

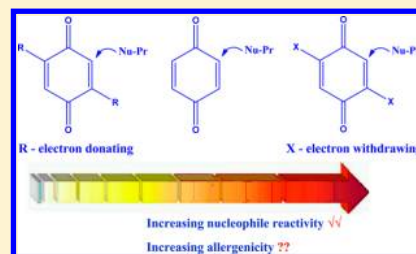
Substituent Effects on the Reactivity of Benzoquinone Derivatives with Thiols

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ABSTRACT: Benzoquinone (BQ) is an extremely potent electrophilic contact allergen that haptens endogenous proteins through Michael addition (MA). It is also hypothesized that BQ may haptenate proteins via free radical formation. The objective of this study was to assess the inductive effects (activating and deactivating) of substituents on BQ reactivity and the mechanistic pathway of covalent binding to a nucleophilic thiol. The BQ binding of Cys34 on human serum albumin was studied, and for reactivity studies, nitrobenzenethiol (NBT) was used as a surrogate for protein binding of the BQ and benzoquinone derivatives (BQD). Stopped flow techniques were used to determine pseudofirst order rate constants (k) of methyl-, *t*-butyl-, and chlorine-substituted BQD reactions with NBT, whereas electron pair resonance (EPR) studies were performed to investigate the presence of the free radical mediated binding mechanism of BQD. Characterization of adducts was performed using mass spectrometry and nuclear magnetic resonance spectroscopy (NMR). The rate constant values demonstrated the chlorine-substituted (activated) BQD to be more reactive toward NBT than the methyl and *t*-butyl-substituted (deactivated) BQD, and this correlated with the respective EPR intensities. The EPR signal, however, was quenched in the presence of NBT suggesting MA as the dominant reaction pathway. MS and NMR results confirmed adduct formation to be a result of MA onto the BQ ring with vinylic substitution also occurring for chlorine-substituted derivatives. The binding positions on BQ and NBT/BQ(D) stoichiometric ratios were affected by whether the inductive effects of the substituents on the ring were positive or negative. The reactivity of BQ and BQD is discussed in terms of the potential relationship to potential allergenic potency.



INTRODUCTION

Benzoquinone (BQ) belongs to a large class of quinonic compounds commonly used for dye production. Quinones have been used as analytical reagents,¹ polymer modifiers,² photoresistors,³ catalysts,⁴ herbicides,⁵ fungicides,^{5,6} and plant-growth regulators.⁷ BQ is also broadly used in tanning, dyes,¹ cosmetics,⁸ and nonprescription forms of skin lightening cosmetics.^{9–11} The widespread domestic and industrial use of BQ and its products results in a significant human population being exposed to quinones. Exposure to BQ leads to allergic contact dermatitis (ACD),¹² which is the clinical manifestation of contact allergy that is estimated to affect a significant proportion (1–4%) of the general population.¹³

Chemical contact allergens must either directly or after metabolic or abiotic activation covalently bind to skin protein to be recognized by the immune system and become allergenic. Early evidence demonstrating chemical reactivity as underpinning the sensitizing potency of a chemical was reported by Landsteiner and Jacobs in 1936.¹⁴ The rationalization of skin sensitizer properties in terms of electrophilicity or proelectrophilicity of the chemicals, enabling them to bind to proteins, is supported by observations from Rosenkranz et al.¹⁵ where 30–40% out of 355 randomly chosen chemicals that are human sensitizers were electrophilic. While the majority of electrophilic chemicals are known to exist within the five common mechanistic applicability domains (i.e., Michael addition (MA),

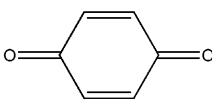
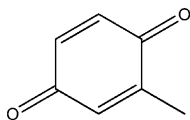
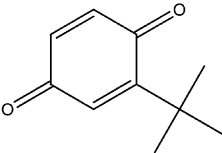
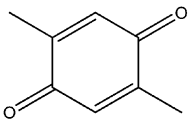
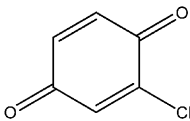
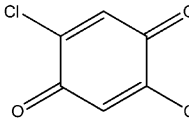
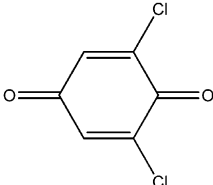
Schiff base formation (SBF), nucleophilic substitution ($S_N1/2$), nucleophilic aryl substitution (S_NAr), and acylation (AA)), other less apparent mechanistic domains may contribute to the reactivity of chemicals and their ultimate allergenicity. We have previously reported the haptenation of mercaptobenzothiazole to proteins via disulfide formation as an example of nonelectrophile–nucleophile interaction.¹⁶ Binding of the skin sensitizer 7-oxodehydroabietic acid to proteins has been reported to be via a free radical mechanism¹⁷ showing it as a viable protein modification pathway. Diverse BQ–protein haptenation pathways/mechanisms may result in different adducts with varying immunogenicity. Understanding and characterization of the dominant reaction pathways will facilitate the filling of knowledge gaps in quantitative mechanistic models for identifying and ranking the potency of skin sensitizers.

The need for reliable nonanimal based methods for the identification of chemical sensitizers and the evaluation of their sensitization capacity have led to the development of a number of *in vitro* and *in chemico* methods. New legislation (e.g., the REACH initiative in the European Union) has led to several initiatives to increase the acceptance of reactivity and (quantitative) structure–activity relationships (QSARs) based

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Table 1. Benzoquinone and Benzoquinone Derivatives That Were Reacted with NBT

 <p>p-benzoquinone (BQ) (2)</p>		
Electron Donating Substituents		
 <p>2-methylbenzoquinone (MBQ) (3)</p>	 <p>2-tertbutylbenzoquinone (tBBQ) (4)</p>	 <p>2,5-dimethylbenzoquinone (DMBQ) (8)</p>
Electron Withdrawing Substituents		
 <p>2-chlorobenzoquinone (CBQ) (5)</p>	 <p>2,5-dichlorobenzoquinone (2,5-DCBQ) (6)</p>	 <p>2,6-dichlorobenzoquinone (2,6-DCBQ) (7)</p>

methods to reduce reliance on animal testing. The present study utilizes NBT in an *in chemico* assay that was developed for screening thiol reactive haptens for their skin sensitizing potency.¹⁸ In this article, we investigate reactivity based QSAR analysis for BQ and BQD (Table 1), which have been hypothesized to react via MA. We also hypothesize that BQ and BQD may also react to NBT via an alternate mechanism involving BQ free radicals.¹⁹ Using BQ as the central molecule, we present the reactivity of BQ and BQD to NBT where we investigate the effect of activating and deactivating substituents on the reactivity and mechanistic pathways of BQ and BQD. BQ-protein haptenation is discussed in terms of the dominant reaction mechanism between Michael addition and a free radical mediated mechanism, and the influence of substituent effects on the binding.

EXPERIMENTAL PROCEDURES

Chemicals. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were used without further purification unless otherwise noted.

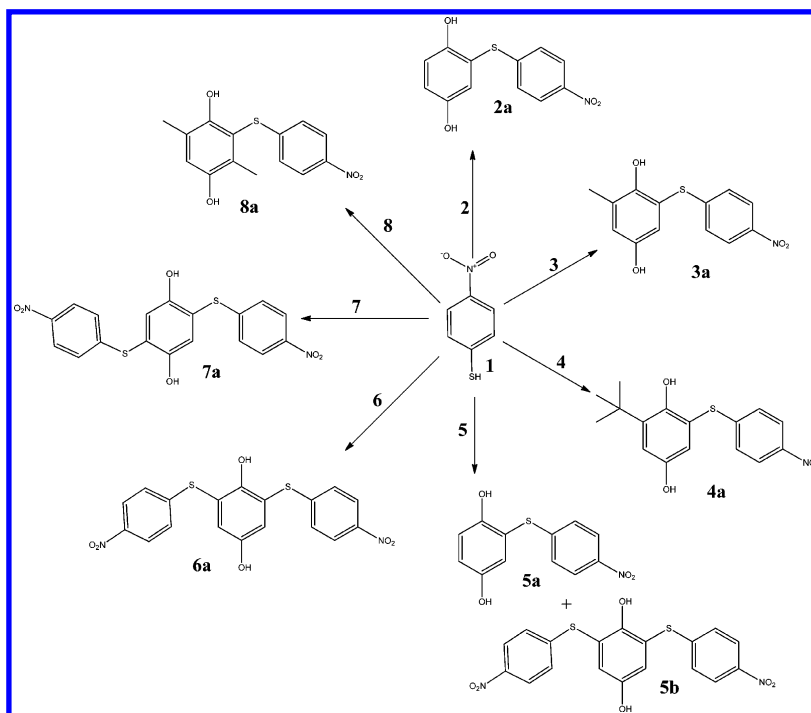
Thiol Binding Determination. The method reported by Schultz et al.²⁰ was used with slight modification. Briefly, Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid; DTNB) and cysteine (5 mM) stock solutions were prepared in a 50 mM ammonium carbonate buffer ($(\text{NH}_4)_2\text{CO}_3$; pH 7.4) buffer. Cysteine (Cys) standards from 50 to 250 μM were reacted with 1 mM DTNB at room temperature in a total volume of 2 mL. Absorbance readings taken at 412 nm ($\epsilon_{412\text{ nm}} = 14,150\text{ M}^{-1}\text{ cm}^{-1}$) after 15 min were plotted against the final cysteine concentration to give the standard curve. This calibration curve was used to determine Cys and glutathione (GSH) binding to BQ following the reaction of 0.125 mM Cys/GSH with 0.625 mM BQ for 2 h with subsequent addition of 0.625 mM DTNB for 15 min. The calibration curve was used to determine human serum albumin (HSA) reduced sulphydryl concentration ($[\text{SH}]/[\text{HSA}]$) following the reaction of 0.8 mg/mL ($\approx 11.9\text{ }\mu\text{M}$) HSA with 0.625 mM DTNB for 15 min with subsequent absorbance readings at 412 nm. BQ (0.625 mM) was incubated with 0.8 mg/mL ($\approx 11.9\text{ }\mu\text{M}$) HSA for 2 h with subsequent addition of 0.625 mM DTNB to measure the loss of Cys34 reactivity.

