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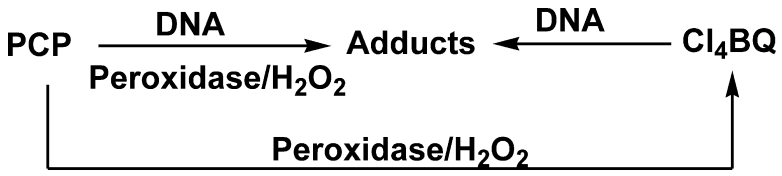

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Nucleobase-Dependent Reactivity of a Quinone Metabolite of Pentachlorophenol

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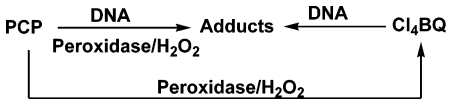
Pentachlorophenol (PCP) is a possible human carcinogen detected widely in the environment. A quinone metabolite of PCP, tetrachloro-1,4-benzoquinone (Cl_4BQ), is a reactive electrophile with the capacity to damage DNA by forming bulky covalent DNA adducts. These quinone adducts may contribute to chlorophenol carcinogenesis, but their structures, occurrence, and biological consequences are not known. Previous studies have indicated that several DNA adducts are formed in vivo in rats exposed to Cl_4BQ, but these adducts were not identified structurally. In the present study, we have elucidated the structure of new agent-specific DNA adducts resulting from the reaction of dGuo, dCyd, and Thd with Cl_4BQ. These have been characterized chemically by liquid chromatography−electrospray ionization mass spectrometry, HPLC, UV, and NMR analysis. Two dGuo adducts and one dCyd adduct resulting from the reaction of double-stranded DNA with Cl_4BQ have been identified. The results indicate that, in the structural context of DNA, Cl_4BQ reacts most readily with dGuo compared to the other DNA bases and that the mode of Cl_4BQ reactivity is dependent on the base structure; i.e., multiple types of adducts are formed. Finally, DNA adducts consistent with Cl_4BQ reactions are observed when DNA or dGuo is treated with PCP and a peroxidase-based bioactivating system.

Introduction

Pentachlorophenol (PCP)[1] is a ubiquitous environmental pollutant with broad applications, including as a biocide, wood preservative, insecticide, and disinfectant (*1*). It has been detected in human urine, blood, breast milk, and adipose tissue (*2*, *3*). Pentachlorophenol also results from biotransformations of environmental pollutants such as hexachlorobenzene and hexachlorocyclohexane (*4*). The International Agency for Research on Cancer (IARC) lists pentachlorophenol as a group 2B (possibly carcinogenic to humans) carcinogen (*5*), and the Environmental Protection Agency (EPA) classifies pentachlorophenol as a group B2 carcinogen (probable human carcinogen) (*6*). Pentachlorophenol has been considered a cytotoxic agent because of its ability to uncouple mitochondrial oxidative phosphorylation (*7*). The carcinogenesis of PCP is thought to involve enzyme-mediated oxidative biotransformations to produce reactive oxygen species (ROS) and the corresponding hydroquinone and semiquinone (*8*). Hydroquinone metabolites can be oxidized further to generate quinones in a process mediated by mammalian peroxidases such as lactoperoxidases and myeloperoxidase (MRP) (Scheme 1) (*9*, *10*). A final quinone metabolite of pentachlorophenol is tetrachlorobenzoquinone (Cl_4BQ), which induces DNA damage in Chinese hamster ovary cells and single-strand breaks from the *para*-isomer but not the corresponding *ortho*-isomer (*10*, *11*). Due to the reactive electrophilic nature of Cl_4BQ, it forms covalent adducts with biomacromolecules such as proteins and DNA, and this process may contribute further to PCP carcinogenesis (*12*, *13*).

Scheme 1



Several covalent DNA adducts are formed when calf thymus DNA is treated with Cl_4BQ, but the structures are not known (*14*). We have demonstrated recently that a dichloroquinone benzetheno-type adduct (**5**) is formed in reactions of the nucleoside dGuo with Cl_4BQ (*15*). Exocyclic etheno-type adducts of quinones have been shown to block DNA polymerase activity and are mutagenic in a site-specific mutagenicity assay (*16*). Furthermore, previous studies revealed that *p*-benzoquinone adducts induce GC → TA transversion and GC → AT transition mutations and that a hydroquinone adduct induces GC → AT transition mutations in a *supF* reporter gene mutation assay (*17*). Taken together, these observations stimulated further investigation of the reaction of Cl_4BQ with nucleic acids and duplex DNA. The present study is focused on the chemical characterization of products of the reactions of Cl_4BQ with purine and pyrimidine nucleosides and nucleotides and a comparison of these findings to reactions that occur in duplex DNA in vitro. We report the formation of adducts resulting from treatment of DNA with either Cl_4BQ directly or PCP and a bioactivating enzyme. The results demonstrate that quinone reactivity is dependent on the base structure and can be linked with phenol reactivity.

Materials and Methods

Chemicals and Reagents. Calf thymus DNA, 2′-deoxycytidine, Cl_4BQ, 2′-deoxyguanosine-3′-monophosphate, DNase I, horseradish

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[1] Abbreviations: APCI, atmospheric pressure chemical ionization; Cl_4BQ, tetrachloro-1,4-benzoquinone; CT DNA, calf thymus deoxyribonucleic acid; 3′-dGMP, 2′-deoxyguanosine 3′-monophosphate; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; MRP, myeloperoxidase; PCP, pentachlorophenol.

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peroxidase (HRP; type VI, 298 U/mg), and phosphodiesterase I were purchased from Sigma Chemicals (Milwaukee, WI). Thymidine was purchased from VWR Chemicals (Eagan, MN). Alkaline phosphatase was purchased from Roche Chemicals (Indianapolis, IN). Strata-X columns (33 μg/mL) were obtained from Phenomenox (Torrance, CA). [$^{13}C_6$]Cl_4BQ was purchased from Cambridge Isotope Laboratories. All chemicals were used as received. The HRP concentration was determined by measuring the absorbance at 402 nm ($E_{402} = 9.5 \times 10^4$ M^{-1} cm^{-1}) as described previously (*10*).

