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DNA Single and Double Strand Breaks Induced by Aliphatic and Aromatic Aldehydes in Combination with Copper(II)

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The aliphatic n-butyr- and n-valeraldehyde as well as the aromatic benz- and anisaldehyde induced DNA strand breaks in PM2 DNA in the presence of $CuCl_2$. Neither aldehydes nor $CuCl_2$ alone showed DNA breakage properties. The maximum of single strand breaks (SSBs) induced by the combination of $CuCl_2$ and aldehydes was dependent on the $CuCl_2$-concentration. The aliphatic aldehydes induced SSBs and double strand breaks (DSBs) at lower concentrations than aromatic aldehydes when optimal $CuCl_2$ concentration were used. Catalase and neocuproine nearly completely inhibited strand break formation induced by aromatic aldehydes/$CuCl_2$. The prevention of strand breaks induced by aliphatic aldehydes/$CuCl_2$ was less effective. While the inhibition by neocuproine was only 25 %, catalase was totally ineffective. In all aldehydes/$CuCl_2$ mixtures the formation of Cu(I) was observed. The results point to different DNA damaging species produced during redox reactions of aromatic and aliphatic aldehydes in combination with $CuCl_2$.

Key words: aldehyde, copper, DNA strand breaks, genotoxicity, free radicals

Abbreviations: AnisA, p-anisaldehyde (4-methoxybenzaldehyde); BCS, bathocuproinedisulfonic acid; BenzA, benzaldehyde; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DSBs, double strand breaks; n-BuA, n-butyraldehyde (butanal); n-VaA, n-valeraldehyde (pentanal); PM2 DNA, supercoiled DNA from the phage PM2; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SSBs, single strand breaks

INTRODUCTION

Aldehydes represent a group of relatively reactive organic compounds. For some aldehydes a carcinogenic potential has been demonstrated. In particular, formaldehyde and acetaldehyde have been examined in respect to their genotoxic and carcinogenic properties.[1] Although long chain aldehydes as well as aromatic aldehydes are used as food additives and in the cosmetic and chemical industries, their biological relevance in toxicological processes is not well known. Aldehydes also occur naturally in a wide range of foods. n-Butyraldehyde (n-BuA), n-valeraldehyde (n-VaA) and benzaldehyde (BenzA) for example have been found in a large number of food products. Other aldehydes, like

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anisaldehyde (AnisA), are occasionally present in high concentrations.[1]

The oxidation of aldehydes is strongly accelerated by some transition metal ions.[2] Initiation by direct interaction between Cu(II) and aldehydes was already proposed in 1931 by Haber and Willstätter.[3] The metal, e.g. Cu(II), initiates the oxidation via a one-electron transfer whereby acyl radicals as primary intermediates and the reduced form of the metal are formed[2]:

$$RCHO + Cu(II) \rightarrow R\dot{C}O + Cu(I) + H^+$$

Cu(I) reduces molecular oxygen to O_2^-, which dismutates to H_2O_2 and O_2. The so called copper driven Fenton reaction produces the ultimately DNA damaging $^{\bullet}OH$ radicals and/or related reactive species.

$$H_2O_2 + Cu(I) \rightarrow Cu(II) + OH^- + {}^{\bullet}OH$$

Because copper has been found to be an essential compound of the chromatin[4–6] and is closely associated with chromosomes and DNA,[7] it is proposed that these reactions can take place directly on the DNA. The hydroxyl radicals are known to induce DNA damages like 5,6-dihydroxy-5,6-dihydrothymine, 8-oxo-7,8-dihydroguanine, or strand breakage. Several studies have provided evidence that Cu(II) is capable of mediating the oxidation of xenobiotics, such as quercetin,[8–10] dietary flavonoids[11] and benzoyl peroxide,[12] by redox mechanism leading to the formation of reactive species. Radicals like the benzoyloxyl radical[13] and reactive oxygen species (ROS) are the results. Antibiotics with multiple oxygen functions, like tetracyclines or penicillins damage DNA synergistically in combination with Cu(II).[14–16] The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) induces DNA strand breaks only in combination with Cu(II).[17] The prevention of tetracycline/Cu(II) and 2,4-D/Cu(II)-induced strand breaks by catalase, or neocuproine points to the involvement of $^{\bullet}OH$-radicals and Cu(I) in the DNA damage. Also, Li and Trush reported that hydroquinone

