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Detection and Characterization of DNA Adducts Formed from Metabolites of the Fungicide *ortho*-Phenylphenol

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The significance of DNA adduction in *ortho*-phenylphenol-induced carcinogenesis remains unclear. Establishing adduct structures may contribute to resolving this issue. The chemical structures of the DNA adduction products resulting from the in vitro reaction of phenylbenzoquinone, the putative ultimate carcinogenic metabolite of the fungicide/disinfectant *ortho*-phenylphenol, are reported here. Three isomeric adducts that resulted from reaction of deoxyguanosine were characterized by UV, LC−ESI−MS, and MS/MS, and 1D and 2D COSY−NMR spectroscopy. The proposed mechanism of product formation is nucleophilic attack by the deoxyguanosine exocyclic amine nitrogen on an electrophilic quinone carbon, followed by stabilization through enolization. Another nucleophilic attack forms a five-membered ring, which aromatizes by dehydration to form the final product. Adducts were also characterized from deoxyadenosine and deoxycytidine, although conversions were at least 10 times lower. Structures are also proposed for these products. Cell culture studies confirmed that HepG2 cells incubated with phenylbenzoquinone at concentrations associated with cytotoxicity form the same DNA adducts.

KEYWORDS: ***ortho*-Phenylphenol; phenylbenzoquinone; DNA adducts; high-performance liquid chromatography−mass spectrometry (HPLC−MS); cell culture**

INTRODUCTION

ortho-Phenylphenol and its sodium salt, sodium *ortho*-phenylphenate, are widely used in agriculture and industry as fungicides and disinfectants (*1*). Specifically, *ortho*-phenylphenol is used on citrus fruit for the prevention of mold, in dishwashing formulations, as a fungistatic wax for coating vegetables, as a preservative in water−oil emulsions, and as a commercial and household disinfectant. Occupational and environmental exposure of human beings to these two compounds is extensive (*1, 2*). Because of their widespread usage and potential for human exposure, the toxicological effects of *ortho*-phenylphenol and sodium *ortho*-phenylphenate have been investigated for decades. Chronic studies by Hiraga and Fujii demonstrated that administration of sodium *ortho*-phenylphenate and *ortho*-phenylphenol produces carcinogenic effects in the bladder and kidney in Fischer 334/DuDrj rats (*3*−*5*). Sodium *ortho*-phenylphenate has greater carcinogenic activity in rats than does the parent acid *ortho*-phenylphenol (*5*). Sodium *ortho*-phenylphenate is also a promoter of skin cancer in CD-1 mice (*6*), and it is suspected to be a potential human carcinogen (*7*).

With regard to the mechanism of *ortho*-phenylphenol carcinogenesis, evidence has accumulated that metabolic activation of *ortho*-phenylphenol occurs via a two-step process involving the cytochrome P-450-mediated formation of phenylhydroquinone and further oxidation of phenylhydroquinone to phenylbenzoquinone by the cytochrome P-450 enzyme system in the liver (*8*−*13*) (**Scheme 1**). Phenylhydroquinone can also be oxidized to the semiquinone and quinone in the bladder and kidney by prostaglandin H synthase, generating metabolites that can potentially interact with DNA (*8*). Many groups have reported that the reactive oxygen species derived from autoxidation of phenylhydroquinone to phenylbenzoquinone cause oxidative DNA damage (*1, 8*). Other reports have indicated that DNA adduction with *ortho*-phenylphenol metabolites plays a role in the genotoxic effects of *ortho*-phenylphenol. In vitro and in vivo studies using the sensitive ^{32}P-postlabeling technique have shown that the *ortho*-phenylphenol metabolites, phenylhydroquinone and phenylbenzoquinone, do bind DNA (*12*−*15*). Horvath et al. studied peroxidative metabolism of phenylhydroquinone and phenylbenzoquinone in HL-60 cells and observed DNA adduction using the ^{32}P-postlabeling method (*14*). Similarly, Pathak et al. demonstrated that application of sodium *ortho*-phenylphenate or phenylhydroquinone to female CD1 mice skin produced DNA adducts speculated to involve phenylbenzoquinone (*12*−*13*). Ushiyama et al. reported DNA adduct formation with *ortho*-phenylphenol metabolites in vivo and in vitro from bladder DNA of male Fischer 334 rats fed a diet containing 2% *ortho*-phenylphenol for 13 weeks (*15*). Phenylbenzoquinone is speculated to be the DNA-binding metabolite of *ortho*-phenylphenol both in vivo and in vitro (*13*).

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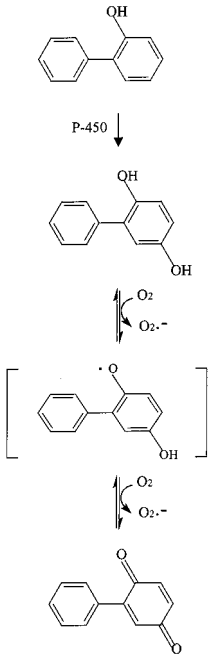
Scheme 1. Proposed Partial Metabolic Pathway of *ortho*-Phenylphenol in Rats



It caused cellular DNA damage more efficiently than phenylhydroquinone, although both of these *ortho*-phenylphenol metabolites induce oxidative damage (*16*−*17*).