Caution: *The work described involves the handling of hazardous agents and was therefore conducted in accordance with the NIH guidelines for the laboratory use of chemical carcinogens (18).*

Instrumentation. HPLC analysis of adducts was carried out using an Agilent 1100 HPLC instrument with a diode array UV detector; the column used in this study was a Luna 5μ C18 (2) 100A 250 × 4.60 mm column (Phenomenox, Torrance, CA). UV absorbance was monitored at 254 nm. Two mobile-phase solvents were used in the HPLC instrument. Solvent A was water, and solvent B was methanol. HPLC method I consisted of 95:5 solvent A/solvent B for 5 min followed by a linear gradient to 5:95 solvent A/solvent B in 25 min and then isocratic elution at 95:5 solvent A/solvent B for 5 min at a flow rate of 0.5 mL/min. Method II was employed for purification and involved a Biotage SP1 high-performance flash chromatography system (Biotage, Charlottesville, VA) equipped with a Biotage C18 reversed-phase flash column (25 + M).

The solvent program consisted of 95:5 solvent A/solvent B for 2 min followed by a linear gradient to 5:95 in 5 min and then isocratic elution at 5:95 solvent A/solvent B for 3 min at a flow rate of 12 mL/min. Liquid chromatography−electrospray ionization mass spectrometry (LC−ESI-MS) was carried out with a Finnigan LCQ Deca instrument (Thermo Electron, San Jose, CA) interfaced to a Waters Alliance 2695 HPLC delivery system and equipped with an SPD-10 A UV detector (Shimadzu Scientific Instruments, Columbia, MD) using the HPLC solvent elution system and column as described above. The ESI source was set in negative ion detection mode with the following ionization parameters: voltage, 4 kV; current, 4 μA; capillary temperature, 275 °C. MS data were collected for a mass range of 100−1000 amu. Tandem mass spectrometry (MS/MS) data were acquired with the following parameters: isolation width, 1.5 amu; normalized collision energy, 40%; activation *Q*, 0.25; activation time, 30 ms. Xcalibur version 1.4 SRI was used to simulate calculated MS spectra. NMR spectra were recorded in d_6-DMSO using a Varian mercury 300 MHz spectrometer. UV absorption spectra of the adducts were recorded using a Cary 50-Bio spectrophotometer.

Reaction of dCyd with Cl_4BQ. To a solution of dCyd (11 mg, 0.05 mmol) in DMSO (1 mL) were added Cl_4BQ (16 mg, 0.06 mmol) and 5 mL of 10 mM Na_3PO_4 buffer (pH 7.4), and the mixture was heated at 65 °C for 2 h. The reaction mixture was concentrated under reduced pressure at 40 °C, and the dCyd−Cl_4BQ adducts were purified by chromatography system II. The dCyd−Cl_4BQ adducts **1**−**3** were isolated as red-orange, yellow, and purple solids, respectively, after removal of solvents.

2,3,5-Trichloro-6-[[1-[4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl]amino][1,4]-benzoquinone (1). ESI-MS (*m*/*z*): 434. MS/MS (*m*/*z*): 282. ^{1}H NMR (300 MHz, d_6-DMSO): δ 8.18 (d, 1H, J = 8.1 Hz, H6); 6.9 (d, 1H, J = 7.8 Hz, H5); 6.5 (s, 1H, 4-NH); 6.15 (t, 1H, J = 6.3 Hz, 1′-CH); 2.04 (m, 1H, 2′$_a$-CH); 2.2(m, 1H, 2′$_b$-CH); 4.15 (m, 1H, 3′-CH); 3.8 (d, 1H, 4′-CH); 3.7 (m, 2H, 5′-CH); 5.1 (t, 1H, OH); 5.2 (d, 1H, OH). UV (λ, nm): 212, 312, 332.

Dichlorohydroxy-2-[[1-[4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl]amino][1,4]-benzoquinone (2). ESI-MS (*m*/*z*): 416. MS/MS (*m*/*z*): 380. ^{1}H NMR (300 MHz, d_6-DMSO): δ 8.2 (d, 1H, J = 7.8 Hz, H6); 6.2 (d, 1H, J = 7.5 Hz, H5); 6.5 (s, 1H, 4-NH); 6.25 (t,1H, J = 5.7 Hz, 1′-CH); 2.02 (m, 1H, 2′$_a$-CH); 2.05 (m, 1H, 2′$_b$-CH); 4.25 (m, 1H, 3′-CH); 3.9 (d, 1H, 4′-CH); 3.6 (m, 2H, 5′-CH); 5.15 (t, 1H, OH); 5.28 (d, 1H, OH). UV (λ, nm): 286, 208.

Chlorohydroxy-2-[4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]-2*H*-benzo[4,5]imidazo[1,2-*c*]pyrimidine-1,6,9-trione (3). ESI-MS (*m*/*z*): 380. MS/MS (*m*/*z*): 264. ^{1}H NMR (300 MHz, d_6-DMSO): δ 7.9 (d, 1H, J = 7.8 Hz, H6); 6.7 (d, 1H, J = 7.8 Hz, H5); 6.3 (t, 1H, 1′-CH); 2.3 (m, 1H, 2′$_a$-CH); 2.4 (m, 1H, 2′$_b$-CH); 4.25 (m, 1H, 3′-CH); 3.9 (d, 1H, 4′-CH); 3.6 (m, 2H, 5′-CH); 5.15 (t, 1H, OH); 5.3 (d, 1H, OH). UV (λ, nm): 248, 300, 364.

Reaction of Thd with Cl_4BQ. 3-(Dichlorohydroxy-3,6-dioxocyclohexa-1,4-dienyl)-1-[4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]-5-methyl-1*H*-pyrimidine-2,4-dione (4). To a solution of Thd (12 mg, 0.05 mmol) in DMSO (1 mL) were added Cl_4BQ (16 mg, 0.06 mmol) and 5 mL of 10 mM Na_3PO_4 buffer (pH 7.4), and the mixture was heated at 65 °C for 2 h. The reaction mixture was concentrated to one-third of its initial volume under reduced pressure at 40 °C, and the Thd−Cl_4BQ adduct was purified by chromatography system II, the solvent removed, and the Thd−Cl_4BQ adduct isolated as a purple solid. ESI-MS (*m*/*z*): 431. MS/MS (*m*/*z*): 395. ^{1}H NMR (300 MHz, d_6-DMSO): δ 11.2 (s, 1H, NH); 7.62 (s, 1H, H6); 6.18 (t, 1H); 5.21 (d, 1H, 1′-H); 5.05 (t, 1H, 5′-OH); 2.1 (m, 2H, 5′-H); 1.78 (s, 3H, CH_3).