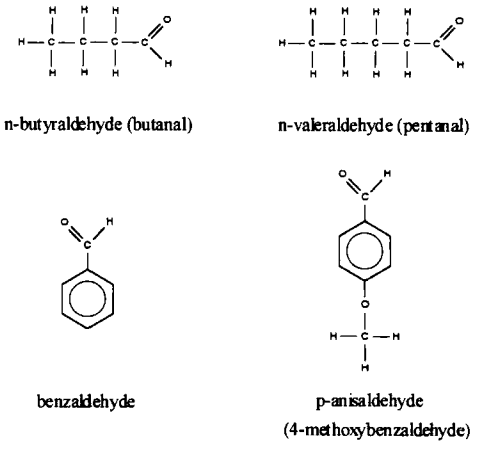

FIGURE 1 Structures of the aldehydes examined.

oxidation is mediated by Cu(II) through a mechanism that involves redox cycling of copper.[7,18–20] They demonstrated that this oxidation resulted in the formation of DNA strand breaks in ΦX-174 plasmid DNA.

It is unknown if all aldehydes oxidize in the presence of Cu(II) and if the reactions lead to $^{\bullet}OH$-radical production, resulting in DNA damage. To acquire information on the genotoxic activities of aldehydes, we selected two aliphatic (n-butyr- and n-valer-) and two aromatic (benz- and p-anis-) aldehydes and studied their reactions in absence and presence of Cu(II).

MATERIALS AND METHODS

Chemicals and reagents

All salts and buffer substances were of analytical grade. Bromophenol blue, bovine serum albumin (BSA) and ethidium bromide were obtained from Serva, Heidelberg (F.R.G.), Seakem agarose was purchased from FMC, Rockland (USA). Benzaldehyde, p-anisaldehyde (4-methoxybenzaldehyde) and neocuproine were obtained from Fluka, Buchs (Ch), n-butyraldehyde (butanal) and $CuCl_2$ from

Riedel de Haen, Seelze (F.R.G.). n-Valeraldehyde (pentanal) was purchased from Merck, Darmstadt (F.R.G.). The aldehydes were stored under nitrogen. Purity of the aldehydes was determined according to their refractive index. Bathocuproinedisulfonic acid was obtained from Sigma, Deisenhofen (F.R.G.). Catalase (specific activity: 65 000 u/mg) was purchased from Boehringer, Mannheim (F.R.G.). Supercoiled DNA from the phage PM2 was produced and isolated in our laboratory according to the method of Espejo and Canelo.[21]

Chemical treatment of PM2 DNA and determination of DNA strand breaks

All solutions were freshly prepared before use. For determination of maximal strand break induction in PM2 DNA (0.2 μg in a final concentration of 5 μg/ml) by 10 mM aldehyde, incubations were performed with various $CuCl_2$ concentrations of up to 2 mM. Incubations occurred for 1 h at 37°C in 10 mM Tris•HCl, pH 7.25. To determine the influence of aldehyde concentration on strand breakage, aldehydes in various concentrations up to 15 mM were incubated with their specific optimal $CuCl_2$ concentration. For inhibition experiments 10 or 35 μg/ml catalase, BSA or 0.4 mM neocuproine in 100 mM sodium phosphat buffer, pH 7.25 were added to the incubation mixture. The reactions were terminated by adding a solution containing 10% SDS, 10% Ficoll, 25% DMSO and 0.04% bromophenol blue in bidistilled water.

To separate the circular supercoiled, circular relaxed and linear PM2 DNA forms horizontal agarose gel electrophoresis was carried out. Gels were formed using 0.5% Seakem agarose dissolved in electrophoresis buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.4). Electrophoresis was run at a constant voltage of 2.7 V/cm for 3.5 h. The gels were stained with ethidium bromide (1 mg/l in water) for 1 h in the dark and then treated with 1 mM $MgSO_4$ for 15 min to remove excess ethidium bromide. Gels were illuminated from below with UV light (Biometra fluo-link TFL-20.M, emitting predominantly at 312 nm) and photographed on Polaroid type 667 films.

