Supporting Information

Halobenzoquinone-Induced Alteration of Gene Expression Associated with Oxidative Stress Signaling Pathways

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Methods

HBQ-Induced Cytotoxicity. The principles and detailed procedures for real-time cell analysis (RTCA; ACEA Biosciences, San Diego, CA) have been reported previously.[1] The unitless parameter, cell index (CI), is the data output of the instrument and corresponds to the density of adherent cells on the bottom of each well. SV-HUC-1 cells were seeded into 96-well E-plates of the instrument at a density of 1.5×10^4 cells per well. When the CI reached a value of 1 after approximately 20 h growth, cells were treated with a range of serially-diluted HBQs (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). The CI of each well was measured hourly for up to 80 h after treatment. Negative controls (no HBQ treatment; culture medium) and solvent controls (1% methanol (v/v) in culture medium) were tested concurrently with the HBQ treatments. Replicates were prepared in triplicate for each experimental condition, and three independent experiments were performed (n = 3).

To confirm that oxidative stress is involved in HBQ-induced cytotoxicity in SV-HUC-1 cells, RTCA experiments described above were repeated following pretreatment with the antioxidant *N*-acetyl-L-cysteine (NAC; Sigma-Aldrich, St. Louis, MO). Cells were pretreated at the time of seeding with 1 mM NAC. When the CI reached a value of 1, the medium in each well was discarded and cells were treated with a range of serially-diluted HBQs (0–150 μM for 2,6-DCBQ, TriCBQ; 0–125 μM for 2,6-DBBQ; 0–100 μM for DCMBQ) for up to 80 h after treatment. NAC controls (1 mM NAC) were included in each experiment in addition to the negative controls and solvent controls described above. Replicates were prepared in triplicate for each experimental condition, and three independent experiments were performed (n = 3).

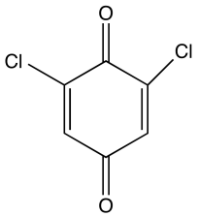
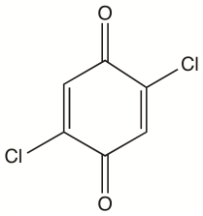
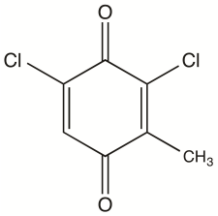
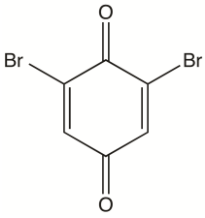
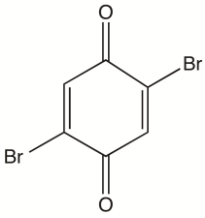
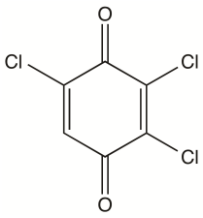
Detection of Reactive Oxygen Species. SV-HUC-1 cells were seeded into solid black 96-well plates (Corning Costar, Fisher Scientific, Ottawa, ON, Canada) at a density of 1.5×10^4 cells per well. After 20 h growth, each well was washed twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Burlington, ON, Canada), loaded with 25 μM DCFH-DA in F-12K medium without FBS, and incubated at 37°C for 45 min. After incubation, each well was washed twice with DPBS and treated with individual HBQs at their respective concentration of equivalent biological response (IC_{20}). The plate was read (λ_{ex} = 485nm, λ_{em} = 535 nm) in a fluorescence microplate reader (Beckman Coulter DTX880 Multimode Detector) at the indicated time points of exposure (0.5, 1, 2, 4, 6, 8, 24, 48, and 72 h). Negative controls (no HBQ treatment; culture

medium) and solvent controls (1% methanol (v/v) in culture medium) were also measured at each time point. Six replicates were used for each experimental condition, and three independent experiments were performed (n = 3).

Real-time PCR with Fast SYBR Green. A 10 μL reaction system was used, consisting of 2 × Fast SYBR Green Master Mix and 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 for each gene.

TaqMan Array. 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.

Table S1. HBQ chemical structure, molecular weight, molecular formula, and purity

HBQs	Molecular Weight	Molecular Formula	Purity	Structure
2,6-dichloro-1,4-benzoquinone [2,6-DCBQ]	176.98	$C_6H_2Cl_2O_2$	98%	
2,5-dichloro-1,4-benzoquinone [2,5-DCBQ]	176.98	$C_6H_2Cl_2O_2$	98%	
2,6-dichloro-3-methyl-1,4-benzoquinone [DCMBQ]	191.01	$C_7H_4Cl_2O_2$	98%	
2,6-dibromo-1,4-benzoquinone [2,6-DBBQ]	263.84	$C_6H_2Br_2O_2$	≥98%	
2,5-dibromo-1,4-benzoquinone [2,5-DBBQ]	263.84	$C_6H_2Br_2O_2$	N.A.*	
2,3,6-trichloro-1,4-benzoquinone [TriCBQ]	211.43	$C_6HCl_3O_2$	≥98%	