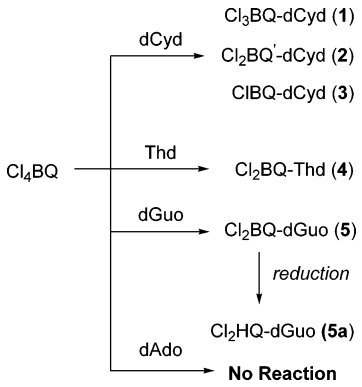
Reaction of [$^{13}C_6$]Cl_4BQ with Thd. 3-([$^{13}C_6$]-2,5-Dichloro-4-hydroxy-3,6-dioxocyclohexa-1,4-dienyl)-1-[4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]-5-methyl-1*H*-pyrimidine-2,4-dione. The procedure was the same as that described for the preparation of **4**, except the reaction was carried out using [$^{13}C_6$]Cl_4-BQ. The product was obtained as a purple solid and was a mixture of the title compound with residual Thd. Efforts to separate the two components or drive the reaction to completion were not successful. ^{1}H NMR data for the reaction product mixture were indistinguishable from those of Thd. ESI MS (*m*/*z*): 437. MS/MS (*m*/*z*): 311. ^{13}C NMR (150 MHz, d_6-DMSO): δ 176 (C2″, C═O), 165 (C5″, C═O), 145 (C4″, CCl), 133 (C3″, COH), 107 (C1″, CN[3]).

Reaction of Cl_4BQ with DNA. Calf thymus DNA (2 mg) in 2 mL of 100 mM Na_3PO_4 buffer was treated with Cl_4BQ (2 mg, 3.3 mM, 0.008 mmol) in 2 mL of DMSO, pH 7.4, at 37 °C for 72 h. The modified DNA was extracted with $CHCl_3$ (3 × 1 mL) and precipitated by adding ethanol (2 mL). The precipitated DNA was washed with 70:30 ethanol/water (1 mL) and with 100% ethanol (1 mL) and dried under a flow of nitrogen. The dry DNA was dissolved in 10 mM Tris/5 mM $MgCl_2$ buffer (1 mL) and digested with DNase I (1000 U, 100 μL) for 10 min; phosphodiesterase (0.05 U, 50 μL) and alkaline phosphate (300 U, 20 μL) were added, and the mixture was heated at 37 °C for 90 min. The resulting mixture was purified by solid-phase extraction using a StrataX column (33 μm) as follows. The column was pretreated with methanol (1 mL) followed by water (1 mL). The digested DNA mixture was loaded onto the column and eluted with 10% MeOH/H_2O followed by 100% MeOH. The product was analyzed using LC−ESI-MS, and HPLC method I. The data are shown in Figure 5.

Reaction of 2′-Deoxyguanosine 3′-Monophosphate (dGMP) with Cl_4BQ. 2′-Deoxyguanosine 3′-monophosphate (5 mg, 0.013 mmol) and Cl_4BQ (5 mg, 0.02 mmol) were dissolved in 10 mM $NH_4^+HCO_2^-$ buffer (1 mL), pH 6.0, and allowed to react for 2 days at 37 °C. The reaction mixture was extracted with ethyl acetate (3 × 1 mL) to remove excess Cl_4BQ. The resulting reaction mixture was treated with alkaline phosphatase (10 U, 1 μL) for 30 min. The presence of **5** and **6** was indicated by HPLC analysis. The corresponding retention times were 29 and 34 min, respectively (HPLC method I). The mixture was analyzed by LC−ESI-MS and UV. The following data were obtained for **5**. ESI-MS (*m*/*z*): 440. MS/MS (*m*/*z*): 324. UV (λ, nm): 256, 302, 338. The following data were obtained for **6**. ESI-MS (*m*/*z*): 458. MS/MS (*m*/*z*): 342. UV (λ, nm): 227, 255, 295.

Reaction of PCP with DNA. To a solution of calf thymus DNA (2 mg) in Na_3PO_4 buffer (100 mM, pH 7.4, 2 mL) were added HRP (50 μM, 50 μL) and aqueous H_2O_2 (5 mM, 1.4 mL), followed by a DMSO solution of PCP (5 mg, 3.75 mM, 5 mL). After 15 min, additional DMSO (2 mL) was added, and the mixture was allowed to react at 37 °C for 1 week. The resulting modified DNA

Scheme 2



was extracted with phenol/$CHCl_3$/isoamyl alcohol (25:24:1) (2 × 1 mL) and $CHCl_3$ (3 × 1 mL) and precipitated by adding ethanol (2 mL). The precipitated DNA was washed with 70:30 ethanol/water followed by 100% ethanol and dried under a flow of nitrogen. The dried DNA was dissolved in 10 mM Tris/5 mM $MgCl_2$ buffer (1 mL) and digested with DNase I (1000 U, 100 μL), phosphodiesterase (0.05 U, 50 μL), and alkaline phosphatase (300 U, 20 μL) at 37 °C for 90 min. The resulting mixture was subjected to solid-phase extraction and analyzed by LC−ESI-MS method I. The data are shown in Figure S5 in the Supporting Information (SI). Control experiments were carried out as described above, but (1) in the absence of DNA, (2) in the absence of DNA and DMSO, and (3) with DMSO, but in the absence of DNA and at varying concentrations (0−75 μM) of HRP (data shown in Figure S6 in the SI).

