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Potent Methyl Oxidation of 5-Methyl-2'-deoxycytidine by Halogenated Quinoid Carcinogens and Hydrogen Peroxide via a Metal-independent Mechanism

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Abstract

Halogenated quinones are a class of carcinogenic intermediates and newly identified chlorination disinfection byproducts in drinking water. We found recently that the highly reactive and biologically important hydroxyl radical ($\cdot\text{OH}$) can be produced by halogenated quinones and H_2O_2 independent of transition metal ions. However, it is not clear whether these quinoid carcinogens and H_2O_2 can oxidize the nucleoside 5-methyl-2'-deoxycytidine (5mdC) to its methyl oxidation products, and if so, what is the underlying molecular mechanism. Here we show that three methyl oxidation products 5-(hydroperoxymethyl)-, 5-(hydroxymethyl)-, and 5-formyl-2'-deoxycytidine could be produced when 5mdC was treated with tetrachloro-1,4-benzoquinone (TCBQ) and H_2O_2 . The formation of the oxidation products was markedly inhibited by typical $\cdot\text{OH}$ scavengers and under anaerobic condition. Analogous effects were observed with other halogenated quinones and the classic Fenton system. Based on these data, we proposed that the oxidation of 5mdC by TCBQ/ H_2O_2 might be through the following mechanism: $\cdot\text{OH}$ produced by TCBQ/ H_2O_2 may first abstract hydrogen from the methyl group of 5mdC, leading to the formation of 5-(2'-deoxycytidyl)methyl radical, which may combine with O_2 to form peroxy radical. The unstable peroxy radical transforms into the corresponding hydroperoxide 5HpmdC, which reacts with TCBQ and results in the formation of 5HmdC and 5fdC. This is the first report that halogenated quinoid carcinogens and H_2O_2 can induce potent methyl oxidation of 5mdC via a metal-independent mechanism, which may partly explain their potential carcinogenicity.

Graphical Abstract

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Keywords

Halogenated quinones; hydroxyl radical; 5-methyl-2'-deoxycytidine; 5-(hydroperoxymethyl)-2'-deoxycytidine; 5-(hydroxymethyl)-2'-deoxycytidine; 5-formyl-2'-deoxycytidine

Introduction

Halogenated quinones are a class of toxicological intermediates which can cause acute nephrotoxicity, hepatotoxicity, and carcinogenesis [1, 2]. They have also been found as reactive intermediates or products in processes used to oxidize or degrade polyhalogenated persistent organic pollutants (POPs) in a variety of chemical and enzymatic systems [3-8]. More recently, several polyhalogenated quinones, which are suspected bladder carcinogens, were observed as new chlorination disinfection byproducts in drinking water [7, 8]. Tetrachloro-1, 4-benzoquinone (TCBQ, also called *p*-chloranil) is one of the major genotoxic and carcinogenic quinoid metabolites of the widely used wood preservative pentachlorophenol (PCP). PCP has been found in over 20% of the National Priorities List sites identified by the U.S. EPA and classified as a group 2B environmental carcinogen [3].

The hydroxyl radical ($\cdot\text{OH}$) is an extremely reactive oxidant, important in biology, medicine, chemistry and environmental science [3, 9-11]. In biology, $\cdot\text{OH}$ is considered as the most reactive and harmful of the so-called reactive oxygen species (ROS), which can cause DNA and other macromolecule oxidation [9-11]. Cancer, Parkinson's disease and arthritis are but a few of the ailments that are related to $\cdot\text{OH}$ generation [9]. One of the most widely accepted mechanisms for $\cdot\text{OH}$ production is via the transition metal-catalyzed Fenton reaction [3, 9-11]. Recently, however, we found that $\cdot\text{OH}$ could be produced by hydrogen peroxide (H_2O_2) with TCBQ and other halogenated quinones independently of transition metal ions [12, 13]. A novel nucleophilic substitution and homolytic decomposition reaction mechanism has been proposed [3, 13]. We also found that alkoxy and carbon-centered quinone ketoxy radicals could be generated via a similar mechanism by halogenated quinone mediated decomposition of organic hydroperoxides [14-16].

