439* (2), 213-221.

70. Echigo, S.; Itoh, S.; Natsui, T.; Araki, T.; Ando, R., Contribution of brominated organic disinfection by-products to the mutagenicity of drinking water. *Water Sci. Technol.* **2004,** *50* (5), 321-8.

71. Plewa, M. J., Wagner, E.D., *Mammalian Cell Cytotoxicity and Genotoxicity of DBPs*. Water Research Foundation: Denver, CO, 2009.

72. IARC *Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Chemicals That Cause Tumours of the Kidney or Urinary Bladder in Rodents and Some Other Substances*; International Agency for Research on Cancer: Lyon, France, 1999.

73. Melnick, R. L.; Nyska, A.; Foster, P. M.; Roycroft, J. H.; Kissling, G. E., Toxicity and carcinogenicity of the water disinfection byproduct, dibromoacetic acid, in rats and mice. *Toxicology* **2007,** *230* (2-3), 126-136.

74. Yokose, Y.; Uchida, K.; Nakae, D.; Shiraiwa, K.; Yamamoto, K.; Konishi, Y., Studies of carcinogenicity of sodium-chlorite in B6c3f1 mice. *Environ. Health Perspec.* **1987,** *76*, 205-210.

75. DeAngelo, A. B.; Daniel, F. B.; Most, B. M.; Olson, G. R., Failure of monochloroacetic acid and trichloroacetic acid administered in the drinking water to produce liver cancer in male F344/N rats. *J. Toxicol. Environ. Health* **1997,** *52* (5), 425-45.

76. Kurokawa, Y.; Takayama, S.; Konishi, Y.; Hiasa, Y.; Asahina, S.; Takahashi, M.; Maekawa, A.; Hayashi, Y., Long-term in vivo carcinogenicity tests of potassium bromate, sodium hypochlorite, and sodium chlorite conducted in Japan. *Environ. Health Perspect.* **1986,** *69*, 221-35.

77. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. List of classsifications, volumes 1-117. International Agency for Research on Cancer. http://monographs.iarc.fr/ENG/Classification/latest_classif.php (accessed January 20, 2017).

78. Nieuwenhuijsen, M. J.; Grellier, J.; Smith, R.; Iszatt, N.; Bennett, J.; Best, N.; Toledano, M., The epidemiology and possible mechanisms of disinfection by-products in drinking water. *Philos. T. R. Soc. A.* **2009,** *367* (1904), 4043-4076.

79. Krewski, D.; Westphal, M.; Andersen, M. E.; Paoli, G. M.; Chiu, W. A.; Al-Zoughool, M.; Croteau, M. C.; Burgoon, L. D.; Cote, I., A framework for the next generation of risk science. *Environ. Health Perspec.* **2014,** *122* (8), 796-805.

80. Aardema, M. J.; MacGregor, J. T., Toxicology and genetic toxicology in the new era of "toxicogenomics": impact of "-omics" technologies. *Mutat. Res. Fund Mol Mech Mutagen.* **2002,** *499* (1), 13-25.

81. Thai, S. F.; Allen, J. W.; DeAngelo, A. B.; George, M. H.; Fuscoe, J. C., Altered gene expression in mouse livers after dichloroacetic acid exposure. *Mutat. Res. Rev. Mutat.* **2003,** *543* (2), 167-180.

82. Tully, D. B.; Luft, J. C.; Rockett, J. C.; Ren, H. Z.; Schmid, J. E.; Wood, C. R.; Dix, D. J., Reproductive and genomic effects in testes from mice exposed to the water disinfectant byproduct bromochloroacetic acid. *Reprod. Toxicol.* **2005,** *19* (3), 353-366.

83. Delker, D.; Hatch, G.; Allen, J.; Crissman, B.; George, M.; Geter, D.; Kilburn, S.; Moore, T.; Nelson, G.; Roop, B.; Slade, R.; Swank, A.; Ward, W.; DeAngelo, A., Molecular biomarkers of oxidative stress associated with bromate carcinogenicity. *Toxicology* **2006,** *221* (2-3), 158-165.

84. Luan, Y.; Suzuki, T.; Palanisamy, R.; Takashima, Y.; Sakamoto, H.; Sakuraba, M.; Koizumi, T.; Saito, M.; Matsufuji, H.; Yamagata, K.; Yamaguchi, T.; Hayashi, M.; Honma, M.,

Potassium bromate treatment predominantly causes large deletions, but not GC>TA transversion in human cells. *Mutat. Res.* **2007,** *619* (1-2), 113-23.

85. Ward, W. O.; Swartz, C. D.; Porwollik, S.; Warren, S. H.; Hanley, N. M.; Knapp, G. W.; McClelland, M.; DeMarini, D. M., Toxicogenomic analysis incorporating operon-transcriptional coupling and toxicant concentration-expression response: analysis of MX-treated *Salmonella*. *BMC. Bioinformatics* **2007,** *8*, 378.

86. Wang, S.; Zhang, H.; Zheng, W.; Wang, X.; Andersen, M. E.; Pi, J.; He, G.; Qu, W., Organic extract contaminants from drinking water activate Nrf2-mediated antioxidant response in a human cell line. *Environ. Sci. Technol.* **2013,** *47* (9), 4768-77.

87. Muellner, M. G.; Attene-Ramos, M. S.; Hudson, M. E.; Wagner, E. D.; Plewa, M. J., Human cell toxicogenomic analysis of bromoacetic acid: a regulated drinking water disinfection by-product. *Environ. Mol. Mutagen.* **2010,** *51* (3), 205-214.

88. Attene-Ramos, M. S.; Wagner, E. D.; Plewa, M. J., Comparative human cell toxicogenomic analysis of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2010,** *44* (19), 7206-7212.

89. Pals, J.; Attene-Ramos, M. S.; Xia, M. H.; Wagner, E. D.; Plewa, M. J., Human cell toxicogenomic analysis linking reactive oxygen species to the toxicity of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2013,** *47* (21), 12514-12523.

90. Prochazka, E.; Escher, B. I.; Plewa, M. J.; Leusch, F. D., In vitro cytotoxicity and adaptive stress responses to selected haloacetic acid and halobenzoquinone water disinfection byproducts. *Chem. Res. Toxicol.* **2015,** *28* (10), 2059-68.

91. Wang, S.; Zheng, W.; Liu, X.; Xue, P.; Jiang, S.; Lu, D.; Zhang, Q.; He, G.; Pi, J.; Andersen, M. E.; Tan, H.; Qu, W., Iodoacetic acid activates Nrf2-mediated antioxidant response in vitro and in vivo. *Environ. Sci. Technol.* **2014,** *48* (22), 13478-88.

92. Stalter, D.; O'Malley, E.; von Gunten, U.; Escher, B. I., Fingerprinting the reactive toxicity pathways of 50 drinking water disinfection by-products. *Water Res.* **2016,** *91*, 19-30.

93. Bull, R. J., *Use of Toxicological and Chemical Models to Prioritize DBP Research*. AWWA Research Foundation: Denver, CO, 2006.

94. Qin, F.; Zhao, Y. Y.; Zhao, Y. L.; Boyd, J. M.; Zhou, W. J.; Li, X.-F., A toxic disinfection by-product, 2,6-Dichloro-1,4-benzoquinone, identified in drinking water. *Angew. Chem. Int. Ed.* **2010,** *49* (4), 790-792.

95. Zhao, Y.; Qin, F.; Boyd, J. M.; Anichina, J.; Li, X.-F., Characterization and determination of chloro- and bromo-benzoquinones as new chlorination disinfection byproducts in drinking water. *Anal. Chem.* **2010,** *82* (11), 4599-4605.

96. Zhai, H.; Zhang, X., Formation and decomposition of new and unknown polar brominated disinfection byproducts during chlorination. *Environ. Sci. Technol.* **2011,** *45* (6), 2194-2201.

97. Zhai, H.; Zhang, X.; Zhu, X.; Liu, J.; Ji, M., Formation of brominated disinfection byproducts during chloramination of drinking water: new polar species and overall kinetics. *Environ. Sci. Technol.* **2014,** *48* (5), 2579-2588.

98. Wang, W.; Qian, Y.; Boyd, J. M.; Wu, M.; Hrudey, S. E.; Li, X.-F., Halobenzoquinones in swimming pool waters and their formation from personal care products. *Environ. Sci. Technol.* **2013,** *47* (7), 3275-3282.

99. Du, H. Y.; Li, J. H.; Moe, B.; McGuigan, C. F.; Li, X.-F., A real-time cell-electronic sensing method for comparative analysis of toxicity of water contaminants. *Anal. Methods* **2013,** *6*, 2053-2058.

100. Moe, B., McGuigan, C. F., Li, X.-F., Dabek-Zlotorzynska, E. and Gabos, S, Cell-electronic sensing of cellular responses to micro- and nanoparticles for environmental applications. *Encyclopedia of Analytical Chemistry* **2014**, 1-28.

101. Wang, W.; Qian, Y. C.; Li, J. H.; Moe, B.; Huang, R. F.; Zhang, H. Q.; Hrudey, S. E.; Li, X.-F., Analytical and toxicity characterization of halo-hydroxyl-benzoquinones as stable halobenzoquinone disinfection byproducts in treated water. *Anal. Chem.* **2014,** *86* (10), 4982-4988.

102. Gillies, R. J.; Didier, N.; Denton, M., Determination of cell number in monolayer-cultures. *Anal. Biochem.* **1986,** *159* (1), 109-113.

103. Yang, M. T.; Zhang, X. R., Comparative developmental toxicity of new aromatic halogenated DBPs in a chlorinated saline sewage effluent to the marine polychaete *Platynereis dumerilii*. *Environ. Sci. Technol.* **2013,** *47* (19), 10868-10876.

104. Whysner, J.; Reddy, M. V.; Ross, P. M.; Mohan, M.; Lax, E. A., Genotoxicity of benzene and its metabolites. *Mutat. Res. Rev. Mutat.* **2004,** *566* (2), 99-130.

105. Whysner, J., Benzene-induced genotoxicity. *J. Toxicol. Environ. Health A.* **2000,** *61* (5-6), 347-351.

106. Yager, J. W.; Eastmond, D. A.; Robertson, M. L.; Paradisin, W. M.; Smith, M. T., Characterization of micronuclei induced in human lymphocytes by benzene metabolites. *Cancer Res.* **1990,** *50* (2), 393-399.

107. O'Brien, P. J., Molecular mechanisms of quinone cytotoxicity. *Chem. Biol. Interact.* **1991,** *80* (1), 1-41.

108. Bolton, J. L.; Trush, M. A.; Penning, T. M.; Dryhurst, G.; Monks, T. J., Role of quinones in toxicology. *Chem. Res. Toxicol.* **2000,** *13* (3), 135-160.

109. Casarett, L. J.; Doull, J.; Klaassen, C. D., *Casarett and Doull's Toxicology: the Basic Science of Poisons*. 7th ed.; McGraw-Hill: New York, 2008.

110. Zhu, B. Z.; Zhao, H. T.; Kalyanaraman, B.; Frei, B., Metal-independent production of hydroxyl radicals by halogenated quinones and hydrogen peroxide: an ESR spin trapping study. *Free Radic. Biol. Med.* **2002,** *32* (5), 465-73.

111. Zhu, B. Z.; Kitrossky, N.; Chevion, M., Evidence for production of hydroxyl radicals by pentachlorophenol metabolites and hydrogen peroxide: a metal-independent organic Fenton reaction. *Biochem. Biophys. Res. Commun.* **2000,** *270* (3), 942-6.

112. Zhu, B. Z.; Kalyanaraman, B.; Jiang, G. B., Molecular mechanism for metal-independent production of hydroxyl radicals by hydrogen peroxide and halogenated quinones. *Proc. Natl. Acad. Sci. USA.* **2007,** *104* (45), 17575-17578.

113. Zhu, B. Z.; Shan, G. Q., Potential mechanism for pentachlorophenol-induced carcinogenicity: a novel mechanism for metal-independent production of hydroxyl radicals. *Chem. Res. Toxicol.* **2009,** *22* (6), 969-977.

114. Zhu, B. Z.; Zhao, H. T.; Kalyanaraman, B.; Liu, J.; Shan, G. Q.; Du, Y. G.; Frei, B., Mechanism of metal-independent decomposition of organic hydroperoxides and formation of alkoxyl radicals by halogenated quinones. *Proc. Natl. Acad. Sci. USA.* **2007,** *104* (10), 3698-3702.

115. Zhu, B. Z.; Shan, G. Q.; Huang, C. H.; Kalyanaraman, B.; Mao, L.; Du, Y. G., Metal-independent decomposition of hydroperoxides by halogenated quinones: Detection and identification of a quinone ketoxy radical. *Proc. Natl. Acad. Sci. USA.* **2009,** *106* (28), 11466-11471.

116. Zhu, B. Z.; Qin, H., Metal-independent decomposition of lipid hydroperoxide 13-HPODE and formation of lipid radicals/4-HNE by the newly identified chlorination disinfection haloquinone byproducts in drinking water. *Free Radical. Biol. Med.* **2012,** *53*, S153-S153.
117. Hayes, J. D.; McLellan, L. I., Glutathione and glutathione-dependent enzymes represent a Co-ordinately regulated defence against oxidative stress. *Free Radic. Res.* **1999,** *31* (4), 273-300.
118. Liu, J.; Song, E. Q.; Liu, L. C.; Ma, X. Y.; Tian, X. G.; Dong, H.; Song, Y., Polychlorinated biphenyl quinone metabolites lead to oxidative stress in HepG2 cells and the protective role of dihydrolipoic acid. *Toxicol. In Vitro* **2012,** *26* (6), 841-848.
119. Siraki, A. G.; Chan, T. S.; O'Brien, P. J., Application of quantitative structure-toxicity relationships for the comparison of the cytotoxicity of 14 p-benzoquinone congeners in primary cultured rat hepatocytes versus PC12 cells. *Toxicol. Sci.* **2004,** *81* (1), 148-159.
120. Smith, A. R.; Shenvi, S. V.; Widlansky, M.; Suh, J. H.; Hagen, T. M., Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress. *Curr. Med. Chem.* **2004,** *11* (9), 1135-1146.
121. Song, Y.; Wagner, B. A.; Witmer, J. R.; Lehmler, H. J.; Buettner, G. R., Nonenzymatic displacement of chlorine and formation of free radicals upon the reaction of glutathione with PCB quinones. *Proc. Natl. Acad. Sci. USA.* **2009,** *106* (24), 9725-9730.
122. Eaton, D. L.; Bammler, T. K., Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol Sci* **1999,** *49* (2), 156-164.
123. Brigelius-Flohe, R.; Kipp, A., Glutathione peroxidases in different stages of carcinogenesis. *Biochim. Biophys. Acta.* **2009,** *1790* (11), 1555-68.
124. Baigi, M. G.; Brault, L.; Neguesque, A.; Beley, M.; El Hilali, R.; Gauzere, F.; Bagrel, D., Apoptosis/necrosis switch in two different cancer cell lines: Influence of benzoquinone- and hydrogen peroxide-induced oxidative stress intensity, and glutathione. *Toxicol. In Vitro* **2008,** *22* (6), 1547-1554.
125. Nakamura, Y.; Kumagai, T.; Yoshida, C.; Naito, Y.; Miyamoto, M.; Ohigashi, H.; Osawa, T.; Uchida, K., Pivotal role of electrophilicity in glutathione S-transferase induction by tert-butylhydroquinone. *Biochemistry-Us* **2003,** *42* (14), 4300-4309.
126. Rubio, V.; Zhang, J.; Valverde, M.; Rojas, E.; Shi, Z. Z., Essential role of Nrf2 in protection against hydroquinone- and benzoquinone-induced cytotoxicity. *Toxicol. In Vitro* **2011,** *25* (2), 521-529.

127. Ma, Q., Role of nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* **2013,** *53*, 401-26.

128. van Ommen, B.; den Besten, C.; Rutten, A. L.; Ploemen, J. H.; Vos, R. M.; Muller, F.; van Bladeren, P. J., Active site-directed irreversible inhibition of glutathione S-transferases by the glutathione conjugate of tetrachloro-1,4-benzoquinone. *J Biol Chem* **1988,** *263* (26), 12939-42.

129. Dierickx, P. J., In vitro binding of 3,4,5,6-tetrachloro-1,2-benzoquinone by rat-liver glutathione S-transferases. *Res. Commun. Chem. Path.* **1983,** *41* (3), 517-520.

130. Li, L. R.; Dong, H.; Song, E. Q.; Xu, X. Y.; Liu, L. C.; Song, Y., Nrf2/ARE pathway activation, HO-1 and NQ01 induction by polychlorinated biphenyl quinone is associated with reactive oxygen species and PI3K/AKT signaling. *Chem-Biol. Interact.* **2014,** *209*, 56-67.

131. Bull, R. J.; Reckhow, D. A.; Li, X.; Humpage, A. R.; Joll, C.; Hrudey, S. E., Potential carcinogenic hazards of non-regulated disinfection by-products: haloquinones, halo-cyclopentene and cyclohexene derivatives, N-halamines, halonitriles, and heterocyclic amines. *Toxicology* **2011,** *286* (1-3), 1-19.

132. Monks, T. J.; Hanzlik, R. P.; Cohen, G. M.; Ross, D.; Graham, D. G., Quinone chemistry and toxicity. *Toxicol. Appl. Pharmacol.* **1992,** *112* (1), 2-16.

133. Gaskell, M.; McLuckie, K. I. E.; Farmer, P. B., Comparison of the repair of DNA damage induced by the benzene metabolites hydroquinone and p-benzoquinone: a role for hydroquinone in benzene genotoxicity. *Carcinogenesis* **2005,** *26* (3), 673-680.

134. Wang, J.; Yu, S. Y.; Jiao, S. H.; Lv, X. W.; Ma, M.; Zhu, B. Z.; Du, Y. G., Characterization of TCHQ-induced genotoxicity and mutagenesis using the pSP189 shuttle vector in mammalian cells. *Mutat. Res. Fund. Mol. M.* **2012,** *729* (1-2), 16-23.

135. Ames, B. N.; Mccann, J.; Yamasaki, E., Methods for detecting carcinogens and mutagens with *salmonella*-mammalian-microsome mutagenicity test. *Mutat. Res.* **1975,** *31* (6), 347-363.

136. McGuigan, C. F., Li, X.-F.; Le, X.C., Cytotoxic and mutagenic effects of haloquinone drinking water disinfection byproducts. *The Toxicologist, Supplement to Toxicological Sciences* **2012,** *126* (1), 450.

137. Anichina, J.; Zhao, Y. L.; Hrudey, S. E.; Le, X. C.; Li, X.-F., Electrospray ionization mass spectrometry characterization of interactions of newly identified water disinfection

byproducts halobenzoquinones with oligodeoxynucleotides. *Environ. Sci. Technol.* **2010,** *44* (24), 9557-9563.

138. Anichina, J.; Zhao, Y. L.; Hrudey, S. E.; Schreiber, A.; Li, X.-F., Electrospray ionization tandem mass spectrometry analysis of the reactivity of structurally related bromo-methyl-benzoquinones toward oligonucleotides. *Anal. Chem.* **2011,** *83* (21), 8145-8151.

139. Nguyen, T. N.; Bertagnolli, A. D.; Villalta, P. W.; Buhlmann, P.; Sturla, S. J., Characterization of a deoxyguanosine adduct of tetrachlorobenzoquinone: dichlorobenzoquinone-1,N2-etheno-2'-deoxyguanosine. *Chem. Res. Toxicol.* **2005,** *18* (11), 1770-6.

140. Vaidyanathan, V. G.; Villalta, P. W.; Sturla, S. J., Nucleobase-dependent reactivity of a quinone metabolite of pentachlorophenol. *Chem. Res. Toxicol.* **2007,** *20* (6), 913-919.

141. Jia, S. P.; Zhu, B. Z.; Guo, L. H., Detection and mechanistic investigation of halogenated benzoquinone induced DNA damage by photoelectrochemical DNA sensor. *Anal. Bioanal. Chem.* **2010,** *397* (6), 2395-2400.

142. Lin, P. H.; La, D. K.; Upton, P. B.; Swenberg, J. A., Analysis of DNA adducts in rats exposed to pentachlorophenol. *Carcinogenesis* **2002,** *23* (2), 365-9.

143. Lin, P. H.; Nakamura, J.; Yamaguchi, S.; Upton, P. B.; La, D. K.; Swenberg, J. A., Oxidative damage and direct adducts in calf thymus DNA induced by the pentachlorophenol metabolites, tetrachlorohydroquinone and tetrachloro-1,4-benzoquinone. *Carcinogenesis* **2001,** *22* (4), 627-34.

144. Lin, P. H.; Nakamura, J.; Yamaguchi, S.; La, D. K.; Upton, P. B.; Swenberg, J. A., Induction of direct adducts, apurinic/apyrimidinic sites and oxidized bases in nuclear DNA of human HeLa S3 tumor cells by tetrachlorohydroquinone. *Carcinogenesis* **2001,** *22* (4), 635-9.

145. Klaunig, J. E.; Kamendulis, L. M.; Hocevar, B. A., Oxidative stress and oxidative damage in carcinogenesis. *Toxicol. Pathol.* **2010,** *38* (1), 96-109.

146. Cheng, K. C.; Cahill, D. S.; Kasai, H.; Nishimura, S.; Loeb, L. A., 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G-T and A-C substitutions. *J. Biol. Chem.* **1992,** *267* (1), 166-72.

147. Dahlhaus, M.; Almstadt, E.; Henschke, P.; Luttgert, S.; Appel, K. E., Oxidative DNA lesions in V79 cells mediated by pentachlorophenol metabolites. *Arch. Toxicol.* **1996,** *70* (7), 457-460.

148. Sze, C. C.; Shi, C. Y.; Ong, C. N., Cytotoxicity and DNA strand breaks induced by benzene and its metabolites in Chinese hamster ovary cells. *J. Appl. Toxicol.* **1996,** *16* (3), 259-264.
149. Dong, H.; Su, C. Y.; Xia, X. M.; Li, L. R.; Song, E. Q.; Song, Y., Polychlorinated biphenyl quinone-induced genotoxicity, oxidative DNA damage and gamma-H2AX formation in HepG2 cells. *Chem-Biol. Interact.* **2014,** *212*, 47-55.
150. Yin, R.; Zhang, D.; Song, Y.; Zhu, B. Z.; Wang, H., Potent DNA damage by polyhalogenated quinones and H(2)O(2) via a metal-independent and Intercalation-enhanced oxidation mechanism. *Sci. Rep.* **2013,** *3*, 1269.
151. Nakamura, J.; La, D. K.; Swenberg, J. A., 5'-nicked apurinic/apyrimidinic sites are resistant to beta-elimination by beta-polymerase and are persistent in human cultured cells after oxidative stress. *J. Biol. Chem.* **2000,** *275* (8), 5323-8.
152. Balasubramanian, B.; Pogozelski, W. K.; Tullius, T. D., DNA strand breaking by the hydroxyl radical is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone. *Proc. Natl. Acad. Sci. USA.* **1998,** *95* (17), 9738-43.
153. Zhao, B.; Yang, Y.; Wang, X.; Chong, Z.; Yin, R.; Song, S. H.; Zhao, C.; Li, C.; Huang, H.; Sun, B. F.; Wu, D.; Jin, K. X.; Song, M.; Zhu, B. Z.; Jiang, G.; Rendtlew Danielsen, J. M.; Xu, G. L.; Yang, Y. G.; Wang, H., Redox-active quinones induces genome-wide DNA methylation changes by an iron-mediated and Tet-dependent mechanism. *Nucleic Acids Res.* **2014,** *42* (3), 1593-605.
154. Zhou, H.; Chen, W. D.; Qin, X.; Lee, K.; Liu, L.; Markowitz, S. D.; Gerson, S. L., MMTV promoter hypomethylation is linked to spontaneous and MNU associated c-neu expression and mammary carcinogenesis in MMTV c-neu transgenic mice. *Oncogene* **2001,** *20* (42), 6009-17.
155. Pandey, A. K.; Gurbani, D.; Bajpayee, M.; Parmar, D.; Ajmani, S.; Dhawana, A., In silico studies with human DNA topoisomerase-II alpha to unravel the mechanism of in vitro genotoxicity of benzene and its metabolites. *Mutat. Res. Fund. Mol. M.* **2009,** *661* (1-2), 57-70.
156. Zhao, Y. L.; Anichina, J.; Lu, X. F.; Bull, R. J.; Krasner, S. W.; Hrudey, S. E.; Li, X.-F., Occurrence and formation of chloro- and bromo-benzoquinones during drinking water disinfection. *Water Res.* **2012,** *46* (14), 4351-4360.

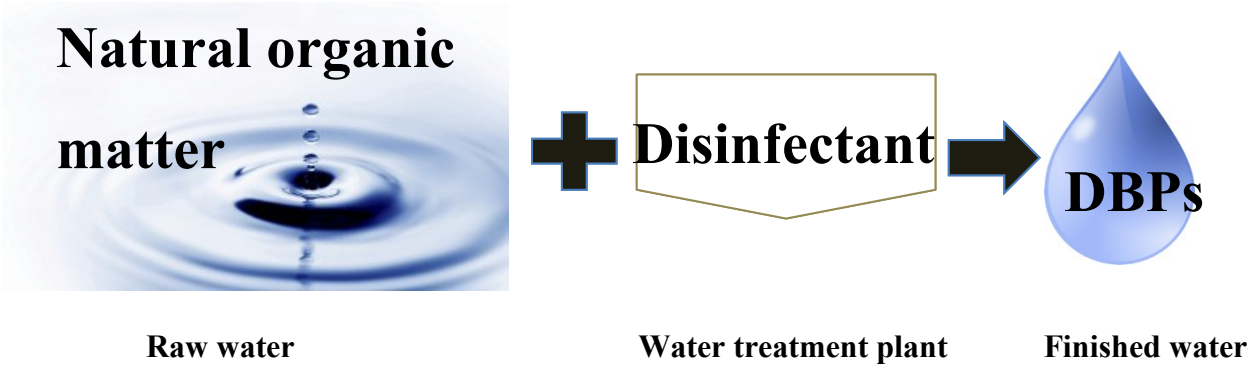


Figure 1.1 Schematic of disinfection byproduct formation during water treatment.

Table 1.1 USEPA Stage 2 DBP Rule [14]

DBP regulations	**maximum contaminant level, MCL (mg/L)**
Total trihalomethanes (THMs)	0.08
Five haloacetic acids (HAAs)	0.06
Bromate	0.01
Chlorite	1.0

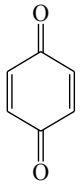
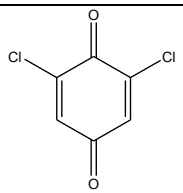
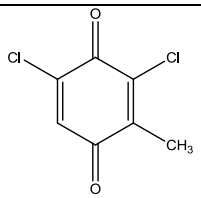
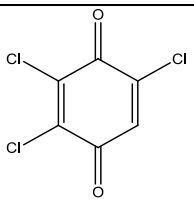
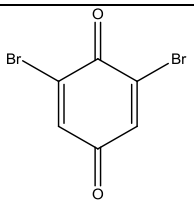
Table 1.2 Relevant Health Canada Guidelines for DBPs [15]

DBP regulations	**maximum acceptable concentrations, MAC, (mg/L)**
Total trihalomethanes (THMs)	0.1
Total haloacetic acids (HAAs)	0.08
Bromate	0.01
Chlorite	1.0
Chlorate	1.0
Formaldehyde	none
N-Nitrosodimethylamine (NDMA)	0.000 04

Table 1.3 Relevant World Health Organization Guidelines for DBPs [16]

DBP regulations	**Guideline value (mg/L)**
Bromate	0.01
Bromodichloromethane	0.06
Bromoform	0.1
Chlorate	0.7
Chlorite	0.7
Chloroform	0.3
Dibromoacetonitrile	0.07
Dibromochloromethane	0.1
Dichloroacetate	0.05
Dichloroacetonitrile	0.02
Monochloroacetate	0.02
N-Nitrosodimethylamine	0.0001
Trichloroacetate	0.2
2,4,6-Trichlorophenol	0.2

Table 1.4 Structure and occurrence of HBQs in drinking water

HBQs	**Molecular Formula**	**Structure**	**Occurrence Frequency**[101, 156]	**Concentration detected, ng/L**[101, 156]
1,4-BQ	$C_6H_4(=O)_2$		-	-
2,6-DCBQ	$C_6H_2Cl_2O_2$		18/18	2.2–275
DCMBQ	$C_7H_4Cl_2O_2$		7/18	0.9–6.5
TriCBQ	$C_6HCl_3O_2$		7/18	2.9–9.1
2,6-DBBQ	$C_6H_2Br_2O_2$		13/18	0.5–37.9

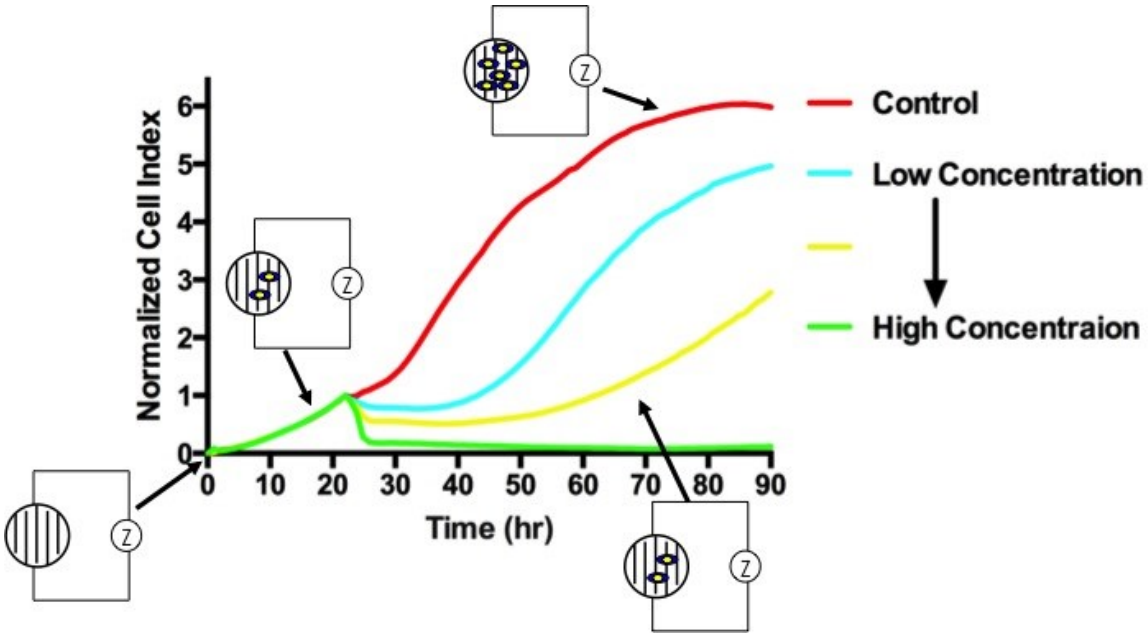


Figure 1.2 The principle of real-time cell analysis. RTCA measures changes in impedance (Z) over time caused by cells adhering to microelectrodes on the bottom of specialized microwell plates. Z is presented as the unitless measure, cell index (CI). Increases in CI correspond to the combined effects 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 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.

Table 1.5 Comparison of chronic cytotoxicity of regulated DBPs and HBQs in CHO cells

DBPs	Chemical class	72h-IC_{50}, mM	Reference
Chloroform	Trihalomethanes	9.62	71
Bromodichloromethane		11.5	
Dibromochloromethane		5.36	
Bromoform		3.96	
Monochloroacetic acid	Haloacetic acids	0.81	43
Dichloroacetic acid		7.3	
Trichlorocetic acid		2.4	
Monobromoacetic acid		0.01	
Dibromoacetic acid		0.59	
Bromate	-	0.963	30
Chlorite	-	No data	
N-Nitrosodimethylamine (NDMA)	Nitrosamines	31	51
2,6-DCBQ	HBQs	41.5×10^{-3}	101
DCMBQ		15.9×10^{-3}	
TriCBQ		72.9×10^{-3}	
2,6-DBBQ		35.5×10^{-3}	

Chapter 2 Cytotoxicity and oxidative damages induced by HBQs [2]

2.1 Introduction

Halobenzoquinones (HBQs) have been recently identified in drinking water as a new class of disinfection byproducts (DBPs) that are produced during common disinfection treatments (e.g. chlorination and chloramination).[1] Disinfection of drinking water most effectively reduces/eliminates microbial risk and prevents waterborne diseases. However, chlorination and other disinfection treatments can result in generation of byproducts from the reactions between the common disinfectants (chlorine, chloramines, ozone, and chlorine dioxide) and natural organic matter in the source water.[2] Halogenated DBPs such as chloroform, halomethanes, and haloacetic acids, have been thoroughly investigated, and yet a large portion of different classes of DBPs remain unknown. For example, of the total organic halides, approximately 70% of halogenated DBPs are unidentified based on a U.S. EPA nationwide occurrence study.[3, 4] Also, toxicological evaluation of the identified DBPs has only been conducted for a small number of DBPs.[2]

The concerns regarding DBPs mainly result from several epidemiological studies that have suggested a potential association between long-term exposure to chlorinated water and increased risk of human bladder cancer.[5, 6] Current regulation of DBPs has mainly focused on trihalomethanes (THMs) and haloacetic acids (HAAs).[7] However, there is no sufficient toxicological evidence that the regulated DBPs could be responsible for the magnitude of increased bladder cancer risk observed.[8] A great deal of effort has been made to identify DBPs of toxicological significance. A quantitative structure–toxicity relationship (QSTR) analysis predicted five classes of DBPs as plausible bladder carcinogens: haloquinones (also called halobenzoquinones, HBQs), halocyclopentenoic acids, organic N-haloamines, nitrosamines/nitrosamides, and halonitriles/haloamides.[9]

Four HBQs have been confirmed as DBPs in treated water.[1, 10] These are 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ), 2,3,6-

[2] This Chapter 2 has been published as Haiying Du, Jinhua Li (co-first author), Birget Moe, Claire F. McGuigan, Shengwen Shen, and Xing-Fang Li. Cytotoxicity and oxidative damage induced by halobenzoquinones to T24 bladder cancer cells. Environ. Sci. Technol., 2013, 47(6):2823-30.

trichloro-1,4-benzoquinone (TriCBQ), and 2,6-dibromobenzoquinone (2,6-DBBQ). The concentrations of these HBQs in drinking water range from 0.5 to 165.1 ng/L with frequent occurrences of 2,6-DCBQ (100%) and 2,6-DBBQ (72%) in the nine water systems we previously studied.[10] Based on TOPKAT software analysis, the chronic lowest observed adverse effect levels (LOAELs) of HBQs are predicted to be 0.049 (2,6-DCBQ), 0.079 (DCMBQ), 0.033 (TriCBQ), and 0.159 (2,6-DBBQ) mg/kg body weight per day, with both DCMBQ and TriCBQ predicted to be carcinogens.[9] On the basis of the literature regarding the known toxicity of benzoquinone and other structurally related compounds, HBQs as DBPs could pose a cancer risk.[8] However, there is little or no experimental data on the toxicity of these compounds. Thus, this study aims to examine the *in vitro* toxicity of these HBQs. These results are useful for assisting with the design of further *in vivo* toxicity studies, contributing to the understanding of the mechanisms of potential toxic effects of human exposure to these compounds.

The cytotoxicity and genotoxicity of benzoquinone has been extensively studied.[11, 12] Multiple pathways are involved in the toxicity of benzoquinones, including redox reactions, alkylation, and reactive metabolites interacting with functional biological macromolecules, resulting in various detrimental effects.[8] Oxidative damage is believed to be one of the most important mechanisms of carcinogenicity of benzoquinone due to the formation of reactive oxygen species (ROS) and/or depletion of cellular reduced glutathione.[12-15] HBQs, as a separate class, have been observed to react with other chemicals to produce alkoxyl radicals and to interact with oligonucleotides in solution.[16, 17] Therefore, I hypothesize that HBQs may cause cytotoxicity via oxidative stress mechanisms.

The objective of this study was to characterize the cytotoxicity and oxidative stress elicited by HBQs on human bladder cells. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and lactate dehydrogenase (LDH) assay were used to examine the cytotoxicity of HBQs to T24 cells when the antioxidant N-acetyl-L-cysteine (NAC) was absent and present. I quantitatively measured the production of ROS after HBQ treatment. In addition, I quantified 8-hydroxydeoxyguanosine (8-OHdG), protein carbonyls, and malondialdehyde (MDA) adducts as markers of oxidative DNA damage, protein oxidation, and lipid peroxidation, respectively, to support the involvement of oxidative damage. The results will contribute to a better understanding of the potential cellular mechanisms of toxicity of HBQs.

2.2 Materials and Methods

2.2.1 Reagents

Standards of 2,6-DCBQ, DCMBQ, TriCBQ, 2,6-DBBQ, and 1,4-benzoquinone (BQ) were purchased from Sigma–Aldrich, Shanghai Acana Pharmtech, INDOFINE Chemical Company and Fluka respectively (Table 2.1). HBQs were dissolved in methanol (HPLC grade, Fisher Scientific) and stored at -20°C in sterile amber glass vials. NAC (Sigma) was dissolved in sterile distilled water and stored at 4°C for up to two weeks.

2.2.2 Cell culture

The human bladder epithelial cancer cell line, T24, was obtained from ATCC (Manassas, VA) and cultured in McCoy's 5A modified medium (ATCC) plus 10% fetal bovine serum (FBS) (Sigma) and 1% Penicillin/Streptomycin (100 U/100μg/mL) (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2. Cells were treated when cells reached 70–80% confluency.

2.2.3 Cell viability[3]

To assess cell viability, two different assays were used: the MTS assay and LDH assay. The CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS based) kit and CytoTox 96 Non-Radioactive Cytotoxicity Assay (LDH based) kit were purchased from Promega, and both assays were performed according to the manufacturer's instructions. T24 was seeded at a density of 7500 cells/100μL/well and reached proper confluency after 24 h incubation. Culture media was then replaced with media containing different concentrations of HBQs (100 μL/well) with or without the addition of 1 mM (v/v) NAC. The concentration of NAC (1 mM) was chosen based on the results of the cytotoxicity of NAC (1–5 mM) on T24 cells. T24 cells treated with 1 mM NAC showed little or no difference to control cells without NAC. Blank and negative controls were also included in parallel. After incubation for 24 h, absorbance at 490 nm was recorded using a Microplate Spectrometer (Bio-Rad, Benchmark Plus).

To determine a proper concentration–response range for each HBQ for the MTS and LDH experiments, I conducted an initial screening test of HBQs at concentrations of 1–500 μM

[3] Dr. Haiying Du helped with optimization of the proper concentration ranges of HBQs.

in culture media. 2,6-DCBQ (50–175 μM), DCMBQ (75–150 μM), TriCBQ (75–200 μM), and 2,6-DBBQ (100–200 μM) were tested in the MTS assay; 2,6-DCBQ (50–175 μM), DCMBQ (50–150 μM), TriCBQ (75–200 μM), and 2,6-DBBQ (25–125 μM) were tested in the LDH assay. For each concentration, six replicates were incubated. Three sets of experiments were performed to generate concentration–response curves (log[HBQ] vs percent viability). Based on the dose–response curves, IC_{50} values were determined. The IC_{50} is defined as the concentration of HBQs causing a 50% decrease in overall cell viability.

2.2.4 Detection of ROS

ROS production was determined using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich) as described elsewhere.[18] Cells were seeded into 96-well plates at a density of 1.5×10^4 cells/100μL/well 24 h before treatment. Briefly, the cells were incubated with media containing 25 μM DCFH-DA at 37 °C for 45 min. After removing the DCFH-DA solution, the cells were washed twice with phosphate buffered saline (PBS) (Invitrogen), and treated with different concentrations of BQ and HBQs (25–150 μM). After incubation for 24 h, the plate was monitored (λ_{ex}= 485 nm, λ_{em}= 535 nm) in a fluorescence microplate reader (Beckman Coulter DTX880 Multimode Detector). The fluorescence intensity was recorded, digitized, and stored using Multimode Analysis Software (Beckman Coulter Incorporated). Data points were exported to Excel 2007 for further analysis.

2.2.5 Determination of 8-OHdG

Oxidative DNA damage was assessed by measuring 8-OHdG with the OxiSelect™ Oxidative DNA Damage ELISA Kit from Cell Biolabs (San Diego, CA). Cell treatments were performed as described above with 25–125 μM HBQs. Experiments were performed three times and in duplicate per concentration. In brief, total genomic DNA was extracted using the DNeasy Blood & Tissue Kit from QIAGEN Sciences (Maryland, USA). DNA yield was determined by measuring its absorbance at 260 nm on a NanoVue Plus (GE Healthcare, UK). The DNA samples were first incubated at 95 ºC for 5 min and rapidly chilled on ice before being digested to nucleosides with 5–20 units of nuclease P1 (Sigma-Aldrich) for 2 h at 37 ºC in 20 mM sodium acetate (pH 5.2) (Sigma-Aldrich), followed by 5-10 units of alkaline phosphatase (Invitrogen) for 1 h at 37 ºC in 100 mM Tris Buffer (pH 7.5) (Fisher). The reaction mixture was centrifuged for 5

min at 6000 g and the supernatant was used for the 8-OHdG ELISA assay. DNA samples and 8-OHdG standards were added onto the 8-OHdG conjugate coated plate. The quantity of 8-OHdG was measured by monitoring absorbance at 450 nm on the Microplate Spectrometer and expressed as 8-OHdG per 10^6 nucleotides.

2.2.6 Determination of protein carbonylation

Protein oxidation was measured using the OxiSelect^{TM} Protein Carbonylation ELISA kit from Cell Biolabs. After treatment for 24 h with different concentrations (25–125μM) of HBQs, cells were lysed using RIPA lysis buffer (Invitrogen) and supernatants were obtained as whole cell extracts. Experiments were performed three times and in duplicate per concentration. The concentration of the protein samples was determined using the Quick Start Bradford protein assay according to the manufacturer's instruction (Bio-Rad Laboratories, Richmond, CA). Protein samples (10 μg/mL) and BSA standards were coated onto a 96-well Protein Binding Plate and incubated for 2 h at 37ºC prior to being analyzed on the microplate spectrometer. The protein carbonyl content was determined by comparing the absorbance data with a standard curve that was prepared from reduced and oxidized BSA standards (absorbance at 450 nm) and was expressed as nmol/mg.

2.2.7 Determination of MDA

The quantity of MDA adducts in samples was determined using the OxiSelect^{TM} MDA Adduct ELISA Kit from Cell Biolabs. Cell treatment and whole cell extract preparation was performed as described above for the protein carbonylation assay. Experiments were performed three times and in duplicate per concentration. Samples were diluted to 10 μg/mL in PBS and 100 μL aliquots were placed onto the 96-well protein binding plate. Absorbance of each well was read on the Microplate Spectrometer at 450 nm. MDA levels in protein samples were calculated against a predetermined MDA-BSA standard curve.

2.2.8 Data analysis

Statistical analysis was performed using GraphPad Prism 5 (Graphpad Software) and OriginPro 8.5 (OriginLab Corporation). Experimental results were expressed as mean ± standard deviation (SD) or as mean ± standard error (SEM). Differences between the treated groups with and

without NAC were determined by Student's *t*-test. One-way analysis of variance (ANOVA, followed by Dunnett's *Post Hoc* Multiple Comparisons) was used for multiple comparisons among treatment and control groups. Differences were considered statistically significant at $P<0.05$.

2.3 Results and Discussion

2.3.1 Effects of HBQs on the viability of T24 cells

I first examined the effects of HBQs on cell viability using the colorimetric MTS assay which is widely used for cytotoxicity testing. The T24 cell line was chosen as our model based on our previous results using T24 cells, which provided higher sensitivity when showing the toxic effects of DBPs such as nitrosamines.[19] In addition, because bladder cancer risk is one of the possible adverse health outcomes associated with chronic exposure to chlorinated drinking water,[5, 6] use of a bladder cell line would serve as a model of target organ toxicity. The results of cytotoxic effects of HBQs in T24 cells are useful for a better understanding of potential human health effects.

T24 cells were measured for the generation of a colorimetric product using the MTS assay after incubation with HBQs for 24 h. Figure 2.1 shows the percentage of viable cells after the cells were incubated with varying concentrations of 2,6-DCBQ, DCMBQ, TriCBQ, and 2,6-DBBQ for 24 h. T24 cells treated with any of the four HBQs had significantly reduced viability; and this effect was concentration-dependent (one-way ANOVA analysis, $P<0.05$). The 24 h IC_{50} values were determined to be 94.5 (86.9–102.7) μM for 2,6-DCBQ, 110.1 (106.5–113.8) μM for DCMBQ, 150.7 (146.8–154.6) μM for TriCBQ, and 142.0 (133.1–151.5) μM for 2,6-DBBQ. The results showed the differential cytotoxicity of the four HBQs, and 2,6-DCBQ had the lowest IC_{50}, indicating it has the greatest cytotoxic effect on T24 cells.

To support our hypothesis that HBQ cytotoxicity is in part caused by oxidative stress, I examined the effects of HBQs on cell viability with and without the additional NAC using the MTS assay. NAC is a thiol antioxidant that acts as cysteine stores in cells and a scavenger of free radicals by directly interacting with ROS.[20, 21] Therefore, the presence of NAC should reduce the cytotoxicity induced by HBQs if oxidative stress is indeed a causative agent. Figure 2.2 shows the viability of T24 cells treated with HBQs when NAC was present or absent. These results

clearly demonstrate that the presence of NAC significantly reduces the cytotoxic effects induced by HBQs, as the presence of NAC during HBQ treatment prevented a loss in cell viability. This strongly implicates oxidative stress as a significant contributor to HBQ cytotoxicity.

To support these findings, I also performed the LDH assay. The LDH assay measures cell membrane integrity,[22] while the MTS assay measures mitochondrial activities,[23] but both are measures of cell viability. Figure 2.3 shows cytotoxicity measured with the LDH assay when T24 cells were treated with HBQs with or without NAC. The LDH results confirm that the presence of NAC significantly reduces the cytotoxicity of the HBQs. The results of both MTS and LDH assays consistently show that NAC treatment prevents HBQ-induced cytotoxicity, supporting that oxidative damage is involved in the cytotoxicity of HBQs.

To further support the oxidative damage contributing to the cytotoxicity of HBQs, I investigated ROS generation and concomitant formation of DNA damage, protein carbonylation, and lipid peroxidation after HBQ treatment.

2.3.2 HBQ-induced ROS in T24 cells

I first evaluated the overall induction of ROS in T24 cells by HBQs. After T24 cells were exposed to a range of concentrations (0 to 150 μM in 25 μM increments) of individual HBQs for 24 h, the amount of intracellular ROS production was measured using the DCFH-DA assay. Figure 2.4 summarizes the fluorescence intensities of the cells after being separately treated with HBQs up to 150 μM. A statistically significant increase of ROS was observed after T24 cells were treated with 25 μM of each of the four HBQs. A concentration-dependent increase in ROS generation was also observed. For each concentration level, the ROS generation by each of the HBQs is higher than the control groups (one-way ANOVA analysis, $P<0.05$). Compared to BQ, the four HBQs generated significantly more ROS at the same concentrations, which is consistent with a previous report.[15] ROS can affect many signal transduction pathways by directly reacting with and modifying all major classes of biomolecules, resulting in changes in their structures and functions.[24] An excessive increase in ROS can cause irreversible oxidative damage to lipids, proteins, and DNA, leading to cell death.[25-27] These results in part support the hypothesis that excessive production of ROS in T24 cells may be one of the mechanisms causing the cytotoxicity of HBQs observed here. Thus, I further examined whether HBQ-induced ROS results in oxidative damage to these three classes of biomolecules.

2.3.3 8-OHdG in genomic DNA extracted from HBQ-treated T24 cells

8-OHdG is an important and the most abundant form of oxidative damage that leads to point mutations in genomic DNA.[28] 8-OHdG has been extensively studied and is commonly used as a sensitive measure of DNA damage.[29] Having established that the four HBQs generated intracellular ROS in T24 cells, I further investigated the generation of 8-OHdG in T24 cells as a result of HBQ treatment.

Figure 2.5 shows the relative increase of 8-OHdG in the genomic DNA of T24 cells after the cells were treated with each of the four HBQs at concentrations ranging from 25 to 125 μM. Cells treated with 75, 100, or 125 μM of 2,6-DCBQ showed significantly (P<0.05) higher levels of 8-OHdG than in the control cells. Cells treated with DCMBQ at all five concentrations (25, 50, 75, 100, and 125 μM) generated an approximately 10-fold increase in the 8-OHdG levels over that in the control cells and also much higher than the other three HBQs. However, there was no statistically significant difference among the treatment groups. Cells treated with 125 μM TriCBQ produced a significantly (P<0.05) higher level of 8-OHdG than in the controls. Treatment of cells with 75, 100, or 125 μM 2,6-DBBQ generated 8-OHdG at levels significantly higher than that in the control cells.

It is documented that point mutations induced by 8-OHdG in nuclear DNA are associated with carcinogenesis.[30, 31] Although the assessment of the levels of 8-OHdG alone is insufficient to understand the whole spectrum of oxidative DNA damage, it can provide evidence to support the potential mechanism of HBQ-induced toxicity. The generation of 8-OHdG induced by DCMBQ is higher than the other three HBQs, while the ROS generation by the four HBQs is not different at the same concentration. This difference is likely influenced by the following factors. First, DCFH-DA is a measure of the total intracellular ROS, including hydroxyl radicals, superoxide radicals, peroxy radicals, singlet oxygen, and hydrogen peroxide.[32] Of these ROS, the hydroxyl radical is the most reactive and is the predominant species that will attack DNA.[33] In addition, ROS-induced DNA damage can include base modification, single- or double-strand breaks, modification of deoxyribose, and DNA cross-links, but among them, 8-OHdG is the most abundant form of oxidative DNA damage.[33, 34] Therefore, the level of intracellular ROS may not be fully consistent with the generation of 8-OHdG. Our results may indicate that 8-OHdG generation is compound-dependent. The effective production of 8-OHdG by DCMBQ is consistent with the previous QSTR prediction that DCMBQ is more likely to be a potent

carcinogen.[8] Our results are undoubtedly supportive of the ROS-related mechanism and genotoxicity induced by HBQs.

2.3.4 Protein carbonyl levels in HBQ-treated T24 cells

Protein modification in response to oxidative stress is another possible consequence of oxidative damage to cells.[35] Protein carbonyls are produced on protein side chains, particularly on the amino acids proline (Pro), arginine (Arg), lysine (Lys), and threonine (Thr).[39] Protein carbonyls are chemically stable and allow for reliable detection.[36] Therefore, I examined the generation of protein carbonyls in HBQ-treated T24 cells.

Figure 2.6 presents the measurements of protein carbonyl levels versus the concentrations of HBQs used to treat T24 cells for 24 h. In general, protein carbonylation in T24 cells increased with the increasing concentrations of HBQs after 24 h of incubation. The observed changes in protein carbonyl levels in treatment groups are significantly higher compared to their respective control groups ($*P<0.05$). Although carbonyls are not an index of all possible oxidative modifications of proteins, the elevated levels of protein carbonyls do indicate both oxidative stress and protein dysfunction, as carbonyl groups are only produced in the presence of severe oxidative stress.[37] These results further support our hypothesis that HBQs induce oxidative stress and that protein modification caused by oxidative damage is a direct result of HBQ exposure in T24 cells.

2.3.5 Malondialdehyde (MDA) protein adducts in HBQ-treated T24 cells

Lipid peroxidation, which derives from polyunsaturated fatty acids (mainly from the membrane structures), is also a common indicator of oxidative stress in cells. Lipid peroxides are unstable and they decompose rapidly to form a series of reactive compounds, such as MDA.[38] An increase in MDA levels indicates excessive lipid peroxidation, which may result in cytotoxic changes in membrane permeability or cell death.[39] In the present study, our measurements of MDA-protein adducts in T24 cells (Figure 2.7) did not show any change when the cells were treated with each of the four HBQs at concentrations up to 125 μM. As I only examined MDA, it is not clear whether HBQs induce other products of lipid peroxidation in T24 cells. These results suggest that 1) lipids may not be the target of HBQ-induced oxidative damage when compared to DNA

and proteins, or 2) MDA may not be the main product of lipid peroxidation induced by HBQs. Further studies are warranted to elucidate these potential mechanisms.

2.4 Conclusion

This study has demonstrated the cytotoxicity of the four HBQ DBPs to T24 bladder cancer cells for the first time. The four HBQs cause significantly reduced viability of T24 cells in a concentration-dependent manner. The presence of the antioxidant NAC significantly reduces the cytotoxic effects induced by HBQs. In addition, the four HBQs can generate intracellular ROS in a concentration-dependent manner and cause oxidative damage to DNA and proteins in T24 cells. These results all suggest that an oxidative stress mechanism is involved in the cytotoxic effects determined in T24 cells after 24 h HBQ exposure. These results support that a ROS-related mechanism at least partially contributes to the HBQ-induced cytotoxicity and genotoxicity. These results are useful to developing a better understanding of the potential cellular mechanisms of toxicity of HBQs.

2.5 References

1. Zhao, Y. L.; Qin, F.; Boyd, J. M.; Anichina, J.; Li, X.-F., Characterization and determination of chloro- and bromo-benzoquinones as new chlorination disinfection byproducts in drinking water. *Anal. Chem.* **2010,** *82*, (11), 4599-4605.

2. Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; Demarini, D. M., Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutat. Res.* **2007,** *636*, (1-3), 178-242.

3. Hua, G. H.; Reckhow, D. A., Comparison of disinfection byproduct formation from chlorine and alternative disinfectants. *Water. Res.* **2007,** *41*, (8), 1667-1678.

4. Krasner, S. W.; Weinberg, H. S.; Richardson, S. D.; Pastor, S. J.; Chinn, R.; Sclimenti, M. J.; Onstad, G. D.; Thruston, A. D., Jr., Occurrence of a new generation of disinfection byproducts. *Environ. Sci. Technol.* **2006,** *40*, (23), 7175-85.

5. Villanueva, C. M.; Cantor, K. P.; Grimalt, J. O.; Malats, N.; Silverman, D.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Marcos, R.; Rothman, N.; Real, F. X.; Dosemeci, M.; Kogevinas, M., Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am. J. Epidemiol.* **2007,** *165*, (2), 148-156.

6. Cantor, K. P.; Villanueva, C. M.; Silverman, D. T.; Figueroa, J. D.; Real, F. X.; Garcia-Closas, M.; Malats, N.; Chanock, S.; Yeager, M.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Samanic, C.; Rothman, N.; Kogevinas, M., Polymorphisms in GSTT1, GSTZ1, and CYP2E1, disinfection by-products, and risk of bladder cancer in Spain. *Environ. Health. Perspec.* **2010,** *118*, (11), 1545-1550.

7. Krasner, S. W., The formation and control of emerging disinfection by-products of health concern. *Philos. Trans. A. Math. Phys. Eng. Sci.* **2009,** *367*, (1904), 4077-95.

8. Bull, R. J.; Reckhow, D. A.; Li, X.; Humpage, A. R.; Joll, C.; Hrudey, S. E., Potential carcinogenic hazards of non-regulated disinfection by-products: haloquinones, halo-cyclopentene and cyclohexene derivatives, N-halamines, halonitriles, and heterocyclic amines. *Toxicology* **2011,** *286*, (1-3), 1-19.

9. Bull, R. J., *Use of Toxicological and Chemical Models to Prioritize DBP Research*. AWWA Research Foundation: Denver, CO, 2006.

10. Zhao, Y. L.; Anichina, J.; Lu, X.-F.; Bull, R. J.; Krasner, S. W.; Hrudey, S. E.; Li, X. F., Occurrence and formation of chloro- and bromo-benzoquinones during drinking water disinfection. *Water Res.* **2012,** *46*, (14), 4351-4360.

11. O'Brien, P. J., Molecular mechanisms of quinone cytotoxicity. *Chem. Biol. Interact.* **1991,** *80*, (1), 1-41.

12. Bolton, J. L.; Trush, M. A.; Penning, T. M.; Dryhurst, G.; Monks, T. J., Role of quinones in toxicology. *Chem. Res. Toxicol.* **2000,** *13*, (3), 135-160.

13. Lin, P. H.; Waidyanatha, S.; Pollack, G. M.; Swenberg, J. A.; Rappaport, S. M., Dose-specific production of chlorinated quinone and semiquinone adducts in rodent livers following administration of pentachlorophenol. *Toxicol. Sci.* **1999,** *47*, (1), 126-133.

14. Jeong, J. K.; Wogan, G. N.; Lau, S. S.; Monks, T. J., Quinol-glutathione conjugate-induced mutation spectra in the supF gene replicated in human AD293 cells and bacterial MBL50 cells. *Cancer Res.* **1999,** *59*, (15), 3641-5.

15. Siraki, A. G.; Chan, T. S.; O'Brien, P. J., Application of quantitative structure-toxicity relationships for the comparison of the cytotoxicity of 14 p-benzoquinone congeners in primary cultured rat hepatocytes versus PC12 cells. *Toxicol Sci.* **2004,** *81*, (1), 148-159.

16. Anichina, J.; Zhao, Y. L.; Hrudey, S. E.; Le, X. C.; Li, X.-F., Electrospray ionization mass spectrometry characterization of interactions of newly identified water disinfection byproducts halobenzoquinones with oligodeoxynucleotides. *Environ. Sci. Technol.* **2010,** *44*, (24), 9557-9563.

17. Zhu, B. Z.; Zhao, H. T.; Kalyanaraman, B.; Liu, J.; Shan, G. Q.; Du, Y. G.; Frei, B., Mechanism of metal-independent decomposition of organic hydroperoxides and formation of alkoxyl radicals by halogenated quinones. *Proc. Natl. Acad. Sci. USA.* **2007,** *104*, (10), 3698-3702.

18. Wang, G.; Gong, Y.; Burczynski, F. J.; Hasinoff, B. B., Cell lysis with dimethyl sulphoxide produces stable homogeneous solutions in the dichlorofluorescein oxidative stress assay. *Free Radic. Res.* **2008,** *42*, (5), 435-441.

19. Boyd, J. M.; Huang, L.; Xie, L.; Moe, B.; Gabos, S.; Li, X.-F., A cell-microelectronic sensing technique for profiling cytotoxicity of chemicals. *Anal. Chim. Acta.* **2008,** *615*, (1), 80-87.

20. Bannai, S., Induction of cystine and glutamate transport activity in human-fibroblasts by diethyl maleate and other electrophilic agents. *J. Biol. Chem.* **1984,** *259*, (4), 2435-2440.
21. Aruoma, O. I.; Halliwell, B.; Hoey, B. M.; Butler, J., The antioxidant action of N-acetylcysteine - its reaction with hydrogen-peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic. Bio. Med.* **1989,** *6*, (6), 593-597.
22. Haslam, G.; Wyatt, D.; Kitos, P. A., Estimating the number of viable animal cells in multi-well cultures based on their lactate dehydrogenase activities. *Cytotechnology* **2000,** *32*, (1), 63-75.
23. Buttke, T. M.; Mccubrey, J. A.; Owen, T. C., Use of an aqueous soluble tetrazolium formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. *J. Immunol. Methods* **1993,** *157*, (1-2), 233-240.
24. Trachootham, D.; Alexandre, J.; Huang, P., Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat. Rev. Drug Discov.* **2009,** *8*, (7), 579-91.
25. Boonstra, J.; Post, J. A., Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. *Gene* **2004,** *337*, 1-13.
26. Schafer, F. Q.; Buettner, G. R., Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Bio. Med.* **2001,** *30*, (11), 1191-1212.
27. Perry, G.; Raina, A. K.; Nunomura, A.; Wataya, T.; Sayre, L. M.; Smith, M. A., How important is oxidative damage? Lessons from Alzheimer's disease. *Free Radic. Bio. Med.* **2000,** *28*, (5), 831-834.
28. Cooke, M. S.; Evans, M. D.; Dizdaroglu, M.; Lunec, J., Oxidative DNA damage: mechanisms, mutation, and disease. *Faseb J.* **2003,** *17*, (10), 1195-1214.
29. Ravanat, J. L., Measuring Oxidized DNA lesions as biomarkers of oxidative stress: an analytical challenge. *FABAD J. Pharm. Sci.* **2005,** *30*, 100-113.
30. Wu, L. L.; Chiou, C. C.; Chang, P. Y.; Wu, J. T., Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. *Clin. Chim. Acta.* **2004,** *339*, (1-2), 1-9.
31. Kryston, T. B.; Georgiev, A. B.; Pissis, P.; Georgakilas, A. G., Role of oxidative stress and DNA damage in human carcinogenesis. *Mutat. Res.* **2011,** *711*, (1-2), 193-201.

