Mechanism of synergistic DNA damage induced by the hydroquinone metabolite of brominated phenolic environmental pollutants and Cu(II): Formation of DNA-Cu complex and site-specific production of hydroxyl radicals

Bo Shao^{a,b}, Li Mao^a, Na Qu^a, Ya-Fen Wang^c, Hui-Ying Gao^a, Feng Li^a, Li Qin^a, Jie Shao^a, Chun-Hua Huang^a, Dan Xu^a, Lin-Na Xie^a, Chen Shen^a, Xiang Zhou^c, Ben-Zhan Zhu^{a,*}

^a State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences and University of Chinese Academy of Sciences, The Chinese Academy of Sciences, Beijing 100085, PR China

^b School of Public Health, Jining Medical University, Jining, Shandong 272067, PR China

^c College of Chemistry and Molecular Sciences, Key Laboratory of Biomedical Polymers of Ministry of Education, Wuhan University, Wuhan, Hubei 430072, PR China

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ABSTRACT

2,6-Dibromohydroquinone (2,6-DBrHQ) has been identified as an reactive metabolite of many brominated phenolic environmental pollutants such as tetrabromobisphenol-A (TBBPA), bromoxynil and 2,4,6-tribromophenol, and was also found as one of disinfection byproducts in drinking water. In this study, we found that the combination of 2,6-DBrHQ and Cu(II) together could induce synergistic DNA damage as measured by double strand breakage in plasmid DNA and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) formation, while either of them alone has no effect. 2,6-DBrHQ/Cu(II)-induced DNA damage could be inhibited by the Cu(I)-specific chelating agent bathocuproine disulfonate and catalase, but not by superoxide dismutase, nor by the typical hydroxyl radical ($\cdot\text{OH}$) scavengers such as DMSO and mannitol. Interestingly, we found that Cu(II)/Cu(I) could be combined with DNA to form DNA-Cu(II)/Cu(I) complex by complementary application of low temperature direct ESR, circular dichroism, cyclic voltammetry and oxygen consumption methods; and the highly reactive $\cdot\text{OH}$ were produced synergistically by DNA-bound-Cu(I) with H_2O_2 produced by the redox reactions between 2,6-DBrHQ and Cu(II), which then immediately attack DNA in a site-specific manner as demonstrated by both fluorescent method and by ESR spin-trapping studies. Further DNA sequencing investigations provided more direct evidence that 2,6-DBrHQ/Cu(II) caused preferential cleavage at guanine, thymine and cytosine residues. Based on these data, we proposed that the synergistic DNA damage induced by 2,6-DBrHQ/Cu(II) might be due to the synergistic and site-specific production of $\cdot\text{OH}$ near the binding site of copper and DNA. Our findings may have broad biological and environmental implications for future research on the carcinogenic polyhalogenated phenolic compounds.

1. Introduction

Polyhalogenated quinoid compounds are a class of toxic intermediates that can cause acute hepatotoxicity, nephrotoxicity, and carcinogenesis [1,2]. They have also been found as reactive oxidation intermediates or products in processes used to oxidize or destroy polychlorinated aromatic pollutants in various chemical and enzymatic systems [3–7]. More recently, more than a dozen of polyhalogenated quinoid compounds and their hydroxylated derivatives, which are suspected to be bladder carcinogens, were identified as new chlorination disinfection byproducts (DBPs) in drinking water [8].

Tetrabromobisphenol-A (TBBPA) is the most widely used brominated flame retardant in the manufacture of industrial equipment and consumer goods in order to prevent or minimize fire damage [9,10]. The annual global consumption of TBBPA was estimated to be more than 170,000 t [11]. Human TBBPA serum levels were measured and found to be in the range from 1 to 3.4 pmol/g lipid [12]. TBBPA has been considered to be an immune-toxicant and thyroid hormone agonist and has the potential to disrupt estrogen signaling, at least in vitro. Although these reports suggest that TBBPA induce many toxic effects, the underlying molecular mechanism remains to be clarified. It has been shown that 2,6-dibromohydroquinone (2,6-DBrHQ) is one of

* Corresponding author.

E-mail address: bzhu@rcees.ac.cn (B.-Z. Zhu).

the major metabolites of TBBPA in rats, and the hepatotoxicity of TBBPA in rats might be due to the *in vivo* generation of the reactive oxygen species as a result of redox reactions involving 2,6-DBrHQ and its corresponding semiquinone radicals [13]. It should be noted that 2,6-DBrHQ was also found to be the metabolite of the widely used herbicide bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and brominated phenols such as 2,4,6-tribromophenol (TBP), which has been used as a flame retardant by itself and also as an important intermediate for other chemical synthesis [14–16].