It is well-known that the genetic material is constructed from the four DNA nucleosides, and 5-methylcytosine (5mCyt) is often considered to be the fifth base of the genome. Cytosine residues in DNA are methylated enzymatically, usually in the CpG dinucleotide [17]. In

humans, approximately 5% cytosine residues are methylated, which play an important role in regulation of transcription during cellular differentiation and silencing of invading viral genomes [18, 19]. The reverse of this process, DNA demethylation is equally important for cleaning genomic slate during embryogenesis or achieving rapid reactivation of silenced gene [20]. Recently, it has been demonstrated that Tet family of proteins have the capacity to convert 5mCyt not only to 5-hydroxymethylcytosine (5HmCyt), but also to 5-formylcytosine (5fCyt) and 5-carboxylcytosine (5caCyt) in vitro [21-23]. In addition, there is evidence for the presence of 5fCyt and 5caCyt in the genomic DNA of mouse ES cells [23-26]. Emerging evidence has shown that 5mCyt derivatives are involved in normal development as well as in many diseases [27-30].

It has been shown that 5HmCyt and 5fCyt were produced when 5mCyt was treated by Fenton-type reagents or after UV-light exposure [31-34]. However, it is still not clear whether halogenated quinones and H₂O₂ can oxidize 5-methyl-2'-deoxycytidine (5mdC) to form its methyl oxidation products or not; and if so, what is the possible underlying mechanism. Therefore, in the present study we addressed the following questions: (i) Can 5HmdC, 5fdC or other methyl oxidation products be produced by halogenated quinones and H₂O₂; (ii) If so, is the oxidation dependent on [•]OH production; (iii) What is the molecular mechanism underlying 5mdC oxidation; and (iv) What are the potential biological and environmental implications?

Materials and Methods

Materials

Analytical grade chemicals, HPLC grade organic solvents, and chelex-100 treated buffer were used throughout. 5mdC purchased from TCI (Shanghai, China) was used as received. All halogenated quinones were purchased from Sigma-Aldrich. Hydrogen peroxide (H₂O₂) was purchased from Beijing Chemical Works (Beijing, China). 5HmdC and 5fdC purchased from Berry & Associates, Inc. (Dexter, MI) were used as standard.

Reactions of XBQs, H₂O₂, and 5mdC

The basic system used in the study consisted of 0.1mM XBQs dissolved in acetonitrile (final acetonitrile concentration in the reaction mixture, 2%), 1.0mM H₂O₂ and 2.0mM 5mdC in 10mM phosphate buffer (pH 7.4) at 37°C for 1h unless otherwise stated. The phosphate buffer used for all experiments was pretreated with chelex-100 ion-exchange resin (Bio-Rad Laboratories, Hercules, CA, 5g/L) overnight to remove trace transition metal probably presented in phosphate buffer as contaminants. The reaction solutions were directly injected into HPLC-triple quadrupole MS system for analysis.

HPLC/triple-quadrupole mass spectrometry (HPLC-MS/MS) analysis of oxidation products of 5mdC

The HPLC separation was conducted on an Agilent 1290 series equipped with two pumps, autosampler, and solvent cabinet (Agilent, Waldbronn, Germany). A reversed-phase ZORBAX Eclipse Plus C18 column (4.6×150mm, 5μm, Agilent) was used, and the mobile phase of 10.0% methanol and 90.0% water (with 0.1% formic acid) was used for isocratic

elution at a flow rate of 1.0 mL/min. The eluate from the HPLC column was directly introduced into an ESI-triple quadrupole mass spectrometer (Agilent 6410B series, Santa Clara, CA) with flow-splitting. The mass spectrometer was operated in the positive ion mode. For Multiple Reaction Monitoring (MRM) analysis, collision energy was performed at 5 eV. The fragmentor voltage was 90 V, nitrogen was used as nebulizer gas and the desolvation gas (nitrogen) was heated to 300 °C and delivered at a flow rate of 9.0 L/min. The capillary voltage was set at 3500 V. The injection volume is 10 µL for the reaction mixture containing nucleotide. Since more than 97% 5mdC could not be oxidized by the reaction, 5mdC was used as an internal standard for calibration and quantification of the formed oxidation products in the reaction mixtures in HPLC-MS/MS analysis. In addition, the characteristic daughter ion m/z 83 was used for the analysis of 5mdC (CID 5 eV). The data were collected by Agilent MassHunter Data Acquisition Workstation.

