ORIGINAL ARTICLE

Marta González-Álvarez · Gloria Alzuet
Joaquín Borrás · Marguerite Pitié · Bernard Meunier

DNA cleavage studies of mononuclear and dinuclear copper(II) complexes with benzothiazolesulfonamide ligands

Abstract Copper-based transition metal complexes performing single- and double-strand scission of DNA have been studied. The dinuclear complexes [Cu$_2$(L)$_2$(OCH$_3$)$_2$(NH$_3$)$_2$] and [Cu$_2$(L)$_2$(OCH$_3$)$_2$(DMSO)$_2$] are more active than the corresponding mononuclear [Cu(L)$_2$(py)$_2$] (where HL = N-(4-methylbenzothiazol-2-yl)benzenesulfonamide), suggesting that the dinuclearity is an important factor in the oxidative cleavage of DNA. The cleavage efficiency of the complexes depends on the reducing agent used in the process, the tandem ascorbate/H$_2$O$_2$ being the most efficient. PAGE analyses have shown that these complexes cleave DNA without sequence selectivity. The DNA degradation process takes place mainly by C1' oxidation, but C4' and C5' oxidations cannot be ruled out as minor pathways. These copper complexes preferably oxidize guanine under mild conditions, but under more drastic conditions the oxidation reactivity appears to be T > G > C > A, suggesting the intervention of hydroxyl radicals as active species.

Electronic Supplementary Material Supplementary material is available for this article if you access the article at http://dx.doi.org/10.1007/s00775-003-0463-6. A link in the frame on the left on that page takes you directly to the supplementary material.

Electronic Supplementary Material Supplementary material is available for this article if you access the article at http://dx.doi.org/10.1007/s00775-003-0463-6. A link in the frame on the left on that page takes you directly to the supplementary material.

M. González-Álvarez · G. Alzuet · J. Borrás (✉)
Departamento de Química Inorgánica,
Facultad de Farmacia, Universidad de Valencia,
Avda. Vicent Estellés s/n, 46100, Burjassot, Spain
E-mail: joaquin.borras@uv.es
Fax: +34-96-3544530

M. Pitié · B. Meunier
Laboratoire de Chimie de Coordination du CNRS,
205 route de Narbonne, 31077, Toulouse cedex 4, France

Introduction

Biological substrate oxidations by metal ions and complexes have grown in importance as links between metal ion damage and several diseases have been elucidated. Nucleic acids are attractive targets for oxidation, resulting in base damage and/or strand scission. Studies relating to DNA cleavage by synthetic reagents are of considerable interest because of their utility as biological tools or as chemotherapeutic agents [1, 2, 3, 4, 5]. Many of these transition metal complexes utilize the redox properties of the metal and dioxygen to produce reactive oxygen species that oxidize DNA, yielding direct strand scission or base modification [6, 7]. Copper complexes are capable of both reactions, bis(o-phenanthroline)copper being the paradigm [8]. Lately, nucleolytic activities of different copper complexes with synthetic and natural ligands have been studied extensively [9, 10, 11, 12]. Karlin and co-workers [13] have shown, for a series of copper(II) complexes with a bis-chelating ligand based on tris(pyridylmethyl)amine moieties, that the nuclearity was crucial in the oxidative cleavage, the synergy between the two metal ions contributing to its high nucleolytic efficiency.

The considerable interest developed in copper complexes as nuclease mimics in recent years prompted us to synthesize copper complexes of N-substituted sulfonamide ligands bearing aromatic rings and characterize their nuclease efficiencies [14, 15]. In the present paper we describe the nuclease activity of the mononuclear complex [Cu(L)$_2$(py)$_2$] and the dinuclear complexes Cu$_2$(L)$_2$(OCH$_3$)$_2$(NH$_3$)$_2$] and [Cu$_2$(L)$_2$(OCH$_3$)$_2$(DMSO)$_2$], with HL = N-(4-methylbenzothiazol-2-yl)benzenesulfonamide.

