

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/260118959>

DNA Cleavage and Detection of DNA Radicals Formed from Hydralazine and Copper (II) by ESR and Immuno-Spin Trapping

ARTICLE in CHEMICAL RESEARCH IN TOXICOLOGY · FEBRUARY 2014

Impact Factor: 3.53 · DOI: 10.1021/tx500011m · Source: PubMed

CITATIONS

4

READS

26

4 AUTHORS:



Birandra K Sinha

U.S. Department of Health and Human Services

132 PUBLICATIONS 4,795 CITATIONS

SEE PROFILE



Fabian Leinisch

National Institute of Environmental Health Sci...

14 PUBLICATIONS 22 CITATIONS

SEE PROFILE



Suchandra Bhattacharjee

National Institutes of Health

15 PUBLICATIONS 226 CITATIONS

SEE PROFILE



Ronald P Mason

National Institute of Environmental Health Sci...

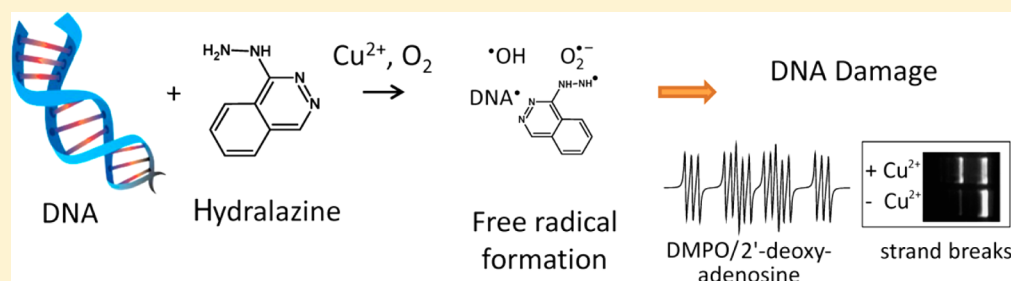
565 PUBLICATIONS 21,036 CITATIONS

SEE PROFILE

DNA Cleavage and Detection of DNA Radicals Formed from Hydralazine and Copper (II) by ESR and Immuno-Spin Trapping

Birandra K. Sinha,* Fabian Leinisch, Suchandra Bhattacharjee, and Ronald P. Mason

Laboratory of Toxicology and Pharmacology, National Institutes of Environmental Health Sciences, Research Triangle Park, North Carolina, United States



ABSTRACT: Metal ion-catalyzed oxidation of hydrazine and its derivatives leads to the formation of the hydrazyl radical and subsequently to oxy-radicals in the presence of molecular oxygen. Here, we have examined the role of Cu^{2+} -catalyzed oxidation of hydralazine in the induction of DNA damage. Neither 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) nor dimethyl sulfoxide (DMSO) was effective in inhibiting hydralazine- Cu^{2+} -induced DNA damage. Singlet oxygen did not appear to participate in this DNA cleavage. The one-electron oxidation of hydralazine also leads to the formation of DNA radicals as confirmed by immuno-spin trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide. Electron spin resonance (ESR) and spin-trapping studies further confirmed the formation of DNA radicals; predominantly, 2'-deoxyadenosine radical adducts were detected, while some radicals were also detected with other nucleosides. Our results suggest that free hydroxyl radicals may not be the main damaging species causing DNA cleavage and that possibly Cu-peroxide complexes, formed from $\text{Cu}^+ \cdot \text{H}_2\text{O}_2$, are responsible for this hydralazine- Cu^{2+} -induced DNA cleavage.

INTRODUCTION

Hydrazine and its derivatives constitute an important class of compounds as they are environmental pollutants, and are used in medicine. Aromatic hydrazines (e.g., agartine) are found in edible mushrooms (*Agaricus bisporus*). Isoniazid is used for the treatment of tuberculosis.¹ Iproniazid is a potent antidepressant; however, it is not currently in use due to its liver toxicity.² Procarbazine is a chemotherapeutic agent used in the treatment of Hodgkin's disease and melanoma.³ Hydralazine is a potent arterial vasodilator and plays an important role in the management of hypertension and congestive heart failure.⁴ Hydrazines in general are toxic and induce a variety of toxic insults. These include liver toxicity, carcinogenicity, and mutagenicity.^{1,2,4-7} Hydralazine, the least toxic of the hydrazine derivatives, induces DNA damage,⁸ causes severe forms of systemic lupus erythematosus,⁹⁻¹¹ and has been shown to increase the incidence of lung tumors in mice.⁴⁻⁶ More recently, hydralazine has been shown to inhibit DNA methyltransferase 1 by preventing the transfer of a methyl group to DNA in several cancer-silencing genes/tumor suppressor genes.^{9,12} It has, therefore, become an important treatment combination in cancer therapy.^{9,12} Furthermore, hydralazine has also been found to inhibit iron-containing prolyl hydroxylase enzymes, which are important for the induction of hypoxia-induced factor (HIF) and vascular endothelial growth factor. HIF1 α is a critical target in cancer

chemotherapy as it is believed to be involved in tumor progression.¹⁰

Because of the significance of hydrazine derivatives as environmental pollutants and food contaminants and their utility in medicine, a large volume of research has been carried out to elucidate the mechanisms of toxicity of these compounds.¹⁻¹⁰ Previous studies from our laboratory and others have clearly shown that hydrazine derivatives generate free radicals upon oxidation, catalyzed by metal ions, cytochrome P450 and peroxidases, including prostaglandin synthase.¹³⁻²² In addition, certain substituted hydrazines have been proposed to alkylate DNA by nucleophilic attack rather than via free radical generation.^{21,22} Irrespective of mechanisms (free radicals or nucleophilic attack), the consequences of such DNA alkylation may lead to carcinogenic events.

We have previously shown that hydralazine undergoes one-electron oxidation by both metal ions (Cu^{2+} and Fe^{3+} ions) and enzymatically (horseradish peroxidase and prostaglandin synthase) to form the hydrazyl radical (HY^\bullet), which then further decomposes to form various products.¹⁴ Reilly and Aust²³ have reported that hydralazine in the presence of peroxidase/ H_2O_2 induces single-strand DNA damage; however, the presence of a donor such as chlorpromazine is required for

Received: January 9, 2014

Published: February 6, 2014

damage to occur. Studies of Yamamoto and Kawanishi²⁴ have shown that the oxidation products of hydralazine and Cu^{2+} induce DNA damage, predominantly at guanine residues, while Fe-catalyzed oxidation of hydralazine induces damage at every nucleotide in DNA.

Copper is an essential trace element that is widely distributed throughout the body and forms the essential redox-active reaction center in a variety of metalloenzymes, including ceruloplasmin, superoxide dismutase, cytochrome oxidase, dopamine β -hydroxylase, tyrosinase, and lysyl oxidase. Nevertheless, the body's total daily requirement for copper estimated at 1–2 mg²⁵ for adult humans is much below that for iron, which is estimated at 4–6 g. Serum levels of copper are significantly elevated in anemia, during pregnancy, and when taking oral contraceptives.²⁶ It is interesting to note that copper metabolism is significantly altered in tumors and that serum concentrations are correlated with tumor incidence, progression, and recurrence in a number of human tumors.^{27–29} Serum ceruloplasmin levels increase by a factor of 4–8 during cancer progression.³⁰ Elevated levels of copper play an important role in tumor angiogenesis and seem to be necessary for endothelial cell activation. Cu^{2+} -dependent DNA fragmentation has been reported to be much more extensive than Fe^{3+} ,³¹ and it is important and relevant to examine the toxicity of hydralazine in the presence of copper as this combination may be more potent for DNA damage and consequently for the formation of tumors than the single agents alone.