Both 2,6-DBrHQ and its oxidation product 2,6-dibromo-1,4-benzoquinone (2,6-DBrBQ) were also identified as DBPs in simulated drinking water, and 2,6-DBrBQ was detected in 11 of the 16 drinking water samples (68.8%) at concentrations up to 37.9 ng/L [8].

Taken together, we can see that 2,6-DBrHQ is not only a common reactive metabolite or decomposition product from several typical brominated phenolic environmental pollutants with high occurrence frequency, it is also a disinfection byproduct in simulated drinking water, highlighting the need for further research.

Copper is an essential trace element for humans [17]. Besides forming the essential redox active center in a variety of metalloproteins, copper has also been found in the nucleus and to be closely associated with the chromosomes and DNA bases, particularly guanine [18]. DNA-associated copper has been suggested to be involved in maintaining normal chromosome structure and in gene regulatory processes [19–21].

Studies have shown that transition metals, particularly copper, are capable of mediating the activation of several classes of less reactive compounds, such as hydroquinones, catechols, and dietary flavonoids and other xenobiotics by a redox Fenton-like mechanism, leading to the formation of more reactive oxygen species (ROS) [22,23]. Among the ROS, hydroxyl radical ($\cdot\text{OH}$) is considered to be the most reactive species that could damage DNA and other macromolecules. Since copper is capable of mediating activation of a variety of phenolic compounds [24], it is reasonable to propose that copper may have the potential to activate 2,6-DBrHQ via a copper-redox reaction, producing more reactive ROS and inducing more severe DNA damage.

Therefore, in this study, we plan to address the following questions. (1) Can 2,6-DBrHQ together with Cu(II) induce synergistic DNA damage; and if so, (2) what is the underlying molecular mechanism?

2. Materials and methods

2.1. Materials

Plasmid pBR322 DNA was purchased from New England Biolabs. 2,6-DBrHQ was synthesized based on the oxidation of 2,6-dibromophenol to 2,6-DBrBQ, then 2,6-DBrBQ was reduced to 2,6-DBrHQ. The carboxyfluorescein (FAM)-labeled DNA was purchased from Takara Biotechnology (Dalian, China). Calf thymus DNA (ct-DNA), cupric sulfate, bovine serum albumin (BSA), catalase from bovine liver (CAT), bathocuproine disulfonate (BCS), reduced glutathione (GSH), superoxide dismutase (SOD) from bovine erythrocytes, mannitol, dimethyl sulfoxide (DMSO), and α -(4-pyridyl-1-oxide)-*N*-tert-butyl nitron (POBN), coumarin-3-carboxylic acid (3-CCA) were purchased from Sigma (St. Louis, MO). All buffer solutions were treated with Chelex overnight to remove adventitious metals.

2.2. DNA strand breakage

Plasmid DNA agarose gel electrophoresis was used to investigate DNA strand breakage induced by 2,6-DBrHQ and Cu(II). The experiments were conducted via incubation of plasmid pBR322 DNA (5 $\mu\text{g}/\text{mL}$) at 37 °C for 30 min in Chelex-treated sodium phosphate buffer (100 mM, pH 7.4) with the 2,6-DBrHQ-containing systems, in the absence or presence of the indicated concentrations of Cu (II) and other agents.

2.3. Analysis of 8-oxodG formation

Calf thymus DNA (100 nM/base) was incubated with 2,6-DBrHQ in the presence or absence of Cu(II) in 100 mM Chelex-treated sodium phosphate buffer, pH 7.4, at 37 °C for the indicated durations. Then the DNA was digested to 2'-deoxyribonucleosides with 1.0 U DNase I, 2.0 U calf intestinal phosphatase, and 0.005 U snake venom phosphodiesterase I at 37 °C overnight. The digests were filtered by ultrafiltration tubes to remove the enzymes and then were subjected to UPLC-ESI-MS/MS analysis for detection of 8-oxodG.

2.4. Oxygen consumption and H_2O_2 generation

The O_2 consumption was measured by Orion 3 (Thermo Scientific) dissolved oxygen meter upon mixing 2,6-DBrHQ and Cu(II) in phosphate buffer (100 mM, pH 7.4) at 37 °C. H_2O_2 generation was determined indirectly by O_2 production upon adding catalase (500 U/mL) to the reaction mixture of 2,6-DBrHQ and Cu(II).

2.5. Electron spin resonance (ESR) spin-trapping studies

The basic system used in this study was composed of Cu(II) (10 μM), 2,6-DBrHQ (1 mM), DMSO (5%, v/v), and the spin trapping agent, α -(4-pyridyl-1-oxide)-*N*-tert-butyl nitron (POBN, 100 mM), in Chelex-treated phosphate buffer (100 mM, pH 7.4) with or without other agents. ESR spectra were recorded at room temperature in a Bruker ESR 300 spectrometer. Typical spectrometer parameters were as follows: scan range, 100 G; field set, 3470 G; time constant, 200 ms; scan time, 100 s; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; receiver gain, 1.25×10^4 ; and microwave power, 9.8 mW.