Preparation of Reduced Thiols. The reconversion of the Cys34 residue on HSA to the free sulphydryl form was performed by incubating equal volumes of 4 mg/mL HSA with 4 mM dithiothreitol (DTT) in $(\text{NH}_4)_2\text{CO}_3$ buffer at room temperature for 3 h, followed by dialysis (molecular mass cutoff of 3500 Da) in 2 L of 50 mM $(\text{NH}_4)_2\text{CO}_3$ for 48 h with three buffer changes during that period to ensure complete removal of DTT. The postdialysis HSA concentrations were determined using the Bradford assay²¹ with human serum albumin (HSA) as the standard protein. HSA thiol concentrations were determined using Ellman's reagent as outlined above, and the extent of thiol blocking with BQ was measured with Ellman's reagent and compared with blocking performed with the thiol specific reagent, N-ethylmaleimide (NEM).

Extent of BQ Binding to Cys34 in HSA. A stock solution consisting of 4 mg/mL HSA was prepared in 50 mM $(\text{NH}_4)_2\text{CO}_3$. Then, 0.8 mg/mL ($\approx 11.9\text{ }\mu\text{M}$) HSA was incubated with 250 μM BQ for 1 h on a shaker at 37 °C followed by dialysis for 48 h. Loss of DTNB reactivity (as described above) was used to determine the loss of thiol reactivity as a result of BQ covalent binding. As controls, HSA thiol concentrations were determined for (i) fresh HSA in buffer that was not incubated or dialyzed, (ii) HSA (in the absence of BQ) that had been incubated under the same conditions (as the BQ-treated HSA) and dialyzed, and (iii) HSA which had been reduced by dithiothreitol (DTT) and dialyzed. A comparison of the kinetics of the reaction of DTNB with (i) reduced HSA, (ii) reduced and dialyzed HSA, and (iii) reduced HSA incubated with BQ with subsequent dialysis was performed. Human albumin has only one free thiol, Cys34. To ascertain the Cys34 as the BQ-targeted residue, the thiol-specific blocking reagent N-ethylmaleimide (NEM) was reacted with reduced HSA (as a positive control) prior to reactions with DTNB. DTT-reduced HSA (0.8 mg/mL, 11.9 μM) was reacted with 250 μM NEM for 30 min in a pH 7.4 $(\text{NH}_4)_2\text{CO}_3$ buffer at room temperature with subsequent dialysis. Aliquots were taken for $[\text{SH}]/[\text{HSA}]$ determination with DTNB. HSA pretreated with BQ and incubated with DTNB was also subjected to the same analysis.

Extent of BQ Binding to Primary Amines in HSA. A stock solution consisting of 0.8 mg/mL HSA prepared in PBS (pH 7.4) was incubated with 250 μM BQ for 1 h at room temperature followed by dialysis for 48 h against PBS buffer. The trinitrobenzene sulfonic acid (TNBS) amine specific probe assay was performed on HSA and HSA-BQ conjugates. HSA (500, 400, 200, 100, and 50 $\mu\text{g/mL}$) was

Scheme 1. Reaction Mechanisms for Electron-Withdrawing and Electron-Donating- Substituted Benzoquinones with NBT



prepared in PBS buffer (pH 7.4). TNBS (5% w/v) was diluted 1:5.48 with 0.1 M borate buffer (pH 9.3). To 500 μ L of HSA and HSA-BQ, 12.5 μ L of TNBS was added, mixed well, and left to react for 30 min, and absorbance was measured at 420 nm.

Whole Human Fresh Blood Collection and Serum Separation. Whole blood was drawn into 10 mL of BD Vacutainer Serum Separation Tubes containing gel and clot activator transport, and inverted four times for the blood to mix with the tube additives. Blood was allowed to sit for 30 min at 37 $^{\circ}$ C for clotting to occur. Serum was separated by centrifugation of the tubes at 1000g for 10 min at 4 $^{\circ}$ C. The serum was transferred into plastic tubes using glass Pasteur pipettes and aliquoted before the determination of protein concentration. The aliquoted samples were stored at -20° C. The Cys-DTNB calibration curve was used to determine serum total sulfhydryl concentration ($[SH_T]$) following the reaction of 0.1 mL of 0.1 mg/mL serum protein with 0.4 mL of 0.5 mM DTNB for 15 min with subsequent absorbance readings at 412 nm. The calibration curve was also used to determine the loss of $[SH_T]$ and the ratio $[SH_T]/[SH_T]$ (SH_T = blocked thiol) when serum was incubated with BQ prior to DTNB reactivity. To measure the binding of BQ and NEM to high molecular weight thiols, dialysis of the serum samples was performed for 48 h against PBS to remove low molecular weight thiols prior to incubation with BQ/NEM and subsequent determination of thiol binding with DTNB. The ratio of blocked thiol postdialysis (SH_d) to total thiol of the control postdialysis SH_{Td} ($= [SH_d]/[SH_{Td}]$) was also determined.

BQ and BQD Binding Kinetic Studies. Reaction kinetics were measured on a Hitech Scientific (Bradford-on-Avon, UK) SF-61DX2 double-mixing stopped-flow spectrophotometer with an F/4 Czerny-Turner MG-60 monochromator and a spectra scan control unit. The signal from the spectrophotometer was amplified and digitized via an Omega Engineering DAS-50/1 16-bit A/D board interfaced to a computer for storage and data analysis. Reaction progress was followed by monitoring the loss of free thiol on NBT at 412 nm, where it has its highest molar absorptivity coefficient (ϵ) as previously described.¹⁸

Test chemicals were dissolved in acetonitrile at concentrations ranging from 0.01 to 10 mM. These solutions (5 μ L) were combined with 5 μ L of 0.1 mM NBT in phosphate buffer (pH 7.4) in a sealed reaction cell with rapid mixing. Absorbance readings were collected after a dead time of 1 ms. Control experiments contained test

chemicals in acetonitrile/phosphate buffer to determine background absorbance. Five replicates were performed for each chemical at each concentration. The temperature was maintained at 25 $^{\circ}$ C in the observation cell with a VWR International (Radnor, PA) circulating water bath.

Electron Paramagnetic Resonance (EPR) Spectroscopy. EPR spectroscopy was carried out using a Bruker (Fremont, CA) EMX spectrometer equipped with a high-sensitivity cavity and an Aqua-X sample holder. Spectra were obtained at room temperature. Typical EPR parameters were as follows: 100 G sweep width (for the spin trapping experiments with 5,5-dimethyl-1-pyrroline *N*-oxide, (DMPO)); 9.77 GHz microwave frequency; 32 mW power; 2×10^5 receiver gain; modulation frequency of 86 kHz; modulation amplitude of 2 G; with the conversion time 5 ms and time constant being 10 ms with 200 X-scans for each 512 point spectrum. The final concentrations of BQD derivatives were 50 μ M, and DMPO was 100 mM unless specifically mentioned. Buffer solutions were prepared by adjusting with 5 M sodium hydroxide solution or 1 M hydrochloric acid. The effect of NBT on the generation of free radicals was studied by incubating 0.1 mM NBT with 1 mM BQD + 100 mM DMPO. Spectral simulations of EPR spectra were performed using the WinEPR program developed by Bruker, and the coefficients of simulated spectra were >0.96 .

Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy. Reagents were from Sigma Aldrich and used as supplied. The reaction mixture of 6-fold molar excess of NBT samples were incubated in phosphate buffer at pH 7.4 for 24 h. The reaction mixture was purified by preparative TLC (10–20% EtOAc/80–90% *n*-hexane) for samples of compounds 3–7 (Scheme 1) to obtain the products in a pure state, while compound 8 was separated using Teledyne Isco Combiflash Rf 200 PSI. After completion, solvents were evaporated using a rotor evaporator, and the products were dissolved in appropriate solvents for spectroscopy analysis. Mass spectra of products from reaction mixtures were taken on a high-resolution ($m/\Delta m = 30\,000$) Thermo Scientific LTQ-Orbitrap Discovery mass spectrometer (San Jose, CA) equipped with an electrospray ionization source. All mass spectrometer samples were dissolved in an MeOH/ H_2O (1:1) mixture. The MS ESI source parameters were set as follows: spray voltage (kV), 2.5; spray current (μ A), 1.96; sheath gas flow rate, 20; auxiliary gas flow rate, 0.01; capillary voltage (V), -16 ;

capillary temperature ($^{\circ}\text{C}$), 300; and tube lens (V), -115 . Detection was carried out in the negative ionization mode ($-\text{ESI}$) for 2 min in the mass range m/z of 100–1000. The detection parameters were set up as follows: Analyzer; FTMS, negative polarity; mass range; normal, resolution; 30 000, scan type; centroid. ^1H NMR spectra were recorded with a Bruker AMX-400 MHz spectrometer using CDCl_3 and D_2O as solvents. Chemical shifts were reported as values in ppm relative to CHCl_3 (7.26) in CDCl_3 and TMS, which was used as an internal standard.²²

2-((4-Nitrophenyl)thio)benzene-1,4-diol (2a and 5a). The title compound was a product of both BQ and CBQ reaction with NBT. Compound 2a was recovered by solvent extraction using *n*-hexane, which was evaporated using a rotor evaporator. ^1H NMR (CDCl_3 , 400 MHz) δ 8.10 (d, $J = 9.0$ Hz, 2H), 7.14 (d, $J = 9.0$ Hz, 2H), 7.05–6.94 (m, 3H). ESI-MS exact mass calculated for $[\text{M}-\text{H}]^-$ requires, m/z 262.01795; found, 262.01779 2a and 262.01746 for 5a, and ESI-MS data was recorded using a scan range of m/z 100–600.

2-Methyl-6-((4-nitrophenyl)thio)benzene-1,4-diol (3a). The title compound was produced as yellow crystals from the reaction of MBQ with NBT. The reaction mixture was purified by preparative TLC with the eluent ethyl acetate/*n*-hexane (1.5:8.5 ratio). ^1H NMR (CDCl_3 , 400 MHz) δ (ppm) 8.32 (d, $J = 8.9$ Hz, 9H), 7.71 (d, $J = 8.9$ Hz, 11H), 6.56 (s, 1H), 5.90 (s, 2H), 2.12 (s, 7H). For MS analysis, the ESI-MS solvent was 50:50 water/methanol, which resulted in the m/z 276.03259, against the exact mass $m/z = 276.03360$ calculated for $[\text{M}-\text{H}]^-$.

2,5-Bis((4-nitrophenyl)thio)benzene-1,4-diol (7a). Data: The reaction mixture was purified by preparative TLC, eluent ethyl acetate/*n*-hexane (1:9 ratio), and orange crystals were obtained. ^1H NMR (CDCl_3 , 400 MHz) δ (ppm) 8.13 (d, $J = 9.1$ Hz, 2H), 7.30 (s, 1H), 7.17 (d, $J = 9.1$ Hz, 2H).

2,5-Dimethyl-3-((4-nitrophenyl)thio)benzene-1,4-diol (8). Data: Flash chromatography eluent, *n*-hexane = 100% with a reaction time of 1 min, then ethyl acetate/*n*-hexane = 1:1 for 12 min. Fine yellow crystals were obtained after drying the solvent on a rotor vapor. ^1H NMR (CDCl_3 , 400 MHz) δ (ppm) 8.08 (d, $J = 8.7$ Hz, 2H), 7.09 (d, $J = 8.7$ Hz, 2H), 6.82 (s, 1H), 4.77 (s, 2H), 2.35–2.15 (m, 6H). ^{13}C NMR (CDCl_3 , 100 MHz) δ (ppm) 125.76 (d, $J = 7.3$ Hz), 124.30, 121.14, 77.34, 16.39, 13.79.

RESULTS

HSA Thiol-BQ Reactions. Loss of thiol reactivity to DTNB was observed with both fresh HSA and DTT-reduced HSA when they were incubated with BQ before reacting with DTNB. In order to confirm the thiol residue as the target for BQ, the thiol blocking reagent *N*-ethylmaleimide (NEM) was incubated with DTT-reduced HSA followed by dialysis before the addition of DTNB. Figure 1 shows that BQ and NEM resulted in $72.3 \pm 2.6\%$ and $90.4 \pm 2.3\%$ loss, respectively, of HSA thiol-DTNB reactivity after 5 min at 21:1 molar ratios of BQ/HSA and NEM/HSA. A paired *t* test of the thiol losses demonstrated a significant difference ($\alpha < 0.05$) between BQ- and NEM-induced thiol loss. Dialysis of both the HSA-BQ and HSA-NEM adducts did not result in the recovery of Cys34 reactivity confirming that both BQ and NEM bind irreversibly to Cys34 on HSA. There was, however, a slight dilution of the protein concentration after dialysis. Preincubation of HSA for 30 min with BQ or NEM with subsequent DTNB reactivity resulted in $85.4 \pm 3.7\%$ and $91.5 \pm 4.2\%$ thiol loss, respectively (Figure 1). The thiol loss between BQ and NEM were not statistically significant ($\alpha = 0.16$) after 30 min.

BQ Reactivity to Primary Amines in HSA. TNBS reactivity of accessible primary amines on nonconjugated HSA was compared to HSA that had been conjugated with BQ. There was $18.4 \pm 2.9\%$ loss of TNBS amine reactivity when BQ was incubated with HSA in 50 mM $(\text{NH}_4)_2\text{CO}_3$, pH 7.4 buffer. Interestingly, BQ amine binding was enhanced to

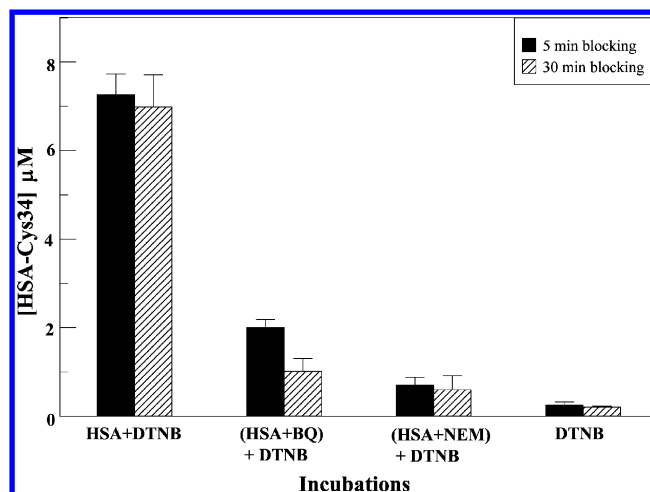
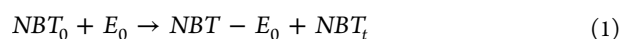


Figure 1. Measurement of BQ binding to HSA Cys34 by the loss of DTNB reactivity. HSA was treated with DTT, dialyzed, and reacted with BQ. NEM was used as a positive control. (a) 0.8 mg/mL HSA reacted with 1 mM DTNB, (b) 0.8 mg/mL HSA preincubated with 1 mM BQ reacted with 1 mM DTNB, (c) 0.8 mg/mL HSA preincubated with 1 mM NEM reacted with 1 mM DTNB, and (d) 1 mM DTNB.

$35.6 \pm 6.6\%$ of TNBS reactive HSA amines when BQ conjugation was performed in PBS (pH 7.4). Dialysis of the HSA-BQ adduct did not result in recovery of primary amine reactivity confirming that BQ also binds irreversibly to primary amines on HSA.

Serum Thiol-BQ Reactivity. The serum total sulfhydryl concentration ($[\text{SH}_\text{T}]$) following reaction with DTNB for 15 min was $221 \mu\text{M}$. Dialysis of the serum samples removed low molecular weight thiols with resultant $[\text{SH}_\text{Td}]$ being $117 \mu\text{M}$. For the undialyzed serum BQ and NEM, thiol loss ($[\text{SH}_\text{T}]/[\text{SH}_\text{Td}]$) after 5 min was 74.4 ± 5.6 and $89.2 \pm 3.7\%$, respectively, showing significant difference ($\alpha < 0.05$) between BQ and NEM. Preincubation for 30 min resulted in 90.7 ± 6.7 and $93.5 \pm 5.9\%$ thiol loss, respectively, which was not statistically significant ($\alpha > 0.05$). Dialysis of the serum samples incubated with BQ and NEM resulted in reduced DTNB reactivity for the control serum sample and all other samples even though the postdialysis thiol loss $[\text{SH}_\text{d}]$ per total serum thiol of the control post dialysis $[\text{SH}_\text{Td}]$ did not change. The thiol loss was reduced to 67.9 ± 4.7 for BQ and $70.1 \pm 3.9\%$ for NEM in 5 min preincubations. When the BQ and NEM preincubation period with the dialyzed serum samples was extended to 30 min, the thiol losses were $76.9 \pm 5.3\%$ and $82.6 \pm 7.1\%$, respectively.