In this article, we have examined the consequences of Cu^{2+} -catalyzed oxidation of hydralazine by assessing the induction of DNA cleavage in supercoiled pHOT1 DNA and the formation of DNA radicals in calf thymus DNA. Furthermore, we have investigated the Cu^{2+} ion/hydralazine stoichiometry that induces maximum DNA damage. Our results indicate that reactive species formed from the oxidation of hydralazine by Cu^{2+} induce significant DNA cleavage in pHOT1 DNA and that this nicking is dependent upon the ratio of Cu^{2+} ions to hydralazine. Using ESR spin-trapping techniques, we found that hydralazine- Cu^{2+} complexes formed significant amounts of 2'-deoxyadenosine radical spin-adducts, while some radical adducts of other bases were also detected. We also found that in calf thymus DNA, the formation of oxy-radicals leads to the generation of DNA radicals, which were trapped by DMPO and were easily detected by the immuno-spin-trapping technique (IST). Again, the maximum DNA radical formation was dependent upon the ratio of Cu^{2+} ions to hydralazine, and this ratio was similar to those found for DNA cleavage.

MATERIALS AND METHODS

Hydralazine, calf thymus DNA, 2,2,6,6-tetramethyl-piperidine (TEMP), and Rose Bengal were obtained from Sigma Aldrich (St. Louis, MO). DMPO was purchased from Dojindo (Rockville, MD) and used without any further purification. DNA was dissolved in 100 mM Chelex-treated PBS buffer (pH 7.4), and all incubations were carried out in this buffer. Supercoiled pHOT1 DNA was obtained from Topgen (Port Orange, FL). Copper(II) sulfate (Sigma Aldrich, St. Louis, MO) was dissolved in doubly distilled water prior to use. A fresh solution of hydralazine was prepared before use. 2'-Deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine, and thymidine were obtained from MP Biomedicals (Irvine, CA).

DNA Cleavage Assay with pHOT1. DNA cleavage in the presence of hydralazine and Cu^{2+} ion was analyzed using pHOT1 supercoiled DNA according to previously published methods.³² Briefly, a 20 μL assay mixture contained 0.5 μg of pHOT1 supercoiled DNA and varying concentrations of hydralazine. The reaction was

initiated by adding Cu^{2+} and incubating the reaction mixture for 30 min at room temperature. The reaction was terminated by adding 2 μL of 0.5 M EDTA. The reaction products were loaded onto 1% agarose gel for DNA separation in Tris-acetate-EDTA buffer (pH 8.0). The DNA was stained with ethidium bromide and photographed. For the effects of scavengers on DNA damage, scavengers were preincubated for 5–10 min before adding Cu^{2+} ions and carrying out gel electrophoresis as before. Quantitation of various forms of DNA cleavage products was carried using Image J software.

Oxygen Consumption with Hydralazine. Oxygen uptake assays were carried out with hydralazine and Cu^{2+} in the presence or absence of DNA with a YSI Model S2 oxygen monitor. Typically, hydralazine, dissolved in 100 mM Chelex-treated PBS buffer (pH 7.4), was equilibrated for 10 min at room temperature before adding Cu^{2+} and monitoring O_2 consumption. When the effects of DNA on oxygen consumption were examined, DNA was first incubated with Cu^{2+} (200 $\mu\text{g}/\text{mL}$ calf thymus DNA and 50 μM Cu^{2+}), the reaction was initiated by adding hydralazine, and O_2 consumption was measured. In some experiments, DNA-hydralazine complexes were preformed¹⁵ before the addition of Cu^{2+} , and no significant differences in O_2 consumption were observed. The effects of scavengers were examined by preincubating DNA-metal ion mixtures with the scavenger for 10 min before adding hydralazine.

ESR Studies. ESR studies for the detection of singlet oxygen³³ and DNA radicals were carried out as described previously.^{34,35} For the detection of singlet oxygen, TEMP (100 mM) was used in the presence of hydralazine (125 μM) and Cu^{2+} (25 μM) in Chelex-treated PBS buffer (pH 7.4). Rose Bengal (20 μM) was used as the positive control in the presence of light (5 min exposure).³⁶ For the spin-trapping studies, reaction mixtures contained in Chelex-treated PBS buffer (pH 7.4) individual 2'-deoxyribonucleosides (5.0 mM), hydralazine (125 μM), and DMPO (100 mM), and the reaction was initiated by adding 25 μM Cu^{2+} . The mixture was transferred to ESR flat cells, and the spectra were recorded with an ELEXSYS E 500 ESR spectrometer (Bruker Biospin, Billerica, MA) equipped with an ER4122SHQ cavity operating at 9.76 GHz at room temperature. The ESR settings were as follows: scan range, 100 G; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; microwave power, 20 mW; receiver gain, 2×10^4 ; time constant, 250 ms; and the conversion time, 250 ms. ESR spectra were simulated as described previously.³⁷

Formation of a DNA-Nitron Adduct with Hydralazine and Cu^{2+} . Calf thymus DNA (0.5 mg–1.0 mg/mL) was mixed with various concentrations of hydralazine, and the reaction was initiated by adding Cu^{2+} followed by the addition of DMPO; the reaction mixtures were then incubated for 30 min at room temperature. Reactions were terminated by adding EDTA solution (0.5 M), and DNA was precipitated in ethanol. DNA was collected by centrifugation and washed with 75% ethanol, dissolved in the Chelex-treated PBS, and analyzed for DNA-nitron adducts.

Immuno-Spin-Trapping Analysis of DNA-Nitron Adducts. For the detection of DNA-nitron adducts formed in calf thymus DNA by hydralazine, a standard ELISA procedure in a 96-well plate (Greiner-Bio-One) was used as described previously.^{38,39} Briefly, DNA samples were diluted to 5 $\mu\text{g}/\text{mL}$ in the Chelex-treated PBS buffer and incubated with Reacti-bind DNA coating solution (Thermo Scientific, Rockford, IL) at 37 °C for 3 h. The plates were washed once with a washing buffer (the Chelex-treated PBS buffer containing 0.05% nonfat dry milk and 0.1% Tween-20) and blocked with a blocking buffer (the Chelex-treated PBS buffer containing 3% nonfat dry milk) for 18 h at 4 °C. The plates were then washed once with the washing buffer and incubated with rabbit anti-DMPO serum (diluted 1:10,000) at 37 °C for 1 h. The plates were washed three times with the washing buffer, 100 μL of the secondary antibody (horseradish peroxidase-conjugated, diluted 1:5000 in the washing buffer) was added, and the plates were incubated at 37 °C for 1 h. Following three washes as before, the antigen-antibody complexes were developed with Immobilon Chemiluminescence substrate (Millipore Corp, Billerica, MA), and the light emitted was recorded as arbitrary light units using X-Fluor software (Tecan US, Research Triangle Park, NC).

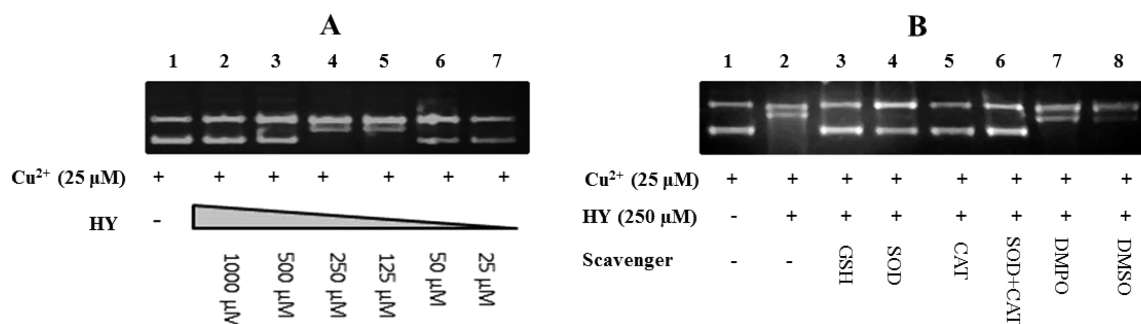


Figure 1. Hydralazine-mediated cleavage (A) in pHOT1 DNA following oxidation with Cu²⁺ (25 μM). The DNA cleavage was examined as described in the Materials and Methods section. Lane 1, DNA in the presence of Cu²⁺ alone; lane 2, hydralazine (1000 μM) and Cu²⁺; lane 3, hydralazine (500 μM) and Cu²⁺; lane 4, hydralazine (250 μM) and Cu²⁺; lane 5, hydralazine (125 μM) and Cu²⁺; lane 6, hydralazine (50 μM) and Cu²⁺; and lane 7, hydralazine (25 μM) and Cu²⁺. (B) Effects of scavengers on the DNA cleavage induced by hydralazine (250 μM) and Cu²⁺ (25 μM). Scavengers were incubated with hydralazine–DNA complexes for 5–10 min before adding Cu²⁺. Lane 1, control DNA; lane 2, hydralazine in the presence of Cu²⁺; lane 3, reduced GSH (1000 μM); lane 4, SOD (50 U/incubation); lane 5, catalase (2500 U/incubation); lane 6, SOD and catalase; lane 7, DMPO (100 mM); and lane 8, DMSO (1 mM).