32. Waris, G.; Ahsan, H., Reactive oxygen species: role in the development of cancer and various chronic conditions. *J. Carcinog.* **2006,** *5*, 14.

33. Klaunig, J. E.; Kamendulis, L. M.; Hocevar, B. A., Oxidative stress and oxidative damage in carcinogenesis. *Toxicol. Pathol.* **2010,** *38*, (1), 96-109.

34. Orrenius, S.; Gogvadze, V.; Zhivotovsky, B., Mitochondrial oxidative stress: implications for cell death. *Annu. Rev. Pharmacol. Toxicol.* **2007,** *47*, 143-83.

35. Dalle-Donne, I.; Rossi, R.; Giustarini, D.; Milzani, A.; Colombo, R., Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta.* **2003,** *329*, (1-2), 23-38.

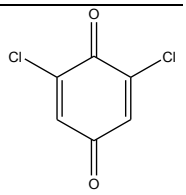
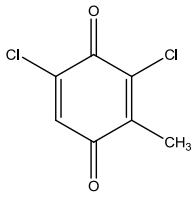
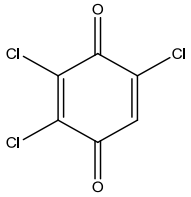
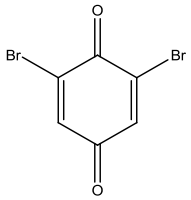
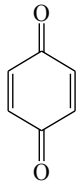
36. Berlett, B. S.; Stadtman, E. R., Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **1997,** *272*, (33), 20313-6.

37. Banfi, C.; Brioschi, M.; Barcella, S.; Veglia, F.; Biglioli, P.; Tremoli, E.; Agostoni, P., Oxidized proteins in plasma of patients with heart failure: role in endothelial damage. *Eur. J. Heart Fail.* **2008,** *10*, (3), 244-51.

38. Col, C.; Dinler, K.; Hasdemir, O.; Buyukasik, O.; Bugdayci, G., Oxidative stress and lipid peroxidation products: effect of pinealectomy or exogenous melatonin injections on biomarkers of tissue damage during acute pancreatitis. *Hepatobiliary Pancreat. Dis. Int.* **2010,** *9*, (1), 78-82.

39. Devasagayam, T. P.; Boloor, K. K.; Ramasarma, T., Methods for estimating lipid peroxidation: an analysis of merits and demerits. *Indian J. Biochem. Biophys.* **2003,** *40*, (5), 300-8.

Table 2.1 Characteristics of the halobenzoquinones analyzed in this chapter

Halobenzoquinones	CAS [#]	Molecular Formula	Source and Purity	Structure
2,6-dichloro-1,4-benzoquinone, 2,6-DCBQ	697-91-6	$C_6H_2Cl_2O_2$	ALDRICH, 98%	
2,6-dichloro-3-methyl-1,4-benzoquinone, DCMBQ	40100-98-9	$C_7H_4Cl_3O_2$	ALDRICH, 98%	
2,3,6-trichloro-1,4-benzoquinone, TriCBQ	634-85-5	$C_6HCl_3O_2$	Shanghai Acana Pharmtech Co., ⩾98%	
2,6-dibromo-1,4-benzoquinone, 2,6-DBBQ	19643-45-9	$C_6H_2Br_2O_2$	INDOFINE Chemical Company, ⩾ 98%	
1,4-benzoquinone, BQ	106-51-4	$C_6H_4(=O)_2$	Fluka, ≥99.5% (HPLC)	

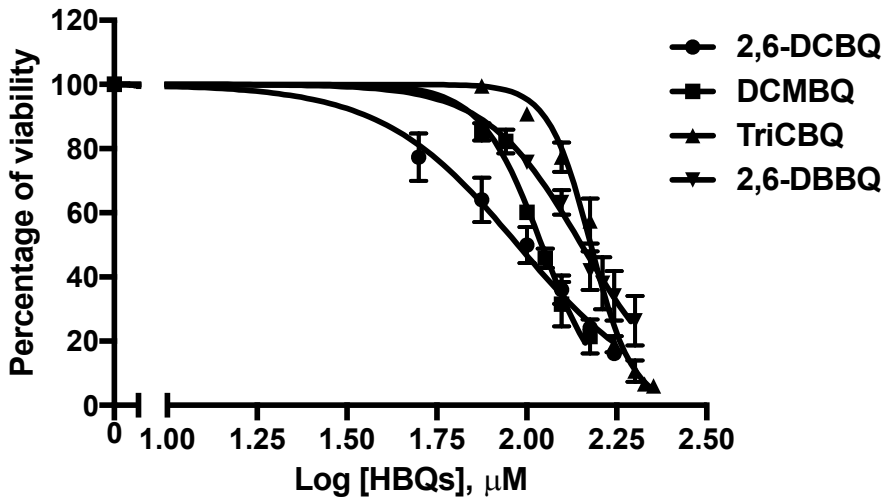


Figure 2.1. Effects of HBQs on the viability of T24 cells after 24 h exposure. The MTS assay was used. All values are expressed as mean ± SEM. For each concentration, six replicates were incubated. Three sets of experiments were performed. The IC_{50} values (with 95% confidence intervals in parentheses) are 94.5 (86.9–102.7) μM for 2,6-DCBQ, 110.1 (106.5–113.8) μM for DCMBQ, 150.7 (146.8–154.6) μM for TriCBQ, and 142.0 (133.1–151.5) μM for 2,6-DBBQ.

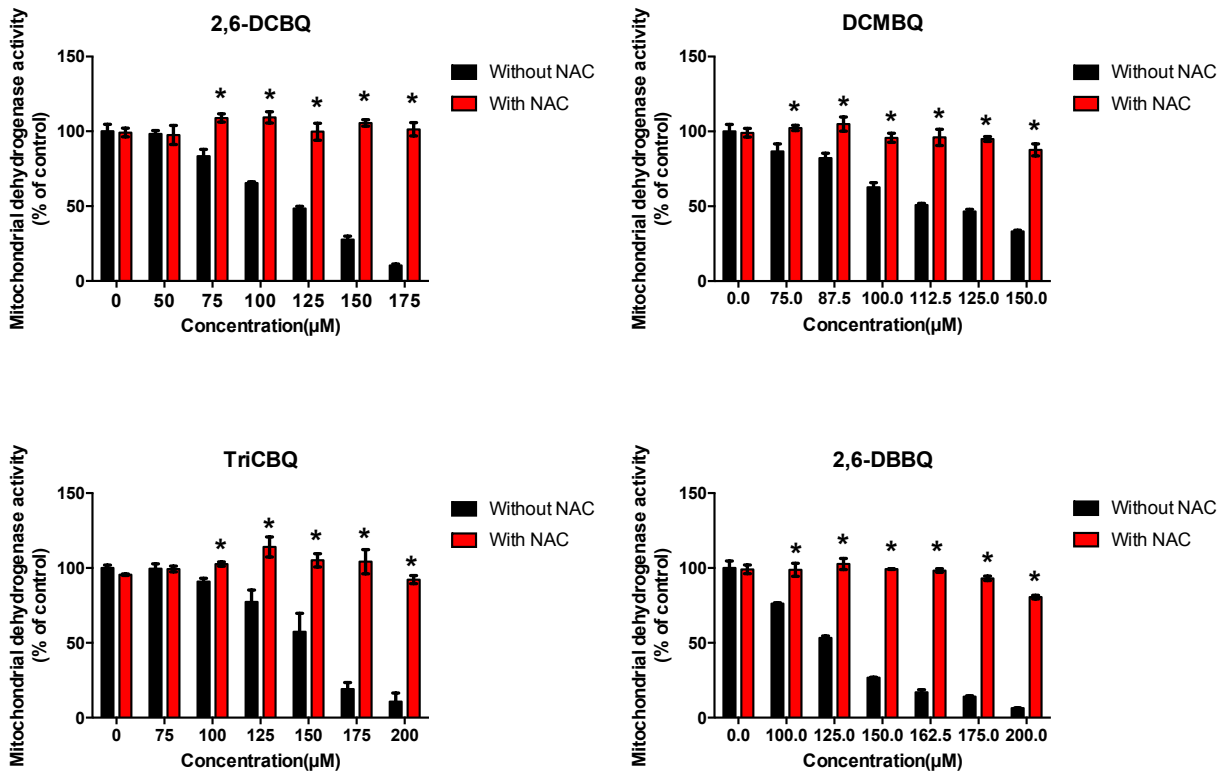


Figure 2.2 Effects of HBQs with or without 1 mM NAC on the viability of T24 cells after 24 h exposure. The MTS assay was used. All values are expressed as mean ± SD. For each concentration, six replicates were incubated. Three sets of experiments were performed. **P*<0.05, HBQ + NAC treatment groups compared with HBQ treatment groups.

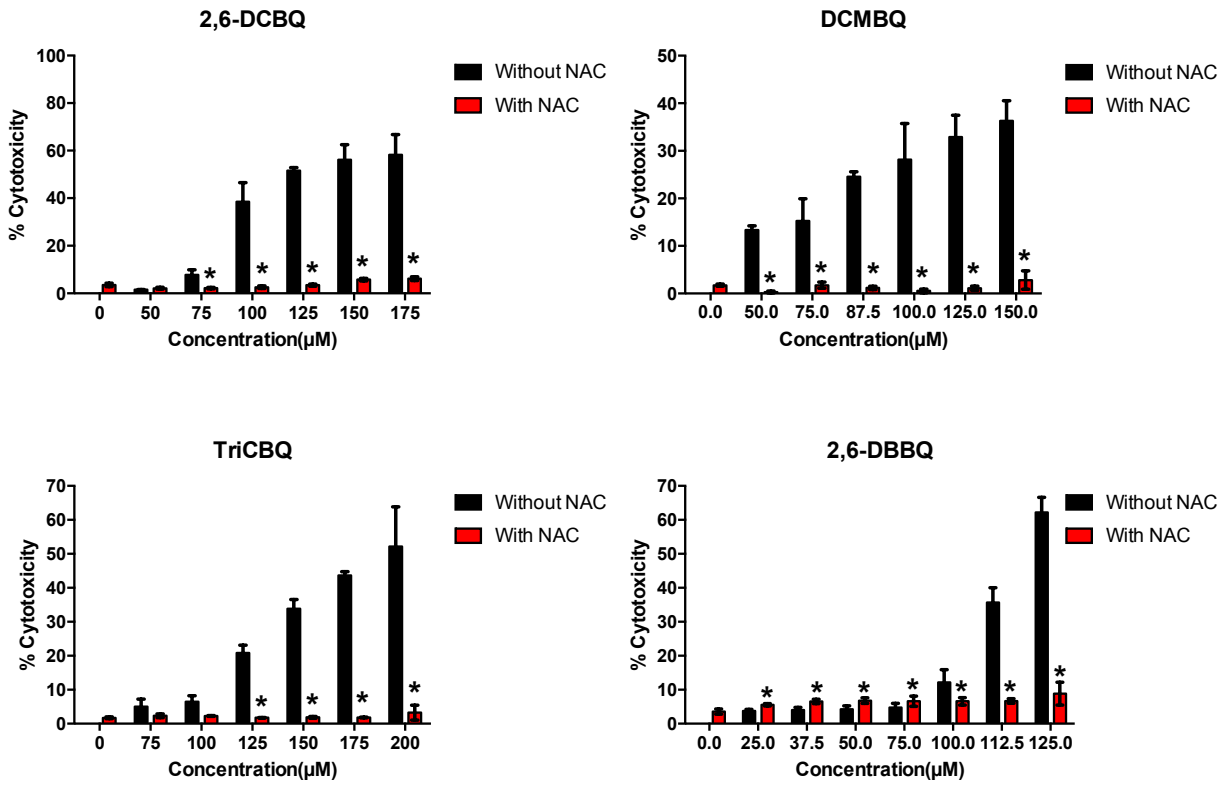


Figure 2.3 Effects of HBQs with or without 1 mM NAC on the viability of T24 cells after 24 h exposure. The LDH assay was used. All values are expressed as mean ± SD. For each concentration, six replicates were incubated. Three sets of experiments were performed. The percent cytotoxicity was calculated using the formula: [(Experimental - Control Cells)/(Maximum LDH Release of Control Cells - Control Cells)]×100. **P*<0.05, HBQ + NAC treatment groups compared with HBQ treatment groups.

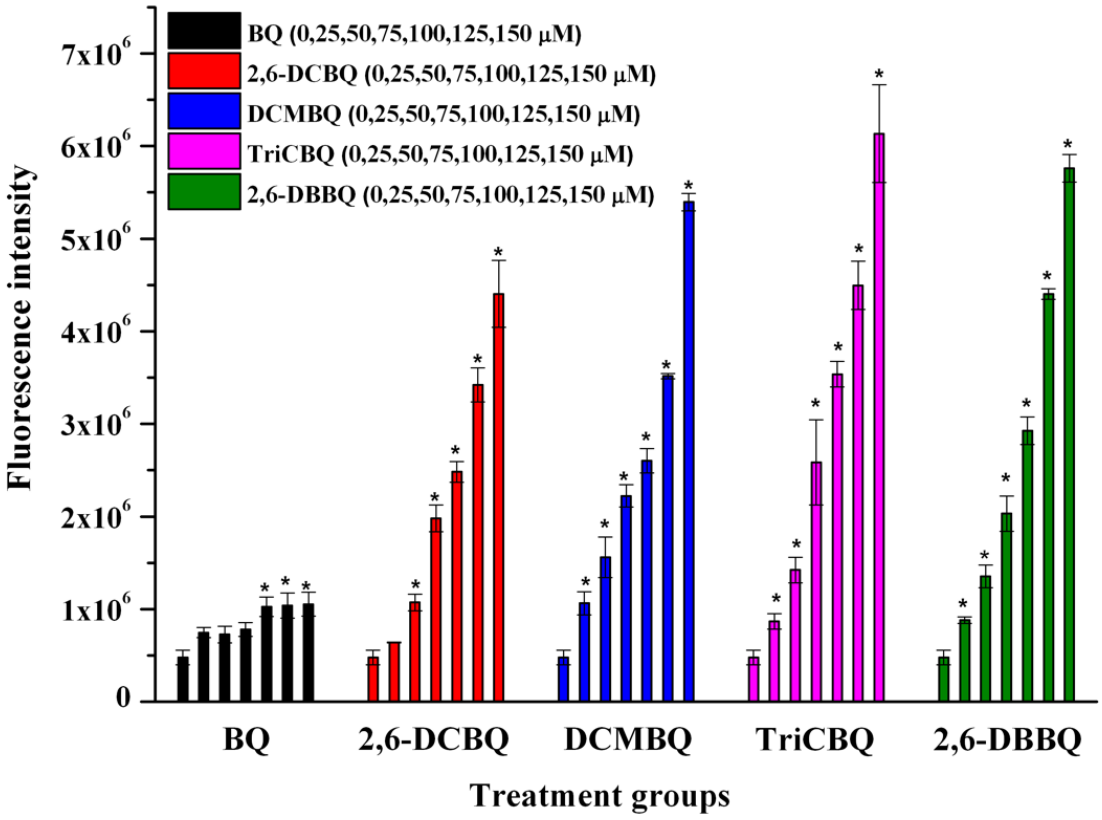


Figure 2.4 Levels of intracellular ROS in T24 cells after 24 h exposure to 25, 50, 75, 100, 125, 150 μM of BQ, 2,6-DCBQ, DCMBQ, TriCBQ, and 2,6-DBBQ. All values are expressed as mean ± SD. For each concentration, six replicates were incubated. Three sets of experiments were performed. Significant differences were observed in all treatment groups of HBQs compared to their respective control group (*P<0.05).

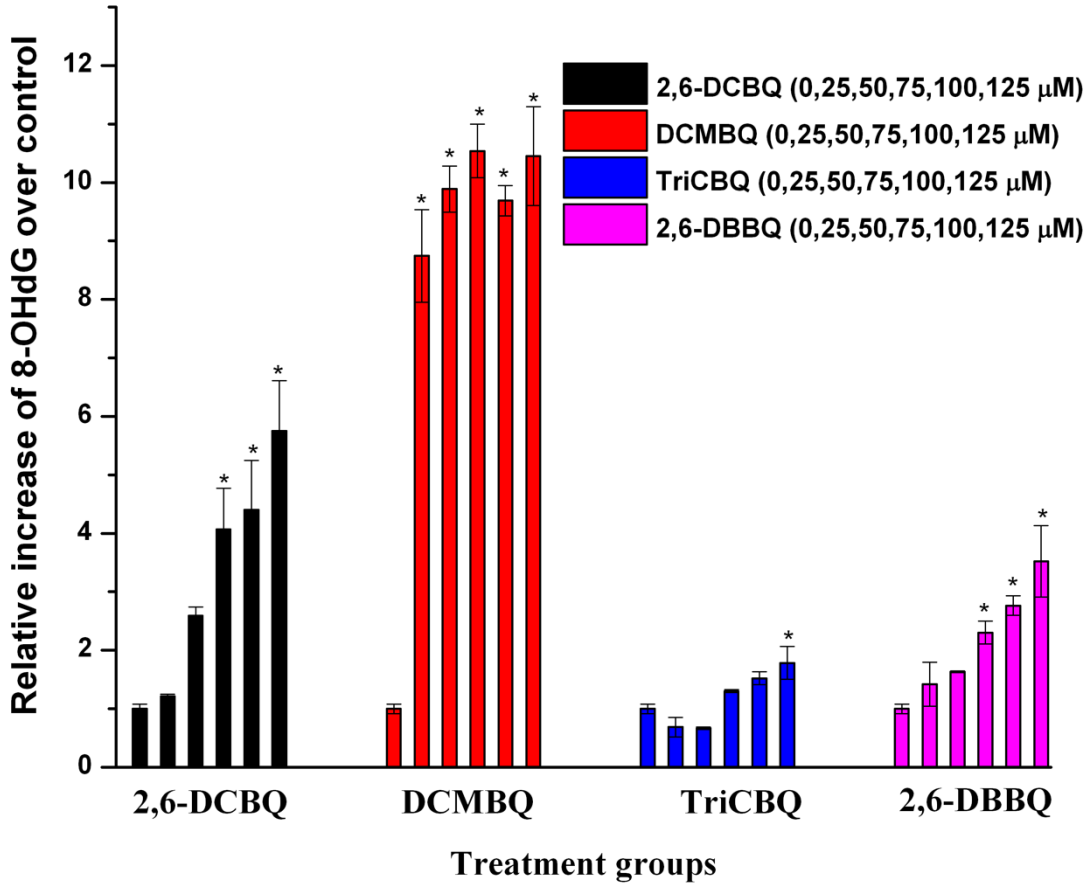


Figure 2.5 Relative increase of 8-hydroxydeoxyguanosine (8-OHdG) in genomic DNA of T24 cells after the cells were treated with one of the four HBQs (25–125 μM). All values are expressed as mean ± SD. For each concentration, two replicates were incubated. Three sets of experiments were performed. The values represent the fold increase of 8-OHdG measured in the treated cells over that in the control cells. Significant differences between treatment groups of HBQs and their respective control group are denoted (*P<0.05).

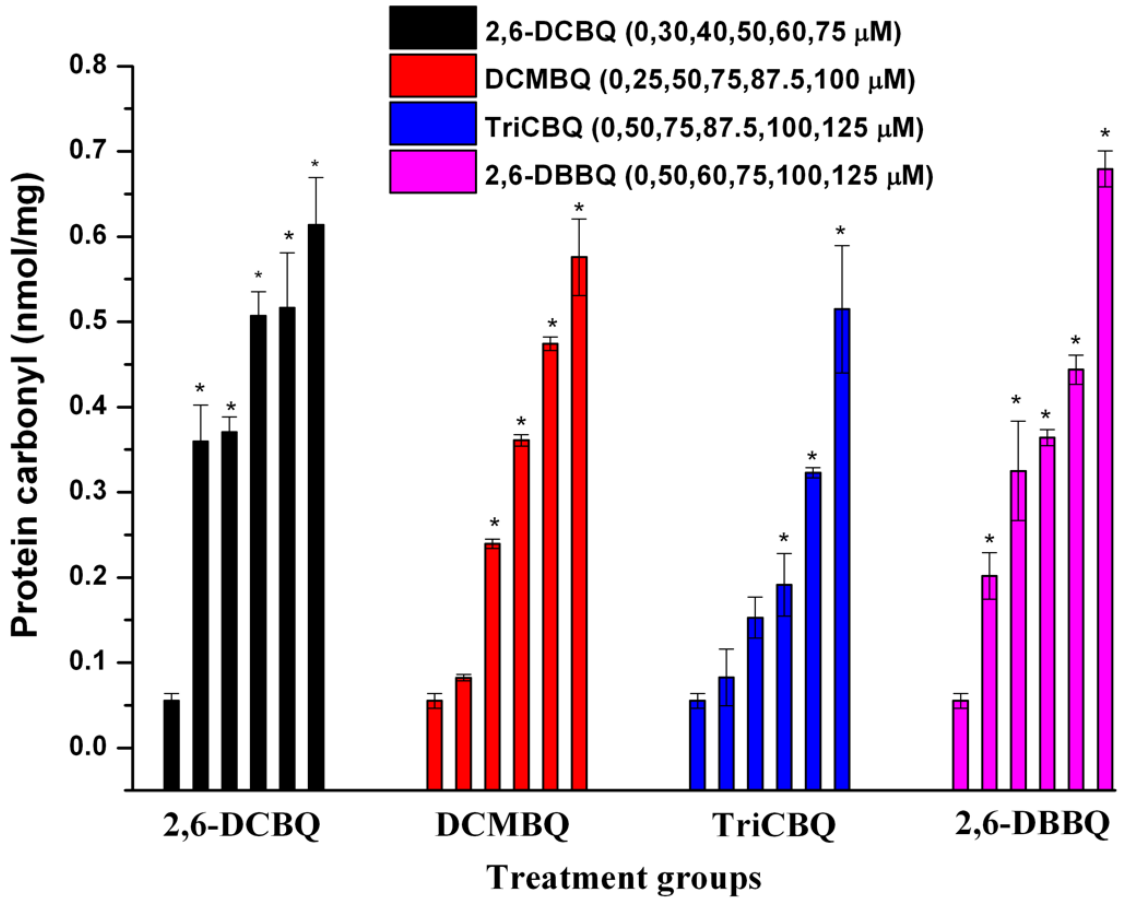


Figure 2.6 Concentrations of protein carbonyl in T24 cells after incubation with one of the four HBQs for 24 h. All values are expressed as mean ± SD. For each concentration, two replicates were incubated. Three sets of experiments were performed. The 24 h treatment of T24 cells with 2,6-DCBQ (30, 40, 50, 60, 75 μM), DCMBQ (50, 75, 87.5, and 100 μM), TriCBQ (87.5, 100, 125 μM), and 2,6-DBBQ (50, 60, 75, 100, and 125 μM) produced statistically higher (*P <0.05) contents of protein carbonyl than in the control cells.

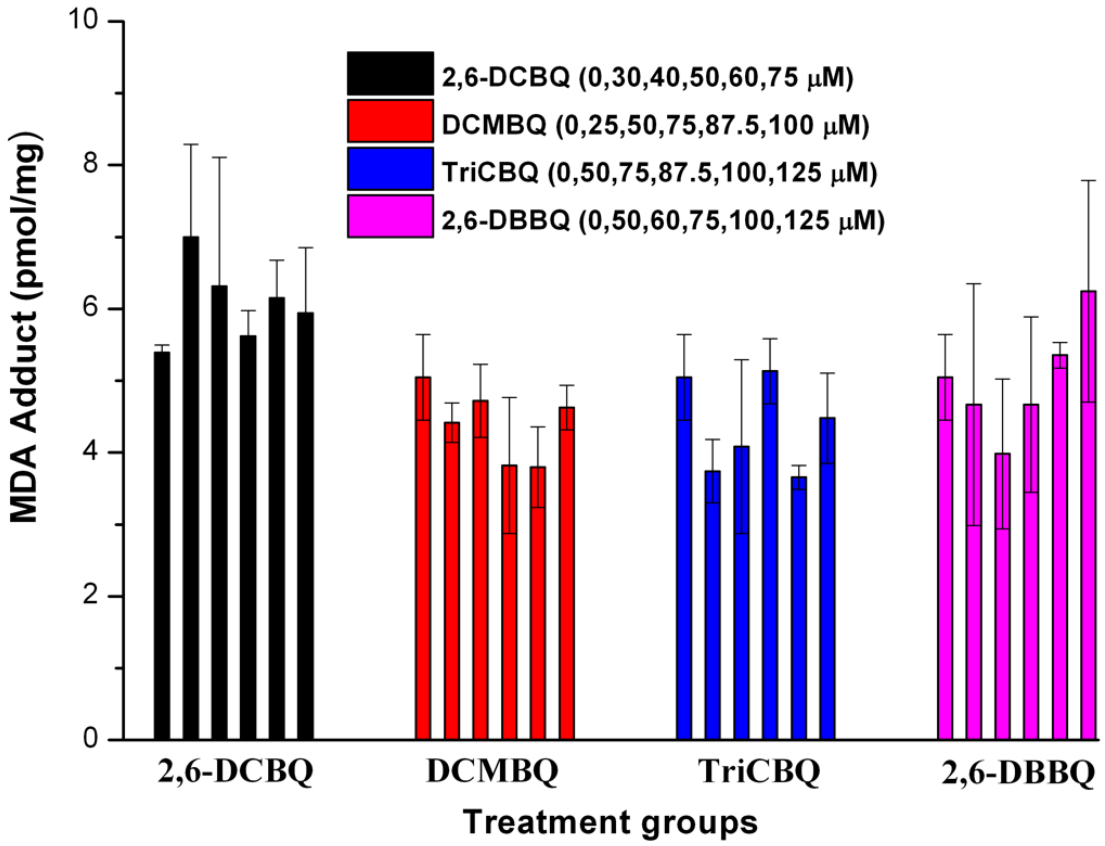


Figure 2.7 Malondialdehyde (MDA) adducts of proteins in T24 cells after incubation with one of the HBQs for 24 h. All values are expressed as mean ± SD. For each concentration, two replicates were incubated. Three sets of experiments were performed. There was no statistically significant difference ($P > 0.05$) between the controls and each of the treated groups.

Chapter 3 Quantitative structure–toxicity relationship of HBQs [4]

3.1 Introduction

As mentioned previously, quantitative structure–toxicity relationship (QSTR) analysis has predicted that HBQs may have the potency to be carcinogenic.[1] This prediction was based on the structural similarity of the HBQs to 1,4-benzoquinone (BQ), a highly reactive metabolite of benzene, a known human carcinogen. Our preliminary studies of these four identified HBQ DBPs, 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), 2,3,6-trichloro-1,4-benzoquinone (TriCBQ), 2,6-dibromo-1,4-benzoquinone (2,6-DBBQ), and 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ), indicate that they induce greater cytotoxicity than the regulated DBPs in the normal mammalian cell line, Chinese hamster ovary (CHO-K1).[2] In Chapter 2, the *in vitro* experiments also demonstrated that HBQs effectively produce a significant amount of cellular reactive oxygen species (ROS) and further induce oxidative stress in the human bladder carcinoma cell line, T24.[3, 4] These findings indicate that HBQs are highly cytotoxic, and oxidative damage is suspected to be one of the key mechanisms of HBQ-induced cytotoxicity.[5] Genotoxicity studies of some HBQs have found that HBQs can interact with DNA both directly and indirectly (via ROS), causing DNA adducts, strand breaks, and apyrimidinic/apurinic (AP) sites.[5] A recent study has shown that 2,6-DCBQ and 2,6-DBBQ can induce activation of the Nrf2/ARE pathway (associated with oxidative stress) and the p53 pathway (associated with DNA damage), with the p53 pathway more sensitive to these HBQ exposures.[6]

The structure of a xenobiotic can heavily influence its reactivity and resulting biological/toxicological effects.[7] Several studies have shown that the halogen substitution groups on DBPs can significantly affect their toxicity, mutagenicity, and genotoxicity.[8-11] For example, the cytotoxicity of iodoacetic acid (IAA) in CHO cells was approximately 3 times and 287 times higher than its brominated and chlorinated analogs, respectively.[8] Haloketones have shown isomer-related effects on cytotoxicity, mutagenicity, and genotoxicity.[12-14] Richardson et al. have observed that the geometric isomer (E)-3-bromo-2-iodopropenoic acid induces greater toxicity

[4] This chapter 3 has been published as Jinhua Li, Birget Moe, Sai Vemula, Wei Wang, and Xing-Fang Li. Emerging disinfection by-products, halobenzoquinones: effects of isomeric structure and halogen substitution on cytotoxicity, formation of reactive oxygen species, and genotoxicity. Environ. Sci. Technol., 2016, 50 (13), 6744–6752.

than its Z isomer in CHO cells.[15] Zhang et al. have also observed different developmental toxicity in a marine polychaete exposed to 2,4-dibromophenol, which was 13.6 times more potent than its isomer, 2,6-dibromophenol, and this was also observed in studies of algal growth inhibition.[10, 16] However, the toxicological effects of HBQs varying in isomeric structure, halogen substitution groups (chloro-, bromo-, iodo-), and number of substitutions (mono-, di-, tri-, tetra-) have not been systematically investigated.

The objective of this study is to systematically examine the effects of isomeric structure and halogen substitution on the cytotoxicity, formation of ROS, and genotoxicity of eight HBQs in CHO-K1 cells. The experimental results will be correlated with physicochemical parameters, including hydrophobicity, electronic properties, and steric properties, to examine potential structure–toxicity relationships. To this end, I have selected eight HBQs: 2,6-DCBQ, 2,6-DBBQ, TriCBQ, tetrachloro-1,4-benzoquinone (TetraCBQ), 2,5-dichloro-1,4-benzoquinone (2,5-DCBQ), 2,5-dibromo-1,4-benzoquinone (2,5-DBBQ), 2-chloro-1,4-benzoquinone (2-CBQ), and 2,3-diiodo-1,4-benzoquinone (2,3-DIBQ). The selection of these HBQs takes into account the known cytotoxicity of the four initially identified HBQ DBPs, the possibility of their formation as DBPs, as well as the availability of standard compounds. Two isomeric pairs 1) 2,6-DCBQ/2,5-DCBQ and 2) 2,6-DBBQ/2,5-DBBQ will be used to examine isomeric structure effects, while the di-chloro-HBQs (2,6-DCBQ, 2,5-DCBQ), di-bromo-HBQs (2,6-DBBQ, 2,5-DBBQ), and a di-iodo-HBQ (2,3-DIBQ) will compare effects of different halogen substitutions. The effects of varying number of chlorine substitutions will be analyzed with mono- (2-CBQ), di- (2,6-DCBQ, 2,5-DCBQ), tri- (TriCBQ), and tetra-substituted (TetraCBQ) chloro-HBQs.

3.2 Materials and Methods

3.2.1 Reagents

Standards of 2,6-DCBQ, 2,5-DCBQ, 2,5-DBBQ, 2-CBQ, and TetraCBQ were purchased from Sigma-Aldrich (St. Louis, MO, USA). TriCBQ and 2,3-DIBQ were purchased from Shanghai Acana Pharmtech (Shanghai, China), and 2,6-DBBQ was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). The chemical structures, molecular formulas, and purity of these HBQs are listed in Table 3.1. HBQs were dissolved in methanol (HPLC grade, Fisher Scientific, Burlington, ON, Canada) as stock solutions and stored at −20°C in sterile amber glass

vials. Dulbecco's phosphate-buffered saline (DPBS) was purchased from Thermo Fisher Scientific (Burlington, ON, Canada).

3.2.2 Cell culture

The CHO-K1 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). CHO-K1 was chosen to evaluate the cytotoxicity of HBQs, since this normal mammalian cell line is widely used in DBP *in vitro* toxicity studies.[17, 18] The cells were cultured in DMEM/F12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON, Canada) and 1% penicillin/streptomycin (100 U/100 µg/mL, Invitrogen, Carlsbad, CA, USA) and were maintained at 37°C in a 5% CO_2 incubator. The cells were sub-cultured twice weekly into standard 10 cm × 20 mm cell culture dishes (Corning Inc., Corning, NY, USA).

3.2.3 Cytotoxicity[5]

Cytotoxicity of HBQs on CHO-K1 cells was analyzed using the xCELLigence real-time cell analysis (RTCA) system (ACEA Biosciences, San Diego, CA). RTCA is a well-established method used in our laboratory to evaluate the cytotoxic effects of environmental contaminants, including nanoparticles, particulate matter, and some DBPs.[19-22] The key component of the RTCA system is the 96-well E-plate, which is embedded with microelectrodes in each micro-well. Live, adherent cells can grow on the surface of the microelectrodes. As the cell population and/or cell attachment increases, impedance continuously increases on the cell-microelectrodes. Impedance is continuously monitored to provide real-time sensing. A 96-well E-plate is electronically connected with the E-plate station located in the CO_2 incubator, which in turn is connected to the system analyzer. The impedance changes caused by cells interacting with the microelectrodes of the RTCA 96-well E-plate are automatically converted to the cell index (CI). CI is calculated via the following equation:

$$CI = \max_{i=1,\ldots N}\left[\frac{R_{cell}(fi)}{R_b(fi)} - 1\right],$$

where Rcell(fi) is the impedance of the microelectrode when cells are bound and Rb(fi) is the

[5] Mr. Sai Vemula contributed to the preliminary experiments of cytotoxicity.

same parameter with no cells bound, and N is the number of frequency points at which impedance is measured (N=3), selected by the instrument to maximize signal. When more cells are attached to the microelectrodes, Rcell is higher. The number of adherent cells in each well positively correlates with CI.

An initial population of 6000 cells (CHO-K1) was seeded into each well of the 96-well E-plate. When the CI reached 1 (around 20 h), the cells in each well were treated with HBQs at a given concentration. This was completed by replacing the culture media with the testing media containing different concentrations of each of the HBQs. The concentration ranges for each HBQ tested in CHO-K1 cells were 2,6-DCBQ (25–75 μM), 2,5-DCBQ (10–50 μM), 2,6-DBBQ (10–75 μM), 2,5-DBBQ (10–50 μM), 2-CBQ (10–50 μM), TriCBQ (25–60 μM), TetraCBQ (15–62.5 μM), and 2,3-DIBQ (5–37.5 μM). The concentration ranges were selected based on preliminary experiments. The CI was recorded hourly for 60 h after HBQ treatment. Triplicate testing was performed for each concentration, and the assay for each HBQ was repeated three times. Solvent controls (cells treated with 1% (v/v) methanol in culture medium, the highest concentration of solvent used) were included to evaluate solvent interference and negative controls (cells treated with culture medium without addition of HBQs) were included to determine the natural growth of cells.

Here, hourly IC_{50} values were determined using the CI values over time for each concentration of HBQ. For each time point, CI values for each treatment concentration, as seen in the cytotoxicity profiles in Figure 3.1, were first transformed to a percent response scale (0-100%) relative to the CI of the untreated controls, which was defined as 100%. Each concentration was transformed to its logarithmic value. A sigmoidal dose–response curve was then generated at each time point after exposure, presenting percent response as a function of the logarithm of the treatment concentration. From these dose–response curves, IC_{50} values were derived. These values were then plotted over the exposure period to generate IC_{50} histograms, as seen in Figure 3.2.

3.2.4 Determination of cellular ROS

ROS production was determined by measuring 2',7'-dichlorofluorescein, the final oxidation product of 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich), as described previously.[23] CHO-K1 cells were plated in a 96-well black plate at a density of 1.5×10^5 cells/mL

and grown to 70–80% confluency. After washing twice with DPBS, cells were treated with 25 μM DCFH-DA in culture medium without FBS at 37 °C for 45 min. Cells were then washed twice with DPBS and treated with HBQ at a range of concentrations at or under their respective 24 h IC_{50} values. After a 6 h, 12 h, or 24 h exposure, the plate was read (λ_{ex} = 485 nm, λ_{em} = 535 nm) in a fluorescence microplate reader (Beckman Coulter DTX880 Multimode Detector). Negative controls and solvent controls were included as described in the above RTCA experiments. Four replicates were used for each concentration, and the experiment was repeated three times.

3.2.5 Determination of 8-hydroxy-2'-deoxyguanosine (8-OHdG)

Oxidative DNA damage was measured using the OxiSelect Oxidative DNA Damage ELISA Kit (Cell Biolabs, San Diego, CA, USA), according to the protocols of the manufacturer. Briefly, CHO-K1 cells were treated at the same concentration of relative biological response (the 24h IC_{50} value of each HBQ), and total genomic DNA was extracted after 12 h and 24 h exposure using the DNeasy Blood & Tissue Kit (QIAGEN, Toronto, ON, Canada). DNA yield was determined by measuring absorbance at 260 nm on a NanoVue Plus (GE Healthcare, UK). The quantity of 8-OHdG was expressed as amount of 8-OHdG/total DNA (pg/μg), and was normalized against the negative control. Replicates were used for each HBQ at each time point, and the experiment was repeated twice.

3.2.6 Assessment of p53 protein

The expression of p53 protein was measured using the high-throughput Pierce p53 Colorimetric In-Cell ELISA Kit (Pierce Biotechnology, Rockford, lL, USA) following the manufacturer's instructions. Briefly, CHO-K1 cells were seeded into 96-well clear plates. Cells were treated with the same concentration of biological response, the 24 h IC_{50} value of each HBQ. After 12 h and 24 h exposure, expression of p53 protein was determined in each well by measuring the absorbance at 450 nm (A_{450}). The cell number in each well was determined using whole-cell stain, with absorbance measured at 615 nm (A_{615}). The level of p53 protein was normalized first as A_{450}/A_{615}, followed by normalization against the negative control. Triplicates were used for each HBQ at each time point, and the experiment was repeated twice.

3.2.7 Physicochemical parameters

Molecular structures of all HBQs were prepared using ChemDraw (PerkinElmer, Waltham, MA, USA). The physicochemical parameters chosen were restricted to those that might be associated with the toxicity of HBQs, as shown in Table 3.2. Calculated LogP and molar refractivity (R) were generated using the ACD/Labs Percepta Platform–PhysChem Module. The energy of the highest occupied molecular orbital (E_{HOMO}), the energy of the lowest unoccupied molecular orbital (E_{LUMO}), and dipole moment (μ) were predicted data generated using MOPAC2012 after optimization of structure geometry using PM7 parameters.[24] Molar surface area (S) was predicted using ChemAxon (http://www.chemaxon.com).

3.2.8 Statistical analysis

Data from the experiments were transferred to Excel spreadsheets and analyzed using GraphPad Prism 6. The analyzed results from separate experiments were averaged and expressed as mean ± standard error of the mean (SEM), with n=3 for the RTCA and ROS assays, and n=2 for the 8-OHdG and p53 assays. The normalized cell index (NCI) data was analyzed to fit concentration–response curves and to obtain IC_{50} values, defined as the concentration of HBQ causing a 50% reduction in relative NCI of the control cells to that of the treated cells at a given time. Fluorescence intensity of ROS production was first normalized against the negative controls, and then analyzed by linear regression to obtain the linear equation. One-way analysis of variance (ANOVA) was used for multiple comparisons among different HBQ treatments. Student's t-test was used to compare cytotoxicity between HBQ isomer pairs. Comparison between physicochemical parameters and toxicological parameters was made by Pearson correlation and linear and/or multilinear regression analyses. The difference was considered to be statistically significant at $P<0.05$.

3.3 Results and Discussion

3.3.1 HBQ-induced cytotoxicity

Figure 3.1 shows the real-time response of CHO-K1 cells to the eight HBQs determined using RTCA. Simultaneous measurement of the control and treated cells over the entirety of the exposure period enabled an accurate comparative measurement of cytotoxicity. This is a key

factor for determining a correlation between the observed cytotoxicity and the physicochemical properties of the structurally similar groups of HBQs under investigation. Furthermore, I found that experiments repeated on different E-plates generated highly reproducible results. The real time continuous response profiles of CHO-K1 cells to the eight HBQs (Figures 3.1 A-H) also clearly demonstrate the compound-, time-, and concentration-dependent cytotoxicity of the eight HBQs, providing dynamic cell response information.

To quantitatively compare the cytotoxic effects of HBQs, I calculated IC_{50} values from the continuous response data at each time point to generate temporal IC_{50} histograms over the 60 h of treatment. Figure 3.2 shows the IC_{50} histograms of each HBQ in CHO-K1 cells. At a given time point, the IC_{50} is determined by the NCI of the treated cells relative to the NCI of control cells without HBQ treatment. The IC_{50} histograms of the eight HBQs in CHO-K1 cells clearly differentiate their cytotoxicity. The 24h IC_{50} values (Table 3.3) for CHO-K1 cells rank the cytotoxicity of the eight HBQs from most toxic to least toxic in the order: 2,3-DIBQ ≥ 2-CBQ > 2,5-DBBQ > TetraCBQ ≈ 2,5-DCBQ ≥ 2,6-DBBQ > 2,6-DCBQ ≥ TriCBQ. A similar trend was observed for 12 h and 6 h exposure, as shown in Table 3.3.

The effect of isomeric structure on the cytotoxicity of HBQs is clearly demonstrated by the IC_{50} histograms of the two pairs of HBQ isomers. Both 2,5-DBBQ and 2,5-DCBQ are significantly more cytotoxic than their corresponding 2,6-HBQ isomers at 6 h, 12 h, and 24 h post exposure (Student's t-test, $P<0.05$). Furthermore, the 24 h IC_{50} values of 2,6-DBBQ and 2,5-DBBQ are significantly lower than those of 2,6-DCBQ and 2,5-DCBQ, respectively, in CHO-K1 cells ($P<0.05$). This observation is consistent with some reported results on isomeric haloketone DBPs. 1,3-dichloropropanone induced levels of mutagenicity 2.4-fold higher than its isomer 1,1-dichloropropanone, and 1,1,3-trichloropropanone induced levels of mutagenicity 2.4-fold higher than its isomer 1,1,1-trichloropropanone using the Ames assay.[14] These results support the importance of considering isomeric structure effects when assessing human exposure and potential health risks of DBPs.

The cytotoxicity of HBQs is dependent on the type of halogen substitution. As shown in Figure 3.2, chloro-, bromo- and iodo-substitutions of HBQ show a trend of cytotoxicity in decreasing order: di-iodo- > di-bromo- > di-chloro-HBQ, when the same isomeric structures are compared. It is important to note that our study was limited to 2,3-DIBQ, as it was the only commercially available iodo-HBQ. This trend is consistent with the reported cytotoxicity of

other DBPs, such as the HAAs, where IAA > bromoacetic acid (BAA) > chloroacetic acid (CAA) in multiple mammalian and human cell lines.[6, 8, 25]

The cytotoxicity of the chloro-HBQs varies with the presence of one, two, three, or four chlorine substitution groups. Comparing the IC_{50} histograms of Figure 3.2, 2-CBQ, with one chloro-substitution on HBQ, is the most cytotoxic of the chloro-HBQs. The 24 h IC_{50} values (Table 3.3) rank cytotoxicity as: 2-CBQ > TetraCBQ ≥ 2,5-DCBQ > 2,6-DCBQ > TriCBQ. Therefore, in addition to the isomeric position, the number of halogen substitutions can also affect cytotoxicity, although there is no clear and specific trend in terms of the number of chloro-substitutions and its comparative cytotoxicity.

A distinct advantage of RTCA testing is the capability to provide dynamic and real time cellular response information. This enables the determination of quantitative IC_{50} histograms as well as provides indications of biological and chemical changes. For example, in Figure 3.2, the IC_{50} histograms of the eight HBQs all show two phases of cellular response to HBQ treatment. This biphasic response is characterized by IC_{50} values that decrease rapidly during the first 12 to 20 h of HBQ exposure (0–20 h), and then slowly increase over the remainder of the exposure period (20–60 h). Biologically, the rapid increase in cytotoxicity during the first phase of exposure indicates HBQ-induced inhibition of cell growth and damage to cells, while the second phase seems to indicate that CHO-K1 cells may be capable of recovering from HBQ exposure at most treatment concentrations. However, the biphasic response can also be interpreted chemically due to the known transformation of HBQs in water to their less toxic products, hydroxyl-HBQs (OH-HBQs). It has been observed that HBQs readily transform to OH-HBQs in water over short incubation times (<12 h), and these OH-HBQ transformation products have been shown to be less toxic than their parent HBQ compounds in CHO-K1 cells.[2] Hence, the observed cellular recovery is likely a result of the presence of less toxic OH-HBQs in solution, indicating both a biological and chemical response.

3.3.2 HBQ-induced ROS production

Having demonstrated that isomeric structure and differences in substitution groups can significantly influence the cytotoxicity of HBQs, I next investigated the effects of the eight HBQs on the generation of ROS. Our previous studies have shown that 2,6-DCBQ, 2,6-DBBQ, DCMBQ, and TriCBQ can induce formation of ROS, 8-OHdG, and carbonyl proteins in T24

cells.[3] Others have shown that TetraCBQ can produce ROS in cells or hydroxyl radicals in solutions.[26-28] ROS generation and resulting oxidative stress is a demonstrated mechanism of HBQ cytotoxicity *in vitro*. Therefore, the correlation between ROS and the physicochemical properties of HBQs is examined to provide further evidence of structure–toxicity relationships of HBQs.

HBQ-induced production of ROS in CHO-K1 cells was determined after the cells were treated with individual HBQs for 6 h, 12 h, and 24 h. ROS formation was measured as increasing intensity of fluorescence, as shown in Figure 3.3. The eight HBQs all show a linear relationship of fluorescence intensity (ROS production) to HBQ concentration with $R^2 \geq 0.86$. To compare the potency of ROS induction by the different HBQs, I performed linear regression analyses of fluorescence intensity versus HBQ concentration and summarized the linear equations in Table 3.4. The slope (k_{ROS}) of each linear regression equation represents the ability of each HBQ to produce ROS: the higher the k value (steeper slope), the higher the ROS production. For example, after 24 h exposure, amongst the eight HBQs, 2,3-DIBQ was the most potent inducer of cellular ROS, with a k_{ROS} value 2.9 times higher than 2,6-DCBQ, the weakest ROS inducer. On the basis of k_{ROS} values, the potency of HBQ-induced ROS production after 24 h exposure follows: 2,3-DIBQ > 2,5-DBBQ > TetraCBQ ≥ 2,5-DCBQ > 2-CBQ > 2,6-DBBQ > TriCBQ > 2,6-DCBQ. In addition, HBQ-induced ROS production was also found to be time-dependent. In Figure 3.4A, the slope of ROS production induced by 2,5-DCBQ is clearly greatest at 12 h exposure, indicating that the highest amount of ROS was produced 12 h after exposure in comparison to the other tested time points. This trend was observed in all eight HBQs: ROS production increased over the first 12 h of exposure, followed by a decrease at 24 h exposure (Figure 3.4B). These results are consistent with our RTCA findings, where a biphasic response was also observed.

The isomeric effects of HBQs on ROS generation in CHO-K1 cells are also clearly supported by the k_{ROS} values. Similar to the cytotoxicity results, both 2,5-DBBQ and 2,5-DCBQ had significantly higher k_{ROS} values than their corresponding 2,6-HBQ isomers, indicating greater ROS generation potency. Specifically, the ratio of the 24 h k_{ROS} values of 2,5-DBBQ to 2,6-DBBQ is 1.4, while the ratio of 2,5-DCBQ to 2,6-DCBQ is 2.1.

The ROS production of HBQs is also dependent on the type of halogen substitution. As shown in Table 3.4, there is a trend of 24 h ROS production in decreasing order: di-iodo- > di-

bromo- > di-chloro-HBQ, when the same isomeric structures are compared. The k_{ROS} of 2,3-DIBQ was 1.2 times higher than 2,5-DBBQ, 1.4 times higher than 2,5-DCBQ, 1.7 times higher than 2,6-DBBQ, and 2.9 times higher than 2,6-DCBQ.

In terms of the effects of the number of chlorination groups on ROS production, 2,5-DCBQ and TetraCBQ can induce significantly higher ROS formation in comparison to 2-CBQ, 2,6-DCBQ, and TriCBQ (one-way ANOVA, $P < 0.001$). Interestingly, although 2-CBQ is the most cytotoxic of the chloro-HBQs (Figure 3.2, Table 3.3), it is not the most potent ROS producer, indicating that there may be mechanisms other than oxidative stress underlying 2-CBQ cytotoxicity. With a pKa of 9.2,[29] 2-CBQ is a neutral molecule at physiological pH (7.4), making it easier for this HBQ to cross the cell membrane. Of the chloro-HBQs with known pKa values tested here, only 2-CBQ had a pKa value above physiological pH (TetraCBQ, 5.6; 2,6-DCBQ, 6.3; TriCBQ, 5.7; 2,5-DCBQ, not available).[30, 31] However, the interactions of HBQs with cellular macromolecules can also play a significant role in the observed toxicity, as HBQ-glutathione conjugation has been reported.[4] All these aspects make understanding *in vitro* HBQ interactions more complex and further research is needed to understand these relationships. Nevertheless, these results are consistent with a previous study that found that TetraCBQ and 2,5-DCBQ can more effectively induce ROS generation in comparison to non-halogen substituted BQs (e.g. phenyl-BQ, butyl-BQ, methyl-BQ, etc.), indicating that the chlorination groups present on BQ can affect ROS generation.[27] Together, these results support that isomeric structures and substitutions of HBQs can significantly affect ROS generation, one of the key mechanisms of HBQ-induced toxicity.

3.3.3 HBQ-induced genotoxicity

Understanding factors that can potentially influence DBP-induced genotoxicity is important because of the known association of DBPs with increased bladder cancer risk. A previous study has shown that 2,6-DCBQ, 2,6-DBBQ, DCMBQ, and TriCBQ can induce formation of the DNA oxidation product, 8-OHdG, in T24 cells.[3] Here, I investigated the genotoxicity of all eight HBQs through analysis of 8-OHdG formation and p53 protein expression in CHO-K1 cells. While 8-OHdG is associated specifically with oxidative stress, p53, a tumor suppressor protein, is crucial in coordinating cellular response to genotoxic stress.[32] Upon activation of the p53 gene, p53 protein will further regulate cell cycle arrest, apoptosis, or senescence.[33, 34]

Figure 3.5 shows the percentage of 8-OHdG production in treated cells over the negative control cells after CHO-K1 cells were treated with each HBQ (24 h IC_{50} value) for 12 h and 24 h. At 12 h post exposure, only cells treated with 2,5-DBBQ or 2-CBQ showed a significant increase in 8-OHdG compared to the negative control ($P<0.05$). At 24 h post exposure, all eight HBQs showed a significant increase in 8-OHdG ($P<0.05$). The potency of 8-OHdG formation at 24 h exposure was determined to be: 2,6-DCBQ > TriCBQ > 2,6-DBBQ ≥ 2,5-DBBQ > 2-CBQ ≈ 2,3-DIBQ ≈ TetraCBQ ≈ 2,5-DCBQ.

Table 3.5 summarizes the percentages of p53 expression in treated cells relative to the untreated control cells after CHO-K1 cells were treated with each HBQ (24 h IC_{50} value) for 12 h and 24 h. After 12 h exposure, only cells treated with 2,5-DBBQ or 2,3-DIBQ expressed increased p53 protein compared with the negative control ($P<0.05$). At 24 h post exposure, a significant increase in p53 expression was detected in cells treated with 2,5-DBBQ, 2,3-DIBQ, 2,6-DCBQ, and 2,5-DCBQ treated cells ($P<0.05$); the other HBQs did not show a significant increase in p53 protein. Increasing p53 protein expression in HBQ-treated CHO-K1 cells at 24 h was determined to be: 2,5-DCBQ ≈ 2,5-DBBQ > 2,6-DCBQ ≈ 2,3-DIBQ > 2,6-DBBQ ≈ 2-CBQ ≈ TriCBQ ≈ TetraCBQ.

The genotoxicity results also show effects of isomeric structure on HBQ-induced toxicity. Interestingly, while HBQ-induced 8-OHdG formation at 24 h exposure was greater for the 2,6-HBQ isomers in comparison to their corresponding 2,5-HBQ isomers, the opposite trend was observed when examining HBQ-induced p53 protein expression at the same exposure time. At 24 h exposure, 2,6-DCBQ generated nearly twice the amount of 8-OHdG in CHO-K1 cells in comparison to 2,5-DCBQ, while 2,6-DBBQ generated slightly higher amounts of 8-OHdG than 2,5-DBBQ. Alternatively, CHO-K1 cells treated with 2,5-DCBQ or 2,5-DBBQ resulted in p53 protein levels approximately twice as high as their corresponding 2,6-HBQ isomers. 2,5-HBQ isomers appear to more effectively activate the p53-mediated DNA damage response. This may partially explain the lower number of 8-OHdG detected in CHO-K1 cells treated with the 2,5-HBQ isomers in comparison to their 2,6-HBQ isomers even though ROS production was greater in 2,5-HBQ-treated cells, as p53 protein has been found to sense oxidative DNA damage and can facilitate excision of 8-OHdG.[35] Genetic activation of the p53 pathway due to 2,6-DCBQ and 2,6-DBBQ exposure was detected by Procházka et al.[6] However, as the assay selected for use in this study only examined downstream p53 protein expression, it is possible that the pathway was

activated in CHO-K1 cells, but did not result in much p53 protein expression. This is supported by the slight increase in p53 at 24 h in CHO-K1 cells treated with 2,6-DCBQ (Table 3.5).

While I found cytotoxicity and ROS production potency to increase: di-iodo- > di-bromo- > di-chloro-HBQ, this trend was not observed in the genotoxicity measured as 8-OHdG formation and p53 protein expression. This also contrasts with studies of the HAAs, where the rank order followed IAA > BAA > CAA in both mutagenicity (Ames assay) and genotoxicity (comet assay) assays in CHO or human cells.[8, 25] Different observation could be due to the use of different assays for genotoxicity assessment, which different endpoints are measured.

3.3.4 HBQ structure–toxicity relationships

To explore potential structural influence on HBQ toxicity, I examined six physicochemical parameters of HBQs in relation to their observed toxicity (IC_{50} values, ROS generation, 8-OHdG and p53) using Pearson correlation analysis. It is widely known that physicochemical properties of chemicals can affect their uptake, metabolism, biochemical interactions in cells, and transport in and out of cells. For our analyses, I selected six common physicochemical parameters[36], as described in Table 3.2. These parameters include hydrophobicity or lipid solubility, (1) logP, quantified by the logarithm of the octanol-water partition coefficient, as well as electronic properties and quantum mechanical descriptors, including (2) E_{HOMO}, (3) E_{LUMO}, and (4) dipole moment, μ. Steric properties, such as molecule size, were assessed using (5) molar refractivity, R, and (6) surface area, S. The primary correlation analysis of all eight HBQs was followed by correlation analysis with the isomer data alone and then with the data obtained from the HBQs with differing types of halogen substitutions to explain the structural trends observed in our toxicity analyses.

Table 3.6 presents the Pearson correlation coefficients from the analysis of the six physicochemical parameters (Table 3.2) and our measured toxicity data ($\log IC_{50}$ derived from Table 3.3, $\log k_{ROS}$ derived from Table 3.4, 8-OHdG derived from Figure 3.5, or p53 derived from Table 3.5) from all eight HBQs. As expected, ROS production ($\log k_{ROS}$) at 24 h is strongly and significantly correlated with both cytotoxicity ($\log IC_{50}$; $r=-0.708$, $P<0.05$) and 8-OHdG production ($r=-0.821$, $P<0.05$). These correlations, however, were not found at 12 h ($P>0.05$). ROS production ($\log k_{ROS}$) at 12 h did correlate with p53 protein expression ($r=0.771$, $P<0.05$).

However, because only two of the HBQs, 2,5-DBBQ and 2,3-DIBQ, had significantly increased p53 levels at 12 h, the confidence in this correlation is not strong.

The Pearson correlation analysis using all eight HBQs found that cytotoxicity correlated with two of the six physicochemical parameters. Neither ROS production nor genotoxicity correlated with any of the structural parameters. Statistically significant correlations between $\log IC_{50}$ and the two descriptors, μ and E_{LUMO}, were found at both 12 h (μ: $r=-0.787$, $P<0.05$, E_{LUMO}: $r=-0.864$, $P<0.01$) and 24 h (μ: $r=-0.721$, $P<0.05$, E_{LUMO}: $r=-0.766$, $P<0.05$) exposure. This indicates that E_{LUMO} and μ are two major structural parameters influencing HBQ-induced cytotoxicity. E_{LUMO} characterizes electron affinity[37] and correlates well with reduction potentials. E_{LUMO} indicates production of the free radical metabolite.[38, 39] Therefore, the observed correlation between E_{LUMO} and HBQ-induced cytotoxicity is consistent with the ability of quinones to undergo one-electron reduction to form semiquinones, which are potent generators of ROS, resulting in oxidative damage and cell death.[40] Our analysis is also consistent with reported results that found a correlation between E_{LUMO} and developmental toxicity in a marine polychaete exposed to aromatic halogenated DBPs.[16]

Dipole moment as another structural parameter influencing HBQ cytotoxicity can be explained. The μ reflects the average charge separation and electron distribution on a molecule.[41] The reactivity of a molecule is strongly influenced by its μ, which is higher in asymmetric molecules than in symmetric ones. It follows that I observed the greatest cytotoxic effects in cells treated with 2,3-DIBQ and 2-CBQ, asymmetric structures that exhibit dramatically higher μ (2.48 Debye for 2,3-DIBQ and 1.73 Debye for 2-CBQ) in comparison to the other HBQs (0.9 for TriCBQ to 0.004 Debye for TetraCBQ). These results indicate that polarized distributions of electrons play an important role in HBQ-induced cytotoxicity, consistent with findings of halobenzene-induced cytotoxicity in hepatocytes.[42]

Both E_{LUMO} and μ correlate with HBQ cytotoxicity ($\log IC_{50}$). Next, I evaluated this correlation using a two-parameter multilinear regression analysis. The multilinear QSTR equation is defined as $\log IC_{50}=0.230-0.403\times E_{LUMO}+0.020\times\mu$, with $R^2=0.620$ and $P=0.089$, indicating lack of association. Of the two descriptors, E_{LUMO} was identified as the stronger of the two within the analysis. Using E_{LUMO} alone for linear regression analysis, I obtained a QSTR equation of $\log IC_{50}=0.52-0.364\times E_{LUMO}$, with $R^2=0.619$ and $P=0.021$. Figure 3.6 shows the plot of the calculated $\log IC_{50}$ values (from our QSTR equation) versus the experimentally determined

$\log IC_{50}$ values from this study. The black line represents the linear regression obtained from the data points of all eight HBQs (n=8). I found that the calculated $\log IC_{50}$ values highly correlated with the experimental $\log IC_{50}$ values ($P<0.05$, $R^2=0.021$). With addition of a 24 h IC_{50} value of 11.4 μM for DCMBQ (E_{LUMO}=-2.631) from a previous study with CHO-K1 cells[2], I re-calculated the linear regression using the new point for DCMBQ (red) and the same data points for the eight HBQs. The red line in Figure 3.6 represents this new linear regression (n=9). Again, I found that the $\log IC_{50}$ values were correlated ($P<0.05$, $R^2=0.543$). These results suggest that E_{LUMO} better correlates with HBQ cytotoxicity, consistent with findings of previous studies of aromatic DBPs.[10, 16]

Pearson correlation analysis performed on the isomer toxicity data alone (2,6-DCBQ, 2,5-DCBQ, 2,6-DBBQ, and 2,5-DBBQ) found that cytotoxicity ($\log IC_{50}$) and ROS production ($\log k_{ROS}$) are correlated with each other at 24 h exposure ($r=-0.979$, $P<0.05$), while both cytotoxicity ($r=0.965$, $P<0.05$) and ROS production ($r=-0.981$, $P<0.01$) are correlated with μ at 24 h (Table 3.7). The μ of 2,5-DBBQ (0.135 Debye) is 6.4 times higher than that of 2,6-DBBQ (0.021). Likewise, the μ of 2,5-DCBQ (0.226 Debye) is 5.3 times higher than the μ of 2,6-DCBQ (0.043). Because reactivity is associated with larger μ, the difference in μ of the isomers explains their differential cytotoxicity, as the 2,5-HBQs exhibited greater cytotoxicity (Figure 3.2) and greater ROS production (Table 3.4) than their corresponding 2,6-HBQ isomers. These results support that μ is an important physicochemical parameter influencing HBQ-isomeric structure effects.