2.6. Low temperature ESR studies

Low temperature ESR spectra were recorded using a Bruker ESR 300 spectrometer at low temperature (77 K). The sample was placed in a standard quartz ESR tube prior to freezing. The sides of the tube were then warmed slightly to allow the frozen sample to slide into a finger Dewar filled with liquid nitrogen for acquisition of spectra. Spectral measurements of the Cu(II) (2 mM) were carried out in 100 mM phosphate buffer, pH 7.4, without or with 2 $\mu\text{g}/\mu\text{L}$ ctDNA.

2.7. Detection of $\cdot\text{OH}$ formation by coumarin-3-carboxylic acid (3-CCA) fluorescent method

A 5 mM stock solution of 3-CCA was prepared in 100 mM phosphate buffer at pH 9 at room temperature. When the 3-CCA was fully dissolved, the pH of the stock solution was adjusted back to 7.4. Working solutions were made by diluting the stock solution with an appropriate buffer at pH 7.4. The excitation and emission wavelength is 388 nm and 446 nm, respectively. Fluorescence was measured using a Thermo Scientific Varioskan Flash (Thermo Fisher scientific, USA).

2.8. Circular dichroism (CD) spectral study

CD of ct-DNA was measured with a MOS-450 Spectrometer, using cells of 10 mm path. The acquire duration was 0.5 s.

2.9. Cyclic voltammetry (CV)

CV was performed in 100 mM PB (pH 7.4) on a CHI 830B electrochemistry analyzer (CH Instruments, Austin, TX) with a Pt counter electrode, an Ag/AgCl reference electrode, and a glass carbon working electrode (CH Instruments Inc). The scan rate was 50 mV/s.

2.10. Detection of site-specificity of DNA damage induced by 2,6-DBrHQ in the presence of Cu(II)

The patterns of DNA cleavage induced by 2,6-DBrHQ in the presence of Cu(II) were determined by DNA sequencing using the modified Maxam–Gilbert procedure [25–27]. In Maxam–Gilbert procedure, DNA can be sequenced by a chemical procedure that breaks a terminally labeled DNA molecule partially at each repetition of a base. The lengths of the labeled fragments then identify the positions of that base. Four reactions were conducted to cleave DNA at different base sites, at guanines, adenines, cytosines, and both cytosines and thymines. When the products of these four reactions are resolved by size, by electrophoresis on a polyacrylamide gel, the DNA sequence can be read from the pattern of radioactive bands [25]. Our reaction mixture containing 2 μ M FAM-labeled DNA and indicated concentration of 2,6-DBrHQ and Cu(II) was incubated in 10 mM PB (pH 7.4) at 37 °C. After 30 min, ethanol (1 mL, 100%) and 10 μ L CH₃COONa–CH₃COOH buffer (1 M, pH 5.0) were added to this mixture. This mixture was frozen at –80 °C for 1 h and then centrifuged for 20 min. After removal of upper solution, the DNA precipitate was vacuum dried, then re-dissolved in 10% (v/v) piperidine and heated at 90 °C for 30 min. After the piperidine treatment, the DNA was precipitated again as the step mentioned above. After dried by vacuum, the DNA precipitate was dissolved in 80% (v/v) formamide for further examination on acrylamide gels. Following the steps above, DNA samples were loaded into a 20% polyacrylamide gel for electrophoresis at 20 V/cm; this electrophoresis occurred at room temperature in 1 $\times$ TBE buffer. The gel was then analyzed using the Pharos FX Molecular Imager (Bio-Rad, USA).

3. Results and discussion

3.1. Synergistic DNA damage, as measured by both strand breakage and 8-oxodG formation, were induced by the combination of both 2,6-DBrHQ and Cu(II)

It is well known that oxidatively generated damage to DNA will lead to strand breakage, base oxidation, tandem lesions, intra- and inter-strand cross-links, DNA-protein cross links [28,29]. According to recent studies, 20 base lesions are accounted for the reactions of hydroxyl radical, singlet oxygen or high intensity UVC laser pulse with nucleobases in cellular DNA. In addition several clustered lesions were found to be generated in cellular DNA as the result of one initial radical hit on either a vicinal base or the 2-deoxyribose [28–31]. The possibility for Cu(II) to act as one-electron oxidant in the presence of H₂O₂ has been proposed in previous studies [32–34]. Most of the one-electron oxidants, with the exception however of ionizing radiation through direct effects, are not able to oxidize 2-deoxyribose and therefore to induce DNA strand breaks [34]. Cu(II) act as one-electron oxidant can be observed in the presence of reducing agents, such as 2,6-DBrHQ, which can reduce Cu(II) to Cu(I). To detect whether synergistic oxidatively generated DNA damage could be induced by 2,6-DBrHQ and Cu(II), we first examined DNA strand breakage and oxidation of 2'-deoxyguanosine (dG) in dsDNA.