The intensities of different PM2 DNA forms were measured directly from the photographs with a Chromoscan scanning densitometer (Joyce & Loebl Ltd., England). Peaks were then integrated and the number of single strand breaks per PM2 DNA molecule were calculated as described by Buschfort and Witte.[14] When single and double strand breaks were simultaneously induced the relative amount of DSBs was calculated as the percent difference of the total amount of added DNA and the remaining supercoiled and relaxed DNA in the experimental samples.

Determination of Cu(I)

Cu(I) generation was determined by using the Cu(I) reagent bathocuproinedisulfonic acid (BCS) according to the method of Shahabudin *et al.*[8] Aldehydes (10 mM final concentration) were mixed with $CuCl_2$ (20 μM final concentration) and BCS solution (0.4 mM final concentration) in 100 mM sodium phosphate buffer, pH 7.25 and incubated at 37°C. The stable BCS-Cu(I) complex was determined by measuring its absorbance at 480 nm. To calculate the aldehyde-specific formation of Cu(I) the reduction of Cu(II) in the BCS solution was subtracted.

RESULTS

Induction of DNA single strand breaks by aldehyde/$CuCl_2$ as a function of $CuCl_2$ concentrations

Induction of single strand breaks (SSBs) in supercoiled double stranded PM2 DNA leads to the formation of open circular (relaxed) DNA.[22,23] Single strand break activities of the two aliphatic aldehydes n-butyraldehyde (n-BuA) and n-valeraldehyde (n-VaA), and the aromatic aldehydes benzaldehyde (BenzA) and p-anisaldehyde (p-AnisA) in combination with $CuCl_2$ were

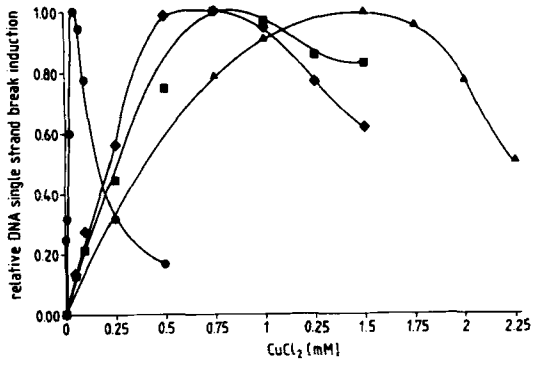

FIGURE 2 Relative DNA single strand break induction in PM2 DNA after 1 hour incubation time. The aldehydes n-butyraldehyde (■), n-valeraldehyde (♦), benzaldehyde (▲) and p-anisaldehyde (●) were incubated at 37 °C with various concentrations of $CuCl_2$ in Tris•HCl (10 mM, pH 7.25).

determined. While $CuCl_2$ and the aldehydes alone did not induce strand breaks, even if incubation times were prolonged from 1 to 3 hours (data not shown), all aldehydes provoked DNA scission in combination with $CuCl_2$. Maximal strand break activity for each aldehyde was observed at specific $CuCl_2$ concentrations (Figure 2). The aliphatic aldehydes showed a maximum of strand scission at 0.75 mM $CuCl_2$, while for p-AnisA and BenzA the strand break maxima were at 0.05 mM and 1.5 mM $CuCl_2$, respectively.

Induction of DNA single and double strand breaks by combination of aldehyde/$CuCl_2$

Extensive DNA damage was observed for varying aldehyde concentrations in combination with their optimal $CuCl_2$ concentration (Figure 3). In addition to the formation of relaxed DNA, linear DNA was formed by the combination of aldehyde/$CuCl_2$ at higher aldehyde concentrations. Linear DNA indicates at least one double strand break (DSB). More than one DSB results in extensive DNA degradation. This was observed with the aliphatic aldehydes/$CuCl_2$ mixtures in concentration higher than 3 mM (data not shown).