RESULTS

We used supercoiled pHOT1 DNA (which contained 80–85% supercoiled DNA, according to the manufacturer and confirmed in our experiments) to examine the induction of DNA cleavage by hydralazine (25 μM–1000 μM) in the presence of 25 μM Cu²⁺ ions. Copper ion alone induced some DNA cleavage (Figure 1A, lane 1), which was significantly enhanced in the presence of hydralazine (Figure 1A). While higher concentrations of hydralazine also induced some DNA cleavage (e.g., 35 ± 5% at 1000 μM), complete DNA cleavage was observed at 125–250 μM hydralazine. At these concentrations of hydralazine, formation of both open circular and linear DNA was observed. Furthermore, as little as 10 μM Cu²⁺ was sufficient to induce this damage (data not shown).

We next examined the effects of various scavengers of oxy-radicals on the hydralazine–Cu²⁺–complex-induced DNA cleavage. As shown in Figure 1B, 1 mM GSH (an OH radical scavenger, lane 3), SOD (a scavenger of superoxide anion radical, 50 U/incubation; lane 4), catalase (a scavenger of hydrogen peroxide, 2500 U/incubation; lane 5), or a combination of SOD and catalase (lane 6) significantly (75 ± 10%) inhibited DNA cleavage. These observations indicate the participation of both the superoxide anion radical (O₂^{•−}) and hydrogen peroxide (H₂O₂) in the DNA damage induced by the Cu²⁺–hydralazine complex (lane 2). It is interesting that DMPO (lane 7) and DMSO (lane 8), both scavengers of free •OH, were not effective in inhibiting this DNA cleavage. This would suggest that the damaging radical (free •OH) if formed is in close proximity to the DNA binding site(s) of hydralazine or the hydralazine–Cu–DNA complex that DMSO/DMPO is unable to inhibit it. Alternatively, it is also possible that the species that induce DNA cleavage are not free •OH and are derived from copper–peroxide complexes.

Scavengers of free •OH were not very effective in preventing DNA cleavage from hydralazine in the presence of Cu²⁺. Since singlet O₂ has been implicated in Cu–H₂O₂-mediated DNA damage,³³ experiments were carried out to determine whether ¹O₂ was formed from hydralazine–Cu²⁺ under our experimental conditions. In order to confirm this, we used TEMP as a probe. TEMP has been successfully used for the detection of singlet O₂^{33,36} from the Cu²⁺–H₂O₂ system. As a positive control, we used Rose Bengal, a dye that has been shown to generate ¹O₂ in the presence of light.³⁶ Figure 2 shows that Rose Bengal

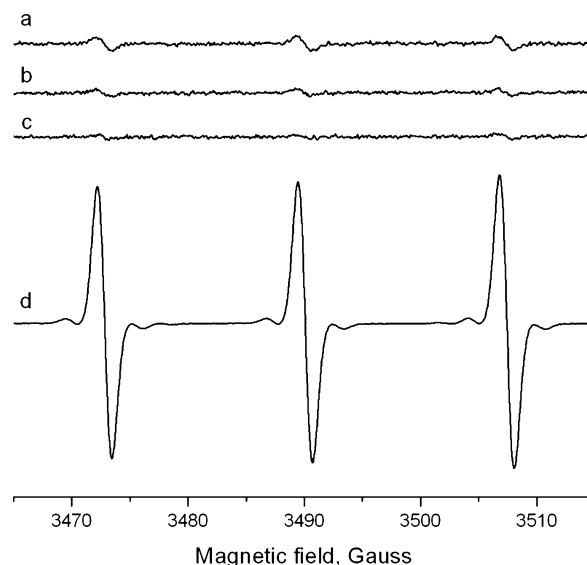


Figure 2. Detection of ¹O₂ from hydralazine (125 μM) in the presence of Cu²⁺ (25 μM) and TEMP (100 mM). (a) Complete system; (b) hydralazine alone; (c) Cu²⁺ alone; and (d) in the presence of Rose Bengal (20 μM) and light for 5 min. No signal for the nitroxide was detected in the absence of light with Rose Bengal. The ESR studies were carried out as described in the Materials and Methods section; a–c, conversion time = time constant = 163 ms. d: 81 ms.

generated large amounts of the TEMP nitroxide (note the difference in receiver gain), the oxidation product of TEMP by ¹O₂. In contrast, the hydralazine–Cu²⁺ system generated very little ¹O₂ under the conditions where significant DNA cleavage was observed. These observations would suggest that ¹O₂ did not participate significantly in inducing DNA cleavage from the hydralazine–Cu²⁺ system under our experimental conditions.

Oxygen Consumption of Hydralazine in the Presence of Cu²⁺. The ratio dependence for the maximum DNA cleavage would suggest that at these ratios the Cu²⁺ ion may undergo recycling for the maximum formation of oxy-radicals. In order to confirm this, we examined O₂ consumption at various metal ion-to-hydralazine ratios. The maximum O₂ consumption was obtained at a ratio of 1:2.5–5 of Cu²⁺/hydralazine (Figure 3A, tracings 1 and 2), while at higher ratios, significantly decreased O₂ was consumed (Figure 3A, tracings