Materials and methods

The ligand HL [N-(4-methylbenzothiazol-2-yl)benzenesulfonamide] and the complexes [Cu(L)$_2$(py)$_2$] (**1**), [Cu$_2$(L)$_2$(OCH$_3$)$_2$(NH$_3$)$_2$] (**2**) and [Cu$_2$(L)$_2$(OCH$_3$)$_2$(DMSO)$_2$] (**3**) were obtained as previously

1

2   **3**
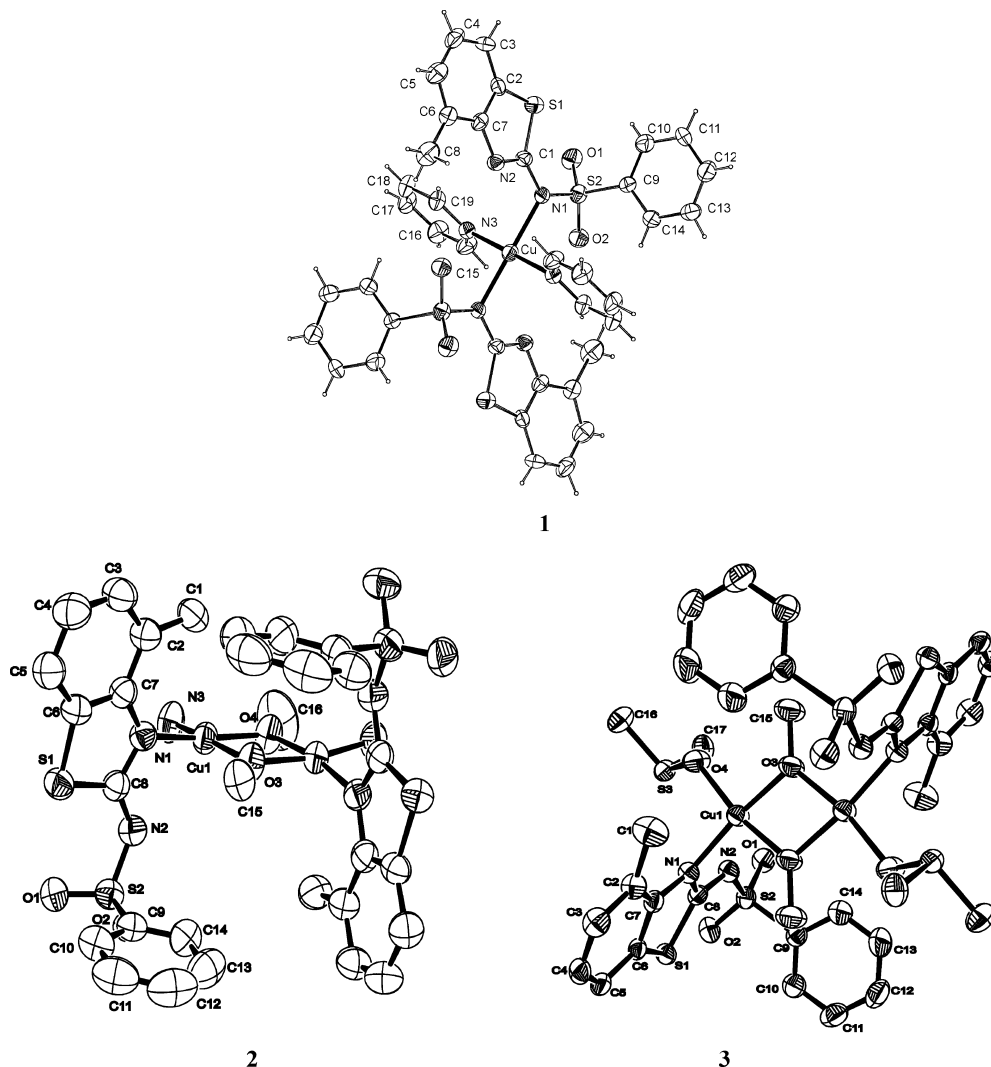
described [16, 17]. They are shown in Fig. 1. All the other chemicals were used as supplied. DNA scission processes were evaluated with commercial pUC18 (Roche Diagnostics, Germany) (0.25 µg/µL in 10 mM Tris and 1 mM EDTA, pH 8.0).

pUC18 DNA cleavage

Reaction mixtures contained 6 µL of a solution of the tested complexes, 3 µL of ascorbate and 3 µL of H_2O_2 respectively dissolved at the desired concentration in 0.1 M cacodylate buffer (pH 6.0), 2 µL of pUC18 DNA and 6 µL of 0.1 M cacodylate buffer (pH 6.0). The mixtures were allowed to stand at room temperature. Agarose gel electrophoresis was performed using 0.8% gels under standard conditions and samples were run on horizontal gels in TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 8.3). Electrophoresis was carried out for 2 h at 80 V; the gels were then stained with ethidium bromide. The bands were photographed on a capturing system (Gelprinter Plus TDI).