Pearson correlation analysis of the toxicity data from the di-halo-HBQs (2,5-DCBQ, 2,6-DCBQ, 2,5-DBBQ, 2,6-DBBQ, and 2,3-DIBQ) found a statistically significant correlation between cytotoxicity ($\log IC_{50}$) and ROS production ($\log k_{ROS}$) ($r=-0.870^{*}$, $P<0.05$) at 24 h. Cytotoxicity ($\log IC_{50}$) at 24 h is also correlated ($P<0.05$) with all six structural parameters: logP, E_{HOMO}, E_{LUMO}, S, R, and μ (Table 3.7). The logP values follow the same increasing rank order as their toxic effects: DIBQ > DBBQ > DCBQ. LogP has also been found to correlate with cytotoxicity of other DBPs, including the cytotoxicity of HAAs in CHO cells: IAA > BAA > CAA. Furthermore, logP correlated well with both cytotoxicity and genotoxicity of HAAs,[8] however, there is no correlation between these five di-halo-HBQs and the genotoxicity in this study. The correlation of logP with developmental toxicity in a marine polychaete and with growth inhibition of marine alga have also been reported after exposure to halophenolic DBPs.[10,

[16] However, in a study from Siraki et al., logP was not correlated with the cytotoxicity of fourteen 1,4-BQ congeners.[27] As I also found that logP was not correlated with cytotoxicity in CHO-K1 cells when all eight HBQs were analyzed together, these results suggest that cytotoxicity is not solely influenced by logP, but other chemical properties of HBQs, such as redox potential, are critically important.

The correlation with the molecular front orbital energies, E_{HOMO} and $E_{LUMO,}$ was expected, as they play a major role in governing many chemical reactions.[43] In contrast to E_{LUMO}, E_{HOMO} indicates electron donating ability and correlates well with oxidation potential. Incidentally, both structural parameters can well represent the redox potential, as HBQs can undergo either reduction or oxidation reactions. This is consistent with a comparative developmental toxicity study of aromatic halogenated DBPs, where both E_{LUMO} and E_{HOMO} were found to be important physicochemical descriptors.[16] Chan et al. also reported that halobenzene-induced cytotoxicity strongly correlated with E_{HOMO}, suggesting oxidation is a mechanism for toxicity.[42]

Steric properties describe the size and 3D structure of a molecule, as well as indicate the steric hindrance and geometric interaction of the molecule with receptors or enzymes.[44] Both the molecular surface area, S, and the molecular refractivity, R, can be used to describe the 3D structure of a molecule. The S and R of halogen-substituted HBQs increase in the same order as their cytotoxicity: DIBQ > DBBQ > DCBQ, as shown in Table 3.2. Hence, it follows that steric properties correlated significantly with their cytotoxicity, as shown in Table 3.7 ($P<0.05$). In general, R, a dual measure of both the volume and polarizability of a molecule, better represents steric properties than molecular volume alone. In a QSTR study, R showed a moderate correlation (not significant) with cytotoxicity induced by BQ compounds in a human hepatocyte cell line.[45] However, I found that halogen-substituted HBQ-induced cytotoxicity significantly correlated with R, demonstrating steric effects on the cytotoxicity of HBQs.

3.4 Conclusions

The results of this study show relatively high genotoxicity of the di-halo-HBQs amongst the eight HBQs, as determined via induction of 8-OHdG formation and p53 protein expression in CHO-K1 cells. 2,6-DCBQ and 2,6-DBBQ are known DBPs in drinking water produced from chlorination and chloramination disinfection processes. The toxicity of 2-CBQ, 2,3-DIBQ, 2,5-DCBQ, and 2,5-DBBQ is systematically examined for the first time in comparison to other

HBQs known to occur in drinking water. On the basis of their cytotoxicity, ROS formation, and genotoxicity in CHO-K1 cells, the iodo-HBQs and 2,5-HBQs are the most toxic in this class of DBPs. The formation and occurrence of iodo-HBQs and 2,5-HBQs in drinking water are unknown. Therefore, further investigation is warranted to confirm their formation and occurrence in drinking water systems. Such information on these toxic HBQs will be important to fully understand HBQ exposure and potential health risks.

3.5 References

1. Bull, R. J.; Reckhow, D. A.; Li, X.; Humpage, A. R.; Joll, C.; Hrudey, S. E., Potential carcinogenic hazards of non-regulated disinfection by-products: haloquinones, halo-cyclopentene and cyclohexene derivatives, N-halamines, halonitriles, and heterocyclic amines. *Toxicology* **2011,** *286*, (1-3), 1-19.

2. Wang, W.; Qian, Y. C.; Li, J. H.; Moe, B.; Huang, R. F.; Zhang, H. Q.; Hrudey, S. E.; Li, X.-F., Analytical and toxicity characterization of halo-hydroxyl-benzoquinones as stable halobenzoquinone disinfection byproducts in treated water. *Anal. Chem.* **2014,** *86*, (10), 4982-4988.

3. Du, H. Y.; Li, J. H.; Moe, B.; McGuigan, C. F.; Shen, S. W.; Li, X.-F., Cytotoxicity and oxidative damage induced by halobenzoquinones to T24 bladder cancer cells. *Environ. Sci. Technol.* **2013,** *47*, (6), 2823-2830.

4. Li, J.; Wang, W.; Zhang, H.; Le, X. C.; Li, X. F., Glutathione-mediated detoxification of halobenzoquinone drinking water disinfection byproducts in T24 cells. *Toxicol. Sci.* **2014,** *141*, (2), 335-343.

5. Li, J.; Wang, W.; Moe, B.; Wang, H.; Li, X. F., Chemical and toxicological characterization of halobenzoquinones, an emerging class of disinfection byproducts. *Chem. Res. Toxicol.* **2015,** *28*, (3), 306-18.

6. Prochazka, E.; Escher, B. I.; Plewa, M. J.; Leusch, F. D., n vitro cytotoxicity and adaptive stress responses to selected haloacetic acid and halobenzoquinone water disinfection byproducts. *Chem. Res. Toxicol.* **2015,** *28*, (10), 2059-68.

7. Casarett, L. J.; Doull, J.; Klaassen, C. D., *Casarett and Doull's Toxicology: the Basic Science of Poisons*. 7th ed.; McGraw-Hill: New York, 2008.

8. Plewa, M. J.; Wagner, E. D.; Richardson, S. D.; Thruston, A. D.; Woo, Y. T.; McKague, A. B., Chemical and biological characterization of newly discovered lodoacid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2004,** *38*, (18), 4713-4722.

9. Kargalioglu, Y.; McMillan, B. J.; Minear, R. A.; Plewa, M. J., Analysis of the cytotoxicity and mutagenicity of drinking water disinfection by-products in Salmonella typhimurium. *Teratogen. Carcin. Mutat.* **2002,** *22*, (2), 113-128.

10. Liu, J.; Zhang, X., Comparative toxicity of new halophenolic DBPs in chlorinated saline wastewater effluents against a marine alga: halophenolic DBPs are generally more toxic than haloaliphatic ones. *Water Re.s* **2014,** *65*, 64-72.

11. Pals, J.; Attene-Ramos, M. S.; Xia, M. H.; Wagner, E. D.; Plewa, M. J., Human cell toxicogenomic analysis linking reactive oxygen species to the toxicity of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2013,** *47*, (21), 12514-12523.

12. Meier, J. R.; Ringhand, H. P.; Coleman, W. E.; Munch, J. W.; Streicher, R. P.; Kaylor, W. H.; Schenck, K. M., Identification of mutagenic compounds formed during chlorination of humic acid. *Mutat. Res.* **1985,** *157*, (2-3), 111-122.

13. Merrick, B. A.; Smallwood, C. L.; Meier, J. R.; Mckean, D. L.; Kaylor, W. H.; Condie, L. W., Chemical-reactivity, cytotoxicity, and mutagenicity of chloropropanones. *Toxicol. Appl. Pharmacol.* **1987,** *91*, (1), 46-54.

14. Lecurieux, F.; Marzin, D.; Erb, F., Study of the genotoxic activity of 5 chlorinated propanones using the Sos chromotest, the Ames-fluctuation test and the newt micronucleus test. *Mutat. Res. Genet. Toxicol.* **1994,** *341*, (1), 1-15.

15. Richardson, S. D.; Fasano, F.; Ellington, J. J.; Crumley, F. G.; Buettner, K. M.; Evans, J. J.; Blount, B. C.; Silva, L. K.; Waite, T. J.; Luther, G. W.; McKague, A. B.; Miltner, R. J.; Wagner, E. D.; Plewa, M. J., Occurrence and mammalian cell toxicity of iodinated disinfection byproducts in drinking water. *Environ. Sci. Technol.* **2008,** *42*, (22), 8330-8338.

16. Yang, M. T.; Zhang, X. R., Comparative developmental toxicity of new aromatic halogenated DBPs in a chlorinated saline sewage effluent to the marine polychaete *Platynereis dumerilii*. *Environ. Sci. Technol.* **2013,** *47*, (19), 10868-10876.

17. Plewa, M. J., and Wagner, E.D., *Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-products*. Water Research Foundation: Denver, CO, 2009.

18. Wagner, E. D.; Osiol, J.; Mitch, W. A.; Plewa, M. J., Comparative in vitro toxicity of nitrosamines and nitramines associated with amine-based carbon capture and storage. *Environ. Sci. Technol.* **2014,** *48*, (14), 8203-8211.

19. Du, H. Y.; Li, J. H.; Moe, B.; McGuigan, C. F.; Li, X.-F., A real-time cell-electronic sensing method for comparative analysis of toxicity of water contaminants. *Anal. Methods* **2013,** *6*, 2053-2058.

20. Moe, B.; Gabos, S.; Li, X.-F., Real-time cell-microelectronic sensing of nanoparticle-induced cytotoxic effects. *Anal. Chim. Acta.* **2013,** *789*, 83-90.

21. Boyd, J. M.; Huang, L.; Xie, L.; Moe, B.; Gabos, S.; Li, X.-F., A cell-microelectronic sensing technique for profiling cytotoxicity of chemicals. *Anal. Chim. Acta.* **2008,** *615*, (1), 80-87.

22. Huang, L.; Xie, L.; Boyd, J. M.; Li, X.-F., Cell-electronic sensing of particle-induced cellular responses. *Analyst* **2008,** *133*, (5), 643-648.

23. Wang, G.; Gong, Y.; Burczynski, F. J.; Hasinoff, B. B., Cell lysis with dimethyl sulphoxide produces stable homogeneous solutions in the dichlorofluorescein oxidative stress assay. *Free Radic. Res.* **2008,** *42*, (5), 435-441.

24. MOPAC2012, James J. P. Stewart, Stewart Computational Chemistry, Colorado Springs, CO, USA, http://openmopac.net/ (2012).

25. Attene-Ramos, M. S.; Wagner, E. D.; Plewa, M. J., Comparative human cell toxicogenomic analysis of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2010,** *44*, (19), 7206-7212.

26. Zhu, B. Z.; Kalyanaraman, B.; Jiang, G. B., Molecular mechanism for metal-independent production of hydroxyl radicals by hydrogen peroxide and halogenated quinones. *Proc. Natl. Acad. Sci. USA.* **2007,** *104*, (45), 17575-17578.

27. Siraki, A. G.; Chan, T. S.; O'Brien, P. J., Application of quantitative structure-toxicity relationships for the comparison of the cytotoxicity of 14 p-benzoquinone congeners in primary cultured rat hepatocytes versus PC12 cells. *Toxicol. Sci.* **2004,** *81*, (1), 148-159.

28. Zhu, B. Z.; Shan, G. Q., Potential Mechanism for Pentachlorophenol-Induced Carcinogenicity: A Novel Mechanism for Metal-Independent Production of Hydroxyl Radicals. *Chem. Res. Toxicol.* **2009,** *22*, (6), 969-977.

29. Uchimiya, M.; Stone, A. T., Aqueous oxidation of substituted dihydroxybenzenes by substituted benzoquinones. *Environ. Sci. Technol.* **2006,** *40*, (11), 3515-3521.

30. Zhao, Y. L.; Qin, F.; Boyd, J. M.; Anichina, J.; Li, X.-F., Characterization and determination of chloro- and bromo-benzoquinones as new chlorination disinfection byproducts in drinking water. *Anal. Chem.* **2010,** *82*, (11), 4599-4605.

31. Fukuzumi, S., Ishikawa, M., and Tanaka, T., Acid-catalysed reduction of p-benzoquinone derivatives by an NADH analogue, 9,10-dihydro-10-methylacridine: the energetic comparison of one-electron vs. two-electron Pathways. *J. Chem. Soc. Perkin Trans. 2* **1989**, 1811-1816.

32. Brooks, C. L.; Gu, W., p53 ubiquitination: Mdm2 and beyond. *Mol. Cell* **2006,** *21*, (3), 307-15.

33. Levine, A. J., p53, the cellular gatekeeper for growth and division. *Cell* **1997,** *88*, (3), 323-31.

34. Horn, H. F.; Vousden, K. H., Coping with stress: multiple ways to activate p53. *Oncogene* **2007,** *26*, (9), 1306-16.

35. Achanta, G.; Huang, P., Role of p53 in sensing oxidative DNA damage in response to reactive oxygen species-generating agents. *Cancer Res.* **2004,** *64*, (17), 6233-9.

36. Hansch, C.; Leo, A.; Hoekman, D. H., *Exploring QSAR*. American Chemical Society: Washington, DC, 1995.

37. Karelson, M.; Lobanov, V. S.; Katritzky, A. R., Quantum-chemical descriptors in QSAR/QSPR studies. *Chem. Rev.* **1996,** *96*, (3), 1027-1043.

38. Maccoll, A., Reduction Potentials of Conjugated Systems. *Nature* **1949,** *163*, 178-179.

39. Mendez-Hernandez, D. D.; Tarakeshwar, P.; Gust, D.; Moore, T. A.; Moore, A. L.; Mujica, V., Simple and accurate correlation of experimental redox potentials and DFT-calculated HOMO/LUMO energies of polycyclic aromatic hydrocarbons. *J. Mol. Model* **2013,** *19*, (7), 2845-8.

40. Bolton, J. L.; Trush, M. A.; Penning, T. M.; Dryhurst, G.; Monks, T. J., Role of quinones in toxicology. *Chem. Res. Toxicol.* **2000,** *13*, (3), 135-160.

41. Cronin, M. T. D.; Livingstone, D., *Predicting chemical toxicity and fate*. CRC Press: Boca Raton, Fla., 2004.

42. Chan, K.; Jensen, N. S.; Silber, P. A.; O'Brien, P. J., Structure-activity relationships for halobenzene induced cytotoxicity in rat and human hepatoctyes. *Chem-Biol. Interact.* **2007,** *165*, (3), 165-174.

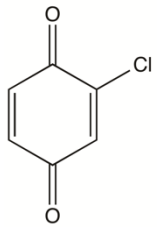
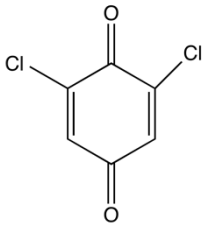
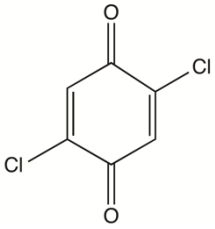
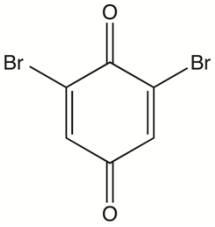
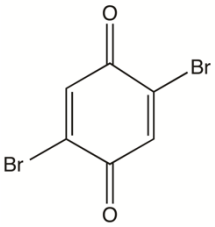
43. Zhou, Z. X.; Parr, R. G., Activation hardness - new index for describing the orientation of electrophilic aromatic-substitution. *J. Am. Chem. Soc.* **1990,** *112*, (15), 5720-5724.

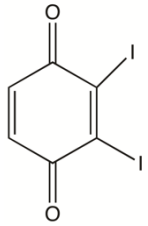
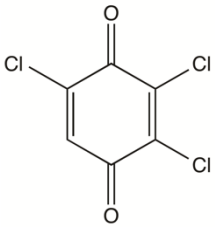
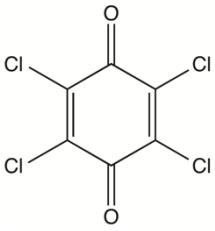
44. Kubinyi, H., *QSAR : Hansch Analysis and Related Approaches*. VCH: Weinheim ; New York, 1993.

45. Chan, K.; Jensen, N.; O'Brien, P. J., Structure-activity relationships for thiol reactivity and rat or human hepatocyte toxicity induced by substituted p-benzoquinone compounds. *J. Appl. Toxicol.* **2008,** *28*, (5), 608-620.

46. Huang, R.; Wang, W.; Qian, Y.; Boyd, J. M.; Zhao, Y.; Li, X.-F., Ultra pressure liquid chromatography-negative electrospray ionization mass spectrometry determination of twelve halobenzoquinones at ng/L levels in drinking water. *Anal. Chem.* **2013,** *85*, (9), 4520-9.

Table 3.1 Chemical structure, molecular formula and purity of HBQs

HBQs	Molecular Formula	Purity	Structure
2-chloro-1,4-benzoquinone [2-CBQ]	$C_6H_3ClO_2$	95%	
2,6-dichloro-1,4-benzoquinone [2,6-DCBQ]	$C_6H_2Cl_2O_2$	98%	
2,5-dichloro-1,4-benzoquinone [2,5-DCBQ]	$C_6H_2Cl_2O_2$	98%	
2,6-dibromo-1,4-benzoquinone [2,6-DBBQ]	$C_6H_2Br_2O_2$	≥98%	
2,5-dibromo-1,4-benzoquinone [2,5-DBBQ]	$C_6H_2Br_2O_2$	N.A.	

2,3-diiodo-1,4-benzoquinone [2,3-DIBQ]	$C_6H_2I_2O_2$	N.A.	
2,3,6-trichloro-1,4-benzoquinone [TriCBQ]	$C_6HCl_3O_2$	≥98%	
2,3,5,6-tetrachloro-1,4-benzoquinone [TetraCBQ]	$C_6Cl_4O_2$	99%	

Note: N.A. means not available from manufacturer, but mass spectra of 2,5-DBBQ and 2,3-DIBQ showed high purity in our previous publication (Figure 1, Huang et al).[46]

Table 3.2 Physicochemical parameters of HBQs

HBQs	log*P*	*R* (cm^3)	E_{HOMO} (EV)	E_{LUMO} (EV)	μ	*S* ($Å^2$)
2-CBQ	0.46	32.4	-10.822	-2.250	1.371	136.45
2,6-DCBQ	0.65	37.0	-10.963	-3.120	0.226	151.60
2,5-DCBQ	0.65	37.0	-10.980	-3.115	0.043	151.54
2,6-DBBQ	0.98	42.7	-10.449	-3.060	0.135	159.29
2,5-DBBQ	0.98	42.7	-10.449	-3.059	0.021	159.34
2,3-DIBQ	2.14	53.2	-9.093	-2.034	2.480	168.76
TriCBQ	1.02	41.6	-10.598	-2.634	0.971	166.30
TetraCBQ	1.39	46.3	-10.621	-2.770	0.004	181.07

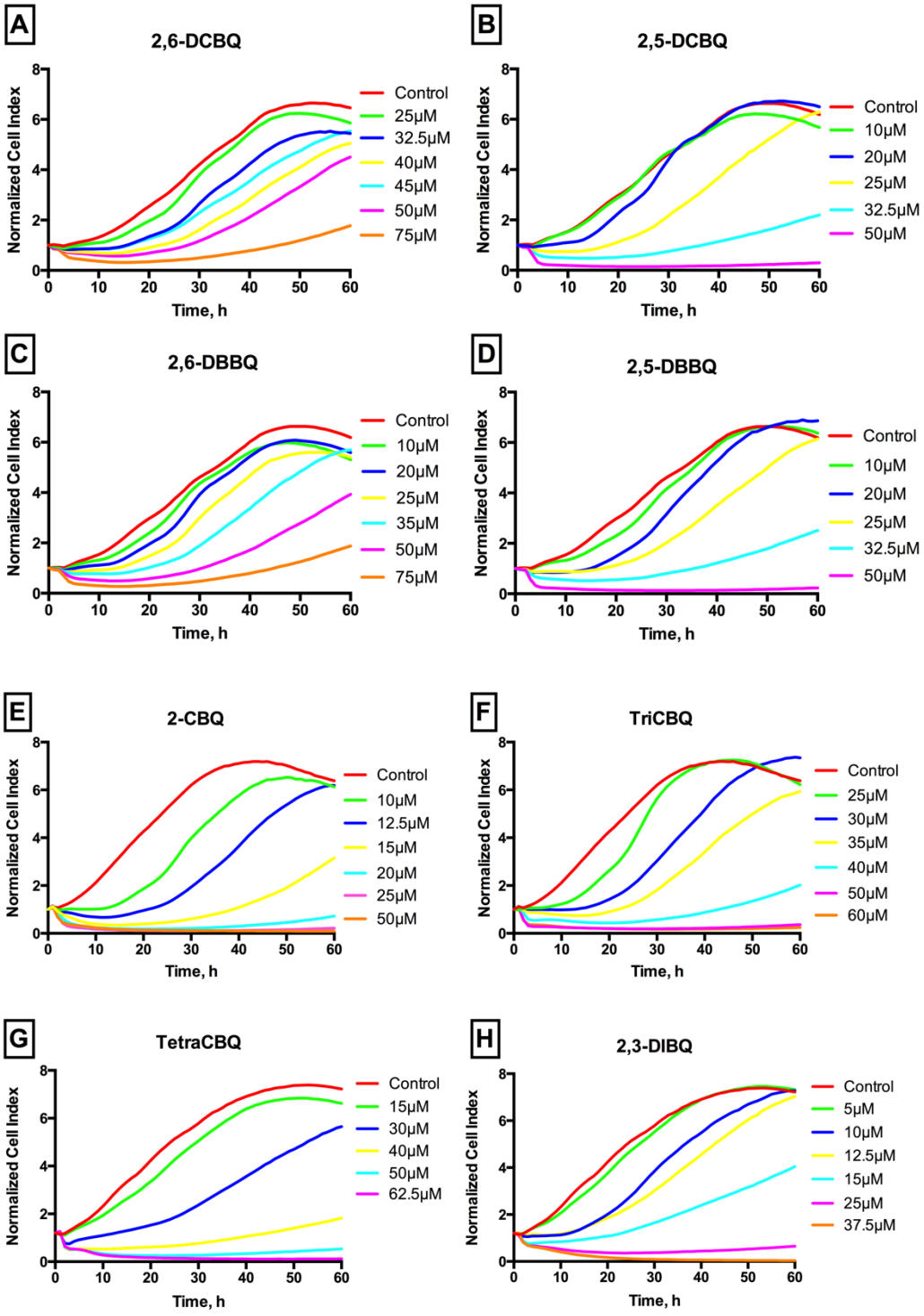


Figure 3.1 RTCA continuous profiles of the normalized cell index (NCI) over time for CHO-K1 cells exposed to HBQs. (A) 2,6-DCBQ, (B) 2,5-DCBQ, (C) 2,6-DBBQ, (D) 2,5-DBBQ, (E) 2-CBQ, (F) TriCBQ, (G) TetraCBQ, and (H) 2,3-DIBQ.

Table 3.3 Comparative IC_{50} Values at 6, 12, and 24 h Post-exposure Determined Using RTCA for Individual HBQs in CHO-K1 Cells

	IC_{50}, µM		
	6 h	**12 h**	**24 h**
2,6-DCBQ	63 ± 3	42 ± 1	32 ± 1
2,5-DCBQ	29 ± 1*	24 ± 1*	24 ± 0*
2,6-DBBQ	51 ± 1	36 ± 1	26 ± 2
2,5-DBBQ	30 ± 1*	21 ± 1*	21 ± 1*
2,3-DIBQ	18 ± 1	11 ± 0	11 ± 1
2-CBQ	17 ± 0	12 ± 0	13 ± 0
TriCBQ	36 ± 1	26 ± 1	35 ± 3
TetraCBQ	34 ± 1	25 ± 1	24 ± 1

Note: Values are the mean ± standard error (SEM), n=3.

* $P < 0.05$, 2,5-HBQ compared to their corresponding 2,6-HBQ isomer

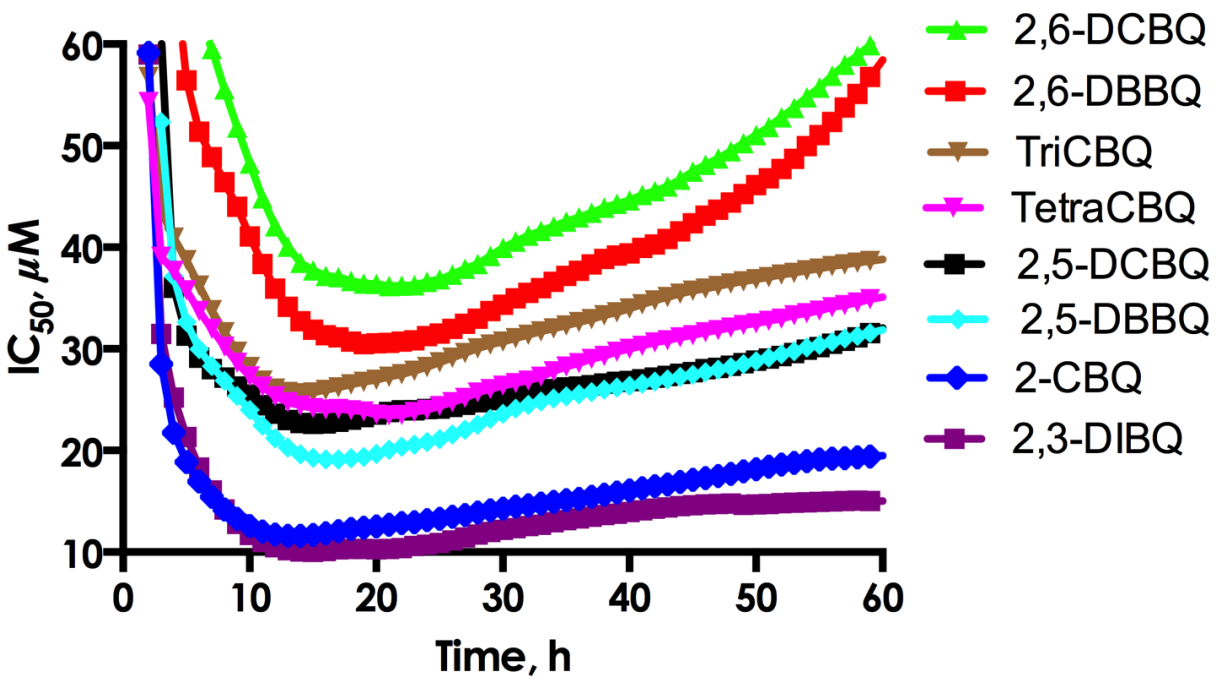


Figure 3.2 IC_{50} histograms of CHO-K1 cells after exposure to eight HBQs.

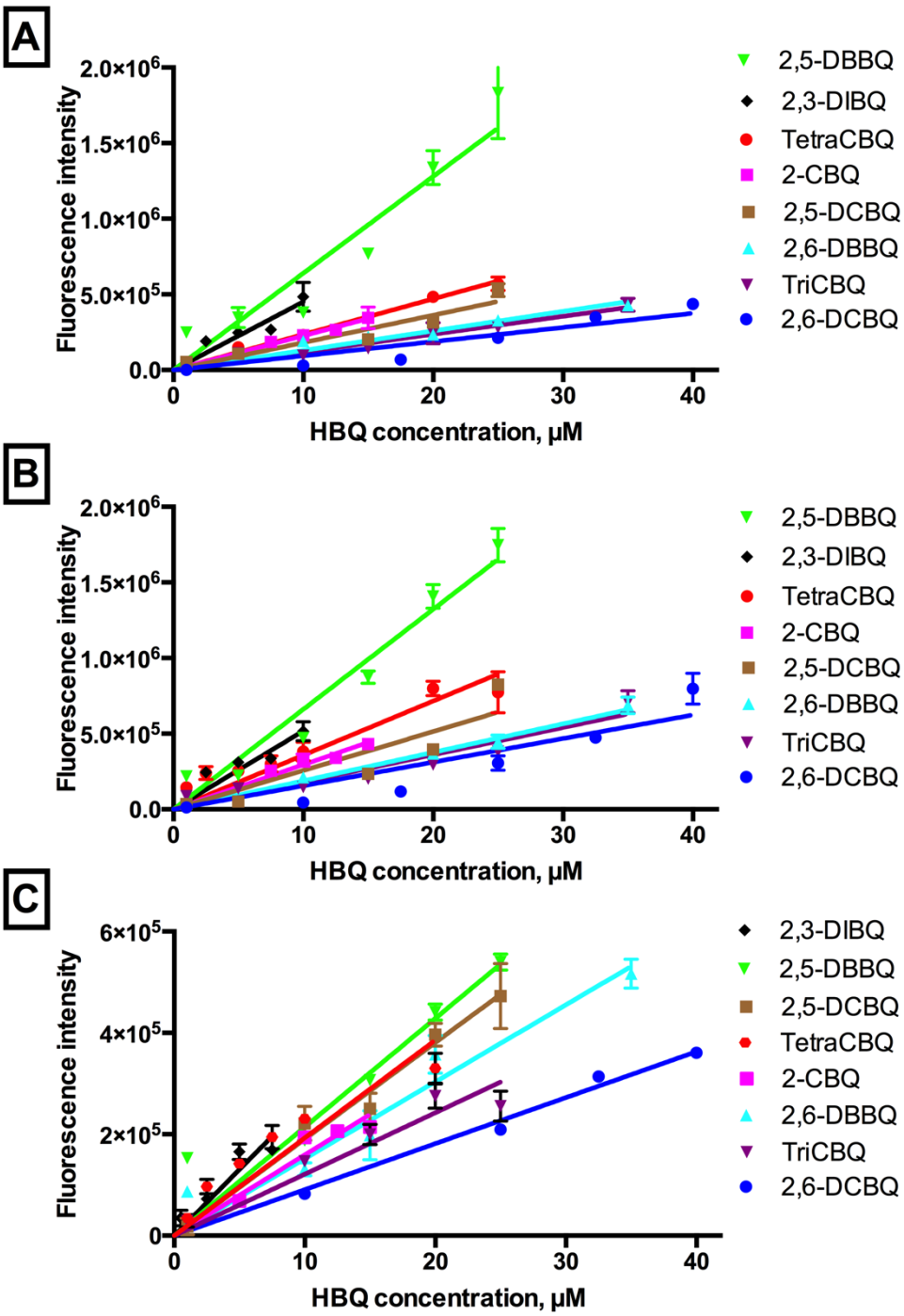


Figure 3.3 Cellular ROS production in CHO-K1 cells with linear regressions at (A) 6, (B) 12, and (C) 24 h post-exposure to HBQs.

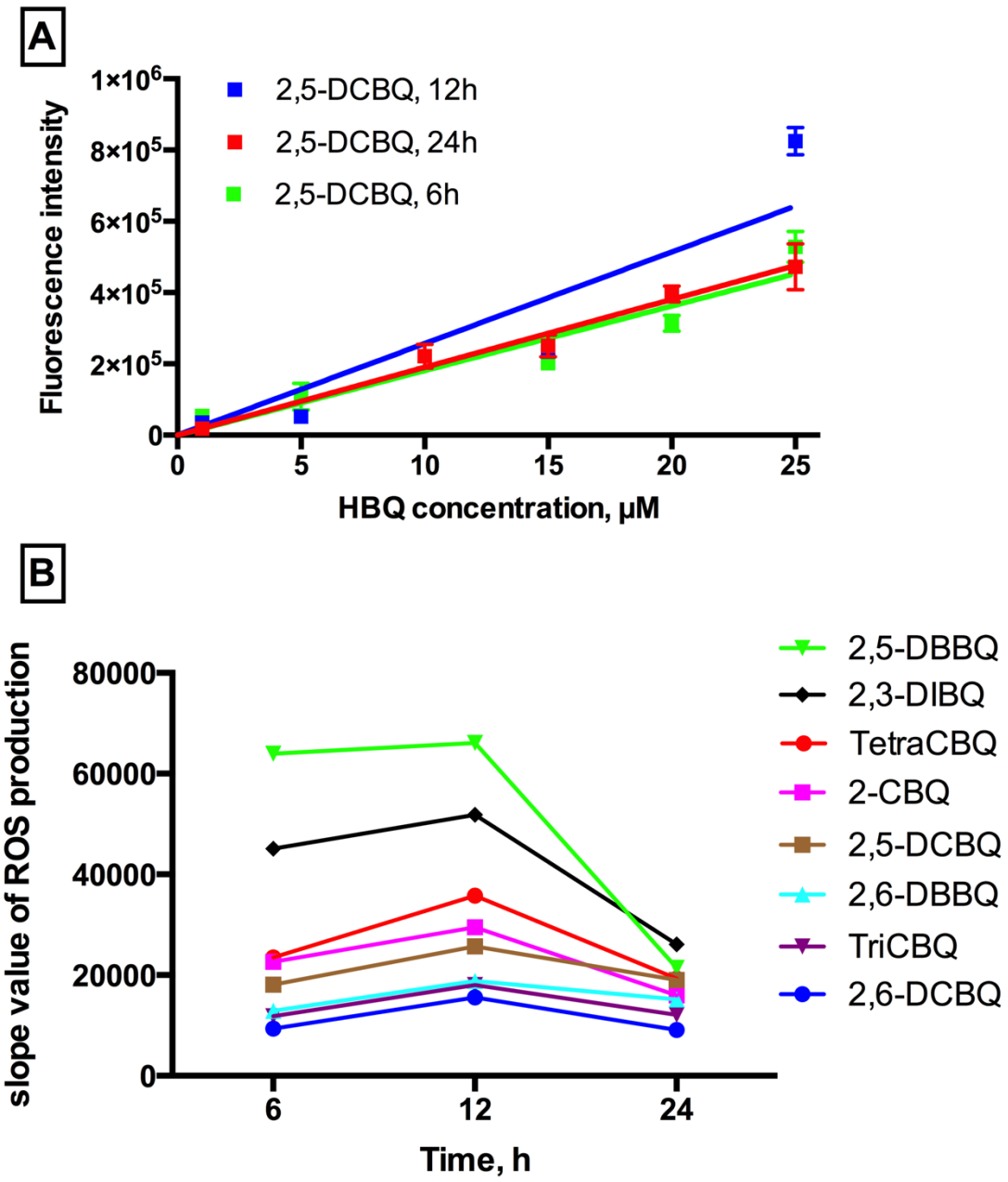


Figure 3.4 Cellular ROS production in CHO-K1 cells at different time points. (A) Cellular ROS production in CHO-K1 cells and linear regression at 6 h, 12 h, and 24 h post-exposure to 2,5-DCBQ; (B) slope value of cellular ROS production at 6 h, 12 h, and 24 h post-exposure to each HBQ.

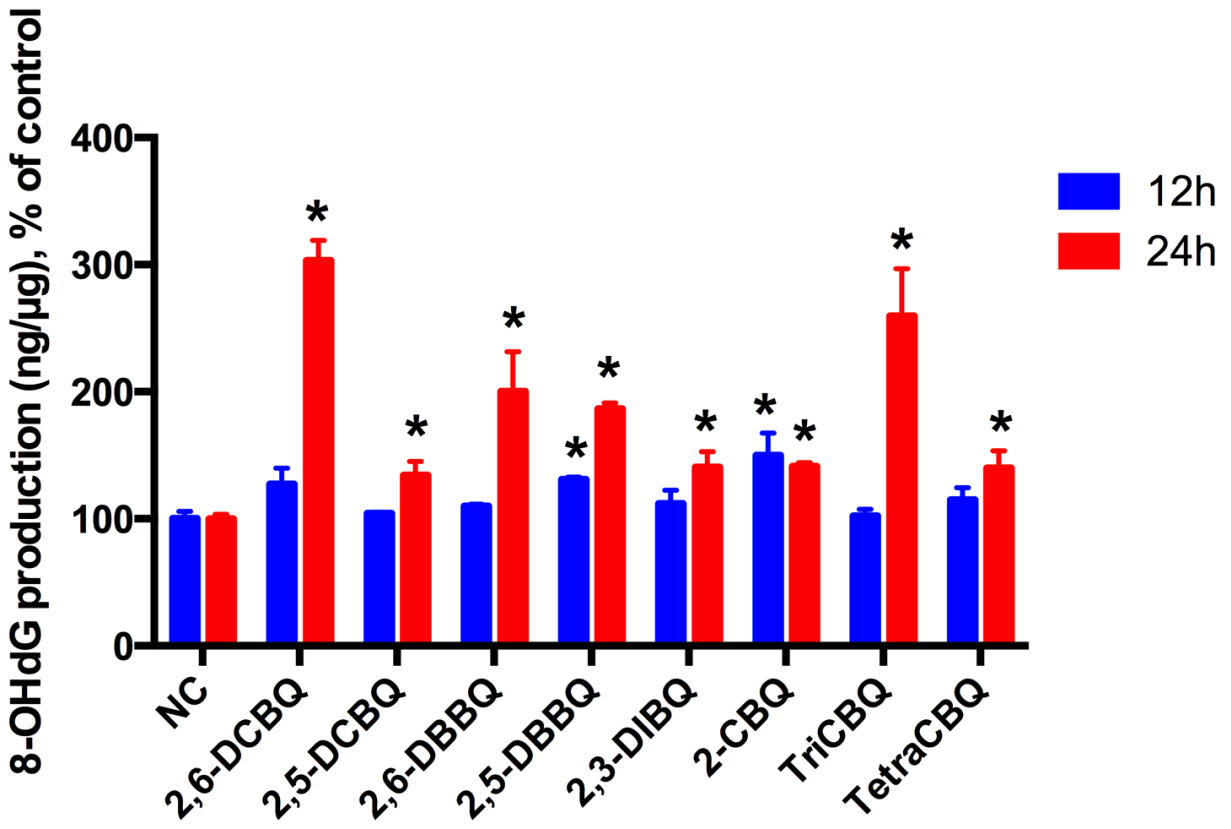


Figure 3.5 Percentage of 8-OHdG production over control after CHO-K1 cells were treated with each HBQ (24 h IC_{50}) at 12 h and 24 h post exposure. All values are expressed as mean ± SEM from two separate experiments with duplicate samples. Significant differences between treatment groups of HBQs and their respective control group are denoted (*P < 0.05).

Table 3.4 Linear regression formula and R^2 of ROS production at 6, 12, and 24 h post-exposure for individual HBQs in CHO-K1 cells

	Concentration	6 h		12 h		24 h	
	(μM)	**Formula**	**R^2**	**Formula**	**R^2**	**Formula**	**R^2**
2,6-DCBQ	0−40	Y = 9377*X	0.9	Y = 15596*X	0.86	Y = 9078*X	0.992
2,5-DCBQ	0−25	Y = 18123*X	0.924	Y = 25704*X	0.848	Y = 19046*X	0.987
2,6-DBBQ	0−35	Y = 12926*X	0.946	Y = 18828*X	0.988	Y = 15177*X	0.947
2,5-DBBQ	0−25	Y = 63998*X	0.906	Y = 66129*X	0.962	Y = 21433*X	0.907
2,3-DIBQ	0−10	Y = 45090*X	0.895	Y = 51824*X	0.866	Y = 26132*X	0.894
2-CBQ	0−15	Y = 22646*X	0.991	Y = 29489*X	0.972	Y = 15942*X	0.947
TriCBQ	0−25	Y = 11832*X	0.951	Y = 18023*X	0.921	Y = 12124*X	0.931
TetraCBQ	0−20	Y = 23515*X	0.993	Y = 35793*X	0.889	Y = 19287*X	0.859

Table 3.5 Percentage of p53 protein over control after CHO-K1 cells were treated with each HBQ (24 h IC_{50}) at 12 h and 24 h post exposure.

	12h	24h
NC	100.0 ± 4.4	100.0 ± 5.5
2,6-DCBQ	93.3 ± 1.7	118.4 ± 1.8*
2,5-DCBQ	108.2 ± 3.8	257.4 ± 19.5*
2,6-DBBQ	106.7 ± 7.0	102.7 ± 5.4
2,5-DBBQ	131.5 ± 11.2*	218.9 ± 15.2*
2,3-DIBQ	114.3 ± 3.7*	114.9 ± 4.0*
2-CBQ	96.4 ± 0.1	98.5 ± 4.6
TriCBQ	98.9 ± 1.5	95.2 ± 1.2
TetraCBQ	98.9 ± 6.1	86.3 ± 4.1

All values are expressed as mean ± SEM from two separate experiments with triplicate samples from each. Significant differences between treatment groups of HBQs and their respective control group are denoted (*$P < 0.05$).

Table 3.6 Pearson correlation coefficient (r) of physicochemical and toxicological parameters of eight HBQs

		$logk_{ROS}$	**8-OHdG**	**p53**	**logP**	**E_{HOMO}**	**E_{LUMO}**	**μ**	**R**	**S**
24 h	$logIC_{50}$	-0.708*	0.726	-0.029	-0.430	-0.639	-0.766*	-0.721*	-0.283	0.169
	$logk_{ROS}$	-	-0.821*	0.310	0.604	0.625	0.392	0.316	0.578	0.315
	8-OHdG	-0.821*	-	-0.194	-0.253	-0.250	-0.395	-0.214	-0.172	-0.041
	p53	0.310	-0.194	-	-0.239	-0.160	-0.468	-0.331	-0.176	-0.276
12 h	$logIC_{50}$	-0.650	-0.319	-0.268	-0.394	-0.577	-0.864**	-0.787*	-0.229	0.131
	$logk_{ROS}$	-	0.256	0.771*	0.507	0.547	0.340	0.255	0.493	0.250
	8-OHdG	0.256	-	-0.052	-0.383	-0.194	0.192	0.078	-0.460	-0.589
	p53	0.771*	-0.052	-	0.344	0.449	-0.090	-0.008	0.431	0.164

* $P < 0.05$ and ** $P < 0.01$, there is a significant correlation.

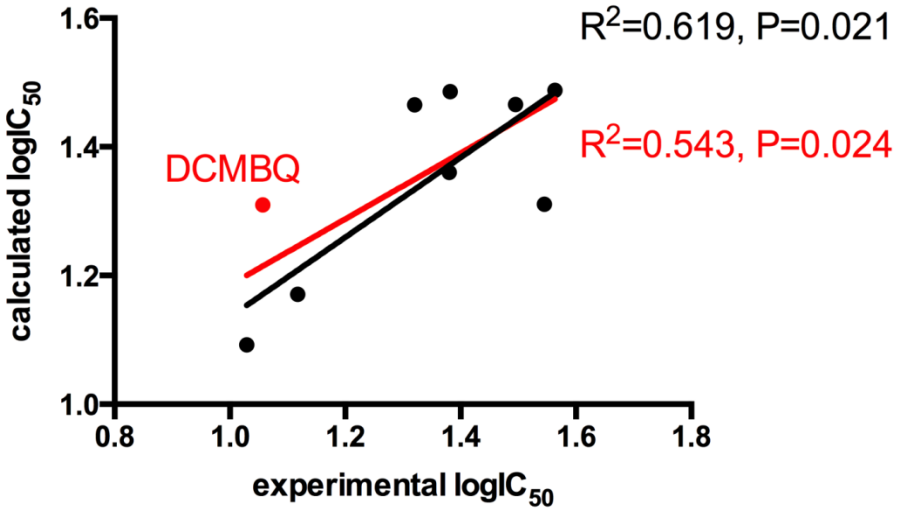


Figure 3.6 Calculated vs experimental $logIC_{50}$ values for CHO-K1 cells treated with HBQs for 24 h. Calculated $logIC_{50}$ values were derived from the QSTR equation: $logIC_{50}=0.52-0.364\times E_{LUMO}$. The black line represents the linear correlation of the eight HBQs under investigation in this study (n=8, $R^2=0.619$, P=0.021). The red line represents the linear correlation of the eights HBQs under investigation in this study with an additional HBQ-DBP, DCMBQ (red point) (n=9, $R^2=0.543$, P=0.024).

Table 3.7 Pearson correlation coefficient (r) of physicochemical and toxicological parameters of isomeric HBQs or halogen-substituted HBQs after 24 h exposure

		logk_{ROS}	**8-OHdG**	**p53**	**log$P$**	**E_{HOMO}**	**E_{LUMO}**	**μ**	**R**	**S**
isomeric HBQs	**logIC_{50}**	-0.979*	0.753	-0.855	-0.570	-0.555	-0.615	0.965*	-0.570	-0.569
	logk_{ROS}	-	-0.858	0.719	-0.480	0.461	0.532	-0.981**	0.480	0.477
halogen-substituted HBQs	**logIC_{50}**	-0.870*	0.697	-0.042	-0.951*	-0.943*	-0.940*	-0.897*	-0.929*	-0.894*
	logk_{ROS}	-	-0.881	0.331	0.674	0.683	0.617	0.530	0.701	0.706

* $P < 0.05$ and ** $P < 0.01$, there is a significant correlation.

Chapter 4 The involvement of cellular glutathione defense system in HBQ-induced cytotoxicity [6]

4.1 Introduction

As introduced previously, four halobenzoquinones (HBQs) have been identified in disinfected drinking water as a new class of DBPs, including 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ), 2,3,6-trichloro-1,4-benzoquinone (TriCBQ), and 2,6-dibromobenzoquinone (2,6-DBBQ).[1] A quantitative structure toxicity relationship (QSTR) model has predicted HBQs as one of the plausible bladder carcinogens and thus proposed to be priority DBPs.[2] In Chapter 2, I have reported that these four HBQs were cytotoxic to T24 human bladder carcinoma cells. The four HBQs can induce cellular oxidative stress via generation of reactive oxygen species (ROS), leading to oxidative damage to DNA and proteins.[3] Additionally, HBQs have been observed to interact with oligonucleotides in solutions, [4] and induce DNA methylation.[5] In Chapter 3, I have found that HBQs could also increase cellular ROS levels in CHO-K1 cells and also increase the oxidative DNA damage marker 8-OHdG.[6] However, the exact mechanism of the cytotoxicity of HBQs is unknown. Because oxidative stress is partly responsible for their toxic effects, HBQs may influence the endogenous antioxidant defense system, which has not been studied up to date.

Glutathione (GSH) and glutathione-related enzymes constitute the primary defense mechanism against cytotoxicity of xenobiotics, especially oxidative stress. GSH is a tri-peptide, comprised of glutamate, cysteine, and glycine. It is an abundant non-protein thiol in cells and is found at the millimolar concentration levels (0.1 to 10 mM), depending on cell types.[7-8] GSH serves as a detoxificant via either a conjugation reaction catalyzed by glutathione S-transferase (GST) or a reduction of hydrogen peroxide by glutathione peroxidase (GPx).[7] Cellular GSH synthesis is associated with two enzymes, glutamatyl cysteine synthetase (GCS) and glutathione synthetase (GS). Here, I hypothesize that GSH defense system plays an important role in protecting cells from cytotoxicity induced by HBQs. This is supported by the literature of

[6] This chapter has been published as Jinhua Li, Wei Wang, Hongquan Zhang, X. Chris Le, and Xing-Fang Li. Glutathione mediated detoxification of halobenzoquinone drinking water disinfection byproducts in T24 cells. Toxicol Sci, 2014, 141(2): 335-343.

toxicity of quinones, since quinones can react readily with sulfur nucleophiles (covalent binding), such as GSH or cysteine residues on proteins, leading to depletion of cellular GSH levels.[9] In this study, I will use buthionine sulfoximine (BSO), an irreversible inhibitor of GCS as BSO can bind to GCS tightly,[10] to explore if the GSH depletion would affect cytotoxicity of HBQs. I also included a parallel assay to examine if extracellular GSH supplementation would affect cytotoxicity of HBQs. The objectives of this study are to explore the role of GSH system in detoxification of HBQs in T24 cells, and to examine the effects of HBQs on cellular GSH levels and glutathione-related enzyme activities including GPx and GST. The results are important to the understanding of mechanisms of toxicity of HBQ DBPs.

4.2 Materials and Methods

4.2.1 Reagents

Standards of 2,6-DCBQ, DCMBQ, TriCBQ, and 2,6-DBBQ were purchased from Sigma–Aldrich, Shanghai Acana Pharmtech, INDOFINE Chemical Company and Fluka respectively. HBQs were dissolved in methanol (HPLC grade, Fisher Scientific) and stored at -20°C in sterile amber glass vials. L-buthionine-sulfoximine (BSO) (Sigma) was dissolved in complete McCoy's 5A medium at a concentration at 20 mM as stock solution and stored at 4°C. L-Glutathione reduced (GSH) was dissolved in complete McCoy's 5A medium at a concentration of 100 mM as stock solution and stored at -20°C. Protease inhibitor cocktail was purchased from Sigma-Aldrich and stored at -20°C. Triton X-100 was purchased from Fisher Scientific.

4.2.2 Cell culture

The human bladder epithelial carcinoma cell line, T24, was obtained from ATCC (Manassas, VA) and cultured in McCoy's 5A modified medium (ATCC) plus 10% fetal bovine serum (FBS) (Sigma) and 1% Penicillin/Streptomycin (100 U/100µg/mL) (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2.

4.2.3 Effects of glutathione depletion on cell viability

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was used to measure cell viability. T24 cells were cultured in the 96

microwell plates. The viability of the cells was measured at 490 nm using a Microplate Spectrometer (Bio-Rad, Benchmark Plus). T24 cells were pretreated with or without 50 μM of BSO for 12 h. When the cells reached 70–80 % confluence, the media were removed and replaced with the media containing varying concentrations of HBQs. After treated with HBQs for 24 h, the viability of the cells was measured. Untreated cells were included as negative control. All experiments were repeated twice in triplicate.

4.2.4 Effects of exogenous glutathione on cell viability

To study the effects of exogenous GSH on cell viability and HBQ-cytotoxicity, MTS assay was used as described above. T24 cells were seeded in the 96 microwell culture plate in the presence of 50 μM of BSO. After 12 h, half of the microwells were replaced with 10 mM of GSH/well in medium, and the other half microwells were replaced with the culture medium without addition of GSH. After another 12 h, the old medium was aspirated, and T24 cells were washed by phosphate buffered saline (PBS) twice and followed by treatment with varying concentrations of the four HBQs. After the cells were treated with HBQ for 24 h, the cell viability was measured. Untreated cells were also measured as negative control. All experiments were repeated twice in triplicate.

4.2.5 Measurement of cellular glutathione levels

T24 cells were seeded in 60 mm dish. After the cells reached 70-80% confluence, they were treated with the concentrations (3/6, 4/6, 5/6 and 1 24h-IC_{50}) of HBQs. Negative control and solvent control (0.4% methanol, v/v) were also included. After 24 hours of HBQ exposure, the cells were trypsinized, collected and centrifuged at 1700 rpm, for 3 minutes. Cell pellets were transferred to 1.5 ml-microcentrifuge tubes, washed twice with ice-cold PBS and resuspended in ice-cold metaphosphoric acid (MPA). After homogenization, the solution was centrifuged at 10,000 × g at 4°C for 10 min and then the supernatant was used for the measurement of GSH levels according to the manufacturer's instructions (Bioxytech-GSH 400, OxisResearch, Portland, OR, USA). The assay was performed in microfuge tubes and transferred to flat-bottom 96-well plates for absorbance measurement at 400 nm. The pellet from the centrifugation was dissolved in 100 μL of 0.1 M NaOH and the protein concentration was determined using the Bio-Rad microprotein assay in a 96-well plate using bovine serum albumin (BSA) as the standard. The

GSH level was expressed as nmol GSH/mg cellular protein or nmol GSH/10^6 cells, and then as the percentage of the control.

4.2.6 Measurement of cellular glutathione S-transferase (GST) activity

T24 cells were seeded and exposed to HBQs as described for the GSH assay. After 24 hours of HBQ exposure, the cells were collected using a rubber policeman and washed with ice-cold PBS and then lysed in GST sample buffer provided in a GST Colorimetric Activity Assay kit (BioVision, Mountain View, CA, USA). A freeze-thaw process was used to break cell membranes: frozen in liquid nitrogen for 15 minutes and thawed on ice. The cell lysate was centrifuged at 10,000 × g for 15 min at 4 ºC and the supernatant was collected and stored at -80 ºC until analysis.

The GST colorimetric activity assay is based on the GST-catalyzed reaction between GSH and a GST substrate, CDNB (1-chloro-2,4-dinitrobenzene). Formation of DNP-GSH can be measured with a spectrophotometer at 340 nm. Measurements were performed every minute over a 5-min period. One unit of GST activity is defined as the amount of enzyme producing 1 mmol of CDNB-GSH/min under the conditions of the assay. Protein concentration was determined by the Bio-Rad microprotein assay in a 96-well plate using BSA as the standard.

4.2.7 Measurement of cellular glutathione peroxidase (GPx) activity

To obtain a cell lysate for the GPx activity assay, T24 cells were exposed to HBQs as described in the GSH assay. The cells were collected by a rubber policeman in ice-cold DPBS, and resuspended in an ice-cold 1 × assay Buffer (HT Glutathione Peroxidase Assay Kit, Trevigen, Gaithersburg, MD, USA) containing 1% protease inhibitor cocktail and 1% Triton X-100. The cell suspensions were incubated on ice with periodic vortexing for 30 minutes. The cell lysate was centrifuged at 10,000 × g for 15 min at 4 ºC, and the supernatant was collected and stored at -80ºC until assayed. The protein concentration of the cleared cell lysate was determined with the Bio-Rad microprotein assay in a 96-well plate using bovine serum albumin as the standard.

GPx activity was measured using a HT Glutathione Peroxidase Assay Kit. This assay measures the GPx activity indirectly by a coupled reaction with GR. Because GPx catalyzes the reduction of hydrogen peroxide using GSH as a substrate and forms GSSG and water, GR is used to reduce GSSG to GSH and decrease the NADPH level. The decreased level of NADPH

measured at 340 nm is directly proportional to GPx activity in the sample. It was measured every minute for 10 time points. One unit of glutathione peroxidase is defined as the amount of enzyme that will cause the oxidation of 1 nmole of NADPH to $NADP^+$ per minute at 25°C. Protein concentration was determined by the Bio-Rad microprotein assay in a 96-well plate using BSA as the standard.

4.2.8 Mass spectrometry analysis of the conjugation between HBQ and GSH[7]

Stock solution of GSH (20 mM) was prepared daily by dissolving GSH in Optima water, and stored at 4 °C. Stock solution of HBQ (10 mM) was prepared monthly by dissolving HBQ in methanol, and stored at -20 °C. The conjugation reactions were performed by mixing GSH with HBQ in water at varying ratios. The final concentrations of GSH and HBQs were 1 mM each. The final ratio of methanol:water in the tested sample was 1:9. The reactions lasted for one h at 37°C. Finally the reacted samples were injected into the high resolution mass spectrometer for analysis of the conjugates.

A hybrid quadrupole time-of-flight (ToF) mass spectrometer (AB Sciex Triple ToF 5600, Concord, ON, Canada) was used to identify the conjugation products of the four HBQs with GSH. Both positive and negative mode was used, and the data indicated negative mode is more suitable. Experiments were performed at ion source voltage of -4500 V, gas I of 60 arbitrary units, gas II of 60 arbitrary units, curtain gas of 25 arbitrary units, source temperature of 450ºC, declustering potential (DP) of -90 V, collision energy (CE) of -40 V, collision energy spread (CES) of 10V.

4.2.9 Data Analysis

Statistical analysis was performed using GraphPad Prism 5 (Graphpad Software) and OriginPro 8.5 (OriginLab Corporation). Experimental results were expressed as mean ± standard deviation (SD). Differences between the treated groups with and without BSO were determined by Student's *t*-test. One-way analysis of variance (ANOVA, followed by Dunnett's *Post Hoc* Multiple Comparisons) was used for multiple comparisons among treatment and control groups. Pearson correlation analysis was used for the study of relationships between GSH level and

[7] Dr. Wei Wang contributed to the data collection and analysis of HBQ-GSH conjugates.

enzyme activity or ROS production. Differences were considered statistically significant at $P<0.05$.

4.3 Results

4.3.1 GSH Mediated detoxification of HBQs in T24 cells

I first hypothesize that GSH plays one of the key roles in detoxification of HBQs. To confirm it, I have examined the cytotoxicity of HBQs to T24 cells when intracellular GSH is depleted and when GSH is supplemented in culture media. First, I identified the optimal concentration of BSO to deplete GSH level, but have minimal toxicity to T24 cells. After T24 cells pretreated with BSO ≤ 50μM, the cell viability maintained more than 90% (Table 4.1). Therefore, BSO (50 μM) was used for the following experiments.

With or without the pretreatment of BSO, the concentration dependent toxic effects of HBQs were obtained as shown in viability curves (Figure 4.1). The IC_{50} values for HBQs are presented in Table 4.2. Without the BSO pretreatment, the IC_{50} values are 2,6-DCBQ (95 μM), DCMBQ (110 μM), TriCBQ (151μM), and 2,6-DBBQ (142 μM). With the BSO pretreatment, the IC_{50} values are 2,6-DCBQ (63 μM), DCMBQ (22 μM), TriCBQ (94μM), and 2,6-DBBQ (70 μM). Compared with no pretreatment of BSO, the IC_{50} of HBQs significantly decreased for 1.5─4.9 fold ($P<0.0001$). The toxicity of HBQ compounds can be ranked as 2,6-DCBQ > DCMBQ > 2,6-DBBQ > TriCBQ in the absence of BSO and as DCMBQ > 2,6-DCBQ ≥ 2,6-DBBQ > TriCBQ in the presence of BSO. The results clearly demonstrate that T24 cells are more sensitive to DCMBQ with pre-treatment of BSO than the other three HBQs, suggesting that pre-depletion of GSH dramatically increases the susceptibility of T24 cells to DCMBQ-cytotoxicity.

After confirming the role of intracellular GSH in detoxification of HBQs, I examined whether exogenous GSH can also assist with detoxification. Figure 4.2 shows the viability of T24 cells treated with HBQs when the cells were with and without pretreatment of GSH. The pretreatment of GSH significantly reduces the cytotoxic effects of HBQs.

Taken together, GSH depletion enhanced the cytotoxicity of HBQs and GSH supplementation attenuated HBQ-induced cytotoxicity in T24 cells, supporting the hypothesis that GSH plays one of the key roles in detoxification of HBQs.

4.3.2 Effects of HBQs on the intracellular levels of free reduced GSH

I further hypothesize that HBQ cytotoxicity is associated with the depletion of intracellular GSH induced by HBQ. To confirm this hypothesis, I studied the effects of HBQs on free GSH levels in T24 cells. Solvent control experiments show that the amount of methanol used in HBQ solutions does not induce statistically significant change in GSH levels (Figure 4.3).

Figure 4.4 presents the cellular GSH levels after HBQ treatments at the same concentration of equivalent biological response (3/6, 4/6, 5/6 and 1 IC_{50}). Because DCMBQ demonstrated significantly reduced IC_{50} when cellular GSH depleted, two lower concentrations (1/20 and ¼ IC_{50}) were used in this study. The reduction in cellular GSH levels is clearly dependent on the concentrations of HBQs ($P<0.05$) (one-way ANOVA analysis of variance, followed by Post Dunnett test). At half of IC_{50} value, the cellular GSH is 57% (2,6-DCBQ), 16% (DCMBQ), 62% (TriCBQ), and 92% (2,6-DBBQ) of the control; at IC_{50} value, the cellular GSH is 23% (2,6-DCBQ), 7% (DCMBQ), 25% (TriCBQ), and 35% (2,6-DBBQ) of the control. These results indicate that at the same concentration of equivalent biological response, DCMBQ displays a higher ability to deplete cellular GSH compared with the other three HBQs.

4.3.3 Effects of HBQs on cellular glutathione S-transferase (GST) activity

To further explain the role of GSH in cellular response to HBQ-treatment, I also evaluated the changes of GSH-associated enzymes in HBQ-treated T24 cells: GST that indicates the conjugation of GSH, and GPx that is involved in the reduction reaction.

To investigate the impact of HBQs on GST activity in T24 cells, I used a colorimetric assay to measure the DNP-GSH conjugate that is catalyzed by GST. Figure 4.5 shows alteration of the cellular GST activity when T24 cells were treated with varying concentrations of individual HBQs at the same concentration used in GSH assay. GST activity increases with the increase of HBQ concentrations used to treat T24 cells. At the same concentration of equivalent biological response (½ 24h-IC_{50} to 24h-IC_{50}), the GST activity over the control significantly increased for several fold ($P < 0.05$): 1.2─2.5 (2,6-DCBQ), 2.5─3.5 (DCMBQ), 1.5─4.5 (TriCBQ), and 1.4─2.0 fold (2,6-DBBQ), when the concentrations of HBQs used were 75 and 95 mM for 2,6-DCBQ, 55─110 μM for DCBMQ, 100─150 μM for TriCBQ, and 118─142 μM for 2,6-DBBQ. In parallel, the solvent control displayed no change on GST activity compared with the negative control (Figure 4.6).