NBT-BQ/BQD Reactions. The previously reported nitro-benzenethiol assay for screening thiol reactive haptens¹⁸ was used in this study to determine the reactivity of BQ(D) electrophilic species (E). The decrease in absorption of NBT at 412 nm ($\epsilon_{412 \text{ nm}} = 11068 \pm 126 \text{ M}^{-1}\text{cm}^{-1}$) was measured after rapid mixing with BQ(D). As an example, Figure 2 shows the consumption of NBT with MBQ. The NBT-E reactions were measured at different time intervals from 0.1 s to 10 min under pseudofirst order conditions ($[\text{NBT}] \ll [\text{E}]$).



The absorbance of the remaining free thiol at time *t*, was used to calculate $[\text{NBT}]_t$ using the molar absorptivity of NBT.

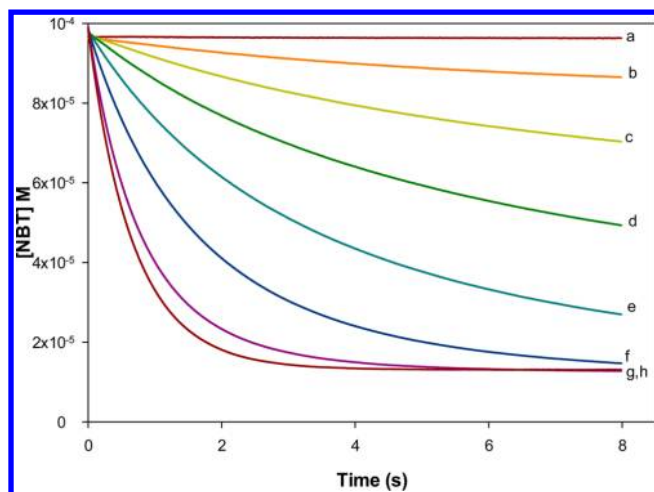


Figure 2. Reaction of MBQ with NBT in 50% acetonitrile in a pH 7.4 phosphate buffer at 25 °C. Absorbance readings were performed at 412 nm. Using $[NBT]_0 = 0.1$ mM. BQ concentrations were varied from (a) 0.0 mM, (b) 0.0125 mM, (c) 0.025 mM, (d) 0.05 mM, (e) 0.1 mM, (f) 0.2 mM, (g) 0.4 mM, and (h) 0.8 mM.

$$[NBT]_t = \frac{A_{NBT_t}}{\epsilon} \quad (2)$$

Figure 3 shows how the apparent rate constant ($k = -k_a[NBT]_0$) and order of reactions with respect to electrophile

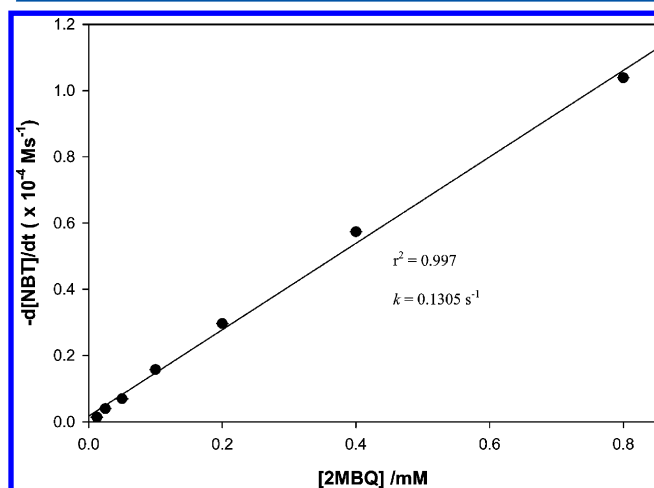


Figure 3. Initial rate vs [MBQ]. Rate constants are calculated from the slope of the $[E]$ vs initial rate for NBT depletion.

were determined from the slope of at least five initial rates (R_i) against five concentration variations of E for a fixed $[NBT]$. Linear plots which were obtained with all NBT-E reactions (Figure 3, $r^2 = 0.997$) confirmed that the reactions were pseudofirst order with respect to BQ(D). The order of reactions of BQ(D) and the apparent rate constants (k) are listed in Table 2. Equation 3 for initial rates, derived from eq 1, was integrated to give eq 4 for the overall reaction where $k' = k_a[E]_0$.

$$R_i = \frac{d[NBT]}{dt} = -k_a[NBT]_0[E]_0 \quad (3)$$

$$\ln\{[NBT] - [NBT]_\infty\} = -k't + \ln[NBT]_0 \quad (4)$$

Table 2. Rate Constants Derived from the Initial Rate Methods for NBT-BQ/BQD Reactions

chemical	k (s^{-1})	r^2	order
BQ (pH 7.4)	6.96×10^{-1}	0.996	1
BQ (pH 5.5)	1.622	0.989	1
(EDG) pH 7.4			
MBQ	7.75×10^{-3}	0.994	1
tBBQ	1.91×10^{-3}	0.991	1
DMBQ	7.22×10^{-5}	0.994	1
(EWG) pH 7.4			
CBQ	1.023	0.996	1
2,6-DCBQ	8.72×10^1	0.987	1
2,5-DCBQ	1.97×10^2	0.989	1
(EDG) pH 5.5			
MBQ	1.31×10^{-1}	0.997	1
tBBQ	3.32×10^{-2}	0.999	1
DMBQ	2.30×10^{-3}	0.995	1

The NBT depletion data for all initial concentrations of the BQD electrophiles (E) were fitted into the integrated rate law (eq 4) and the k values given in Table 3 calculated for the given

Table 3. Rate Constants and Half Lives for BQ and BQD on pH 7.4 and 5.5

chemical	k_a (s^{-1})	k_s (s^{-1})	$t_{1/2}(s)$	k_i (s^{-1})
BQ (pH 7.4)	1.66×10^3	1.50×10^3	4.44×10^{-4}	1.06×10^{-2}
BQ (pH 5.5)	2.61×10^4	2.26×10^4	3.07×10^{-5}	6.94×10^{-1}
(EDG) pH 7.4				
MBQ	3.87×10^2	3.23×10^2	2.14×10^{-3}	5.74×10^{-3}
tBBQ	2.39×10^1	2.31×10^1	3.00×10^{-2}	8.06×10^{-4}
DMBQ	7.47×10^{-1}	7.27×10^{-1}	9.54×10^{-1}	2.93×10^{-4}
(EWG) pH 7.4				
CBQ	2.18×10^5	2.62×10^5	2.64×10^{-6}	3.78×10^{-1}
2,6-DCBQ	1.60×10^6	1.21×10^6	5.74×10^{-7}	2.98
2,5-DCBQ	2.92×10^6	2.83×10^6	2.45×10^{-7}	2.99×10^{-1}
(EDG) pH 5.5				
MBQ	1.51×10^3	1.60×10^3	4.34×10^{-4}	1.71×10^{-3}
tBBQ	3.60×10^2	3.37×10^2	2.06×10^{-3}	3.33×10^{-2}
DMBQ	1.05×10^1	1.25×10^1	5.56×10^{-2}	1.48×10^{-3}

$[E]$ variations for all BQD. NBT is amenable to oxidative reactions such as disulfide formation, which can compete with NBT reactions with the BQD. The side reactions for NBT were minimized by varying initial $[E]_0$ and plotting the slopes, $(-k_s[E]_0)$, obtained from plots of data fitted into eq 4 against $[E]_0$. By assuming that $k_i \ll k_s$ within the overall reaction time, where k_i is the rate of loss of NBT due to side reactions, and k_s is the rate of NBT reaction with E, eq 5 is reduced to eq 6.

$$R = \frac{-d[NBT]}{dt} = k_s[E]_0[NBT]_0 + k_i[NBT] \quad (5)$$

$$R = \frac{d[NBT]}{dt} = k_s[E]_0[NBT]_0 \quad (6)$$

Pseudofirst-order rate constants were calculated as the slopes of the resultant linear curves ($[E]$ vs $-k_a[E]_0$), and the intercept gave the pseudofirst-order rate constant k_i for the side reaction. The rate constants k_a , k_s and k_i are listed in Table 3. The rate constant values for k_a and k_s were not significantly different for all BQD. There was an increase in the disulfide formation or

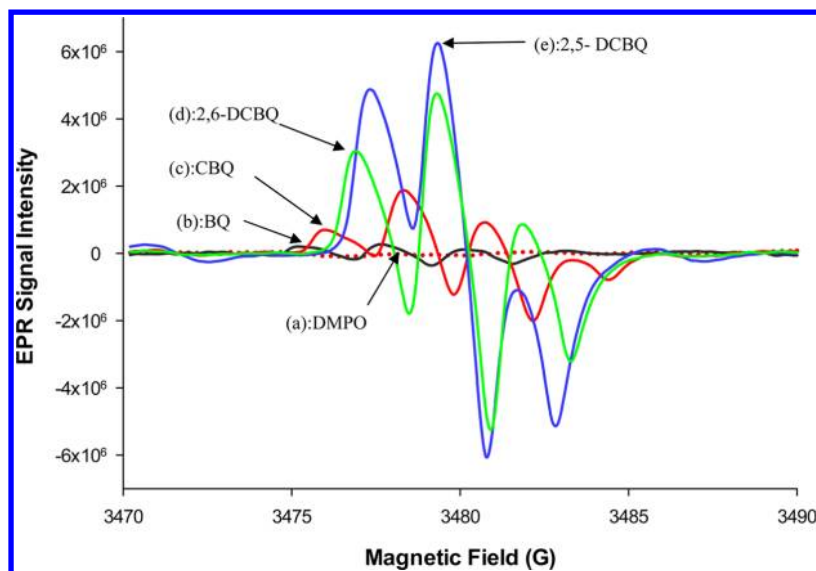


Figure 4. Effects of chlorine substitution on BQ on EPR signal intensity for (a) DMPO, (b) BQ, (c) CBQ, (d) 2,6-DCBQ, and (e) 2,5-DCBQ in 50 mM phosphate buffer, 100 mM DMPO, and 50 μ M of the corresponding BQD at 25 $^{\circ}$ C.