Assays to determine the influence of different activators in the DNA scission process were carried out in the presence of the copper complex and H_2O_2/ascorbate, ascorbate, H_2O_2, 3-mercaptopropionic acid or dithiothreitol. Quenching and analyses followed the same procedure as described above.

To test for the presence of reactive oxygen species (ROS) formed during strand scission, the reactive oxygen intermediate scavengers superoxide dismutase (15 units), sodium azide (10 mM),

DMSO (0.4 M) or D-mannitol (0.4 M) were added alternatively to the reaction mixtures. Samples were treated as described above.

HPLC analysis of released DNA nucleobases and oxidized sugars

Experiments were carried out in 200 µL volumes. All the indicated concentrations are final concentrations. Complexes **1**, **2** or **3** (15.7 µM) and ascorbate and/or H_2O_2 (1 mM) were added to a solution of calf thymus DNA (345 µM in nucleotides) in cacodylate buffer (0.1 M, pH 6.0). Samples were incubated for 1 h at 25 °C and then the reaction was stopped by a fast-freezing step in liquid nitrogen.

Some samples were precipitated with 100 µL of sodium acetate buffer (3 M, pH 5.2) and 1.2 mL of ethanol for 4 h at −70 °C and then centrifuged. Pellets were rinsed with 1 mL of ethanol (twice) and then lyophilized in a SpeedVac concentrator. An aliquot of 50 µL of HEPES-NaOH buffer (0.1 M, pH 8.0) was added. Then the samples were heated for 15 min at 90 °C, and afterwards 150 µL of H_2O were added. The reaction was stopped by fast freezing in liquid nitrogen.

Samples were analyzed at 260 and 280 nm on a Nucleosyl C18 10µ HPLC analytical column (250×4.6 mm) coupled to a diode array detector (Waters 994) with 95% triethylammonium acetate buffer (0.1 M, pH 6.5)/5% acetonitrile isocratic elution (1 mL/min). Nucleobases, 5-MF and furfural released during DNA oxidation or the heating step were analyzed by injection of authentic

samples and from their UV-visible profiles. They were quantified by comparison of peak areas versus standard injections of samples at different concentrations. Retention times were: G = 5.7 min, T = 6.3 min, A = 12.9 min, 5-MF = 14.1 min, furfural = 13.2 min.

Characterization of malondialdehyde

Malondialdehyde was characterized according to Murugesan et al. [18]. Standard malondialdehyde solutions were prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane. Calf thymus DNA cleavage by copper complexes was carried out for 1 h at 25 °C on a 100 μL scale. Then, 900 μL of an aqueous solution of 0.6% 2-thiobarbituric acid (TBA) were added and the samples were heated for 20 min at 85 °C. In control experiments the reducing agent was added after TBA but before the heating step. UV-vis spectra of the reaction mixtures were taken and the quantity of the malondialdehyde chromophore ($\epsilon = 150,000$ M^{-1} cm^{-1} at 531 nm) was determined from TBA reaction with a standard. The quantity of malondialdehyde formed during the oxidation step was obtained by subtraction of the malondialdehyde formed in the control experiment from the value obtained under DNA cleavage conditions.

Cleavage pattern of ODN I-ODN II by copper complexes

3′-TGTGCCACGGACTGACGCAATCGTTAAATTGACAC-5′ODN I
5′-ACACGGTGCCTGACTGCGTTAGCAATTTAACTGTG-3′ODN II

The oligonucleotides (ODN I, ODN II) were purified before use on a 20% polyacrylamide gel. DNA was then dissolved in water. Concentrations were determined spectrophotometrically at 260 nm using calculated extinction coefficients [19]. ODN I was radiolabeled by incubation with [γ-^{32}P]ATP and T4 kinase according to the supplier. The 5′-^{32}P-labeled DNA was isolated by passage over a Microspin G25 column (Pharmacia) [20].

Duplex structures containing a 5′-^{32}P-labeled oligonucleotide and a complementary sequence were obtained in cacodylate buffer (0.1 M, pH 6.0) by heating to 90 °C for 5–10 min followed by slow cooling to room temperature.