Although evidence exists that covalent binding occurs between *ortho*-phenylphenol metabolites and DNA in various model systems, evidence demonstrating the occurrence of DNA-adduction in rat urinary bladder has not been convincing. In particular, Smith et al. conducted a thorough but unproductive search for in vivo formation of adducts in rat urinary bladder epithelium (*18*). Kowk et al. suggested that the molecular target involved in *ortho*-phenylphenol-induced bladder carcinogenesis might be protein, and that DNA adducts play a minor role, after they reported negative results for DNA adduction in the urinary bladder of male Fischer 334 rats by the very sensitive accelerator mass spectrometry technique (*19*). ^{32}P-Postlabeling and TLC by Smith and co-workers showed evidence for the formation of multiple adducts by reaction of deoxyguanosine-3′ monophosphate with phenylbenzoquinone, although they reported negative results in ^{32}P-postlabeling studies of *ortho*-phenylphenol binding to bladder DNA (*18*). However, the limited amount of bladder tissue available for analysis, the poor sensitivity of liquid scintillation counting for detecting radiolabeled DNA adducts, and the relatively low concentration of *ortho*-phenylphenol metabolites formed under in vivo conditions may account for the negative results observed (*19*).

The detection of DNA adducts formed from *ortho*-phenylphenol metabolites by ^{32}P-postlabeling supports the possibility of a genotoxic mechanism for *ortho*-phenylphenol's tumorigenicity. However, previous studies concerning DNA adducts from *ortho*-phenylphenol metabolites have not established the structures of the adducts because of the limitations of the ^{32}P-postlabeling technique. It has been reported that preferential damage occurs more frequently at guanine sites for *ortho*-phenylphenol metabolites (*15*−*16*). Identification and characterization of the chemical structures of these DNA adducts may facilitate efforts to establish the occurrence of DNA adduction and its biological significance in complex biological systems. The more thoroughly studied benzene/quinone system may provide a useful analogy for considering these questions. Formation of DNA adducts with quinone was reported to play a causative role in human carcinogenesis (*20*−*21*). A mechanism has been proposed for the reaction of guanosine with quinone (*22*). The reaction is initiated by nucleophilic attack of the exocyclic amine nitrogen, N^2, of the deoxyguanosine on the electrophilic carbon from the quinone, followed by stabilization through enolization. Another nucleophilic attack forms a five-member ring, followed by loss of water, thus forming an aromatic system that stablizes the molecule. Phenylbenzoquinone is a Michael acceptor structurally similar to quinone, and it may be capable of Michael addition to DNA. The present study investigated the capability of phenylbenzoquinone to form adducts with DNA in solution and in tissue culture, and uses chemical techniques to define the structures of the resulting products.

MATERIALS AND METHODS

Safety. Caution: Phenylbenzoquinone is highly toxic and should be handled carefully.

Chemicals and Reagents. 2′-Deoxyguanosine, 2′-deoxyadenosine, 2′-deoxycytidine, thymidine, calf thymus DNA, and nuclease P1 were purchased from Sigma Chemical Co. (St. Louis, MO). Phenylbenzoquinone, anhydrous dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), KH_2PO_4, sodium acetate, and formic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). HepG2 hepatoma cells (HB-8065) were purchased from American Type Culture Collection (Manssas, VA).

Preparation of Phenylbenzoquinone Modified Nucleosides. 60 mg (0.225 mmol) of 2′-deoxyguanosine (or 2′-deoxyadenosine, 2′-deoxycytidine) was dissolved in 4 mL of phosphate buffer (0.05 M, pH 7.2). To this solution, 50.6 mg (0.275 mmol) of phenylbenzoquinone in 100 μL of THF was added. The reaction mixture was incubated at 37 °C for 48 h with agitation, followed by centrifugation at 2000×*g*. The upper solution was filtered through a 0.2-μm filter cartridge prior to preparative HPLC. Preparative HPLC purification of the PBQ−^{2}N-dG adducts was conducted on a 250 mm × 10 mm i.d., 5-μm Semi Prep LC-18 column (Supelco, Bellefonte, PA) at a flow rate of 2 mL/min using the following gradient program: 15% THF and 5% methanol in water, followed by a linear gradient to 5% THF and 75% methanol in water over the course of 40 min. The adduct peaks collected were further purified through use of a 250 mm × 4.6 mm i.d., 5-μm Luna C18 analytical column (Phenomenex, Torrance, CA) at a flow rate of 1 mL/min using the following gradient program: 40% methanol in water, followed by a linear gradient to 80% methanol in water for 20 min; and then a linear gradient to 95% methanol in water over the course of 40 min.

Preparation of Phenylbenzoquinone Modified Calf Thymus DNA. Calf thymus DNA type I was suspended at a concentration of 0.5 mg/mL in 0.05 M potassium phosphate buffer (pH 7.2) containing 3 mg/mL of phenylbenzoquinone. The incubation was carried out at 37 °C for 48 h and was subsequently placed in ice for 30 min. DNA was precipitated by adding 1 mL of ice-cold ethanol and 35 μL of ice-cold 3.0 M sodium acetate. The sample was centrifuged at 14000×*g* for 15 min; the supernatant was removed, and the DNA pellet was washed with 1 mL of cold 70% ethanol in water. The sample was again centrifuged, and the supernatant was removed. The DNA was solubilized in 400 μL of water. Formic acid (12 μL of 88%) was added to a 200-μL aliquot of the modified calf-thymus DNA sample, and this solution was heated at 80 °C for 1 h and filtered through a 0.2-μM filter cartridge before analysis by LC−ESI−MS.

UV Spectrometry. UV spectra were obtained on-line using an Agilent 1100 LC system equipped with a Hewlett-Packard 1050 diode array detector. The wavelength was set at 276 nm for monitoring DNA adducts.