4.3.4 Effects of HBQs on cellular glutathione peroxidase (GPx) activity

Figure 4.7 shows the cellular GPx activities at varying concentrations of HBQs treatments. No change of GPx activities was observed in the cells exposed to 0.4% methanol compared with the negative control (Figure 4.8). The effects of HBQs on GPx activities are compound dependent. When the cells were treated with each HBQs at the concentrations of 1/2 IC_{50}-IC_{50}, 2,6-DCBQ and TriCBQ did not change GPx activity, whereas DCMBQ (1/4 IC_{50}-IC_{50}) and 2,6-DBBQ (1/2 IC_{50}-IC_{50}) significantly reduced GPx activity, compared to that of the negative control ($P < 0.05$). At the same concentration of equivalent biological response (½ 24h-IC_{50} to 24h-IC_{50}), GPx activity was deceased to 75%-83% for DCMBQ and 65%-72% for 2,6-DBBQ compared to the negative control.

4.3.5 Correlation between cellular GSH level and GST activity, GPx activity or ROS production

Finally, I conducted a Pearson correlation analysis between cellular GSH level and GST activity, GPx activity or ROS production (Table 4.3). The cellular GSH level is inversely correlated with ROS production and cellular GST activity in the cells treated with the four HBQs; the correlation was highly significant for 2,6-DCBQ and DCMBQ, while moderate for TriCBQ and 2,6-DBBQ. The cellular GSH level was significantly correlated with cellular GPx activity in DCMBQ-treated cells, but not in the cells treated with the other three HBQs. To sum up, HBQs can deplete the cellular GSH level; induce production of ROS and GST activity.

4.4 Discussion

I first examined the role of GSH in the defense against HBQ-induced cytotoxicity in bladder cancer cell line T24, because our previous studies have shown T24 cells were more sensitive to some DBPs, including nitrosamines and phenazine, and it is relevant to the potential risk of bladder cancer due to exposure to DBPs.[11-13] The involvement of GSH in detoxifying the HBQ-induced cytotoxicity was first supported by two sets of evidence: GSH depletion enhanced the cytotoxicity induced by HBQs and GSH supplementation reduced HBQ-induced cytotoxicity. A decrease of intracellular GSH was observed in T24 cells exposed to HBQs. These observations indicate that HBQ cytotoxicity is associated with depleting intracellular GSH. I then examined whether the GSH depletion was due to conjugation or reduction reaction through the analysis of

the effects of HBQs on GST and GPx enzyme activities. Our results indicate that GST plays more important role compared with GPx, which lead us to propose a hypothesis that GSH depletion is mainly due to conjugation. To confirm this hypothesis, I examined HBQ-GSH conjugation using mass spectrometry analysis. After incubating GSH with individual HBQs for one hour, I identified the peaks corresponding to the conjugation products (HBQ-GSH). Figure 4.9 shows the measured (black) and theoretical (red) accurate mass and isotope pattern of the conjugation products of GSH with 2,6-DCBQ, DCMBQ, TriCBQ and 2,6-DBBQ, respectively. The accurate mass measurements are in agreement with the theoretical mass and isotopic patterns (red) of the conjugates consisting of one molecule of HBQ bound with one molecule of GSH. The mass accuracies are all better than 10 ppm. Our results are consistent with the determination of GSH conjugation with quinones.[14-15]

The pretreatment of cells with 50 μM BSO increased the cytotoxicity of DCMBQ up to 5 fold and that of other three HBQs by 2 fold. This result is consistent with others' studies that BSO sensitizes T24 cells to increase cytotoxicity of other compounds,[16-17] and increases ML-1 cells' susceptibility to toxic effects of hydroquinone.[18] GSH supplementation reduces cytotoxic effects of HBQs, suggesting that T24 cells can uptake GSH. Other cells, kidney cells and the small intestinal cells can also uptake GSH.[19-20] The protection of extracellular GSH against HBQ-cytotoxicity is consistent with our previous result that N-acetylcysteine(NAC) treatment prevents HBQ-induced cytotoxic effects,[3] and also consistent with other findings that exogenous GSH can protect cells and mice from toxicity.[21-23]

HBQs depleted cellular GSH in a concentration-dependent manner. Several studies have shown that GSH depletion is one of the key mechanisms for hydroquinone (HQ) and benzoquinone (BQ) toxicity in some human cells.[18, 24-26] One previous study reported that cellular GSH was decreased to 38% of the control, after HepG2 cells were incubated with HQ (50 μM) for 1 h.[24] In another study, relative GSH content over the control was decreased to 80% for HQ and 60% for BQ, after Beas-2B cells were treated with 20 μM of HQ or BQ for 2 h.[25] Compared with these results of HQ and BQ, HBQs displayed a much higher ability to deplete cellular GSH. At the highest concentration (IC_{50}) value I used, the cellular GSH is depleted to 7% - 35% of the control. Among the four HBQs, DCMBQ has the highest ability to deplete cellular GSH levels. I have previously observed that DCMBQ can generate higher levels of 8-OHdG adducts in T24 compared with the other three HBQs.[3] It is likely that higher depletion of GSH by

DCMBQ may make T24 cells more sensitive to DNA damage and lead to enhanced formation of DNA adducts. Quinones are Michael acceptors and react readily with sulfur nucleophiles like GSH, leading to depletion of cellular GSH levels.[9] Moreover, conjugation of HQ and BQ to GSH resulted in the formation of less toxic metabolites with increased solubility and excretion.[27] Our mass spectrometry results of interactions of HBQs with GSH support the HBQ-GSH conjugation. Therefore, the conjugation between HBQs and GSH is proposed to be one of the major pathways of HBQ-induced depletion of cellular GSH levels.

The findings of the effects of HBQs on the activities of GST and GPx are supported by the study of BQ-treated MCF7 cells that no significant change occurs in the gene expression of GPx whereas GST gene expression was significantly enhanced after 24 h treatment.[28] The induction of intracellular antioxidants, such as detoxifying enzymes (e.g. GST), is critical for detoxification of quinones and benzene.[15, 25, 29] The induction of GST was also observed in inorganic arsenic, and similar pathway is also at work for many heavy metal ions, e.g. Cd^{2+}.[30-31] The induction of GST and other GSH-related enzymes are mostly regulated by the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which is the principal regulator of the antioxidant response element (ARE) – driven cellular defense system.[32] The lower cellular GPx activity in HBQ-treated T24 cells suggests that GPx was consumed to defend against cellular ROS. As a cofactor, GSH is indispensable in the process that GPx catalyzed hydrogen peroxide or hydroperoxide into water.[33-34] Because HBQs significantly depleted GSH levels, little cellular GSH left for GPx to use. Also, catalase (CAT) is able to convert hydrogen peroxide into water, while CAT does not need GSH as a cofactor.[33] Therefore, the effects of HBQs on cellular GPx activity were not as significant as on GST activity.

Based on the results, I propose the possible pathways of GSH-mediated detoxification of HBQs (Figure 4.10). Low concentrations of HBQs can cause cellular ROS production, GSH depletion, and oxidative stress, which lead to the activation of related genes (like Nrf2/ARE) and to the induction of cellular antioxidants (like GST) to detoxify HBQs. The data presented here support a possible pathway for the toxic mechanisms of HBQs, a class of drinking water DBPs.

4.5 References

1. Zhao, Y. L.; Qin, F.; Boyd, J. M.; Anichina, J.; Li, X.-F., Characterization and determination of chloro- and bromo-benzoquinones as new chlorination disinfection byproducts in drinking water. *Anal. Chem.* **2010,** *82* (11), 4599-4605.
2. Bull, R. J., Reckhow, D.A. , Rotello, V. , Bull, O.M., and J. Kim, *Use of Toxicological and Chemical Models to Prioritize DBP Research*. Awwa Research Foundation: Denver CO, 2006.
3. Du, H. Y.; Li, J. H.; Moe, B.; McGuigan, C. F.; Shen, S. W.; Li, X.-F., Cytotoxicity and oxidative damage induced by halobenzoquinones to T24 bladder cancer cells. *Environ. Sci. Technol.* **2013,** *47* (6), 2823-2830.
4. Anichina, J.; Zhao, Y. L.; Hrudey, S. E.; Le, X. C.; Li, X.-F., Electrospray ionization mass spectrometry characterization of interactions of newly identified water disinfection byproducts halobenzoquinones with oligodeoxynucleotides. *Environ. Sci. Technol.* **2010,** *44* (24), 9557-9563.
5. Zhao, B.; Yang, Y.; Wang, X.; Chong, Z.; Yin, R.; Song, S. H.; Zhao, C.; Li, C.; Huang, H.; Sun, B. F.; Wu, D.; Jin, K. X.; Song, M.; Zhu, B. Z.; Jiang, G.; Rendtlew Danielsen, J. M.; Xu, G. L.; Yang, Y. G.; Wang, H., Redox-active quinones induces genome-wide DNA methylation changes by an iron-mediated and Tet-dependent mechanism. *Nucleic Acids Res.* **2013,** *42* (3), 1593-1605.
6. Li, J.; Moe, B.; Vemula, S.; Wang, W.; Li, X.-F., Emerging disinfection byproducts, halobenzoquinones: effects of isomeric structure and halogen substitution on cytotoxicity, formation of reactive oxygen species, and genotoxicity. *Environ. Sci. Technol.* **2016,** *50* (13), 6744-52.
7. Hayes, J. D.; McLellan, L. I., Glutathione and glutathione-dependent enzymes represent a Co-ordinately regulated defence against oxidative stress. *Free Radic. Res.* **1999,** *31* (4), 273-300.
8. Ehrlich, K.; Viirlaid, S.; Mahlapuu, R.; Saar, K.; Kullisaar, T.; Zilmer, M.; Langel, U.; Soomets, U., Design, synthesis and properties of novel powerful antioxidants, glutathione analogues. *Free Radic. Res.* **2007,** *41* (7), 779-787.
9. Bolton, J. L.; Trush, M. A.; Penning, T. M.; Dryhurst, G.; Monks, T. J., Role of quinones in toxicology. *Chem. Res. Toxicol.* **2000,** *13* (3), 135-160.

10. Griffith, O. W., Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic. Biol. Med.* **1999,** *27* (9-10), 922-935.

11. Boyd, J. M.; Huang, L.; Xie, L.; Moe, B.; Gabos, S.; Li, X.-F., A cell-microelectronic sensing technique for profiling cytotoxicity of chemicals. *Anal. Chim. Acta.* **2008,** *615* (1), 80-87.

12. Villanueva, C. M.; Cantor, K. P.; Grimalt, J. O.; Malats, N.; Silverman, D.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Marcos, R.; Rothman, N.; Real, F. X.; Dosemeci, M.; Kogevinas, M., Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am. J. Epidemiol.* **2007,** *165* (2), 148-156.

13. Zhou, W. J.; Lou, L. J.; Zhu, L. F.; Li, Z. M.; Zhu, L. Z., Formation and cytotoxicity of a new disinfection by-product (DBP) phenazine by chloramination of water containing diphenylamine. *J. Environ. Sci.* **2012,** *24* (7), 1217-1224.

14. Rossi, L.; Moore, G. A.; Orrenius, S.; Obrien, P. J., Quinone toxicity in hepatocytes without oxidative stress. *Arch. Biochem. Biophys.* **1986,** *251* (1), 25-35.

15. Nakamura, Y.; Kumagai, T.; Yoshida, C.; Naito, Y.; Miyamoto, M.; Ohigashi, H.; Osawa, T.; Uchida, K., Pivotal role of electrophilicity in glutathione S-transferase induction by tert-butylhydroquinone. *Biochem-Us* **2003,** *42* (14), 4300-4309.

16. Jiang, J.; Yuan, X.; Zhao, H.; Yan, X.; Sun, X.; Zheng, Q., Licochalcone A inhibiting proliferation of bladder cancer T24 cells by inducing reactive oxygen species production. *Biomed. Mater. Eng.* **2014,** *24* (1), 1019-25.

17. Byun, S. S.; Kim, S. W.; Choi, H.; Lee, C.; Lee, E., Augmentation of cisplatin sensitivity in cisplatin-resistant human bladder cancer cells by modulating glutathione concentrations and glutathione-related enzyme activities. *Bju. Int.* **2005,** *95* (7), 1086-90.

18. Trush, M. A.; Twerdok, L. E.; Rembish, S. J.; Zhu, H.; Li, Y. B., Analysis of target cell susceptibility as a basis for the development of a chemoprotective strategy against benzene-induced hematotoxicities. *Environ. Health. Perspec.* **1996,** *104*, 1227-1234.

19. Deneke, S. M.; Fanburg, B. L., Regulation of cellular glutathione. *Am. J. Physiol.* **1989,** *257* (4), L163-L173.

20. vanKlaveren, R. J.; Demedts, M.; Nemery, B., Cellular glutathione turnover in vitro, with emphasis on type II pneumocytes. *Eur. Respir. J.* **1997,** *10* (6), 1392-1400.

21. Lash, L. H.; Hagen, T. M.; Jones, D. P., Exogenous glutathione protects intestinal epithelial-cells from oxidative injury. *Proc. Natl. Acad. Sci. USA.* **1986,** *83* (13), 4641-4645.
22. Jin, Y. P.; Zhao, F. H.; Zhong, Y. A.; Yu, X. Y.; Sun, D.; Liao, Y. J.; Lv, X. Q.; Li, G. X.; Sun, G. F., Effects of exogenous GSH and methionine on methylation of inorganic arsenic in mice exposed to arsenite through drinking water. *Environ. Toxicol.* **2010,** *25* (4), 361-366.
23. Hagen, T. M.; Brown, L. A.; Jones, D. P., Protection against paraquat-induced injury by exogenous gsh in pulmonary alveolar type-II Cells. *Biochem. Pharmacol.* **1986,** *35* (24), 4537-4542.
24. Luo, L. H.; Jiang, L. P.; Geng, C. Y.; Cao, J.; Zhong, L. F., Hydroquinone-induced genotoxicity and oxidative DNA damage in HepG2 cells. *Chem-Biol. Interact.* **2008,** *173* (1), 1-8.
25. Rubio, V.; Zhang, J.; Valverde, M.; Rojas, E.; Shi, Z. Z., Essential role of Nrf2 in protection against hydroquinone- and benzoquinone-induced cytotoxicity. *Toxicol. In Vitro* **2011,** *25* (2), 521-529.
26. Smith, M. T., Benzene, NQO1, and genetic susceptibility to cancer. *Proc. Natl. Acad. Sci. USA.* **1999,** *96* (14), 7624-7626.
27. Snyder, C. A.; Sellakumar, A. R.; James, D. J.; Albert, R. E., The carcinogenicity of discontinuous inhaled benzene exposures in Cd-1 and C57b1/6 mice. *Arch. Toxicol.* **1988,** *62* (5), 331-335.
28. Baigi, M. G.; Brault, L.; Neguesque, A.; Beley, M.; El Hilali, R.; Gauzere, F.; Bagrel, D., Apoptosis/necrosis switch in two different cancer cell lines: Influence of benzoquinone- and hydrogen peroxide-induced oxidative stress intensity, and glutathione. *Toxicol. In Vitro* **2008,** *22* (6), 1547-1554.
29. Zhou, H. F.; Kepa, J. K.; Siegel, D.; Miura, S.; Hiraki, Y.; Ross, D., Benzene metabolite hydroquinone up-regulates chondromodulin-I and inhibits tube formation in human bone marrow endothelial cells. *Mol. Pharmacol.* **2009,** *76* (3), 579-587.
30. Schuliga, M.; Chouchane, S.; Snow, E. T., Upregulation of glutathione-related genes and enzyme activities in cultured human cells by sublethal concentrations of inorganic arsenic. *Toxicol. Sci.* **2002,** *70* (2), 183-192.
31. Prins, J. M.; Fu, L.; Guo, L.; Wang, Y., Cd(2+)-induced alteration of the global proteome of human skin fibroblast cells. *J. Proteome. Res.* **2014,** *13* (3), 1677-87.

32. Biswas, S. K.; Rahman, I., Environmental toxicity, redox signaling and lung inflammation: the role of glutathione. *Mol. Aspects. Med.* **2009,** *30* (1-2), 60-76.
33. Li, S. J.; Yan, T.; Yang, J. Q.; Oberley, T. D.; Oberley, L. W., The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. *Cancer Res.* **2000,** *60* (14), 3927-3939.
34. Brigelius-Flohe, R.; Kipp, A., Glutathione peroxidases in different stages of carcinogenesis. *Biochim. Biophys. Acta.* **2009,** *1790* (11), 1555-68.

Table 4.1 Percentage of T24 viable cells after exposure to varying concentrations of BSO for 12 h of pretreatment

[BSO], μM	Viable cells (% of control)
0	100
1	100 ± 1
5	99 ± 5
10	96 ± 5
25	95 ± 8
30	95 ± 6
40	90 ± 8
50	92 ± 7
100	41 ± 5

Note: Viability was measured using the MTS assay. Mean ± SD, N = 3 from three independent experiments.

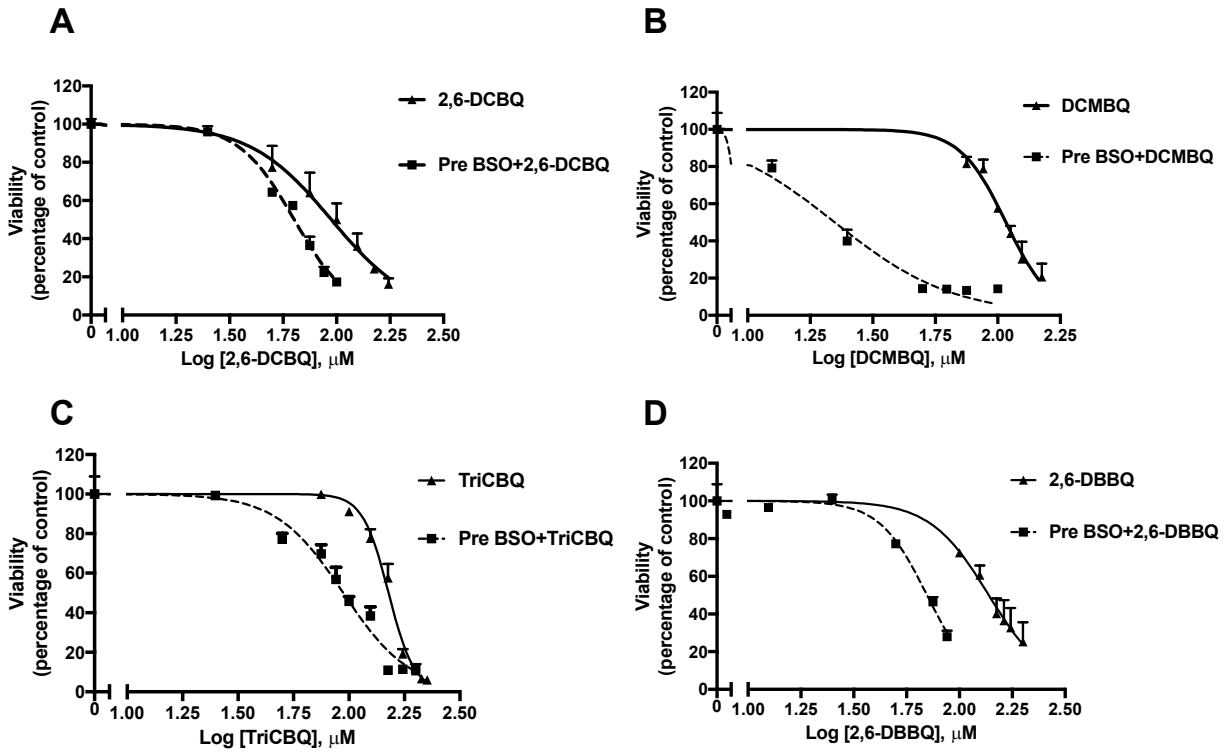


Figure 4.1 Effects of BSO and HBQs on viability of T24 cells. T24 cells were separately exposed to four HBQs for 24 h in the absence and presence of pretreatment of 50 μM BSO (red trace) in the culture media. Quantitative determination of viable cells was performed with MTS assay. Error bars represent SD of triplicate data and three separate experiments (N = 3).

Table 4.2 IC_{50} values of HBQs in the absence and presence of pretreatment with BSO

HBQ compounds	IC_{50}, μM (Mean ± SD, N = 3)		Enhancement factor in cytotoxicity	P value
	Without pretreatment BSO	Pretreatment with BSO		
2,6-DCBQ	95 ± 8	63 ± 2	1.5	<0.0001
DCMBQ	110 ± 4	22 ± 2	4.9	<0.0001
TriCBQ	150 ± 4	94 ± 5	1.6	<0.0001
2,6-DBBQ	142 ± 9	70 ± 3	2.0	<0.0001

Note: Quantitative determination of viable cells was performed with the MTS assay.

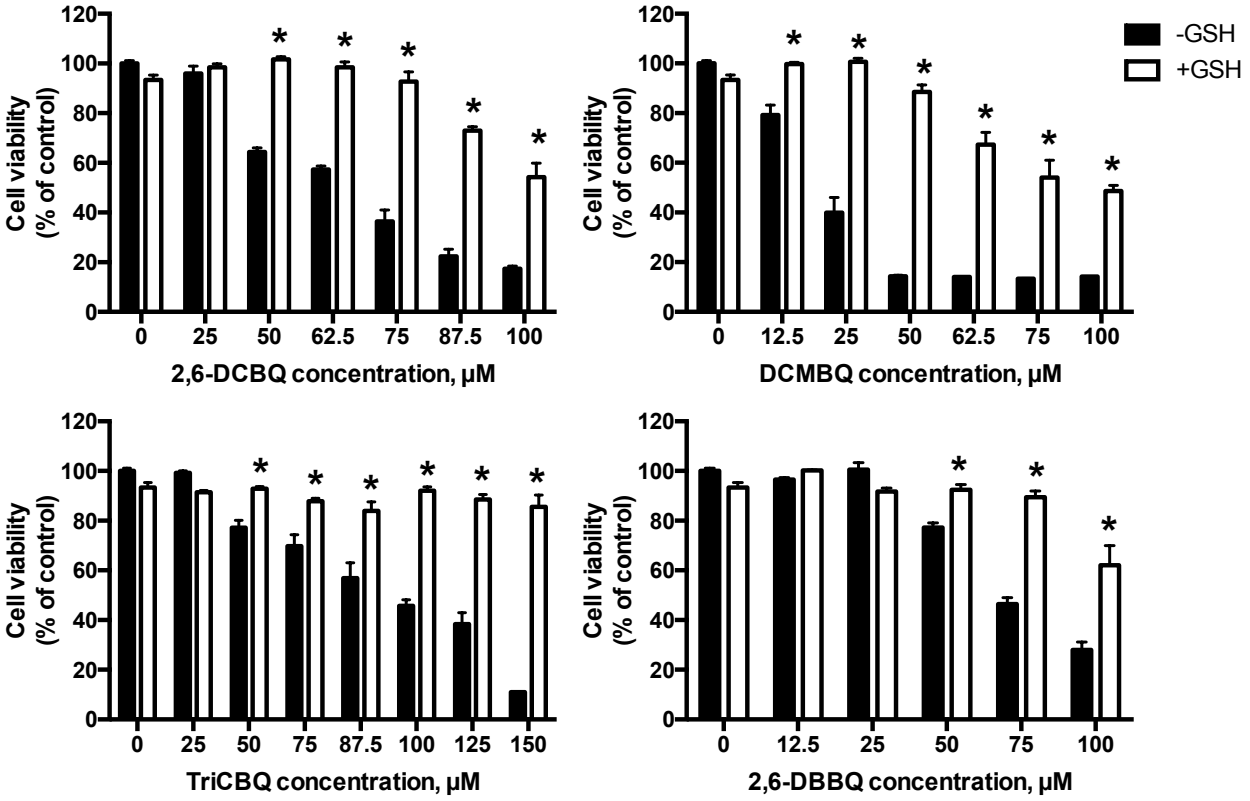


Figure 4.2 Effects of exogenous GSH on HBQ-cytotoxicity in T24 cells. T24 cells were exposed to 4 HBQs for 24 hours after the cells were with or without the pretreatment of 10 mM of exogenous GSH. Quantitative determination of viable cells was performed by MTS assay. Error bars represent SD of triplicate data and three separate experiments (N = 3).

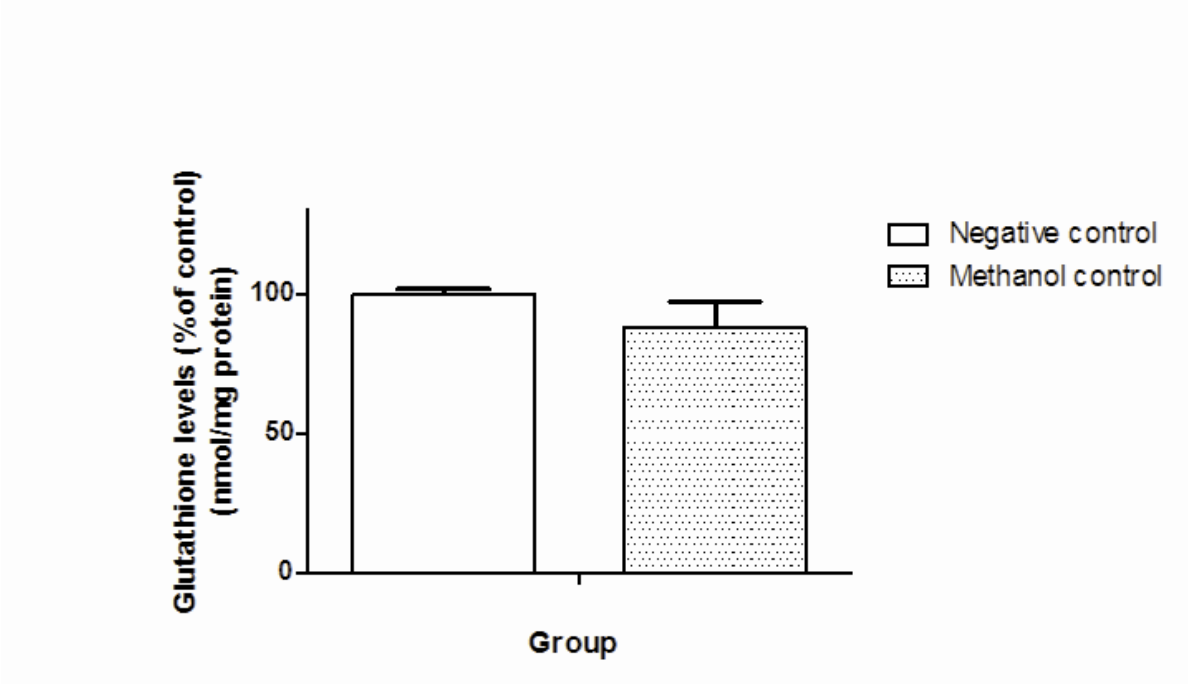


Figure 4.3 Effect of solvent control, 0.4% methanol (v/v), on the cellular glutathione levels in T24 cells. GSH levels were determined after 24 h of exposure to methanol and compared to the levels of negative control (untreated) cells. Error bar represent mean standard error of triplicate data and two separate experiments (N=2).

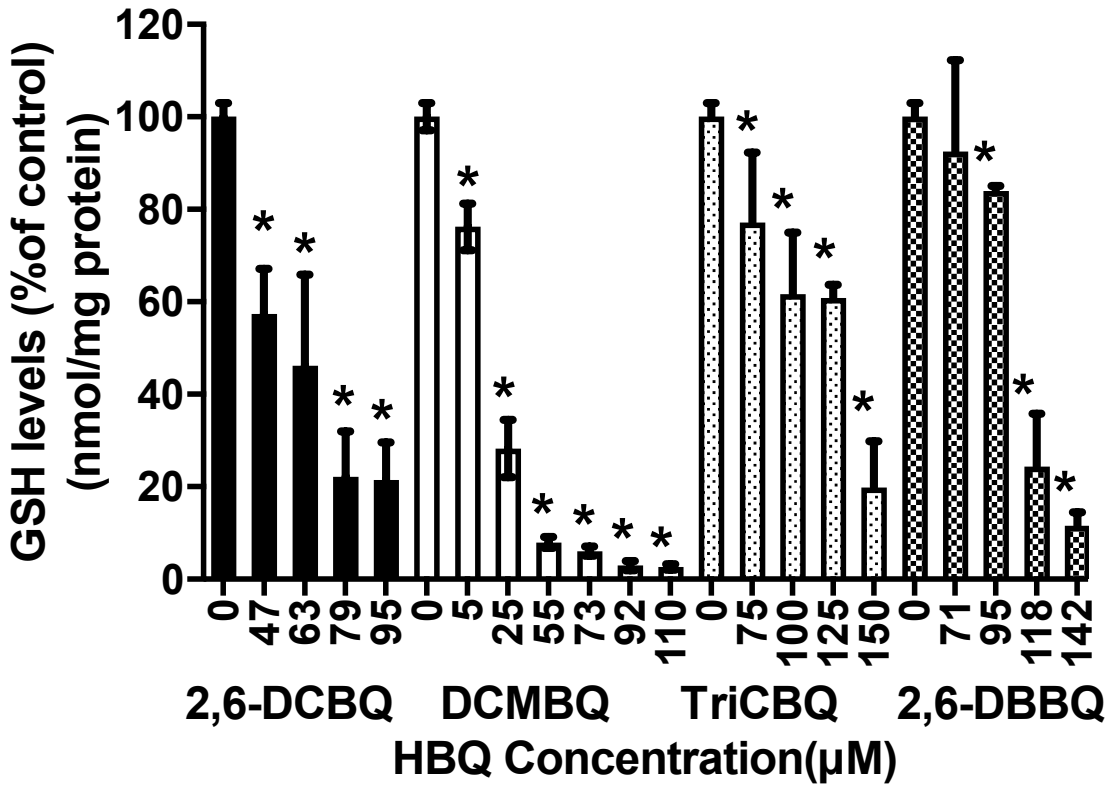


Figure 4.4 Effect of HBQs on the cellular glutathione levels in T24 cells. GSH levels were determined after 24 h of exposure to HBQs and compared to the levels of the negative control. Error bar represents SD of triplicate data and two separate experiments (N=2). $^{*}P<0.05$, HBQ treatment groups compared with negative control.

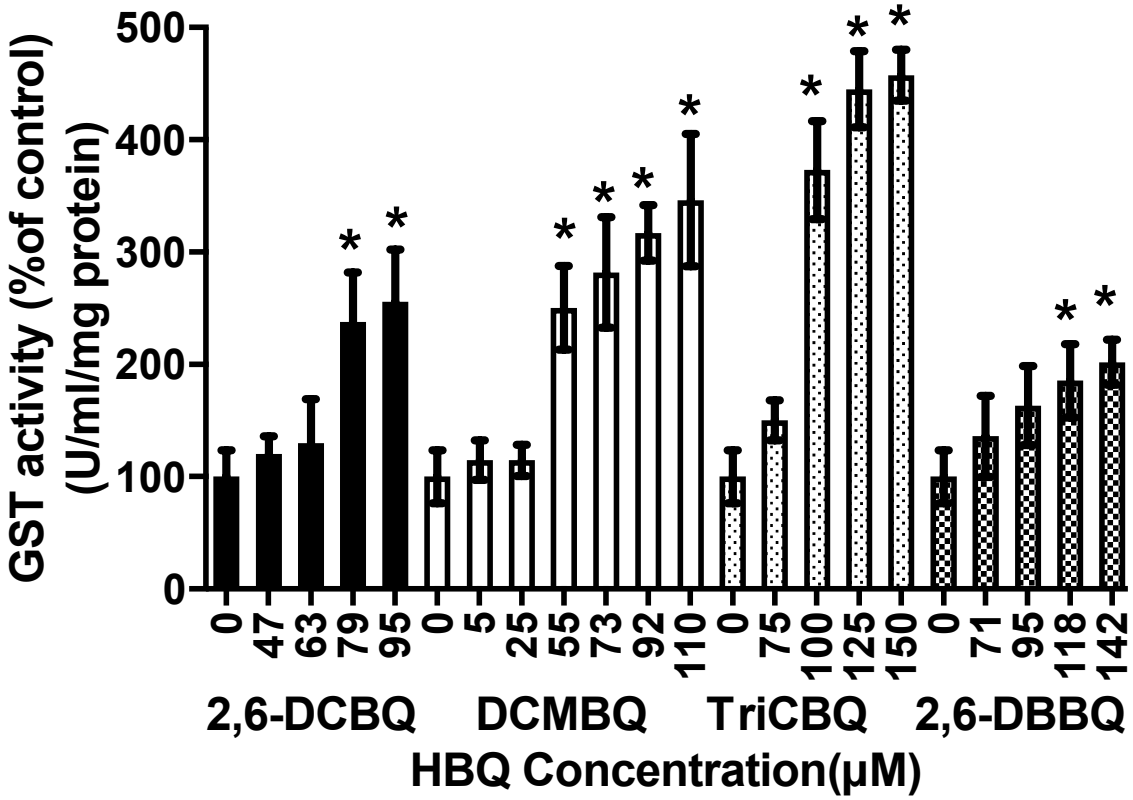


Figure 4.5 Effect of HBQ compounds on cellular glutathione S-transferase (GST) activity. T24 cells were exposed to HBQs for 24 hours. GST activity was measured using a colorimetric assay, and the 340 nm absorbance obtained was the measure of the formation of the DNP-GSH conjugate which was compared to the amount of cellular total protein. Bar graphs show the differences in the means between HBQ-exposed cells and control cells (* $P < 0.05$). Error bars indicate SD of six determinations from two separate experiments.

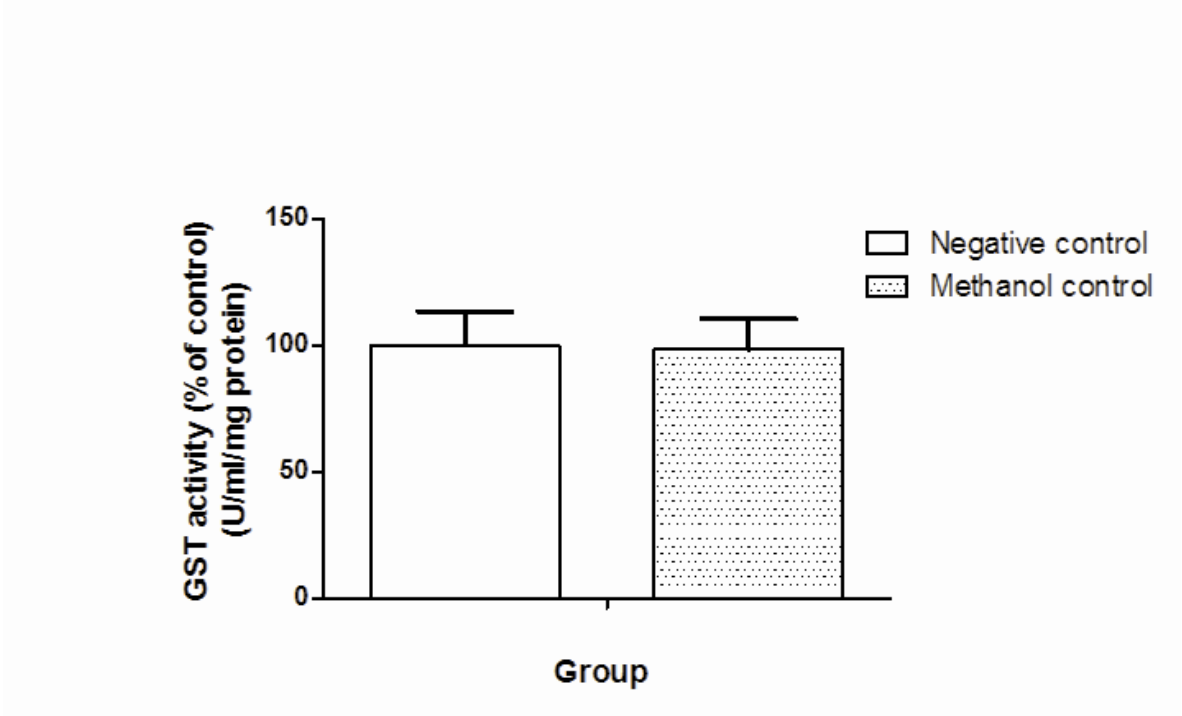


Figure 4.6 Effect of methanol control on cellular glutathione S-transferase (GST) activity. T24 cells were exposed to HBQs for 24 h. GST activity was measured using a colorimetric assay. The 340 nm absorbance obtained from the assay was the measure of the formation of the CDNB glutathione conjugate which was compared to the amount of cellular total protein. Error bars indicate mean standard error of six determinations from two separate experiments.

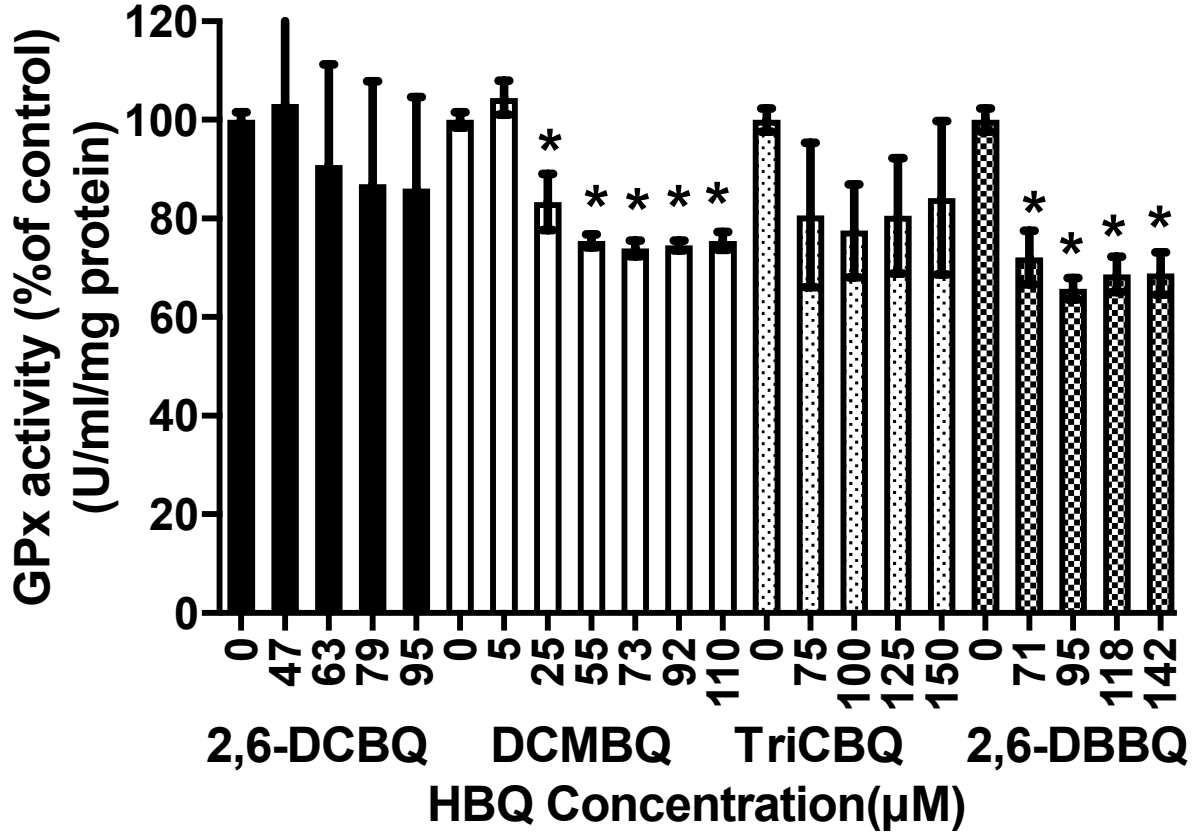


Figure 4.7 Effect of 4 HBQs on cellular glutathione peroxidase (GPx) activity. T24 cells were exposed to HBQs for 24 hours. The GPx activity was investigated indirectly with GR in a couple-enzyme reaction. The bar graphs show the differences in the means between HBQ exposed-cells and control cells (* $P < 0.05$). Error bars indicate SEM of six determinations from two separate experiments.

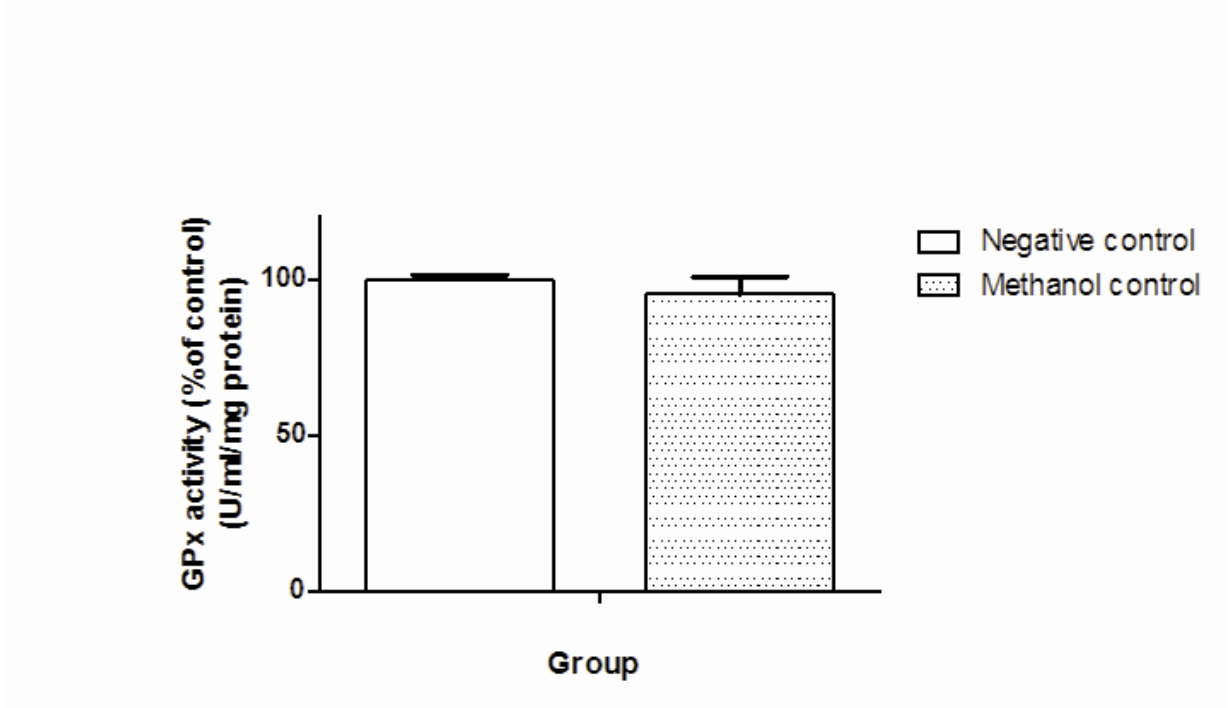


Figure 4.8 Effect of methanol control on glutathione peroxidase (GPx) activity. T24 cells were exposed to HBQs for 24 h. The GPx activity was investigated indirectly with GR in a coupled enzyme reaction. Error bars indicate mean standard error of six determinations from two separate experiments.

Table 4.3 Pearson correlation analysis of cellular GSH level with cellular GST activity, GPx activity and ROS production after 24h in HBQ-treated T24 cells

	HBQ group	GST activity (log value)[a]		GPx activity (log value)[a]		ROS production (log value)[b]	
		r	*P* value	*r*	*P* value	*r*	*P* value
GSH level (log value)[a]	2,6-DCBQ	-1.00**	0.00	0.81	0.19	-0.96*	0.04
	DCMBQ	-0.96**	0.00	0.91*	0.01	-0.96**	0.01
	TriCBQ	-0.62	0.38	-0.78	0.22	-0.89	0.11
	2,6-DBBQ	-0.92	0.08	0.03	0.97	-0.95	0.05

Note. [a] Data presented in this study. Data is from six determinations from two separate experiments. [b] Data summarized from our previous study.[3] * Correlation is significant at the level of 0.05. ** Correlation is significant at the level of 0.01.

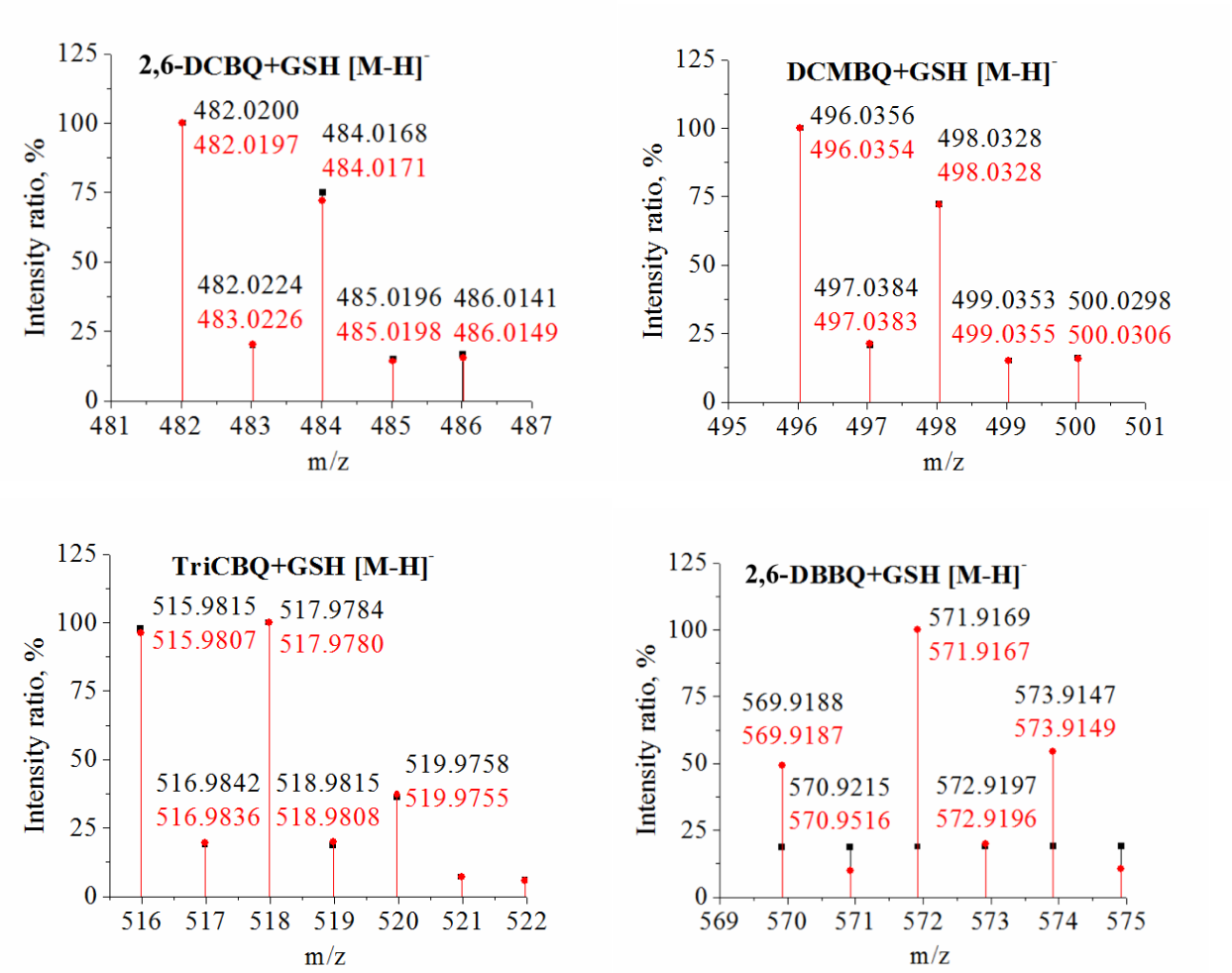


Figure 4.9 Isotope pattern of conjugation products of HBQs with GSH. The measured results were acquired by high resolution mass spectrometry analysis. Black traces are the measured accurate mass, and red traces are the theoretical accurate mass. The accurate mass measurements are in agreement with the theoretical mass and isotopic patterns of the conjugates consisting of one molecule of HBQ bound with one molecule of GSH.

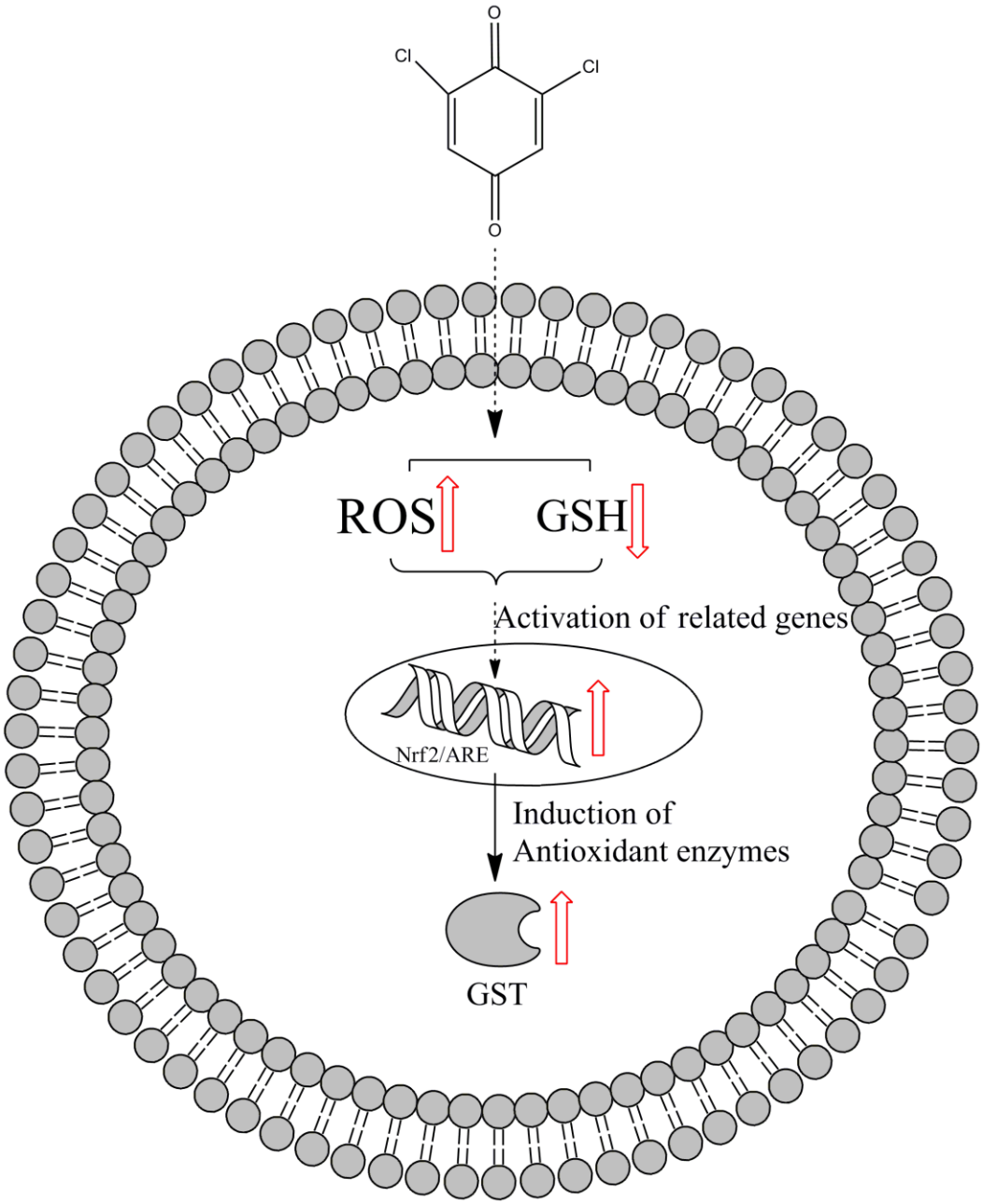


Figure 4.10 Possible protection pathway involved GSH against HBQ-cytotoxicity. Exposure to HBQs cause cellular GSH depletion and ROS production, generating oxidative stress, which lead to activation of related genes (like Nrf2/ARE) and induction of cellular antioxidants (like GST) to detoxify HBQs in T24 cells.

Chapter 5 Multidrug resistance proteins (MRPs) protect cells from the toxic effects of HBQs

5.1 Introduction

In the past several years, halobenzoquinones (HBQs) have been consistently reported as water disinfection byproducts (DBPs) in drinking water and swimming pool water in North America,[1-4] including 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), 2,6-dibromo-1,4-benzoquinone (2,6-DBBQ), 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ), and 2,3,6-trichloro-1,4-benzoquinone (TriCBQ). In Chapters 2–4, I have shown that HBQs are highly cytotoxic,[5-8] and can induce the production of reactive oxygen species (ROS), deplete cellular glutathione (GSH),[6] and damage DNA and proteins in mammalian cell lines.[5, 7] While much *in vitro* research has focused on understanding the toxicodynamics of HBQs, less attention has been paid to their toxicokinetics, including HBQ transport. This is significant, as transporter-mediated distribution of chemicals is an important factor and can drive the biological activity of xenobiotics.[9]

The multidrug resistance proteins (MRPs/ABCCs), are members of the ATP-binding cassette (ABC) transporter protein subfamily "C", and several members play a critical role in the cellular export of a variety of endogenous and exogenous compounds, including pharmaceuticals, carcinogens, environmental toxicants, and their conjugated metabolites.[10, 11] There are twelve members of ABC subfamily C found in humans and of these, nine are drug transporters. Of these nine, MRP1 (ABCC1), MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4), and MRP5 (ABCC5) are the best characterized for their role in the transport of xenobiotics, and enable the cellular export of numerous conjugated (e.g., glutathione-, glucuronide-, sulfate-) and unconjugated chemicals.[11, 12] Each MRP can also exhibit different localization in human tissues or within polarized epithelia.[11] In the kidney, several MRPs localize on either the apical or the basolateral sides of renal epithelial cells. MRP2 and MRP4 are localized on the apical surface of proximal tubule cells and are capable of effluxing compounds into urine for potential elimination, while MRP1 and MRP3 are found on the basolateral surface of renal epithelial cells resulting in the reabsorption into blood.[9] The localization of MRPs in renal cells is of particular significance

for DBPs, as they might promote or prevent elimination of DBPs into urine for transport to the bladder, a target organ of DBP-induced health effects.

Little is known about the involvement of transporter proteins in the toxicity of DBPs, in general, although transporters are known to play critical roles in chemical toxicity. The influence of MRPs on the cytotoxicity of different chemicals has been clearly demonstrated using cell lines stably over-expressing different MRPs or empty vector.[13-15] Thus, I hypothesize that MRPs play roles in the cytotoxic effects of HBQs. To confirm the hypothesis, I used previously established HEK293 cells stably expressing MRP1, MRP3, MRP4, MRP5, and HEK293 with empty vector (HEK-MRP1, -MRP3, -MRP4, -MRP5, and -V) to compare the cytotoxicity of four HBQs with or without overexpressed MRPs. I found that of the four MRPs examined, only MRP4 significantly reduced the cytotoxicity of all four HBQs. To confirm the role of MRP4 in the reduction of cytotoxicity of HBQs, I further examined the effect of MRP4 on the cellular ROS production induced by HBQs. Because previous studies have shown that HBQs can readily conjugate with GSH and HBQ cytotoxicity is GSH-dependent,[6] I further examined the effects of GSH levels on MRP4-protection against HBQs. The structural diversity of the HBQs makes them an excellent candidate class of DBPs to assess the importance of MRP transport in DBP testing, as chemical structure is an important determinant for both MRP specificity[11, 12] and HBQ biological activity.[16] The results of this study contribute to the understanding of the mechanisms of detoxification of HBQs.

5.2 Materials and Methods

5.2.1 Materials and reagents

Standards of 2,6-DCBQ and DCMBQ were purchased from Sigma-Aldrich (St. Louis, MO, USA), and standards of TriCBQ and 2,6-DBBQ were purchased from Shanghai Acana Pharmtech (Shanghai, China) and INDOFINE Chemical Company (Hillsborough, NJ, USA), respectively. HBQs were dissolved in methanol (HPLC grade; ThermoFisher Scientific, Burlington, ON) and stored at -20 °C in sterile amber glass vials as stock solutions. Dulbecco's phosphate-buffered saline (DPBS) was purchased from ThermoFisher Scientific. Stock solutions of 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) were prepared by dissolving

DCFH-DA in dimethyl sulfoxide (DMSO) with storage at -20 °C. Buthionine sulfoximine (BSO; Sigma-Aldrich) was dissolved in culture medium as a stock solution and kept refrigerated at 4°C.

5.2.2 Cell lines and cell culture

Stable human embryonic kidney, HEK293, cell lines expressing empty vector (pcDNA3.1; HEK-V) or human MRP1 (HEK-MRP1) were kind gifts from Dr. Susan P.C. Cole (Queen's University, Kingston, ON, Canada), and were generated as described previously.[17] Stable HEK293 cells expressing human MRP3 (HEK-MRP3), MRP4 (HEK-MRP4), or MRP5 (HEK-MRP5) were generated in a similar manner, and reported previously.[13] All cell lines were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 600 μg/ml of G418 (ThermoFisher Scientific) and 7.5% fetal bovine serum (FBS; ThermoFisher Scientific) in a 37°C humidified incubator with 5% CO_2.

5.2.3 Cytotoxicity testing

The cytotoxicity of 2,6-DCBQ, DCMBQ, TriCBQ, and 2,6-DBBQ was measured in HEK-V, HEK-MRP1, HEK-MRP3, HEK-MRP4, and HEK-MRP5 stable cell lines. Each cell line was seeded into a 96-well plate at a density of 1×10^4 cells/well and incubated for 24 h. For HBQ treatment, the old medium was aspirated from each well and replaced with culture medium containing between 0.1–300 μM of each HBQ. Cell viability was measured after 24 h exposure using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Blank controls (media + HBQ treatment, no cells), negative controls (cells + media, no HBQ treatment), and vehicle controls (cell + media containing 1% v/v methanol) were also included. Five replicates were used for each concentration, and independent experiments were repeated three times (n=3).

To determine whether MRP4-mediated protection from HBQs is GSH-dependent, HEK-V and HEK-MRP4 cells were pretreated with BSO, an irreversible inhibitor of the rate-limiting enzymatic step of GSH synthesis.[18] BSO, at a final concentration of 1 mM, was added to each well at the time of cell seeding and incubated for 24 h. The culture medium containing BSO was then removed and replaced with fresh medium containing HBQs (0.1–300 μM). Cell viability was measured at 24 h exposure, as described above.

Cytotoxicity data was analyzed to determine IC_{50} values (the concentration causing a 50% decrease in cell viability) using the sigmoidal dose–response equation in GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Relative resistance values were calculated as the ratio of the IC_{50} values of HEK-MRP cells to HEK-V cells, as the equation (Equation 1) describes below:

$$\text{Relative Resistance (RR)} = \frac{\text{IC}_{50}\text{ values of HEK\_MRP}}{\text{IC}_{50}\text{ values of HEK\_V}}$$

5.2.4 Measurement of HBQ-induced cellular ROS levels

Intracellular ROS production was determined by measuring the fluorescence of 2',7'-dichlorofluorescein (DCF), the final product of DCFH-DA. The basis of the assay is that DCFH-DA diffuses into the cell and gets deacetylated to a non-fluorescent compound which is oxidized to DCF in the presence of ROS. Because DCF is a fluorescent compound, fluorescence generation is proportional to the amount of ROS present within the cells.[19] Briefly, HEK-V and HEK-MRP4 cells were seeded into 96-well black plates at a density of 1.0×10^4 cells/well and incubated for 24 h. The cells were then washed with DPBS and incubated with 25 μM of DCFH-DA for 45 min. After the DCFH-DA solution was removed, the cells were washed with DPBS and treated with equimolar concentrations of 2,6-DCBQ, DCMBQ, TriCBQ, or 2,6-DBBQ (25, 50, and 100 μM). Fluorescence (λex = 485 nm, λem = 535 nm) was monitored at 24 h post-exposure using a fluorescence microplate reader (Beckman Coulter DTX880 Multimode Detector) with Multimode Analysis Software. Blank controls (media + DCFH-DA, no cells) and negative controls (cells + media + DCFH-DA) were also included. Three independent experiments (n=3) were performed, using five replicates for each testing concentration.