Results

Reactions of Cl_4BQ with Pyrimidine Nucleosides. The reaction of dCyd with Cl_4BQ resulted in the formation of multiple products (Scheme 2). These were observed as HPLC peaks with retention times t_R of 29, 24, and 20 min for **1**−**3** (∼99%), which were red-orange, yellow, and purple, respectively (Figure S1 in the SI). Of the isolated material, adduct **3** accounted for about 99%, and adducts **1** and **2** for 1% of the yield. In the UV spectrum of **1**, multiple electronic transitions were observed as shoulders at 212, 312, and 332 nm and a well-defined band at 282 nm for **1** (Figure S2 in the SI). Three well-defined absorption bands were observed at 248, 300, and 364 nm for **3** in contrast to two bands at 286 and 208 nm for **2** (Figure S2). The MS spectrum of **1** has a molecular ion peak ($[M - H]^-$) of *m*/*z* 434 with an isotope distribution pattern consistent with the presence of three chlorine atoms (Figure 1A). Tandem MS analysis of *m*/*z* 434 produces a fragment with *m*/*z* 282, representing loss of HCl and deoxyribose (Figure 1C). Adduct **2** has a two-chlorine isotopic pattern with *m*/*z* 416 (Figure 2A) and an MS/MS fragment corresponding to loss of one molecule of HCl (*m*/*z* 380) and two molecules of HCl (*m*/*z* 344, Figure 2C). For **3**, a molecular ion of *m*/*z* 380 and an isotope distribution pattern consistent with the presence of one chlorine atom were observed (Figure 3A). The MS fragmentation pattern for **3** indicates the loss of deoxyribose (*m*/*z* 264) instead of loss of HCl (*m*/*z* 344), as in the other two products (Figure 3C). On the basis of these data, the structures for **1**−**3** were formulated as Cl_3BQ−dCyd, Cl_2BQ'−dCyd, and ClBQ−dCyd, respectively, as illustrated in Chart 1, with the relative isomeric relationships of the OH and Cl groups unknown. For further analysis, the dCyd adducts were purified by reversed-phase HPLC. ^{1}H NMR spectral data of the purified dCyd adducts are shown in Table 1. The chemical shifts of the C6 protons were observed at δ 8.18, 8.20, and 7.90 ppm for adducts **1**−**3**, respectively. The chemical shifts of the C4 protons were

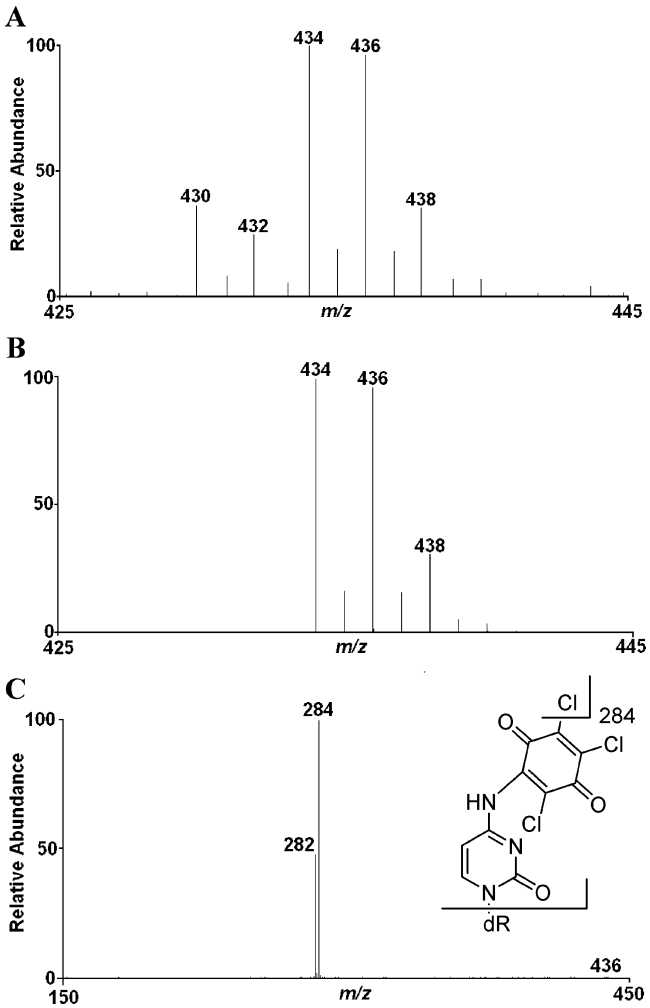


Figure 1. Typical MS/MS spectrum of adduct **1** from the reaction of 2′-deoxycytidine with Cl_4BQ in 10 mM phosphate buffer, pH 7.4, at 37 °C for 2 h: (A) adduct **1** has a molecular ion, $[M - H]^-$, of *m*/*z* 434, (B) simulated spectrum of adduct **1**, (C) MS/MS data of the fragment ion *m*/*z* 284.

δ 6.5 in both **1** and **2**, while for adduct **3** no C4 proton was observed, which is consistent with a cyclized structure. Adduct **3** does not exhibit any fluorescence, although the analogous hydroxyl−benzetheno adduct produced in the reaction of dCyd with *p*-benzoquinone is fluorescent (*19*).

The reaction of Cl_4BQ with Thd resulted in the formation of an adduct that eluted at 19 min using HPLC method 1 (figure not shown). The retention time of the adduct was identical to that of unmodified Thd. However, LC−ESI-MS analysis indicated the presence of a new species with a molecular ion of *m*/*z* 431 ($[M - H]^-$) containing two Cl atoms (Figure 4A), consistent with a Cl_2(OH)BQ adduct (**4**). The MS/MS spectrum exhibited a molecular ion peak at *m*/*z* 395 ($[M - HCl - H]^-$) (Figure 4C). The ^{1}H NMR and absorption spectra of material obtained after chromatography were identical to those of Thd. These results indicate that the single HPLC peak is comprised of a mixture of unreacted Thd and a small amount of adduct. Reasons for the low observed levels of this adduct may be relative instability of this adduct, which does not appear to cyclize under these conditions, and diminished relative reactivity of Thd toward Cl_4BQ compared to that of other nucleosides. We explored many strategies to obtain pure **4**, such as different chromatography methods with varying buffer pH (6.0−7.5), and by increasing the reaction time to consume unreacted Thd, but were unable to obtain the adduct free from Thd. The reaction