HPLC−ESI−MS/MS. The mass spectrometric data were acquired on either a Finnigan TSQ7000 triple-quadrupole mass spectrometer or a Finnigan LCQ ion trap instrument equipped with a Finnigan electrospray ion source. On-line chromatography was performed using an Agilent 1100 LC system. The LC/MS system was tuned for optimum response through use of a 100-μL flow injection of a 100 ppm solution

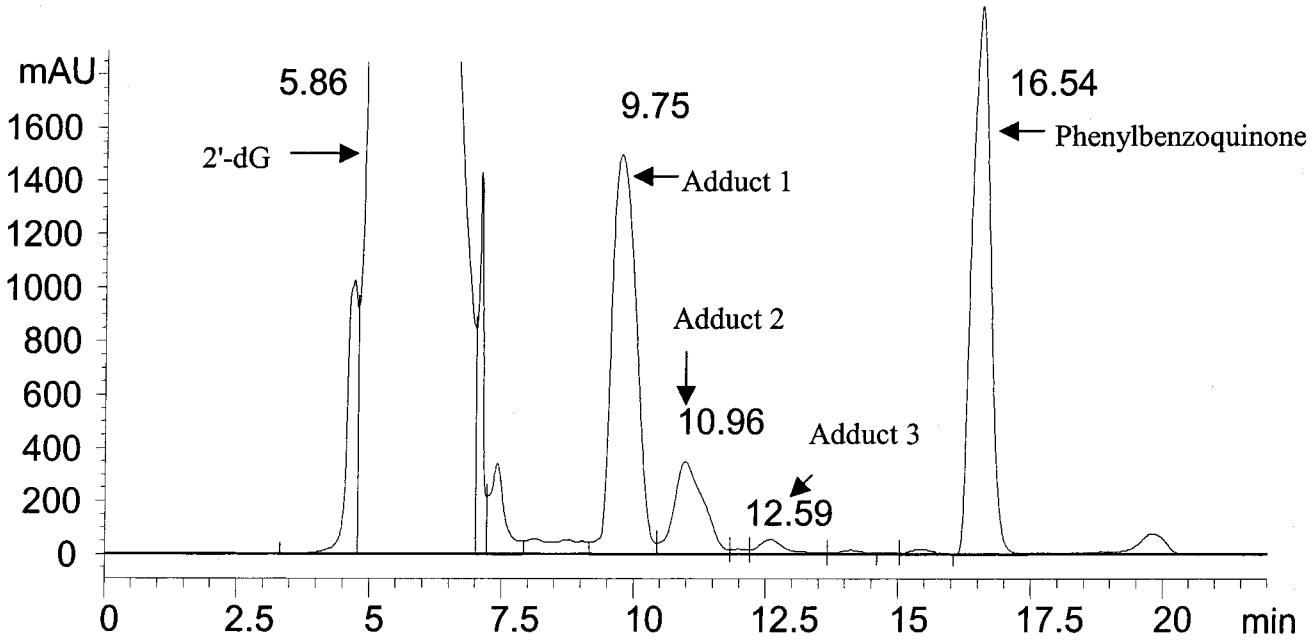


Figure 1. Preparative HPLC profile obtained after incubation of phenylbenzoquinone with 2′-deoxyguanosine (0.05 M phosphate buffer, pH 7.2) at 37 °C for 48 h. HPLC was conducted on a Supelco Semi-Prep C18 column with diode array detection at 276 nm. Michael addition at the *meta* position on phenylbenzoquinone forms adduct 1, which is believed to be the predominant product. Michael addition at the *para* and *ortho* positions of phenylbenzoquinone forms adducts 2 and 3, respectively.

of 2′-deoxyguanosine in methanol/0.01 M acetic acid (35:65). A gradient program was used with solution A (2% of ethanol, 1% of acetic acid, and 1% sodium acetate in water) and solution B (methanol) as 60% A and 40% B, followed by a linear gradient to 20% A and 80% B over the course of 20 min, and then a linear gradient to 5% A and 95% B for another 20 min. A 250 mm × 4.6 mm i.d., 5-μm Luna C18 analytical column (Phenomenex, Torrance, CA) was used, and the flow rate was 0.2 mL/min. Nitrogen was used as the sheath gas, the capillary temperature for the ESI source was set at 250 °C, and the ESI spray voltage was 4500 V.

NMR Spectrometry. ^{1}H NMR and two-dimensional homonuclear H, H-correlated NMR (COSY) spectra were obtained on a Bruker AM-600 spectrometer operated at 600 MHz. Me_2SO-d_6 was used as the solvent. All chemical shifts are in ppm downfield from tetramethylsilane (TMS).

Cell Culture. HepG2 hepatoma cells derived from a human hepatocellular carcinoma (American Type Culture Collection, Manssas, VA) were suspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, and seeded at 10^7 cells/10 mL/150 cm^2 flask. After 24 h of incubation at 37 °C, and under 5% CO_2, 95% humidity, the medium was replaced by fresh medium containing various concentrations of phenylbenzoquinone dissolved in DMSO. The treated cells, vehicle control (0.1% DMSO), and medium alone were then incubated for 48 h before they were washed twice with phosphate buffered saline and harvested by scraping off with cold 0.2 M sucrose/0.05 M Tris−HCl (pH 7.4). The cells were centrifuged for 10 min at 1000×g and homogenized in buffered sucrose with cooling. DNA was isolated by repetitive extraction with phenol/chloroform/isoamyl alcohol and was then precipitated with 2 vol of 100% ice-cold ethanol and 1/30 vol of 3 M sodium acetate (pH 5.3). Precipitated DNA was rinsed with 70% ethanol and dissolved in 2 mM Tris (pH 7.4). The purity for the DNA free of RNA and protein was checked by the 260:230 nm and 260:280 nm absorbance ratios of DNA, which were always ∼ 2.3 and 1.8, respectively. The purified DNA samples were then hydrolyzed by nuclease P1 and subjected to HPLC−ESI−MS/MS at selected reaction monitoring (SRM) mode.