Results and Discussion

Three methyl oxidation products were identified during metal-independent oxidation of 5mdC by TCBQ and H₂O₂

Three major oxidation products of 5mdC were observed by HPLC-MS/MS analysis, when 5mdC (2.0 mM) was incubated with the combination of TCBQ (0.1 mM) and H₂O₂ (1.0 mM) together, but not by either of them alone (Figure S1). Two of these products showed the same chromatographic retention time (1.7 and 4.3 min), and the same MRM fragmentation pattern (m/z 258 → 142 and 256 → 140) as the authentic standard 5-(hydroxymethyl)-2'-deoxycytidine (5HmdC), and 5-formyl-2'-deoxycytidine (5fdC), and therefore they were identified as 5HmdC and 5fdC, respectively.

Interestingly and unexpectedly, a new peak with the retention time at 1.8 min was also observed in the HPLC profile besides 5HmdC and 5fdC. This was identified by LC/MS/MS method as the corresponding hydroperoxide, 5-(hydroperoxymethyl)-2'-deoxycytidine (5HpmdC), which was previously identified as one of the oxidation products during photosensitized one-electron oxidation of 5mdC [35], but was not reported when 5mC was treated by Fenton-type reagents or after UV-light exposure [31-34]. We found that 5HpmdC produced in our TCBQ/H₂O₂ system was the same as that produced in the photosensitized oxidation of 5mdC, since they showed the same chromatographic retention time and the same MRM fragmentation pattern (Figure S2). The high resolution MS data further confirmed that the compound is indeed 5HpmdC (experimental value: m/z = 274.10390; theoretical value: m/z = 274.10336). To our surprise, 5HpmdC, together with 5HmdC and 5fdC, could also be observed when 5mdC was oxidized by the classic Fenton system (Fe^{II}EDTA/H₂O₂) in this study. To our knowledge, this is the first report that 5HpmdC could be produced by two distinct •OH-producing systems, the metal-dependent Fenton system and the metal-independent TCBQ/H₂O₂ system.

The formation of the three methyl oxidation products of 5mdC was found to be time and dose-dependent on TCBQ (or Fe^{II}EDTA) and H₂O₂ (Figure 1, for details, see Supporting Information). Besides the above three methyl oxidation products, other •OH-oxidized products of 5mdC were also investigated in TCBQ/H₂O₂ system (see Supporting Information).

Both $\cdot\text{OH}$ and molecular oxygen (O_2) played a critical role in the formation of 5mdC oxidation products by TCBQ and H_2O_2

Since we have shown recently that $\cdot\text{OH}$ can be produced through homolytical decomposition of H_2O_2 by TCBQ independent of transition metal ions [12, 13], we hypothesized that $\cdot\text{OH}$ may play a critical role in methyl oxidation of 5mdC. To test whether $\cdot\text{OH}$ was involved or not, three typical $\cdot\text{OH}$ scavengers including DMSO, ethanol and sodium formate were chosen. We found that the three $\cdot\text{OH}$ scavengers can significantly suppress the oxidation of 5mdC by TCBQ and H_2O_2 (Figure 2A). Analogous inhibitory effects were observed in $\text{Fe}^{\text{II}}\text{EDTA}/\text{H}_2\text{O}_2$ system (Figure 2B). These results indicate that $\cdot\text{OH}$ indeed played an essential role in the formation of 5mdC oxidation products.

The most feasible pathway for $\cdot\text{OH}$ to oxidize the methyl group of 5mdC should be the abstraction of a hydrogen atom by $\cdot\text{OH}$ from the methyl group with the concomitant formation of a carbon-centered 5-(2'-deoxycytidyl)methyl radical, which then combines with molecular oxygen (O_2) to form its corresponding peroxy radical. The unstable peroxy radical transforms into the corresponding hydroperoxide 5HpmdC. If the above hypothesis were right, then O_2 should also play an important role in the formation of the methyl oxidation products. To test whether this is the case, the same experiments were conducted in an Anaerobic Chamber. Interestingly, we found that the generation of 5mdC oxidation products in both TCBQ/ H_2O_2 and $\text{Fe}^{\text{II}}\text{EDTA}/\text{H}_2\text{O}_2$ system decreased markedly by 70% and 50%, respectively, under anaerobic condition (Figure 2C). These results clearly suggest that O_2 is also important for the formation of 5mdC oxidation products.