*N.A. = not available from the manufacturer. The mass spectra of 2,5-DBBQ showed high purity in our previous publication (Huang, et al.).[2]

Table S2. Primer sequences used for real-time PCR

Gene	Forward (5'-3')	Reverse (5'-3')
NFE2L2	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

Table S3. Genes included for analysis in the TaqMan Array

	Functional Group	**Gene**	**Symbol**
1	Transcription factor and its inhibitor	Nuclear factor-erythroid 2-related factor (Nrf2)	*NFE2L2*
		Kelch-like ECH-associated protein 1	*Keap1*
2	GSH utilization	Glutathione transferase	*GST: GSTP1, GSTT1, GSTZ1, GSTM1, GSTA1, GSTA4*
		Glutathione peroxidase	*GPX: GPX1, GPX2, GPX4*
3	GSH production	Glutamate–cysteine ligase	*GCL: GCLM* (*GCL* modifier subunit), *GCLC* (*GCL* catalytic subunit)
		Glutathione reductase	*GSR*
		Glutathione synthetase	*GSS*
4	Thioredoxin system	Thioredoxin reductase	*TXNRD: TXNRD1, TXNRD2*
		Peroxiredoxin	*PRDX: PRDX1, PRDX2, PRDX3, PRDX4, PRDX5*
		Sulfiredoxin	*SRXN1*
5	Membrane transporters	Multidrug resistance protein/ATP-binding cassette transporter subfamily C	*MRP/ABCC: ABCC1, ABCC2, ABCC3, ABCC4, ABCC5*
6	Antioxidants	NADPH:quinone oxidoreductase 1	*NQO1*
		Catalase	*CAT*
		Superoxide dismutase	*SOD: SOD1, SOD2, SOD3*
		Prostaglandin-endoperoxide synthase 2 [Cyclooxygenase-2]	*PTGS2* [*COX-2*]
		Heme oxygenase (decycling) 1	*HMOX1*

7	Stress responsive genes	N-acetyltransferase	*NAT: 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	Cyclin-dependent kinase inhibitor 1, CDK-interacting protein 1[p21 / WAF1]	*CDKN1A*

Table S4. Significant gene expression changes vs concurrent negative controls after 2 h and 8 h HBQ exposure in SV-HUC-1 cells

Gene	Time	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	***2h***	1.309	0.034			1.437	0.003			1.589	0.005	1.443	0.005
	8h			1.153	0.030	-1.173	< 0.001	1.305	0.016			-1.085	< 0.001
KEAP1	***2h***			-1.337	0.003			-1.204	0.001				
	8h			1.208	0.007	-1.066	< 0.001					-1.108	< 0.001
GSTP1	***2h***	1.191	0.041	-1.224	0.011	1.419	0.031	1.151	0.045			1.281	0.019
	8h	-1.084	< 0.001			-1.095	< 0.001	-1.064	0.001	-1.071	< 0.001	-1.251	< 0.001
GSTT1	***2h***			-1.470	0.001			-1.414	< 0.001				
	8h	-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	***2h***			-1.286	0.019								
	8h	-1.174	< 0.001	1.134	0.036	-1.132	< 0.001			-1.315	0.004	-1.243	< 0.001
GSTA4	***2h***	-1.121	0.006	-1.373	0.014			-1.110	0.006			-1.142	0.006
	8h							1.477	0.039				
GPX1	***2h***	-1.099	< 0.001	-1.276	0.004			-1.090	< 0.001			-1.042	< 0.001
	8h	-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	***2h***	2.031	0.004			3.867	0.003			1.495	0.034	1.593	0.012
	8h	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	***2h***					1.286	0.043						
	8h	-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	***2h***	1.512	0.003			1.477	< 0.001	1.330	0.001			1.394	0.015