RESULTS

Characterization of DNA Adducts Formed Between Phenylbenzoquinone and 2′-Deoxyguanosine (PBQ−^{2}N-dG). The adduct peaks were collected from the preparative column and further purified by an analytical Phenomenex Luna C18 column on the Agilent 1100 HPLC system. Each peak was identified by comparison with the standard and confirmed by ESI(+)−MS as follows: unreacted 2′-deoxyguanosine, retention time (RT) 5−7 min; adduct 1, RT 9.75 min; adduct 2, RT 10.96 min; adduct 3, RT 12.59 min; and unreacted phenylbenzoquinone, RT 16.54 min (**Figure 1**). These three adducts have identical mass spectra and the full-scan ESI(+) mass spectra contained abundant protonated molecules $[MH]^+$ (**Figure 2**). The mass spectra were identified as m/z 434 $(M + H)^+$; m/z 456 $(M + Na)^+$; m/z 318 $(M - \text{sugar} + H)^+$; m/z 867 $(2M + H)^+$; and m/z 889 $(2M + Na)^+$. The loss of 116 u is indicative of the occurrence of 2′-deoxynucleosides with an unmodified 2′-deoxyribofuranosyl moiety. This reveals that DNA adducts are solely modified on the base moiety. The molecular weight is consistent with the addition of one molecule of phenylbenzoquinone to the base moiety, followed by dehydration (−18). UV absorption was scanned for these DNA adducts from 190 to 500 nm. All of the three adducts have the maximum absorption at 254, 276 nm (pH 7.2). These UV patterns resembled those of N^2-substituted deoxyguanosines (*23*). Phenylbenzoquinone is a di-α,β-unsaturated carbonyl compound that can undergo Michael addition. We propose that the DNA adduction between phenylbenzoquinone and 2′-deoxyguanosine is introduced by Michael addition at exocyclic N^2 position (**Figure 3**). Michael addition was first initiated between the electron-deficient carbons from the quinone and nucleophilic exocyclic nitrogen atom from 2′-deoxyguanosine. Another nucleophilic attack forms a five-member ring, followed by the loss of water, thus forming an aromatic system that stablizes the molecule. In this mechanism, phenylbenzoquinone has three electron-deficient carbons available for the nucleophilic attack by the exocyclic nitrogen atom from 2′-deoxyguanosine, which in turn can result in the formation of three adduct isomers. Theoretically, nucleophilic attack at carbon 5 (*para* to phenyl group) or carbon 6 (*meta* to phenyl group) on phenylbenzo-

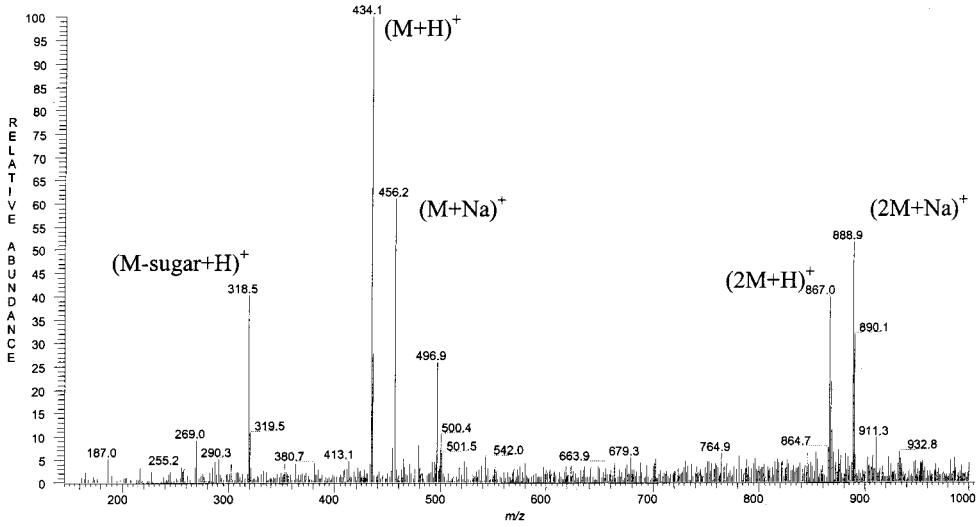


Figure 2. ESI–MS(+) mass spectrum of PBQ–^{2}N-dG adducts derived from the reaction of phenylbenzoquinone with 2′-deoxyguanosine (0.05 M phosphate buffer, pH 7.2, 37 °C) for 48 h in the full-scan mode (*m*/*z* 150–1000). A solution of 2% ethanol, 1% acetic acid, and 1% sodium acetate in water was used as mobile phase in the HPLC.