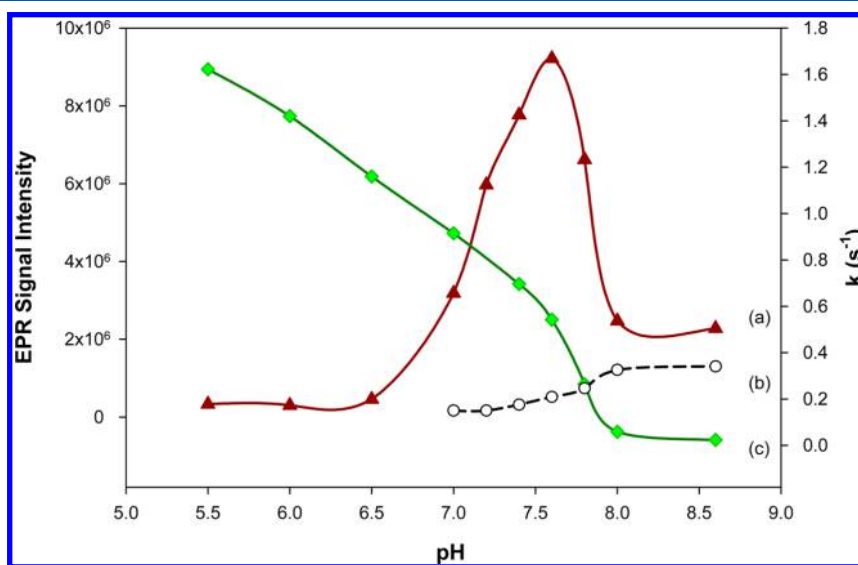


Figure 5. Relationship between EPR signal intensity and rate constants due to pH variation for $[2,6\text{-DCBQ}]_0 = 50 \mu\text{M}$ in 50 mM phosphate buffer and 100 mM DMPO at 25 $^{\circ}$ C. (a) EPR signal due to DMPO-SQ adduct formation, (b) EPR signal due to DMPO-OH, and (c) the effect of pH on the rate of reaction between 2,6-DCBQ and NBT. There is an inverse relationship between the rate constant and the EPR DMPO-SQ signal.

loss of NBT for chlorine-substituted BQ as indicated by higher k_i values (ranging from 2.98 s^{-1} for CBQ to 0.387 s^{-1} for 2,6-DCBQ). The rate constants in Tables 2 and 3 demonstrate that the reactivity of BQ decreased significantly when electron-donating groups were added with the observed order $(\text{CH}_3)_3\text{C} < \text{CH}_3 < \text{H}$. The reaction half-lives ($t_{1/2}$) ranged from 0.4 ms (BQ) to 0.95 s (tBBQ). The reactivity of BQ was enhanced significantly when the substituents were electron-withdrawing. The order of reactivity was $\text{BQ} < \text{CBQ} < 2,6\text{-DCBQ} < 2,5\text{-DCBQ}$ as demonstrated by the k values in Tables 2 and 3. In comparison, the rate constants for electron-withdrawing substituents (EWG) were much higher than those of electron-donating substituents (EDG) at pH 7.4. For example, the rate constant for CBQ was 132-fold higher than that for MBQ, and that of 2,5-DCBQ was 2.5×10^6 higher than that of DMBQ. The kinetics at pH 5.5 for BQD with EWG were too fast to be measured by the stopped-flow spectrophotometer.

Benzoquinone Derivatives and Free Radical Formation.

Figure 4 (a–e) shows the EPR signals that were observed with EWG-substituted BQD. No EPR signals were observed with free DMPO and all EDG-substituted BQD. The EPR signal intensity was in the order $2,5\text{-DCBQ} > 2,6\text{-DCBQ} > \text{CBQ} > \text{BQ}$. This trend in the EPR intensity signal was the same trend that was observed with pseudofirst order rate constants. Reduction of the BQ resulted in a weak signal of approximately 1:2:1, a triplet line spectrum ($a^{\text{H}2} = a^{\text{H}3} = a^{\text{H}5} = a^{\text{H}6} = 2.4 \text{ G}$ at pH 7.4). Under the same conditions (50 mM DMPO and pH 7.4), there was a 5-fold increase in SQ yield from reduced CBQ compared to that from the reduced BQ radical. This demonstrated that the chlorine substituent has an effect on the SQ radical yield. CBQ reduction gave a quartet of lines, with a peak height ratio of 1:3:3:1 and hyperfine splitting of $a^{\text{H}3} = 2.2 \text{ G}$, $a^{\text{H}5} = 2.4 \text{ G}$, and $a^{\text{H}6} = 2.3 \text{ G}$ as shown in Figure 4. The addition of another chlorine atom (2,6-DCBQ) resulted

in a 3-fold increase in signal intensity of the corresponding SQ radical compared to the EPR signal from CBQ. 2,6-DCBQ gave the expected triplet line spectrum (1:2:1) $a^{\text{H}3} = a^{\text{H}5} = 2.5$ G. There was an observed 1.3-fold increase in the signal intensity signifying an increase in stabilization effect when the second chlorine was placed in the fifth position of the ring to form 2,5-DCBQ. A triplet line spectrum (1:2:1) was also observed with 2,5-DCBQ, with $a^{\text{H}3} = a^{\text{H}6} = 2.2$ G. There was an observed shift in peaks to higher magnetic fields and g -values whenever a chlorine atom was added or changed positions on the BQ, and the same trend of increase (depending on the number and position of the chlorine atom) was observed in EPR signal intensity.

Effect of pH on Radical Formation. The determination of the effect of pH on radical formation was done using phosphate buffer from pH 5.5 to pH 8.6. Using 2,6-DCBQ as an example, Figure 5 shows that there was no EPR signal detected when pH was below 6.5. The increase in pH from 6.5 introduces more OH^- ions into the system, which act as a reducing agent to quinones giving semiquinone radicals.²³ The peak signal intensity went up from pH 6.5 to 7.6 and then down after pH 7.6. Under the aerobic conditions of these experiments, the oxygen acts as a fast and efficient radical quencher while forming a superoxide or peroxy radical ($\text{ROO}\cdot$).²³ The decrease in signal intensity peak after pH 7.4 is attributed to the peroxy radicals, which are reported to form from dissolved oxygen.²⁴ The spectrum was composed of a quartet of lines, with a peak height ratio of 1:2:2:1, with parameters including hyperfine constants $a^{\text{H}} = 14.8$ G, and was attributed to the DMPO-OH. These parameters are typical of a DMPO-OH adduct EPR signal generated by the reaction of OH with DMPO.²⁵ The peaks at pH values > 7.6 increased in intensity then went down as the DMPO-OH adduct peaks appeared and increased in intensity. A proposed scheme leading to DMPO-OH formation is shown in Figure 6. The electron g -factors of BQD radicals

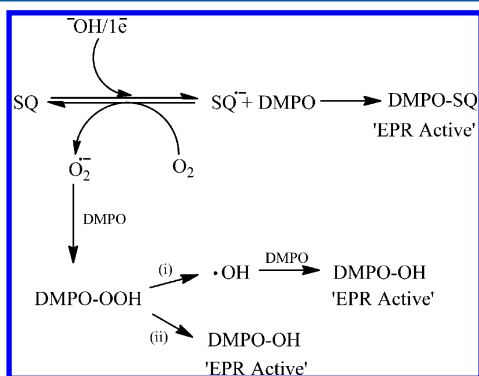


Figure 6. Schematic routes which lead to DMPO-SQ and DMPO-OH EPR active adduct formation.

changed with increasing number of chlorine atoms and position(s) of the chlorine substituents on the BQ ring (Table 4). Compared to results for the BQ radical ($g = 2.001382$), the CBQ had $g = 2.001534$ and $\Delta g = 0.000152$, and the 2,6-DCBQ had $g = 2.001585$ and $\Delta g = 0.000203$; and changing chlorine position to 2,5-DCBQ gave $g = 2.002077$ and $\Delta g = 0.000695$. This increase in g -value was harmonious with the increased spin orbit coupling that is expected with increasing number of chlorine atoms.