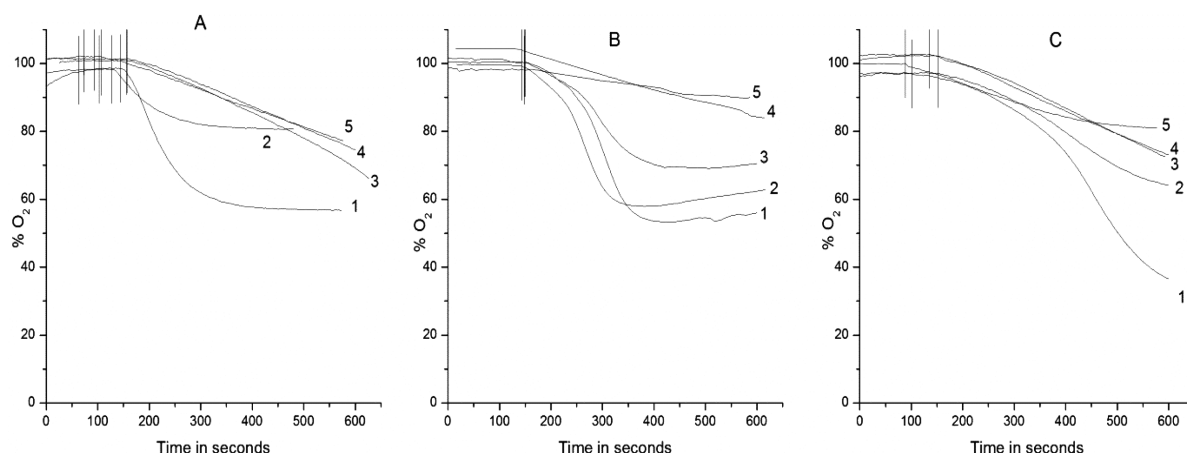


Figure 3. Oxygen consumption (A) with different concentrations of hydralazine in the presence of Cu^{2+} ($50 \mu\text{M}$). The oxygen consumption was carried out as described in the Materials and Methods section. Tracing 1, $125 \mu\text{M}$ hydralazine; tracing 2, $50 \mu\text{M}$ hydralazine; tracing 3, $250 \mu\text{M}$ hydralazine; tracing 4, $500 \mu\text{M}$ hydralazine; and tracing 5, $1000 \mu\text{M}$ hydralazine. (B) Effects of oxy-radical scavengers on oxygen consumption from $125 \mu\text{M}$ hydralazine and $50 \mu\text{M}$ Cu^{2+} . Tracing 1, control; tracing 2, 100 mM DMPO; tracing 3, catalase (6000 U/mL); tracing 4, SOD (500 U/mL); and tracing 5, $1000 \mu\text{M}$ GSH. (C) Effect of DNA ($200 \mu\text{g/mL}$) on the oxygen consumption by various concentrations of hydralazine in the presence of Cu^{2+} ($50 \mu\text{M}$). Tracing 1, $250 \mu\text{M}$ hydralazine; tracing 2, $125 \mu\text{M}$ hydralazine; tracing 3, $500 \mu\text{M}$ hydralazine; tracing 4, $1000 \mu\text{M}$ hydralazine; and tracing 5, $50 \mu\text{M}$ hydralazine. DNA was added to the hydralazine solutions and incubated for 10 min before adding Cu^{2+} and measuring the O_2 consumption.

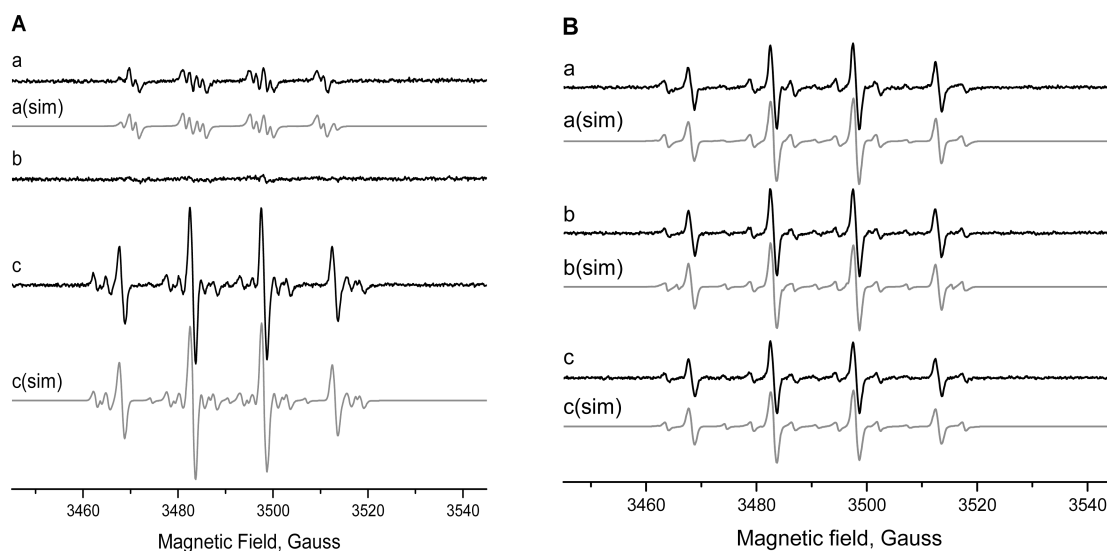


Figure 4. Detection of free radicals in the presence of DMPO (100 mM). Panel A: (a) hydralazine ($125 \mu\text{M}$) and Cu^{2+} ($25 \mu\text{M}$); (b) +SOD (500 U/mL); and (c) $2'$ -deoxyadenosine (5 mM). Panel B: radical adducts obtained from (a) $2'$ -deoxyguanosine (5 mM), (b) $2'$ -deoxycytidine (5 mM), and (c) thymidine (5 mM) from hydralazine and Cu^{2+} . These studies were carried out at pH 7.4 as described in the Materials and Methods section.

3–5). These observations are similar to our cleavage data where complete DNA degradation was observed (e.g., 125 – $250 \mu\text{M}$ hydralazine). This consumption of O_2 was inhibited by the presence of SOD (250 U/mL), while catalase ($6,000 \text{ U/mL}$) was only partially effective (Figure 3B). GSH ($1000 \mu\text{M}$) was also very effective in inhibiting O_2 consumption, while DMPO had no effect (Figure 3B). These observations implicate $\text{O}_2^{\cdot -}$ as the intermediate in the formation of H_2O_2 and other reactive intermediates from hydralazine and Cu^{2+} .

The effect of DNA on O_2 consumption was also examined, and the data is presented in Figure 2c. Addition of DNA increased O_2 consumption from hydralazine and Cu^{2+} , and the maximum O_2 was consumed at a Cu^{2+} /hydralazine ratio of 1:5. Again, these observations are consistent with our cleavage reactions suggesting that Cu^{2+} is accessible for cycling to generate the maximum reactive intermediates.

ESR Spin-Trapping Studies. In order to detect free radicals formed in DNA following oxidation of hydralazine, spin-trapping studies with DMPO were carried out with nucleosides and calf thymus DNA (native and sonicated DNA). Typically, the reaction mixtures contained 5 mM individual nucleosides, dissolved in Chelex-treated PBS buffer (pH 7.4), $125 \mu\text{M}$ hydralazine, and 100 mM DMPO. The reactions were initiated by adding $25 \mu\text{M}$ Cu^{2+} and recording the spectra.

Incubation of hydralazine (0.5 mM or more) with Cu^{2+} ($25 \mu\text{M}$) rapidly formed DMPO-adducts as previously reported;¹⁴ however, when the hydralazine concentration was decreased to $125 \mu\text{M}$ (ratio of 5:1 with Cu^{2+}), the DMPO-adduct (94%) formed had the following coupling constants $a^{\text{N}} = 14.14 \text{ G}$; $a^{\text{H}}_{\beta} = 11.3 \text{ G}$; and $a^{\text{H}}_{\gamma} = 1.24 \text{ G}$ (Figure 3A). These coupling constants are similar to those described for DMPO-adducts of superoxide anion radicals.⁴⁰ When SOD (500 U/mL) was

included in the reaction mixtures, very little DMPO-adduct was detected, indicating that the DMPO-adducts trapped were derived from the trapping of superoxide anion radicals (Figure 3B). Formation of DMPO adducts required the presence of both hydralazine and Cu^{2+} .

Additionally, when the incubations were carried out for more than 20 min, a small amount (<10%) of another DMPO-adduct with coupling constants of $a^{\text{N}} = 15.4$ G and $a^{\text{H}} = 22.86$ G was also trapped. The identity of this radical is not known, but it appears to be derived from the trapping of a carbon-centered radical with DMPO. Finkelstein et al.⁴⁰ have described the appearance over time of a DMPO-adduct with $a^{\text{N}} = 15.31$ and $a^{\text{H}} = 22.0$ during the trapping of a superoxide anion radical and have indicated that it was formed from the decomposition of DMPO. These coupling constants are similar to those of the DMPO-adduct trapped in our system and thus may represent this decomposition product of DMPO.

Inclusion of 2'-deoxyadenosine in the reaction mixtures containing hydralazine and Cu^{2+} also resulted in the formation of several different DMPO-adducts, including the 2'-deoxyadenosine radical adduct (Figure 4A, c). This radical adduct (40%) had the following coupling constants: $a^{\text{N1}} = 15.42$ G; $a^{\text{N2}} = 2.58$ G; and $a^{\text{H}} = 19.95$ G. These coupling constants are similar to those described for the N-centered exocyclic NH_2 of the 2'-deoxyadenosine moiety.^{34,41} DMPO-OH (53%), DMPO-decomposition products, and some nitroxide radical were also present. Simulated spectra of these radical adducts with their coupling constants are shown in Figure 4.