Analogous oxidation of 5mdC was also observed with other halogenated quinones

5mdC can also be effectively oxidized to 5HpmdC, 5HmdC and 5fdC when TCBQ was substituted by other XBQs. These include 2-chloro- (2-CBQ), 2,3-dichloro- (2,3-DCBQ), 2,5-dichloro- (2,5-DCBQ), 2,6-dichloro- (2,6-DCBQ), 2,3,5-trichloro- (TriCBQ), tetrafluoro- (TFBQ) and tetrabromo-1,4-benzoquinone (TBrBQ) (Figure 3). The reactivity of XBQs depends on the type, number, and position of halogen substitution, and ranks in the descending order of 2,3-DCBQ > TCBQ, TBrBQ > TriCBQ > TFBQ > 2,5-DCBQ > 2,6-DCBQ > 2-CBQ. In contrast, 1,4-benzoquinone (*p*-BQ) that lacks any halogen substitution induced much less oxidation products under the same experiment conditions. These results clearly indicate the importance of halogen substitution of XBQs played in the enhanced oxidation of 5mdC by XBQs/ H_2O_2 . Surprisingly, 2,3-DCBQ displays strongest oxidation capacity among the 9 tested XBQs, which is more than twice more efficient than that of TCBQ/ H_2O_2 , and similar to that of $\text{Fe}^{\text{II}}\text{EDTA}$ mediated Fenton reaction. Further studies are needed to understand why 2,3-DCBQ is so special.

Proposed mechanism for the formation of 5mdC oxidation products by TCBQ/ H_2O_2

Based on the above data and other previous studies [10], we proposed that the formation of 5HpmdC, 5HmdC and 5fdC from 5mdC by TCBQ/ H_2O_2 might be through the following metal-independent mechanism (Scheme 1): First, $\cdot\text{OH}$ produced by TCBQ/ H_2O_2 may abstract hydrogen atom from the methyl group of 5mdC, resulting in the formation of the carbon-centered 5-(2'-deoxycytidyl)methyl radical, which then combines with O_2 to form its corresponding peroxy radical. The unstable peroxy radical can transform into the

corresponding hydroperoxide 5HpmdC. A further nucleophilic reaction may take place between 5HpmdC and TCBQ, forming a quinone-peroxide reaction intermediate, which can decompose homolytically to produce 5-CH₂O[•] alkoxyl radical (Scheme 1) [14]. The generated 5-CH₂O[•] alkoxyl radical may transform into 5HmdC and 5fdC via dismutation. The combination of 5-(2'-deoxycytidylyl)methyl radical and [•]OH can also lead to the formation of 5HmdC. An alternative pathway for the generation of 5HmdC and 5fdC might be due to reduction and dehydration of 5HpmdC. To our knowledge, this is the first report that halogenated quinoid carcinogens and H₂O₂ can induce potent methyl oxidation of 5mdC via a metal-independent mechanism.

It should be noted that, although we tried hard to identify the proposed carbon-centered 5-(2'-deoxycytidylyl)methyl radical by ESR spin-trapping methods, using various spin trapping agents such as the well-known dimethyl pyridine N-oxide (DMPO) and α-(4-pyridyl-1-oxide)-N-tert-butyl nitron (POBN), we failed to detect this radical so far. The reason might be that either the radical adduct formed may not be stable enough, or the concentration of the radical adduct was just too low to be detected by ESR. Further studies are needed to solve this problem.