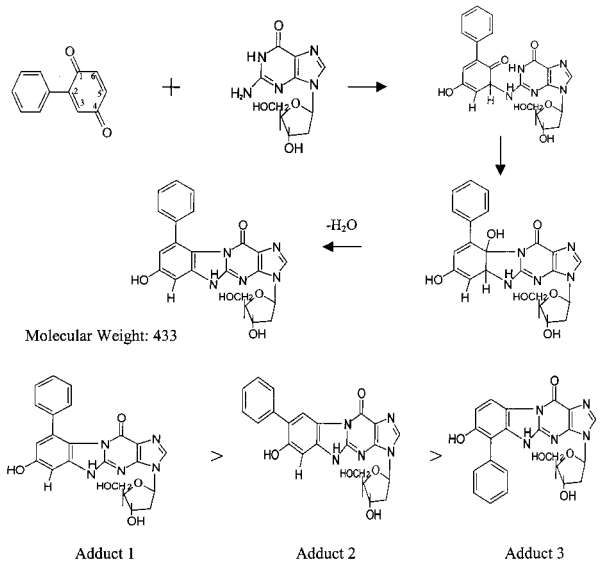


Figure 3. Mechanism for the formation of PBQ–^{2}N-dG adduct. Michael addition is first initiated between the electron-deficient quinone carbon and the nucleophilic exocyclic nitrogen atom of deoxyguanosine. Another nucleophilic attack forms a five-member ring, followed by the loss of water, thus forming an aromatic system that stablizes the molecule. Michael addition at the *meta* position on phenylbenzoquinone forms adduct 1, which is believed to be the predominant product. Michael addition at the *para* or *ortho* position of PBQ forms adducts 2 and 3, respectively.

quinone will form a sterically favorable structure, whereas nucleophilic attack at carbon 3 (*ortho* to phenyl group) will form a sterically unfavorable structure due to the steric hindrance from bulky phenyl group (**Figure 3**). On the basis of Michael addition of phenylbenzoquinone with nucleophilic thiol reagents, Maggiora and co-workers suggested that donor substituents and conjugating substituents on phenylbenzoquinone give regioselective nucleophilic addition in the order of *meta* > *para* > *ortho* (*24*). We found three adduct peaks in the analytical HPLC spectra, and they were demonstrated to share the same molecular weight. Therefore, we propose that the three DNA adduct isomers were formed in the order of *meta* > *para* > *ortho*, which correspond to the three peaks shown in **Figure 1** as adduct 1, adduct 2, and adduct 3, respectively. The structures of the three DNA adducts are also shown in **Figure 3**. To confirm the mechanism for the DNA adduction and the structure of the adduct isomers, the largest adduct peak (adduct 1 in **Figure 1**) was collected from preparative HPLC in repetitive experiments, pooled, and lyophilized to complete dryness for NMR analysis.

^{1}H NMR and two-dimensional homonuclear H, H-correlated NMR (COSY) spectra were obtained. The proton NMR spectrum of PBQ−^{2}N-dG isomer 1 showed the presence of the three major parts of the nucleoside adduct, i.e., the 2′-deoxyribose, the phenyl-1,4-benzoquinone, and the guanine base (**Figure 4**). The identity of the protons was confirmed by their coupling patterns in both the ^{1}H spectrum and the COSY spectrum. The proton assignment was shown in **Figure 4**. The LC/MS and NMR results confirmed unequivocally that phenylbenzoquinone was adducted at the exocyclic N^2 position on 2′-deoxyguanosine by Michael addition. An analogous mechanism was proposed by Jowa to explain benzene/quinone DNA adduct formation (*22*).

DNA adducts formed from phenylbenzoquinone-modified 2′-deoxyguanosine (PBQ−^{2}N-dG) were found in the yield of approximately 0.1% in aqueous solution at pH 7.2. These three adducts, which can be detected as early as 8 h after the start of the incubation, reached a plateau by 48 h. Even after extensive collection and lyophilization, the three DNA adduct isomers cannot be differentiated by NMR because of the limited amount of the DNA adducts.

DNA adducts formed from phenylbenzoquinone-modified 2′-deoxyadenosine (PBQ−^{6}N-dA) and 2′-deoxycytidine (PBQ−^{4}N-dC) (0.05 M phosphate buffer, pH 7.2, 37 °C for 48 h) were also detected by HPLC−ESI−MS/MS at selected reaction monitoring (SRM) mode. We report the structures for PBQ−^{6}N-dA adduct and PBQ−^{4}N-dC adduct as shown in **Figure 5**. They are formed by the same Michael addition mechanism as for PBQ−^{2}N-dG (**Figure 3**). Fewer PBQ−^{6}N-dA adducts (∼10-fold fewer, gauged from the HPLC peak area) and PBQ−^{4}N-dC adducts (∼26-fold fewer, gauged from the HPLC peak area) were formed than for PBQ−^{2}N-dG.

Detection of Adducts in Phenylbenzoquinone Modified Calf Thymus DNA. Calf thymus DNA was subjected to incubation with phenylbenzoquinone. The DNA was then hydrolyzed and submitted to HPLC−ESI−MS/MS at SRM