EPR Spectrum Simulations. The main objective with respect to spectrum simulations was to obtain a satisfactory

Table 4. Summary of Peak Shift and g -Factor Values

chemical	peak shift (mT)	g -factor
BQ	0 (standard)	2.001382
CBQ	0.0782	2.001534
2,6-DCBQ	0.176	2.001585
2,5-DCBQ	0.215	2.002077

simulation of the room-temperature EPR spectrum for BQ with EWG using CBQ and 2,6-DCBQ as examples (see Figure 7).

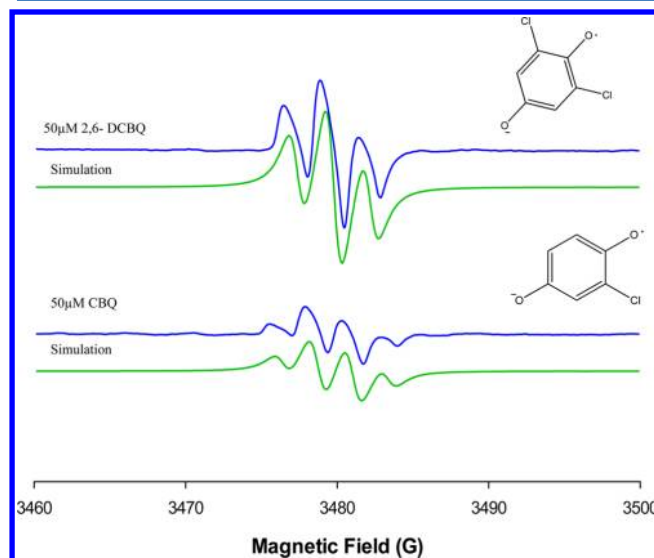


Figure 7. Computer simulations on the effects of chlorine substitution on BQ on EPR signal intensity data shown in Figure 5c (CBQ) and d (2,6-DCBQ). This model gave a reasonably good fit to the experimental data in both hyperfine splitting constants and the signal intensity.

The fitting of the data were simple, and the best fit values were obtained with hyperfine splitting constants $a^{\text{H}3} = a^{\text{H}5} = 2.5$ G for 2,6-DCBQ and $a^{\text{H}3} = 2.2$ G, $a^{\text{H}5} = 2.4$ G and $a^{\text{H}6} = 2.3$ G for CBQ. The adopted values for the hyperfine splitting constant are most likely valid for the BQD radical formation at physiological pH.

NBT Effect on SQ Radical Formation. Figure 8 shows that the addition of NBT to a DMPO and 2,6-DCBQ mixture decreased the amount of EPR signal formed even when the DMPO was in excess over both the NBT and the 2,6-DCBQ. Control experiments showed no reactivity between DMPO and NBT. Figure 8 shows that mixing NBT and 2,6-DCBQ in equimolar quantities resulted in about 50% loss of EPR signal. When NBT was 3-fold in excess over 2,6-DCBQ, the EPR signal loss was >90% in agreement with the NBT-2,6-DCBQ reactivity that was observed. The loss of EPR signal is due to NBT-BQ adduct formation (Scheme 2) overpowering the production of DMPO-SQ adduct formation. This shows that the rate of production of free radicals from 2,6-DCBQ is much slower than the rate of adduct formation between NBT and the 2,6-DCBQ. The NBT quenches the system of any available 2,6-DCBQ; thus, no radicals are produced. This suggests that the free radical mechanism is overshadowed by the MA and therefore has minimal contribution to the rate constants obtained for the rapid depletion of NBT.

Characterization of BQD-NBT Adducts. On the basis of NMR and MS characterization of the NBT-BQ(D) reaction

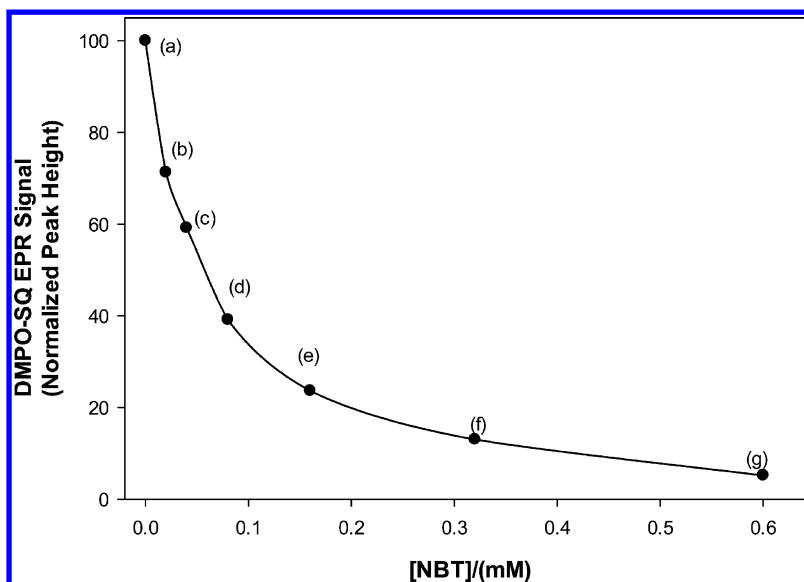
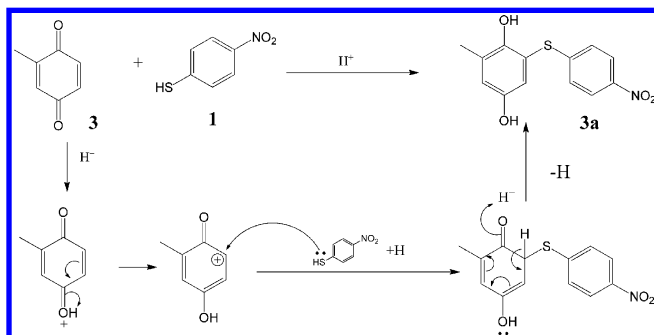


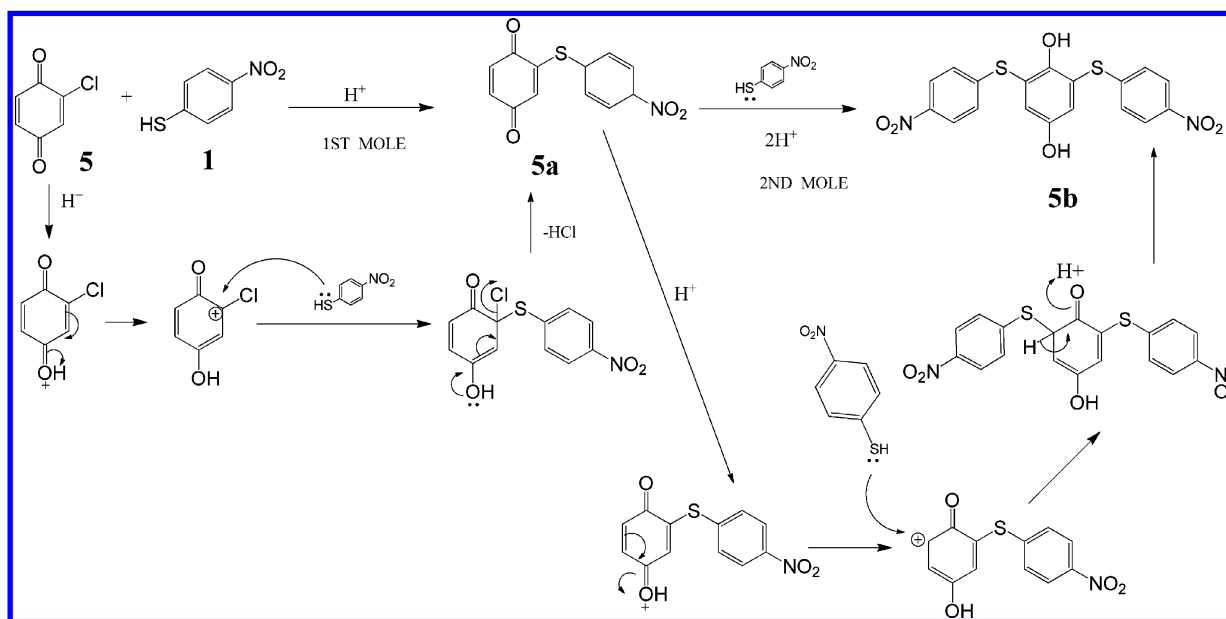
Figure 8. Plot of the depletion of the DMPO-SQ signal vs [NBT] at 25 °C. [DMPO]₀ = 100 mM, [2,6-DCBQ]₀ = 0.05 mM, and varied [NBT] = (a) 0 mM, (b) 0.02 mM, (c) 0.04 mM, (d) 0.08 mM, (e) 0.16 mM, (f) 0.32 mM, and 0.6 mM in 100 mM phosphate buffer at pH 7.4.