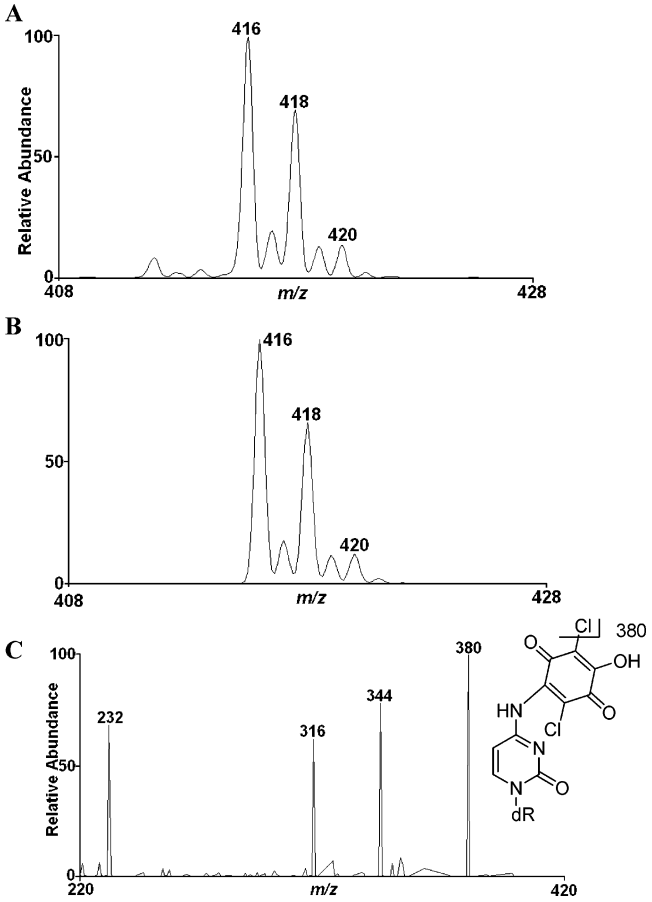


Figure 2. Spectra for analysis of adduct **2** from the reaction of 2′-deoxycytidine with Cl_4BQ in 10 mM phosphate buffer, pH 7.4, at 37 °C for 2 h: (A) adduct **2** has a molecular ion, $[M - H]^-$, of *m*/*z* 416, (B) simulated MS data, (C) MS/MS data with fragment ion *m*/*z* 380.

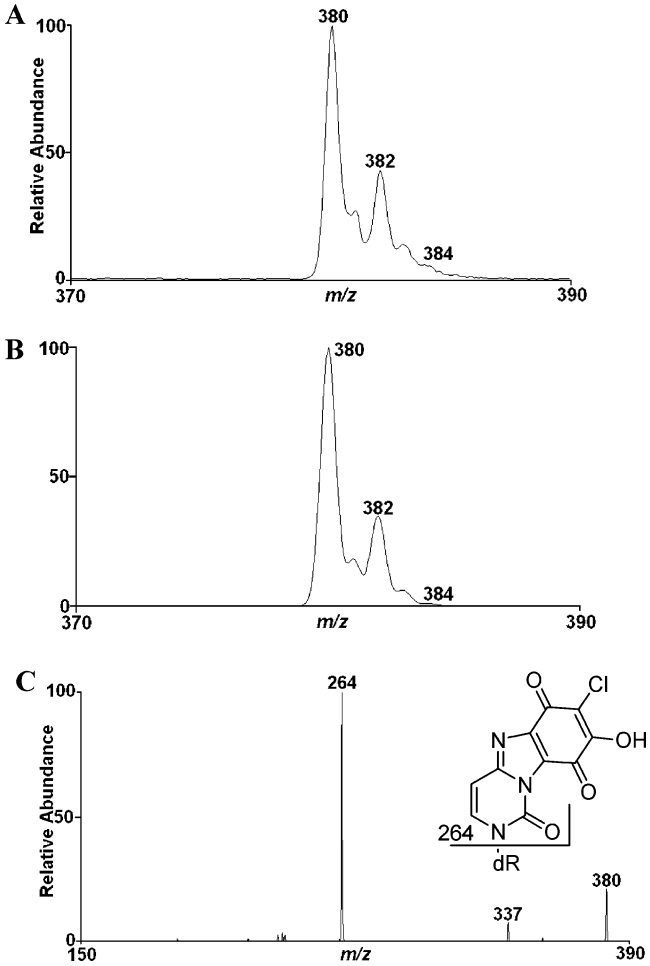


Figure 3. Spectra for analysis of adduct **3** from the reaction of 2′-deoxycytidine with Cl_4BQ in 10 mM phosphate buffer, pH 7.4, at 37 °C for 2 h: (A) adduct **3** has a molecular ion, $[M - H]^-$, of *m*/*z* 380, (B) simulated MS data, (C) MS/MS data with fragment ion *m*/*z* 264.

of Thd with ^{13}C-labeled Cl_4BQ was carried out so that NMR experiments could be performed to distinguish whether the linkage of Cl_4BQ was to N^3 or O^2. Six ^{13}C resonances were observed, consistent with an unsymmetrical quinone structure (Figure S3 in the SI). The ^{13}C chemical shift for the conjugated carbonyl group was 176 ppm, as observed previously for **5** (*18*), and on the basis of a ^{13}C peak at 110 ppm, we formulated the structure containing a N^3-linkage.