Plasmid DNA (pBR322) agarose gel electrophoresis has been considered as a model system to detect DNA strand breakage. The conversion of covalently closed circular double-stranded supercoiled plasmid DNA (form I) to a relaxed open circle form (form II) and a linear form (form III) was used to investigate whether DNA strand breakage can be induced by 2,6-DBrHQ and Cu(II) [35].

We found that only slight DNA strand breakage can be induced by 2,6-DBrHQ alone (100 μ M); however, in the presence of trace amount of Cu(II) (5 μ M), 2,6-DBrHQ-induced DNA damage increased significantly. Even very low concentration of 2,6-DBrHQ (1 μ M) could induce clear DNA strand breakage in the presence of 50 μ M Cu(II). The linear form (III) of DNA (i.e., double strand breakage) could be clearly observed when treated with 2,6-DBrHQ (100 μ M) in the presence of

Fig. 1. Synergistic DNA strand breakage was induced by the combination of 2,6-DBrHQ and Cu(II). A and B: DNA strand breakage was induced by 2,6-DBrHQ and Cu(II) in a concentration-dependent manner. C: The time course of the DNA strand breakage induced by 2,6-DBrHQ in the presence and absence of Cu(II). D: The role of reactive oxygen species and Cu(I) in 2,6-DBrHQ/Cu(II)-induced DNA strand breakage. The reaction mixtures contained Cu(II) (10 μ M) and 2,6-DBrHQ (100 μ M); Other agents: SOD, 1000 U/mL; Catalase, 500 U/mL; bathocuproine disulfonate (BCS), 40 μ M; reduced glutathione (GSH), 1 mM; DTPA, 40 μ M; Mannitol, 100 mM and DMSO, 5%. All reactions were conducted in Chelex-treated phosphate buffer (100 mM, pH 7.4) at 37 °C for 30 min. All reaction mixtures contained 5 μ g/mL plasmid DNA. Different DNA Forms: Form I, closed circular double-stranded supercoiled DNA; Form II, relaxed open circle DNA; and Form III, linear DNA.

10 μ M Cu(II). DNA cleavage was also found to depend on the concentrations of both 2,6-DBrHQ and Cu(II) and the treatment time (Fig. 1A–C). We found that DNA cleavage increases with increasing concentrations of 2,6-DBrHQ (1.0–100 μ M) in the presence of fixed concentration of Cu(II) (50 μ M); DNA cleavage induced by fixed concentration of 2,6-DBrHQ (100 μ M) also increased with the increasing concentrations of Cu(II) 2.5–10 μ M (Fig. 1A–C). These results showed that synergistic DNA cleavages can be induced by combining 2,6-DBrHQ and Cu(II) together.

In most cases the main DNA target is guanine with subsequent formation of 8-oxo-7,8-dihydroguanine (8-OxodG) and 4,6-diamino-5-formamidopyrimidine [29]. 8-OxodG is the most commonly measured biomarker of oxidative DNA damage [36], which causes DNA misreplication that might lead to mutation or cancer [37], 8-OxodG can be characterized and accurately quantified using highly sensitive and specific ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS). Analogous synergism was also observed for the formation of 8-oxodG from oxidation of ct-DNA by 2,6-DBrHQ/Cu(II) (Fig. 2).

3.2. The role of reactive oxygen species and Cu(I) on 2,6-DBrHQ/Cu(II)-induced synergistic DNA damages

To investigate the role of ROS and Cu(I) on 2,6-DBrHQ/Cu(II)-induced synergistic formation of DNA strand breakage and 8-oxodG, we studied the effects of CAT, SOD, Cu(I)-chelator BCS, Cu(II)-chelator DTPA, GSH and two typical $\cdot$ OH scavengers (mannitol and DMSO)

Fig. 2. Synergistic 8-oxodG formation was induced by the combination of 2,6-DBrHQ and Cu(II). A and B: 8-OxodG formation was induced by 2,6-DBrHQ and Cu(II) in a concentration-dependent manner. Cu(II), 10 μM; 2,6-DBrHQ, 200 μM; C: The time course of 8-oxodG formation induced by 2,6-DBrHQ in the presence and absence of Cu(II). D: The role of reactive oxygen species and Cu(I) in 2,6-DBrHQ/Cu(II)-induced 8-oxodG formation. The reaction mixtures contained Cu(II) (10 μM) and 2,6-DBrHQ (200 μM); Other agents: SOD, 1000 U/mL; Catalase, 500 U/mL; BCS, 40 μM; GSH, 1 mM; DTPA, 40 μM; Mannitol, 100 mM and DMSO, 5%. All reactions were conducted in Chelex-treated phosphate buffer (100 mM, pH 7.4) at 37°C for 30 min. All reaction mixtures contained 0.2 mg/mL calf thymus DNA (ct-DNA).