Scheme 2. Proposed MA Reaction between MBQ and *p*-Nitrobenzenethiol



products, NBT was shown to react with BQ 2 to give a 1:1 ratio product 2-((4-nitrophenyl) thiol) benzene-1,4-diol 2a in phosphate buffer at pH 5.5 after 1 h. The electron-donating-group-substituted benzoquinones reacted through MA (Scheme 2) giving a 1:1 ratio with NBT; MBQ 3 gave 2-methyl-6-((4-nitrophenyl)thio)benzene-1,4-diol 3a, tBBQ 4 gave 2-(*tert*-butyl)-6-((4-nitrophenyl)thio)benzene-1,4-diol 4a, and DMBQ 8 gave 2,5-dimethyl-3-((4-nitrophenyl)thio)-benzene-1,4-diol 8a. The reaction between CBQ 5 and NBT was involved in 2 reaction mechanisms in the following order: MA reaction followed by vinylic substitution (Scheme 3). The reaction between NBT and CBQ gave 2 products as confirmed by ESI-MS (Figure 9(a)): a monosubstituted benzoquinone, 2-((4-nitrophenyl)thio)benzene-1,4-diol 5a, and a disubstituted benzoquinone, 2,6-bis((4-nitrophenyl)thio)benzene-1,4-diol 5b. The reaction between the disubstituted chloro-benzoqui-

Scheme 3. Proposed Reaction between CBQ and *p*-Nitrobenzenethiol



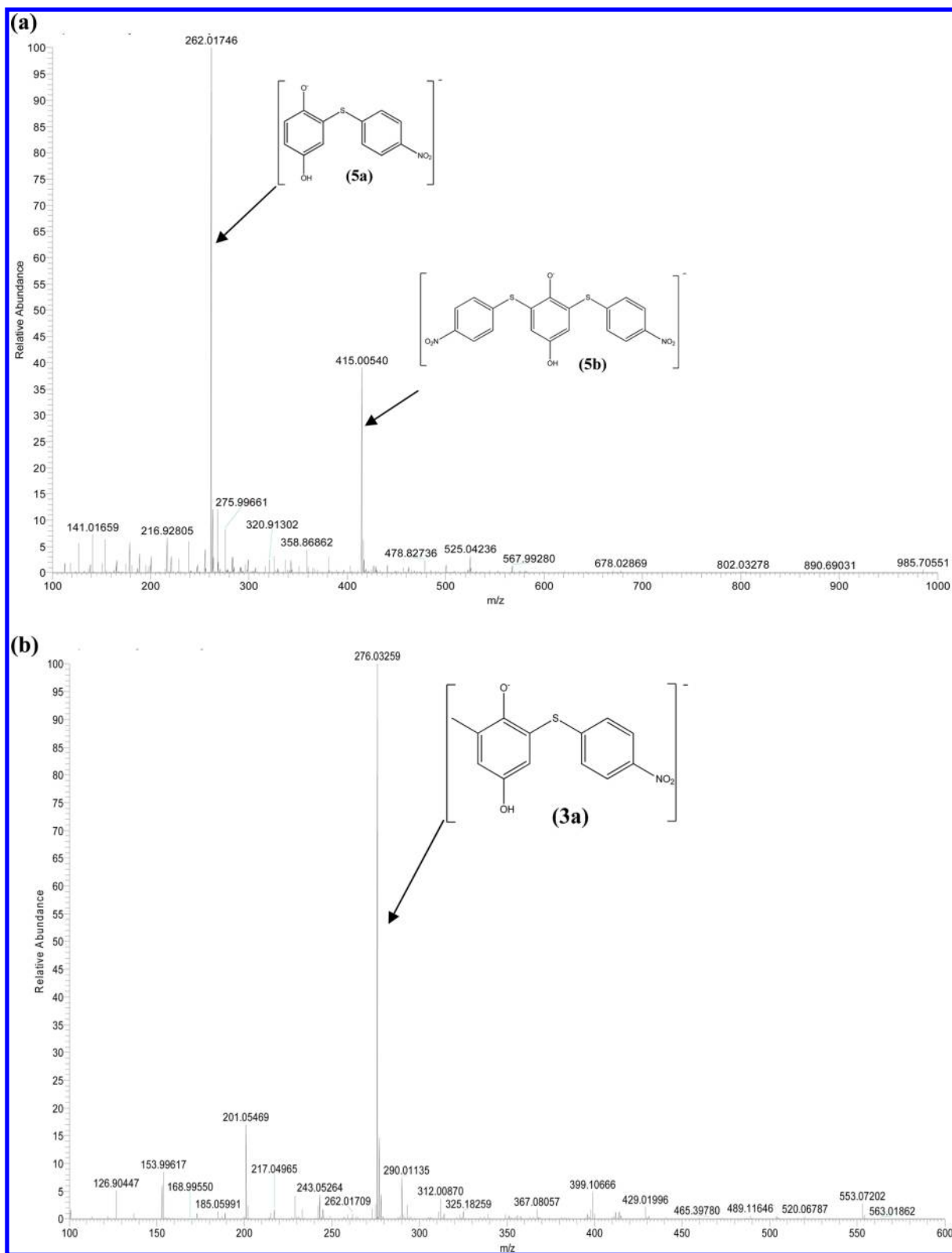


Figure 9. Negative ESI-MS spectra of the *p*-nitrobenzenethiol adduct(s) formation with (a) CBQ giving a mono-NBT-hydroxyl BQ adduct (*m/z* 415.00540) and a di-NBT-hydroxyl BQ adduct (*m/z* 262.01746), and (b) MBQ giving a mono-NBT-hydroxyl MBQ adduct (*m/z* 276.03259).

nones 2,5-DCBQ and 2,6-DCBQ gave corresponding disubstituted benzoquinone with chlorines being substituted by NBT in a 1:2 ratio (BQD/NBT). The reaction between 2,5-DCBQ **6** and NBT gave 2,5-bis((4-nitrophenyl)thio)benzene-1,4-diol **6a**, while 2,6-DCBQ **7** gave a corresponding disubstituted

benzoquinone, 2, 5-bis((4-nitrophenyl)thio)benzene-1,4-diol **7a**.

The BQ(D)-NBT reaction is acid catalyzed as shown by the faster reaction rate at lower pH. Acid facilitated the creation of a carbocation center on which the nucleophile attacks

(Schemes 2 and 3). The reactions between electron-donating-substituted benzoquinone and NBT were direct 1:1 ratio MA reactions (Scheme 3). NBT went onto position 6 for monosubstituted BQ and position 3 for disubstituted BQ (Scheme 1), and this was confirmed by ESI-MS (Figure 9b). For electron-withdrawing-substituted benzoquinones, the carbocation center was created on the carbon on which the chlorine atom was attached, and this was the target for the $-SH$ group on NBT followed by the substitution of the chlorine atom. In contrast to the methyl and *tert*-butyl-substituted BQ, the reaction mechanism for the chlorine-substituted BQs was direct nucleophilic vinylic substitution (SNV). As a result of the electron-withdrawing-substituents, CBQ gave 2-((4-nitrophenyl)thio)benzene-1,4-diol **5a** as a product, and 2,5-DCBQ gave 2,5-bis((4-nitrophenyl)thio)benzene-1,4-diol **7a**.

DISCUSSION

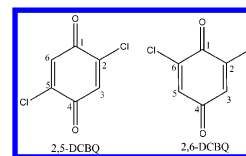
Murine local lymph node (LLNA) studies rank BQ as an extreme dermal sensitizer with an EC₃ (threshold) value of 0.013%.²⁶ BQ covalent binding to proteins resulting in immunogenic complexes has been attributed to BQ's reactivity as a Michael acceptor. New evidence^{27,28} on both the quinone's unique electrophilic chemistry and high reactivity, however, provides an alternative hypothesis on the possible contributory role of other reaction pathways to BQ's overall reactivity toward nucleophiles such as cysteinyl amino acids on proteins.²⁹ The impact of substituents on BQ's reactivity and ultimate *in vivo* allergenicity has not been previously assessed.

Binding experiments with HSA demonstrated that Cys34 (the only reduced thiol in HSA) on the protein is the preferred target for BQ binding and that the bond formed is an irreversible covalent bond. BQ was shown to have high affinity for thiols when serum was used as the source for thiols. Comparison of BQ and NEM-blocked thiols pre- and postdialysis showed that the ratios $[SH_r]/[SH_T]$ and $[SH_d]/[SH_{Td}]$ were equal, demonstrating the binding of BQ to both high and low molecular weight thiols such as cysteine and glutathione. The binding of BQ to cysteine and glutathione may result in reduced BQ available for protein binding, but its extreme potency²⁶ in the LLNA assay shows that the concentrations absorbed through the skin during the *in vivo* assay are high enough to overcome any clearance that may result from low molecular weight thiol binding. The observed 85.5% loss of reactivity to thiols compared to the 18% loss of reactivity to primary amines under identical conjugation conditions demonstrates that BQ is much more reactive to thiols than amines, in agreement with previously reported observations.³⁰

The thiol nucleophile, NBT, was used to measure the reactivity of six BQD. NBT has previously been utilized as a model nucleophile where the relative reactivity of electrophilic allergens to NBT was highly correlated to LLNA EC₃ values.¹⁸ All of the BQD tested rapidly reacted with NBT. There was, however, a 90-fold decrease in reactivity of the BQ ring when MBQ was reacted with NBT. The methyl group donates electrons into the BQ π system leading to an increase in electron density on the π system with consequent reduction of the partial positive charges on the electrophilic carbons on BQ. The π electrons also repelled the electrons on the $-SH$ group on NBT, thus slowing down the reaction. The positive inductive effect became more apparent when the methyl group was replaced with the *tert*-butyl group. The reaction dropped by approximately 4-fold from MBQ to tBBQ. This was

due to the higher electron-donating ability of *tert*-butyl than the methyl group. The steric hindrance offered by *tert*-butyl, to the incoming NBT, compared to the methyl group could also have contributed the decreased reactivity of tBBQ. For NBT-DMBQ reactions, the further 26-fold loss of reactivity relative to tBBQ can be attributed to the increased positive inductive effect of two methyl groups in close proximity to the reacting β carbocations. The repulsive force of the π electron cloud around the electrophilic carbon was increased by having two methyl groups on the ring.