	8h	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	***2h***			-1.492	0.016			-1.302	< 0.001				
	8h	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	***2h***			-1.383	< 0.001			-1.163	< 0.001				
	8h	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	***2h***			-1.823	< 0.001	1.199	0.017	-1.097	< 0.001				
	8h					-1.043	0.004	-1.116	< 0.001	-1.176	0.003	-1.216	0.002
NQO1	***2h***	1.788	< 0.001	2.796	< 0.001	1.898	0.005	1.252	0.002	2.004	0.031	1.575	0.006
	8h	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	***2h***	1.171	0.015			1.326	0.001						
	8h	1.430	0.012	1.528	0.010	1.550	0.046	1.565	0.007	1.348	0.023	1.370	0.027
PTGS2	***2h***	2.364	< 0.001	1.365	0.007	2.378	0.006	1.352	0.035	3.403	0.004	1.693	0.035
	8h	2.089	< 0.001	1.903	< 0.001	1.638	0.001	2.673	< 0.001	3.262	< 0.001	1.184	< 0.001
SOD1	***2h***												
	8h			1.117	0.042	-1.257	0.002	1.195	0.013			-1.258	0.002
SOD2	***2h***												
	8h			1.275	0.021	-1.162	0.002	1.251	0.009			-1.199	< 0.001
HMOX1	***2h***	6.662	< 0.001	10.172	< 0.001	5.508	< 0.001	10.992	< 0.001	6.869	< 0.001	4.920	< 0.001
	8h	13.693	< 0.001	41.132	< 0.001	7.390	< 0.001	50.238	< 0.001	18.845	< 0.001	10.317	< 0.001
NAT1	***2h***												
	8h	-1.044	< 0.001			-1.088	0.002					-1.153	0.001
NAT2	***2h***	-1.443	0.021	-1.935	0.020								
	8h	-2.698	0.012	-2.649	< 0.001			-2.303	< 0.001			-3.210	0.004
CYBB	***2h***	2.244	0.003	3.519	0.017	1.746	0.020	3.770	0.006			1.719	0.002

	8h	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	***2h***							-1.164	< 0.001				
	8h	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	***2h***	-1.061	< 0.001	-1.488	< 0.001			-1.496	< 0.001			-1.107	0.004
	8h	-1.155	0.001	-1.425	0.001	-1.205	< 0.001	-1.621	0.002	-1.426	< 0.001	-1.339	0.007
OGG1	***2h***	-1.054	< 0.001	-1.561	< 0.001	1.148	0.011	-1.490	< 0.001	1.111	0.004		
	8h	-1.044	< 0.001	-1.402	0.001	-1.073	< 0.001	-1.597	< 0.001	-1.046	< 0.001	-1.204	0.005
CDKN1A	***2h***	1.445	0.015	1.281	< 0.001	1.431	0.009	1.447	0.049	1.307	< 0.001	1.204	0.018
	8h	1.389	0.029	1.820	0.003			2.530	0.011	1.477	0.016		
TXNRD1	***2h***	2.516	0.002	1.886	0.005	2.313	0.004	1.972	0.004	2.570	0.002	1.971	0.004
	8h	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	***2h***	-1.024	< 0.001	-1.737	0.001			-1.382	< 0.001				
	8h	-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	***2h***							-1.096	0.001				
	8h	1.090	0.013			-1.042	< 0.001	1.095	0.010	1.149	0.028	-1.102	0.004
PRDX2	***2h***	-1.045	0.001			1.115	0.003	-1.188	< 0.001				
	8h					-1.101	< 0.001	-1.063	< 0.001	-1.050	< 0.001	-1.092	< 0.001
PRDX3	***2h***	-1.046	< 0.001			1.174	0.009	-1.138	< 0.001				
	8h			-1.047	< 0.001	-1.062	< 0.001	-1.137	< 0.001	-1.075	< 0.001	-1.150	0.002
PRDX4	***2h***							-1.353	< 0.001				
	8h			-1.216	< 0.001	-1.292	0.004	-1.303	< 0.001	-1.061	< 0.001	-1.336	0.002
PRDX5	***2h***					1.1878	0.022						
	8h	-1.139	0.001			-1.200	0.002			-1.120	0.001	-1.329	0.002
SRXN1	***2h***					1.369	< 0.001	-1.197	< 0.001	1.315	< 0.001	1.228	0.007

	8h	1.739	0.007	1.987	0.008			2.283	0.009	1.755	0.011		
ABCC1	***2h***			-1.210	< 0.001			-1.245	< 0.001				
	8h							1.061	0.018				
ABCC2	***2h***			-1.331	0.001								
	8h	1.529	0.010	2.083	0.001	1.231	0.027	3.051	0.002	1.740	< 0.001		
ABCC3	***2h***			-1.665	< 0.001			-1.67	< 0.001				
	8h	1.778	0.004			1.300	0.016			1.851	0.019	1.266	0.011
ABCC4	***2h***	-1.140	< 0.001	-1.167	< 0.001	-1.008	< 0.001	-1.089	< 0.001			-1.045	< 0.001
	8h												
ABCC5	***2h***			-1.300	< 0.001			-1.197	< 0.001				
	8h					-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.

	1	2	3	4	5	6	7	8	9	10	11	12
A	*18S rRNA*	*GAPDH*	*HPRT1*	*GUSB*	*NFE2L2*	*KEAP1*	*18S rRNA*	*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 S1. The 96-well format of the customized TaqMan Array. *18S rRNA*, *GAPDH*, *HPRT1*, and *GUSB* were used as the reference controls. Samples are in duplicate format.

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.