5.2.5 Data analysis

Statistical analysis was performed using GraphPad Prism 5.0. Experimental results were expressed as the mean ± standard error (SEM). Student's t-test was used to compare 1) the IC_{50} values and 2) fluorescence intensity of the empty vector cells (HEK-V) with those overexpressing the four MRPs (HEK-MRP1, -MRP3, -MRP4, MRP5). Differences were considered statistically significant at $P < 0.05$.

5.3 Results and Discussion

5.3.1 MRP-mediated protection of cells from the cytotoxicity of HBQs

To determine the functions of MRP1, MRP3, MRP4, or MRP5 in protecting cells from HBQs, I treated HEK-V and HEK-MRP cells with a range of concentrations (0.1–300 μM) of individual HBQs (2,6-DCBQ, DCMBQ, TriCBQ, and 2,6-DBBQ) to obtain dose–response curves for 24 h exposure. Figures 5.1–5.4 show the paired dose–response curves of HEK-MRPs and HEK-V cells after they were individually treated with 2,6-DCBQ, DCMBQ, TriCBQ, and 2,6-DBBQ. In Figure 5.3, it is clear that the presence of MRP4 causes a shift of the dose–response curve to the right for each HBQ, indicating that higher concentrations are needed to induce a cytotoxic response in the HEK-MRP4 cell line than in the HEK-V cell line. The IC_{50} value, the concentration of HBQ that results in a 50% inhibition of cell viability, was determined for each cell pair to assess the differential cytotoxicity. Table 5.1 presents the 24 h IC_{50} values for each HBQ (2,6-DCBQ, DCMBQ, TriCBQ, and 2,6-DBBQ) in each cell line (HEK-V, HEK-MRP1, -MRP3, -MRP4, and -MRP5). The IC_{50} values of all four HBQs are significantly higher in HEK-MRP4 cells compared to those in HEK-V cells ($P<0.05$), indicating that MRP4 is able to decrease HBQ-induced cytotoxicity (Table 5.1; Figure 5.3). To quantitatively present the differential cytotoxicity of the cell pairs, the relative resistance for each MRP cell line was calculated from the IC_{50} values. The relative resistance is defined as the ratio of the 24 h IC_{50} value of each HEK-MRP cell line to the HEK-V cell line (Equation 1) and represents the level of protection (or resistance to cytotoxicity) induced by the presence of the MRP. In comparison to HEK-V cells, MRP4 overexpression provided a reduction in cytotoxicity, as shown by the relative resistance of 1.3 (2,6-DBBQ), 1.5 (TriCBQ), 1.6 (2,6-DCBQ), and 2.8 (DCMBQ). These results support the role of MRP4 in protecting cells from HBQs, potentially through cellular efflux. This is the first time that MRPs are shown to be able to protect cells from HBQs.

5.3.2 MRP4 reduced ROS generation induced by HBQ

Having identified that MRP4 protect cells from the toxic effects of the four HBQs, I hypothesized that the presence of MRP4 should also reduce the production of intracellular ROS. This was also based on the fact that the generation of cellular ROS is significantly correlated with HBQ-induced cytotoxicity.[6, 8, 16] To test this, I treated both HEK-V and HEK-MRP4 cells

with HBQs at equimolar concentrations (25, 50, and 100 μM) and measured intracellular ROS levels after 24 h exposure. DCFH-DA, a non-fluorescent probe readily taken up by cells, was used to determine ROS production by measuring the fluorescence of its hydrolysis product, DCF. Because another fluorescent compound, 5(or 6)-carboxy-2',7'-dichlorofluorescein (CDF), has the same basic structure as DCF and is a known substrate of MRP2 and MRP3,[20] I first confirmed that DCF would not be effluxed by MRP4. This data indicated that DCF was not transported by MRP4, as the fluorescence level of DCF in HEK-MRP4 cells was similar to that in the negative control HEK-V cells. These results are shown in Figure 5.5.

Figure 5.6 shows the level of intracellular ROS production in HEK-V and HEK-MRP4 cells after 24 h treatment of individual 2,6-DCBQ, DCMBQ, TriCBQ, or 2,6-DBBQ. ROS production was found to be concentration-dependent in both cell lines, as was also observed in other HBQ studies.[5, 16] The relative ROS levels were significantly decreased in HEK-MRP4 cells ($P<0.05$) in comparison to HEK-V cells, indicating that MRP4 efflux provides protection against HBQ-induced biological activity. These results are consistent with a study which found that treatment of bovine aortic endothelial cells (BAECs) with the nonspecific MRP inhibitor, MK571, increased the cytotoxicity and cellular superoxide production induced by menadione (a quinone).[21] The decreased presence of intracellular ROS in HEK-MRP4 cells after HBQ exposure provides further support for MRP4 protecting cells from these compounds.

5.3.3 The detoxification of HBQs by MRP4 is GSH-dependent

MRP4 can efflux conjugated (e.g., GSH-, glucuronide-, sulfate-) or unconjugated organic anions.[11] Because HBQs have been found to readily form HBQ-GSH conjugates in solution and HBQ toxicity is dependent on cellular GSH level,[6] I hypothesized that MRP4 can efflux HBQs as HBQ-GSH conjugates. To test that MRP4 efflux of HBQs is dependent on cellular GSH levels, HEK-V and HEK-MRP4 cells were depleted of cellular GSH through the addition of BSO prior to HBQ treatment. Because BSO is an irreversible inhibitor of GSH synthesis,[18] cells pre-treated with BSO will prevent production and accumulation of cellular GSH. To ensure that cell viability was not affected by BSO pre-treatment, the cell viability of BSO-alone treated cells was compared with BSO-free controls in both cell lines (Figure 5.7). No significant difference in viability was observed at the BSO concentrated used.

Figure 5.8 shows the concentration–response curves of each HBQ in both GSH-depleted HEK-V and HEK-MRP4 cell lines. Table 5.2 presents the IC_{50} values of the four HBQs in GSH-depleted HEK-MRP4, compared with those in HEK-V. The IC_{50} values of each HBQ in GSH-depleted HEK-MRP4 have no statistical difference (t-test, $P>0.05$) from those of HEK-V. These results confirm that GSH-depletion in HEK-MRP4 cells diminishes the ability of MRP4 to protect against the toxic effects of HBQs. A comparison of the relative resistance of MRP4 in HEK-MRP4 cells (Table 5.1) with that in the GSH-depleted HEK-MRP4 cells (Table 5.2) further supports the effects of GSH on the function of MRP4 towards HBQs, as the relative resistance of MRP4 for each HBQ decreased after GSH depletion in HEK-MRP4 cells. Because HBQ-GSH conjugation occurs rapidly,[6] it is likely that the observed MRP4-mediated detoxification of HBQs occurs, at least in part, via HBQ-GSH conjugates.

MRP4 may also export HBQs in their anionic forms in cells in addition to transporting GSH conjugates. The pKa values of the four HBQs are in the range of 5.7 to 6.3,[2] thus, the HBQs are mostly in their ionized form at neutral pH inside the cells and in the culture medium. Previous studies have reported that HBQs can undergo hydrolysis degradation to form halo-hydroxyl-benzoquinones (OH-HBQs) in drinking water,[3, 22] which can easily transform to their anionic forms. Therefore, it is plausible that MRP4 may efflux the HBQs either in their parental anionic forms or in their transformed product anionic forms. Together, this study demonstrates that the function of MRP4 against cytotoxicity of HBQs is GSH-dependent. MRP4 may transport unconjugated compounds through a GSH-stimulated mechanism, in addition to directly transporting GSH conjugates.[13, 23-26]

Many classes of DBPs, including haloacetamides, haloacetic acids, haloacetonitriles, and haloacetaldehydes, are known to react with or deplete GSH.[27-30] GSH, which is found at millimolar concentrations in cells, provides a thiol reservoir to protect cells, especially against oxidative stress.[31-32] The reactivity of quinones with the thiol groups of GSH likely explains the observed effects of GSH on MRP4-mediated cellular protection from the HBQs, as thiol reactivity has previously been shown to be a molecular initiating event for the toxicity of three individual DBPs (bromoacetic acid, bromoacetonitrile, and bromoacetamide).[30] The present study suggests that MRP4 may also influence the cellular toxicity of other DBPs that have high thiol reactivity.

5.3.4 The protection of MRP4 is HBQ compound-dependent

Although MRP4 demonstrated the protection of cells from the four HBQs, the resistance of MRP4 to each HBQ is different. Of the four HBQs tested in this study, MRP4 conferred the highest level of resistance to DCMBQ, with MRP4 overexpression resulting in a 2.8-fold reduction in cytotoxicity. Surprisingly, DCMBQ was also the only HBQ to be statistically impacted by the presence of the other MRPs (MRP1 and 3). The IC_{50} values of DCMBQ in HEK-MRP1 and HEK-MRP3 cells were significantly higher (t-test; $P<0.05$) than those in the HEK-V cells (Table 5.1; bolded) with relative resistance values of 1.8 and 1.6, respectively. Of the four HBQs, DCMBQ was determined to be the most cytotoxic in HEK-V cells (Table 5.1). However, in the presence of any of the examined MRPs, DCMBQ was either equally or less cytotoxic than 2,6-DCBQ or 2,6-DBBQ on the basis of their 24 h IC_{50} values (Table 5.1). Differences in MRP1, MRP3, and/or MRP4 protein levels may help explain past cytotoxicity studies which observed inconsistent rank order of the four HBQs when tested in different cell lines.[5, 22] In addition, DCMBQ produced a significantly higher amount of ROS compared to the other three HBQs ($P<0.05$) at equimolar treatment concentrations in HEK-V cells, consistent with the greater cytotoxicity observed for DCMBQ in HEK-V cells (Table 5.1).

Among the four HBQs, DCMBQ was the most impacted by GSH depletion. The relative resistance of MRP4 against DCMBQ was significantly decreased from 2.8-fold to 1.3-fold after GSH depletion in HEK-MRP4 cells. This is consistent with our previous findings in T24 cells, where DCMBQ exhibited a 5-fold reduction in IC_{50} value after GSH depletion.[6] In addition, DCMBQ was also determined to be the most potent HBQ to deplete cellular GSH levels. At a treatment concentration equal to half the IC_{50} value of DCMBQ, the intracellular GSH level was determined to be only 16% of that of the untreated control. This data supports that the protection conferred by MRP4 against HBQ-induced cytotoxicity is dependent on cellular GSH levels, especially for DCMBQ.

The relatively high reactivity of DCMBQ with GSH in comparison to the other tested HBQs can be partially explained by the chemical properties related to HBQ structures. The energy of the lowest unoccupied molecular orbital (E_{LUMO}) has been used as a major predictor of GSH/thiol reactivity both *in chemico* and *in vitro*.[30, 33-36] In general, compounds with low E_{LUMO} values are soft electrophiles and prefer to react with soft nucleophiles. In biological systems, thiol groups are the most predominant soft nucleophiles.[34] In a previous study that examined

quantitative structure–activity relationships (QSAR) of GSH reactivity with p-benzoquinone compounds, an equation relating E_{LUMO} with GSH activity was obtained as log $k_{GSH} = -18.38 - 16.78\ E_{LUMO} - 3.19\ (E_{LUMO})^2$; n = 10, $r^2 = 0.80$, P = 0.008.[35] Using this QSAR model and predicted E_{LUMO} values for the four HBQs,[16] the calculated log k_{GSH} values are 2.9 for 2,6-DCBQ, 3.1 for 2,6-DBBQ, and 3.7 for both TriCBQ and DCMBQ. This indicates that TriCBQ and DCMBQ are more reactive towards GSH than 2,6-DCBQ and 2,6-DBBQ. In our study, however, it is unknown why DCMBQ exhibited greater sensitivity to GSH depletion than TriCBQ. The methyl group may influence the reaction between HBQ and GSH, as the methyl group receives electrons more easily and affects the electron density of the benzoquinone ring. More research into the mechanisms of HBQ-GSH interaction is needed in the future to understand the observed results.

5.4 Conclusion

This study clearly demonstrates the role of transport proteins in DBP toxicity, suggesting that future studies should examine how cells handle and eliminate DBPs to fully understand the mechanisms of DBP toxicity. In this study, stable MRP overexpressing cell lines were used to evaluate the toxicokinetics of a structurally diverse class of DBPs. MRP4-expression in HEK cells is an important factor of HBQ toxicity. A unique characteristic of MRP4 in relation to other MRPs is its dual membrane localization in polarized cell types. MRP4 is expressed at the basolateral surface of hepatocytes, which can facilitate cellular efflux to sinusoidal blood for entry into the systematic circulation.[23, 37] MRP4 is also localized on the apical surface of renal proximal tubule cells, which can facilitate cellular efflux of substrates into urine,[23, 37] and therefore, plays a role in xenobiotic transport to the bladder. Thus, because of its unique localization, MRP4 is an ideal candidate for the urinary elimination of DBPs.

Understanding how MRP4 relates to DBP distribution in the human body, particularly within elimination pathways in urine, will help elucidate the relationship between DBPs and increased bladder cancer risk. However, it is important to note that the *ABCC4* gene, which encodes the MRP4 protein, is highly polymorphic. This may alter the ability of MRP4 to efflux DBPs, causing interindividual susceptibility. Nevertheless, the paired cell lines used in this study provided a rapid method to assess the influence of MRPs on the cytotoxicity of HBQs and can easily be adapted for screening of other DBPs. The utility of the method is highlighted by the

DCMBQ results, which demonstrated how small structural differences within a class of DBPs can have far-reaching biological consequences for the compounds within that class due to the compound-specific nature of MRP interactions. The routine assessment of how transport pathways influence DBP toxicity is currently an underserved area of research; however, such toxicokinetic information will be critical for understanding the potential human health impacts of DBPs.

These results are also important because of the localization of these various MRPs in the kidney. While MRP4 is an apical transporter in the proximal tubule that promotes efflux into urine, MRP1 and MRP3 are basolateral transporters in nephron epithelial cells, therefore effluxing solutes into blood,[9, 38] meaning that DCMBQ can potentially be reabsorbed back into the bloodstream for further distribution within the human body. More research into the biological implications of DCMBQ reabsorption is needed.

5.5 References

1. Qin, F.; Zhao, Y. Y.; Zhao, Y. L.; Boyd, J. M.; Zhou, W. J.; Li, X.-F., A toxic disinfection by-product, 2,6-Dichloro-1,4-benzoquinone, identified in drinking water. *Angew. Chem. Int. Ed.* **2010,** *49*, (4), 790-792.
2. Zhao, Y. L.; Qin, F.; Boyd, J. M.; Anichina, J.; Li, X.-F., Characterization and determination of chloro- and bromo-benzoquinones as new chlorination disinfection byproducts in drinking water. *Anal. Chem.* **2010,** *82*, (11), 4599-4605.
3. Zhao, Y. L.; Anichina, J.; Lu, X. F.; Bull, R. J.; Krasner, S. W.; Hrudey, S. E.; Li, X.-F., Occurrence and formation of chloro- and bromo-benzoquinones during drinking water disinfection. *Water Res.* **2012,** *46*, (14), 4351-4360.
4. Wang, W.; Qian, Y. C.; Boyd, J. M.; Wu, M. H.; Hrudey, S. E.; Li, X.-F., Halobenzoquinones in swimming pool waters and their formation from personal care products. *Environ. Sci. Technol.* **2013,** *47*, (7), 3275-3282.
5. Du, H. Y.; Li, J. H.; Moe, B.; McGuigan, C. F.; Shen, S. W.; Li, X.-F., Cytotoxicity and oxidative damage induced by halobenzoquinones to T24 bladder cancer cells. *Environ. Sci. Technol.* **2013,** *47*, (6), 2823-2830.
6. Li, J.; Wang, W.; Zhang, H.; Le, X. C.; Li, X.-F., Glutathione-mediated detoxification of halobenzoquinone drinking water disinfection byproducts in T24 cells. *Toxicol. Sci.* **2014,** *141*, (2), 335-343.
7. Li, J.; Moe, B.; Vemula, S.; Wang, W.; Li, X.-F., Emerging disinfection byproducts, halobenzoquinones: effects of isomeric structure and halogen substitution on cytotoxicity, formation of reactive oxygen species, and genotoxicity. *Environ. Sci. Technol.* **2016**.
8. Li, J.; Wang, W.; Moe, B.; Wang, H.; Li, X.-F., Chemical and toxicological characterization of halobenzoquinones, an emerging class of disinfection byproducts. *Chem. Res. Toxicol.* **2015,** *28*, (3), 306-18.
9. Klaassen, C. D.; Lu, H., Xenobiotic transporters: ascribing function from gene knockout and mutation studies. *Toxicol. Sci.* **2008,** *101*, (2), 186-96.
10. Leslie, E. M.; Deeley, R. G.; Cole, S. P. C., Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol. Appl. Pharmacol.* **2005,** *204*, (3), 216-237.

11. Deeley, R. G.; Westlake, C.; Cole, S. P. C., Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. *Physiol. Rev.* **2006,** *86*, (3), 849-899.
12. Keppler, D., Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy. *Handb. Exp. Pharmacol.* **2011,** *201*, 299-323.
13. Banerjee, M.; Carew, M. W.; Roggenbeck, B. A.; Whitlock, B. D.; Naranmandura, H.; Le, X. C.; Leslie, E. M., A novel pathway for arsenic elimination: human multidrug resistance protein 4 (MRP4/ABCC4) mediates cellular export of dimethylarsinic acid (DMAV) and the diglutathione conjugate of monomethylarsonous acid (MMAIII). *Mol. Pharmacol.* **2014,** *86*, (2), 168-79.
14. Carew, M. W.; Naranmandura, H.; Shukalek, C. B.; Le, X. C.; Leslie, E. M., Monomethylarsenic diglutathione transport by the human multidrug resistance protein 1 (MRP1/ABCC1). *Drug Metab. Dispos.* **2011,** *39*, (12), 2298-2304.
15. Massimi, I.; Ciuffetta, A.; Temperilli, F.; Ferrandino, F.; Zicari, A.; Pulcinelli, F. M.; Felli, M. P., Multidrug resistance protein-4 influences aspirin toxicity in human cell line. *Mediat. Inflamm.* **2015,** *2015*, 607957.
16. Li, J.; Moe, B.; Vemula, S.; Wang, W.; Li, X.-F., Emerging disinfection byproducts, halobenzoquinones: effects of isomeric structure and halogen substitution on cytotoxicity, formation of reactive oxygen species, and genotoxicity. *Environ. Sci. Technol.* **2016,** *50*, (13), 6744-52.
17. Conseil, G.; Cole, S. P. C., Two polymorphic variants of ABCC1 selectively alter drug resistance and inhibitor sensitivity of the multidrug and organic anion transporter multidrug resistance protein 1. *Drug Metab. Dispos.* **2013,** *41*, (12), 2187-2196.
18. Griffith, O. W., Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic. Biol. Med.* **1999,** *27*, (9-10), 922-935.
19. Wang, G.; Gong, Y.; Burczynski, F. J.; Hasinoff, B. B., Cell lysis with dimethyl sulphoxide produces stable homogeneous solutions in the dichlorofluorescein oxidative stress assay. *Free Radic. Res.* **2008,** *42*, (5), 435-441.
20. Wang, W.; Qian, Y. C.; Li, J. H.; Moe, B.; Huang, R. F.; Zhang, H. Q.; Hrudey, S. E.; Li, X.-F., Analytical and toxicity characterization of halo-hydroxyl-benzoquinones as stable

halobenzoquinone disinfection byproducts in treated water. *Anal. Chem.* **2014,** *86*, (10), 4982-4988.

21. van de Water, F. M.; Masereeuw, R.; Russel, F. G. M., Function and regulation of multidrug resistance proteins (MRPs) in the renal elimination of organic anions. *Drug Metab. Rev.* **2005,** *37*, (3), 443-471.

22. Zamek-Gliszczynski, M. J.; Xiong, H.; Patel, N. J.; Turncliff, R. Z.; Pollack, G. M.; Brouwer, K. L., Pharmacokinetics of 5 (and 6)-carboxy-2',7'-dichlorofluorescein and its diacetate promoiety in the liver. *J. Pharmacol. Exp. Ther.* **2003,** *304*, (2), 801-9.

23. Takahashi, K.; Shibata, T.; Oba, T.; Ishikawa, T.; Yoshikawa, M.; Tatsunami, R.; Takahashi, K.; Tampo, Y., Multidrug-resistance-associated protein plays a protective role in menadione-induced oxidative stress in endothelial cells. *Life Sci.* **2009,** *84*, (7-8), 211-7.

24. Russel, F. G.; Koenderink, J. B.; Masereeuw, R., Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules. *Trends Pharmacol. Sci.* **2008,** *29*, (4), 200-7.

25. Roggenbeck, B. A.; Banerjee, M.; Leslie, E. M., Cellular arsenic transport pathways in mammals. *J. Environ. Sci.* **2016,** *49*, 38-58.

26. Rius, M.; Nies, A. T.; Hummel-Eisenbeiss, J.; Jedlitschky, G.; Keppler, D., Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane. *Hepatology* **2003,** *38*, (2), 374-384.

27. Rius, M.; Hummel-Eisenbeiss, J.; Hofmann, A. F.; Keppler, D., Substrate specificity of human ABCC4 (MRP4)-mediated cotransport of bile acids and reduced glutathione. *Am. J. Physiol. Gastrointest Liver Physiol.* **2006,** *290*, (4), G640-9.

28. Pals, J. A.; Wagner, E. D.; Plewa, M. J., Energy of the lowest unoccupied molecular orbital, thiol reactivity, and toxicity of three monobrominated water disinfection byproducts. *Environ. Sci. Technol.* **2016,** *50*, (6), 3215-3221.

29. Hughes, T. B.; Miller, G. P.; Swamidass, S. J., Site of reactivity models predict molecular reactivity of diverse chemicals with glutathione. *Chem. Res. Toxicol.* **2015,** *28*, (4), 797-809.

30. LoPachin, R. M.; Gavin, T.; DeCaprio, A.; Barber, D. S., Application of the hard and soft, acids and bases (HSAB) theory to toxicant-target interactions. *Chem. Res. Toxicol.* **2012,** *25*, (2), 239-251.

31. Chan, K.; Jensen, N.; O'Brien, P. J., Structure-activity relationships for thiol reactivity and rat or human hepatocyte toxicity induced by substituted p-benzoquinone compounds. *J. Appl. Toxicol.* **2008,** *28*, (5), 608-620.
32. Siraki, A. G.; Chan, T. S.; O'Brien, P. J., Application of quantitative structure-toxicity relationships for the comparison of the cytotoxicity of 14 p-benzoquinone congeners in primary cultured rat hepatocytes versus PC12 cells. *Toxicol. Sci.* **2004,** *81*, (1), 148-159.
33. Schmidt, M. M.; Dringen, R., Differential effects of iodoacetamide and iodoacetate on glycolysis and glutathione metabolism of cultured astrocytes. *Front. Neuroenergetics.* **2009,** *1*, 1.
34. Ahmed, A. E.; Jacob, S.; Campbell, G. A.; Harirah, H. M.; Perez-Polo, J. R.; K, M. J., Fetal origin of adverse pregnancy outcome: the water disinfectant by-product chloroacetonitrile induces oxidative stress and apoptosis in mouse fetal brain. *Brain Res. Dev. Brain Res.* **2005,** *159*, (1), 1-11.
35. Sood, C.; O'Brien, P. J., 2-Chloroacetaldehyde-induced cerebral glutathione depletion and neurotoxicity. *Br. J. Cancer. Suppl.* **1996,** *27*, S287-93.
36. Meister, A.; Anderson, M. E., Glutathione. *Annu Rev Biochem* **1983,** *52*, 711-760.
37. Ehrlich, K.; Viirlaid, S.; Mahlapuu, R.; Saar, K.; Kullisaar, T.; Zilmer, M.; Langel, U.; Soomets, U., Design, synthesis and properties of novel powerful antioxidants, glutathione analogues. *Free Radic. Res.* **2007,** *41*, (7), 779-787.
38. Wen, J.; Luo, J.; Huang, W.; Tang, J.; Zhou, H.; Zhang, W., The pharmacological and physiological role of multidrug-resistant protein 4. *J Pharmacol. Exp. Ther.* **2015,** *354*, (3), 358-375.

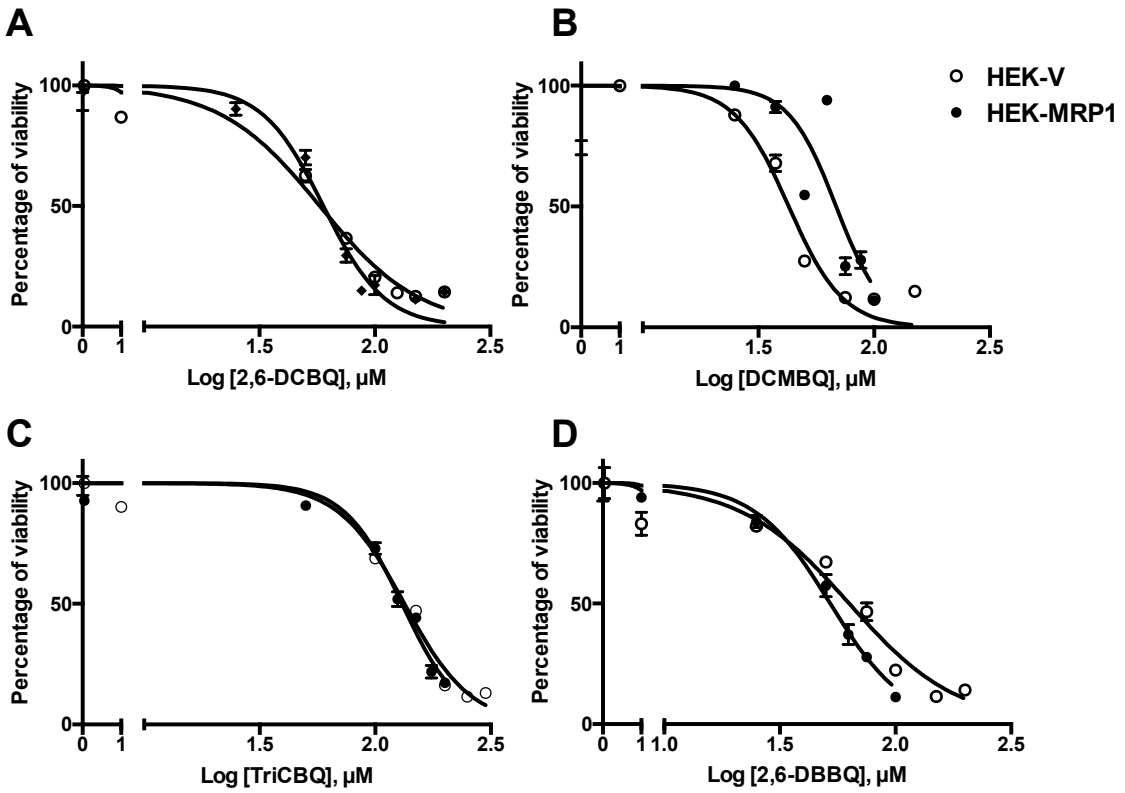


Figure 5.1 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cell viability of HEK-MRP1 and HEK-V cells after 24 h exposure. Cell viability was determined using a tetrazolium-based cytotoxicity assay. Data are expressed as the mean ± SD of quintuplicate determinations in a representative experiment; similar results were obtained in three or more repeated experiments.

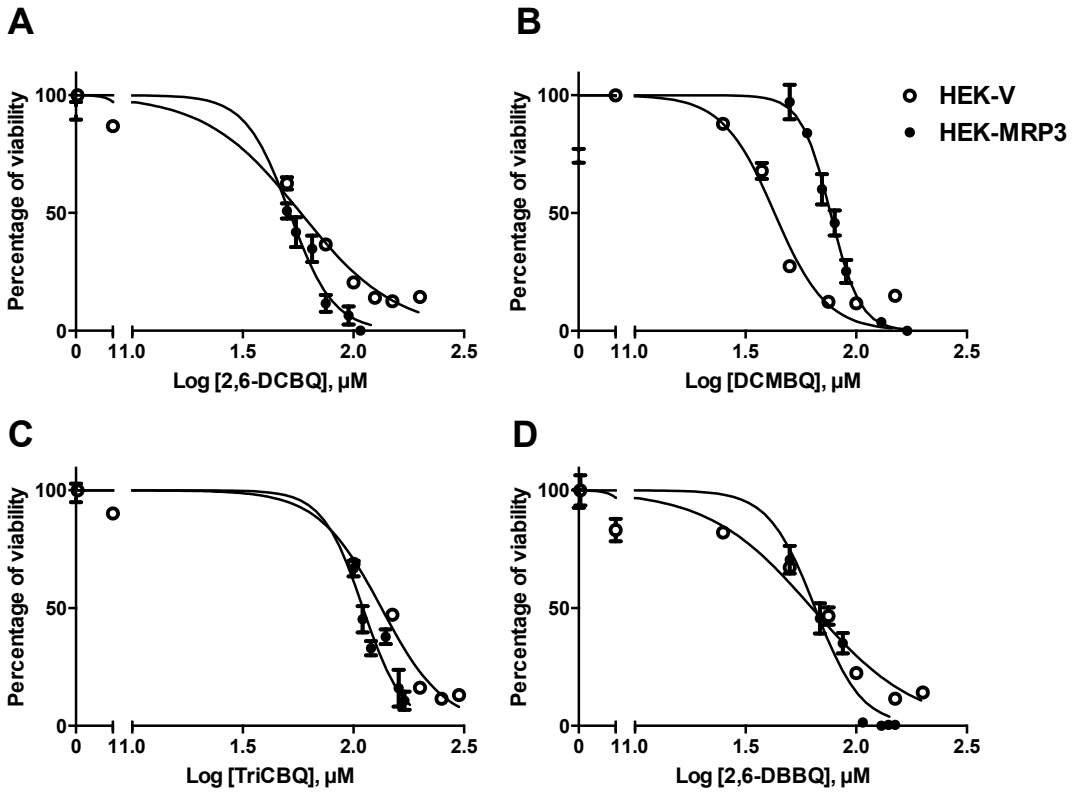


Figure 5.2 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cell viability of HEK-MRP3 and HEK-V cells after 24 h exposure. Cell viability was determined using a tetrazolium-based cytotoxicity assay. Data are expressed as the mean ± SD of quintuplicate determinations in a representative experiment; similar results were obtained in three or more repeated experiments.

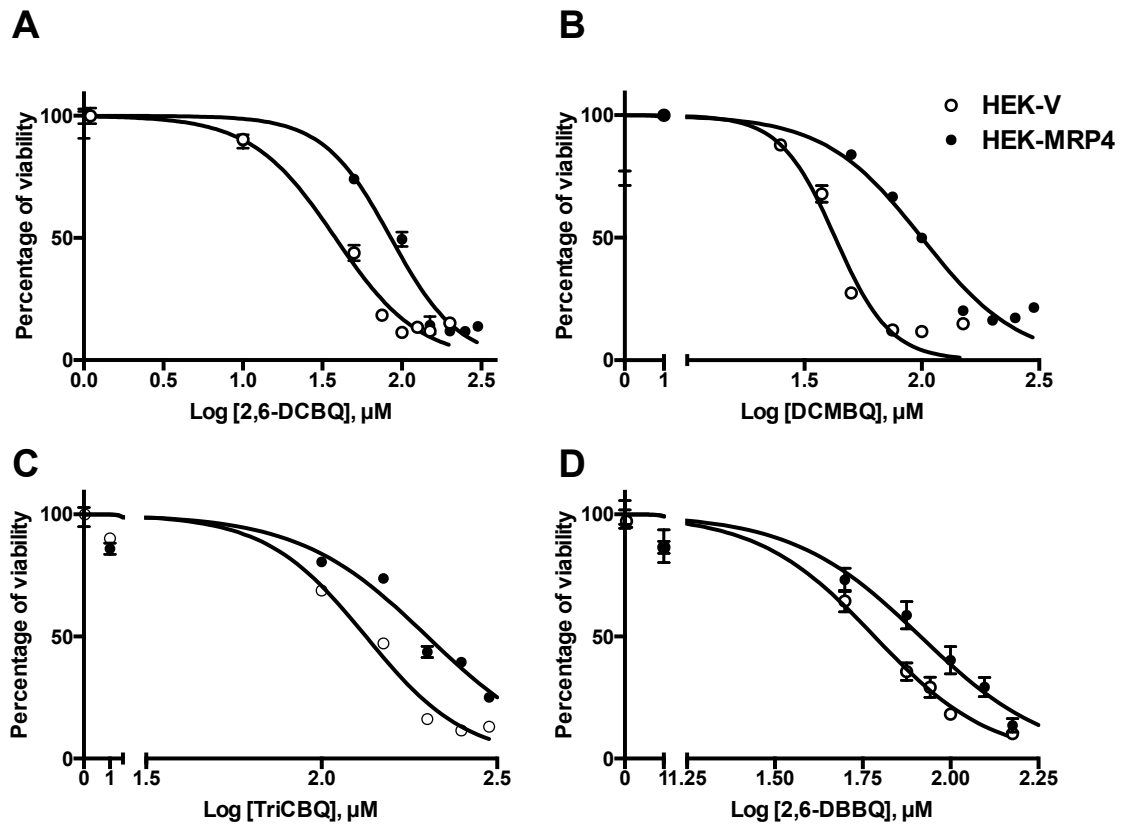


Figure 5.3 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cell viability of HEK-MRP4 and HEK-V cells after 24 h exposure. Cell viability was determined using a tetrazolium-based cytotoxicity assay. Data are expressed as the mean ± SD of quintuplicate determinations in a representative experiment; similar results were obtained in three or more repeated experiments.

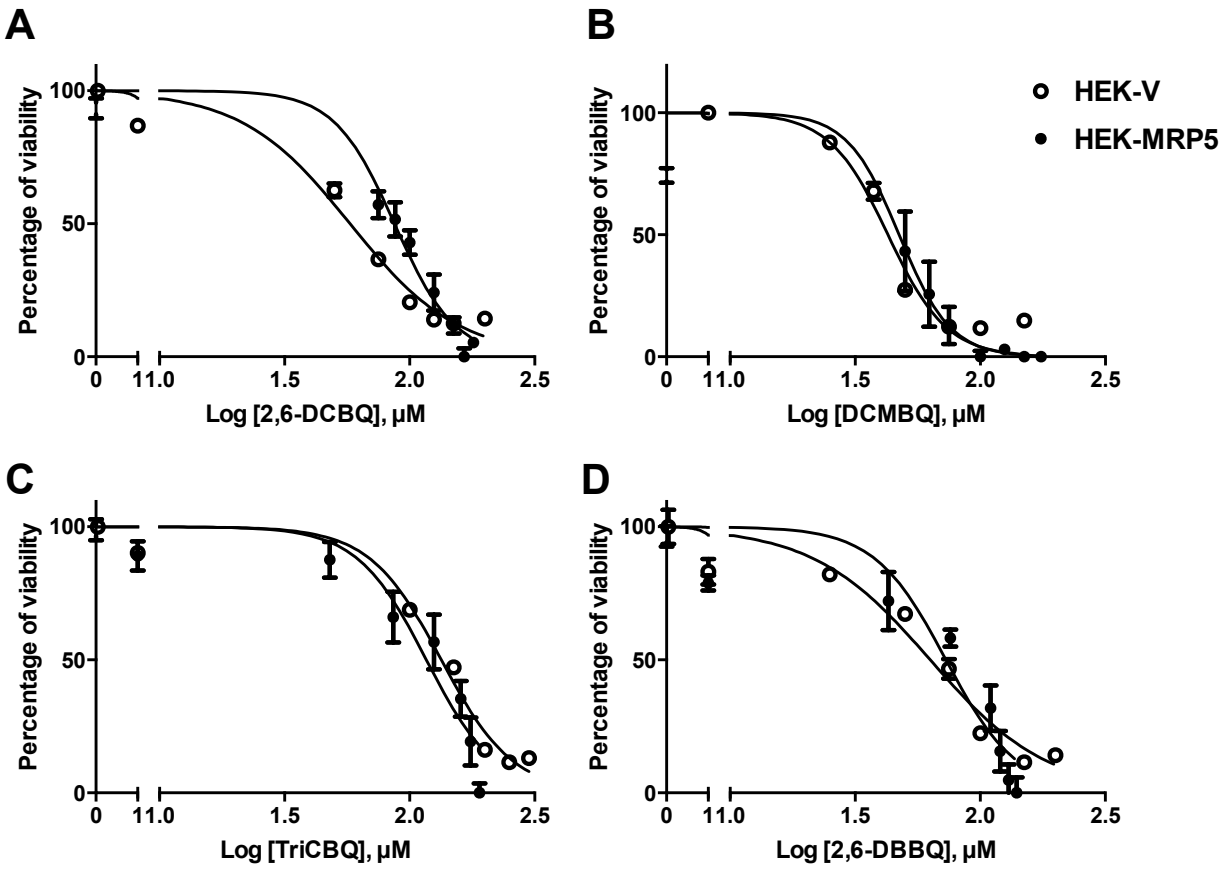


Figure 5.4 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cell viability of HEK-MRP5 and HEK-V cells after 24 h exposure. Cell viability was determined using a tetrazolium-based cytotoxicity assay. Data are expressed as the mean ± SD of quintuplicate determinations in a representative experiment; similar results were obtained in three or more repeated experiments.

Table 5.1 IC_{50} values and the relative resistance of human MRP-transfected HEK293 cells to each HBQ after 24 h exposure.

	HEK-V	**HEK-MRP1**		**HEK-MRP3**		**HEK-MRP4**		**HEK-MRP5**	
HBQ	IC_{50}, μM	IC_{50}, μM	RR[a]	IC_{50}, μM	RR[a]	IC_{50}, μM	RR[a]	IC_{50}, μM	RR[a]
2,6-DCBQ	58 ± 8	59 ± 5	1.1 ± 0.1	49 ± 7	0.8 ± 0.1	**87 ± 3***	1.6 ± 0.2	83 ± 13	1.5 ± 0.1
DCMBQ	37 ± 5	**65 ± 6***	1.8 ± 0.1	**58 ± 9***	1.6 ± 0.2	**97 ± 2***	2.8 ± 0.4	74 ± 14	2.0 ± 0.2
TriCBQ	120 ± 6	130 ± 5	1.1 ± 0.1	110 ± 1	0.9 ± 0.0	**180 ± 16***	1.5 ± 0.1	150 ± 30	1.1 ± 0.1
2,6-DBBQ	63 ± 1	54 ± 3	0.9 ± 0.0	53 ± 11	0.8 ± 0.2	**85 ± 1***	1.3 ± 0.0	80 ± 10	1.3 ± 0.1

Data are the mean ± SEM (n=3)

[a] RR = Relative Resistance - ratio of the IC_{50} values of HEK-MRP to HEK-V

*P < 0.05, Students's t-test: HEK-MRP vs. HEK-V.

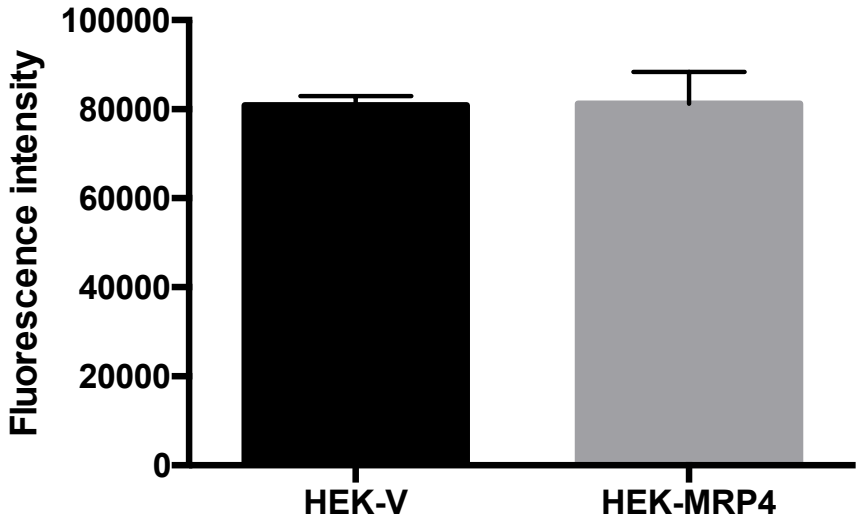


Figure 5.5 The fluorescence level of DCF in negative control cells of both HEK-MRP4 cells and HEK-V cells after 24 h of incubation. Data are expressed as the mean ± SEM (n=3).

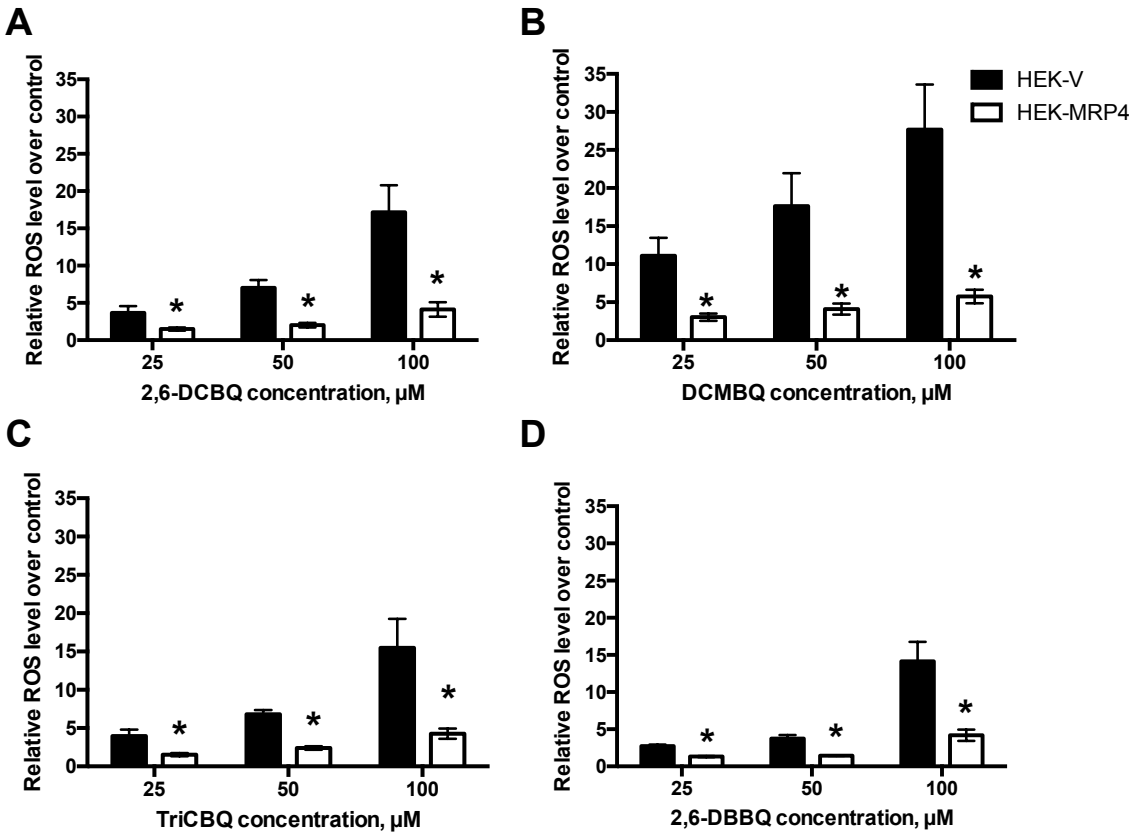


Figure 5.6 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cellular ROS levels of HEK-MRP4 and HEK-V cells after 24 h exposure. Cellular ROS levels were determined using a DCFH-DA-based fluorescence assay. The results were calculated as the ratio of the fluorescence intensity of HBQ-treated cells over negative control cells. Data are expressed as the mean ± SEM (n=3). * $P < 0.05$, Student's t-test: HEK-MRP4 vs. HEK-V.

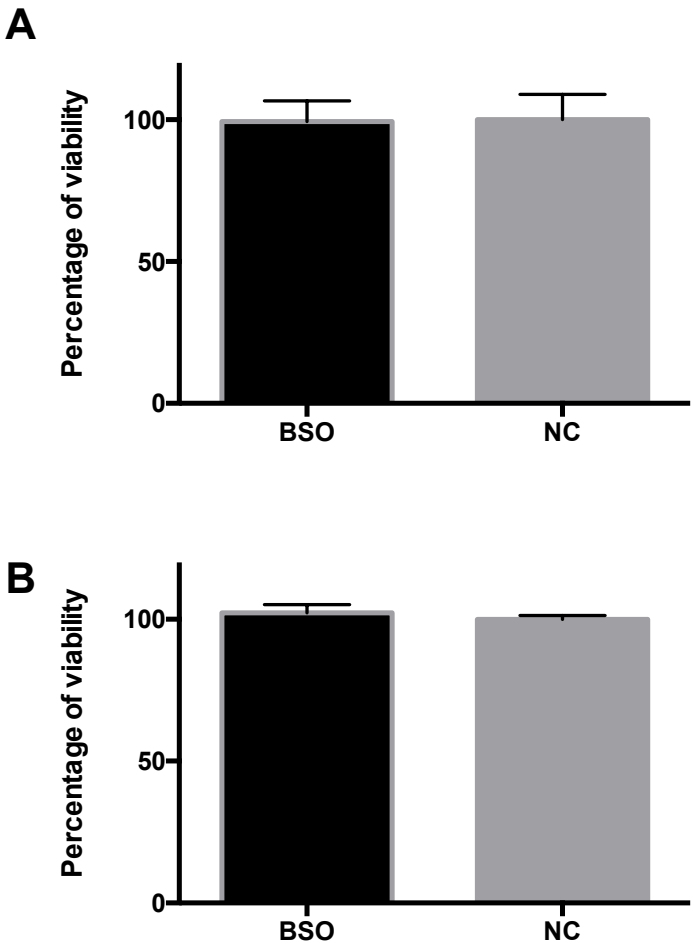


Figure 5.7 Cell viability of the negative control (NC) and control cells treated with 1 mM BSO after 24 h culture of (A) HEK-V and (B) HEK-MRP4 cells. Cell viability was determined using a tetrazolium-based cytotoxicity assay. Data are expressed as the mean ± SEM (n=3).

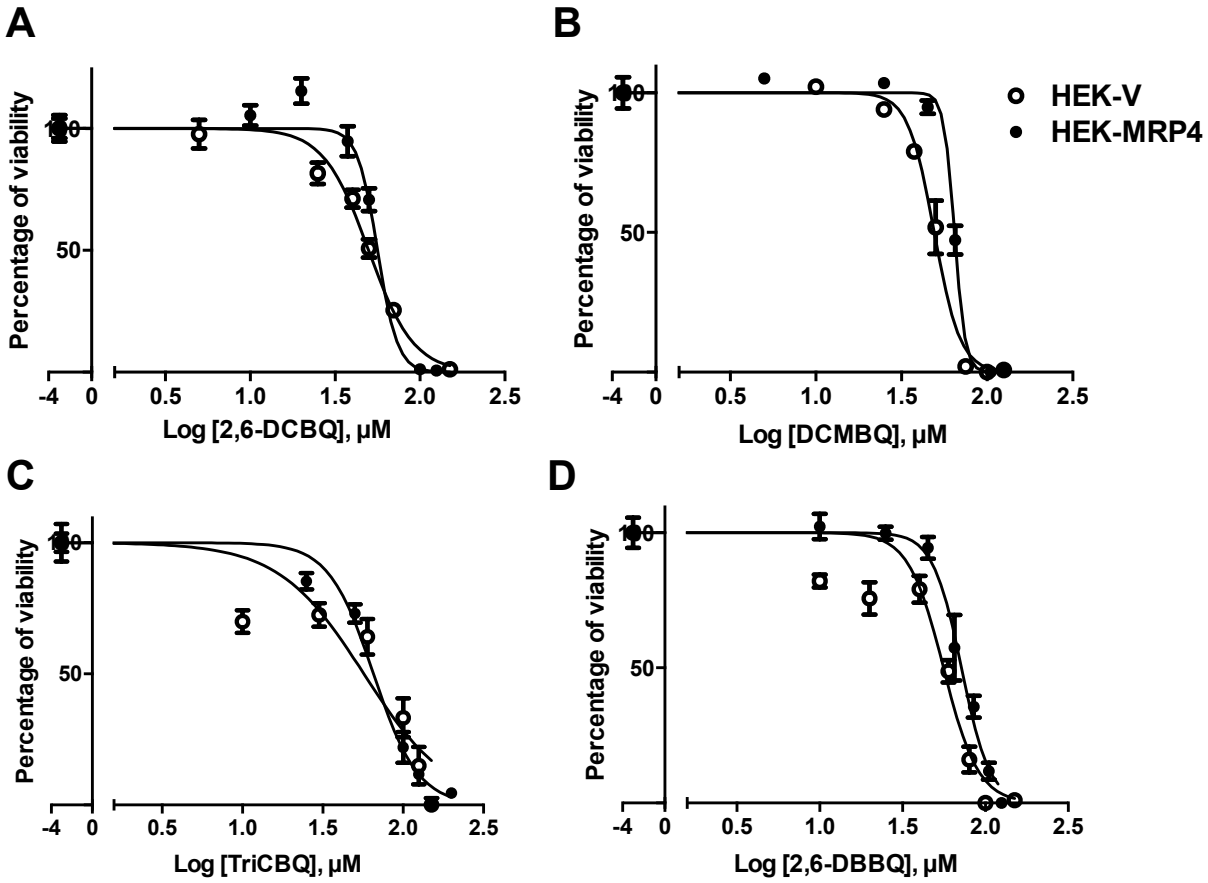


Figure 5.8 Effects of (A) 2,6-DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) 2,6-DBBQ on the cell viability of GSH-depleted HEK-MRP4 and HEK-V cells after 24 h exposure. Both HEK-MRP4 and HEK-V cells were incubated with 1 mM of BSO for 24 h, and then treated with each HBQ for 24 h. Cell viability was determined using a tetrazolium-based cytotoxicity assay. Data are expressed as the mean ± SEM (n=3).

Table 5.2 IC_{50} values and the relative resistance of GSH-depleted human MRP4-transfected HEK293 cells to each HBQ after 24 h exposure.

HBQ	IC_{50}, μM		Relative Resistance[a]
	HEK-V	HEK-MRP4	
2,6-DCBQ	44 ± 5	53 ± 7	1.2 ± 0.3
DCMBQ	38 ± 9	49 ± 9	1.3 ± 0.3
TriCBQ	63 ± 7	100 ± 18	1.3 ± 0.3
2,6-DBBQ	45 ± 5	59 ± 6	1.1 ± 0.3

Data are the mean ± SEM (n=4).

[a] Ratio of the IC_{50} values of HEK-MRP4 to HEK-V

Chapter 6 Activation of Nrf2/ARE pathway by HBQs

6.1 Introduction

Halobenzoquinones (HBQs) are a new class of DBPs, recently identified, that occur widely in treated tap water and recreational waters.[1-3] Little *in vitro* experimental data are available concerning the toxicological effects of HBQs. HBQs showed greater cytotoxicity than some regulated DBPs when Chinese hamster ovary (CHO) cells were used as a model.[4, 5] HBQs also induced cytotoxic effects in T24 human bladder carcinoma cells, with half-inhibitory concentrations (IC_{50}) at micromolar levels (Chapter 2). *In vitro* studies have shown that oxidative stress is one of the main mechanisms of HBQ-induced cytotoxicity (Chapter 2 and 4). HBQs have been found to produce cellular reactive oxygen species (ROS), deplete the cellular antioxidant glutathione (GSH), affect cellular antioxidant enzymes, and cause oxidative damage to cellular DNA and proteins.[6, 7] These findings suggest that HBQs are likely to be genotoxic and carcinogenic through oxidative stress-induced DNA damage; however, the molecular basis for this is still not clear.

Oxidative stress is recognized as one of the major contributors to the development of cancer.[8, 9] In response to increased levels of ROS and oxidative stress, several cell signaling pathways are involved to induce adaptive stress responses through modulation of stress–response genes.[10] The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling pathway is one of the most important mechanisms protecting cells against oxidative stress.[33, 34] As a transcription factor, Nrf2 regulates the expression of a series of antioxidant genes through its interaction with the antioxidant response element (ARE), which is located on the regulatory region of these genes.[11] In addition, *in vivo* studies have shown that the susceptibility to chemical-induced urinary bladder cancer was significantly increased in Nrf2-deficient mice compared with wild-type mice.[12] It has been reported that the Nrf2 pathway was activated in human cells or in rats by other DBPs, such as haloacetic acids (HAAs).[13, 14] However, the participation of the Nrf2 signaling pathway in the cellular response to HBQs, particularly the Nrf2-regulated genes, is still unclear.

Microarrays are a useful tool to identify multiple pathways or monitor whole gene expression simultaneously after exposure to environmental toxicants.[15] Compared to microarrays,

PCR arrays are often a better tool, offering more sensitive and reliable data on transcriptome profiles, as they are able to provide quantitative data on gene expression using real-time PCR even though less data are generated.[16] Although microarray analysis has been applied to study the potential toxicity pathways of a few DBPs, such as HAAs,[13, 17-20] bromate,[21, 22] and 3-chloro-4-(dichloromethyl)-5-hydroxy-5H-furan-2-one (MX),[23] none of these studies have used bladder cells which are the main target organ of DBPs.

The objective of this study was to use a PCR array to investigate the changes in transcriptome profiles of 44 genes related to the Nrf2 signaling pathway in SV-HUC-1 cells exposed to HBQs. I selected the immortalized human urinary tract epithelial cells, SV-HUC-1, for this study, as SV-HUC-1 is a SV40 transformed cell line and has similar properties to human bladder cells.[24] Therefore, the use of this cell line will provide more accurate information on gene expression compared to human cancer cell lines. This study will also provide an understanding of the response of human bladder cells to HBQs, providing insights into the molecular basis of antioxidant defense against HBQ-induced oxidative stress.

6.2 Materials and Methods

6.2.1 Reagents

Standards of 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), 2,5-dichloro-1,4-benzoquinone (2,5-DCBQ), and 2,5-dibromo-1,4-benzoquinone (2,5-DBBQ) were purchased from Sigma-Aldrich (St. Louis, MO). 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ) and 2,3,6-trichloro-1,4-benzoquinone (TriCBQ) were purchased from Shanghai Acana Pharmtech (Shanghai, China), while 2,6-dibromo-1,4-benzoquinone (2,6-DBBQ) was purchased from Indofine Chemical Company (Hillsborough, NJ). HBQ stock solutions were prepared by dissolving each HBQ in methanol (HPLC grade, Fisher Scientific, Ottawa, ON, Canada). Stock solutions were stored at -20°C in sterile amber glass vials.

6.2.2 Cell culture

The SV-HUC-1 human uroepithelial cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and was grown in F-12K medium (Kaighn's Modification of Ham's F-12 Medium) (ATCC) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1%

penicillin/streptomycin (100 U/100 μg/ml) (Invitrogen, Burlington, ON, Canada) at 37°C in a 5% CO_2 incubator.

6.2.3 Cell viability

Cell viability was examined using real-time cell analysis (RTCA; ACEA Biosciences, San Diego, CA). The principle of and detailed procedures for RTCA have been reported previously.[25] The SV-HUC-1 cells were seeded into 96-well E-plates, at a density of 15,000 cells per well. Briefly, when the cell index (CI) reached a value of 1 after approximately 20 h of growth, cells were treated with individual HBQs at a series of concentrations (0–75 μM for 2,6-DCBQ, 2,6-DBBQ, DCMBQ, TriCBQ; 0–50 μM for 2,5-DCBQ; 0–20 μM for 2,5-DBBQ) for 80 h. The experiments were continuously monitored for up to 80 h after treatment. Negative controls (no HBQ treatment) and solvent controls (culture medium containing 1% v/v methanol) were also included. Replicates were prepared in triplicate for each experimental condition, and three independent experiments were completed. Cell viability is defined as the relative normalized CI of the treated cells to that of the control cells at a given time.

To confirm that oxidative stress is involved in HBQ-induced cytotoxicity in SV-HUC-1 cells, cells were pretreated (at the time of seeding) with the antioxidant N-acetyl-L-cysteine (NAC). The concentration of NAC, 1 mM, was chosen based on the results of the cytotoxicity of NAC alone (0.01−10 mM) on SV-HUC-1 cells. When the CI reached a value of 1, the old medium in each well was discarded and cells were treated with individual HBQs at a series of concentrations (0–150 μM for 2,6-DCBQ and TriCBQ, 0–125 μM for 2,6-DBBQ, and 0–100 μM for DCMBQ) for 80 h in the presence or absence of NAC. NAC-only controls were included in addition to the negative controls and solvent controls. Replicates were prepared in triplicate for each experimental condition, and three independent experiments were performed.

6.2.4 Reactive oxygen species (ROS) detection

ROS formation was determined using 2', 7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich), a commonly used fluorogenic dye for the quantification of intracellular ROS generation.[26] SV-HUC-1 cells were seeded into solid black 96-well plates (Corning Costar, Fisher Scientific) at a density of 1.5×10^4 cells per well. When the cells reached 70–80% confluency, wells were washed twice with Dulbecco's phosphate buffered saline (DPBS;

Invitrogen), loaded with 25 μM DCFH-DA in F-12K medium without FBS, and incubated at 37°C for 45 min. After incubation, the wells were washed twice with DPBS and treated with individual HBQs at their respective concentration of equivalent biological response (IC_{20}). At the indicated time points of exposure (0.5, 1, 2, 4, 6, 8, 24, 48, and 72 h), the plate was read (λex = 485nm, λem = 535 nm) in a fluorescence microplate reader (Beckman Coulter DTX880 Multimode Detector). Negative controls (no HBQ treatment) and solvent controls (culture medium containing 1% v/v methanol) were also included at each time point. Six replicates were used for each experimental condition, and three independent experiments were completed.

6.2.5 RNA extraction and reverse transcription PCR (RT-PCR)

Total RNA was extracted using TRIzol (Invitrogen) as described in the manufacturer's instructions, and RNA yield was quantified with a NanoVue Plus (GE Healthcare, UK). The resultant RNA samples were stored at -80 °C. RNA was reverse transcribed into cDNA using SuperScriptTM III Reverse Transcriptase (Invitrogen) with Random Primers (Invitrogen) according to the manufacturer's instructions. The cDNA samples were stored in sterile microcentrifuge tubes at -20°C.

6.2.6 Real-time PCR for time-course gene expression

SV-HUC-1 cells were seeded into 6-well plates (Corning Costar, Fisher Scientific) at a density of 1×10^6 cells per well. After 24 h of culture, cells were treated with each HBQ at the concentration of equivalent biological response (IC_{20}). At each indicated time point of exposure (0.5, 1, 2, 4, 6, 8, and 24 h), cells were collected. Total RNA was isolated and reverse transcribed to cDNA as described previously. The cDNA was used as a template for real-time quantitative PCR analysis using the Fast SYBR® Green PCR kit (Applied Biosystems, Carlsbad, CA). The primers were synthesized by Integrated DNA Technologies (Coralville, IA), and the sequences are listed in Table 6.1. A 10 μL of reaction system was used, consisting of 2 × Fast SYBR Green Master Mix, 500 nM of each primer and cDNA. The amplification reaction conditions were 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Melt curves were conducted after amplification. Real-time fluorescence detection was carried out using a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Carlsbad, CA).

Two representative genes, Nrf2 as transcription factor and NQO1 as the Nrf2 response gene, were used here to select the proper time points for further array experiments. The gene expression was determined using cycle threshold (Ct) values. A comparative Ct method was used for relative quantification. The Ct values were first normalized to the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the same samples (ΔCt) and were then expressed as the fold-change over control groups ($2^{-\Delta\Delta Ct}$). Concurrent negative controls were prepared at each treatment time. Duplicate samples were used in individual experiments, and three independent experiments were completed.