(Figs. 1D and 2D). We found that both kinds of DNA damage were completely inhibited by catalase, BCS, DTPA and GSH, but not by SOD, suggesting the involvement of both H₂O₂ and Cu(I). To our surprise, two typical •OH scavengers, DMSO and mannitol, showed little or no inhibitory effect on DNA strand breakage induced by 2,6-DBrHQ/Cu(II).

3.3. Synergistic production of •OH by 2,6-DBrHQ and Cu(II) by electron spin resonance (ESR) spin-trapping and fluorescent methods

We found that 2,6-DBrHQ alone can induce marked consumption of O₂ with the concomitant generation of H₂O₂ as shown by addition of catalase (Fig. S1). Interestingly, the O₂ consumption and H₂O₂ generation mediated by 2,6-DBrHQ was not significantly enhanced in the presence of Cu(II) (Fig. S1).

Our direct ESR study demonstrated that 2,6-DBrHQ is rapidly autoxidized to 2,6-DBrQ via its corresponding semiquinone radical, 2,6-DBSQ• (with g value = 2.0062). The rate of 2,6-DBSQ• formation

was clearly enhanced in the presence of 10 μM Cu(II). However, the formation and decay of 2,6-DBSQ• was not affected in the presence of DNA (Fig. S2). These results suggest that 2,6-DBrSQ• itself might not be the main reactive radical species responsible for 2,6-DBrHQ/Cu(II)-induced synergistic DNA damage.

The kinetics of formation and decay of 2,6-DBrSQ• (λ_{max} = 444 nm) in the presence and absence of Cu(II) and DNA under anaerobic conditions were also investigated by UV-visible method (Fig. S3). The autoxidation of 2,6-DBrHQ was completely inhibited under anaerobic conditions. And the presence of Cu(II) significantly increased the 2,6-DBrSQ•, suggesting that 2,6-DBrHQ is the Cu(II) reductant in the 2,6-DBrHQ/Cu(II) reaction. However, in the presence of both Cu(II) and DNA, the generation of 2,6-DBrSQ• decreased, which might be due to Cu(II) binding with DNA.

Since both H₂O₂ and a Cu(II)/Cu(I) redox cycle have been demonstrated to occur during the Cu(II)-mediated oxidation of 2,6-DBrHQ, more reactive oxygen species such as •OH may be generated from the

Fig. 3. Synergistic production of $\cdot\text{OH}$ from 2,6-DBrHQ and Cu(II) by ESR spin-trapping method using POBN as spin-trapping agent. **A** and **B**: ESR spectra for the synergistic production of POBN-CH₃ by 2,6-DBrHQ and Cu(II), and the inhibition by various agents. The reaction mixtures contained Cu(II) (10 μM) and 2,6-DBrHQ (1 mM); Other agents: SOD, 1000 U/mL; Catalase, 500 U/mL; BCS, 40 μM ; GSH, 1 mM; DTPA, 40 μM . **C** and **D**: POBN-CH₃ produced by 2,6-DBrHQ and Cu(II) in a concentration-dependent manner. Cu(II), 10 μM ; 2,6-DBrHQ, 200 μM ; All reaction mixtures contained 100 mM POBN, 5% DMSO. Reactions were conducted at room temperature in Chelex-treated phosphate buffer (100 mM, pH 7.4) in 30 min. Values represent a single scan with arbitrary units.

further reduction of H₂O₂ by Cu(I), and participate in the observed synergistic DNA damage.

To examine whether $\cdot\text{OH}$ could be generated from 2,6-DBrHQ/Cu(II) system, ESR spin trapping method using α -(4-pyridyl-1-oxide)-N-tert-butyl nitron (POBN) as spin-trapping agent was employed. It has been shown that interaction of $\cdot\text{OH}$ with DMSO results in the formation of a methyl radical ($\cdot\text{CH}_3$) [38]. Because of a high reaction rate constant (approximately $10^7 \text{ M}^{-1} \text{ s}^{-1}$) for POBN with $\cdot\text{CH}_3$, POBN in conjunction with DMSO has often been employed in ESR spin trapping studies to assess the formation of $\cdot\text{OH}$ [38].