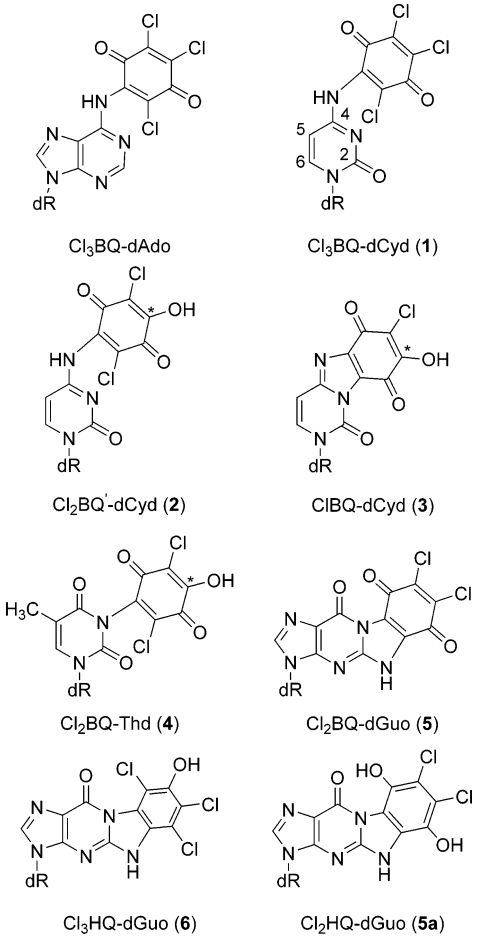
Reactions of Cl_4BQ with Purine Nucleosides. A dichloro benzetheno-type adduct results from the reaction of dGuo with Cl_4BQ, and this adduct has been identified previously as **5** and characterized by NMR, UV, and mass spectrometry (*15*). Although dGuo reacts with Cl_4BQ, no adducts were observed in the reaction of dAdo with Cl_4BQ under the same conditions (2 h, 65 °C). No reaction was observed using ammonium formate (pH 6.0) or sodium phosphate (pH 7.4) as the buffer, nor at 37 or 65 °C, or when using nucleotide precursors such as 5′-dAMP.

Reaction of Cl_4BQ with DNA. Calf thymus DNA was treated with Cl_4BQ, and the resulting modified DNA was isolated, enzymatically hydrolyzed, and analyzed by LC−ESI-MS and LC−ESI-MS/MS. Figure 5 shows LC−MS data for digested DNA, where panels A−C show the extracted ion chromatograms for *m*/*z* 324, 342, and 380, respectively. MS analysis of DNA samples indicated the formation of a new adduct with *m*/*z* 458 with a corresponding three-chlorine isotope distribution pattern. MS/MS fragmentation resulted in loss of deoxyribose (*m*/*z* 342). Such an adduct, with *m*/*z* 458, could result from reaction with dGuo or dAdo with possible structures shown in Chart 1. As dAdo and the corresponding nucleotide did not react with Cl_4BQ, we hypothesized that the new adduct with *m*/*z* 458 might be due to reaction with dGuo. However, in the reaction of dGuo with Cl_4BQ, **5** was observed exclusively. To probe the identity of the previously unknown dGuo adduct, we investigated reactions of Cl_4BQ with guanine nucleotides.

The reaction of 3′-dGMP with Cl_4BQ at pH 6.0 yields two different products with retention times of 29 and 34 min and *m*/*z* 440 and 458, respectively (data not shown). Tandem MS analysis of these two compounds indicates fragment ions with peaks of *m*/*z* 324 and 342, resulting from the loss of deoxyribose (*m*/*z* 116). The two adducts produced from 3′-dGMP exhibit different UV spectra with electronic transitions at 256, 302, and 338 nm for **5** and 225, 254, and 295 nm for **6** (Figure S4 in the SI). The reaction at pH 7.4 yields a reduced form of the benzetheno adduct with *m*/*z* 440. The reaction of 5′-dGMP with Cl_4BQ was carried out, but only **5** was formed.

Peroxidase-Mediated PCP Activation and Reaction with DNA. To study whether the peroxidase-mediated metabolism of PCP results in the formation of adducts attributable to reactions of nucleic acids with the quinone metabolite, we investigate whether similar adduct profiles result from the reaction of PCP with dGuo and DNA in the presence of HRP and peroxide. Initially, we carried out the reaction under conditions analogous to those of previous studies involving the reaction of PCP (100 μM), HRP (260 μM), and H_2O_2 (1 mM), but no adducts were observed by LC−ESI MS, and the

Chart 1[a]



[a] An asterisk indicates the relative positions of OH and Cl atoms are unknown.

Table 1. Comparison of Chemical Shifts (ppm) of ^{1}H NMR Peaks (d_6-DMSO) for Adducts 1−3

proton	1	2	3
4	6.50 (s, 1H)	6.50 (s, 1H)	
5	6.90 (d, 1H,7.8 Hz)	6.20 (d, 1H, 7.5 Hz)	6.70 (d, 1H, 7.8 Hz)
6	8.18 (d, 1H, 8.1 Hz)	8.20 (d, 1H, 7.8 Hz)	7.90 (d, 1H, 7.8 Hz)
1′	6.15 (t,1H, 6.3 Hz)	6.25 (t,1H, 5.7 Hz)	6.30 (t,1H, 6.3 Hz)
2′$_a$	2.04 (m, 1H)	2.02 (m, 1H)	2.30 (m, 1H)
2′$_b$	2.20(m, 1H)	2.05 (m, 1H)	2.40 (m, 1H)
3′	4.15 (m, 1H)	4.25 (m, 1H)	4.25 (m, 1H)
4′	3.80 (d, 1H)	3.90 (d, 1H)	3.90 (d, 1H,3.9 Hz)
5′	3.70 (m, 2H)	3.60 (m, 2H)	3.60 (m, 2H)
OH	5.10 (t, 1H)	5.15 (t, 1H)	5.15 (t, 1H)
	5.20 (d, 1H)	5.28 (d, 1H)	5.30 (d, 1H)

previously identified 8-PCP−dGuo adduct only was observed by LC−APCI-MS (*20*). At high DMSO concentration (50%) and extended reaction time (one week), however, analysis of the mixtures from reactions of PCP with DNA in the presence of HRP (50 μM) and peroxide (5 mM) reveals an LC−MS peak (retention time 28 min, *m*/*z* 440, fragment ion *m*/*z* 324) that represents **5a**, the reduced form of **5** (Figure S5 in the SI) (*15*). Consistent with previous observations (*15*), presumably **5** is formed initially and is reduced under the reaction conditions or during analysis. Under the same high DMSO reaction conditions, but in the absence of DNA, Cl_4BQ formation is observed independently. The generation of Cl_4BQ in the absence or presence of DMSO correlates with the HRP concentration but saturates at 50 μM HRP for the high DMSO conditions compared to 25 μM in the absence of DMSO (Figure S6 in the SI). Furthermore, in these control experiments, production of