When incubations were carried out with other nucleosides, various DMPO-adducts were detected, and these coupling constants are in agreement with those previously published^{34,41} and are summarized in Table 1. It should be noted that these radical adducts were minor products (less than 10% of the total adducts formed) as DMPO-OH was the major adduct detected.

Table 1. Parameters of DMPO-Adducts Formed from Nucleosides Following Treatment with Hydralazine (125 μM) and Cu^{2+} (25 μM) at Room Temperature^a

substrate	hyperfine coupling constants (G)			assignment
	a^{N}	a^{H}	a^{N}	
2'-deoxyadenosine	15.42	19.95	2.58	N-centered, exocyclic NH_2
2'-deoxycytidine	15.38	18.95	2.02	N-centered
thymidine	15.88	19.5	2.71	N-centered
2'-deoxyguanosine	15.6	20.27	2.56	N-centered, N_3 position

^aESR-spin trapping studies were carried out as described in the Materials and Methods section.

It is interesting to note that when the spin-trapping experiments were carried out under conditions such as those described by Yamamoto and Kawanishi,²⁴ no radicals originating from nucleosides were trapped with DMPO. More importantly, when incubations were carried out at 37 °C, only exocyclic NH_2 from 2'-deoxyadenosine was detected with DMPO, suggesting that this radical (or the DMPO-adduct) was significantly more stable than those formed from other nucleosides. When calf thymus DNA or sonicated calf thymus DNA was used, no radical was detected with DMPO.

Formation of DNA-Nitrone Adducts. Because oxy-radicals are formed from hydralazine- Cu^{2+} -DNA complexes, it is possible that this oxy-radical formation would also lead to the

formation of DNA radical(s) in spite of our negative ESR spin-trapping results. This possibility was investigated by examining the formation of stable DNA-nitrone adducts by a highly sensitive immuno-spin-trapping method.^{38,39} Data presented in Figure 5 clearly show the formation of stable nitrone adducts in

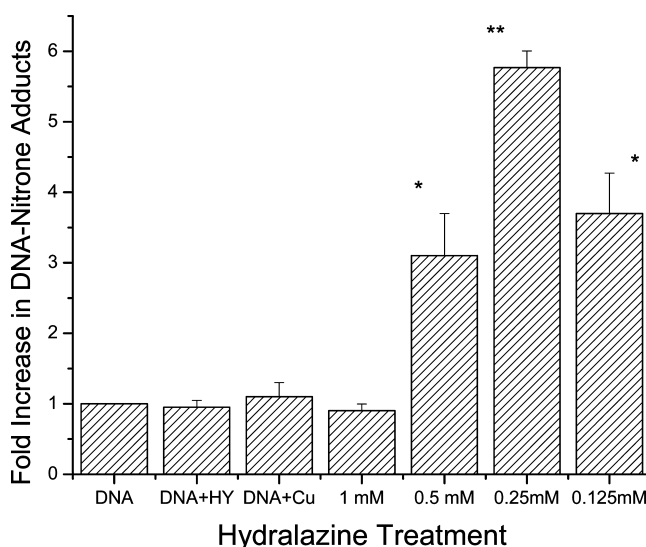


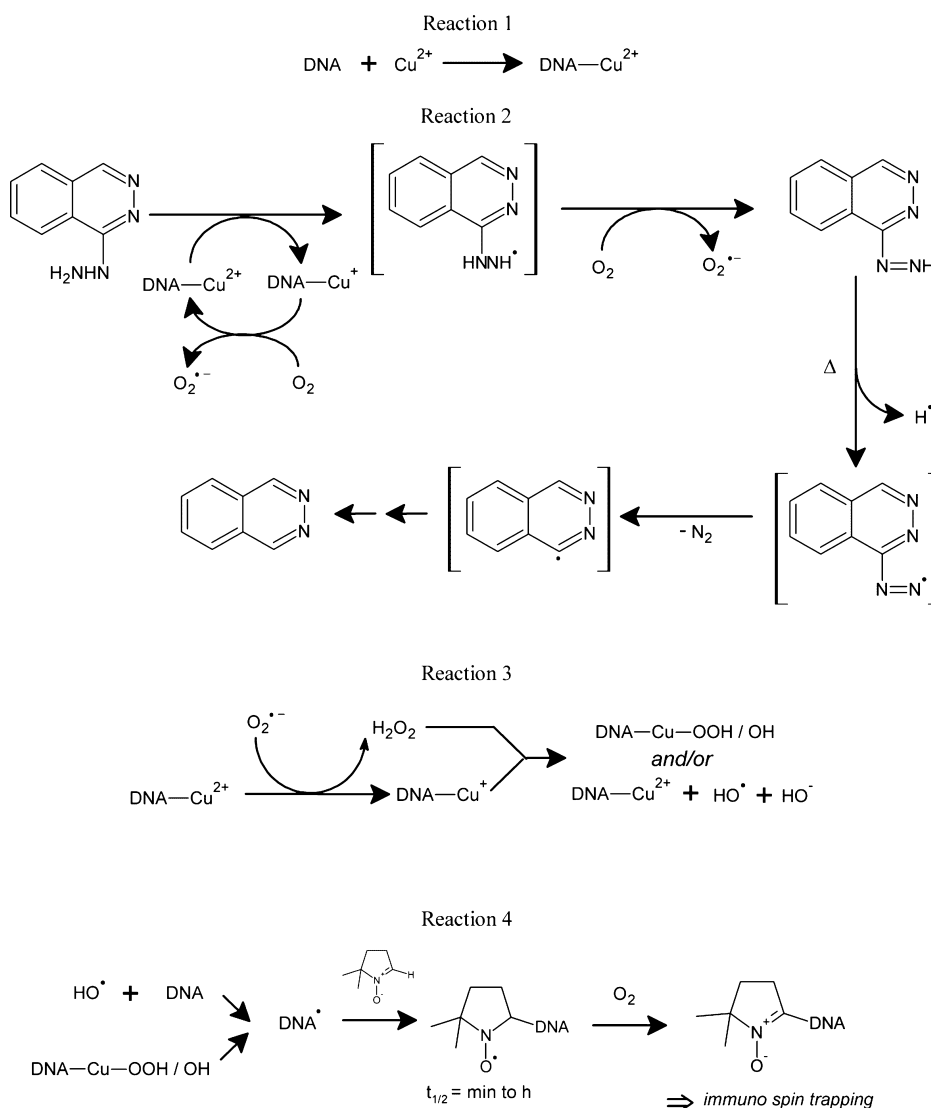
Figure 5. Immunological detection of the DNA radicals formed in calf thymus DNA (0.5 mg/mL) by various concentrations of hydralazine in the presence of Cu^{2+} (50 μM) and DMPO (100 mM). The detection of these DNA radicals was carried out as detailed in the Materials and Methods section. Values with asterisks were significantly different (* < 0.05; ** < 0.005, $n = 3$) from DNA alone or DNA in the presence of hydralazine and DNA in the presence of Cu^{2+} . The significance was determined by Student's t test.

calf thymus DNA. Interestingly, at high hydralazine concentrations, very few DNA-nitrone adducts were formed, and the highest amounts of adducts were detected at a Cu^{2+} /hydralazine ratio of 1:5, a ratio at which the maximum O_2 consumption was also observed in the presence of DNA (Figure 3C, tracing 1). The time course indicated that this DNA-nitrone adduct formation was rapid and reached a maximum within 5–10 min (data not shown). Furthermore, when we examined the dependence of this adduct formation on DMPO concentrations, the highest amounts of adducts were formed at 50–100 mM, and no significant adduct formation was observed at 1–10 mM of DMPO.