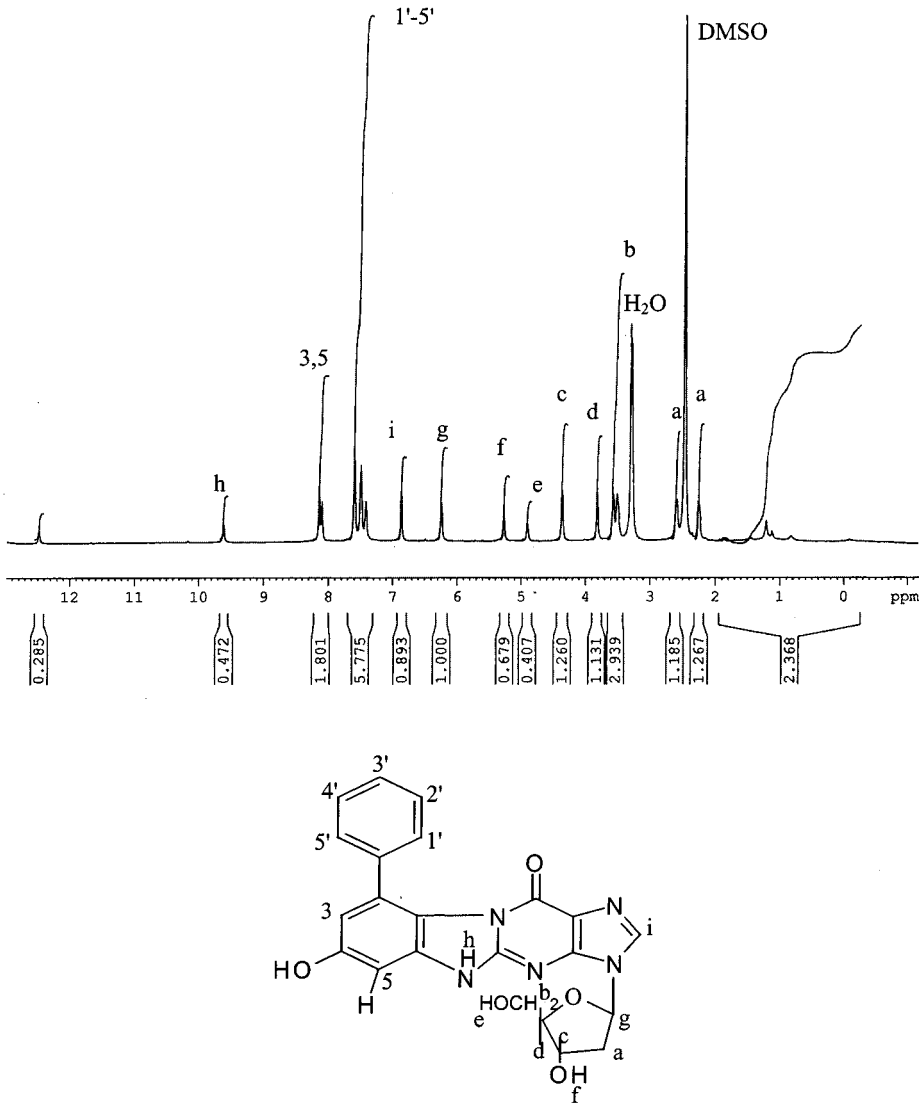
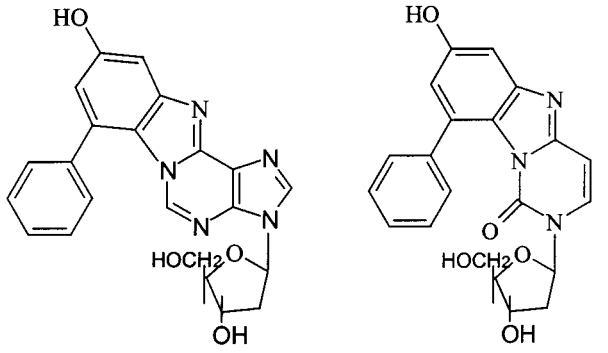


Figure 4. Spin decoupling ^{1}H NMR spectrum of PBQ–^{2}N-dG adduct obtained from incubation of phenylbenzoquinone with 2′-deoxyguanosine (0.05 M phosphate buffer, pH 7.2) at 37 °C for 48 h. The sample was purified by preparative and analytical HPLC before determination of the spectra. The Bruker AM-600 NMR spectrometer was operated at 600 MHz and Me_2SO-d_6 was used as solvent.



PBQ-^{6}N-dA adduct PBQ-^{4}N-dC adduct

Figure 5. Structures for PBQ–^{6}N-dA adduct and PBQ–^{4}N-dC adduct. The DNA adducts were obtained from incubation of phenylbenzoquinone with 2′-deoxyadenosine or 2′-deoxycytidine (0.05 M phosphate buffer, pH 7.2) at 37 °C for 48 h.

mode. The molecular ion (*m*/*z* 434) detected in the first stage quadrupole was directed into a collision induced decomposition (CID) chamber for decomposition through use of a pressure of 1.5×10^{-3} mbar Ar in the collision cell and a collision energy of 25 eV. Daughter ion $[M - \text{sugar} + H]^+$ (*m*/*z* 318) was monitored at the third stage quadrupole mass analyzer. The loss of 116 u indicates occurrence of 2′-deoxynucleosides with an unmodified 2′-deoxyribofuranosyl moiety. Owing to the specificity of this scanning, interferences were eliminated, yielding neat spectra revealing only the 2′-deoxynucleoside derivatives of interest. The PBQ−^{2}N-dG adducts were detected from phenylbenzoquinone-modified calf thymus DNA sample (**Figure 6**).

Detection of Adducts in Phenylbenzoquinone-Modified HepG2 Hepatoma Cell Culture. A series of 50 μM, 25 μM, 12.5 μM, and 6.25 μM phenylbenzoquinone in DMSO were added to HepG2 hepatoma cell cultures. After 48 h incubation, approximately 50% suppression of cell proliferation was observed in cell cultures treated with 50 μM phenylbenzoquinone compared to that of the control. DNA from these cultures was extracted and hydrolyzed by nuclease P1 and subjected to HPLC−ESI−MS/MS at selected ion monitoring (SIM) mode. The same PBQ−^{2}N-dG adducts were detected from cell cultures treated with 50 μM phenylbenzoquinone as from phenylbenzoquinone-modified calf thymus DNA at SIM and total ion chromatography (TIC) by HPLC−ESI−MS