We found that only slight POBN-CH₃ formation can be induced by 2,6-DBrHQ alone. The central signal in the spectrum was identified as 2,6-DBSQ $\cdot$, which was also observed in the absence of POBN/DMSO (see above). However, in the presence of 10 μM Cu(II), 2,6-DBrHQ-induced formation of POBN-CH₃ significantly increased, which was also found to depend on the concentrations of both 2,6-DBrHQ and Cu(II), as well as reaction time (Fig. 3).

Similar to the above results on DNA damage, we found that $\cdot\text{OH}$ formation by 2,6-DBrHQ/Cu(II) was completely inhibited by catalase, BCS, DTPA and GSH, but not by SOD, suggesting $\cdot\text{OH}$ formation results from the interaction between H₂O₂ and Cu(I), which is a copper-mediated Fenton-like reaction (Fig. 3A, B).

Besides the classic ESR spin-trapping method, $\cdot\text{OH}$ formation can be also detected by a more simple fluorescent method with coumarin-3-carboxylic acid (3-CCA) as an $\cdot\text{OH}$ probe, since the 3-CCA hydroxylation product 7-OH-3-CCA showed strong fluorescence [39,40]. Synergistic $\cdot\text{OH}$ generation from 2,6-DBrHQ/Cu(II) was also observed by the fluorescent method with 3-CCA (Fig. S4). It should be noted that minor amounts of $\cdot\text{OH}$ could also be generated by 2,6-DBrHQ alone as shown by both methods, which can be attributed to a novel metal-independent mechanism for $\cdot\text{OH}$ production by halogenated quinoid compounds in the presence of H₂O₂ [41–46].

Since 8-oxo-dG could also be generated by singlet oxygen other than $\cdot\text{OH}$ [29], we studied possible singlet oxygen production by 2,6-DBrHQ/Cu(II) using TEMP (2,2,6,6-tetramethylpiperidine) as the trapping agent. No singlet oxygen was detected by this method.

To further investigate the role of singlet oxygen on 2,6-DBrHQ/Cu(II)-induced synergistic formation of DNA strand breakage, we also examined whether NaN₃, methionine and DABCO could provide any protection (Fig. S5). As expected, no inhibition on 2,6-DBrHQ/Cu(II)-induced DNA damage was observed for the two classic singlet oxygen quenchers (NaN₃ and DABCO), suggesting that singlet oxygen is not the source of DNA damage induced by 2,6-DBrHQ/Cu(II). Interestingly, the DNA damage can be completely inhibited by methionine, which has

been considered as an excellent target for oxidation by $\cdot\text{OH}$, singlet oxygen and one-electron oxidants [47]. Meanwhile, the moderate Cu(II)-binding ability of methionine may also plays an important role in DNA damage inhibition [48].

These results demonstrated clearly that synergistic formation of $\cdot\text{OH}$ can be induced by combining both 2,6-DBrHQ and Cu(II) together, which might be responsible for 2,6-DBrHQ/Cu(II)-induced synergistic DNA damage.

3.4. Direct experimental evidence for the formation of a DNA-Cu(II)/(I) complex

From the above results, we can see that synergistic damaging effect on DNA by 2,6-DBrHQ/Cu(II) was well correlated with the redox reaction between Cu(II) and 2,6-DBrHQ, and H_2O_2 and Cu(I) generated in the reaction played an important role in DNA damage (Scheme 1). We also found that $\cdot\text{OH}$ can be produced by 2,6-DBrHQ and Cu(II) together by both ESR spin-trapping and fluorescent methods. However, we found that the two typical $\cdot\text{OH}$ scavengers, DMSO and mannitol, showed little or no inhibitory effect on both DNA strand breakage and the formation of 8-oxodG. Therefore, it is interesting to know what the underlying molecular mechanism is.

It has been suggested that copper-mediated DNA damage might be through a site-specific pathway: Cu(II) may bind to DNA to form a DNA-Cu(II) complex, which can be reduced to DNA-Cu(I); DNA-Cu(I) may react with H_2O_2 to form a DNA-Cu(I)-OOH complex, which may release $\cdot\text{OH}$ to attack an adjacent DNA constituent before being captured by $\cdot\text{OH}$ scavengers [49]. However, there was only very limited direct experimental evidence for the interaction between DNA and Cu(II)/Cu(I). It is not clear whether a DNA-Cu(II)/Cu(I) complex was formed or not; and if so, what is its redox activity as compared to Cu(II)/Cu(I) alone.

To test whether DNA could combine with Cu(II)/Cu(I) to form a DNA-Cu(II)/Cu(I) complex, a complementary application of low temperature direct ESR, circular dichroism, cyclic voltammetry and oxygen consumption methods were employed (Fig. 4).