DISCUSSION

Hydrazine and its derivatives react with metal ions (Cu^{2+} and Fe^{3+}) to generate the hydrazyl radical, which then reacts with molecular O_2 to generate superoxide anion radicals, H_2O_2 and, finally, $\cdot\text{OH}$.^{13–15} Our previous studies^{13–15} as well as this study shows that the hydrazyl radical, superoxide anion radicals, and $\cdot\text{OH}$ are formed from hydralazine under aerobic conditions at pH 7.4 during one-electron oxidation of hydralazine. In this study, hydralazine in the presence of Cu^{2+} ions induced significant DNA cleavage in the supercoiled pHOT1 DNA. Furthermore, we found that the DNA damage was $\text{O}_2^{\cdot-}$ - and H_2O_2 -dependent as both SOD and catalase were effective in preventing DNA damage from hydralazine and Cu^{2+} ions. While it has been suggested that hydroxyl radicals, once formed, will react with any species present in high concentrations in a diffusion-controlled manner, two typical

Scheme 1. Structures of Hydralazine and DNA Nitron, and the Proposed Formation of Various Free Radical Species from Hydralazine That May Induce DNA Cleavage Following Oxidation by the Cu^{2+} Ion



hydroxyl radical scavengers (DMSO and DMPO) exhibited no significant inhibition of the DNA damage induced by hydralazine- Cu^{2+} complexes in our hands. There are a number of reports in which the addition of $\cdot\text{OH}$ scavengers (ethanol, mannitol, sodium formate, and DMSO) conferred only partial protection or had no effect on DNA damage.^{33,42–47} Because of the small or insignificant protection against DNA damage by hydroxyl radical scavengers, it has been proposed that the active species causing DNA damage are more likely copper-peroxide complexes (Scheme 1) with reactivity similar to that of the hydroxyl radical and/or singlet oxygen rather than that of a free hydroxyl radical.^{44,47} In this study, we found no inhibition of Cu^{2+} -hydralazine-induced DNA damage by DMSO and DMPO as observed by others.^{44,47} These observations lead us to suggest that free hydroxyl radicals are not the main DNA-damaging species and that participation of Cu^{+} -peroxo species (Scheme 1) in inducing DNA cleavage cannot be ruled out this time.

Reduced glutathione was extremely effective in preventing DNA cleavage. This would suggest that GSH rapidly traps any Cu^{+} formed, resulting in the formation of a stable Cu^{+} -GSH complex and preventing the formation of reactive species. It is

of interest to note that studies reported from our laboratory have shown that Cu^{+} -GSH complexes are stable and do not undergo oxidation to generate Cu^{2+} . Using the spin-trapping technique, we have shown that these Cu^{+} -GSH complexes do not form DMPO-OH spin adducts in the presence of H_2O_2 .⁴⁸ Concentrations of GSH in cells and *in vivo* are extremely high, and it would appear that reactive species generated from hydralazine-Cu complexes may be ineffective in causing any DNA damage. However, it should be noted that many reactive species formed from various toxic drugs (e.g., cisplatin and adriamycin) induce significant damage at submicromolar concentrations *in vivo*. Furthermore, it is now well established that oxidative stress causes a significant decrease in reduced GSH in cells and *in vivo*.^{49,50} Repeated use of hydralazine in the presence of elevated levels of copper ions *in vivo* would result in the depletion of GSH, causing increased toxicity of hydralazine.

It is noteworthy that DNA-cleavage studies, oxygen-consumption studies, ESR spin-trapping studies, and immunological detection of DNA-DMPO adducts all show a very similar stoichiometry for Cu^{2+} ion with hydralazine for the maximum oxy-radical formation, DNA cleavage, oxygen uptake, and nitron formation at a ratio of 5–10. The reason for this is

not clear at the present; however, it would suggest that hydralazine at these concentrations (e.g., 125–250 μM) is extremely effective in cycling Cu^{2+} ions for Fenton-type chemistry and facilitates the maximum formation of oxy-radicals. We found that this ratio of hydralazine to Cu^{2+} ions of between 5 and 10 is extremely effective in inducing DNA damage when this ratio is maintained throughout the range of concentrations, e.g., 50 μM hydralazine and 10 μM Cu^{2+} or 1 mM hydralazine and 100 μM Cu^{2+} (data not shown). It is possible, then, that hydralazine, at higher concentrations, acts as an antioxidant inhibiting the cycling of copper ions and formation of oxy-radicals, and, consequently, decreasing O_2 consumption, DNA cleavage, and DNA radical formation, as observed in this study. This would be similar to ascorbic acid, which can act both as an antioxidant and as a pro-oxidant, causing significant DNA damage in the presence of Fe or Cu ions.⁵¹ It should be noted that hydralazine concentrations greater than 100 μM are extremely difficult to achieve in patients undergoing treatment with hydralazine even at the highest doses of hydralazine used.⁵² Furthermore, due to acetylation and rapid metabolism, the concentration of hydralazine is significantly lower⁵² than 100 μM , and thus, at these lower concentrations, hydralazine is expected to act more as a pro-oxidant, inducing significant oxidatively generated damage *in vivo* in the presence of Cu^{2+} .

The most important finding is that hydralazine in the presence of Cu^{2+} induces significant DNA radical formation, detected as DNA-DMPO nitron adducts as shown in Scheme 1. The implications of this finding are that DNA radicals, once formed, may induce the formation of 8-oxo-7,8-dihydroguanine (8-oxoGua) and DNA-aldehydes.^{53,54} Formation of DNA aldehydes (or 4,6-diamino-5-formidopyrimidine and/or 2,6-diamino-4-hydroxy-5-formamidopyrimidine) is common to many oxidizing systems such as ionizing radiation and some chemotherapeutic agents, e.g., bleomycin.^{55,56} We have previously shown that hydralazine binds to DNA and induces DNA aldehyde formation in the presence of metal ions.¹⁵ As copper is distributed throughout the body, DNA damage involving copper would be relatively favored in tissues/cells where copper concentration is elevated. Furthermore, in tissues where O_2 concentration is low, the oxidation of Cu (I) by H_2O_2 would be favored, resulting in DNA damage as studied in this article. Repeated use of drugs such as hydralazine is expected to further enhance DNA damage, resulting in more serious adverse effects, e.g., tumor formation, and possibly have implications in autoimmune disease.

Using spin-trapping studies, we show here the direct trapping of DMPO-nucleoside adducts from hydralazine- Cu^{2+} complexes. Furthermore, we clearly show that the Cu^{2+} -hydralazine system induces adduct formation predominantly at the 2'-deoxyadenosine moiety of the DNA. Thomas et al.⁵⁷ have shown that hydralazine binds to the poly dA-poly dT sequences of DNA, and it is possible that hydralazine- Cu^{2+} also binds to these sequences in DNA and induces radical formation in 2'-deoxyadenosine similar to that induced by the Cu^{2+} - H_2O_2 system.³⁵ Yamamoto and Kawanishi²⁴ have presented evidence for the formation of nucleoside radicals from hydralazine-peroxidase/ H_2O_2 and hydralazine- Cu^{2+} complexes. However, no nucleoside radical adducts of DMPO were trapped in their studies. Furthermore, both 2'-deoxyguanosine and 2'-deoxyadenosine were equally effective in producing radicals.²⁴ In contrast, our studies suggest that the exocyclic- NH_2 adenosine radical was predominantly formed and trapped (40%) by

DMPO and that only a small amount of radicals (5%) was detected from 2'-deoxyguanosine. However, it should be noted that other nucleoside radicals may have been formed in calf thymus DNA but not trapped/detected at 37 °C, as these radical adducts of DMPO were not stable at 37 °C as observed with our spin-trapping studies.

Work is in progress to understand how DNA radical formation may be linked to the inhibition of DNA methyltransferase 1. In this regard, formation of 8-oxoGua has been implicated in decreasing the ability of DNA methyltransferase 1 to methylate neighboring cytosines.^{58,59} Furthermore, the formation of 8-oxoGua in the CpG island significantly alters the binding of the enzyme to DNA, resulting in the inhibition of methylation.^{60,61} It is not known at this time whether the hydralazyl radical, hydralazine diazonium radical,²³ or the phthalazinyl radical derived from the diazonium radical (Scheme 1) can directly alkylate DNA. Furthermore, it is also not known if the diazonium radical can generate oxy-radicals that could induce DNA damage as was found in this study.