For cleavage experiments, 6 μL of the copper complexes (final concentration 6 μM) were combined with 8 μL of labeled DNA sample in cacodylate buffer (0.1 M, pH 6.0). Strand scission was initiated by addition of ascorbate (100 μM), ascorbate/H_2O_2 (100 μM/100 μM) or H_2O_2 (1 mM). Final volumes were 20 μL. Reactions were quenched after 1 h incubation at 25 °C from DNA precipitation with 20 μL of sodium acetate (3 M, pH 5.2), 2 μg of tRNA and 120 μL of ethanol for 3 h at −20 °C, then centrifuged. Pellets were rinsed with 100 μL of ethanol (twice) and lyophilized for 5 min in a SpeedVac concentrator.

With the aim to studying the DNA cleavage mechanism, additional treatments were performed on particular samples:

1. Addition of 50 μL of aqueous piperidine (1 M) followed by heating for 30 min at 90 °C; these samples were then lyophilized.
2. After the heating step with piperidine and precipitation, some of these samples were dissolved in 20 μL of H_2O. Then 3′-end phosphates were removed by treatment with 10 U T4 polynucleotide kinase for 30 min at 37 °C followed by precipitation of samples with 40 μL of NaOAc/tRNA and 300 μL of ethanol.
3. Heating for 30 min at 90 °C in 55 μL of HEPES-NaOH buffer (0.1 M, pH 8.0) followed by precipitation with 160 μL of sodium acetate buffer (3 M, pH 5.2) and 1.2 mL of ethanol.

All these samples were dissolved in 5 μL of loading buffer [H_2O/formamide (1:3), 0.01% xylene cyanol and bromophenol blue]. Then they were separated by denaturing 20% polyacrylamide gel electrophoresis and visualized by autoradiography and PhosphorImager (Molecular Dynamics). Fragments of DNA were identified by comparison with Maxam and Gilbert sequencing ladders [21]. Quantification of the products relied on ImageQuant Software.

Results and discussion

Nuclease activity

To determine their ability for DNA strand scission, the copper complexes (1 equiv) were incubated with pUC18 plasmid DNA using ascorbate/H_2O_2 activation (75 equiv of each of these reagents were used when unspecified). For comparison, reactions were carried out also with Cu(II) alone since it is known that copper(II) ions in the presence of ascorbate or hydrogen peroxide cause extensive DNA cleavage [22].

As shown in Fig. 2 (lanes 2 and 3), the complex [Cu(L)$_2$(py)$_2$] (1) presents nuclease activity and the supercoiled plasmid (form I) appears converted into the nicked circular (form II) and linear (form III) forms through single-strand cleavage events. For the same complex concentration (12 μM), a comparison of lanes 2 (0.5 h) and 3 (2 h) shows the dependence of the reaction time on the cleavage for complex 1 that produces more DNA cleavage products when the reaction time increased. The fact that the nicked products are formed before the linear products is in accordance with the proposition that this last form results from two sequential single-strand cleavage events. Increase of the complex concentration also caused increasing DNA cleavage (results not shown). Under the same experimental conditions, Cu(II) (lane 4) also presents nuclease activity. However, its efficiency was found to be lower than that of complex 1.

These concentration and time dependences of the cleavage activity were also observed in the case of plasmid oxidation by complexes 2 and 3. Both compounds also performed single-strand cleavage in the presence of the ascorbate/H_2O_2 tandem but are clearly more active.
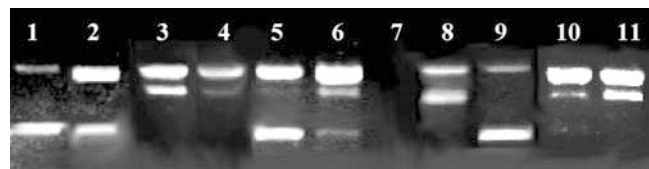

Fig. 2 Agarose gel electrophoresis of pUC18 plasmid DNA treated with complexes 1, 2, 3 or CuSO$_4$·5H$_2$O in the presence of the ascorbate/H_2O_2 tandem for different incubations times. *1*, Control DNA; *2*, 12 μM complex 1, 75 equiv tandem, 0.5 h; *3*, 12 μM complex 1, 75 equiv tandem, 2 h; *4*, 12 μM CuSO$_4$·5H$_2$O, 75 equiv tandem, 2 h; *5*, 3 μM complex 2, 75 equiv tandem, 1 h; *6*, 4.5 μM complex 2, 75 equiv tandem, 1 h; *7*, 6 μM complex 2, 75 equiv tandem, 1 h; *8*, 6 μM complex 2, 25 equiv tandem, 5 min; *9*, 6 μM CuSO$_4$·5H$_2$O, 25 equiv tandem, 5 min; *10*, 6 μM complex 3, 75 equiv tandem, 1.5 h; *11*, 12 μM complex 3, 75 equiv tandem, 1.5 h