6.2.7 Taqman® Array for pathway gene expression analysis

After 2 h and 8 h exposure to each HBQ at concentrations equal to their respective IC_{20} value, total RNA was isolated and reverse transcribed to cDNA. A customized Taqman Array in a 96-well fast plate was synthesized (ThermoFisher Scientific, Waltham, MA), using the plate format shown in Figure 6.1. A list of genes included in this array with their function is displayed in Table 6.2. Four reference control genes [18S rRNA, GAPDH, hypoxanthine phosphoribosyltransferase 1 (HPRT1), β-glucuronidase (GUSB)] and 44 target genes were present in duplicate in a single plate. The plate layout file was first imported into the real-time PCR system computer. A volume of 10 μL of reaction system was added into each well, consisting of 2 × Taqman Fast Universal PCR Master Mix and cDNA. After the plate was covered and briefly centrifuged, the real-time PCR reaction was run on the plate: 50°C for 2 min and 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Real-time PCR analysis was conducted on a StepOnePlus™ Real-Time PCR system. The comparative Ct method was again used for relative quantification. The Ct values were first normalized to control genes in the same samples (ΔCt) and expressed as the fold-change over control groups ($2^{-\Delta\Delta Ct}$). Concurrent negative controls were analyzed at each treatment time. Duplicate samples were used in individual experiments, and two independent experiments were completed.

6.2.8 Data analysis

Data are expressed as the mean ± SEM. The normalized cell index (CI) data were used to fit dose–response curves and obtain the half inhibitory concentration, IC_{50}. Student's t-test was performed to determine the statistical significance of the gene expression between control groups

and HBQ treatment groups. Statistical analysis was conducted using GraphPad Prism 5.0 software (San Diego, CA). $P < 0.05$ was considered as statistically significant.

6.3 Results and Discussion

6.3.1 Optimization of experimental conditions for the PCR array study

To obtain reliable gene expression profiles, it was important to first optimize the conditions for several important experimental parameters, including 1) the treatment concentrations of the HBQs and 2) the exposure time.

Treatment concentrations: An optimized treatment concentration is required to avoid false negative and false positive gene responses, which can occur when too low or too high testing concentrations are selected. Thus, not only is it important that the HBQ treatment concentrations exhibit low cytotoxicity, but it is also important that the selected treatment concentrations generate sufficient amounts of intracellular ROS to elicit a measurable biological response, as oxidative stress is needed to induce the Nrf2/ARE pathway.

To select low cytotoxic concentrations of each HBQ, RTCA was used to monitor cell responses to different concentrations of HBQs for 80 h. The cytotoxicity response profiles for SV-HUC-1 cells exposed to each HBQ are shown in Figure 6.2. From these response profiles, it is clear that HBQ-induced cytotoxicity in SV-HUC-1 cells is concentration-dependent. The concentration–response information was used to determine IC_{20} values, which are concentrations that elicit a 20% decrease in CI in comparison to untreated control cells. The IC_{20} values, therefore, exhibit low cytotoxicity and allow for a comparison between HBQ-induced effects on the basis of equivalent concentrations of biological response. The 24 h IC_{20} values for each HBQ can be found in Table 6.3. Co-treatment of the HBQs with 1 mM NAC also confirmed the influence of oxidative stress on HBQ-induced cytotoxicity in SV-HUC-1 cells, as NAC functions as either a source of thiol in cells or as an antioxidant to scavenge ROS directly in cells.[27, 28] A significantly larger concentration range of HBQs were needed to induce a full concentration–response measured using RTCA (Figure 6.3) and resulted in 24, 48, and 72 h IC_{50} values that were significantly higher than the HBQs in the absence of NAC (Figure 6.4). The influence of oxidative stress on HBQ-induced cytotoxicity in normal human bladder cells has not been previously demonstrated, but is consistent with results from T24 bladder carcinoma cells.[6]

To ensure that the selected IC_{20} values for each HBQ will be able to elicit a measurable biological response in the gene expression assays, it is important to confirm their ability to generate sufficient ROS to induce oxidative stress. Thus, I measured cellular ROS levels after HBQ treatment from 0.5 h to 72 h. Figure 6.5 summarizes the time-course of cellular ROS generation after each HBQ treatment at their respective IC_{20} values. All six HBQs share a similar time trend of ROS production: ROS production increases from 0.5 h, peaks at 8 h, and then slowly decreases after that time. For all time points, ROS generation by each of the HBQs is statistically greater than the control groups (one-way ANOVA, $P < 0.05$). This is consistent with a study of the quinone compound menadione, where ROS production was also found to increase over time until peaking at 8 h after treatment in HepG2 cells.[29] On the basis of RTCA and ROS results, the IC_{20} values are an optimal testing concentration for the gene expression assays.

Time points: To select the optimal testing time points for the PCR array, I examined the time-dependent effects of HBQ treatment on the expression of two representative genes. The two genes examined were Nrf2, representing the transcription factor, and NQO1, representing the Nrf2-response gene. Exposure to 24-h IC_{20} value concentrations of HBQs resulted in an initial increase in Nrf2 expression during the first 2 h exposure, with a subsequent decrease in expression after 4 h (Figure 6.6A). Because 2 h exposure to each HBQ significantly increased Nrf2 gene expression ($P<0.05$), it was chosen as the early response time point for the array experiments. Each HBQ also increased the Nrf2 downstream target gene, NQO1, in a time-dependent manner (Figure 6.6B). The expression of NQO1 increased gradually from 2 h, peaked at 8 h, and subsequently decreased at 24 h. Exposure to each HBQ significantly increased NQO1 gene expression at 2, 4, 6, 8, and 24 h for 2,6-DCBQ, DCMBQ, and 2,6-DBBQ, and 4, 6, 8, and 24 h for TriCBQ. Thus, the exposure time of 8 h was chosen as the late response time point for the array experiments, as the results reveal the maximum response of Nrf2-target genes after 8 h exposure to HBQs.

6.3.2 Overview of transcriptome changes induced by HBQs

After optimizing the experimental conditions necessary to assess gene expression, I next analyzed the transcriptome changes that occur after HBQ treatment in SV-HUC-1 cells. The expression of 44 genes in SV-HUC-1 cells were examined after 2 h or 8 h exposure to the 24-h IC_{20} concentrations of each HBQ, using a customized Taqman PCR Array. This array focuses on

the Nrf2/ARE antioxidant pathway and nine functional gene groups, including transcription factors, the GSH production/utilization pathway, the thioredoxin system pathway, stress responsive genes, other antioxidants genes, membrane transporters, cell cycle regulation, and oxidative DNA damage repair. I proposed that each of the six HBQs share a similar pathway and that differential gene expression exists between HBQ isomers.

Each HBQ altered the transcription of multiple genes after 2 h or 8 h exposure. It is not surprising since it is already known that HBQs could induce oxidative damage.[6] The genes that exhibited significant differences in expression levels ($P<0.05$) after 2 h or 8 h exposure to each HBQ can be found in Table 6.4 and 6.5 respectively, with the genes listed according to their functional gene groups. After 2 h exposure, each HBQ exhibited altered expression levels in 9 to 28 genes. Among these, only five genes were shared by all six HBQs: thioredoxin reductatse 1 (TXNRD1), NADPH:quinone oxidoreductase 1 (NQO1), hemeoxygenase-1 (HMOX1), prostaglandin-endoperoxide synthase 2 (PTGS2), and cyclin-dependent kinase inhibitor 1 (CDKN1A). After 8 h exposure, between 29 to 31 genes exhibited altered expression levels after exposure to each HBQ treatment. Of these significantly altered genes, there were 19 altered genes common to all six HBQs. These 19 genes are shown in Figure 6.7, along with the 5 common genes altered after 2 h HBQ exposure, and are clustered by their functional gene groups.

6.3.3 Common genes with altered expression at both early and late time points: the 4-gene set

Of the genes with altered expression levels common to all six HBQs, only four were found to be altered at both the early and late exposure time points: NQO1, HMOX1, TXNRD1, and PTGS2 (Figure 6.7). Each gene was found to be up-regulated by all six HBQs, as shown in Figure 6.8. The significant up-regulation of NQO1 gene at both exposure time points was consistent with the gene expression levels of NQO1 in the time-course measurement performed during parameter optimization (Figure 6.6B), where gene levels of NQO1 increased 2-fold at 2 h and increased 4-fold at 8 h exposure. However, it was surprising that NQO1 was not the most prominent gene exhibiting increased expression since NQO1 is a detoxification enzyme specific to quinones. NQO1 catalyzes a two-electron reduction of quinones to their less toxic hydroquinone form, which is considered a major detoxification process of quinones.[30] The gene exhibiting the highest up-regulation was HMOX1, with expression levels 7- to 50-fold higher after 8 h HBQ exposure,

which was in turn about 5- to 10-fold higher than the expression level after 2 h HBQ exposure. The HMOX1 gene encodes the hemeoxygenase-1 (HO-1) enzyme, which is able to cleave heme and produce carbon monoxide, biliverdin, and Fe (II).[31, 32] Although HO-1 is not a direct antioxidant, its protective role against oxidative stress is believed to be through its products, which are the signal molecules in the anti-inflammatory pathway.[33, 34] In addition, Nrf2 can indirectly reduce ROS levels by regulating free Fe (II) homeostasis through HMOX1. The significant increase of HMOX1 gene expression suggests that iron regulation may play a role in detoxification of HBQs by controlling ROS levels. The induction of HMOX1 genes was also observed in menadione-treated HepG2 cells,[29] in PCB quinone-treated HepG2 cells (which also caused increased NQO1 gene expression),[35] and in macrophages and epithelial cells after exposure to diesel exhaust containing quinone chemicals.[36] Therefore, the up-regulation of HMOX1 could be considered as an important indicator of the cellular detoxification of HBQs via Nrf2.

The gene expression levels of TXNRD1 increased 2- to 3-fold after 2 h HBQ exposure, and increased 2- to 5-fold after 8 h exposure to each HBQ. TXNRDs are a class of enzymes that reduce oxidized thioredoxin (Trx).[37] Because the altered transcription of TXNRD1 is shared by all six tested HBQs at both time points, Trx may be a much more important target for HBQs instead of GSH. The Trx system is the other thiol-dependent antioxidant system present in mammalian cells in addition to the GSH antioxidant system.[38] The Trx system is composed of Trx, TXNRD, peroxiredoxin (PRDX), and sulfiredoxin (SRXN). Nrf2-regulated genes in this system include TXNRD1, SRXN, PRDX1 and PRDX6.[11]

The PTGS2 gene encodes the cyclooxygenase-2 (COX-2) enzyme in humans. COX-2 catalyzes the conversion of arachidonic acid to prostaglandin H2 (PGH2), which can further transform to prostaglandins (PGs), a mainly anti-inflammatory component.[39] So the up-regulation of PTGS2 suggests a possible inflammatory response after HBQ treatment in human bladder cells. This is significant, as inflammation has been linked to the development of every stage of cancer,[40] and the development of various types of cancer, including bladder cancer.[41] Furthermore, the induction of COX-2 itself has been linked to the development of bladder cancer,[42, 43] which is the main target of DBP exposure in several epidemiological studies. These results suggest that HBQs can induce expression of the PTGS2 gene, which may further link HBQs with the potential development of bladder cancer.

6.3.4 Other common genes with altered expression at 2 h exposure

After 2 h HBQ exposure, there was a single gene with significantly increased expression common to all six HBQs, but was unique to the early exposure time point: the CDKN1A gene. CDKNIA encodes the cyclin-dependent kinase inhibitor 1 protein or p21 protein, which functions as a checkpoint of cell cycle regulation at the G1 and S phases and is regulated by p53.[44] This indicates that SV-HUC-1 cells may promote cell cycle arrest to protect against HBQ-induced DNA damage. Cell cycle arrest induced by 2,6-DCBQ and 2,6-DBBQ exposure in human neural stem cells has recently been reported.[45] Furthermore, a significant increase in p53 protein expression has also been detected in CHO cells treated with HBQs (2,5-DBBQ, 2,6-DCBQ, and 2,5-DCBQ) at 24 h post exposure.[46] Activation of the p53 pathway after 2,6-DCBQ and 2,6-DBBQ exposure in Caco-2 cells was also detected using a β-lactamase reporter system.[47] Therefore, it is consistent that the p53 pathway was activated in SV-HUC-1 cells after HBQ-treatment. It is also possible that p21 can activate the Nrf2/ARE antioxidant response pathway to protect cells from oxidative stress by disrupting Nrf2-Keap1 binding.[48, 49] Taken together, p53 may be activated by HBQs to further induce p21, which may cause cell cycle arrest to stop DNA damage, while p53 may also simultaneously activate the Nrf2 pathway to protect against oxidative stress induced by HBQs.

6.3.5 Other common genes with altered expression at 8 h exposure

The longer HBQ exposure time point exhibited far more significantly modified genes shared by the six HBQs than the early exposure time point. In addition to the commonly shared 4-gene set, genes associated with the GSH redox pathway, thioredoxin pathway, antioxidant genes, stress responsive genes, and DNA damage repair genes were also involved.

Within the GSH synthesis pathway, all six HBQs up-regulated GCLM, GCLC, and GSR by 2-fold after 8 h exposure. The up-regulation of GCLM, GCLC, and GSR indicates that Nrf2 increased GSH synthesis to supplement cellular GSH levels in response to HBQ-induced GSH depletion, as the ability of HBQs to deplete GSH has been demonstrated in HBQ-treated T24 cells.[7] Other quinones, such as 2,3-dimethoxy-1,4-naphthoquinone, have increased GCLC mRNA content by around 4-fold after 6 h exposure in rat lung epithelial L2 cells.[50] IAA-DBPs have also been found to increase the mRNA expression of the GCLC gene in HepG2 cells,[14] suggesting that GSH production genes might be used as biomarkers upon DBP exposure,

although the testing of more DBPs is necessary to confirm this hypothesis.

Within the GSH utilization pathway, all six HBQs increased GPX2 gene expression by 6- to 12-fold and down-regulated GPX1, GPX4, and GSTT1 after 8 h HBQ exposure. GPX2 is the only gene that is regulated by Nrf2/ARE in the GPX family,[51, 52] so GPX2 is the main glutathione peroxidase enzyme that is responsive to the oxidative stress. Additionally, GPX2 is distributed in the gastrointestinal tract,[51, 52] which is the main site that absorbs the DBPs through ingestion of disinfected water. This could explain why only GPX2 is up-regulated upon exposure to HBQ-DBPs. Cantor et al. found that the relationship between bladder cancer risk and THMs is related to the polymorphisms in GSTT1 positive and GSTZ1 CT/TT genotypes.[53] In this study, GSTT1 and GSTZ1 genes were mostly down-regulated after HBQ treatment. However, the significant increase of GPX2 and decrease of GSTT1 indicates that oxidative damage from the production of hydrogen peroxide could be the main reaction instead of the reactions between HBQs and GSH after cells are exposed to low-cytotoxic HBQ concentrations. This is because glutathione transferases (GST) directly neutralize free radicals to form GSH conjugates, and glutathione peroxidase (GPx) reduces hydrogen peroxide.[54] These results also suggest that GPX2 and GSTT1 could be used as biomarkers for late HBQ exposure.

Among the antioxidant genes and stress responsive genes, the CAT and CYGB genes were both up-regulated 2-fold, the CYBB gene was up-regulated by 3- to 15-fold, and the NOX5 gene was down-regulated. The CYGB gene encodes cytoglobin protein, a ubiquitous hemoglobin in humans that serves as a scavenger of ROS or nitric oxides.[55] The increase of CYGB gene levels suggests that the increase of stress-responsive hemoprotein might be a late adaptive response to HBQ treatment, and is consistent with the results of a study of MCF7 human breast cancer cells exposed to hydrogen peroxide, which also observed increased CYGB expression.[56] The increase of CAT gene expression suggests that CAT may have worked with GPX2 to eliminate hydrogen peroxide at the late 8 h exposure time instead of the early 2 h exposure time. NADPH oxidases (NOXs) are the enzymes capable of generating superoxide. In this study, I included NOX5 (encoded by the NOX5 gene) and NOX2 (encoded by the CYBB gene). These results indicate that superoxide radicals are produced at the late exposure time by the up-regulation of the CYBB gene, while suppression of the NOX5 gene could reduce its production upon HBQ exposure.

The DNA damage repair gene OGG1 was down-regulated by all six tested HBQs at 8 h

exposure time. As mentioned above, HBQs could cause oxidative DNA damage as they have been observed to significantly increase cellular 8-hydroxydeoxyguanosine (8-OHdG) levels,[6, 46] a key biomarker of oxidative DNA damage.[57] 8-OHdG is repaired primarily by 8-oxoguanine DNA glycosylase, which is an enzyme encoded by the 8-oxoguanine glycosylase (OGG1) gene involved in the DNA base excision repair (BER) pathway.[58] Decreased levels of OGG1 have been shown to be associated with tumor development.[59] Thus, the suppression of OGG1 suggests that HBQs may cause DNA damage through inhibition of the DNA repair gene. However, other genes may be responsible for DNA damage repair, such as the MutY homologue (MYH) gene.[59]

6.3.6 Differences of transcriptome changes between HBQ isomers

2,5-HBQs have been shown to induce higher cytotoxicity and greater ROS production than their corresponding 2,6-HBQ isomers.[46] Thus, determining the underlying differences in biological activity between isomer pairs may help elucidate the differences in cellular response that have been observed. Figure 6.9 shows the difference in gene expression between the HBQ isomer pairs in SV-HUC-1 cells. Interestingly, 2,6-DCBQ and 2,6-DBBQ both up-regulate Nrf2, while 2,5-DCBQ and 2,5-DBBQ both down-regulate Keap1 at the 2 h exposure time point. This indicates that at early exposure times, HBQs are able to activate the Nrf2-Keap1 signal pathway; however, it appears this activation may be achieved in different ways for different HBQ isomers. While the 2,6-HBQs may directly activate Nrf2, the 2,5-HBQs may suppress Keap1 to indirectly activate the Nrf2 pathway. These findings are consistent with a recent work by Procházka et al. that indicated that 2,6-DCBQ and 2,6-DBBQ both activate Nrf2, as determined using an ARE-dependent β-lactamase-based reporter gene assay.[47] Tetrachlorobenzoquinone (TetraCBQ) has also been observed to activate Nrf2, but not Keap1, in HepG2 cells.[60] Furthermore, it has been found that the ability of compounds to activate the Nrf2 pathway correlates with their reactivity toward the thiol groups of Keap1 cysteine residues, which inhibits the ubiquitination of Nrf2. For example, the binding site for *tert*-butylhydroquinone and 1,2-naphthoquinone was shown to be Cys-151 of Keap1.[61] Therefore, 2,5-HBQs may bind to Keap1 cysteine residues to activate the Nrf2 pathway. Another interesting finding among the HBQ isomers is that the 2,6-HBQs significantly increased the gene expression levels of GPX2 and CAT at 2 h, while the 2,5-HBQs did not. It is known that GPX2 and CAT are responsible for reducing hydrogen peroxide, so the higher level of these two genes would be helpful to decrease the cellular ROS induced by 2,6-

HBQs. This could be used to explain the higher toxicity induced by 2,5-HBQs compared with their 2,6-HBQ isomers. The third and most important finding was that 2,5-DCBQ and 2,5-DBBQ induced significantly higher expression levels of seven genes (2 h: HMOX1; 8 h: GCLC, GSR, TXNRD1, CYBB, CYGB, OGG1, HMOX1) than their corresponding 2,6-HBQ isomers ($P<0.05$). This result indicated that 2,5-HBQ caused higher level of oxidative stress, so that the adaptive genes also increased higher than 2,6-HBQ. As shown in Figure 6.5, 2,5-DCBQ and 2,5-DBBQ produced more ROS in comparison to the other HBQs at each time point, which matches the same pattern of gene expression observed for these seven genes. Thus, these seven genes have potential for use as molecular markers for comparison of 2,5-HBQs with their 2,6-HBQ isomers.

6.3.7 Identification of potential pathways

To identify the significant canonical pathways affected by HBQ exposure, Ingenuity Pathway Analysis (IPA) software was used. Since the canonical pathways that responded to exposure to each tested HBQ at both time points were similar, 2,6-DCBQ was used as an example (Table 6.6). At both exposure times points, the Nrf2-mediated oxidative stress response was the canonical pathway with the most statistically significant level, as I predicted. A typical Nrf2 pathway after 2 h or 8 h HBQ exposure is shown in Figure 6.10, and most of the Nrf2 target genes show significantly increased expression. Other canonical pathways that responded to HBQ exposure at both exposure time points were similar, and included glutathione redox reductions, superoxide radical degradation, and xenobiotic metabolism signaling, indicating that superoxide radicals are the major radical produced by HBQs and GSH works to detoxify HBQs.

6.4 Conclusion

In summary, this study showed that significantly high levels of cellular ROS can be produced by HBQs in SV-HUC-1 cells, and 8 h is the peak time of ROS production for each HBQ. This is the first study that has shown HBQ-induced oxidative stress in human urinary tract cells. Additionally, I explored the toxicity pathways activated upon exposure of SV-HUC-1 cells to low cytotoxic concentrations of HBQs at early and late response time points (2 h and 8 h), using a TaqMan PCR Array containing 44 target genes. Each tested HBQ at both exposure time points induced significant alterations in gene expression levels. The higher number of significantly

altered genes at both exposure time points suggests that the Nrf2/ARE antioxidant response pathway is the main signaling pathway responsive to HBQ exposure. Cell cycle regulation and the oxidative DNA damage/repair pathways were also activated to protect cells against low cytotoxic concentrations of HBQs. The HMOX1 gene showed a fast and sensitive response to HBQ exposure at both the early and late time points, followed by the NQO1, TXNRD1, and PTGS2 genes. The data here suggest that inflammation is caused in cells exposed to HBQs, and the Trx system is involved in detoxification of HBQs. Several genes were identified as potential molecular markers for comparison of 2,5-HBQs with their corresponding 2,6-HBQ isomers at late exposure time points. This study provides an understanding of the molecular basis upon the response of human bladder cells to exposure to low cytotoxic concentrations of HBQ-DBPs, and provides insights into the antioxidant defense systems activated against oxidative stress induced by HBQ-DBPs. This study also highlights the need for more comprehensive studies to more fully understand the toxicity pathways involved in HBQ exposure. As gene expression can only indirectly estimate levels of encoded proteins, further studies are needed to investigate these transcriptome profiles by examining intracellular protein levels and function in the future.

6.5 References

1. Zhao, Y. L.; Qin, F.; Boyd, J. M.; Anichina, J.; Li, X.-F., Characterization and determination of chloro- and bromo-benzoquinones as new chlorination disinfection byproducts in drinking water. *Anal. Chem.* **2010,** *82*, (11), 4599-4605.
2. Zhao, Y. L.; Anichina, J.; Lu, X. F.; Bull, R. J.; Krasner, S. W.; Hrudey, S. E.; Li, X.-F., Occurrence and formation of chloro- and bromo-benzoquinones during drinking water disinfection. *Water Res.* **2012,** *46*, (14), 4351-4360.
3. Wang, W.; Qian, Y. C.; Boyd, J. M.; Wu, M. H.; Hrudey, S. E.; Li, X.-F., Halobenzoquinones in swimming pool waters and their formation from personal care products. *Environ. Sci. Technol.* **2013,** *47*, (7), 3275-3282.
4. Wang, W.; Qian, Y. C.; Li, J. H.; Moe, B.; Huang, R. F.; Zhang, H. Q.; Hrudey, S. E.; Li, X. F., Li, X.-F., Analytical and toxicity characterization of halo-hydroxyl-benzoquinones as stable halobenzoquinone disinfection byproducts in treated water. *Anal. Chem.* **2014,** *86*, (10), 4982-4988.
5. Li, J.; Wang, W.; Moe, B.; Wang, H.; Li, X. F., Chemical and toxicological characterization of halobenzoquinones, an emerging class of disinfection byproducts. *Chem. Res. Toxicol.* **2015,** *28*, (3), 306-18.
6. Du, H. Y.; Li, J. H.; Moe, B.; McGuigan, C. F.; Shen, S. W.; Li, X.-F., Cytotoxicity and oxidative damage induced by halobenzoquinones to T24 bladder cancer cells. *Environ. Sci. Technol.* **2013,** *47*, (6), 2823-2830.
7. Li, J.; Wang, W.; Zhang, H.; Le, X. C.; Li, X.-F., Glutathione-mediated detoxification of halobenzoquinone drinking water disinfection byproducts in T24 cells. *Toxicol. Sci.* **2014,** *141*, (2), 335-343.
8. Klaunig, J. E.; Kamendulis, L. M.; Hocevar, B. A., Oxidative stress and oxidative damage in carcinogenesis. *Toxicol. Pathol.* **2010,** *38*, (1), 96-109.
9. Storz, P., Reactive oxygen species in tumor progression. *Front. Biosci.* **2005,** *10*, 1881-96.
10. Jennings, P.; Limonciel, A.; Felice, L.; Leonard, M. O., An overview of transcriptional regulation in response to toxicological insult. *Arch. Toxicol.* **2013,** *87*, (1), 49-72.
11. Ma, Q., Role of Nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* **2013,** *53*, 401-26.

12. Iida, K.; Itoh, K.; Kumagai, Y.; Oyasu, R.; Hattori, K.; Kawai, K.; Shimazui, T.; Akaza, H.; Yamamoto, M., Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Re.s* **2004,** *64*, (18), 6424-31.
13. Pals, J.; Attene-Ramos, M. S.; Xia, M. H.; Wagner, E. D.; Plewa, M. J., Human cell toxicogenomic analysis linking reactive oxygen species to the toxicity of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2013,** *47*, (21), 12514-12523.
14. Wang, S.; Zheng, W.; Liu, X.; Xue, P.; Jiang, S.; Lu, D.; Zhang, Q.; He, G.; Pi, J.; Andersen, M. E.; Tan, H.; Qu, W., Iodoacetic acid activates Nrf2-mediated antioxidant response in vitro and in vivo. *Environ. Sci. Technol.* **2014,** *48*, (22), 13478-88.
15. Waters, M. D.; Fostel, J. M., Toxicogenomics and systems toxicology: aims and prospects. *Nat. Rev. Genet.* **2004,** *5*, (12), 936-948.
16. Arikawa, E.; Sun, Y.; Wang, J.; Zhou, Q.; Ning, B.; Dial, S. L.; Guo, L.; Yang, J., Cross-platform comparison of SYBR Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. *BMC. Genomics.* **2008,** *9*, 328.
17. Thai, S. F.; Allen, J. W.; DeAngelo, A. B.; George, M. H.; Fuscoe, J. C., Altered gene expression in mouse livers after dichloroacetic acid exposure. *Mutat. Res. Rev. Mutat.* **2003,** *543*, (2), 167-180.
18. Tully, D. B.; Luft, J. C.; Rockett, J. C.; Ren, H. Z.; Schmid, J. E.; Wood, C. R.; Dix, D. J., Reproductive and genomic effects in testes from mice exposed to the water disinfectant byproduct bromochloroacetic acid. *Reprod. Toxicol.* **2005,** *19*, (3), 353-366.
19. Muellner, M. G.; Attene-Ramos, M. S.; Hudson, M. E.; Wagner, E. D.; Plewa, M. J., Human cell toxicogenomic analysis of bromoacetic acid: a regulated drinking water disinfection by-product. *Environ. Mol. Mutagen.* **2010,** *51*, (3), 205-214.
20. Attene-Ramos, M. S.; Wagner, E. D.; Plewa, M. J., omparative human cell toxicogenomic analysis of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2010,** *44*, (19), 7206-7212.
21. Delker, D.; Hatch, G.; Allen, J.; Crissman, B.; George, M.; Geter, D.; Kilburn, S.; Moore, T.; Nelson, G.; Roop, B.; Slade, R.; Swank, A.; Ward, W.; DeAngelo, A., Molecular biomarkers of oxidative stress associated with bromate carcinogenicity. *Toxicology* **2006,** *221*, (2-3), 158-165.

22. Luan, Y.; Suzuki, T.; Palanisamy, R.; Takashima, Y.; Sakamoto, H.; Sakuraba, M.; Koizumi, T.; Saito, M.; Matsufuji, H.; Yamagata, K.; Yamaguchi, T.; Hayashi, M.; Honma, M., Potassium bromate treatment predominantly causes large deletions, but not GC>TA transversion in human cells. *Mutat. Res.* **2007,** *619*, (1-2), 113-23.

23. Ward, W. O.; Swartz, C. D.; Porwollik, S.; Warren, S. H.; Hanley, N. M.; Knapp, G. W.; McClelland, M.; DeMarini, D. M., Toxicogenomic analysis incorporating operon-transcriptional coupling and toxicant concentration-expression response: analysis of MX-treated Salmonella. *BMC. Bioinformatics* **2007,** *8*, 378.

24. Christian, B. J.; Loretz, L. J.; Oberley, T. D.; Reznikoff, C. A., Characterization of human uroepithelial cells immortalized in vitro by simian virus 40. *Cancer Res.* **1987,** *47*, (22), 6066-73.

25. Du, H. Y.; Li, J. H.; Moe, B.; McGuigan, C. F.; Li, X. F., A real-time cell-electronic sensing method for comparative analysis of toxicity of water contaminants. *Anal. Methods* **2013,** *6*, 2053-2058.

26. Wang, G.; Gong, Y.; Burczynski, F. J.; Hasinoff, B. B., Cell lysis with dimethyl sulphoxide produces stable homogeneous solutions in the dichlorofluorescein oxidative stress assay. *Free Radic. Res.* **2008,** *42*, (5), 435-441.

27. Aruoma, O. I.; Halliwell, B.; Hoey, B. M.; Butler, J., The antioxidant action of N-acetylcysteine - its reaction with hydrogen-peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic. Bio. Med.* **1989,** *6*, (6), 593-597.

28. Zafarullah, M.; Li, W. Q.; Sylvester, J.; Ahmad, M., Molecular mechanisms of N-acetylcysteine actions. *Cell. Mol. Life Sci.* **2003,** *60*, (1), 6-20.

29. Deferme, L.; Briede, J. J.; Claessen, S. M. H.; Jennen, D. G. J.; Cavil, R.; Kleinjans, J. C. S., Time series analysis of oxidative stress response patterns in HepG2: a toxicogenomics approach. *Toxicology* **2013,** *306*, 24-34.

30. Bolton, J. L.; Dunlap, T., Formation and biological targets of quinones: cytotoxic versus cytoprotective effects. *Chem. Res. Toxicol.* **2017,** *30*, (1), 13-37.

31. Tenhunen, R.; Marver, H. S.; Schmid, R., Enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc. Natl. Acad. Sci. USA.* **1968,** *61*, (2), 748-&.

32. Gorrini, C.; Harris, I. S.; Mak, T. W., Modulation of oxidative stress as an anticancer strategy. *Nat. Rev. Drug Discov*. **2013,** *12*, (12), 931-947.

33. Chang, M.; Xue, J.; Sharma, V.; Habtezion, A., Protective role of hemeoxygenase-1 in gastrointestinal diseases. *Cell. Mol. Life Sci.* **2015,** *72*, (6), 1161-73.

34. Itoh, K.; Mochizuki, M.; Ishii, Y.; Ishii, T.; Shibata, T.; Kawamoto, Y.; Kelly, V.; Sekizawa, K.; Uchida, K.; Yamamoto, M., Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-Delta(12,14)-prostaglandin j(2). *Mol. Cell Biol.* **2004,** *24*, (1), 36-45.

35. Li, L.; Dong, H.; Song, E.; Xu, X.; Liu, L.; Song, Y., Nrf2/ARE pathway activation, HO-1 and NQO1 induction by polychlorinated biphenyl quinone is associated with reactive oxygen species and PI3K/AKT signaling. *Chem. Biol. Interact.* **2014,** *209*, 56-67.

36. Li, N.; Alam, J.; Venkatesan, M. I.; Eiguren-Fernandez, A.; Schmitz, D.; Di Stefano, E.; Slaughter, N.; Killeen, E.; Wang, X. R.; Huang, A.; Wang, M. Y.; Miguel, A. H.; Cho, A.; Sioutas, C.; Nel, A. E., Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells: Protecting against the proinflammatory and oxidizing effects of diesel exhaust chemicals. *J. Immunol.* **2004,** *173*, (5), 3467-3481.

37. Mustacich, D.; Powis, G., Thioredoxin reductase. *Biochem. J.* **2000,** *346*, 1-8.

38. Lu, J.; Holmgren, A., The thioredoxin antioxidant system. *Free Radic. Biol. Med.* **2014,** *66*, 75-87.

39. Seibert, K.; Zhang, Y.; Leahy, K.; Hauser, S.; Masferrer, J.; Perkins, W.; Lee, L.; Isakson, P., Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc. Natl. Acad. Sci. USA.* **1994,** *91*, (25), 12013-7.

40. Grivennikov, S. I.; Greten, F. R.; Karin, M., Immunity, Inflammation, and Cancer. *Cell* **2010,** *140*, (6), 883-899.

41. Michaud, D. S., Chronic inflammation and bladder cancer. *Urol. Oncol.* **2007,** *25*, (3), 260-8.

42. Komhoff, M.; Guan, Y.; Shappell, H. W.; Davis, L.; Jack, G.; Shyr, Y.; Koch, M. O.; Shappell, S. B.; Breyer, M. D., Enhanced expression of cyclooxygenase-2 in high grade human transitional cell bladder carcinomas. *Am. J Pathol.* **2000,** *157*, (1), 29-35.

43. Shariat, S. F.; Matsumoto, K.; Kim, J.; Ayala, G. E.; Zhou, J. H.; Jian, W.; Benedict, W. F.; Lerner, S., Correlation of cyclooxygenase-2 expression with molecular markers, pathological features and clinical outcome of transitional cell carcinoma of the bladder. *J. Urol.* **2003,** *170*, (3), 985-9.

44. Dutto, I.; Tillhon, M.; Cazzalini, O.; Stivala, L. A.; Prosperi, E., Biology of the cell cycle inhibitor p21(CDKN1A): molecular mechanisms and relevance in chemical toxicology. *Arch. Toxicol.* **2015,** *89*, (2), 155-178.
45. Fu, K. Z. L., J.; Vemula, S.; Moe, B.; Li, X.-F., Effects of halobenzoquinone and haloacetic acid water disinfection byproducts on human neural stem cells. *J. Environ. Sci.* **2017**.
46. Li, J.; Moe, B.; Vemula, S.; Wang, W.; Li, X.-F., Emerging disinfection byproducts, halobenzoquinones: effects of isomeric structure and halogen substitution on cytotoxicity, formation of reactive oxygen species, and genotoxicity. *Environ. Sci. Technol.* **2016,** *50*, (13), 6744-52.
47. Prochazka, E.; Escher, B. I.; Plewa, M. J.; Leusch, F. D., In vitro cytotoxicity and adaptive stress responses to selected haloacetic acid and halobenzoquinone water disinfection byproducts. *Chem. Res. Toxicol.* **2015,** *28*, (10), 2059-68.
48. Villeneuve, N. F.; Sun, Z.; Chen, W.; Zhang, D. D., Nrf2 and p21 regulate the fine balance between life and death by controlling ROS levels. *Cell Cycle* **2009,** *8*, (20), 3255-6.
49. Chen, W.; Sun, Z.; Wang, X. J.; Jiang, T.; Huang, Z.; Fang, D.; Zhang, D. D., Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Mol. Cell* **2009,** *34*, (6), 663-73.
50. Shi, M. M.; Kugelman, A.; Iwamoto, T.; Tian, L.; Forman, H. J., Quinone-induced oxidative stress elevates glutathione and induces gamma-glutamylcysteine synthetase activity in rat lung epithelial L2 cells. *J. Biol. Chem.* **1994,** *269*, (42), 26512-7.
51. Kulinskii, V. I.; Kolesnichenko, L. S., Glutathione system. I. Synthesis, transport, glutathione transferases, glutathione peroxidases. *Biomed. Khim.* **2009,** *55*, (3), 255-77.
52. Arthur, J. R., The glutathione peroxidases. . *Cell. Mol. Life Sci.* **2000,** *57*, (13-14), 1825-35.
53. Cantor, K. P.; Villanueva, C. M.; Silverman, D. T.; Figueroa, J. D.; Real, F. X.; Garcia-Closas, M.; Malats, N.; Chanock, S.; Yeager, M.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Samanic, C.; Rothman, N.; Kogevinas, M., Polymorphisms in GSTT1, GSTZ1, and CYP2E1, disinfection by-products, and risk of bladder cancer in Spain. *Environ. Health Perspec.* **2010,** *118*, (11), 1545-1550.
54. Hayes, J. D.; McLellan, L. I., Glutathione and glutathione-dependent enzymes represent a Co-ordinately regulated defence against oxidative stress. *Free Radic. Res.* **1999,** *31*, (4), 273-300.

55. Burmester, T.; Ebner, B.; Weich, B.; Hankeln, T., Cytoglobin: A novel globin type ubiquitously expressed in vertebrate tissues. *Mol. Biol. Evol.* **2002,** *19*, (4), 416-421.

56. Chua, P. J.; Yip, G. W. C.; Bay, B. H., Cell cycle arrest induced by hydrogen peroxide is associated with modulation of oxidative stress related genes in breast cancer cells. *Exp. Biol. Med.* **2009,** *234*, (9), 1086-1094.

57. Valavanidis, A.; Vlachogianni, T.; Fiotakis, C., 8-hydroxy-2' -deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. *J. Environ. Sci. Health C* **2009,** *27*, (2), 120-139.

58. Boiteux, S.; Radicella, J. P., The human OGG1 gene: structure, functions, and its implication in the process of carcinogenesis. *Arch. Biochem. Biophys.* **2000,** *377*, (1), 1-8.

59. Evans, M. D.; Dizdaroglu, M.; Cooke, M. S., Oxidative DNA damage and disease: induction, repair and significance. *Mutat. Res. Rev. Mutat.* **2004,** *567*, (1), 1-61.

60. Su, C.; Zhang, P.; Song, X.; Shi, Q.; Fu, J.; Xia, X.; Bai, H.; Hu, L.; Xu, D.; Song, E.; Song, Y., Tetrachlorobenzoquinone activates Nrf2 signaling by Keap1 cross-linking and ubiquitin translocation but not Keap1-Cullin3 complex dissociation. *Chem. Res. Toxicol.* **2015,** *28*, (4), 765-74.

61. Kobayashi, M.; Li, L.; Iwamoto, N.; Nakajima-Takagi, Y.; Kaneko, H.; Nakayama, Y.; Eguchi, M.; Wada, Y.; Kumagai, Y.; Yamamoto, M., The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. *Mol. Cell. Biol.* **2009,** *29*, (2), 493-502.

Table 6.1 Primer sequences used for real-time PCR

Gene Name	Forward (5'-3')	Reverse (5'-3')
Nrf2	TCC AGT CAG AAA CCA GTG GAT	GAA TGT CTG CGC CAA AAG CTG
NQO1	ACT GCC CTC TTG TGG TGC AT	GCT CGG TCC AAT CCC TTC AT
GAPDH	TGC ACC ACC AAC TGC TTA GC	GGC ATG GAC TGT GGT CAT GAG

Gene Symbols

	1	2	3	4	5	6	7	8	9	10	11	12
A	18S	GAPDH	HPRT1	GUSB	NFE2L2	KEAP1	18S	GAPDH	HPRT1	GUSB	NFE2L2	KEAP1
B	GSTP1	GSTT1	GSTZ1	GSTM1	GSTA1	GSTA4	GSTP1	GSTT1	GSTZ1	GSTM1	GSTA1	GSTA4
C	GCLM	GCLC	GPX1	GPX2	GPX4	GSR	GCLM	GCLC	GPX1	GPX2	GPX4	GSR
D	GSS	NQO1	NAT1	NAT2	TXNRD1	TXNRD2	GSS	NQO1	NAT1	NAT2	TXNRD1	TXNRD2
E	CAT	SOD1	SOD2	SOD3	PTGS2	PRDX1	CAT	SOD1	SOD2	SOD3	PTGS2	PRDX1
F	PRDX2	PRDX3	PRDX4	PRDX5	SRXN1	LPO	PRDX2	PRDX3	PRDX4	PRDX5	SRXN1	LPO
G	MPO	CYBB	CYGB	NOX5	HMOX1	OGG1	MPO	CYBB	CYGB	NOX5	HMOX1	OGG1
H	CDKN1A	ABCC1	ABCC2	ABCC3	ABCC4	ABCC5	CDKN1A	ABCC1	ABCC2	ABCC3	ABCC4	ABCC5

Figure 6.1 The 96-well format of the Taqman PCR Array. 18S, GAPDH, HPRT1, and GUSB were used as the reference controls. Samples are in duplicate format.

Table 6.2 A list of genes used for the TaqMan PCR array

	Gene Functional Groups	**Gene Name**	**Gene Symbols**
1	Transcription factor and its inhibitor	Nuclear factor-erythroid 2-related factor	Nrf2
		Kelch-like ECH-associated protein 1	Keap1
2	GSH utilization	Glutathione S-transferase	GST, Including subtypes: GSTP1, GSTT1, GSTZ1, GSTM1, GSTA1, GSTA4
		Glutathione peroxidase	GPx, Including subtypes: GPx1 GPx2 GPx4
3	GSH production	Glutamate–cysteine ligase	GCL, Including subtypes: GCLM (GCL modifier subunit) GCLC (GCL

			catalytic subunit)
		Glutathione reductase	QSR
		Glutathione synthetase	GSS
4	Thioredoxin system	Thioredoxin reductase 1	TXNRD1
		Peroxiredoxin	PRDX, Including subtypes: PRDX1, PRDX2, PRDX3, PRDX4, PRDX5
		Sulfiredoxin	SRXN1
5	Membrane transporters	Multidrug resistance protein	MRP, Including subtypes: MRP1 MRP2 MRP3 MRP4
6	Antioxidants	NADPH:quinone oxidoreductase 1	NQO1
		Catalase	CAT
		Superoxide dismutase	SOD, Including subtypes: SOD1, SOD2, SOD3
		Prostaglandin-endoperoxide synthase 2*or*	PTGS2 *or* COX-2

		Cyclooxygenase-2	
		Heme oxygenase (decycling) 1	HMOX1
7	Stress responsive genes	N-acetyltransferase	NAT, Including subtypes: NAT1 NAT2
		Lactoperoxidase	LPO
		Myeloperoxidase	MPO
		Cytochrome b-245 beta chain	CYBB
		Cytoglobin	CYGB
		NADPH oxidase, EF-hand calcium binding domain 5	NOX5
8	Oxidative DNA damage repair	8-Oxoguanine glycosylase	OGG1
9	Cell cycle regulation	p21 / WAF1 also known as cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1	CDKN1A

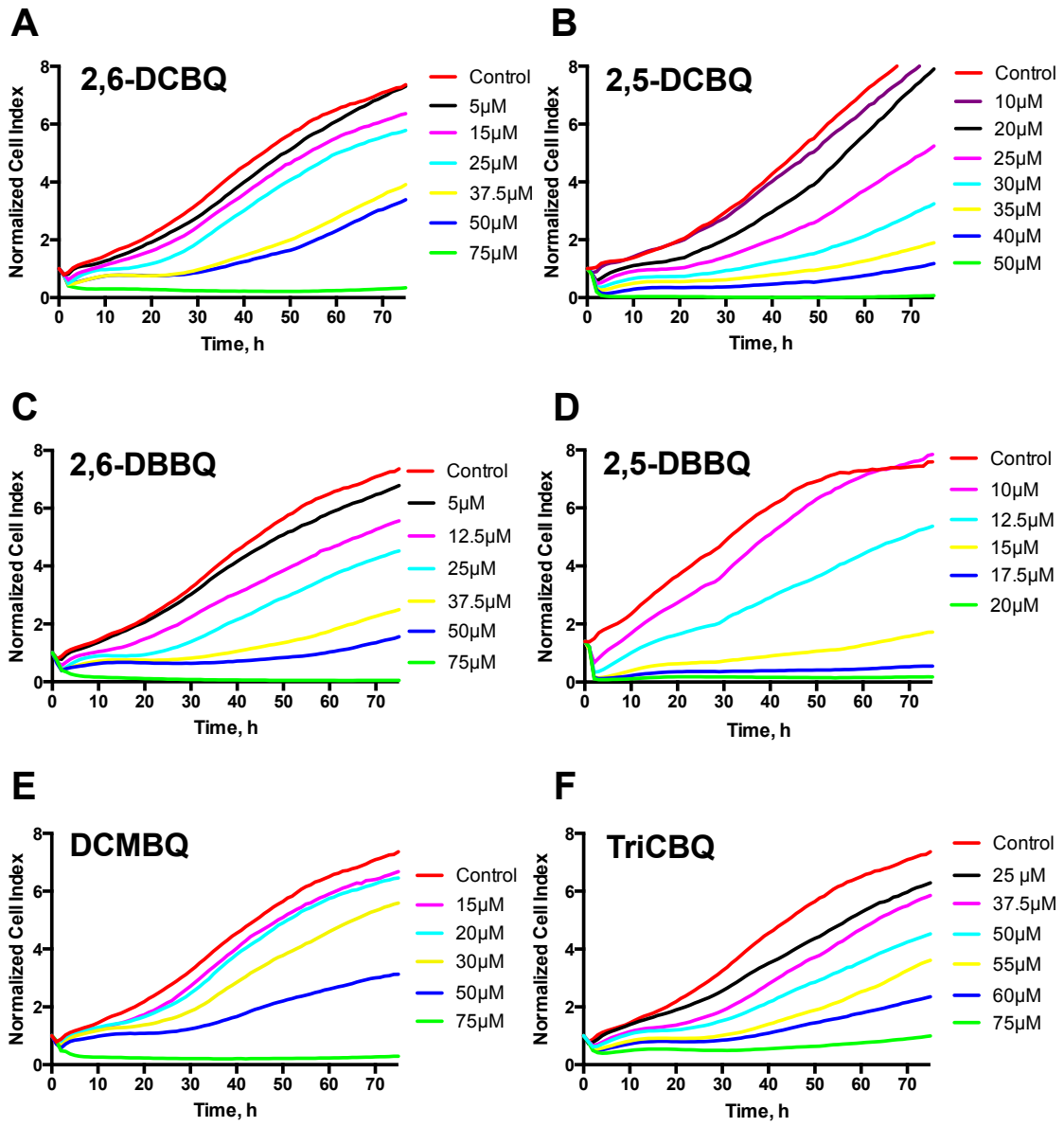


Figure 6.2 Cytotoxicity response profiles of the normalized cell index (CI) over time for SV-HUC-1 cells exposed to HBQs. (A) 2,6-DCBQ, (B) 2,5-DCBQ, (C) 2,6-DBBQ, (D) 2,5-DBBQ, (E) DCMBQ, and (F) TriCBQ.

Table 6.3 24-h IC_{20} values (μM ± SEM) for each HBQ in SV-HUC-1 cells

	2,6-DCBQ	*2,5-DCBQ*	*2,6-DBBQ*	*2,5-DBBQ*	*DCMBQ*	*TriCBQ*
IC_{20} Value	13 ± 2	17 ± 1	10 ± 1	12 ± 1	17 ± 3	24 ± 3

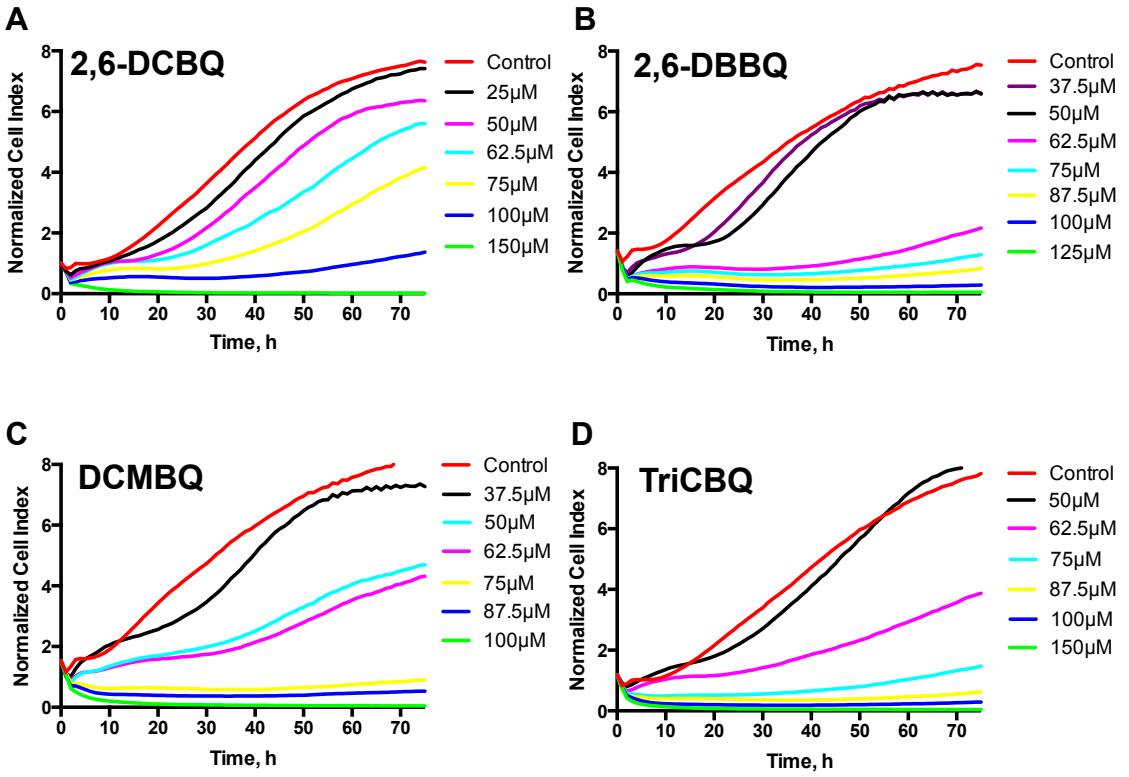


Figure 6.3 Cytotoxicity response profiles of the normalized cell index (NCI) over time for SV-HUC-1 cells exposed to HBQ with the addition of 1 mM N-acetyl-L-cysteine (NAC). (A) 2,6-DCBQ, (B) 2,6-DBBQ, (C) DCMBQ, and (D) TriCBQ.

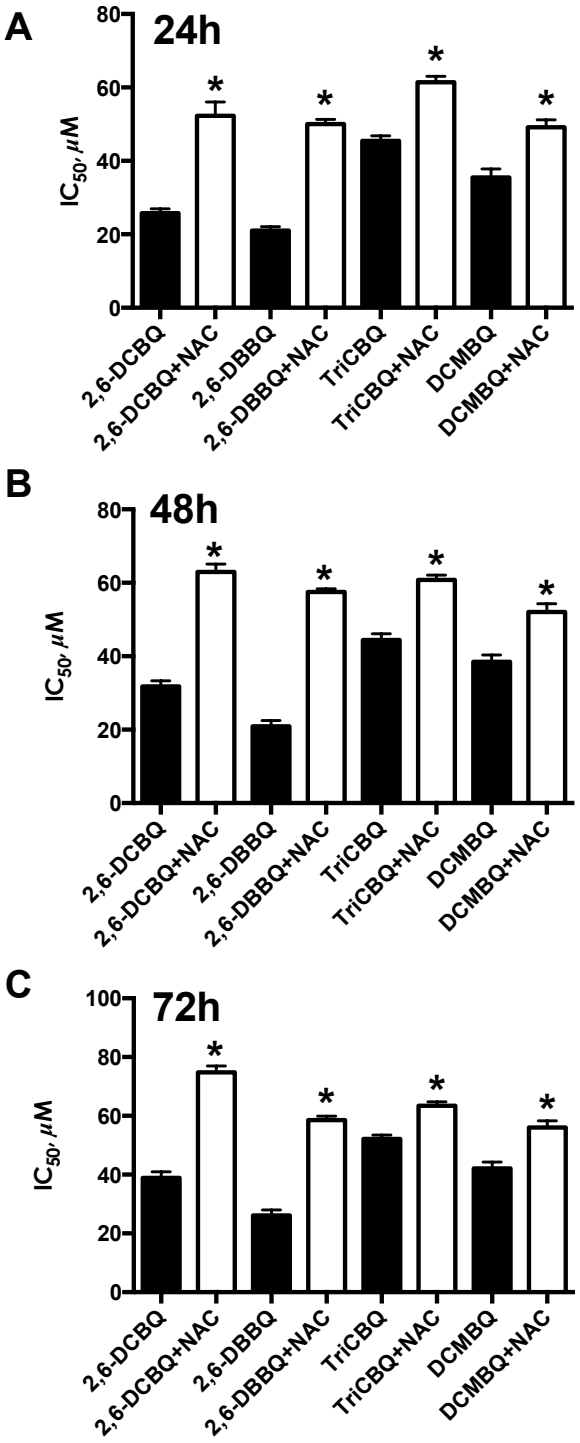


Figure 6.4 IC_{50} values of HBQs with or without 1 mM NAC in SV-HUC-1 cells after 24 h, 48 h, and 72 h exposure. All values are expressed as mean ± SD from RTCA. *P < 0.05, HBQ + NAC treatment groups compared with HBQ treatment groups.

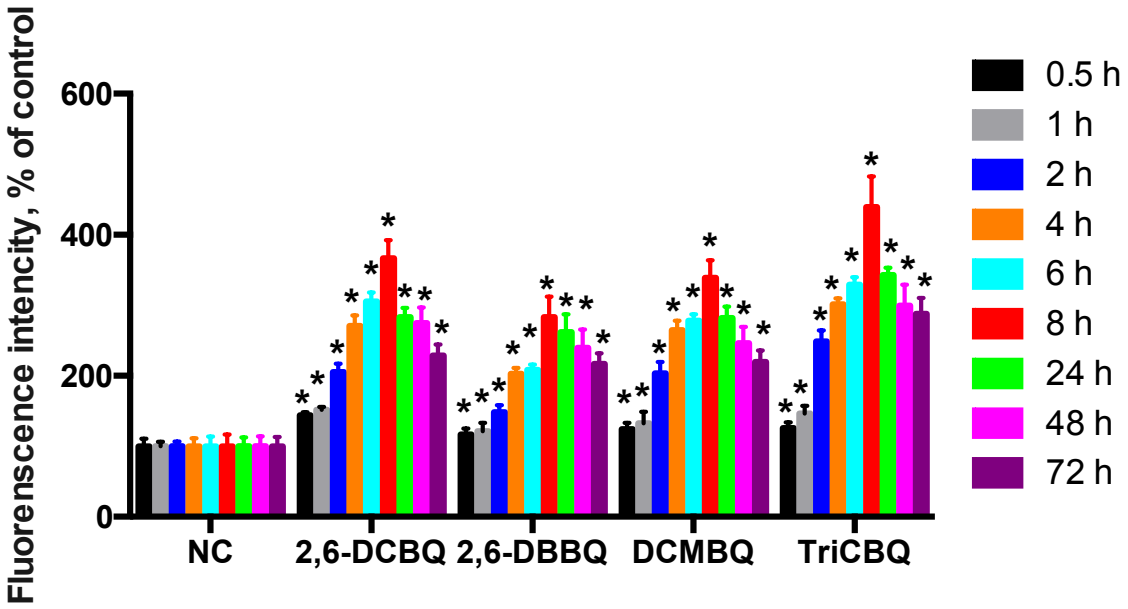


Figure 6.5 Time-course of ROS generation in SV-HUC-1 cells after each HBQ treatment at their respective IC_{20} values. All values are expressed as mean ± SEM of six replicates determined in a representative experiment, and similar results were obtained in three independent experiments.

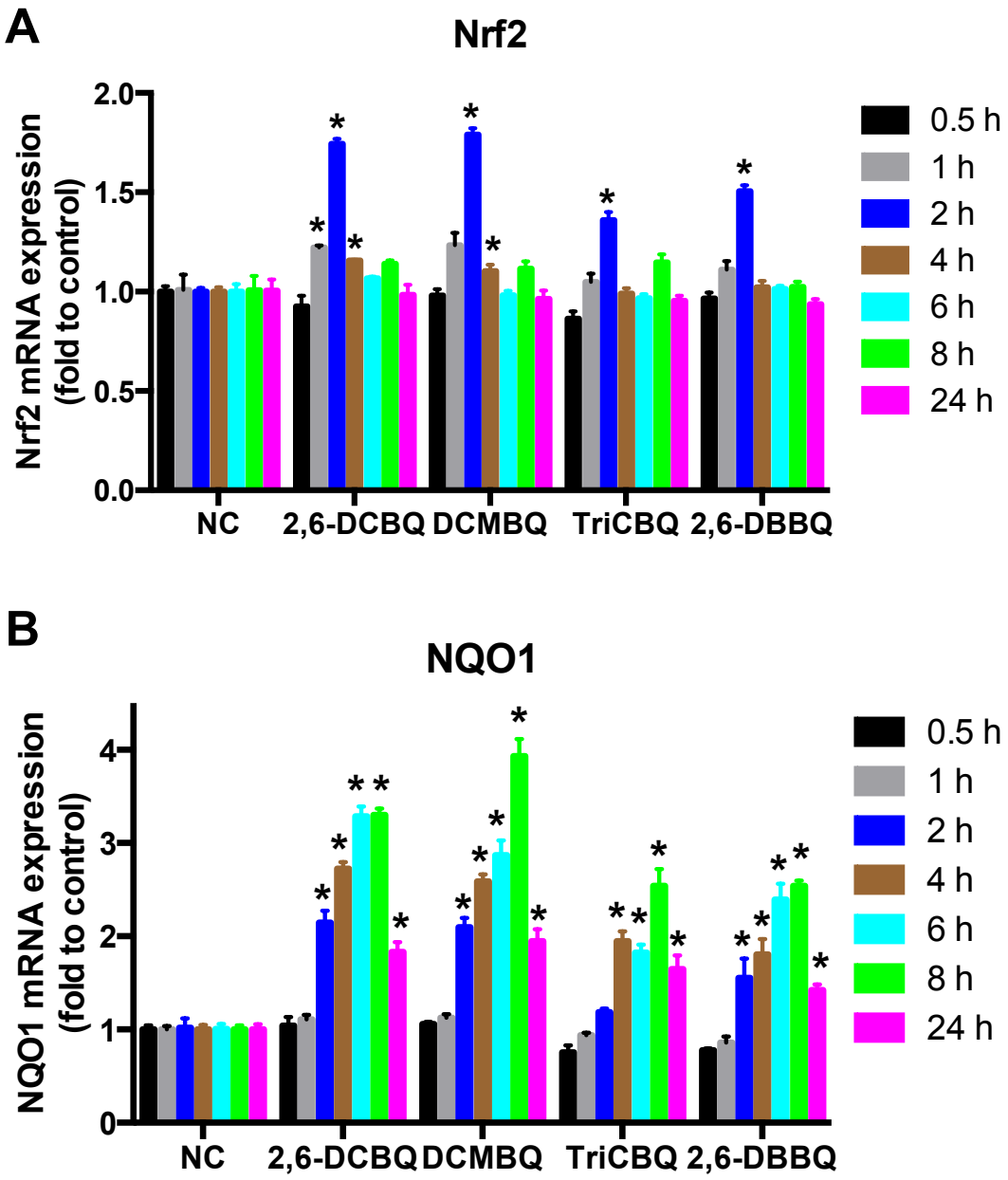


Figure 6.6 Time-course of HBQ-induced (A) Nrf2 and (B) NQO1 gene response in SV-HUC-1 cells. The mRNA expression was determined by real-time PCR and normalized by GAPDH. Data are shown as mean ± SEM, n=3. *P < 0.05 vs concurrent negative control (NC).