AUTHOR INFORMATION

Corresponding Author

*Laboratory of Toxicology and Pharmacology, NIEHS, 111 T. W. Alexander Drive, Research Triangle Park, NC 27709. E-mail: sinha1@niehs.nih.gov.

Funding

This research was supported [in part] by the intramural research program of the NIH, National Institute of Environmental Health Sciences.

Notes

Statements contained herein do not necessarily represent the statements, opinions, or conclusions of NIEHS, NIH, or the U.S. Government.

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Jean Corbett for assisting with oxygen uptake experiments and Dr. Ann Motten and Mrs. Mary Mason for critically editing the manuscript. We also thank Drs. Fiona Summers and Douglas da Silva Ganini for their critical review of the manuscript.

ABBREVIATIONS

DMPO, dimethyl-1-pyrrolidine *N*-oxide; GSH, reduced glutathione; DMSO, dimethylsulfoxide; SOD, superoxide dismutase; OH, hydroxyl radical; IST, immuno-spin trapping; TEMP, 2,2,6,6-tetramethyl-piperidine

REFERENCES

- (1) Mitchell, J. R., Zimmerman, H. J., Ishak, K. G., Thorgeirsson, U. P., Timberell, J. A., Snodgrass, W. R., and Nelson, S. D. (1976) Isoniazid liver injury-clinical spectrum, pathology, and probable pathogenesis. *Ann. Intern. Med.* 94, 181–192.
- (2) Nelson, S. D., Mitchell, J. R., Timberell, J. A., Snodgrass, W. R., and Corcoran, G. B. (1976) Isoniazid and Iproniazid-activation of metabolites to toxic intermediates in man and rat. *Science* 193, 901–903.
- (3) Martz, G., Dalessandri, A., Keel, H. J., and Bollag, W. (1963) Preliminary clinical results with a new antitumor agent RO-4-6467 (NSC-77213). *Cancer Chemother. Rep.* 33, 5–14.
- (4) De Flora, S., Zanicchi, P., Bennicelli, C., Camoirano, A., Cavanna, M., Sciaba, L., Faggin, P., and Brambilla, G. (1982) *In vivo* and *in vitro*

genotoxicity of three antihypertensive hydrazine derivatives (hydralazine, dihydralazine, and endralazine). *Environ. Mutagen.* 4, 605–619.

(5) Toth, B. (1978) Tumorigenic effects of 1-hydrazinophthalazine hydrochloride in mice. *J. Natl. Cancer Inst.* 61, 1363–1365.

(6) Toth, B. (1980) Actual cancer-causing hydrazines, hydrazides, and hydrazones. *J. Cancer Res. Clin. Oncol.* 97, 97–108.

(7) Parodi, S., De Flora, S., Cavanna, M., Pino, A., Bennicelli, C., and Brambilla, G. (1981) DNA damaging activity in vivo and bacterial mutagenicity of sixteen hydrazine derivatives as related to quantitatively to their carcinogenicity. *Cancer Res.* 41, 1469–1482.

(8) Freese, E., Sklarow, S., and Fresse, E. B. (1968) DNA damage caused by antidepressant hydrazines and related drugs. *Mutat. Res.* 5, 343–348.

(9) Arce, C., Segura-Pacheco, B., Perez-Cardenas, E., Taja-Chayeb, L., Candelaria, M., and Duenas-Gonzalez, A. (2006) Hydralazine targets: From blood vessels to epigenome. *J. Transl. Med.* 4, 10–22.

(10) Knowles, H. J., Tian, Y.-M., Mole, D. R., and Harris, A. L. (2004) Novel mechanism of action of hydralazine: Induction of hypoxia-inducible factor I alpha, vascular endothelial growth factor, and angiogenesis by inhibition of prolyl hydroxylases. *Circ. Res.* 95, 162–169.

(11) Timbrell, J. A., Facchini, V., Harland, S. J., and Mansilla-Tinoco, R. (1984) Hydralazine-induced Lupus: is there a toxic metabolic pathway? *Eur. J. Clin. Pharmacol.* 27, 555–559.

(12) Cuz-Hernandez, E., Perez-Cardena, E., Taja-Chayeb, L., Chavez-Blanco, A., Trejo-Becerril, C., Trujillo, J., Franco-Medina, J. L., and Duenas-Gonzalez, A. (2011) DNA demethylating activity of hydralazine in cancer cell lines. *Life Sci. Med. Res.*, 2–8.

(13) Sinha, B. K., and Motten, A. G. (1982) Oxidative metabolism of hydralazine: evidence for nitrogen-centered radicals formation. *Biochim. Biophys. Res. Commun.* 105, 1044–1051.

(14) Sinha, B. K. (1983) Enzymatic activation of hydrazine derivatives: a spin-trapping study. *J. Biol. Chem.* 258, 796–801.

(15) Sinha, B. K., and Patterson, M. A. (1983) Free radical metabolism of hydralazine: binding and degradation of nucleic acids. *Biochem. Pharmacol.* 32, 3279–3284.

(16) Sinha, B. K. (1984) Metabolic activation of Procarbazine: evidence for carbon-centered radical intermediates. *Biochem. Pharmacol.* 33, 2777–2781.

(17) Kalyanaraman, B., and Sinha, B. K. (1985) Free radical-mediated activation of hydrazine derivatives. *Environ. Health Perspect.* 64, 179–184.

(18) Sinha, B. K. (1987) Activation of hydrazine derivatives to free radicals in the perfused rat liver: A spin-trapping study. *Biochim. Biophys. Acta* 924, 261–269.

(19) Rangelova, K., Suarez, J., Magliozzo, R. S., and Mason, R. P. (2008) Spin trapping investigations of peroxide- and isoniazid-induced radicals in Mycobacterium tuberculosis catalase-peroxidase. *Biochemistry* 47, 11377–11385.

(20) Fiala, E. S. (1975) Investigations into the metabolism and mode of action of the colon carcinogen, DMH. *Cancer* 36, 2407–2412.

(21) Leite, L. C. C., and Augusto, O. (1989) DNA alterations induced by the carbon-centered radical derived from the oxidation 2-phenylhydrazine. *Arch. Biochem. Biophys.* 270, 560–572.

(22) Gamberini, M., Cidade, M. R., Valotta, L. A., Armelin, M. C. S., and Leite, L. C. C. (1998) Contribution of hydrazines-derived alkyl radicals to cytotoxicity and transformation induced in normal c-myc-overexpressing mouse fibroblasts. *Carcinogenesis* 19, 147–155.

(23) Reily, C. A., and Aust, S. D. (1997) Peroxide substrates stimulate the oxidation of hydralazine to metabolites which cause single-strand breaks in DNA. *Chem. Res. Toxicol.* 10, 328–334.

(24) Yamamoto, K., and Kawanishi, S. (1991) Free radical production and site-specific DNA damage induced by hydralazine in the presence of metal ions or peroxidase/hydrogen peroxide. *Biochem. Pharmacol.* 41, 905–914.

(25) (2000) *Copper: Environmental Health Criteria*, World Health Organization (WHO) Geneva, Switzerland.

(26) Markovitz, H., Gubler, C. J., Mahoney, J. P., Cartwright, G. E., and Wintrobe, M. M. (1955) Studies on copper metabolism XIV.

Copper ceruloplasmin and oxidase activity in serum of normal subject, pregnant women, and patients with infection, hepatolenticular degeneration and nephric syndrome. *J. Clin. Invest.* 34, 1498–1508.

(27) Zowczak, M., Iskara, M., Torlinski, S., and Cofta, S. (2001) Analysis of serum copper and zinc concentrations in cancer patients. *Biol. Trace Elem. Res.* 82, 1–8.