Figure 2 (lanes 5–7) presents DNA cleavage by the complex $[Cu_2(L)_2(OCH_3)_2(NH_3)_2]$ (**2**). This dinuclear complex behaves as an efficient chemical nuclease. Within 1 h, complex **2** (4.5 μM, lane 6) converts the supercoiled plasmid to forms II and III. At higher complex concentration (6 μM), complete conversion of form I to smaller linear fragments (smearing) takes place (lane 7). The intense nuclease activity of complex **2** is evidenced in lane 8, indicating that in 5 min and a reduced concentration of reducing agent (25-fold molar excess relative to the complex), 6 μM of complex **2** are able to completely cleave supercoiled DNA to nicked and linear forms. No cleavage was found under the same conditions with $CuSO_4$ (lane 9).

Similar results have been obtained with $[Cu_2(L)_2(OCH_3)_2(DMSO)_2]$ (**3**). Figure 2 shows that complex **3** acts as a remarkable chemical nuclease. After 1.5 h of reaction, 6 μM of complex convert supercoiled DNA to the nicked form and a small amount of the linear form (lane 10); 12 μM of complex produce the conversion of form I to forms II and III (lane 11). At a concentration of 18 μM, smaller linear fragments are produced, as indicated by a smear on the gel (not shown). By comparison of lanes 4 and 11 it was found that the nuclease activity of complex **3** was much higher than that of Cu(II).

DNA cleavage efficiency is dependent on ascorbate and H_2O_2 concentration. Using a concentration of a 5-fold excess relative to the complex and an incubation time of 1 h, 18 μM of complex **3** can cleave DNA to nicked and linear forms. A concentration of a 10-fold excess relative to the complex produces smears (results not shown).

Influence of different reducing agents

In order to elucidate the influence of the nature of the reducing agent, the cleavage process was studied using 3-mercaptopropionic acid (MPA)/air, dithiothreitol/air, H_2O_2 alone, ascorbate/air and ascorbate/H_2O_2 systems as activators. Figure 3 shows the typical results obtained for complex **2**. For the three copper complexes, tandem H_2O_2/ascorbate (lane 6) was the most effective initiating agent followed by ascorbate (lane 3). The other three agents, H_2O_2 (lane 2), MPA (lane 4) and dithiothreitol (lane 5), were less active. However, MPA/air was always more effective than H_2O_2 alone.

Several authors have studied the influence of different reducing agents on the cleavage of DNA by copper(II) complexes [15, 23, 24, 25, 26]. Detmer et al. [23] have found that the tandem ascorbate/H_2O_2 is a more effective activating agent than ascorbate alone. At similar concentrations, ascorbate/H_2O_2 produces a higher quantity of free radicals. Chiou et al. [22] have indicated that ascorbate is more effective in cleaving DNA than other reducing agents such as MPA and dithiothreitol, even though the latter compounds possess higher reducing powers than ascorbate. The uniqueness of
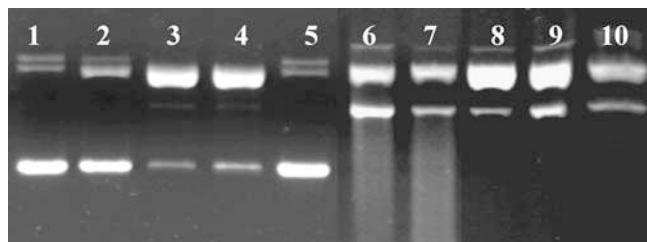