Our low temperature ESR studies found that Cu(II) has a characteristic ESR spectrum at low temperature (77 K), showing the typical pattern of copper (Fig. 4A). When DNA (2 mg/mL) was added, the ESR peak height of Cu(II) significantly decreased and the g value of Cu(II) changed from 2.1510 to 2.0956, suggesting that Cu(II) may be complexed with DNA (Fig. 4A). The Cu(II)-DNA complex band shapes arise because Cu(II) binding is anisotropic, giving rise to two or three different g-tensors. The change upon introducing DNA suggests that the Cu(II) environment increases in anisotropy.

Circular dichroism (CD) is a phenomenon originating from interactions of chiral molecules with circularly polarized electromagnetic rays.

Scheme 1. Proposed mechanism of DNA damage induced by 2,6-DBrHQ/Cu(II): formation of DNA-Cu(II)/Cu(I) complex and $\cdot\text{OH}$ generated site-specifically near the binding sites between DNA and Cu(II).

Absorption of right- and left-handed circularly polarized light by chiral molecules differs and the difference is called CD [50]. CD spectroscopy is widely used in the study of DNA conformation. As expected, a significant Cotton effect of ct-DNA at ~ 248 nm and ~ 278 nm was observed. After addition of Cu(II), its absolute magnitude of CD spectra significantly decreased, suggesting that a ct-DNA-Cu(II) complex might be formed (Fig. 4B).

It should be interesting to know whether Cu(I) can interact with DNA, and if so, what are the difference between Cu(I) and Cu(II). From the above results, we knew that 2,6-DBrHQ can reduce Cu(II) to Cu(I). Interestingly, very different behavior was observed in the CD spectra of ct-DNA in the presence of 2,6-DBrHQ/Cu(II). CD spectra of DNA was significantly shifted toward longer wavelength (~ 268 nm, ~ 300 nm) as compared with ct-DNA-Cu(II) complex (~ 248 nm, ~ 282 nm), and the intensity of negative peak (~ 268 nm) increased most significantly (Fig. 4B). Similar CD spectra change was also observed when 2,6-DBrHQ was substituted by another typical reducing agent hydroxylamine (Fig. S6).

In order to make this result more convincing, Cu(I) binding DNA was more directly probed by adding Cu(I) salts in anaerobic media, which also reproduce the above phenomenon (Fig. 5). These results strongly suggest that Cu(I) can also combine with ct-DNA with stronger effect on DNA conformation as compared to Cu(II), possibly by binding N7 atom of guanine [51,52].

Cyclic voltammetry (CV) is one of the most effective electroanalytical methods available for the mechanism probing of redox systems. In part, the basis for their effectiveness is the capability for rapid observing redox behavior over the entire potential range available [53]. As shown in Fig. 4C, the aqua copper ion shows a well-behaved quasi-reversible wave and potential appropriate for reversible Cu(II)/Cu(I) cycling. However, the current of Cu(II) solution after addition of DNA decreased remarkably. This behavior suggested that electrons transfer between Cu(II)-DNA complex and glassy carbon electrode was hindered efficiently, which is known as a common circumstance for macromolecule-bound redox ions. Consequently, the above results indicated that Cu(II) may probably bind to DNA, inducing decrease of its electroactivity.

It is well-known that the redox-cycling between ascorbic acid and Cu(II) can induce marked O_2 consumption, which can be used as a model to study the redox activity of Cu(II) or Cu(II)-complexes; We found that, in the presence of ct-DNA, O_2 consumption-induced by ascorbic acid/Cu(II) decreased as the concentration of ct-DNA increases, indicating that Cu(II) may combine with DNA to form a DNA-Cu(II) complex, and the redox activity of the DNA-Cu(II) complex is lower than that of Cu(II) alone (Fig. 4D). Analogous effect was also observed in 2,6-DBrHQ/Cu(II) system (Fig. S7).

The above results clearly showed that a DNA-Cu(II)/Cu(I) complex was indeed formed and its redox activity is different from that of Cu(II)/Cu(I) alone. This represents the first systematic investigation on the interactions between Cu(II)/Cu(I) and DNA, and provided more direct and stronger experimental evidence for the formation of a DNA-Cu(II)/Cu(I) complex with a unique redox activity by complementary application of various advanced analytical methods, which may have important implications for future study on Cu-mediated biological effects.