A one hour incubation of 1 mM aliphatic aldehydes in combination with 0.75 mM $CuCl_2$ resulted in a 90% and 98% reduction of supercoiled DNA for n-BuA and n-VaA, respectively. A concentration of 3 mM transformed the native DNA almost completely into the relaxed and linear form (Figure 3). The DSB formation by aliphatic aldehydes increased with increasing concentrations from 0.3 mM and 0.5 mM for n-VaA and n-BuA, respectively. At least, one DSB in more than 60% of the PM2 DNA molecules was induced with 10 mM n-VaA. The same concentration of n-BuA formed one DSB in 35% of the PM2 DNA molecules.

DSB formation by aromatic aldehydes/$CuCl_2$ was observed at higher concentrations than by aliphatic aldehydes. At a concentration of 1 mM aromatic aldehydes in combination with their optimal $CuCl_2$ concentration showed a reduction of up to 25% (p-AnisA) and 50% (BenzA) of supercoiled PM2 DNA (Figure 3). At 10 mM the supercoiled DNA was completely degraded. Linear DNA molecules were detected at 5 mM BenzA and 7.5 mM p-AnisA. The amount of linear PM2 DNA at 10 mM aldehyde concentration was 10% with BenzA or 25% with p-AnisA.

Formation of ROS and Cu(I) in aldehyde/$CuCl_2$ induced DNA damage

To investigate if Cu(I) and/or ROS (especially hydroxyl radicals) are involved in DNA breakage, catalase and the Cu(I) scavenger neocuproine were added to the incubation mixtures. The effects of these additives are shown in Table 1. The inhibition of DNA strand breaks by catalase and the Cu(I) chelator was different for aliphatic and aromatic aldehydes. Strand break formation by BenzA- and p-AnisA/$CuCl_2$ was almost completely suppressed by catalase or neocuproine. The enzyme inhibition (35 μg/ml) was 82% and 97% for BenzA and p-AnisA, respectively, whereas for the aliphatic aldehydes inhibition was not observed (or only to a small extent) by the enzymatic function of catalase. Bovine serum albumin (BSA) (35 μg/ml) inhibited strand breakage induced by aliphatic aldehydes/$CuCl_2$ to the