With BQD with electron-withdrawing substituents the pseudofirst order rate constants for NBT reactivity at pH 7.4 increased in the following order BQ < CBQ < 2,6-DCBQ < 2,5-DCBQ (Table 4). The effect of the increased negative inductive effect on the rate constants is apparent from CBQ to the dichloro-substituted BQD. When 2,6-DCBQ was reacted with NBT, there was an 85-fold increase in NBT reactivity compared to that of CBQ. There was an observed increase in rate constants by 2-fold between 2,6-DCBQ and 2,5-DCBQ with the latter being faster. The difference in reactivity between the dichloro-substituted derivatives may be attributed to the fact the 2,5-DCBQ has its chlorine atoms in para positions to each other, which makes the carbon atoms on which the chlorine atoms are attached more partially positive due to the inductive withdrawal of electrons, unlike the 2,6-DCBQ, which has chlorine atoms in meta positions to each other and do not influence each other by inductive electron withdrawal. This makes 2,5-DCBQ more reactive than 2,6-DCBQ. This strongly agrees with the findings of Enoch et al.³¹ in which a direct relationship between electrophilic effects of a chemical and rate reaction by Michael addition reaction were observed and used in an electrophilicity index for the prediction of skin sensitization potential. For both 2,6-DCBQ and 2,5-DCBQ, the $-SH$ on NBT adds on to a position on which the chlorine atom is attached by MA, and then nucleophilic elimination of the chlorine atoms occurs (reaction of the 1st mole in Scheme 3).



The structure for 2,5-DCBQ is such that it will undergo MA and then nucleophilic substitution onto positions 2 and 5, which have the higher electrophilicity. 2,6-DCBQ where positions 2 and 6 are not influenced by the negative inductive effect of the chlorine atoms possesses a lower partial positive charge at these positions. This makes 2,5-DCBQ more reactive than 2,6-DCBQ. Increased rate constants increased several-fold for MBQ, DMBQ, and tBBQ when NBT reactivity was measured at pH 5.5, suggesting that the MA reactions are acid-catalyzed. Figure 5c shows an inverse relationship between rate constant and pH. The acid catalysis is more pronounced for CBQ, 2,5-DCBQ, and 2,6-DCBQ reactions performed at pH 5.5, which could not be measured using the stopped-flow technique. The fact that rate constants for MBQ, DMBQ, and tBBQ had increased several-fold under pH 5.5 but were still lower than those of CBQ, 2,5-DCBQ, and 2,6-DCBQ at pH 7.4 demonstrates the agreement on the effect of electron-donating versus electron-withdrawing substituents on BQ reactivity under pH values 7.4 and 5.5.

BQ has been hypothesized to haptenate proteins via MA. The production of semiquinone (SQ) radicals as a mechanism of BQ(D) protein haptenation was investigated, and EPR signal intensity was of the order 2,5-DCBQ > 2,6-DCBQ > CBQ > BQ. No EPR signals were observed with methyl- and *tert*-butyl-substituted BQD. BQ-generated radicals are known to form through the one electron reduction of BQ, followed by hemolytic cleavage of the O–O bonds on the carbonyl functional group. The resulting SQ radical may potentially bind directly to a protein.³² Thus, the presence of the SQ radical indicated the potential of free radical mechanisms as another mechanism of BQ(D) covalent modification of proteins. Increased production of free radicals from chloro-substituted BQD (Figure 4) is a result of the high negative inductive effect arising from the highly electronegative chlorines. This activates the BQ in contrast to the positive inductive effect of the electron-donating groups. The increase in *g*-values from the monochloro-substituted to the dichloro-substituted BQD is harmonious with the increased spin orbit coupling that will be expected with increasing number of chlorine atoms. Halogenation of BQ has been known to increase the persistence of SQ radicals.³³ The EPR results show that the yield of the SQ radicals resulting from chlorine-substituted BQD depends on pH and on the position and number of chlorines on the SQ ring.

The trend in the EPR intensity signal agreed with the observed order of reactivity indicating that inductive effects have a dominant influence on the chemistry of the BQD compared with other factors such as steric hindrance. The effect of pH on radical formation (Figure 5) highlights the sensitivity of the SQ radicals to either acidic or basic conditions. The increase in pH from 6.5 to 7.6 might have introduced more OH[−] ions into the system, which act as a reducing agent to quinones giving semiquinone radicals.²³ Beyond pH 7.6, the OH[−] effect might have been overcome by the presence of produced oxygen, which has been reported to act as a fast and efficient SQ radical quencher, forming a superoxide radical or peroxy radical (ROO·)²³ in the process (Figure 6). Peroxy radicals have been shown to produce dissolved oxygen in alkaline conditions, thus availing more oxygen, which quenches the SQ radical. The observed decrease in SQ radical peak intensity at pH > 7.6 (Figure 4) as the peaks of DMPO-OH adduct simultaneously started appearing can explain the minimum or no role of OH[−] and oxygen quenching in the temporary increase in SQ radical intensity at pH values 7.6 and below followed by oxygen quenching at pH values beyond 7.6.

The contribution of a free radical pathway to protein haptenation may not be significant because of the lack of production of SQ radicals at acidic pH, in contrast to the increased NBT reactivity that was observed at pH 5.5. Skin pH is around 5.5, and the lack of formation of radicals shown herein means that BQ and BQD binding to proteins will occur via MA with additional vinylic substitution where the substituents such as chlorine are good leaving groups. The dominance of MA versus free radical formation was also demonstrated with the inclusion of NBT to the (DMPO + BQ) mixture (pH 7.4), which resulted in quenching of the EPR signal even when the DMPO ≫ NBT and the BQD. The loss of EPR signal was due to NBT-BQ adduct formation (Scheme 2), which formed at a rate that was faster than the production of DMPO-SQ adduct formation. It may have been possible that NBT quenching of the EPR signal was due to the SQ radical forming quickly and then reacting with NBT to form an EPR

silent adduct. This argument was, however, dismissed when MS and NMR characterization of adducts formed in the reaction mixture showed no presence of NBT-SQ adducts. NBT mops the system of any available BQD; thus, no radicals are produced. This aspect proves that the free radical mechanism plays a minimal role, at best, in BQD binding. The dominance of MA as a pathway is further supported by quantitative mechanistic read-across-based estimation of BQ EC₃ values that utilized known potency data for sensitizers in the MA domain. A theoretical EC₃ value of 0.013% was obtained in agreement with an actual LLNA EC₃ value of 0.01%.²⁶

The inclusion of substituents on BQ has been shown to have a profound effect on the overall chemistry of BQ. This is manifested in an increased or decreased reactivity of BQD toward NBT and the emergence of other mechanistic pathways such as free radical mechanism and vinylic substitution (Scheme 3). NMR results also demonstrate the variations in the stoichiometry of the NBT-BQD reactions as well as the shifting of positions for nucleophilic addition. From the hapten hypothesis¹⁴ perspective, the effect of vinylic substitution (Scheme 3) for the chloro-substituted BQD would not lead to any immunogenic adducts that are different from those formed with BQ as this step is subsequent to MA, which results in adduct formation. The substituted chlorine leaves as a salt, which would not be expected to modify any proteins.

CONCLUSIONS

It can be concluded that MA is the predominant mechanism for BQ and methyl-BQD haptenation of proteins and that MA followed by vinylic substitution is dominant for chlorine-BQD. On the basis of the findings in these studies, SQ radical-induced binding of proteins may play a minimal to no role in the development of BQD sensitization. Subsequent studies such as the LLNA will be able to confirm the effect of substituents on the allergenic potency of BQD.

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Notes

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ABBREVIATIONS

ACD, allergic contact dermatitis; AA, acylation; BQ, *p*-benzoquinone; BQD, benzoquinone derivatives; CBQ, chlorobenzoquinone; Cys, cysteine; 2,5-DCBQ, 2,5-dichlorobenzoquinone; 2,6-DCBQ, 2,6-dichlorobenzoquinone; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; GSH, glutathione; HSA, human serum albumin; 2-MBQ, 2-methylbenzoquinone; MA, Michael addition; NEM, *N*-ethylmaleimide; NBT, nitrobenzenethiol; S_NAr, nucleophilic aryl substitution; S_N1/2, nucleophilic substitution; TNBS, trinitrobenzene sulfonic acid; QSARs quantitative structure–activity relationships; 2-

tBBQ, 2-tertbutyl benzoquinone; SQ, semiquinone; SBF, Schiff base formation; SS, skin sensitization; SH, sulphhydryl

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