(28) Gupta, S. K., Shukla, V. K., Vaidya, M. P., Roy, S. K., and Gupta, S. (1993) Serum and tissue trace elements in colorectal cancer. *J. Surg. Oncol.* 52, 172–175.

(29) Yoshida, D., Ikeda, Y., and Nakazawa, S. (1993) Quantitative analysis of copper, zinc, and copper/zinc ratio in selected human brain tumors. *J. Neurooncol.* 16, 109–115.

(30) Nasulewicz, A., Mazur, A., and Opolski, A. (2004) Role of copper in tumor angiogenesis-clinical implications. *J. Trace Elem. Med. Biol.* 18, 1–8.

(31) Aruoma, O. I., Halliwell, B., Gajewski, E., and Dizdareoglu, M. (1991) Copper-ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide. *Biochem. J.* 273, 601–604.

(32) Sinha, B. K., Antholine, W. M., Kalyanaraman, B., and Eliot, H. M. (1990) Copper ion-dependent oxy-radical mediated DNA damage from dihydroxy derivative of etoposide. *Biochim. Biophys. Acta* 1096, 81–83.

(33) Yamamoto, K., and Kawanishi, S. (1989) Hydroxyl free radical is not the main active species in site-specific DNA damage induced by copper (II) ion and hydrogen peroxide. *J. Biol. Chem.* 264, 15435–15440.

(34) Bhattacharjee, S., Deterding, L. J., Chatterjee, S., Jiang, J., Ehrenshaft, M., Lardinois, O., Ramirez, D. C., Tomer, K. B., and Mason, R. P. (2011) Site-specific radical formation in DNA induced by the Cu(II)-H₂O₂ oxidizing system, using ESR, immuno-spin trapping, LC-MS and MS/MS. *Free Radical Biol. Med.* 50, 1536–1545.

(35) Bhattacharjee, S., Chatterjee, S., Jiang, J., Sinha, B. K., and Mason, R. P. (2012) Detection and imaging of the free radical DNA in cells-Site-specific radical formation induced by Fenton chemistry and its repair in cellular DNA as seen by electron spin resonance, immuno-spin trapping and confocal microscopy. *Nucleic Acids Res.* 40, 5477–5486.

(36) Nakamura, K., Ishiyama, K., Ikai, H., Kanno, T., Sasaki, K., Niwano, Y., and Kohno, K. (2011) Reevaluation of analytical methods for photogenerated singlet oxygen. *J. Clin. Biochem. Nutr.* 49, 87–95.

(37) Dulling, D. R. (1994) Simulation of multiple isotropic spin traps ESR spectra. *J. Magn. Reson. Ser. B* 104, 105–110.

(38) Mason, R. P. (2004) Using anti-5, 5-dimethyl-1-pyrroline N-oxide (anti-DMPO) to detect protein radical in time and space with immuno-spin trapping. *Free Radical Biol. Med.* 36, 1214–1223.

(39) Ramirez, D. C., Gomez-Mejiba, S. E., and Mason, R. P. (2007) Immuno-spin trapping analysis of DNA radicals. *Nat. Protocol.* 2, 512–522.

(40) Finkelstein, E., Rosen, G. M., Rauckman, E. J., and Paxton, J. (1979) Spin trapping of superoxide. *Mol. Pharmacol.* 16, 676–686.

(41) Hawkins, C. L., and Davies, M. J. (2001) Hypochlorite-induced damage to nucleosides: formation of chloramine and nitrogen-centered radicals. *Chem. Res. Toxicol.* 14, 1071–1081.

(42) Fujimoto, S., Adachi, Y., Ishimitsu, S., and Ohara, A. (1986) Release of bases from deoxyribonucleic acid by ascorbic acid in the presence of Cu²⁺. *Chem. Pharm. Bull.* 34, 4848–4851.

(43) Sagripanti, J.-S., and Kraemer, K. H. (1989) Site-specific oxidative DNA damage at polyguanosine produced by copper plus hydrogen peroxide. *J. Biol. Chem.* 264, 1729–1734.

(44) Oikawa, S., and Kawanishi, S. (1996) Copper-mediated DNA damage by metabolites of p-dichlorobenzene. *Carcinogenesis* 17, 2733–2739.

(45) Oikawa, S., and Kawanishi, S. (1998) Distinct mechanisms of site-specific DNA damage induced by endogenous reductants in the presence of iron (III) and copper (II). *Biochim. Biophys. Acta* 1399, 19–30.

(46) Frelon, S., Douki, T., Favier, A., and Cadet, J. (2003) Hydroxyl radical is not the main reactive species involved in the degradation of

DNA bases by copper in the presence of hydrogen peroxide. *Chem. Res. Toxicol.* 16, 191–197.

(47) Imlay, J. A., Chin, S. M., and Linn, S. (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science* 240, 640–642.

(48) Hanna, P. M., and Mason, R. P. (1992) Direct evidence for inhibition of free radical formation from Cu(I) and hydrogen peroxide by glutathione and other potential ligands using the EPR- spin-trapping technique. *Arch. Biochem. Biophys.* 295, 205–213.

(49) Kidd, P. M. (1997) Glutathione: Systemic protectant against oxidative and free radical damage. *Altern. Med. Rev.* 2, 155–176.

(50) Vaziri, N. D., Wang, X. Q., Oveisi, F., and Rad, B. (2000) Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats. *Hypertension* 36, 142–146.

(51) Halliwell, B. (1999) Vitamin C: Antioxidant or pro-oxidant in vivo? *Free Radical Res.* 25, 439–454.

(52) Shepherd, A. M. M., Luden, T. M., McNay, J. L., and Lin, M.-S. (1980) Hydralazine kinetics after single and repeated oral doses. *Clin. Pharmacol. Ther.* 28, 804–811.

(53) Fujita, S., and Steenken, S. (1982) Pattern of OH radical addition to uracil and methyl- and carbon substituted uracils: Electron transfer of OH adducts with N,N,N'-tertramethylphenyldiamine and tetranitromethane. *J. Am. Chem. Soc.* 103, 2540–2545.

(54) Cadet, J., Douki, T., and Ravanat, J.-L. (2008) Oxidatively generated damage to the guanine moiety of DNA: mechanistic aspects and formation in cells. *Acc. Chem. Res.* 41, 1075–1083.

(55) Burger, R. M., Peisac, J., and Horwitz, S. B. (1982) Stoichiometry of DNA strand scission and aldehyde formation by bleomycin. *J. Biol. Chem.* 257, 8612–8614.

(56) Gutteridge, J. M., West, M., Eneff, K., and Floyd, R. A. (1990) Bleomycin-iron damage with formation of 8-hydroxydeoxyguanosine and base propenals. Implications that xanthine oxidase generates superoxide from DNA degradation products. *Free Radical Res. Commun.* 10, 159–165.

(57) Thomas, T. J., Seibold, J. R., Adams, L. E., and Hess, E. V. (1995) Triplex-DNA stabilization by hydralazine and the presence of anti-(triplex DNA) antibodies in patients treated with hydralazine. *Biochem. J.* 311, 183–188.

(58) Tudek, B. (2003) Imidazole ring-opened DNA purines and their biological significance. *J. Biochem. Mol. Biol.* 36, 12–19.

(59) Turk, P. W., Laayoun, A., Smith, S. S., and Weitzman, S. A. (1995) DNA adduct 8-hydroxyl-2'-deoxyguanosine (8-hydroxyguanine) affects function of DNA methyltransferase. *Carcinogenesis* 16, 1253–1255.

(60) Cerda, S., and Weitzman, S. A. (1997) Influences of oxygen radical injury on DNA methylation. *Mutat. Res.* 386, 141–152.

(61) Maltseva, D. V., Baykov, A. A., Jeltsch, A., and Gromova, E. S. (2009) Impact of 7,8-dihydro-8-oxoguanine on methylation of CpG sites by Dnmt3a. *Biochemistry* 48, 1361–1368.