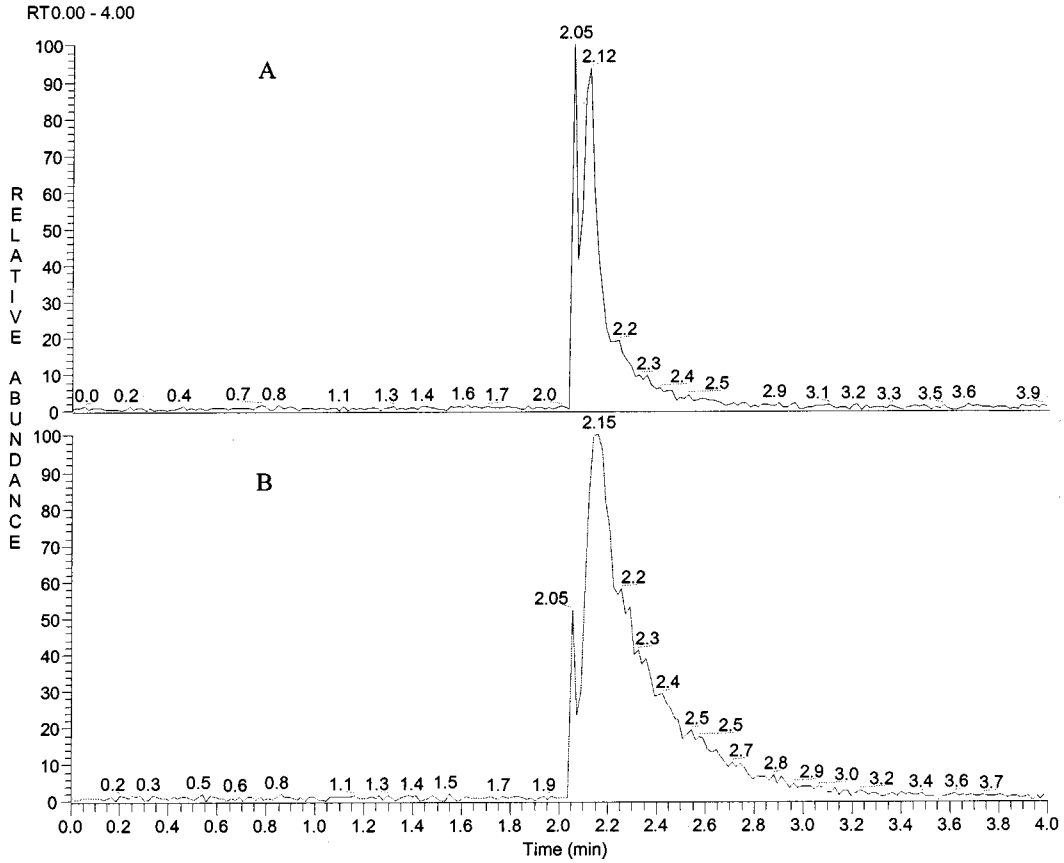


Figure 6. Direct-injection detection of phenylbenzoquinone-modified calf thymus DNA (incubated in 0.05 M phosphate buffer, pH 7.2, 37 °C for 48 h) at selected reaction monitoring (SRM) mode. Panel A, molecular ion (*m*/*z* 434) was selectively detected in first stage quadrupole. Panel B, daughter ion (*m*/*z* 318) was scanned in the third stage quadrupole after decomposition of the molecular ions in the collision cell.

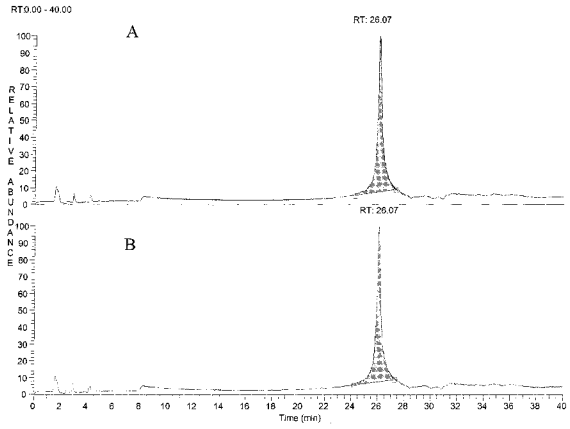


Figure 7. PBQ–^{2}N-dG adducts detected from HepG2 cells incubated with 50 μM phenylbenzoquinone in DMSO for 48 h. Panel A, selected ion monitoring (SIM) for the molecular ion of PBQ–^{2}N-dG adducts from 50 μM phenylbenzoquinone-modified HepG2 cell cultures. Panel B, total ion chromatography (TIC) of PBQ–^{2}N-dG adducts from 50 μM phenylbenzoquinone-modified HepG2 cell cultures.

(**Figure 7**). No adducts were found in the vehicle, untreated control cells, or cultures treated with a lower amount of phenylbenzoquinone.

DISCUSSION

Covalent modification of DNA by carcinogens or their metabolites represents an early critical step in multistage chemical carcinogenesis. If not repaired before DNA replication, DNA adducts can cause mispairing, resulting in mutations and chromosomal alternations (*20*). Analysis of DNA adducts is a challenge, as the specificity and sensitivity of the method must be sufficient to allow identification and quantitation of the modified nucleobases. ^{32}P-postlabeling is one of the most sensitive methods commonly used for detecting DNA adducts. It can typically detect one adduct per 10^8 normal bases or fewer (*25*−*26*). Unfortunately, ^{32}P-postlabeling provides no structural information on the analytes, which are usually detected as "spots" on the TLC plate. This may hinder understanding of the significance of DNA adducts in complex biological specimens (*18*). Gas chromatography/mass spectrometry is less sensitive but more specific than ^{32}P-postlabeling, because adducts can be identified by their molecular weight and GC retention times. But, because DNA nucleobases are polar, chemical derivatization is usually required (*27*). This step represents a major challenge due to poor derivatization yield and unstability of the DNA adducts.