3.5. The 2,6-DBrHQ/Cu(II)-induced DNA damage was found to be due to $\cdot\text{OH}$ generated site-specifically near the binding sites between DNA and Cu(II)

To provide more direct evidence that the 2,6-DBrHQ/Cu(II)-induced DNA damage was due to $\cdot\text{OH}$ generated site-specifically near the binding sites between DNA and Cu(II)/Cu(I), we compared two typical $\cdot\text{OH}$ generating systems: The classic Fenton system $\text{Fe(II)EDTA}/\text{H}_2\text{O}_2$ and the Fenton-like system $\text{Cu(II)}/\text{Asc}/\text{H}_2\text{O}_2$. $\cdot\text{OH}$ production for the two systems was measured with 3-CCA fluorescence method, either in the presence, or in the absence of DNA. We found that in the classic

Fig. 4. Direct experimental evidence for the formation of a DNA-Cu(II)/(I) complex as characterized by complementary application of four different analytical methods. **A:** low-temperature direct ESR measurements. The reaction mixtures contained 2 mM Cu(II) with or without 2 mg/mL ct-DNA. **B:** circular dichroism spectra of ct-DNA (30 μ g/mL) in the absence or presence of Cu(II) (0.1 mM)/2,6-DBrHQ (0.1 mM). **C:** cyclic voltammograms for Cu(II) (50 μ M) in the absence or presence of Cu(II)/ascorbate-mediated O_2 consumption by DNA. The mixtures contained 5 μ M Cu(II), 250 μ M Asc and indicated concentration of ct-DNA.

Fig. 5. Circular dichroism spectra of ct-DNA (25 μ g/mL) in the absence or presence of Cu(I) (0.1 mM) or Cu(II) (0.1 mM) under anaerobic conditions.

Fenton system (Fe(II)EDTA/ H_2O_2), the generation of $\cdot OH$ was inhibited only slightly by addition of DNA (Fig. 6). This can be well explained by the fact that since there is only very weak interactions between DNA and Fe(II)EDTA, the $\cdot OH$ produced by Fe(II)EDTA/ H_2O_2 system was equally dispersed in reaction solution, like free $\cdot OH$, which would attack on 3-CCA and DNA randomly and non-discriminatively, and thus only a small number of these $\cdot OH$ can be quenched by DNA. In agreement with this result, we found that DMSO and mannitol could inhibit the DNA damage when Cu(II) is replaced by Fe(III)-EDTA under the same experimental conditions as in Fig. 1 (Fig. S8).

In contrast, in Cu-mediated Fenton-like system (Cu(II)/Asc/ H_2O_2), we found that the generation of $\cdot OH$ was inhibited much more significantly by addition of DNA (Fig. 6). The reasonable explanation should be that, in Cu(II)/Asc/ H_2O_2 system, due to the relatively strong binding between copper and DNA as shown above, the DNA-Cu(I) complex will react with H_2O_2 to produce $\cdot OH$ near the Cu-binding site of DNA. Then the $\cdot OH$ produced in such a site-specific manner will readily attack copper-binding site of DNA prior to attack 3-CCA. In another word, DNA will be more susceptible to attack by $\cdot OH$ site-specifically produced by DNA-Cu(I) and H_2O_2 .

3.6. Site-specificity of DNA damage induced by 2,6-DBrHQ in the presence of Cu(II)

Whether the proposed binding of copper ions to DNA would induce some site-specificity in the formation of strand breaks? To further elucidate this question, we investigated the site-specificity of DNA cleavage induced by 2,6-DBrHQ/Cu(II) using FAM-labeled DNA fragments (Fig. 7). We found that, in the presence of 50 μ M Cu(II), 200 μ M 2,6-DBrHQ frequently caused cleavage at thymine (T), cytosine (C) and guanine (G) residues in 76 mer DNA fragment, not adenine. According to previous studies, piperidine-labile DNA lesions were produced preferentially at sites of two or more adjacent guanine residues in Cu(II) containing system. Cu(II) can bind strongly to the N-7 and O-6 atoms of guanine and the N-3 cytosine in G-C base pairs [54]. The modified bases may include 8-oxodG, 5,6-dihydroxy-5,6-dihydrothymine, 5-formyluracil and 4,6-diamino-5-formamidopyrimidine [55–57]. A similar pattern of DNA cleavage was obtained in 23 mer DNA fragment (Fig. S9).

specific chelating agent and catalase, but not by the typical $\cdot\text{OH}$ scavengers. By complementary application of various advanced analytical methods, we found that Cu(II)/Cu(I) could be complexed with DNA to form DNA-Cu(II)/Cu(I) complex with a different redox activity as compared to Cu(II)/Cu(I) ; and the highly reactive $\cdot\text{OH}$ were produced synergistically by DNA-bound-Cu(I) with H_2O_2 produced by 2,6-DBrHQ/ Cu(II) , which then immediately attack DNA in a site-specific manner. Based on these data, we proposed that the synergistic DNA damage induced by 2,6-DBrHQ/ Cu(II) might be due to the synergistic and site-specific production of $\cdot\text{OH}$ near the binding site of copper and DNA. Similar mode of action might be applied for copper-mediated biological damage with other reactive quinoid environmental pollutants. Therefore, our findings may have broad biological and environmental implications for future research on the carcinogenic polyhalogenated phenolic compounds.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2016.12.050.

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