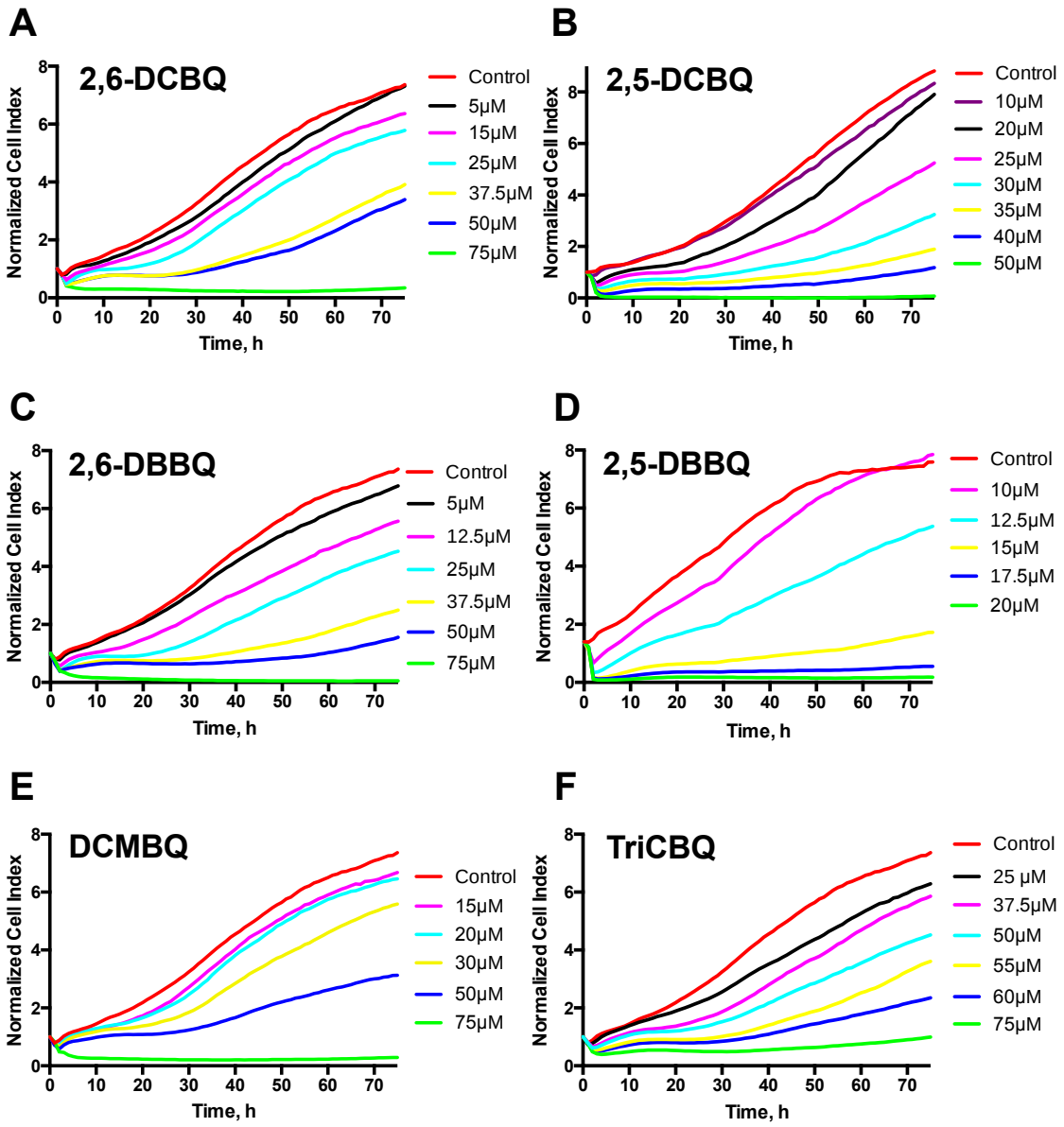


Figure S2. Cytotoxicity response profiles of the normalized cell index (CI) over time for SV-HUC-1 cells exposed to (A) 2,6-DCBQ, (B) 2,5-DCBQ, (C) 2,6-DBBQ, (D) 2,5-DBBQ, (E) DCMBQ, and (F) TriCBQ.