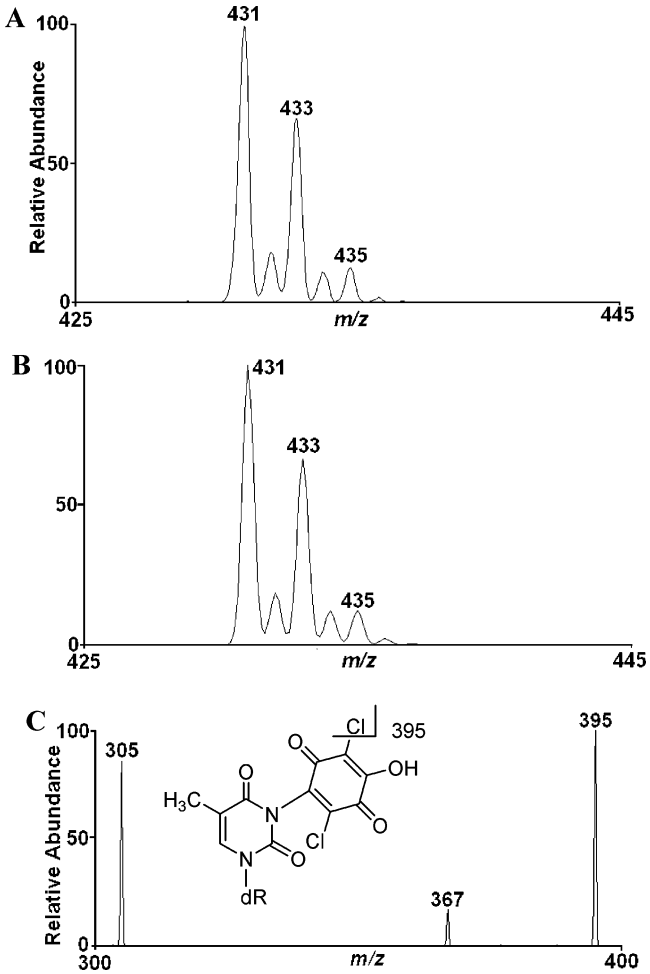


Figure 4. Typical MS/MS analyses of **4** from the reaction of Thd with Cl_4BQ in 10 mM phosphate buffer, pH 7.4, at 37 °C for 2 h: (A) adduct **4** has a molecular ion, $[M - H]^-$, of *m*/*z* 431, (B) simulated MS data, (C) MS/MS data with fragment ion *m*/*z* 395.

Cl_4BQ was observed to be time dependent, consistent with a previous report that did not involve DMSO (*10*). The presence of DMSO did not appear to influence the relative rate of Cl_4-BQ formation. Finally, a minor peak with an earlier retention time (26 min) and MS and MS/MS data identical to those of **5a** also was observed in reactions with DNA. Its structure is not known, and it does not coelute with any of the identified Cl_4BQ−DNA adducts, but it is also observed in reactions of PCP, peroxidase, and dGuo. Also observed in this experiment was 8-PCP−dGuo, an adduct that was previously identified by Manderville and co-workers (*20*). On the basis of relative peak areas, but not accounting for potential differences in efficiencies of ionization of these two adducts, the relative ratio of **5** to 8-PCP−dGuo is 5:1, while under the original conditions, only 8-PCP−dGuo is formed. These data demonstrate that **5** can be produced in the peroxidase-mediated reaction of PCP with DNA.

Discussion

Previous reports involving ^{32}P-postlabeling experiments indicate that, in rodents chronically exposed to PCP, multiple DNA adducts are formed in the liver and these adducts correlate with products of cell-free Cl_4BQ−DNA reactions (*21*). While the product of the reaction of dGuo with Cl_4BQ has been identified (*15*), the characterization of Cl_4BQ-generated adducts has been technically challenging for various reasons. Initially

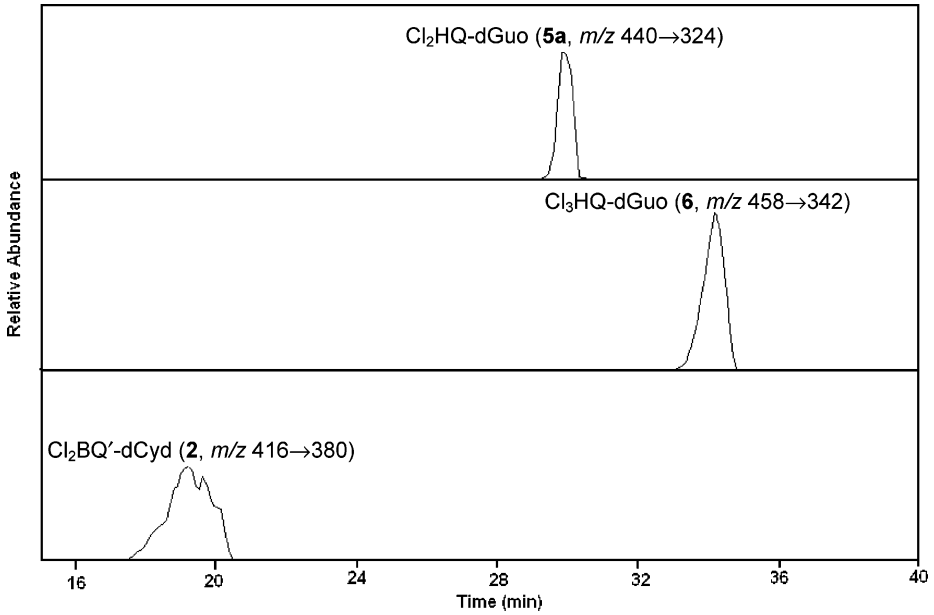


Figure 5. LC−ESI-MS/MS chromatograms from the reaction of DNA with Cl_4BQ in 10 mM phosphate buffer, pH 7.4, at 37 °C for 72 h. Selected ion monitoring (SIM) was carried out for each of the Cl_4BQ-derived adducts identified in experiments with isolated nucleosides. Data for the three adducts observed under these conditions, **2**, **5a**, and **6**, are shown here. Structures are indicated in Chart 1 and the corresponding *m*/*z* values in each panel. Assuming similar MS response factors, the relative ratio of concentrations of **5a** to **6** to **2** is 1.00:0.7:0.2.