LC−ESI−MS/MS methods developed in this laboratory were successfully applied to characterization of DNA adducts in samples exposed to *ortho*-phenylphenol-derived quinone. The SRM method was conducted using a Finnigan triple-stage quadrupole mass spectrometer, taking advantage of the fact that one major fragmentation process − loss of deoxyribose − can be observed for all of the adducts under CID conditions. Protonated molecules of the adducts were selected and focused in the first quadrupole, Q1, their fragmentation was achieved by CID in Q2, and the daughter ions generated from loss of deoxyribose were selectively detected in Q3. The collision energy and core energy were optimized for obtaining the highest response, and the instrument was tuned using standard solutions of the analyte analogues introduced by infusion.

In this study, we structurally characterized PBQ−^{2}N-dG adducts using various spectral techniques, such as UV, HPLC−MS, and NMR. We verified that phenylbenzoquinone, the putative ultimate carcinogen derived from *ortho*-phenylphenol, can form DNA adducts with 2′-deoxyguanosine by the same mechanism as that of the benzene-derived quinones proposed by Jowa (*22*). Phenylbenzoquinone belongs to the quinone class of chemicals known to have high affinity for electron-rich sites, a class that is capable of covalent binding to DNA and proteins (*21*). We also found that guanine is the preferred nucleobase for the DNA adduction, since 2′-deoxyguanosine forms much greater amounts of DNA adducts with phenylbenzoquinone than do 2′-deoxyadenosine or 2′-deoxycytidine. No DNA adducts are formed based on the Michael addition mechanism in phenylbenzoquinone-modified thymidine, presumably because there are no exocyclic amine nitrogen atoms present in thymidine.

The generation of DNA lesions, namely PBQ−^{2}N-dG adducts, was also observed in phenylbenzoquinone-treated hepatoma cells by HPLC−ESI−MS/MS in cell exposure studies. These adducts are identical to those detected from phenylbenzoquinone-treated calf thymus DNA. Suppression of cell proliferation was observed in HepG2 cell cultures with 50 μM phenylbenzoquinone incubated for 48 h where PBQ−^{2}N-dG adducts were detected by HPLC−MS/MS. HepG2 cell cultures with 25 μM phenylbenzoquinone incubated for 48 h exhibited slight suppression of cell proliferation, while cell growth in cultures with 12.5 μM and 6.25 μM phenylbenzoquinone incubated for 48 h were almost similar to that of the control. PBQ−^{2}N-dG adducts were detected in HepG2 cell cultures treated with 50 μM phenylbenzoquinone, but no detectable adducts were found by HPLC−MS/MS from HepG2 cell cultures treated with 25, 12.5, and 6.25 μM phenylbenzoquinone. This may be due to the limited sensitivity of HPLC−MS/MS. It is also observed that cell injury in HepG2 cell cultures treated with 25 μM phenylbenzoquinone can recover after prolonged incubation. This may be because more than 48 h of incubation may trigger the DNA repair mechanism, which results in the recovery of injured cells through loss of DNA adducts (*8*). A trace amount of 8-hydroxy-2′-deoxyguanosine, an adduct used as biomarker for phenylbenzoquinone-induced oxidative DNA damage, was found in phenylbenzoquinone-treated calf thymus DNA, but not in phenylbenzoquinone-treated HepG2 cell culture. This can be explained by the fact that hepatocytes have effective defense mechanisms adequate to cope with oxidative DNA damage (*8*). Phenylbenzoquinone adduction to guanine residues may lead to mutagenic effects, such as the misreading of PBQ−^{2}N-dG affected templates in DNA when replicating DNA-containing PBQ−^{2}N-dG moieties (*8*). This DNA damage could cause cell death.

Although phenylbenzoquinone has produced cytotoxic and genotoxic effects in various model systems, evidence demonstrating the role of DNA-adduction in these processes has not been convincing (*18*−*19*). Several studies support the hypothesis that DNA-adduction may occur. Horvath et al. studied the peroxidative metabolism of phenylhydroquinone and phenylbenzoquinone in HL-60 cells and observed DNA adduction using the ^{32}P-postlabeling method (*14*). Similarly, Pathak et al. demonstrated that application of sodium *ortho*-phenylphenate or phenylhydroquinone to female CD1 mice skin produced DNA adducts speculated to involve phenylbenzoquinone (*12*−*13*). However, Smith et al. conducted a thorough but unproductive search for in vivo formation of adducts in rat urinary bladder epithelium (*18*). Kowk et al. reported negative results for DNA adduction in the urinary bladder of male F334 rats by the very sensitive accelerator mass spectrometry technique, leading to the suggestions that the molecular targets involved in *ortho*-phenylphenol-induced bladder carcinogenesis are primarily protein, and that DNA adducts play a minor role (*19*). Our data demonstrate that phenylbenzoquinone can covalently bind to nucleophilic sites on DNA *in vitro*. Identification and characterization of the chemical structure of these adducts may facilitate efforts to establish the occurrence and biological significance of this process in more complex biological systems and at more biologically relevant exposures.

ABBREVIATIONS USED

PHS, prostaglandin H synthase; LC−MS/MS, liquid chromatography tandem mass spectrometry; ESI, electrospray ionization; TMS, tetramethylsilane; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; SRM, selected reaction monitoring; SIM, selected ion monitoring; TIC, total ion chromatography; CID, collision-induced decomposition; RT, retention time.

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