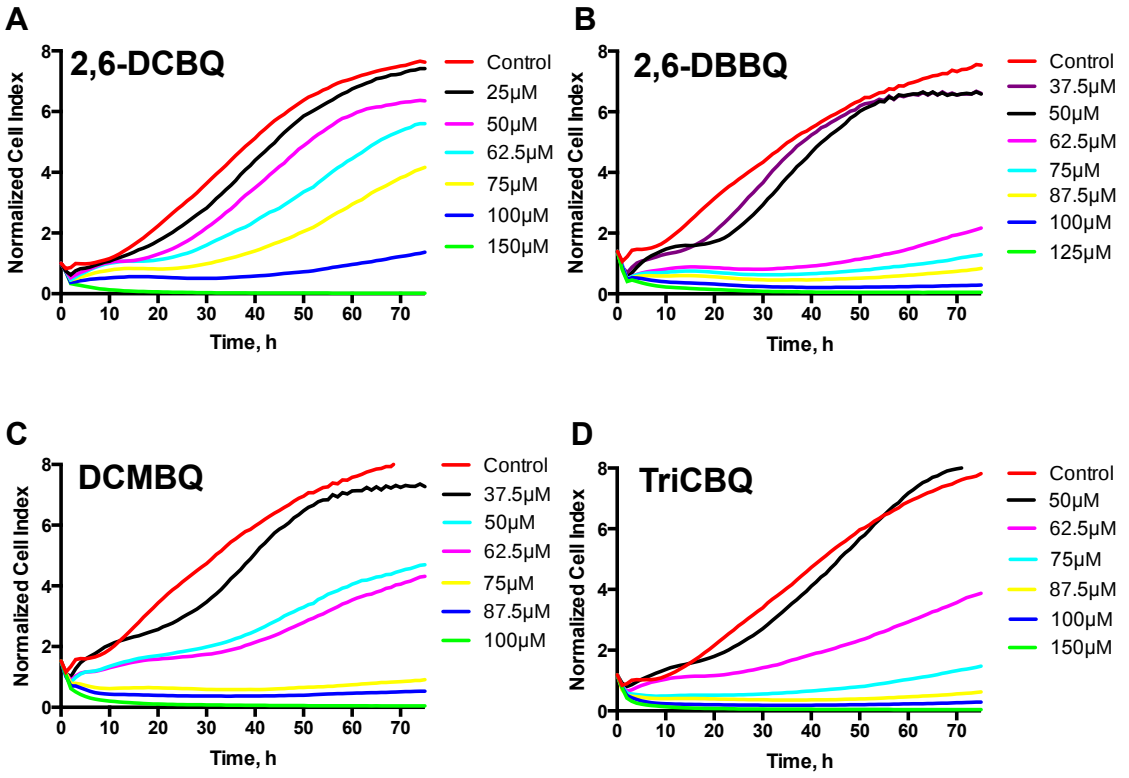


Figure S3. Cytotoxicity response profiles of the normalized cell index (CI) over time for SV-HUC-1 cells exposed to (A) 2,6-DCBQ, (B) 2,6-DBBQ, (C) DCMBQ, and (D) TriCBQ with 1 mM *N*-acetyl-L-cysteine (NAC) pretreatment.

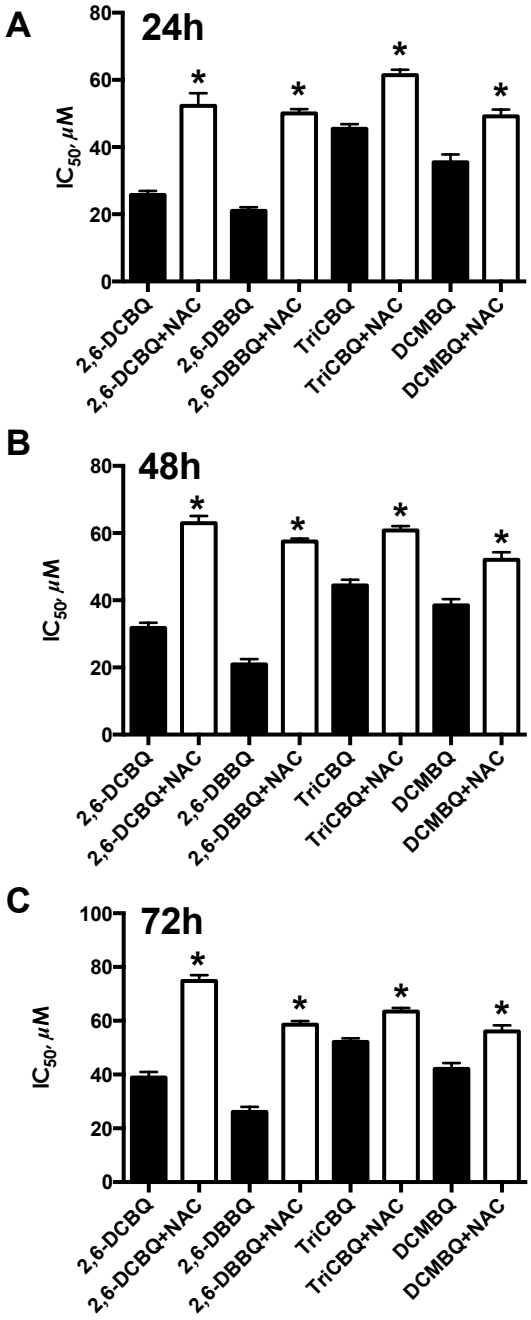


Figure S4. IC_{50} values of the HBQs with or without 1 mM *N*-acetyl-L-cysteine (NAC) pretreatment in SV-HUC-1 cells after (A) 24 h, (B) 48 h, or (C) 72 h exposure. Values are expressed as the mean ± SD, n = 3. *P < 0.05, HBQ + NAC treatment group vs. HBQ treatment group.