Fig. 3 Agarose gel electrophoresis of pUC18 plasmid DNA treated with complex **2** (6 μM) and different activating agents (90 μM) with or without different oxidation inhibitors; $T_{inc} = 1$ h. *1*, Control DNA; *2*, H_2O_2; *3*, ascorbate; *4*, 3-mercaptopropionic acid (MPA); *5*, dithiothreitol; *6*, ascorbate/H_2O_2; *7*, ascorbate/H_2O_2 + SOD; *8*, ascorbate/H_2O_2 + sodium azide; *9*, ascorbate/H_2O_2 + DMSO; *10*, ascorbate/H_2O_2 + D-mannitol

ascorbate as a reductant in the scission reaction may depend on its ability to generate hydrogen peroxide in the presence of oxygen and metal ions, whereas other reducing agents having a thiol group are known to produce superoxide which rapidly undergoes dismutation in aqueous solution. The DNA cleavage process by [Cu(phen)$_2$] studied by Sigman [25] has shown that the efficiency of cleavage using MPA and air is higher than that of H_2O_2 alone. From these results, Sigman deduced that the reduction of Cu(II) to Cu(I) is an essential step in the cleavage process. Later, Karlin and co-workers [26] reported the nuclease activity of a trinuclear Cu(II) complex in the presence of reagents such as MPA and hydrogen peroxide. For this trinuclear complex the action of MPA/air and H_2O_2 alone is practically similar. This fact induced Karlin to suggest that a reduced form of the trinuclear complex is not a prerequisite for DNA cleavage chemistry and that perhaps a copper(II)-(hydro)peroxo intermediate is responsible for the cleavage of the plasmid. Our results for complexes **1**, **2** and **3** are in agreement with those of Sigman. As a consequence, the reduction of Cu(II) to Cu(I) must play an important role in the cleavage process by **1**, **2** and **3**, as was proposed for [Cu(phen)$_2$].

Influence of potential inhibitors

In order to identify the nature of the reactive oxygen species that could mediate DNA strand scission, we decided to investigate the influence of different potentially inhibiting agents. Figure 3 shows typical results obtained with complex **2** activated by the ascorbate/H_2O_2 tandem. The hydroxyl radical scavengers dimethyl sulfoxide and D-mannitol inhibit the scission reaction, suggesting that OH· radicals are involved in the mechanism (compare lanes 6, 9 and 10). Sodium azide ($^1O_2$ scavenger) is also effective in blocking the DNA strand scission by the copper complex, but this result should not be interpreted as an indication that $^1O_2$ is also participating in the process; it is probably due to the affinity of sodium azide for transition metals (lane 8). Superoxide dismutase (SOD) showed no significant

effect on the cleavage reaction mediated by complexes **1** or **2** (lane 7), but the involvement of $O_2 \cdot^-$ somewhere along the reaction pathway cannot be ruled out since the reaction of SOD with $O_2 \cdot^-$ produces hydrogen peroxide which appears to be required for DNA cleavage. One can only suggest that, for these complexes (**1** and **2**), superoxide anion itself is not the active species [26]. It is remarkable that under the same conditions the activity of complex **3** decreases in the presence of SOD (result not shown). This result indicates that in this case the superoxide anion might be the active species involved in DNA cleavage.

Mechanism of DNA degradation

In order to study the mechanisms of DNA degradation, and to compare the reactivity of these copper complexes with that of a simple copper salt, we first searched for oxidative markers of C1', C5' and C4' oxidations of DNA previously observed with other copper compounds [9]. C1' oxidation produces the direct release of nucleobases during the oxidation step associated with formation of 5-MF after a heating step, products that can be easily characterized by HPLC. C5' oxidation can be characterized (HPLC) by the release of nucleobases and furfural (generated by two β-elimination steps occurring during the heating step) and oxidation at C4' is often associated with 3-phosphoglycolate fragments and also, in many cases, with the presence of base propenals detectable by HPLC. In order to confirm the observed mechanisms of DNA oxidation, PAGE analysis of cleavage patterns resulting from oxidation of a 35-mer double-stranded target by complexes **1**, **2** and **3** was realized.

HPLC characterization of marker products resulting from C1' and C5' oxidations

For the characterization of the DNA oxidation markers, calf thymus DNA was used and experimental conditions were chosen to have less than 10% of DNA degradation (estimated from nucleobase release) in order to decrease the formation of secondary oxidation products.

After DNA oxidation, a precipitation step quenched the reaction by removing the reductant (or H_2O_2 generated during the reaction) and the nucleobases directly released during the oxidation step. Nucleobases and marker products of the oxidation of 2-deoxyribose released before or after heating were analyzed by HPLC. DNA cleavage was initiated by ascorbate, H_2O_2 or a H_2O_2/ascorbate tandem (see Supplementary material). As previously observed during plasmid degradation, the best cleavage conditions for the three complexes and a copper salt are obtained in the presence of the H_2O_2/ascorbate tandem. Quantifications obtained in the case of this latter activation mode are shown in Fig. 4.