Table 6.4 Significant gene expression changes vs concurrent negative controls after 2 h HBQ exposure in SV-HUC-1 cells

Gene	2,6-DCBQ		2,5-DCBQ		2,6-DBBQ		2,5-DBBQ		DCMBQ		TriCBQ	
	fold change	*P* value	fold change	*P* value	fold change	*P* value	fold change	*P* value	fold change	*P* value	fold change	*P* value
NFE2L2	1.309	0.034			1.437	0.003			1.589	0.005	1.443	0.005
KEAP1			-1.337	0.003			-1.204	0.001				
GSTP1	1.191	0.041	-1.224	0.011	1.419	0.031	1.151	0.045			1.281	0.019
GSTT1			-1.470	0.001			-1.414	< 0.001				
GSTZ1	-1.013	< 0.001	-1.286	0.019								
GSTA4	-1.121	0.006	-1.373	0.014			-1.110	0.006			-1.142	0.006
GPX1	-1.099	< 0.001	-1.276	0.004			-1.090	< 0.001			-1.042	< 0.001
GPX2	2.031	0.004			3.867	0.003			1.495	0.034	1.593	0.012
GPX4					1.286	0.043						
GCLM	1.512	0.003			1.477	< 0.001	1.330	0.001			1.394	0.015
GCLC			-1.492	0.016			-1.302	< 0.001				
GSR			-1.383	< 0.001			-1.163	< 0.001				
GSS			-1.823	< 0.001	1.199	0.017	-1.097	< 0.001				
NQO1	1.788	< 0.001	2.796	< 0.001	1.898	0.005	1.252	0.002	2.004	0.031	1.575	0.006
CAT	1.171	0.015			1.326	0.001						
PTGS2	2.364	< 0.001	1.365	0.007	2.378	0.006	1.352	0.035	3.403	0.004	1.693	0.035
HMOX1	6.662	< 0.001	10.172	< 0.001	5.508	< 0.001	10.992	< 0.001	6.869	< 0.001	4.920	< 0.001

NAT1	-1.005	< 0.001										
NAT2	-1.443	0.021	-1.935	0.020								
CYBB	2.244	0.003	3.519	0.017	1.746	0.020	3.770	0.006			1.719	0.002
CYGB	-1.013	0.001					-1.164	< 0.001				
NOX5	-1.061	< 0.001	-1.488	< 0.001			-1.496	< 0.001			-1.107	0.004
TXNRD1	2.516	0.002	1.886	0.005	2.313	0.004	1.972	0.004	2.570	0.002	1.971	0.004
TXNRD2	-1.024	< 0.001	-1.737	0.001			-1.382	< 0.001				
PRDX1							-1.096	0.001				
PRDX2	-1.045	0.001			1.115	0.003	-1.188	< 0.001				
PRDX3	-1.046	< 0.001			1.174	0.009	-1.138	< 0.001				
PRDX4							-1.353	< 0.001				
PRDX5					1.1878	0.022						
SRXN1					1.369	< 0.001	-1.197	< 0.001	1.315	< 0.001	1.228	0.007
OGG1	-1.054	< 0.001	-1.561	< 0.001	1.148	0.011	-1.490	< 0.001	1.111	0.004		
CDKN1A	1.445	0.015	1.281	< 0.001	1.431	0.009	1.447	0.049	1.307	< 0.001	1.204	0.018
ABCC1	-1.027	< 0.001	-1.210	< 0.001			-1.245	< 0.001				
ABCC2			-1.331	0.001							-1.032	0.002
ABCC3			-1.665	< 0.001			-1.67	< 0.001				
ABCC4	-1.140	< 0.001	-1.167	< 0.001	-1.008	< 0.001	-1.089	< 0.001			-1.045	< 0.001
ABCC5			-1.300	< 0.001			-1.197	< 0.001				

Functional gene groups: membrane transporter, oxidative DNA damage repair, cell cycle regulation, stress responsive gene, antioxidants, thioredoxin system, transcription factors, GSH utilization, and GSH production.

Table 6.5 Significant gene expression changes vs concurrent negative controls after 8 h HBQ exposure in SV-HUC-1 cells

Gene	2,6-DCBQ		2,5-DCBQ		2,6-DBBQ		2,5-DBBQ		DCMBQ		TriCBQ	
	fold change	*P* value	fold change	*P* value	fold change	*P* value	fold change	*P* value	fold change	*P* value	fold change	*P* value
NFE2L2			1.153	0.030	-1.173	< 0.001	1.305	0.016			-1.085	< 0.001
KEAP1			1.208	0.007	-1.066	< 0.001					-1.108	< 0.001
GSTP1	-1.084	< 0.001			-1.095	< 0.001	-1.064	0.001	-1.071	< 0.001	-1.251	< 0.001
GSTT1	-1.091	< 0.001	-1.330	< 0.001	-1.323	< 0.001	-1.456	< 0.001	-1.201	0.003	-1.362	0.002
GSTZ1	-1.174	< 0.001	1.134	0.036	-1.132	< 0.001			-1.315	0.004	-1.243	< 0.001
GSTA4							1.477	0.039				
GPX1	-1.266	< 0.001	-1.098	< 0.001	-1.149	< 0.001	-1.181	< 0.001	-1.232	< 0.001	-1.306	< 0.001
GPX2	11.462	< 0.001	9.022	< 0.001	8.370	< 0.001	8.701	< 0.001	9.191	< 0.001	5.421	< 0.001
GPX4	-1.251	< 0.001	-1.093	0.001	-1.224	0.003	-1.240	< 0.001	-1.236	< 0.001	-1.371	0.003
GCLM	2.016	0.002	2.372	< 0.001	1.557	0.010	2.605	0.008	2.170	< 0.001	1.616	0.024
GCLC	1.697	0.003	2.134	0.002	1.220	0.020	2.375	0.003	1.583	0.012	1.285	0.019
GSR	1.400	0.014	1.666	0.004	1.194	0.018	1.937	0.003	1.503	0.014	1.275	0.012
GSS					-1.043	0.004	-1.116	< 0.001	-1.176	0.003	-1.216	0.002
NQO1	3.663	< 0.001	3.631	< 0.001	3.029	< 0.001	3.397	< 0.001	4.130	< 0.001	3.147	< 0.001
CAT	1.430	0.012	1.528	0.010	1.550	0.046	1.565	0.007	1.348	0.023	1.370	0.027
HMOX1	13.693	< 0.001	41.132	< 0.001	7.390	< 0.001	50.238	< 0.001	18.845	< 0.001	10.317	< 0.001
SOD1			1.117	0.042	-1.257	0.002	1.195	0.013			-1.258	0.002
SOD2			1.275	0.021	-1.162	0.002	1.251	0.009			-1.199	< 0.001

PTGS2	2.089	< 0.001	1.903	< 0.001	1.638	0.001	2.673	< 0.001	3.262	< 0.001	1.184	< 0.001
NAT1	-1.044	< 0.001			-1.088	0.002			-1.023	< 0.001	-1.153	0.001
NAT2	-2.698	0.012	-2.649	< 0.001			-2.303	< 0.001			-3.210	0.004
CYBB	3.536	0.005	16.999	< 0.001	14.008	< 0.001	20.085	< 0.001	2.433	< 0.001	13.962	< 0.001
CYGB	1.501	0.026	1.930	0.002	1.297	0.002	1.651	< 0.001	2.200	< 0.001	1.183	0.005
NOX5	-1.155	0.001	-1.425	0.001	-1.205	< 0.001	-1.621	0.002	-1.426	< 0.001	-1.339	0.007
TXNRD1	1.955	< 0.001	3.553	< 0.001	1.590	< 0.001	4.785	0.001	2.575	< 0.001	1.789	< 0.001
TXNRD2	-1.159	< 0.001	-1.455	< 0.001	-1.164	0.002	-1.593	< 0.001	-1.094	< 0.001	-1.262	< 0.001
PRDX1	1.090	0.013			-1.042	< 0.001	1.095	0.010	1.149	0.028	-1.102	0.004
PRDX2			-1.039	< 0.001	-1.101	< 0.001	-1.063	< 0.001	-1.050	< 0.001	-1.092	< 0.001
PRDX3	-1.029	< 0.001	-1.047	< 0.001	-1.062	< 0.001	-1.137	< 0.001	-1.075	< 0.001	-1.150	0.002
PRDX4	-1.024	< 0.001	-1.216	< 0.001	-1.292	0.004	-1.303	< 0.001	-1.061	< 0.001	-1.336	0.002
PRDX5	-1.139	0.001			-1.200	0.002			-1.120	0.001	-1.329	0.002
SRXN1	1.739	0.007	1.987	0.008			2.283	0.009	1.755	0.011		
OGG1	-1.044	< 0.001	-1.402	0.001	-1.073	< 0.001	-1.597	< 0.001	-1.016	< 0.001	-1.204	0.005
CDKN1A	1.389	0.029	1.820	0.003			2.530	0.011	1.477	0.016		
ABCC1							1.061	0.018				
ABCC2	1.529	0.010	2.083	0.001	1.231	0.027	3.051	0.002	1.740	< 0.001		
ABCC3	1.778	0.004			1.300	0.016			1.851	0.019	1.266	0.011
ABCC5					-1.100	0.001			1.178	0.046	-1.116	< 0.001

Functional gene groups: membrane transporter, oxidative DNA damage repair, cell cycle regulation, stress responsive gene, antioxidants, thioredoxin system, transcription factors, GSH utilization, and GSH production.

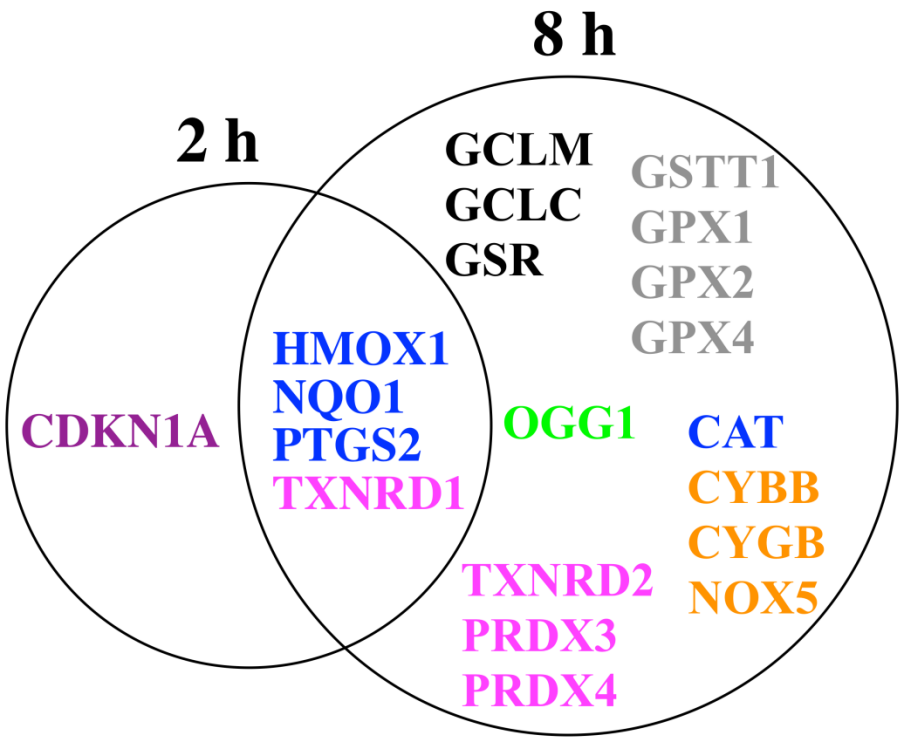


Figure 6.7 Commonly shared genes by the 6 HBQs at 2 h and/or 8 h exposure times. Functional gene groups: ■oxidative DNA damage repair, ■cell cycle regulation, ■stress responsive gene, ■antioxidants, ■thioredoxin system, ■GSH utilization, and ■GSH production.

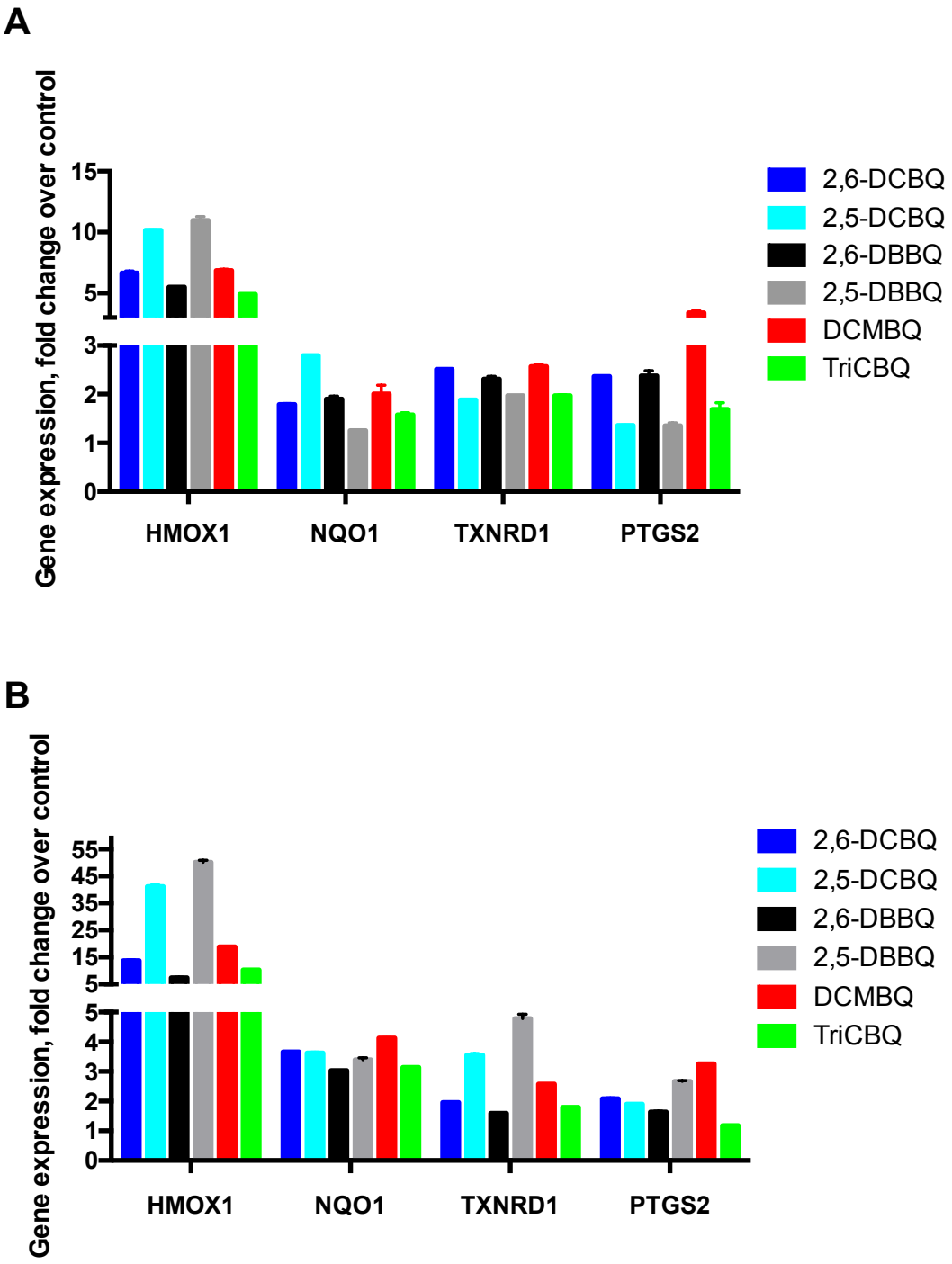


Figure 6.8 Identification of 4 genes that were specifically altered by the 6 tested HBQs in SV-HUC-1 cells. (A) 2 h exposure and (B) 8 h exposure. Data of fold-changes in the expression are shown as mean ± SEM, n=2.

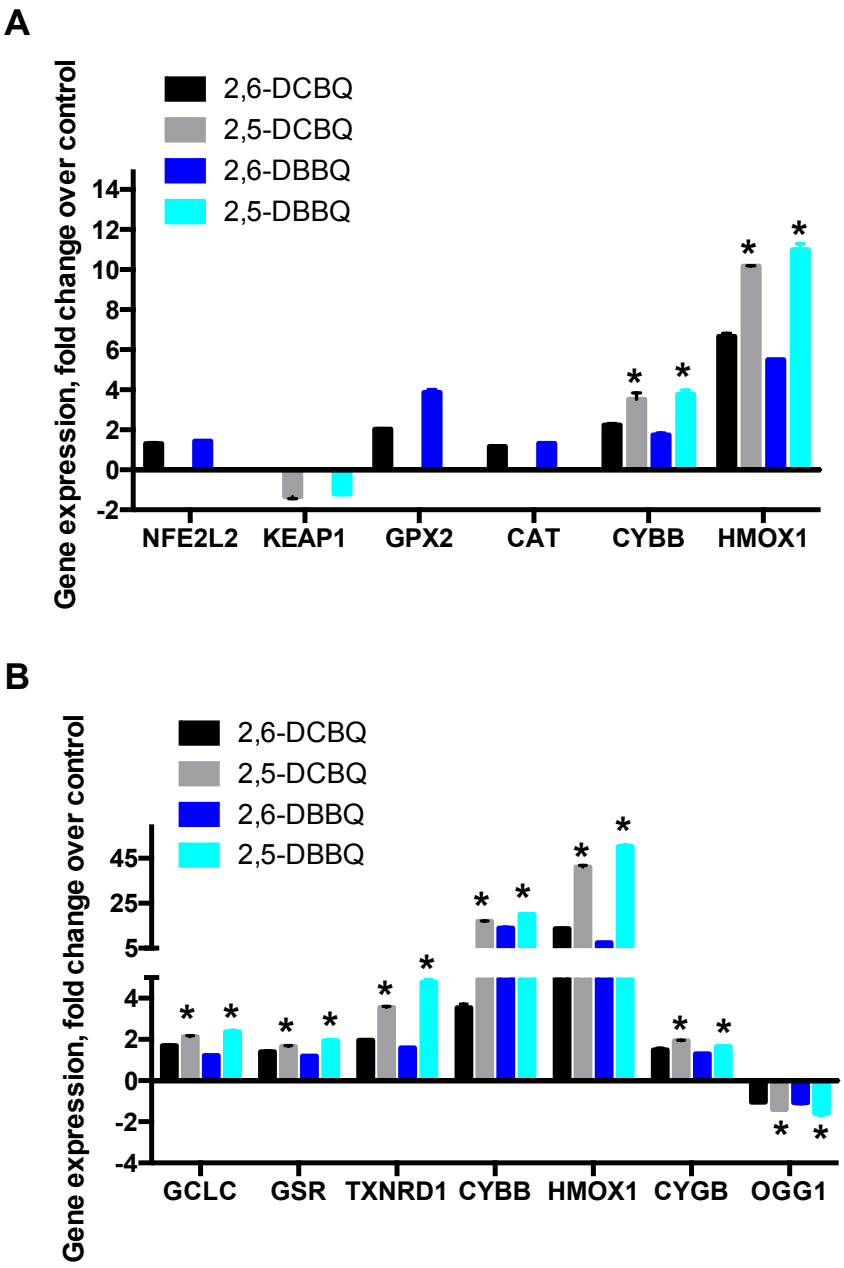


Figure 6.9 The comparison of significantly altered genes between HBQ isomers in SV-HUC-1 cells. (A) 2 h exposure and (B) 8 h exposure. Data of fold-changes in the expression are shown as mean ± SEM, n=2. *P < 0.05, 2,5-HBQ vs their corresponding 2,6-HBQ isomers.

Table 6.6 Canonical Pathways after HBQ exposure

	2 h		8 h	
	Canonical Pathways	P value	Canonical Pathways	P value
2,6-DCBQ	Nrf2-mediated oxidative stress response	2.93E-31	Nrf2-mediated oxidative stress response	3.15E-28
	Superoxide radicals degradation	1.04E-15	Glutathione redox reductions	5.03E-13
	Xenobiotic metabolism signaling	8.91E-14	Xenobiotic metabolism signaling	7.84E-13
	Glutathione redox reductions	9.89E-13	Superoxide radicals degradation	5.56E-10

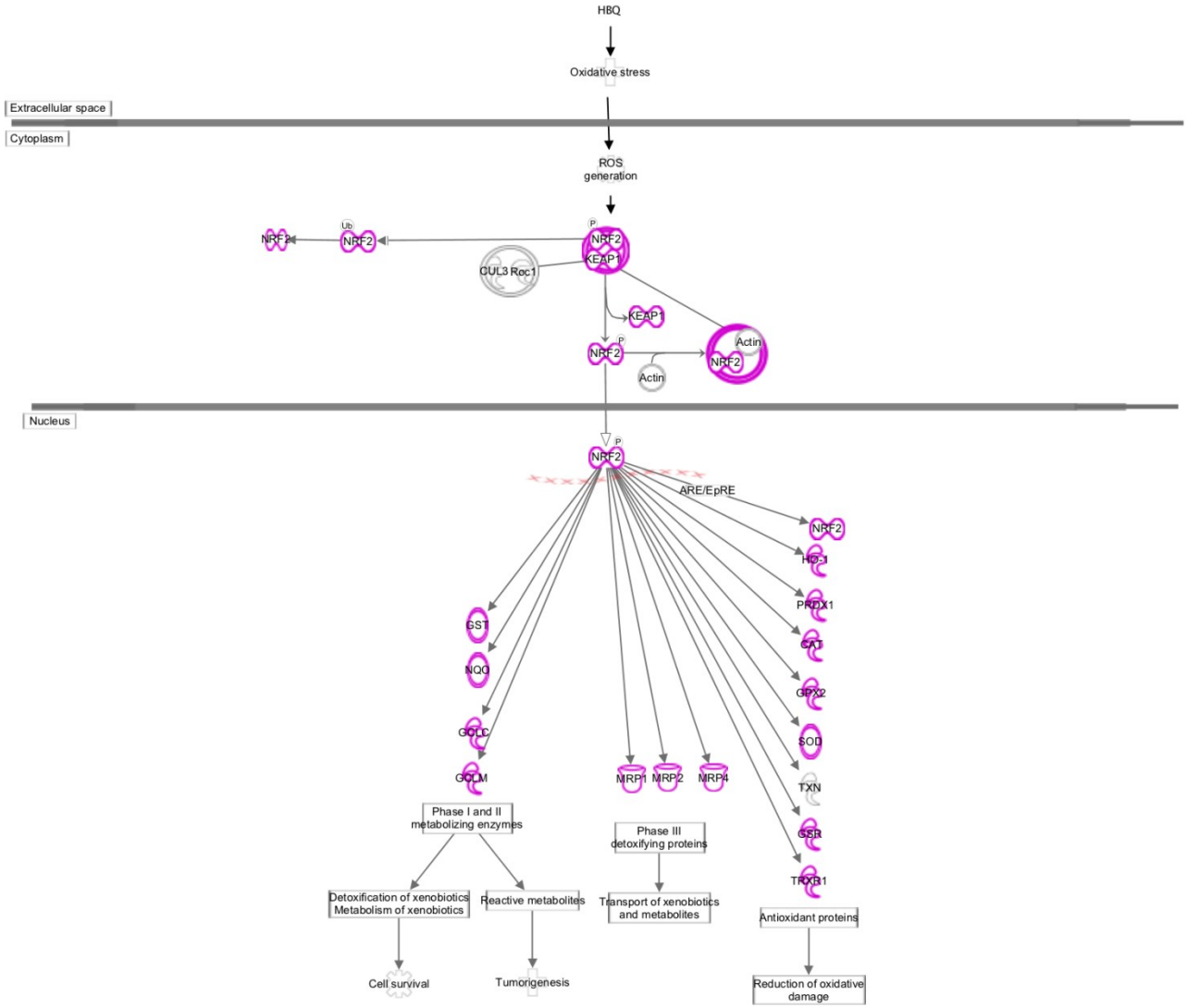


Figure 6.10 The typical Nrf2-mediated oxidative stress response pathway after HBQ treatment at 2 h or 8 h. The significantly altered genes are labeled in magenta.

Chapter 7 Conclusions and Future Work

7.1 Introduction

Drinking water disinfection prevents waterborne diseases and is a very successful public health measure.[1] However, disinfection byproducts (DBPs) are unintentionally formed during the disinfection processes. Many DBPs have been shown to be cytotoxic, mutagenic, genotoxic, and carcinogenic;[2] however, the effect of long-term exposure to low doses of these chemicals on humans is unknown. Epidemiological studies have only been able to consistently link consumption of chlorinated drinking water with an increased risk of developing bladder cancer,[3-4] but it is still unclear which DBP or DBP mixtures are responsible for this increased risk. What is clear is that the regulated DBPs such as trihalomethanes (THMs) and haloacetic acids (HAAs), which are also the highest proportion of identified DBPs, are not sufficiently potent carcinogens to be responsible for the observed risk of bladder cancer.[2] Because everyone who consumes municipal water is exposed to DBPs, the identification of DBPs of health relevance is an important research area addressing DBP health concerns.

Halobenzoquinones (HBQs) are a newly identified class of DBPs.[5-7] The quantitative structure–toxicity relationship (QSTR) analysis has predicted that HBQs are up to 1000 times more toxic than the regulated DBPs.[8] The available toxicological data of compounds with similar structure, such as benzoquinones or other halogenated quinones, indicate that HBQs are likely to be potent carcinogens.[9] However, little toxicological data are available for HBQs.

My thesis research aimed to fill the research gaps in the knowledge about HBQ DBPs, through examination of the cytotoxicity and oxidative damage induced by HBQs in human bladder cells (Chapter 2); investigation of the structure–toxicity relationship of HBQs in CHO cells (Chapter 3); investigation of the involvement of the cellular glutathione defense system in HBQ cytotoxicity (Chapter 4); investigation of the role of MRPs in protecting cells from HBQ cytotoxicity (Chapter 5); and identification of toxicity response genes and toxicity pathways influenced by HBQs in human bladder cells (Chapter 6). This chapter presents summaries of the major findings from my research (Chapters 2-6), conclusions based on my thesis work as a whole, and suggestions for future research directions.

7.2 Advancements in Knowledge

7.2.1 Determination of the cytotoxicity and oxidative damage induced by HBQs (Chapter 2)

Oxidative damage is believed to be one of the most important mechanisms of carcinogenicity of benzoquinone through the formation of reactive oxygen species (ROS) and/or depletion of cellular glutathione in the reduced state.[10-14] HBQs, as a separate class, have been observed to react with other chemicals to produce alkoxyl radicals and to interact with oligonucleotides in solution.[15-16] Therefore, I hypothesized that HBQs cause cytotoxicity via oxidative stress in T24 human bladder cancer cells. To test this hypothesis, I have examined the effects of HBQs on T24 cell viability with and without the addition of N-acetyl-L-cysteine (NAC) using the MTS assay after 24 h exposure. NAC is a thiol antioxidant that acts as a cysteine source in cells and a scavenger of free radicals by directly interacting with ROS.[17-18] The results clearly demonstrate that the presence of NAC significantly reduces the cytotoxic effects induced by HBQs. I also examined HBQ-induced ROS production in T24 cells after 24 h exposure, and measured 8-hydroxydeoxyguanosine (8-OHdG), protein carbonyls, and malondialdehyde (MDA) adducts of proteins, markers of oxidative damage to DNA, proteins, and lipids, respectively. All four HBQs (25 μM to 150 μM) generated significant intracellular ROS in T24 cells in a concentration-dependent manner. HBQs produced significant 8-OHdG in genomic DNA of T24 cells, with the highest levels of 8-OHdG (approximately 10-fold increase) induced by DCMBQ. The protein carbonyl levels in HBQ-treatment groups are significantly higher compared to their respective control groups. However, MDA-protein adducts (a marker of lipid oxidation) in T24 cells did not show any change when the cells were treated with each of the four HBQs at concentrations up to 125 μM. These results suggest the involvement of the ROS-induced oxidative damage to DNA and proteins in the observed toxicity of HBQs in T24 cells, supporting my initial hypothesis. This is the first time the cytotoxic effects of these four HBQ DBPs in human bladder cells were observed, and also the first time that oxidative stress as a potential mechanism underlying HBQ-induced cytotoxicity was demonstrated.

7.2.2 Investigation of the structure–toxicity relationship of HBQs (Chapter 3)

After showing the cytotoxicity and oxidative damage induced by HBQ DBPs, my second aim was to identify the structure–toxicity relationship of HBQs since HBQs are a structurally diverse

class of DBPs. The structure of a xenobiotic can heavily influence its reactivity and resulting biological/toxicological effects.[19] Several studies have shown that the halogen substitution groups on DBPs can significantly affect their toxicity, mutagenicity, and genotoxicity.[20-23] Here, I reported a systematic study of isomeric structure and the type and number of halogen substitutions of HBQs on their cytotoxicity, formation of ROS, and genotoxicity. Dynamic responses and IC_{50} histograms were obtained using real time cell analysis, clearly ranking the cytotoxicity of the HBQs in Chinese hamster ovary (CHO-K1) cells over 60 h. Strong isomeric structure effects were seen with 2,5-HBQ isomers inducing greater cytotoxicity than their corresponding 2,6-HBQ isomers ($P<0.05$). HBQ-halogen substitution groups also influence cytotoxicity, as cytotoxicity increases across the di-halogenated HBQs: iodo- > bromo- > chloro-HBQs ($P<0.05$). Determination of HBQ-induced ROS further supports isomeric structure and halogen substitution effects. HBQ-induced genotoxicity was seen as increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and p53 protein. Pearson correlation analysis of the HBQ toxicity measurements with their physicochemical properties demonstrates that dipole moment and the lowest unoccupied molecular orbital energy are two major structural descriptors ($r=-0.721$ or -0.766, $P < 0.05$). Dipole moment also strongly correlates with isomer toxicity. This study suggests that formation and occurrence of highly toxic iodo-HBQs and 2,5-HBQs warrants further investigation to fully assess the impact of HBQs in drinking water.

7.2.3 Investigation of the involvement of cellular glutathione defense system in HBQ-induced cytotoxicity (Chapter 4)

Based on the results from Chapter 2 and Chapter 3, I hypothesized that HBQs influence the endogenous antioxidant defense system as a part of the oxidative stress-induced cytotoxicity. Glutathione (GSH) and glutathione-related enzymes constitute the primary defense mechanism against the cytotoxicity of xenobiotics, especially against oxidative stress.[24-25] GSH serves as a detoxificant via either a conjugation reaction catalyzed by glutathione S-transferase (GST) or a reduction of hydrogen peroxide by glutathione peroxidase (GPx).[24] To test this hypothesis, I examined the cytotoxicity of HBQs to T24 cells when intracellular GSH was depleted and when GSH was supplemented in the culture media. When intracellular GSH was depleted by buthionine sulfoximine (BSO), an irreversible inhibitor of GSH synthesis, HBQ cytotoxicity was significantly enhanced. Compared with no treatment with BSO, the IC_{50} of HBQs significantly

decreased by 1.5–4.9-fold ($P<0.0001$). The supplementation of GSH in the culture media significantly reduces the cytotoxic effects of HBQs ($P<0.05$). Furthermore, I examined the effects of HBQs on cellular GSH levels and glutathione-related enzyme activities including GPx and GST. The reduction in cellular GSH levels is clearly dependent on the concentrations of HBQs ($P<0.05$). GST activity increases with the increase in HBQ concentrations. The effects of HBQs on GPx activity are compound-dependent; DCMBQ and 2,6-DBBQ decrease cellular GPx activities, whereas 2,6-DCBQ and TriCBQ have no significant effects at the concentrations tested. Pearson correlation analysis shows that the cellular GSH level is inversely correlated with ROS production and cellular GST activity in HBQ-treated cells. These results indicate that GST plays a more important role compared with GPx, which led me to propose a hypothesis that GSH depletion is mainly due to conjugation. To test this hypothesis, I examined HBQ-GSH conjugation using mass spectrometry analysis. The result suggested the conjugates consisted of one molecule of HBQ bound with one molecule of GSH. Taken together, these results support my hypothesis that GSH and GSH-related enzymes mediate the detoxification mechanism of HBQs in T24 cells.

7.2.4 Investigation of multidrug resistance protein (MRP)-mediated export of HBQs (Chapter 5)

My *in vitro* research in Chapter 2, Chapter 3, and Chapter 4 has focused on understanding the toxicodynamics of HBQs; however, the proteins responsible for the cellular export of DBPs for ultimate detoxification and elimination are unknown. This is significant, as transporter-mediated distribution of chemicals is an important factor driving the biological activity of several xenobiotics.[26] Human multidrug resistance proteins (MRPs) are an important class of transporters that play a critical role in the elimination of numerous drugs, carcinogens, toxicants, and their conjugated metabolites.[27-28] In this chapter, I used human embryonic kidney (HEK) 293 cells stably expressing selected MRPs to examine their role in cellular protection against HBQ-DBPs. By comparing the cytotoxicity of HBQs in HEK-MRP1, -MRP3, -MRP4, and -MRP5 cells with cells expressing empty vector alone (HEK-V), MRP4 was found to have the most significant effect on reducing the toxicity of HBQs. The protection of cells by MRP4 has been confirmed through the decrease of HBQ-induced cellular ROS levels in HEK-MRP4 cells compared with HEK-V cells. Furthermore, because of evidence that suggests HBQs readily

conjugate with GSH in solution,[29] I also examined whether MRP4-mediated HBQ detoxification is GSH-dependent. The results demonstrated an increased cytotoxicity in the GSH-depleted HEK-MRP4 cells, suggesting that a potential of MRP4 to export HBQ-GSH conjugates. This study demonstrates a role for MRP4 in protecting against HBQ-induced cytotoxicity and oxidative stress for the first time. The cell models used in this study could serve as a rapid method for testing of MRP protection against other DBPs.

7.2.5 Identification of Nrf2/ARE pathway activated by HBQs (Chapter 6)

HBQ DBPs have been demonstrated to have the ability to produce high levels of ROS and induce cellular oxidative DNA and protein damage, suggesting that HBQs are likely to be genotoxic and carcinogenic through DNA damage induced by oxidative stress (Chapters 2-5). However, the molecular basis behind this is still not clear. The Nrf2/ARE signal pathway is one of the most important mechanisms against oxidative stress.[30-31] Based on the results described above, I proposed that HBQs are able to influence the Nrf2/ARE antioxidant response pathway. To determine if this specific molecular pathway is involved, I used TaqMan PCR Array with a low-cytotoxic dose of HBQ at two different time points (2 h as early response time and 8 h as late response time) in human uroepithelial cells SV-HUC-1. This TaqMan PCR Array is a set of optimized real-time PCR primer assays on 96-well plates for pathway or disease focused genes as well as appropriate RNA quality controls. The major transcripts differentially up-regulated at both exposure times were HMOX1, followed by NQO1, PTGS2 and TXNRD1. The most influenced toxicity pathway was the Nrf2/ARE antioxidant response pathway. Other pathways such as cell cycle regulation, oxidative DNA damage/repair pathway, glutathione redox reductions, superoxide radical degradation, and xenobiotic metabolism signaling were also involved to protect cells against low cytotoxic dose of HBQs. The data also indicates that inflammation is caused in cells exposed to HBQs, and the thioredoxin system was involved in detoxification of HBQs. Our study provides an understanding of the molecular basis of human bladder cells exposure to low-cytotoxic level of HBQ DBPs.

7.3 Conclusions

HBQs are an emerging class of DBPs, and are predicted as bladder carcinogens more potent than the regulated DBPs. However, there is little existing toxicological data on HBQs. My research

has expanded this knowledge through new *in vitro* assays and toxicological results that contribute to the understanding of cytotoxic effects and the mechanisms of potential toxic effects of HBQs in *in vitro* systems for the first time. HBQs are cytotoxic to CHO cells, T24 human bladder cancer cells, and SV-HUC-1 human bladder cells. Oxidative damage is the main mechanism for HBQ-induced cytotoxicity, because HBQs produced high levels of cellular total ROS, weakened the GSH antioxidant system, and caused damage to cellular oxidative DNA and proteins. With toxicogenomic analysis, HBQs showed that they are able to activate the Nrf2/ARE antioxidant pathway, which further supports that oxidative stress is a major mechanism for HBQ-induced cytotoxicity. In addition, MRP4 played a role in the detoxification of HBQs, suggesting a possible mechanism for the elimination of HBQs from the human body. These studies contribute to the understanding of cytotoxicity, oxidative stress, antioxidant defense, possible elimination pathway, and major gene response pathway following human exposure to HBQ DBPs.

My research results contribute to a better understanding of the potential health effects of human exposure to HBQ DBPs, and provide useful information for the regulatory consideration of HBQ DBPs. In addition, my *in vitro* results could be used to design experiments for future *in vivo* testing, allowing for more efficient use of animals.

7.4 Perspectives

Toxicity of DBPs in drinking water is a challenging question. My research has demonstrated that HBQ DBPs are cytotoxic and genotoxic. It is critically important to have balanced assessment of the risks posed by water-borne pathogens and DBPs. Microbial pathogens pose acute and significantly greater risk to human health in comparison to DBPs. When drinking water is not properly disinfected, it can cause a public health emergency. The importance of proper drinking water disinfection is signified by the outbreak in Walkerton, Ontario, in May 2000. In this rural community of approximately 5000 people, seven people died and 2300 people became ill after consuming treated drinking water that was contaminated with enterohemorrhagic *E. coli* O157:H7.[32] This human health tragedy was a direct result of improper chlorination of a well that failed to kill the bacteria.

It is also important to consider the concentration ranges I chose to use for my HBQ DBP experiments. I tested concentrations (mg/L) that are much higher than the concentrations of HBQ

DBPs reported in actual finished water samples (ng/L). It was necessary to examine the higher concentration ranges in my *in vitro* assays because high concentrations were needed to evoke observable toxic effects. Furthermore, it is unknown whether chronic exposure to low doses of HBQs in drinking water would result in human health effects. Water treatment plants may optimize parameters to reduce the formation of HBQs to reduce human exposure to and minimize potential risk from HBQ DBPs.

On the basis of this evidence, it is abundantly clear that drinking water needs to be disinfected, as the acute human health risks from water-borne pathogens greatly outweigh the known risks presented by DBPs.

7.5 Future work

There are many potential directions for future studies arising from my research work. One direction is to test the toxicity of HBQ mixtures, since DBPs are a mixture of different classes of compounds. However, the composition of these HBQ mixtures is not constant, and may be influenced by source water, disinfectant, disinfection time, and pH. In addition, HBQs are not stable and they may transform to hydroxyl-HBQs (OH-HBQs) at neutral pH (such as in cell culture medium),[33] resulting in a mixture of HBQs and OH-HBQs. Therefore, my results may represent the mixture of HBQ and its transformed species. However, it is not clear how quickly this transformation occurs or to what degree HBQs transform either in culture medium or inside the cells. Although it is not possible to test HBQ DBPs as a mixture now, further testing with mixtures could be taken into consideration to better correlate with real environmental conditions.

Although I analyzed the quantitative structure–toxicity relationship of eight HBQs in Chapter 3, additional work could be performed to obtain higher power results. This study is limited by the availability of HBQ standards. For example, for the comparison of HBQ isomers, only two pairs of HBQ isomer standard compounds are commercially available. For the comparison among different halogen substitutions, I could only compare between 2,6-DCBQ/2,5-DCBQ, 2,6-DBBQ/2,5-DBBQ, and 2,3-DIBQ. The iodo-substitution of DIBQ is on a different position compared with DCBQ and DBBQ, because 2,3-DIBQ is the only available diiodo-HBQ. In the future, if more HBQ standard compounds are available, further testing could be performed. In addition, the physicochemical parameters were chosen according to the literature. In the future, more physicochemical parameters could be included to find other

potential structural predictors for HBQ toxicity. Also, I only included IC_{50}, ROS, 8-OHdG, and p53 as my toxicological parameters according to my previous results. Other toxicological experiments could be performed to establish a better model for the structure–toxicity relationship analysis.

Since I only explored the potential elimination of HBQs in Chapter 5, the absorption, disposition, and metabolism of HBQs may be examined in the future. With an *in vitro* cell system, the absorption and metabolism could be performed by analyzing the cell culture supernatant and cell lysates collected from HBQ-treated cultures. The complex of cell culture medium and cell lysates make the testing difficult. It may be accomplished with improved analytical techniques for the future identification and quantification of HBQs and their metabolites and transformation products. For the metabolism, the primary liver cells would be a good model to use instead of immortalized cell lines (such as HepG2 cells).

Although I explored the potential mechanisms of HBQ toxicity in this work, additional mechanistic investigations may be performed. As I found in Chapter 6, the p53 pathway, cell cycle pathway, DNA damage repair pathway, and inflammation were also involved in HBQ toxicity. Therefore, future work could be performed using PCR array to explore these pathways. In addition, the effects of HBQs on cell cycle progression could be examined with flow cytometry analysis to confirm whether HBQs are truly able to cause cell cycle arrest. For the DNA damage, more experiments could be performed in addition to PCR array. For example, the Ames assay could be performed to examine the mutagenic potential of HBQs, and the comet assay could be used to examine the genotoxic potential of HBQs. In addition to RNA, protein levels and ideally protein activity should be evaluated to confirm the finding, because if the RNA level increases but it is not translated to protein it really does not change anything. These results would be helpful to understand the association between DBPs and cancer risk.

7.6 References

1. White, G. C., *Handbook of Chlorination; for Potable Water, Wastewater, Cooling Water, Industrial Processes, and Swimming Pools*. Van Nostrand Reinhold Co.: New York,, 1972.

2. Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; Demarini, D. M., Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutat. Res.* **2007,** *636* (1-3), 178-242.

3. Villanueva, C. M.; Cantor, K. P.; Grimalt, J. O.; Malats, N.; Silverman, D.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Marcos, R.; Rothman, N.; Real, F. X.; Dosemeci, M.; Kogevinas, M., Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am. J. Epidemiol.* **2007,** *165* (2), 148-156.

4. Cantor, K. P.; Lynch, C. F.; Hildesheim, M. E.; Dosemeci, M.; Lubin, J.; Alavanja, M.; Craun, G., Drinking water source and chlorination byproducts I. Risk of bladder cancer. *Epidemiology* **1998,** *9* (1), 21-28.

5. Zhao, Y. L.; Qin, F.; Boyd, J. M.; Anichina, J.; Li, X.-F., Characterization and determination of chloro- and bromo-benzoquinones as new chlorination disinfection byproducts in drinking water. *Anal. Chem.* **2010,** *82* (11), 4599-4605.

6. Qin, F.; Zhao, Y. Y.; Zhao, Y. L.; Boyd, J. M.; Zhou, W. J.; Li, X.-F., A toxic disinfection by-product, 2,6-Dichloro-1,4-benzoquinone, identified in drinking water. *Angew. Chem. Int. Ed.* **2010,** *49* (4), 790-792.

7. Zhao, Y. L.; Anichina, J.; Lu, X. F.; Bull, R. J.; Krasner, S. W.; Hrudey, S. E.; Li, X. F., Li, X.-F., Occurrence and formation of chloro- and bromo-benzoquinones during drinking water disinfection. *Water Res.* **2012,** *46* (14), 4351-4360.

8. Bull, R. J., *Use of Toxicological and Chemical Models to Prioritize DBP Research*. AWWA Research Foundation: Denver, CO, 2006.

9. Li, J.; Wang, W.; Moe, B.; Wang, H.; Li, X. F., Chemical and toxicological characterization of halobenzoquinones, an emerging class of disinfection byproducts. *Chem. Res. Toxicol.* **2015,** *28* (3), 306-18.

10. O'Brien, P. J., Molecular mechanisms of quinone cytotoxicity. *Chem. Biol. Interact.* **1991,** *80* (1), 1-41.

11. Bolton, J. L.; Trush, M. A.; Penning, T. M.; Dryhurst, G.; Monks, T. J., Role of quinones in toxicology. *Chem. Res. Toxicol.* **2000,** *13* (3), 135-160.

12. Lin, P. H.; Waidyanatha, S.; Pollack, G. M.; Swenberg, J. A.; Rappaport, S. M., Dose-specific production of chlorinated quinone and semiquinone adducts in rodent livers following administration of pentachlorophenol. *Toxicol. Sci.* **1999,** *47* (1), 126-133.

13. Jeong, J. K.; Wogan, G. N.; Lan, S. S.; Monks, T. J., Quinol-glutathione conjugate-induced mutation spectra in the supF gene replicated in human AD293 cells and bacterial MBL50 cells. *Cancer Res.* **1999,** *59* (15), 3641-3645.

14. Siraki, A. G.; Chan, T. S.; O'Brien, P. J., Application of quantitative structure-toxicity relationships for the comparison of the cytotoxicity of 14 p-benzoquinone congeners in primary cultured rat hepatocytes versus PC12 cells. *Toxicol. Sci.* **2004,** *81* (1), 148-159.

15. Anichina, J.; Zhao, Y.; Hrudey, S. E.; Le, X. C.; Li, X.-F., Electrospray ionization mass spectrometry characterization of interactions of newly identified water disinfection byproducts halobenzoquinones with oligodeoxynucleotides. *Environ. Sci. Technol.* **2010,** *44* (24), 9557-63.

16. Zhu, B. Z.; Zhao, H. T.; Kalyanaraman, B.; Liu, J.; Shan, G. Q.; Du, Y. G.; Frei, B., Mechanism of metal-independent decomposition of organic hydroperoxides and formation of alkoxyl radicals by halogenated quinones. *Proc. Natl. Acad. Sci. USA*. **2007,** *104* (10), 3698-3702.

17. Aruoma, O. I.; Halliwell, B.; Hoey, B. M.; Butler, J., The antioxidant action of N-acetylcysteine - its reaction with hydrogen-peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic. Bio. Med.* **1989,** *6* (6), 593-597.

18. Bannai, S., Induction of cystine and glutamate transport activity in human-fibroblasts by diethyl maleate and other electrophilic agents. *J. Biol. Chem.* **1984,** *259* (4), 2435-2440.

19. Casarett, L. J.; Doull, J.; Klaassen, C. D., *Casarett and Doull's Toxicology: the Basic Science of Poisons*. 7th ed.; McGraw-Hill: New York, 2008.

20. Plewa, M. J.; Wagner, E. D.; Richardson, S. D.; Thruston, A. D.; Woo, Y. T.; McKague, A. B., Chemical and biological characterization of newly discovered lodoacid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2004,** *38* (18), 4713-4722.

21. Kargalioglu, Y.; McMillan, B. J.; Minear, R. A.; Plewa, M. J., Analysis of the cytotoxicity and mutagenicity of drinking water disinfection by-products in Salmonella typhimurium. *Teratogen. Carcin. Mutat.* **2002,** *22* (2), 113-128.

22. Liu, J.; Zhang, X., Comparative toxicity of new halophenolic DBPs in chlorinated saline wastewater effluents against a marine alga: halophenolic DBPs are generally more toxic than haloaliphatic ones. *Water Res.* **2014,** *65*, 64-72.
23. Pals, J.; Attene-Ramos, M. S.; Xia, M. H.; Wagner, E. D.; Plewa, M. J Human cell toxicogenomic analysis linking reactive oxygen species to the toxicity of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2013,** *47* (21), 12514-12523.
24. Hayes, J. D.; McLellan, L. I., Glutathione and glutathione-dependent enzymes represent a Co-ordinately regulated defence against oxidative stress. *Free Radic. Res.* **1999,** *31* (4), 273-300.
25. Ehrlich, K.; Viirlaid, S.; Mahlapuu, R.; Saar, K.; Kullisaar, T.; Zilmer, M.; Langel, U.; Soomets, U., Design, synthesis and properties of novel powerful antioxidants, glutathione analogues. *Free Radic. Res.* **2007,** *41* (7), 779-787.
26. Klaassen, C. D.; Lu, H., Xenobiotic transporters: ascribing function from gene knockout and mutation studies. *Toxicol. Sci.* **2008,** *101* (2), 186-96.
27. Leslie, E. M.; Deeley, R. G.; Cole, S. P. C., Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol. Appl. Pharmacol.* **2005,** *204* (3), 216-237.
28. Deeley, R. G.; Westlake, C.; Cole, S. P. C., Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. *Physiol. Rev.* **2006,** *86* (3), 849-899.
29. Li, J.; Wang, W.; Zhang, H.; Le, X. C.; Li, X.-F., Glutathione-mediated detoxification of halobenzoquinone drinking water disinfection byproducts in T24 cells. *Toxicol. Sci.* **2014,** *141* (2), 335-343.
30. Jennings, P.; Limonciel, A.; Felice, L.; Leonard, M. O., An overview of transcriptional regulation in response to toxicological insult. *Arch Toxicol* **2013,** *87* (1), 49-72.
31. Ma, Q., Role of Nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* **2013,** *53*, 401-26.
32. Hrudey, S. E.; Payment, P.; Huck, P. M.; Gillham, R. W.; Hrudey, E. J., A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water. Sci. Technol*. **2003**, 47 (3), 7-14.
33. Wang, W.; Qian, Y. C.; Li, J. H.; Moe, B.; Huang, R. F.; Zhang, H. Q.; Hrudey, S. E.; Li, X.-F., Analytical and toxicity characterization of halo-hydroxyl-benzoquinones as stable

halobenzoquinone disinfection byproducts in treated water. *Anal. Chem.* **2014,** *86* (10), 4982-4988.

Bibliography

Aardema, M. J.; MacGregor, J. T., Toxicology and genetic toxicology in the new era of "toxicogenomics": impact of "-omics" technologies. *Mutat. Res. Fund. Mol. Mech. Mutagen.* **2002,** *499* (1), 13-25.

Achanta, G.; Huang, P., Role of p53 in sensing oxidative DNA damage in response to reactive oxygen species-generating agents. *Cancer. Res.* **2004,** *64* (17), 6233-9.

Ahmed, A. E.; Jacob, S.; Campbell, G. A.; Harirah, H. M.; Perez-Polo, J. R.; K, M. J., Fetal origin of adverse pregnancy outcome: the water disinfectant by-product chloroacetonitrile induces oxidative stress and apoptosis in mouse fetal brain. *Brain Res. Dev. Brain Res.* **2005,** *159* (1), 1-11.

Akin, E. W.; Hoff, J. C.; Lippy, E. C., Waterborne outbreak control - which disinfectant. *Environ. Health. Perspect.* **1982,** *46*, 7-12.

American Water Works Association., *Water Quality and Treatment: a handbook of community water supplies*. 4th ed.; McGraw-Hill: New York, 1990.

Ames, B. N.; Mccann, J.; Yamasaki, E., Methods for detecting carcinogens and mutagens with *salmonella*-mammalian-microsome mutagenicity test. *Mutat. Res.* **1975,** *31* (6), 347-363.

Anichina, J.; Zhao, Y. L.; Hrudey, S. E.; Le, X. C.; Li, X. F., Li, X.-F., Electrospray ionization mass spectrometry characterization of interactions of newly identified water disinfection byproducts halobenzoquinones with oligodeoxynucleotides. *Environ. Sci. Technol.* **2010,** *44* (24), 9557-9563.

Anichina, J.; Zhao, Y. L.; Hrudey, S. E.; Schreiber, A.; Li, X.-F., Electrospray ionization tandem mass spectrometry analysis of the reactivity of structurally related bromo-methyl-benzoquinones toward oligonucleotides. *Anal. Chem.* **2011,** *83* (21), 8145-8151.

Arikawa, E.; Sun, Y.; Wang, J.; Zhou, Q.; Ning, B.; Dial, S. L.; Guo, L.; Yang, J., Cross-platform comparison of SYBR Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. *BMC. Genomics* **2008,** *9*, 328.

Arthur, J. R., The glutathione peroxidases. *Cell. Mol. Life Sci.* **2000,** *57* (13-14), 1825-35.

Aruoma, O. I.; Halliwell, B.; Hoey, B. M.; Butler, J., The antioxidant action of N-acetylcysteine - its reaction with hydrogen-peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic. Bio. Med.* **1989,** *6* (6), 593-597.

Attene-Ramos, M. S.; Wagner, E. D.; Plewa, M. J., Comparative human cell toxicogenomic analysis of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2010,** *44* (19), 7206-7212.

Baigi, M. G.; Brault, L.; Neguesque, A.; Beley, M.; El Hilali, R.; Gauzere, F.; Bagrel, D., Apoptosis/necrosis switch in two different cancer cell lines: Influence of benzoquinone- and hydrogen peroxide-induced oxidative stress intensity, and glutathione. *Toxicol. In Vitro* **2008,** *22* (6), 1547-1554.

Baird, L.; Dinkova-Kostova, A. T., The cytoprotective role of the Keap1-Nrf2 pathway. *Arch. Toxicol.* **2011,** *85* (4), 241-272.

Balasubramanian, B.; Pogozelski, W. K.; Tullius, T. D., DNA strand breaking by the hydroxyl radical is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone. *Proc. Natl. Acad. Sci. USA.* **1998,** *95* (17), 9738-43.

Banerjee, M.; Carew, M. W.; Roggenbeck, B. A.; Whitlock, B. D.; Naranmandura, H.; Le, X. C.; Leslie, E. M., A novel pathway for arsenic elimination: human multidrug resistance protein 4 (MRP4/ABCC4) mediates cellular export of dimethylarsinic acid (DMAV) and the diglutathione conjugate of monomethylarsonous acid (MMAIII). *Mol. Pharmacol.* **2014,** *86* (2), 168-79.

Banfi, C.; Brioschi, M.; Barcella, S.; Veglia, F.; Biglioli, P.; Tremoli, E.; Agostoni, P., Oxidized proteins in plasma of patients with heart failure: role in endothelial damage. *Eur. J. Heart Fail.* **2008,** *10* (3), 244-51.

Bannai, S., Induction of cystine and glutamate transport activity in human-fibroblasts by diethyl maleate and other electrophilic agents. *J. Biol. Chem.* **1984,** *259* (4), 2435-2440.

Bellar, T. A.; Lichtenberg, J. J.; Kroner, R. C., Occurrence of organohalides in chlorinated drinking waters. *J. Am. Water Works Assoc.* **1974,** *66* (12), 703-706.

Berlett, B. S.; Stadtman, E. R., Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **1997,** *272* (33), 20313-6.

Biswas, S. K.; Rahman, I., Environmental toxicity, redox signaling and lung inflammation: The role of glutathione. *Mol. Aspects. Med.* **2009,** *30* (1-2), 60-76.

Boiteux, S.; Radicella, J. P., The human OGG1 gene: structure, functions, and its implication in the process of carcinogenesis. *Arch. Biochem. Biophys.* **2000,** *377* (1), 1-8.
Bolton, J. L.; Dunlap, T., Formation and biological targets of quinones: cytotoxic versus cytoprotective effects. *Chem. Res. Toxicol.* **2017,** *30* (1), 13-37.
Bolton, J. L.; Trush, M. A.; Penning, T. M.; Dryhurst, G.; Monks, T. J., Role of quinones in toxicology. *Chem. Res. Toxicol.* **2000,** *13* (3), 135-160.
Boonstra, J.; Post, J. A., Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. *Gene* **2004,** *337*, 1-13.
Boorman, G. A.; Dellarco, V.; Dunnick, J. K.; Chapin, R. E.; Hunter, S.; Hauchman, F.; Gardner, H.; Cox, M.; Sills, R. C., Drinking water disinfection byproducts: Review and approach to toxicity evaluation. *Environ. Health Perspec.* **1999,** *107*, 207-217.
Boyd, J. M.; Huang, L.; Xie, L.; Moe, B.; Gabos, S.; Li, X.-F., A cell-microelectronic sensing technique for profiling cytotoxicity of chemicals. *Anal. Chim. Acta.* **2008,** *615* (1), 80-87.
Brigelius-Flohe, R.; Kipp, A., Glutathione peroxidases in different stages of carcinogenesis. *Biochim. Biophys. Acta* **2009,** *1790* (11), 1555-68.
Brooks, C. L.; Gu, W., p53 ubiquitination: Mdm2 and beyond. *Mol. Cell* **2006,** *21* (3), 307-15.
Bull, R. J., *Use of Toxicological and Chemical Models to Prioritize DBP Research*. AWWA Research Foundation: Denver, CO, 2006.
Bull, R. J.; Birnbaum, L. S.; Cantor, K. P.; Rose, J. B.; Butterworth, B. E.; Pegram, R.; Tuomisto, J., Water chlorination: essential process or cancer hazard? *Fund. Appl. Toxicol.* **1995,** *28* (2), 155-166.
Bull, R. J.; Reckhow, D. A.; Li, X.; Humpage, A. R.; Joll, C.; Hrudey, S. E., Potential carcinogenic hazards of non-regulated disinfection by-products: haloquinones, halo-cyclopentene and cyclohexene derivatives, N-halamines, halonitriles, and heterocyclic amines. *Toxicology* **2011,** *286* (1-3), 1-19.
Burmester, T.; Ebner, B.; Weich, B.; Hankeln, T., Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. *Mol. Biol. Evol.* **2002,** *19* (4), 416-421.
Buttke, T. M.; Mccubrey, J. A.; Owen, T. C., Use of an aqueous soluble tetrazolium formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. *J. Immunol. Methods* **1993,** *157* (1-2), 233-240.

Byun, S. S.; Kim, S. W.; Choi, H.; Lee, C.; Lee, E., Augmentation of cisplatin sensitivity in cisplatin-resistant human bladder cancer cells by modulating glutathione concentrations and glutathione-related enzyme activities. *Bju. Int.* **2005,** *95* (7), 1086-90.

Cantor, K. P.; Lynch, C. F.; Hildesheim, M. E.; Dosemeci, M.; Lubin, J.; Alavanja, M.; Craun, G., Drinking water source and chlorination byproducts I. Risk of bladder cancer. *Epidemiology* **1998,** *9* (1), 21-28.

Cantor, K. P.; Villanueva, C. M.; Silverman, D. T.; Figueroa, J. D.; Real, F. X.; Garcia-Closas, M.; Malats, N.; Chanock, S.; Yeager, M.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Samanic, C.; Rothman, N.; Kogevinas, M., Polymorphisms in GSTT1, GSTZ1, and CYP2E1, disinfection by-products, and risk of bladder cancer in Spain. *Environ. Health Perspec.* **2010,** *118* (11), 1545-1550.

Cao, N.; Miao, T.; Li, K.; Zhang, Y.; Yang, M., Formation potentials of typical disinfection byproducts and changes of genotoxicity for chlorinated tertiary effluent pretreated by ozone. *J. Environ. Sci.* **2009,** *21* (4), 409-13.

Carew, M. W.; Naranmandura, H.; Shukalek, C. B.; Le, X. C.; Leslie, E. M., Monomethylarsenic diglutathione transport by the human multidrug resistance protein 1 (MRP1/ABCC1). *Drug Metab. Dispos.* **2011,** *39* (12), 2298-2304.

Casarett, L. J.; Doull, J.; Klaassen, C. D., *Casarett and Doull's Toxicology: the Basic Science of Poisons*. 7th ed.; McGraw-Hill: New York, 2008.

Centers for Disease Control and Prevention. *Control of infectious diseases*; Morbidity and Mortality Weekly Reports: 48(29): 621-629, 1999.

Chan, K.; Jensen, N.; O'Brien, P. J., Structure-activity relationships for thiol reactivity and rat or human hepatocyte toxicity induced by substituted p-benzoquinone compounds. *J. Appl. Toxicol.* **2008,** *28* (5), 608-620.

Chan, K.; Jensen, N. S.; Silber, P. A.; O'Brien, P. J., Structure-activity relationships for halobenzene induced cytotoxicity in rat and human hepatoctyes. *Chem-Biol. Interact.* **2007,** *165* (3), 165-174.

Chang, M.; Xue, J.; Sharma, V.; Habtezion, A., Protective role of hemeoxygenase-1 in gastrointestinal diseases. *Cell. Mol. Life Sci.* **2015,** *72* (6), 1161-73.

Chen, W.; Sun, Z.; Wang, X. J.; Jiang, T.; Huang, Z.; Fang, D.; Zhang, D. D., Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Mol. Cell* **2009,** *34* (6), 663-73.

Cheng, K. C.; Cahill, D. S.; Kasai, H.; Nishimura, S.; Loeb, L. A., 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G-T and A-C substitutions. *J. Biol. Chem.* **1992,** *267* (1), 166-72.

Chua, P. J.; Yip, G. W. C.; Bay, B. H., C Cell cycle arrest induced by hydrogen peroxide is associated with modulation of oxidative stress related genes in breast cancer cells. *Exp. Biol. Med.* **2009,** *234* (9), 1086-1094.

Col, C.; Dinler, K.; Hasdemir, O.; Buyukasik, O.; Bugdayci, G., Oxidative stress and lipid peroxidation products: effect of pinealectomy or exogenous melatonin injections on biomarkers of tissue damage during acute pancreatitis. *Hepatobiliary Pancreat. Dis. Int.* **2010,** *9* (1), 78-82.

Cole, S. P., Targeting multidrug resistance protein 1 (MRP1, ABCC1): past, present, and future. *Annu. Rev. Pharmacol. Toxicol.* **2014,** *54*, 95-117.

Conseil, G.; Cole, S. P. C., Two polymorphic variants of ABCC1 selectively alter drug resistance and inhibitor sensitivity of the multidrug and organic anion transporter multidrug resistance protein 1. *Drug Metab. Dispos.* **2013,** *41* (12), 2187-2196.

Cooke, M. S.; Evans, M. D.; Dizdaroglu, M.; Lunec, J., Oxidative DNA damage: mechanisms, mutation, and disease. *Faseb J.* **2003,** *17* (10), 1195-1214.

Cronin, M. T. D.; Livingstone, D., *Predicting Chemical Toxicity and Fate*. CRC Press: Boca Raton, Fla., 2004.

Dahlhaus, M.; Almstadt, E.; Henschke, P.; Luttgert, S.; Appel, K. E., Oxidative DNA lesions in V79 cells mediated by pentachlorophenol metabolites. *Arch. Toxicol.* **1996,** *70* (7), 457-460.

Dalle-Donne, I.; Rossi, R.; Giustarini, D.; Milzani, A.; Colombo, R., Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta.* **2003,** *329* (1-2), 23-38.