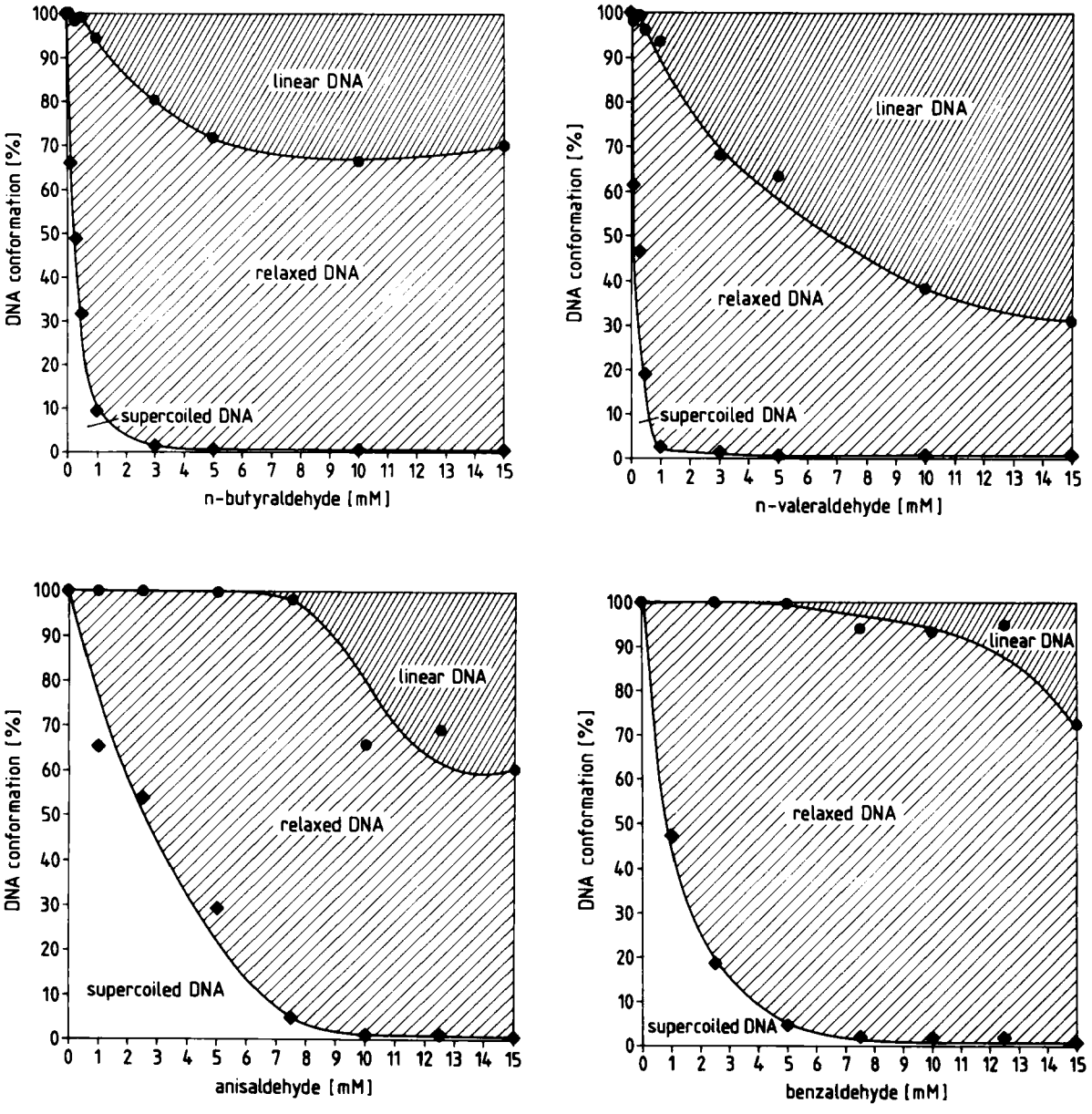

FIGURE 3 Relative amount of the three PM2 DNA conformations: supercoiled (□); relaxed (/ / /); linear (///) DNA. PM2 DNA was incubated with aldehydes and their optimal $CuCl_2$ concentrations of 0.05 mM for p-anisaldehyde, 0.75 mM for n-butyraldehyde and n-valeraldehyde and 1.5 mM for benzaldehyde. Incubation was carried out for 1 h at 37 °C in Tris•HCl (10 mM, pH 7.25).

same extent as catalase. Addition of H_2O_2 to the catalase containing reaction mixture resulted in O_2 production which was measured with a Clark oxygen electrode (data not shown). This confirmed that catalase itself was not inhibited.

The inhibition effect of neocuproine on the strand break induction by aromatic aldehydes/Cu(II) was nearly complete (96% and 97%), whereas the Cu(I) chelator showed only a weak inhibitory effect of about 25% and 20% on the

TABLE 1 Inhibition of DNA single strand break formation by catalase, bovine serum albumin (BSA) and neocuproine. The aldehyde concentrations were 10 mM, the $CuCl_2$ concentration was 0.1 mM. Incubation was carried out for 1 h at 37 °C in sodium phosphate buffer (100 mM, pH 7.25).

	% inhibition			
additive	n-butyraldehyde	n-valeraldehyde	benzaldehyde	p-anisaldehyde
catalase (65 000 U/mg)				
35 µg/ml	31	37	82	97
10 µg/ml	23	22	70	96
BSA				
35 µg/ml	31	31	11	−10
10 µg/ml	0	−3	−6	−9
neocuproine				
0.4 mM	25	20	95	96

formation of strand breaks due to aliphatic aldehydes/Cu(II).

During the reaction of aldehydes with $CuCl_2$ Cu(I) was formed in all samples. Table 2 shows the Cu(I)-BCS complex formation after 30 and 60 minutes incubation with 10 mM aldehyde and 20 µM $CuCl_2$. Within this time the Cu(I) formation rates were nearly identical for aliphatic aldehydes. Approximately 20% of the Cu(II) was reduced by n-BuA and n-VaA within 60 minutes. BenzA reduced about twice as much as these, while p-AnisA as the best reductant reduced nearly all of the added Cu(II) within this time period.