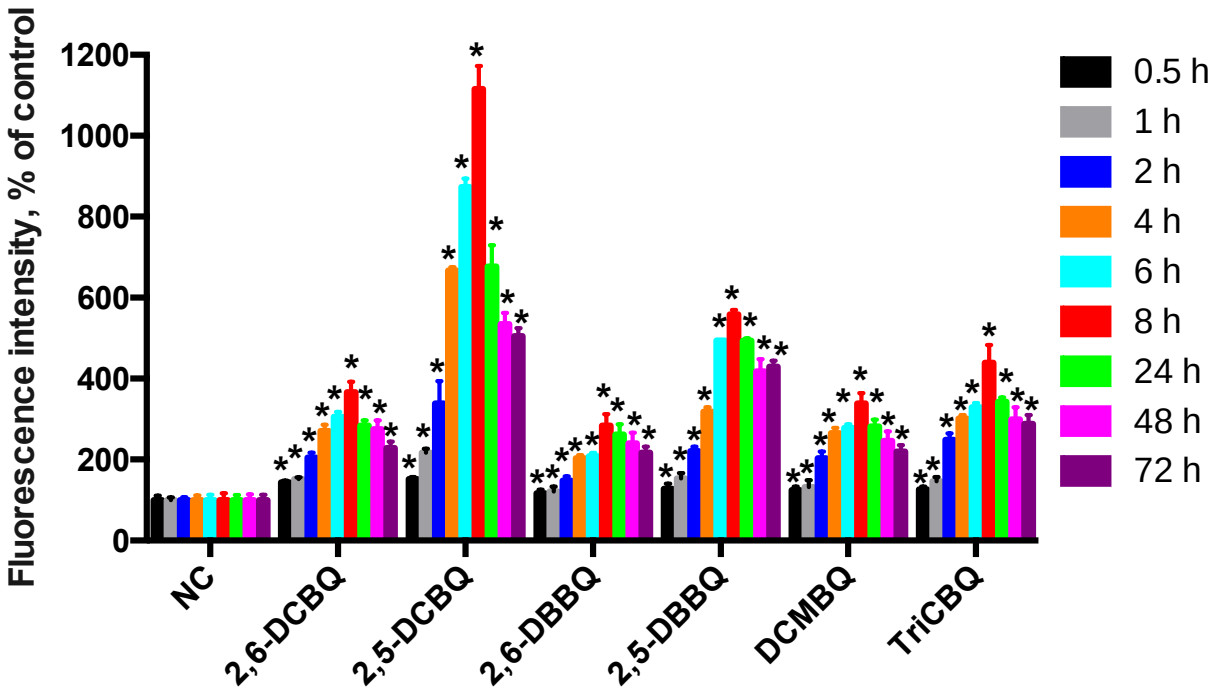


Figure S5. Time-course of ROS generation in SV-HUC-1 cells after each HBQ treatment at their respective IC_{20} values. All values are expressed as mean ± SEM of six replicates determined in a representative experiment, and similar results were obtained in three independent experiments (*n* = 3). NC = negative control. *P < 0.05, ROS generation at each time point compared with their concurrent control at the corresponding time point.