DeAngelo, A. B.; Daniel, F. B.; Most, B. M.; Olson, G. R., Failure of monochloroacetic acid and trichloroacetic acid administered in the drinking water to produce liver cancer in male F344/N rats. *J. Toxicol. Environ. Health* **1997,** *52* (5), 425-45.

Deeley, R. G.; Westlake, C.; Cole, S. P. C., Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. *Physiol. Rev.* **2006,** *86* (3), 849-899.

Deferme, L.; Briede, J. J.; Claessen, S. M. H.; Jennen, D. G. J.; Cavil, R.; Kleinjans, J. C. S., Time series analysis of oxidative stress response patterns in HepG2: A toxicogenomics approach. *Toxicology* **2013,** *306*, 24-34.

Delker, D.; Hatch, G.; Allen, J.; Crissman, B.; George, M.; Geter, D.; Kilburn, S.; Moore, T.; Nelson, G.; Roop, B.; Slade, R.; Swank, A.; Ward, W.; DeAngelo, A., Molecular biomarkers of oxidative stress associated with bromate carcinogenicity. *Toxicology* **2006,** *221* (2-3), 158-165.

DeMarini, D. M.; Abu-Shakra, A.; Felton, C. F.; Patterson, K. S.; Shelton, M. L., Mutation spectra in salmonella of chlorinated, chloraminated, or ozonated drinking water extracts: comparison to MX. *Environ. Mol. Mutagen.* **1995,** *26* (4), 270-85.

Deneke, S. M.; Fanburg, B. L., Regulation of cellular glutathione. *Am. J. Physiol.* **1989,** *257* (4), L163-L173.

Devasagayam, T. P.; Boloor, K. K.; Ramasarma, T., Methods for estimating lipid peroxidation: an analysis of merits and demerits. *Indian J. Biochem. Biophys.* **2003,** *40* (5), 300-8.

Dierickx, P. J., Invitro Binding of 3,4,5,6-Tetrachloro-1,2-Benzoquinone by Rat-Liver Glutathione S-Transferases. *Res. Commun. Chem. Path.* **1983,** *41* (3), 517-520.

Dong, H.; Su, C. Y.; Xia, X. M.; Li, L. R.; Song, E. Q.; Song, Y., Polychlorinated biphenyl quinone-induced genotoxicity, oxidative DNA damage and gamma-H2AX formation in HepG2 cells. *Chem-Biol. Interact.* **2014,** *212*, 47-55.

Du, H. Y.; Li, J. H.; Moe, B.; McGuigan, C. F.; Li, X.-F., A real-time cell-electronic sensing method for comparative analysis of toxicity of water contaminants. *Anal. Methods* **2013,** *6*, 2053-2058.

Du, H. Y.; Li, J. H.; Moe, B.; McGuigan, C. F.; Shen, S. W.; Li, X.-F., Cytotoxicity and oxidative damage induced by halobenzoquinones to T24 bladder cancer cells. *Environ. Sci. Technol.* **2013,** *47* (6), 2823-2830.

Duirk, S. E.; Lindell, C.; Cornelison, C. C.; Kormos, J.; Ternes, T. A.; Attene-Ramos, M.; Osiol, J.; Wagner, E. D.; Plewa, M. J.; Richardson, S. D., Formation of toxic iodinated disinfection by-products from compounds used in medical imaging. *Environ. Sci. Technol.* **2011,** *45* (16), 6845-6854.

Dutto, I.; Tillhon, M.; Cazzalini, O.; Stivala, L. A.; Prosperi, E., Biology of the cell cycle inhibitor p21(CDKN1A): molecular mechanisms and relevance in chemical toxicology. *Arch. Toxicol.* **2015,** *89* (2), 155-178.

Eaton, D. L.; Bammler, T. K., Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol. Sci.* **1999,** *49* (2), 156-164.

Echigo, S.; Itoh, S.; Natsui, T.; Araki, T.; Ando, R., Contribution of brominated organic disinfection by-products to the mutagenicity of drinking water. *Water Sci. Technol.* **2004,** *50* (5), 321-8.

Ehrlich, K.; Viirlaid, S.; Mahlapuu, R.; Saar, K.; Kullisaar, T.; Zilmer, M.; Langel, U.; Soomets, U., Design, synthesis and properties of novel powerful antioxidants, glutathione analogues. *Free Radic. Res.* **2007,** *41* (7), 779-787.

Evans, M. D.; Dizdaroglu, M.; Cooke, M. S., Oxidative DNA damage and disease: induction, repair and significance. *Mutat. Res-Rev. Mutat.* **2004,** *567* (1), 1-61.

Fu, K. Z. L., J.; Vemula, S.; Moe, B.; Li, X.-F., Effects of halobenzoquinone and haloacetic acid water disinfection byproducts on human neural stem cells. *J. Environ. Sci.* **2017**.

Fukuzumi, S., Ishikawa, M., and Tanaka, T., Acid-catalysed reduction of p-benzoquinone derivatives by an NADH analogue, 9,10-dihydro-10-methylacridine: the energetic comparison of one-electron vs. two-electron Pathways. *J. Chem. Soc. Perkin Trans.* **1989**, *2,* 1811-1816.

Gaskell, M.; McLuckie, K. I. E.; Farmer, P. B., Comparison of the repair of DNA damage induced by the benzene metabolites hydroquinone and p-benzoquinone: a role for hydroquinone in benzene genotoxicity. *Carcinogenesis* **2005,** *26* (3), 673-680.

Giller, S.; LeCurieux, F.; Gauthier, L.; Erb, F.; Marzin, D., Genotoxicity assay of chloral hydrate and chloropicrine. *Mutat. Res. Lett.* **1995,** *348* (4), 147-152.

Gillies, R. J.; Didier, N.; Denton, M., Determination of cell number in monolayer-cultures. *Anal. Biochem.* **1986,** *159* (1), 109-113.

Gorrini, C.; Harris, I. S.; Mak, T. W., Modulation of oxidative stress as an anticancer strategy. *Nat. Rev. Drug Discov*. **2013,** *12* (12), 931-947.

Grellier, J.; Bennett, J.; Patelarou, E.; Smith, R. B.; Toledano, M. B.; Rushton, L.; Briggs, D. J.; Nieuwenhuijsen, M. J., Exposure to disinfection by-products, fetal growth, and prematurity a systematic review and meta-analysis. *Epidemiology* **2010,** *21* (3), 300-313.

Griffith, O. W., Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic. Bio. Med.* **1999,** *27* (9-10), 922-935.

Grivennikov, S. I.; Greten, F. R.; Karin, M., Immunity, inflammation, and cancer. *Cell* **2010,** *140* (6), 883-899.

Gunter, S.; Haupter, S.; Schutz, P.; Kreis, P., Comparative evaluation of the genotoxic properties of potassium bromate and potassium superoxide in V79 Chinese hamster cells. *Mutat. Res-Gen. Tox. En.* **1999,** *439* (2), 213-221.

Hagen, T. M.; Brown, L. A.; Jones, D. P., Protection against paraquat-induced injury by exogenous gsh in pulmonary alveolar type-II Cells. *Biochem. Pharmacol.* **1986,** *35* (24), 4537-4542.

Hansch, C.; Leo, A.; Hoekman, D. H., *Exploring QSAR*. American Chemical Society: Washington, DC, 1995.

Harrington-Brock, K.; Doerr, C. L.; Moore, M. M., Mutagenicity of three disinfection by-products: di- and trichloroacetic acid and chloral hydrate in L5178Y/TK+/- -3.7.2C mouse lymphoma cells. *Mutat. Res-Gen. Tox. En.* **1998,** *413* (3), 265-276.

Haslam, G.; Wyatt, D.; Kitos, P. A., Estimating the number of viable animal cells in multi-well cultures based on their lactate dehydrogenase activities. *Cytotechnology* **2000,** *32* (1), 63-75.

Hayes, J. D.; McLellan, L. I., Glutathione and glutathione-dependent enzymes represent a Co-ordinately regulated defence against oxidative stress. *Free Radic. Res.* **1999,** *31* (4), 273-300.

Health Canada (2014). Guidelines for Canadian Drinking Water Quality—Summary Table. Water and Air Quality Bureau, Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa, Ontario.

Hildesheim, M. E.; Cantor, K. P.; Lynch, C. F.; Dosemeci, M.; Lubin, J.; Alavanja, M.; Craun, G., Drinking water source and chlorination byproducts II. Risk of colon and rectal cancers. *Epidemiology* **1998,** *9* (1), 29-35.

Horn, H. F.; Vousden, K. H., Coping with stress: multiple ways to activate p53. *Oncogene* **2007,** *26* (9), 1306-16.

Hrudey, S. E.; Payment, P.; Huck, P. M.; Gillham, R. W.; Hrudey, E. J., A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water. Sci. Technol*. **2003**, 47 (3), 7-14.

Hrudey, S. E., Chlorination disinfection by-products, public health risk tradeoffs and me. *Water Res.* **2009,** *43* (8), 2057-2092.

Hrudey, S. E., *Epidemiological Inference and Evidence on DBPs and Human Health*. IWA Publishing: London, U.K., 2012.

Hrudey, S. E.; Backer, L. C.; Humpage, A. R.; Krasner, S. W.; Michaud, D. S.; Moore, L. E.; Singer, P. C.; Stanford, B. D., Evaluating evidence for association of human bladder cancer with drinking-water chlorination disinfection by-products. *Journal Toxicol. Environ. Health. Part B, Crit. Rev.* **2015,** *18* (5), 213-241.

Hua, G. H.; Reckhow, D. A., Comparison of disinfection byproduct formation from chlorine and alternative disinfectants. *Water Res.* **2007,** *41* (8), 1667-1678.

Huang, L.; Xie, L.; Boyd, J. M.; Li, X. F., Cell-electronic sensing of particle-induced cellular responses. *Analyst* **2008,** *133* (5), 643-648.

Hughes, T. B.; Miller, G. P.; Swamidass, S. J., Site of reactivity models predict molecular reactivity of diverse chemicals with glutathione. *Chem. Res. Toxicol.* **2015,** *28* (4), 797-809.

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. List of classsifications, volumes 1-117. International Agency for Research on Cancer. http://monographs.iarc.fr/ENG/Classification/latest_classif.php (accessed January 20, 2017).

IARC *Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Chemicals that Cause Tumours of the Kidney or Urinary Bladder in Rodents and Some Other Substances*; International Agency for Research on Cancer: Lyon, France, 1999.

Iida, K.; Itoh, K.; Kumagai, Y.; Oyasu, R.; Hattori, K.; Kawai, K.; Shimazui, T.; Akaza, H.; Yamamoto, M., Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Res.* **2004,** *64* (18), 6424-31.

Itoh, K.; Mochizuki, M.; Ishii, Y.; Ishii, T.; Shibata, T.; Kawamoto, Y.; Kelly, V.; Sekizawa, K.; Uchida, K.; Yamamoto, M., Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-Delta(12,14)-prostaglandin j(2). *Mol. Cell Biol.* **2004,** *24* (1), 36-45.

Jennings, P.; Limonciel, A.; Felice, L.; Leonard, M. O., An overview of transcriptional regulation in response to toxicological insult. *Arch. Toxicol.* **2013,** *87* (1), 49-72.

Jeong, C. H.; Postigo, C.; Richardson, S. D.; Simmons, J. E.; Kimura, S. Y.; Marinas, B. J.; Barcelo, D.; Liang, P.; Wagner, E. D.; Plewa, M. J., Occurrence and comparative toxicity of haloacetaldehyde disinfection byproducts in drinking water. *Environ. Sci. Technol.* **2015**, *49*, 13749−13759.

Jeong, C. H.; Richardson, S. D.; Marinas, B. J.; Wagner, E. D.; Plewa, M. J., Mammalian cell cytotoxicity and genotoxicity of haloacetaldehyde drinking water disinfection byproducts. *Environ. Mol. Mutagen* **2010,** *51* (7), 726.

Jeong, C. H.; Wagner, E. D.; Siebert, V. R.; Anduri, S.; Richardson, S. D.; Daiber, E. J.; McKague, A. B.; Kogevinas, M.; Villanueva, C. M.; Goslan, E. H.; Luo, W. T.; Isabelle, L. M.; Pankow, J. F.; Grazuleviciene, R.; Cordier, S.; Edwards, S. C.; Righi, E.; Nieuwenhuijsen, M. J.; Plewa, M. J., Occurrence and toxicity of disinfection byproducts in European drinking waters in relation with the HIWATE epidemiology study. *Environ. Sci. Technol.* **2012,** *46* (21), 12120-12128.

Jeong, J. K.; Wogan, G. N.; Lau, S. S.; Monks, T. J., Quinol-glutathione conjugate-induced mutation spectra in the supF gene replicated in human AD293 cells and bacterial MBL50 cells. *Cancer Res.* **1999,** *59* (15), 3641-5.

Jia, S. P.; Zhu, B. Z.; Guo, L. H., Detection and mechanistic investigation of halogenated benzoquinone induced DNA damage by photoelectrochemical DNA sensor. *Anal. Bioanal. Chem.* **2010,** *397* (6), 2395-2400.

Jiang, J.; Yuan, X.; Zhao, H.; Yan, X.; Sun, X.; Zheng, Q., Licochalcone A inhibiting proliferation of bladder cancer T24 cells by inducing reactive oxygen species production. *Biomed. Mater. Eng.* **2014,** *24* (1), 1019-25.

Jin, Y. P.; Zhao, F. H.; Zhong, Y. A.; Yu, X. Y.; Sun, D.; Liao, Y. J.; Lv, X. Q.; Li, G. X.; Sun, G. F., Effects of exogenous GSH and methionine on methylation of inorganic arsenic in mice exposed to arsenite through drinking water. *Environ. Toxicol.* **2010,** *25* (4), 361-366.

Karelson, M.; Lobanov, V. S.; Katritzky, A. R., Quantum-chemical descriptors in QSAR/QSPR studies. *Chem. Rev.* **1996,** *96* (3), 1027-1043.

Kargalioglu, Y.; McMillan, B. J.; Minear, R. A.; Plewa, M. J., Analysis of the cytotoxicity and mutagenicity of drinking water disinfection by-products in Salmonella typhimurium. *Teratogen. Carcin. Mutat.* **2002,** *22* (2), 113-128.

Keppler, D., Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy. *Handb. Exp. Pharmacol.* **2011,** *201*, 299-323.

Kimura, S. Y.; Vu, T. N.; Komaki, Y.; Plewa, M. J.; Marinas, B. J., Acetonitrile and N-chloroacetamide formation from the reaction of acetaldehyde and monochloramine. *Environ. Sci. Technol.* **2015,** *49* (16), 9954-63.

King, W. D.; Marrett, L. D., Case-control study of bladder cancer and chlorination by-products in treated water (Ontario, Canada). *Cancer Cause Control* **1996,** *7* (6), 596-604.

Klaassen, C. D.; Lu, H., Xenobiotic transporters: ascribing function from gene knockout and mutation studies. *Toxicol Sci.* **2008,** *101* (2), 186-96.
Klaunig, J. E.; Kamendulis, L. M.; Hocevar, B. A., Oxidative stress and oxidative damage in carcinogenesis. *Toxicol. Pathol.* **2010,** *38* (1), 96-109.
Kobayashi, M.; Li, L.; Iwamoto, N.; Nakajima-Takagi, Y.; Kaneko, H.; Nakayama, Y.; Eguchi, M.; Wada, Y.; Kumagai, Y.; Yamamoto, M., The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. *Mol. Cell Biol.* **2009,** *29* (2), 493-502.
Komhoff, M.; Guan, Y.; Shappell, H. W.; Davis, L.; Jack, G.; Shyr, Y.; Koch, M. O.; Shappell, S. B.; Breyer, M. D., Enhanced expression of cyclooxygenase-2 in high grade human transitional cell bladder carcinomas. *Am. J. Pathol.* **2000,** *157* (1), 29-35.
Kopfler, F. C.; Melton, R. G.; Lingg, R. D.; Coleman, W. E., GC/MS determination of volatiles for the national organics reconnaissance survey (NORS) of drinking water, In: Identification and Analysis of Organic Pollutants in Water, Keith, L.H., (Ed.), Ann Arbor Science, Ann Arbor, MI. **1976**, 87-104.
Krasner, S. W., The formation and control of emerging disinfection by-products of health concern. *Philos. Trans. A. Math. Phys. Eng. Sci.* **2009,** *367* (1904), 4077-95.
Krasner, S. W.; Mcguire, M. J.; Jacangelo, J. G.; Patania, N. L.; Reagan, K. M.; Aieta, E. M., The Occurrence of disinfection by-products in United States drinking water. *J. Am. Water Works Assoc.* **1989,** *81* (8), 41-53.
Krasner, S. W.; Weinberg, H. S.; Richardson, S. D.; Pastor, S. J.; Chinn, R.; Sclimenti, M. J.; Onstad, G. D.; Thruston, A. D., Occurrence of a new generation of disinfection byproducts. *Environ. Sci. Technol.* **2006,** *40* (23), 7175-7185.
Krasner, S. W.; Weinberg, H. S.; Richardson, S. D.; Pastor, S. J.; Chinn, R.; Sclimenti, M. J.; Onstad, G. D.; Thruston, A. D., Jr., Occurrence of a new generation of disinfection byproducts. *Environ. Sci. Technol.* **2006,** *40* (23), 7175-85.
Krewski, D.; Westphal, M.; Andersen, M. E.; Paoli, G. M.; Chiu, W. A.; Al-Zoughool, M.; Croteau, M. C.; Burgoon, L. D.; Cote, I., A framework for the next generation of risk science. *Environ. Health Perspec.* **2014,** *122* (8), 796-805.
Kryston, T. B.; Georgiev, A. B.; Pissis, P.; Georgakilas, A. G., Role of oxidative stress and DNA damage in human carcinogenesis. *Mutat. Res.* **2011,** *711* (1-2), 193-201.

Kubinyi, H., *QSAR : Hansch Analysis and Related Approaches*. VCH: Weinheim ; New York, 1993.

Kulinskii, V. I.; Kolesnichenko, L. S., Glutathione system. I. Synthesis, transport, glutathione transferases, glutathione peroxidases. *Biomed Khim.* **2009,** *55* (3), 255-77.

Kundu, B.; Richardson, S. D.; Granville, C. A.; Shaughnessy, D. T.; Hanley, N. M.; Swartz, P. D.; Richard, A. M.; DeMarini, D. M., Comparative mutagenicity of halomethanes and halonitromethanes in Salmonella TA100: structure-activity analysis and mutation spectra. *Mutat. Res. Fund. Mol Mech. Mutagen.* **2004,** *554* (1-2), 335-350.

Kurokawa, Y.; Takayama, S.; Konishi, Y.; Hiasa, Y.; Asahina, S.; Takahashi, M.; Maekawa, A.; Hayashi, Y., Long-term in vivo carcinogenicity tests of potassium bromate, sodium hypochlorite, and sodium chlorite conducted in Japan. *Environ. Health Perspec.* **1986,** *69*, 221-35.

Laingam, S.; Froscio, S. M.; Bull, R. J.; Humpage, A. R., In vitro toxicity and genotoxicity assessment of disinfection by-products, organic N-chloramines. *Environ. Mol. Mutagen.* **2012,** *53* (2), 83-93.

Laingam, S.; Froscio, S. M.; Humpage, A. R., Flow-cytometric analysis of in vitro micronucleus formation: comparative studies with WIL2-NS human lymphoblastoid and L5178Y mouse lymphoma cell lines. *Mutat. Res.* **2008,** *656* (1-2), 19-26.

Lalezary, S.; Pirbazari, M.; Mcguire, M. J., Oxidation of 5 earthy musty taste and odor compounds. *J. Am. Water Works Assoc.* **1986,** *78* (3), 62-69.

Lash, L. H.; Hagen, T. M.; Jones, D. P., Exogenous glutathione protects intestinal epithelial-cells from oxidative injury. *Proc. Natl. Acad. Sci. USA.* **1986,** *83* (13), 4641-4645.

Lecurieux, F.; Marzin, D.; Erb, F., Study of the genotoxic activity of 5 chlorinated propanones using the Sos chromotest, the Ames-fluctuation test and the newt micronucleus test. *Mutat. Res. Genet. Toxicol.* **1994,** *341* (1), 1-15.

Leslie, E. M., Arsenic-glutathione conjugate transport by the human multidrug resistance proteins (MRPs/ABCCs). *J. Inorg. Biochem.* **2012,** *108*, 141-149.

Leslie, E. M.; Deeley, R. G.; Cole, S. P. C., Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol. Appl. Pharmacol.* **2005,** *204* (3), 216-237.

Levine, A. J., p53, the cellular gatekeeper for growth and division. *Cell* **1997,** *88* (3), 323-31.

Li, J.; Moe, B.; Vemula, S.; Wang, W.; Li, X.-F., Emerging disinfection byproducts, halobenzoquinones: effects of isomeric structure and halogen substitution on cytotoxicity, formation of reactive oxygen species, and genotoxicity. *Environ. Sci. Technol.* **2016,** *50* (13), 6744-52.

Li, J.; Wang, W.; Moe, B.; Wang, H.; Li, X.-F., Chemical and toxicological characterization of halobenzoquinones, an emerging class of disinfection byproducts. *Chem. Res. Toxicol.* **2015,** *28* (3), 306-18.

Li, J.; Wang, W.; Zhang, H.; Le, X. C.; Li, X.-F., Glutathione-mediated detoxification of halobenzoquinone drinking water disinfection byproducts in T24 cells. *Toxicol. Sci.* **2014,** *141* (2), 335-343.

Li, L.; Dong, H.; Song, E.; Xu, X.; Liu, L.; Song, Y., Nrf2/ARE pathway activation, HO-1 and NQO1 induction by polychlorinated biphenyl quinone is associated with reactive oxygen species and PI3K/AKT signaling. *Chem. Biol. Interact.* **2014,** *209*, 56-67.

Li, N.; Alam, J.; Venkatesan, M. I.; Eiguren-Fernandez, A.; Schmitz, D.; Di Stefano, E.; Slaughter, N.; Killeen, E.; Wang, X. R.; Huang, A.; Wang, M. Y.; Miguel, A. H.; Cho, A.; Sioutas, C.; Nel, A. E., Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells: protecting against the proinflammatory and oxidizing effects of diesel exhaust chemicals. *J. Immunol.* **2004,** *173* (5), 3467-3481.

Li, S. J.; Yan, T.; Yang, J. Q.; Oberley, T. D.; Oberley, L. W., The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. *Cancer Res.* **2000,** *60* (14), 3927-3939.

Lin, P. H.; La, D. K.; Upton, P. B.; Swenberg, J. A., Analysis of DNA adducts in rats exposed to pentachlorophenol. *Carcinogenesis* **2002,** *23* (2), 365-9.

Lin, P. H.; Nakamura, J.; Yamaguchi, S.; La, D. K.; Upton, P. B.; Swenberg, J. A., Induction of direct adducts, apurinic/apyrimidinic sites and oxidized bases in nuclear DNA of human HeLa S3 tumor cells by tetrachlorohydroquinone. *Carcinogenesis* **2001,** *22* (4), 635-9.

Lin, P. H.; Nakamura, J.; Yamaguchi, S.; Upton, P. B.; La, D. K.; Swenberg, J. A., Oxidative damage and direct adducts in calf thymus DNA induced by the pentachlorophenol metabolites, tetrachlorohydroquinone and tetrachloro-1,4-benzoquinone. *Carcinogenesis* **2001,** *22* (4), 627-34.

Lin, P. H.; Waidyanatha, S.; Pollack, G. M.; Swenberg, J. A.; Rappaport, S. M., Dose-specific production of chlorinated quinone and semiquinone adducts in rodent livers following administration of pentachlorophenol. *Toxicol. Sci.* **1999,** *47* (1), 126-133.
Liu, J.; Song, E. Q.; Liu, L. C.; Ma, X. Y.; Tian, X. G.; Dong, H.; Song, Y., Polychlorinated biphenyl quinone metabolites lead to oxidative stress in HepG2 cells and the protective role of dihydrolipoic acid. *Toxicol. In Vitro* **2012,** *26* (6), 841-848.
Liu, J.; Zhang, X., Comparative toxicity of new halophenolic DBPs in chlorinated saline wastewater effluents against a marine alga: halophenolic DBPs are generally more toxic than haloaliphatic ones. *Water Res.* **2014,** *65*, 64-72.
Liviac, D.; Creus, A.; Marcos, R., Genotoxicity analysis of two halonitromethanes, a novel group of disinfection by-products (DBPs), in human cells treated in vitro. *Environ. Res.* **2009,** *109* (3), 232-8.
Liviac, D.; Creus, A.; Marcos, R., Genotoxicity testing of three monohaloacetic acids in TK6 cells using the cytokinesis-block micronucleus assay. *Mutagenesis* **2010,** *25* (5), 505-9.
LoPachin, R. M.; Gavin, T.; DeCaprio, A.; Barber, D. S., Application of the hard and soft, acids and bases (HSAB) theory to toxicant-target interactions. *Chem. Res. Toxicol.* **2012,** *25* (2), 239-251.
Lu, J.; Holmgren, A., The thioredoxin antioxidant system. *Free Radic. Biol. Med.* **2014,** *66*, 75-87.
Luan, Y.; Suzuki, T.; Palanisamy, R.; Takashima, Y.; Sakamoto, H.; Sakuraba, M.; Koizumi, T.; Saito, M.; Matsufuji, H.; Yamagata, K.; Yamaguchi, T.; Hayashi, M.; Honma, M., Potassium bromate treatment predominantly causes large deletions, but not GC>TA transversion in human cells. *Mutat. Res.* **2007,** *619* (1-2), 113-23.
Luo, L. H.; Jiang, L. P.; Geng, C. Y.; Cao, J.; Zhong, L. F., Hydroquinone-induced genotoxicity and oxidative DNA damage in HepG2 cells. *Chem-Biol. Interact.* **2008,** *173* (1), 1-8.
Ma, Q., Role of nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* **2013,** *53*, 401-26.
Maccoll, A., Reduction potentials of conjugated systems. *Nature* **1949,** *163*, 178-179.
Massimi, I.; Ciuffetta, A.; Temperilli, F.; Ferrandino, F.; Zicari, A.; Pulcinelli, F. M.; Felli, M. P., Multidrug resistance protein-4 influences aspirin toxicity in human cell line. *Mediat. Inflamm.* **2015,** *2015*, 607957.

Mcgeehin, M. A.; Reif, J. S.; Becher, J. C.; Mangione, E. J., Case-control study of bladder cancer and water disinfection methods in Colorado. *Am. J. Epidemiol.* **1993,** *138* (7), 492-501.
McGuigan, C. F.; Li, X. F., Cytotoxicity and genotoxicity of phenazine in two human cell lines. *Toxicol. In Vitro.* **2014,** *28* (4), 607-615.
McGuigan, C. F. L., X.-F.; Le, X.C., Cytotoxic and mutagenic effects of haloquinone drinking water disinfection byproducts. *The Toxicologist, Supplement to Toxicological Sciences* **2012,** *126* (1), 450.
Meier, J. R.; Ringhand, H. P.; Coleman, W. E.; Munch, J. W.; Streicher, R. P.; Kaylor, W. H.; Schenck, K. M., Identification of mutagenic compounds formed during chlorination of humic acid. *Mutat. Res.* **1985,** *157* (2-3), 111-122.
Meister, A.; Anderson, M. E., Glutathione. *Annu. Rev. Biochem.* **1983,** *52*, 711-760.
Melnick, R. L.; Nyska, A.; Foster, P. M.; Roycroft, J. H.; Kissling, G. E., Toxicity and carcinogenicity of the water disinfection byproduct, dibromoacetic acid, in rats and mice. *Toxicology* **2007,** *230* (2-3), 126-136.
Mendez-Hernandez, D. D.; Tarakeshwar, P.; Gust, D.; Moore, T. A.; Moore, A. L.; Mujica, V., Simple and accurate correlation of experimental redox potentials and DFT-calculated HOMO/LUMO energies of polycyclic aromatic hydrocarbons. *J. Mol. Model.* **2013,** *19* (7), 2845-8.
Merrick, B. A.; Smallwood, C. L.; Meier, J. R.; Mckean, D. L.; Kaylor, W. H.; Condie, L. W., Chemical-reactivity, cytotoxicity, and mutagenicity of chloropropanones. *Toxicol. Appl. Pharmacol.* **1987,** *91* (1), 46-54.
Michaud, D. S., Chronic inflammation and bladder cancer. *Urol. Oncol.* **2007,** *25* (3), 260-8.
Moe, B.; Gabos, S.; Li, X.-F., Real-time cell-microelectronic sensing of nanoparticle-induced cytotoxic effects. *Anal. Chim. Acta.* **2013,** *789*, 83-90.
Moe, B., McGuigan, C. F., Li, X.-F., Dabek-Zlotorzynska, E. and Gabos, S, Cell-electronic sensing of cellular responses to micro- and nanoparticles for environmental applications. *Encyclopedia of Analytical Chemistry* **2014**, 1-28.
Monks, T. J.; Hanzlik, R. P.; Cohen, G. M.; Ross, D.; Graham, D. G., Quinone chemistry and toxicity. *Toxicol. Appl. Pharmacol.* **1992,** *112* (1), 2-16.

Muellner, M. G.; Attene-Ramos, M. S.; Hudson, M. E.; Wagner, E. D.; Plewa, M. J., Human cell toxicogenomic analysis of bromoacetic acid: a regulated drinking water disinfection by-product. *Environ. Mol. Mutagen.* **2010,** *51* (3), 205-214.

Mustacich, D.; Powis, G., Thioredoxin reductase. *Biochem. J.* **2000,** *346*, 1-8.

Nakamura, J.; La, D. K.; Swenberg, J. A., 5'-nicked apurinic/apyrimidinic sites are resistant to beta-elimination by beta-polymerase and are persistent in human cultured cells after oxidative stress. *J. Biol. Chem.* **2000,** *275* (8), 5323-8.

Nakamura, Y.; Kumagai, T.; Yoshida, C.; Naito, Y.; Miyamoto, M.; Ohigashi, H.; Osawa, T.; Uchida, K., Pivotal role of electrophilicity in glutathione S-transferase induction by tert-butylhydroquinone. *Biochem. Us.* **2003,** *42* (14), 4300-4309.

National Cancer Institute. *Report on Carcinogenesis Bioassay of Chloroform*; Carcinogenesis Program, Division of Cancer Cause and Prevention: Bethesda, MD, 1976.

Nguyen, T.; Yang, C. S.; Pickett, C. B., The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. *Free Radic. Bio. Med.* **2004,** *37* (4), 433-441.

Nguyen, T. N.; Bertagnolli, A. D.; Villalta, P. W.; Buhlmann, P.; Sturla, S. J., Characterization of a deoxyguanosine adduct of tetrachlorobenzoquinone: dichlorobenzoquinone-1,N2-etheno-2'-deoxyguanosine. *Chem. Res. Toxicol.* **2005,** *18* (11), 1770-6.

Nieuwenhuijsen, M. J.; Grellier, J.; Smith, R.; Iszatt, N.; Bennett, J.; Best, N.; Toledano, M., The epidemiology and possible mechanisms of disinfection by-products in drinking water. *Philos. T. R. Soc. A.* **2009,** *367* (1904), 4043-4076.

Nieuwenhuijsen, M. J.; Toledano, M. B.; Elliott, P., Uptake of chlorination disinfection by-products; a review and a discussion of its implications for exposure assessment in epidemiological studies. *J. Expo. Anal. Env. Epid.* **2000,** *10* (6), 586-599.

Noot, D. K.; Anderson, W. B.; Daignault, S. A.; Williams, D. T.; Huck, P. M., Evaluating Treatment Processes with the Ames Mutagenicity Assay. *J. Am. Water Works Ass.* **1989,** *81* (9), 87-102.

O'Brien, P. J., Molecular mechanisms of quinone cytotoxicity. *Chem. Biol. Interact.* **1991,** *80* (1), 1-41.

Orrenius, S.; Gogvadze, V.; Zhivotovsky, B., Mitochondrial oxidative stress: implications for cell death. *Annu. Rev. Pharmacol. Toxicol.* **2007,** *47*, 143-83.

Pals, J.; Attene-Ramos, M. S.; Xia, M. H.; Wagner, E. D.; Plewa, M. J., Human cell toxicogenomic analysis linking reactive oxygen species to the toxicity of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2013,** *47* (21), 12514-12523.

Pals, J. A.; Wagner, E. D.; Plewa, M. J., Energy of the lowest unoccupied molecular orbital, thiol reactivity, and toxicity of three monobrominated water disinfection byproducts. *Environ. Sci. Technol.* **2016,** *50* (6), 3215-3221.

Pandey, A. K.; Gurbani, D.; Bajpayee, M.; Parmar, D.; Ajmani, S.; Dhawana, A., In silico studies with human DNA topoisomerase-II alpha to unravel the mechanism of in vitro genotoxicity of benzene and its metabolites. *Mutat. Res-Fund. Mol. M.* **2009,** *661* (1-2), 57-70.

Patterson, K. S.; Lykins, B. W.; Richardson, S. D., Mutagenicity of drinking-water following disinfection. *J Water Supply Res. T.* **1995,** *44* (1), 1-9.

Perry, G.; Raina, A. K.; Nunomura, A.; Wataya, T.; Sayre, L. M.; Smith, M. A., How important is oxidative damage? Lessons from Alzheimer's disease. *Free Radic. Bio. Med.* **2000,** *28* (5), 831-834.

Plewa, M. J., and Wagner, E.D., *Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-products*. Water Research Foundation: Denver, CO, 2009.

Plewa, M. J.; Kargalioglu, Y.; Vankerk, D.; Minear, R. A.; Wagner, E. D., Development of a quantitative comparative cytotoxicity and genotoxicity assays for environmental hazardous chemicals. *Water Sci. Technol.* **2000,** *42* (7-8), 109-116.

Plewa, M. J.; Kargalioglu, Y.; Vankerk, D.; Minear, R. A.; Wagner, E. D., Mammalian cell cytotoxicity and genotoxicity analysis of drinking water disinfection by-products. *Environ. Mol. Mutagen.* **2002,** *40* (2), 134-142.

Plewa, M. J.; Muellner, M. G.; Richardson, S. D.; Fasanot, F.; Buettner, K. M.; Woo, Y. T.; Mckague, A. B.; Wagner, E. D., Occurrence, synthesis, and mammalian cell cytotoxicity and genotoxicity of haloacetamides: An emerging class of nitrogenous drinking water disinfection byproducts. *Environ. Sci. Technol.* **2008,** *42* (3), 955-961.

Plewa, M. J.; Simmons, J. E.; Richardson, S. D.; Wagner, E. D., Mammalian cell cytotoxicity and genotoxicity of the haloacetic acids, a major class of drinking water disinfection by-products. *Environ. Mol. Mutagen.* **2010,** *51* (8-9), 871-878.

Plewa, M. J.; Wagner, E. D.; Jazwierska, P.; Richardson, S. D.; Chen, P. H.; McKague, A. B., Halonitromethane drinking water disinfection byproducts: chemical characterization and mammalian cell cytotoxicity and genotoxicity. *Environ. Sci. Technol.* **2004,** *38* (1), 62-68.
Plewa, M. J.; Wagner, E. D.; Metz, D. H.; Kashinkunti, R.; Jamriska, K. J.; Meyer, M., Differential toxicity of drinking water disinfected with combinations of ultraviolet radiation and chlorine. *Environ. Sci. Technol.* **2012,** *46* (14), 7811-7817.
Plewa, M. J.; Wagner, E. D.; Mitch, W. A., Comparative mammalian cell cytotoxicity of water concentrates from disinfected recreational pools. *Environ. Sci. Technol.* **2011,** *45* (9), 4159-4165.
Plewa, M. J.; Wagner, E. D.; Richardson, S. D.; Thruston, A. D.; Woo, Y. T.; McKague, A. B., Chemical and biological characterization of newly discovered lodoacid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2004,** *38* (18), 4713-4722.
Plewa, M. J., Wagner, E.D., *Mammalian Cell Cytotoxicity and Genotoxicity of DBPs*. Water Research Foundation: Denver, CO, 2009.
Postigo, C.; Jeong, C. H.; Richardson, S. D.; Wagner, E. D.; Plewa, M. J.; Simmons, J. E.; Barcelo, D., Analysis, Occurrence, and Toxicity of Haloacetaldehydes in Drinking Waters: Iodoacetaldehyde as an Emerging Disinfection By-Product. *Recent Advances in Disinfection by-Products* **2015,** *1190*, 25-43.
Prins, J. M.; Fu, L.; Guo, L.; Wang, Y., Cd(2+)-induced alteration of the global proteome of human skin fibroblast cells. *J. Proteome. Res.* **2014,** *13* (3), 1677-87.
Prochazka, E.; Escher, B. I.; Plewa, M. J.; Leusch, F. D., n vitro cytotoxicity and adaptive stress responses to selected haloacetic acid and halobenzoquinone water disinfection byproducts. *Chem. Res. Toxicol.* **2015,** *28* (10), 2059-68.
Qin, F.; Zhao, Y. Y.; Zhao, Y. L.; Boyd, J. M.; Zhou, W. J.; Li, X.-F., A toxic disinfection by-product, 2,6-Dichloro-1,4-benzoquinone, identified in drinking water. *Angew. Chem. Int. Ed.* **2010,** *49* (4), 790-792.
Rahman, M. B.; Driscoll, T.; Cowie, C.; Armstrong, B. K., Disinfection by-products in drinking water and colorectal cancer: a meta-analysis. *Int. J. Epidemiol.* **2010,** *39* (3), 733-745.
Ravanat, J. L., Measuring Oxidized DNA lesions as biomarkers of oxidative stress: an analytical challenge. *FABAD J. Pharm. Sci.* **2005,** *30*, 100-113.
Richardson, S. D., *Disinfection By-Products: Formation and Occurrence in Drinking Water.* Elsevier: Burlington, MA, 2011; Vol. 2.

Richardson, S. D.; Fasano, F.; Ellington, J. J.; Crumley, F. G.; Buettner, K. M.; Evans, J. J.; Blount, B. C.; Silva, L. K.; Waite, T. J.; Luther, G. W.; McKague, A. B.; Miltner, R. J.; Wagner, E. D.; Plewa, M. J., Occurrence and mammalian cell toxicity of iodinated disinfection byproducts in drinking water. *Environ. Sci. Technol.* **2008,** *42* (22), 8330-8338.

Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; Demarini, D. M., Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutat. Res.* **2007,** *636* (1-3), 178-242.

Richardson, S. D.; Ternes, T. A., Water analysis: emerging contaminants and current issues. *Anal. Chem.* **2011,** *83* (12), 4614-4648.

Richardson, S. D.; Ternes, T. A., Water analysis: emerging contaminants and current issues. Anal. Chem. ***2014***, 86 (6), 2813-48.

Rius, M.; Nies, A. T.; Hummel-Eisenbeiss, J.; Jedlitschky, G.; Keppler, D., Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane. *Hepatology* **2003,** *38* (2), 374-384.

Rius, M.; Hummel-Eisenbeiss, J.; Hofmann, A. F.; Keppler, D., Substrate specificity of human ABCC4 (MRP4)-mediated cotransport of bile acids and reduced glutathione. *Am. J. Physiol. Gastrointest Liver Physiol.* **2006,** *290*, (4), G640-9.

Roggenbeck, B. A.; Banerjee, M.; Leslie, E. M., Cellular arsenic transport pathways in mammals. *J. Environ. Sci.* **2016,** *49*, 38-58.

Rook, J. J., Formation of haloforms during chlorination of natural waters. *Water Treat. Exam.* **1974,** *23*, 234-243.

Rossi, L.; Moore, G. A.; Orrenius, S.; Obrien, P. J., Quinone toxicity in hepatocytes without oxidative stress. *Arch. Biochem. Biophys.* **1986,** *251* (1), 25-35.

Rubio, V.; Zhang, J.; Valverde, M.; Rojas, E.; Shi, Z. Z., Essential role of Nrf2 in protection against hydroquinone- and benzoquinone-induced cytotoxicity. *Toxicol. In Vitro* **2011,** *25* (2), 521-529.

Russel, F. G.; Koenderink, J. B.; Masereeuw, R., Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules. *Trends Pharmacol. Sci.* **2008,** *29* (4), 200-7.

Schafer, F. Q.; Buettner, G. R., Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Bio. Med.* **2001,** *30* (11), 1191-1212.

Schmidt, M. M.; Dringen, R., Differential effects of iodoacetamide and iodoacetate on glycolysis and glutathione metabolism of cultured astrocytes. *Front. Neuroenergetics.* **2009,** *1*, 1.
Schuliga, M.; Chouchane, S.; Snow, E. T., Upregulation of glutathione-related genes and enzyme activities in cultured human cells by sublethal concentrations of inorganic arsenic. *Toxicol. Sci.* **2002,** *70* (2), 183-192.
Seibert, K.; Zhang, Y.; Leahy, K.; Hauser, S.; Masferrer, J.; Perkins, W.; Lee, L.; Isakson, P., Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc. Natl. Acad. Sci. USA.* **1994,** *91* (25), 12013-7.
Shariat, S. F.; Matsumoto, K.; Kim, J.; Ayala, G. E.; Zhou, J. H.; Jian, W.; Benedict, W. F.; Lerner, S., Correlation of cyclooxygenase-2 expression with molecular markers, pathological features and clinical outcome of transitional cell carcinoma of the bladder. *J. Urol.* **2003,** *170* (3), 985-9.
Shi, M. M.; Kugelman, A.; Iwamoto, T.; Tian, L.; Forman, H. J., Quinone-induced oxidative stress elevates glutathione and induces gamma-glutamylcysteine synthetase activity in rat lung epithelial L2 cells. *J. Biol. Chem.* **1994,** *269* (42), 26512-7.
Siraki, A. G.; Chan, T. S.; O'Brien, P. J., Application of quantitative structure-toxicity relationships for the comparison of the cytotoxicity of 14 p-benzoquinone congeners in primary cultured rat hepatocytes versus PC12 cells. *Toxicol. Sci.* **2004,** *81* (1), 148-159.
Smith, A. R.; Shenvi, S. V.; Widlansky, M.; Suh, J. H.; Hagen, T. M., Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress. *Curr. Med. Chem.* **2004,** *11* (9), 1135-1146.
Smith, M. T., Benzene, NQO1, and genetic susceptibility to cancer. *Proc. Natl. Acad. Sci. USA.* **1999,** *96* (14), 7624-7626.
Snyder, C. A.; Sellakumar, A. R.; James, D. J.; Albert, R. E., The carcinogenicity of discontinuous inhaled benzene exposures in Cd-1 and C57b1/6 mice. *Arch. Toxicol.* **1988,** *62* (5), 331-335.
Song, Y.; Wagner, B. A.; Witmer, J. R.; Lehmler, H. J.; Buettner, G. R., Nonenzymatic displacement of chlorine and formation of free radicals upon the reaction of glutathione with PCB quinones. *Proc. Natl. Acad. Sci. USA.* **2009,** *106* (24), 9725-9730.
Sood, C.; O'Brien, P. J., 2-Chloroacetaldehyde-induced cerebral glutathione depletion and neurotoxicity. *Br. J. Cancer. Suppl.* **1996,** *27*, S287-93.

Stalter, D.; O'Malley, E.; von Gunten, U.; Escher, B. I., Fingerprinting the reactive toxicity pathways of 50 drinking water disinfection by-products. *Water Res.* **2016,** *91*, 19-30.
Stewart, J.J.P. MOPAC2012, Stewart Computational Chemistry, Colorado Springs, CO, USA, http://openmopac.net/ (2012).
Storz, P., Reactive oxygen species in tumor progression. *Front. Biosci.* **2005,** *10*, 1881-96.
Su, C.; Zhang, P.; Song, X.; Shi, Q.; Fu, J.; Xia, X.; Bai, H.; Hu, L.; Xu, D.; Song, E.; Song, Y., Tetrachlorobenzoquinone activates Nrf2 signaling by Keap1 cross-linking and ubiquitin translocation but not Keap1-Cullin3 complex dissociation. *Chem. Res. Toxicol.* **2015,** *28* (4), 765-74.
Sze, C. C.; Shi, C. Y.; Ong, C. N., Cytotoxicity and DNA strand breaks induced by benzene and its metabolites in Chinese hamster ovary cells. *J. Appl. Toxicol.* **1996,** *16* (3), 259-264.
Takahashi, K.; Shibata, T.; Oba, T.; Ishikawa, T.; Yoshikawa, M.; Tatsunami, R.; Takahashi, K.; Tampo, Y., Multidrug-resistance-associated protein plays a protective role in menadione-induced oxidative stress in endothelial cells. *Life Sci.* **2009,** *84* (7-8), 211-7.
Tardiff, R. G.; Carson, M. L.; Ginevan, M. E., Updated weight of evidence for an association between adverse reproductive and developmental effects and exposure to disinfection by-products. *Regul. Toxicol. Pharm.* **2006,** *45* (2), 185-205.
Tenhunen, R.; Marver, H. S.; Schmid, R., Enzymatic Conversion of Heme to Bilirubin by Microsomal Heme Oxygenase. *Proc. Natl. Acad. Sci. USA.* **1968,** *61* (2), 748-&.
Thai, S. F.; Allen, J. W.; DeAngelo, A. B.; George, M. H.; Fuscoe, J. C., Altered gene expression in mouse livers after dichloroacetic acid exposure. *Mutat. Res-Rev. Mutat.* **2003,** *543* (2), 167-180.
Trachootham, D.; Alexandre, J.; Huang, P., Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat. Rev. Drug Discov.* **2009,** *8* (7), 579-91.
Trush, M. A.; Twerdok, L. E.; Rembish, S. J.; Zhu, H.; Li, Y. B., Analysis of target cell susceptibility as a basis for the development of a chemoprotective strategy against benzene-induced hematotoxicities. *Environ. Health Perspec.* **1996,** *104*, 1227-1234.
Tully, D. B.; Luft, J. C.; Rockett, J. C.; Ren, H. Z.; Schmid, J. E.; Wood, C. R.; Dix, D. J., Reproductive and genomic effects in testes from mice exposed to the water disinfectant byproduct bromochloroacetic acid. *Reprod. Toxicol.* **2005,** *19* (3), 353-366.

Uchimiya, M.; Stone, A. T., Aqueous oxidation of substituted dihydroxybenzenes by substituted benzoquinones. *Environ. Sci. Technol.* **2006,** *40* (11), 3515-3521.
Vaidyanathan, V. G.; Villalta, P. W.; Sturla, S. J., Nucleobase-dependent reactivity of a quinone metabolite of pentachlorophenol. *Chem. Res. Toxicol.* **2007,** *20* (6), 913-919.
Valavanidis, A.; Vlachogianni, T.; Fiotakis, C., 8-hydroxy-2' -deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. *J. Environ. Sci. Health C* **2009,** *27* (2), 120-139.
U. S. Environmental Protection Agency, National primary drinking water regulations: Stage 1 disinfectant and disinfection byproducts rule. http://water.epa.gov/lawsregs/rulesregs/sdwa/stage1/index.cfm.
U. S. Environmental Protection Agency, National primary drinking water regulations: Stage 2 disinfectants and disinfection byproducts rule. http://water.epa.gov/lawsregs/rulesregs/sdwa/stage2/index.cfm.
van Ommen, B.; den Besten, C.; Rutten, A. L.; Ploemen, J. H.; Vos, R. M.; Muller, F.; van Bladeren, P. J., Active site-directed irreversible inhibition of glutathione S-transferases by the glutathione conjugate of tetrachloro-1,4-benzoquinone. *J. Biol. Chem.* **1988,** *263* (26), 12939-42.
vanKlaveren, R. J.; Demedts, M.; Nemery, B., Cellular glutathione turnover in vitro, with emphasis on type II pneumocytes. *Eur. Respir. J.* **1997,** *10* (6), 1392-1400.
Villanueva, C. M.; Cantor, K. P.; Grimalt, J. O.; Malats, N.; Silverman, D.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Marcos, R.; Rothman, N.; Real, F. X.; Dosemeci, M.; Kogevinas, M., Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am. J. Epidemiol.* **2007,** *165* (2), 148-156.
Villeneuve, N. F.; Sun, Z.; Chen, W.; Zhang, D. D., Nrf2 and p21 regulate the fine balance between life and death by controlling ROS levels. *Cell Cycle* **2009,** *8* (20), 3255-6.
Wagner, E. D.; Hsu, K. M.; Lagunas, A.; Mitch, W. A.; Plewa, M. J., Comparative genotoxicity of nitrosamine drinking water disinfection byproducts in Salmonella and mammalian cells. *Mutat. Res.* **2012,** *741* (1-2), 109-15.
Wagner, E. D.; Osiol, J.; Mitch, W. A.; Plewa, M. J., Comparative in vitro toxicity of nitrosamines and nitramines associated with amine-based carbon capture and storage. *Environ. Sci. Technol.* **2014,** *48* (14), 8203-8211.

Wang, G.; Gong, Y.; Burczynski, F. J.; Hasinoff, B. B., Cell lysis with dimethyl sulphoxide produces stable homogeneous solutions in the dichlorofluorescein oxidative stress assay. *Free Radic. Res.* **2008,** *42* (5), 435-441.

Wang, J.; Yu, S. Y.; Jiao, S. H.; Lv, X. W.; Ma, M.; Zhu, B. Z.; Du, Y. G., Characterization of TCHQ-induced genotoxicity and mutagenesis using the pSP189 shuttle vector in mammalian cells. *Mutat. Res-Fund. Mol. M.* **2012,** *729* (1-2), 16-23.

Wang, S.; Zhang, H.; Zheng, W.; Wang, X.; Andersen, M. E.; Pi, J.; He, G.; Qu, W., Organic extract contaminants from drinking water activate Nrf2-mediated antioxidant response in a human cell line. *Environ. Sci. Technol.* **2013,** *47* (9), 4768-77.

Wang, S.; Zheng, W.; Liu, X.; Xue, P.; Jiang, S.; Lu, D.; Zhang, Q.; He, G.; Pi, J.; Andersen, M. E.; Tan, H.; Qu, W., Iodoacetic acid activates Nrf2-mediated antioxidant response in vitro and in vivo. *Environ. Sci. Technol.* **2014,** *48* (22), 13478-88.

Wang, W.; Qian, Y.; Boyd, J. M.; Wu, M.; Hrudey, S. E.; Li, X.-F., Halobenzoquinones in swimming pool waters and their formation from personal care products. *Environ. Sci. Technol.* **2013,** *47* (7), 3275-3282.

Wang, W.; Qian, Y. C.; Li, J. H.; Moe, B.; Huang, R. F.; Zhang, H. Q.; Hrudey, S. E.; Li, X.-F., Analytical and toxicity characterization of halo-hydroxyl-benzoquinones as stable halobenzoquinone disinfection byproducts in treated water. *Anal. Chem.* **2014,** *86* (10), 4982-4988.

Ward, W. O.; Swartz, C. D.; Porwollik, S.; Warren, S. H.; Hanley, N. M.; Knapp, G. W.; McClelland, M.; DeMarini, D. M., Toxicogenomic analysis incorporating operon-transcriptional coupling and toxicant concentration-expression response: analysis of MX-treated Salmonella. *BMC. Bioinformatics* **2007,** *8*, 378.

Waris, G.; Ahsan, H., Reactive oxygen species: role in the development of cancer and various chronic conditions. *J. Carcinog.* **2006,** *5*, 14.

Waters, M. D.; Fostel, J. M., Toxicogenomics and systems toxicology: Aims and prospects. *Nat. Rev. Genet.* **2004,** *5* (12), 936-948.

Water and Air Quality Bureau, Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa, Ontario.

Wen, J.; Luo, J.; Huang, W.; Tang, J.; Zhou, H.; Zhang, W., The pharmacological and physiological role of multidrug-resistant protein 4. *J Pharmacol. Exp. Ther.* **2015,** *354* (3), 358-375.

Wendel, F. M.; Ternes, T. A.; Richardson, S. D.; Duirk, S. E.; Pals, J. A.; Wagner, E. D.; Plewa, M. J., Comparative toxicity of high-molecular weight iopamidol disinfection byproducts. *Environ. Sci. Technol. Lett.* **2016,** *3* (3), 81-84.

White, G. C., *Handbook of Chlorination; for Potable Water, Wastewater, Cooling Water, Industrial Processes, and Swimming Pools*. Van Nostrand Reinhold Co.: New York,, 1972.

Whysner, J., Benzene-induced genotoxicity. *J. Toxicol. Env. Health A* **2000,** *61* (5-6), 347-351.

Whysner, J.; Reddy, M. V.; Ross, P. M.; Mohan, M.; Lax, E. A., Genotoxicity of benzene and its metabolites. *Mutat. Res-Rev. Mutat.* **2004,** *566* (2), 99-130.

World Health Organization., *Guidelines for Drinking-Water Quality*. 4th ed.; World Health Organization: Geneva, 2011.

World Health Organization.; UNICEF., *Progress on sanitation and drinking water: 2015 update and MDG assessment*. New York, Geneva, 2015; p 90.

Wu, L. L.; Chiou, C. C.; Chang, P. Y.; Wu, J. T., Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. *Clin. Chim. Acta.* **2004,** *339* (1-2), 1-9.

Wu, Q. Y.; Li, Y.; Hu, H. Y.; Ding, Y. N.; Huang, H.; Zhao, F. Y., Removal of genotoxicity in chlorinated secondary effluent of a domestic wastewater treatment plant during dechlorination. *Environ. Sci. Pollut. Res. Int.* **2012,** *19* (1), 1-7.

Wu, Q. Y.; Li, Y.; Hu, H. Y.; Sun, Y. X.; Zhao, F. Y., Reduced effect of bromide on the genotoxicity in secondary effluent of a municipal wastewater treatment plant during chlorination. *Environ. Sci. Technol.* **2010,** *44* (13), 4924-9.

Yager, J. W.; Eastmond, D. A.; Robertson, M. L.; Paradisin, W. M.; Smith, M. T., Characterization of Micronuclei Induced in Human-Lymphocytes by Benzene Metabolites. *Cancer Res.* **1990,** *50* (2), 393-399.

Yang, C. Y.; Xiao, Z. P.; Ho, S. C.; Wu, T. N.; Tsai, S. S., Association between trihalomethane concentrations in drinking water and adverse pregnancy outcome in Taiwan. *Environ. Res.* **2007,** *104* (3), 390-395.

Yang, M. T.; Zhang, X. R., Comparative developmental toxicity of new aromatic halogenated DBPs in a chlorinated saline sewage effluent to the marine polychaete *Platynereis dumerilii*. *Environ. Sci. Technol.* **2013,** *47* (19), 10868-10876.
Yin, R.; Zhang, D.; Song, Y.; Zhu, B. Z.; Wang, H., Potent DNA damage by polyhalogenated quinones and H(2)O(2) via a metal-independent and Intercalation-enhanced oxidation mechanism. *Sci. Rep.* **2013,** *3*, 1269.
Yokose, Y.; Uchida, K.; Nakae, D.; Shiraiwa, K.; Yamamoto, K.; Konishi, Y., Studies of carcinogenicity of sodium-chlorite in B6c3f1 mice. *Environ. Health Perspec.* **1987,** *76*, 205-210.
Zafarullah, M.; Li, W. Q.; Sylvester, J.; Ahmad, M., Molecular mechanisms of N-acetylcysteine actions. *Cell. Mol. Life Sci.* **2003,** *60* (1), 6-20.
Zamek-Gliszczynski, M. J.; Xiong, H.; Patel, N. J.; Turncliff, R. Z.; Pollack, G. M.; Brouwer, K. L., Pharmacokinetics of 5 (and 6)-carboxy-2',7'-dichlorofluorescein and its diacetate promoiety in the liver. *J. Pharmacol. Exp. Ther.* **2003,** *304* (2), 801-9.
Zhai, H.; Zhang, X., Formation and decomposition of new and unknown polar brominated disinfection byproducts during chlorination. *Environ. Sci. Technol.* **2011,** *45* (6), 2194-2201.
Zhai, H.; Zhang, X.; Zhu, X.; Liu, J.; Ji, M., Formation of brominated disinfection byproducts during chloramination of drinking water: new polar species and overall kinetics. *Environ. Sci. Technol.* **2014,** *48* (5), 2579-2588.
Zhao, B.; Yang, Y.; Wang, X.; Chong, Z.; Yin, R.; Song, S. H.; Zhao, C.; Li, C.; Huang, H.; Sun, B. F.; Wu, D.; Jin, K. X.; Song, M.; Zhu, B. Z.; Jiang, G.; Rendtlew Danielsen, J. M.; Xu, G. L.; Yang, Y. G.; Wang, H., Redox-active quinones induces genome-wide DNA methylation changes by an iron-mediated and Tet-dependent mechanism. *Nucleic Acids Res.* **2014,** *42* (3), 1593-605.
Zhao, Y.L.; Qin, F.; Boyd, J. M.; Anichina, J.; Li, X.-F., Characterization and determination of chloro- and bromo-benzoquinones as new chlorination disinfection byproducts in drinking water. *Anal. Chem.* **2010,** *82* (11), 4599-4605.
Zhao, Y. L.; Anichina, J.; Lu, X. F.; Bull, R. J.; Krasner, S. W.; Hrudey, S. E.; Li, X.-F., Occurrence and formation of chloro- and bromo-benzoquinones during drinking water disinfection. *Water Res.* **2012,** *46* (14), 4351-4360.
Zhou, H.; Chen, W. D.; Qin, X.; Lee, K.; Liu, L.; Markowitz, S. D.; Gerson, S. L., MMTV promoter hypomethylation is linked to spontaneous and MNU associated c-neu expression and mammary carcinogenesis in MMTV c-neu transgenic mice. *Oncogene* **2001,** *20* (42), 6009-17.

Zhou, H. F.; Kepa, J. K.; Siegel, D.; Miura, S.; Hiraki, Y.; Ross, D., Benzene metabolite hydroquinone up-regulates chondromodulin-I and inhibits tube formation in human bone marrow endothelial cells. *Mol. Pharmacol.* **2009,** *76* (3), 579-587.

Zhou, W. J.; Lou, L. J.; Zhu, L. F.; Li, Z. M.; Zhu, L. Z., Formation and cytotoxicity of a new disinfection by-product (DBP) phenazine by chloramination of water containing diphenylamine. *J. Environ. Sci.* **2012,** *24* (7), 1217-1224.

Zhou, Z. X.; Parr, R. G., Activation hardness - new index for describing the orientation of electrophilic aromatic-substitution. *J. Am. Chem. Soc.* **1990,** *112* (15), 5720-5724.

Zhu, B. Z.; Kalyanaraman, B.; Jiang, G. B., Molecular mechanism for metal-independent production of hydroxyl radicals by hydrogen peroxide and halogenated quinones. *Proc. Natl. Acad. Sci. USA.* **2007,** *104* (45), 17575-17578.

Zhu, B. Z.; Kitrossky, N.; Chevion, M., Evidence for production of hydroxyl radicals by pentachlorophenol metabolites and hydrogen peroxide: a metal-independent organic Fenton reaction. *Biochem. Bioph. Res. Commun.* **2000,** *270* (3), 942-6.

Zhu, B. Z.; Qin, H., Metal-independent decomposition of lipid hydroperoxide 13-HPODE and formation of lipid radicals/4-HNE by the newly identified chlorination disinfection haloquinone byproducts in drinking water. *Free Radical. Biol. Med.* **2012,** *53*, S153-S153.

Zhu, B. Z.; Shan, G. Q., Potential mechanism for pentachlorophenol-induced carcinogenicity: a novel mechanism for metal-independent production of hydroxyl radicals. *Chem. Res. Toxicol.* **2009,** *22* (6), 969-977.

Zhu, B. Z.; Shan, G. Q.; Huang, C. H.; Kalyanaraman, B.; Mao, L.; Du, Y. G., Metal-independent decomposition of hydroperoxides by halogenated quinones: Detection and identification of a quinone ketoxy radical. *Proc. Natl. Acad. Sci. USA.* **2009,** *106* (28), 11466-11471.

Zhu, B. Z.; Zhao, H. T.; Kalyanaraman, B.; Frei, B., Metal-independent production of hydroxyl radicals by halogenated quinones and hydrogen peroxide: an ESR spin trapping study. *Free Radic. Biol. Med.* **2002,** *32* (5), 465-73.

Zhu, B. Z.; Zhao, H. T.; Kalyanaraman, B.; Liu, J.; Shan, G. Q.; Du, Y. G.; Frei, B., Mechanism of metal-independent decomposition of organic hydroperoxides and formation of alkoxyl radicals by halogenated quinones. *Proc. Natl. Acad. Sci. USA.* **2007,** *104* (10), 3698-3702.