Potential biological and environmental implications

In conclusion, these findings demonstrate that not only TCBQ, but also other XBQs together with H₂O₂ can effectively oxidize 5mdC to form its methyl oxidation products not requiring the involvement of redox-active transition metal ions, and the oxidation efficiency of some XBQs is comparable to that of classic iron-mediated Fenton reaction. These results may partly explain the potential carcinogenicity of not only PCP but also other widely used polyhalogenated aromatic compounds which can be metabolized in vivo or dechlorinated chemically to tetra-, di-, or monohalogenated quinones [4-6, 36, 37]. Recently, four chloro- and bromo-benzoquinones were identified as new chlorination disinfection byproducts in drinking water, which may be responsible for the observed bladder cancer risk [7, 8]. Regarding the wide distribution of XBQs in the environment and the fact that the established 5mdC oxidation occurs even at physiologically relevant concentrations of XBQ (as low as 10 μM), our new findings may have potential biological and environmental implications for future study of these ubiquitous halogenated quinoid carcinogens. Further investigations are needed to study whether these oxidation reactions occur in cellular and animal models. If the conversion of 5mCyt to 5HmCyt and other oxidation derivatives by redox-active quinones were confirmed in mammalian cells, this study would provide a new understanding of a non-enzymatic pathway for DNA demethylation induced by environmental pollutants, which might play important roles in development and many diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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Abbreviations

TCBQ	tetrachloro-1,4-benzoquinone (<i>p</i> -chloranil)
PCP	pentachlorophenol
$\cdot\text{OH}$	hydroxyl radical
ROS	reactive oxygen species
H_2O_2	hydrogen peroxide
5mCyt	5-methylcytosine
5HmCyt	5-hydroxymethylcytosine
5fCyt	5-formylcytosine
5caCyt	5-carboxylcytosine
MRM	multiple reaction monitoring
5mdC	5-methyl-2'-deoxycytidine
5HmdC	5-(hydroxymethyl)-2'-deoxycytidine
5fdC	5-formyl-2'-deoxycytidine
5HpmdC	5-(hydroperoxymethyl)-2'-deoxycytidine
<i>p</i>-BQ	1,4-benzoquinone
2-CBQ	2-chloro-1,4-benzoquinone
2,3-DCBQ	2,3-dichloro-1,4-benzoquinone
2,5-DCBQ	2,5-dichloro-1,4-benzoquinone
2,6-DCBQ	2,6-dichloro-1,4-benzoquinone
TriCBQ	2,3,5-trichloro-1,4-benzoquinone
TFBQ	tetrafluoro-1,4-benzoquinone
TBrBQ	tetrabromo-1,4-benzoquinone

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Highlights

Three methyl oxidation products were generated in the mixture of 5mCyt, TCBQ and H_2O_2 .

The oxidation was markedly inhibited by $\cdot OH$ scavengers and under anaerobic condition.

Analogous effects were observed in the classic Fenton system.

The proposed mechanism may partly explain halogenated quinones' carcinogenicity.

Figure 1. Time course and dose-dependent formation of methyl oxidation products of 5mdC by TCBQ (or Fe^{II}EDTA) with H₂O₂
 Time-dependent formation of 5mdC oxidation products by TCBQ (A) or Fe^{II}EDTA (B) with H₂O₂. Dose-dependent formation of methyl oxidation products of 5mdC on TCBQ (C) or Fe^{II}EDTA (D). Dose-dependent formation of methyl oxidation products of 5mdC on H₂O₂ (E and F). The reaction solution contained 0.01mM TCBQ (or Fe^{II}EDTA), 0.1mM H₂O₂ and 0.1mM 5mdC in 10mM phosphate buffer, pH7.4, at 37°C.

Figure 2. Both $\cdot\text{OH}$ and O_2 played a critical role in the formation of oxidation products of 5mdC by TCBQ/H₂O₂ and Fe^{II}EDTA/H₂O₂

Inhibition effect by $\cdot\text{OH}$ scavengers on the formation of 5mdC oxidation products by TCBQ/H₂O₂ (A) and Fe^{II}EDTA/ H₂O₂ (B); the formation of 5mdC oxidation products under anaerobic condition by TCBQ/H₂O₂ and Fe^{II}EDTA (C). The reaction solution contained 2.0mM 5mdC, 0.1mM TCBQ (or Fe^{II}EDTA), and 1.0mM H₂O₂.

Figure 3. (A) Methyl oxidation products of 5mdC could also be produced by other XBQs and H_2O_2

All the reaction mixture contained 2.0mM 5mdC, 1.0mM H_2O_2 and 0.1mM XBQs (Fe^{II} or Fe^{II} /EDTA). **(B)** The chemical structures of XBQs used in this study.

Scheme 1.

Proposed mechanism for the formation of 5mCyt oxidation products by TCBQ and H_2O_2 via a metal-independent pathway.