DISCUSSION

Our investigation demonstrates the synergistic DNA damaging effect of aldehydes in combination with Cu(II). Aliphatic as well as the aromatic aldehydes induced DNA single and double strand breaks in the presence of Cu(II) in a concentration dependent manner. These Cu(II) induced oxidation processes of aldehydes have not previously been observed under physiological conditions. The fact that each aldehyde shows its own Cu(II) optimum during DNA strand break induction suggests that catalytic amounts of the metal are insufficient to support the oxidation processes. Stochiometric amounts of copper seem to be necessary for at least some reaction steps.

Strand break formation induced by aromatic aldehydes/Cu(II) was nearly completely prevented by catalase and neocuproine (Table 1). Catalase as well as the Cu(I) chelator neocuproine are potent inhibitors of the copper driven Fenton reaction so that DNA damaging $^{\bullet}OH$-radicals are

TABLE 2 Cu(I) production after 30 and 60 minutes measured as Cu(I)-bathocuproinedisulfonic acid (BCS) complex in aldehyde/$CuCl_2$ mixtures with 0.4 mM BCS. The aldehyde concentrations were 10 mM, the $CuCl_2$ concentration was 20 µM. Incubation was carried out for 1 h at 37 °C in sodium phosphate buffer (100 mM, pH 7.25).

	after 30 min		after 60 min	
	Cu(I) [µM]	Cu(I) [%]	Cu(I) [µM]	Cu(I) [%]
n-BuA	1.48	8.5	3.41	20.1
n-VaA	0.96	5.5	3.63	21.4
BenzA	2.07	11.9	6.44	38.0
p-AnisA	16.07	92.7	15.85	93.4

not formed.[30,31] The inhibition by catalase and neocuproine points to •OH radicals formed during the Cu(I) driven Fenton reaction as the causative species to induce DNA strand breaks. These results are in agreement with the previously observed one electron oxidation of aldehydes by Cu(II) with the primary intermediary products acyl radical and Cu(I)[2]. The reduction of O_2 by Cu(I) leads to ROS whereby •OH radicals seem to be the main reactive agents responsible for DNA strand breakage. The formation of DSBs in our PM2 DNA assay induced by aromatic aldehydes/Cu(II) are non-random events which points to site-specific, or sequence-specific DNA cleavage. If ROS are involved, site-specific DSBs may be the result of two SSBs in corresponding DNA strands. Copper is preferentially complexed in DNA between the GC bases.[24,25] Repeated site-specific redox reactions of DNA bound copper result in repeated induction of DNA strand breaks,[26] especially when highly reactive intermediates with strand breaking properties are formed. This site-specific mechanism of strand break formation was described for several Cu(II) catalyzed redox systems like ascorbic acid/Cu(II),[27] 2,4-dichlorophenoxyacetic acid/Cu(II),[17] hydrazine/Cu(II)[28] and hydroxylamine/Cu(II) or 4-hydroxyaminoquinoline-1-oxide/Cu(II).[29]

For aliphatic aldehydes in combination with Cu(II) the production of •OH radicals should play a minor role in DNA strand breakage, because neither catalase nor neocuproine showed a noteworthy effect in inhibition experiments, even though the formation of Cu(I) was demonstrated (Table 2). Catalase, which was not inhibited by the reaction mixture, did not prevent strand breakage by its enzymatic function because a comparable inhibition effect was shown with BSA (Table 1). Nevertheless, oxygen seems to be needed for the reactions because strand break formation of aliphatic aldehydes/Cu(II) is completely suppressed in the absence of oxygen (data not shown). DSB formation was more pronounced with aliphatic than with aromatic aldehydes using the optimal Cu(II) concentrations. The absence of ROS during oxidation with aliphatic aldehydes suggests a DSB mechanism which may be different than the induction of DSBs due to the formation of adjacent SSBs by •OH radicals.

In summary, the different inhibitory effects of catalase and neocuproine on DNA strand breakage by aliphatic and aromatic aldehydes/Cu(II) suggest that for aromatic aldehydes •OH radicals are the ultimate DNA strand breaking molecules during oxidation, whereas ROS seem to play a minor role in the DNA damage by aliphatic aldehydes/Cu(II). The identification of the DNA modifications induced by aliphatic or aromatic aldehydes in combination with Cu(II) should give important information on the DNA damaging mechanisms.

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