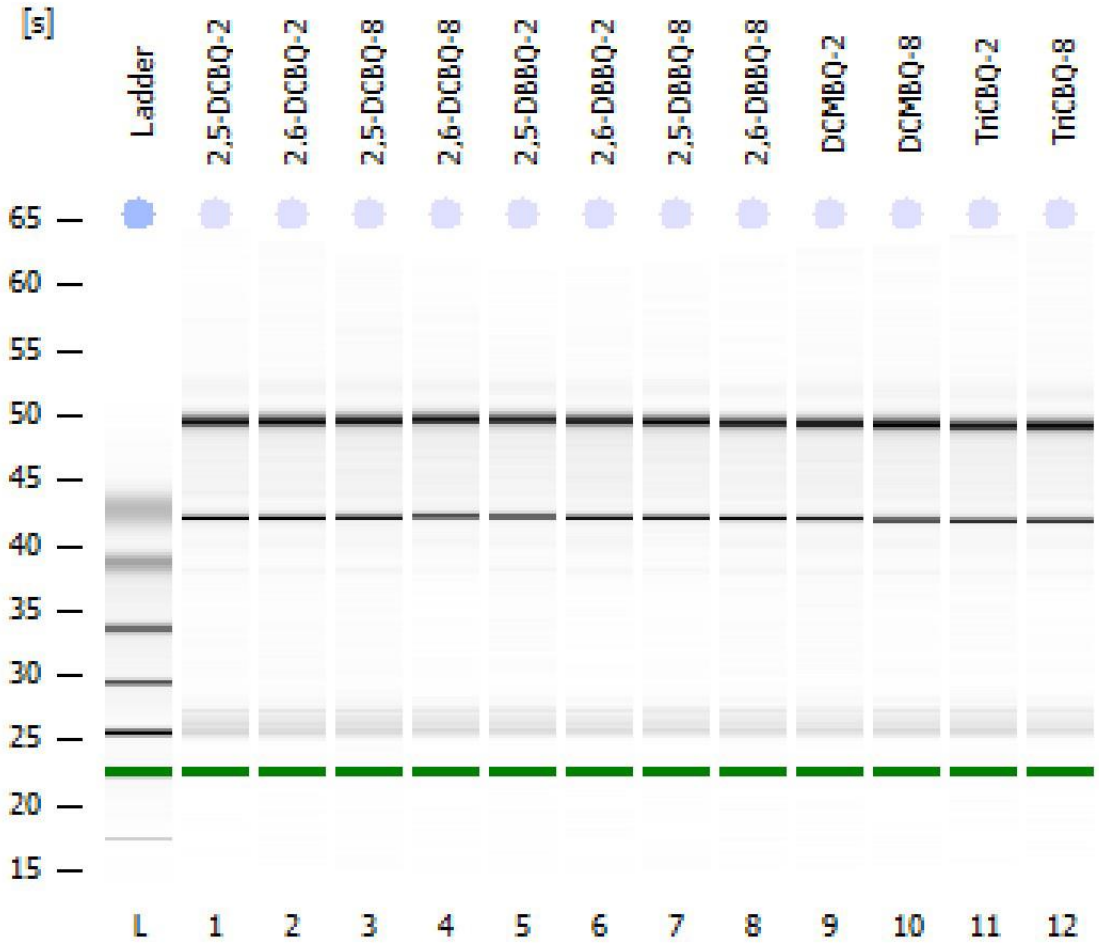


Figure S6. Densitometry plot of the capillary electrophoresis data of the RNA isolated from HBQ-treated SV-HUC-1 cells, obtained using an Agilent Bioanalyzer 2100.

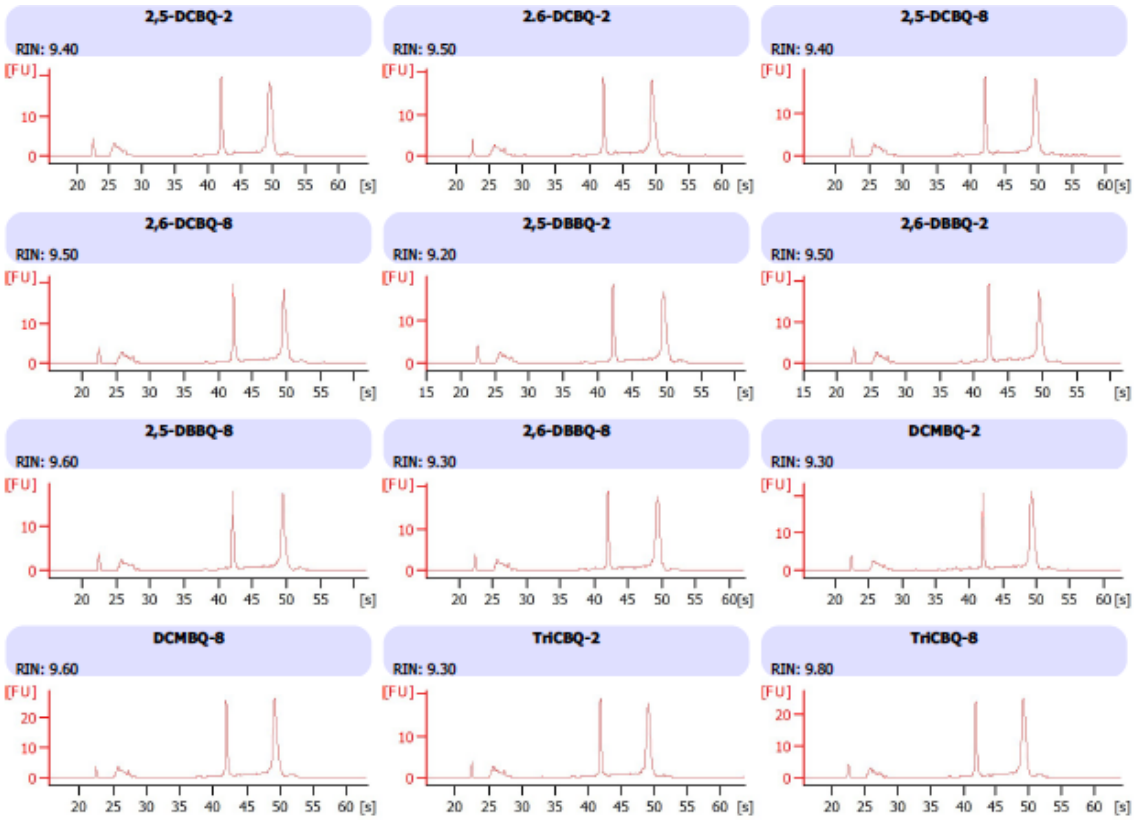


Figure S7. Typical capillary electropherograms of the RNA isolated from HBQ-treated SV-HUC-1 cells, obtained using an Agilent Bioanalyzer 2100. The RNA Integrity Number (RIN) values were greater than 9.0 in all samples, indicating high RNA integrity.