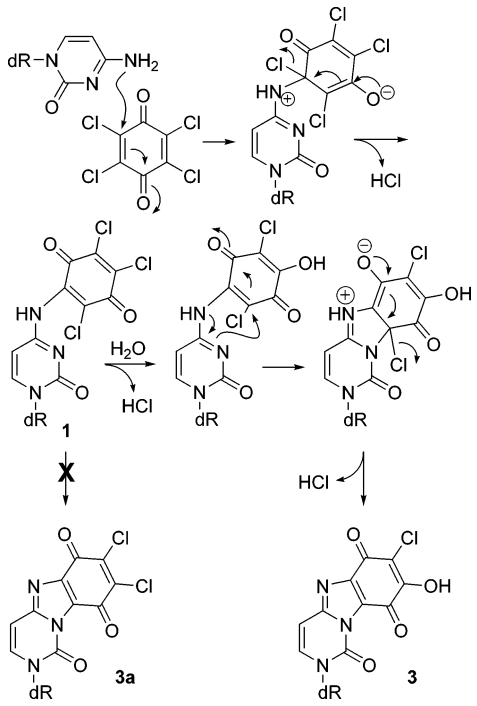
formed Cl_4BQ adducts may be unstable and susceptible to further nucleophilic attack or may be redox sensitive under reaction conditions, on a chromatographic support, or in an electrospray ionization source. Furthermore, they generally lack characteristic ^1H NMR signals. We investigated reactions of Cl_4BQ with individual nucleosides and DNA in an effort to chemically characterize the corresponding reaction products and to develop experimental approaches for continued investigation of PCP reaction pathways in biological systems, such as in the presence of enzymes, in cells, and in vivo.

The reaction of dCyd with Cl_4BQ yields three different adducts, identified as Cl_3BQ−dCyd (**1**), Cl_2BQ'−dCyd (**2**), and ClBQ−dCyd (**3**), with **3** being the major adduct. Analogous exocyclic adducts were observed previously in reactions of dGuo, dAdo, and dCyd with *p*-benzoquinone (*19*, *22*, *23*). On the basis of the structure illustrated as **3** (Chart 1), its formation is presumably associated with the initial formation of Cl_3BQ−dCyd followed by hydrolysis and subsequent cyclization. The distribution of Cl atoms for **1**−**3** suggests a putative mechanism for their formation involving initial addition by the N^4-amino group of cytosine followed by loss of HCl to produce **1** (Scheme 3). At this stage, a Cl atom is lost and a second conjugate addition at the dCyd 3-position results in ring closure and generation of **3**. Interestingly, none of the hypothetical adduct Cl_2BQ−dCyd (**3a**) was observed, which would, on the basis of previous studies involving dGuo, be the expected adduct. Lack of **3a** tentatively suggests that hydrolysis precedes cyclization.

The adduct from the reaction of Thd with Cl_4BQ was characterized as Cl_2BQ−Thd (**4**). This adduct has a ^1H NMR spectrum that is indistinguishable from that of unreacted Thd and has the same retention time by HPLC. On the basis of ^{13}C NMR chemical shifts for [$^{13}C_6$]**4**, the structure is formulated as containing a N^3-linkage, consistent with previous reports of reactions of Thd with reactive electrophiles such as butadiene monoxide (BM) and esterone-*o*-quinones (*24*, *25*).

The structures of Cl_4BQ adducts for each of the nucleosides indicate notable and unexpected base-dependent differences in their reactivities. In the reaction of Cl_4BQ with DNA, three

Scheme 3



products were observed in DNA hydrolysates, the major ones being benzetheno-type dGuo adducts Cl_2BQ−dGuo (**5**) and Cl_3-HQ−dGuo (**6**), with **2** as a minor product. No adducts of dAdo were observed in spite of the known formation of benzetheno-type adducts in reactions of dAdo with *p*-benzoquinone (*23*).

It has been demonstrated here and previously (*15*) that Cl_4-BQ forms covalent adducts with DNA and that Cl_4BQ is a metabolite of PCP, but Cl_4BQ-derived adducts have not been identified previously in reactions of PCP with DNA. In this study, quinone-derived products were observed in the reaction of PCP with DNA or dGuo in the presence of an activating system, but required the addition of DMSO and extended reaction times. The failure of reactions under low DMSO

conditions is presumably associated with the low aqueous solubility of Cl_4BQ. While the high DMSO levels are not biological, the compartmentalized and complex environment of the cell may have the capacity to solubilize the transiently produced quinone. Previously reported in vivo levels (8 adducts/10^7 nucleotides) for adducts that coelute with quinone−DNA products suggest the potential for a significant nuclear dose of Cl_4BQ (*21*). Regarding the duration of this reaction, it was observed that dietary PCP administration (1000 ppm) for 27 weeks, but not single or multiple acute doses, produced adducts in rat liver. The data obtained in this study suggest that **5**/Cl_2HQ−dGuo (**5a**), **6**, and 8-PCP−dGuo are potential structures for the observed in vivo adducts; further studies are needed to directly correlate the in vitro and in vivo results and to determine the mutagenic potential of the adducts that are present.

New Cl_4BQ−dGuo, dCyd, and Thd adducts have been identified in reactions of Cl_4BQ with nucleosides, nucleotides, and DNA. The structures of adducts reflect different proclivities for conjugate addition vs hydrolysis reactions for each base. Furthermore, the presence of Cl substituents on the quinone alters the reactivity of the electrophile relative to that for unsubstituted quinones, such that quinone-containing adducts with the potential to undergo further reactions with nucleophiles are formed. Adducts resulting from PCP metabolism and subsequent DNA alkylation were observed in the presence of a peroxidase, and while the efficiency of this process is low, the observation of Cl_4BQ-specific adducts indicates the potential biological relevance of this pathway. The data obtained in the present study are important for understanding potential contributions of chloroquinone-specific DNA adducts to chlorophenol carcinogenesis, their chemical reactivities, and for the development of biomarkers for carcinogen exposure.

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Supporting Information Available: HPLC chromatogram of **1**−**3**, ^{13}C NMR data of **4**, TIC of the Cl_4BQ−DNA adduct resulting from PCP/HRP, and data for control reactions involving PCP and HRP. This material is available free of charge via the Internet at http://pubs.acs.org.

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