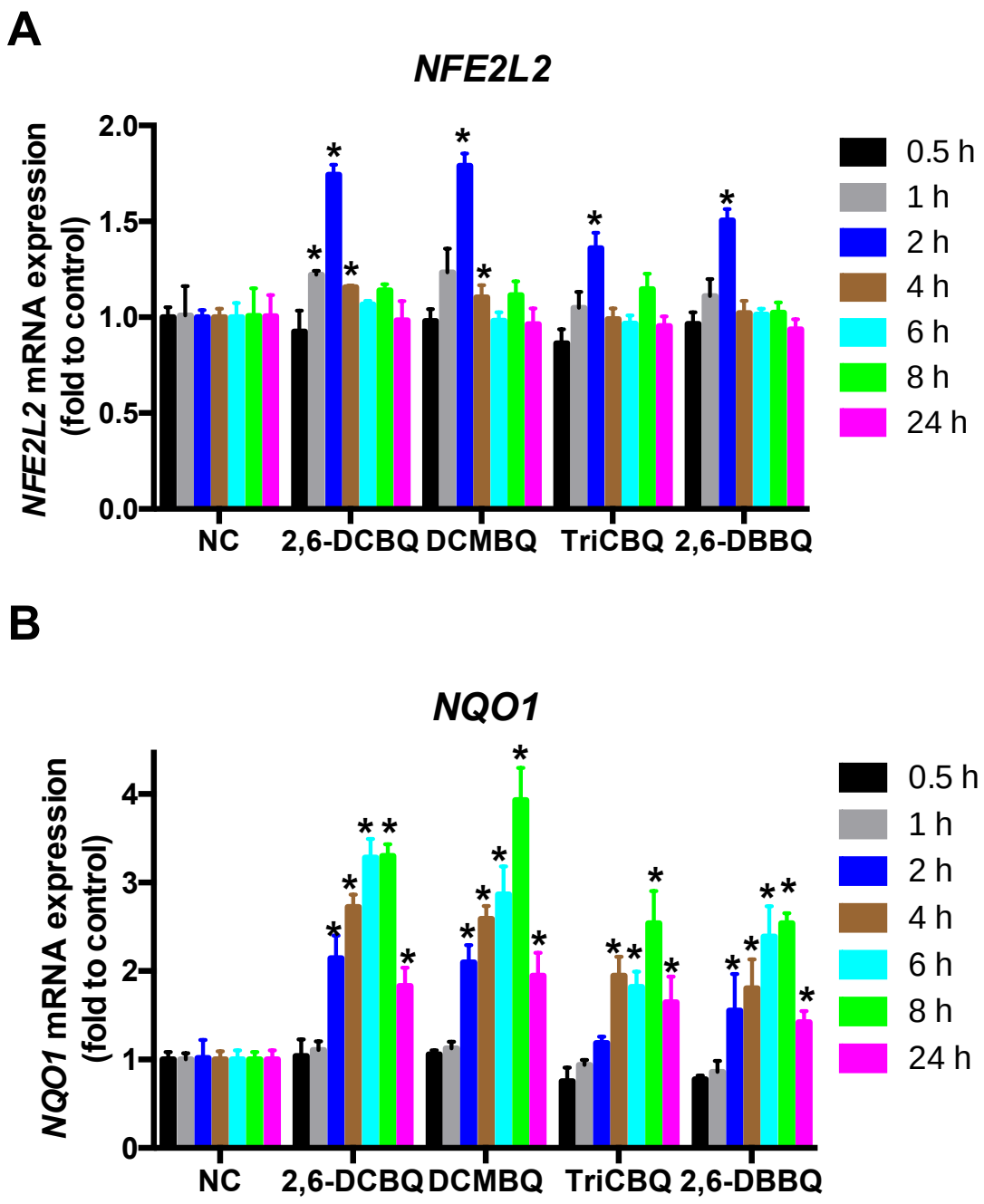


Figure S8. Time-course of HBQ-induced (A) *NFE2L2* and (B) *NQO1* gene response in SV-HUC-1 cells. SV-HUC-1 cells were treated with HBQ concentrations equal to 24h-IC_{20} values. mRNA expression was determined by real-time PCR and normalized by *GAPDH*. Values are expressed as the mean ± SD, n = 3. *P < 0.05, HBQ treatment group vs concurrent negative control (NC).

References

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