DNA degradation by complexes **1**, **2** and **3** in the presence of air and a reductant gave nucleobase release
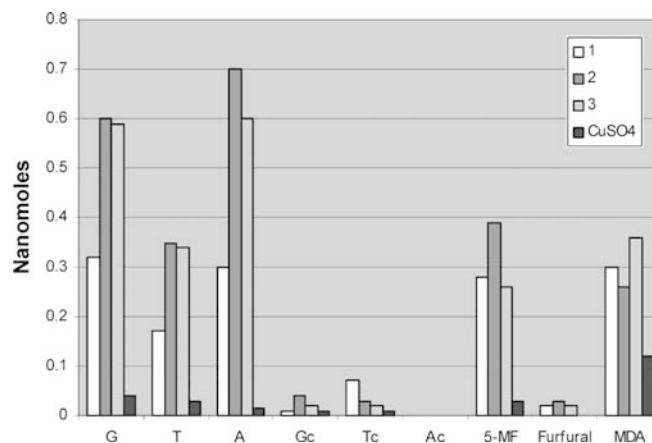

Fig. 4 Comparative study of nucleobases and oxidation markers released for 15.7 μM CuSO$_4$ or complexes **1**, **2** or **3**. The oxidation reactions (1 h at 25 °C) of calf thymus DNA (345 μM in nucleotides) were initiated by 1 mM of H_2O_2/ascorbate tandem in 0.1 M cacodylate buffer (pH 6.0). The reaction volume was 200 μL. *G, T, A*: quantification of nucleic acid bases released before the heating step. *Gc, Tc, Ac, 5-MF, furfural*: quantification of nucleobases, 5-MF and furfural released during the heating step (0.1 M HEPES-NaOH buffer, pH 8.0, 15 min at 90 °C). *MDA*: malondialdehyde estimated with thiobarbituric acid

before and, to a lesser extent, after the heating step. 5-MF and a small quantity of furfural were mainly observed after the thermal step. Control experiments showed that nucleobases, 5-MF and furfural were not released in the absence of copper complexes or reductant (data not shown).

Furfural and 5-MF are markers of C1' and C5' DNA oxidation, respectively. C1' oxidation gave direct nucleobase release during the oxidation step, whereas two β-elimination steps (occurring during the heating step) are necessary to release the nucleobases after C5' oxidation [9].

A previous study concerning the stability of 5-MF produced during DNA oxidation by Mn-TMPyP/KHSO$_5$ has shown that the maximum release of 5-MF occurred after 10–15 min of heating at 90 °C at pH 8.0 [27]. Thus, heating steps were conducted for 15 min at 90 °C in HEPES-NaOH buffer at pH 8.0 (5-MF and furfural are heat sensitive). The quantities observed were only a part of the total 5-MF or furfural that had actually been produced during DNA oxidation. Another part might not be released or could be degraded during the heating step (estimated as 33%, according to Pratviel et al. [27]) or during the relatively long oxidation step.

Figure 4 represents the quantity of the nucleobases released before the heating step, and nucleobases, 5-MF and furfural released after this step for complexes **1**, **2**, **3** and the copper salt. The facility of production of free nucleobases was A≈G > T (calf thymus DNA contains 39% GC). It was not possible to determine the quantity of released cytosine.

Results show that the best cleavage conditions for the three complexes and the copper salt have been obtained in

Fig. 5 PAGE analysis of cleavage of the ODN I-ODN II duplex (5′-end-labeled on ODN I) by complexes **1**, **2** or **3** or CuSO$_4$ (6 μM). The duplex (0.1 μM) cleavage reactions (1 h, 25 °C) were initiated with ascorbate (100 μM), ascorbate/ H$_2$O$_2$ (100 μM/100 μM) or H$_2$O$_2$ (1 mM) in 0.1 M cacodylate buffer, pH 6.0. Lanes $A + G$, G and C: Maxam-Gilbert A + G, G and C ladders, respectively. Heat treatment in 1 M piperidine is indicated at the top of the gel. Marks near the bottom of the gel show typical double bands resulting from 3′-phosphate and 3′-phosphoglycolate fragments
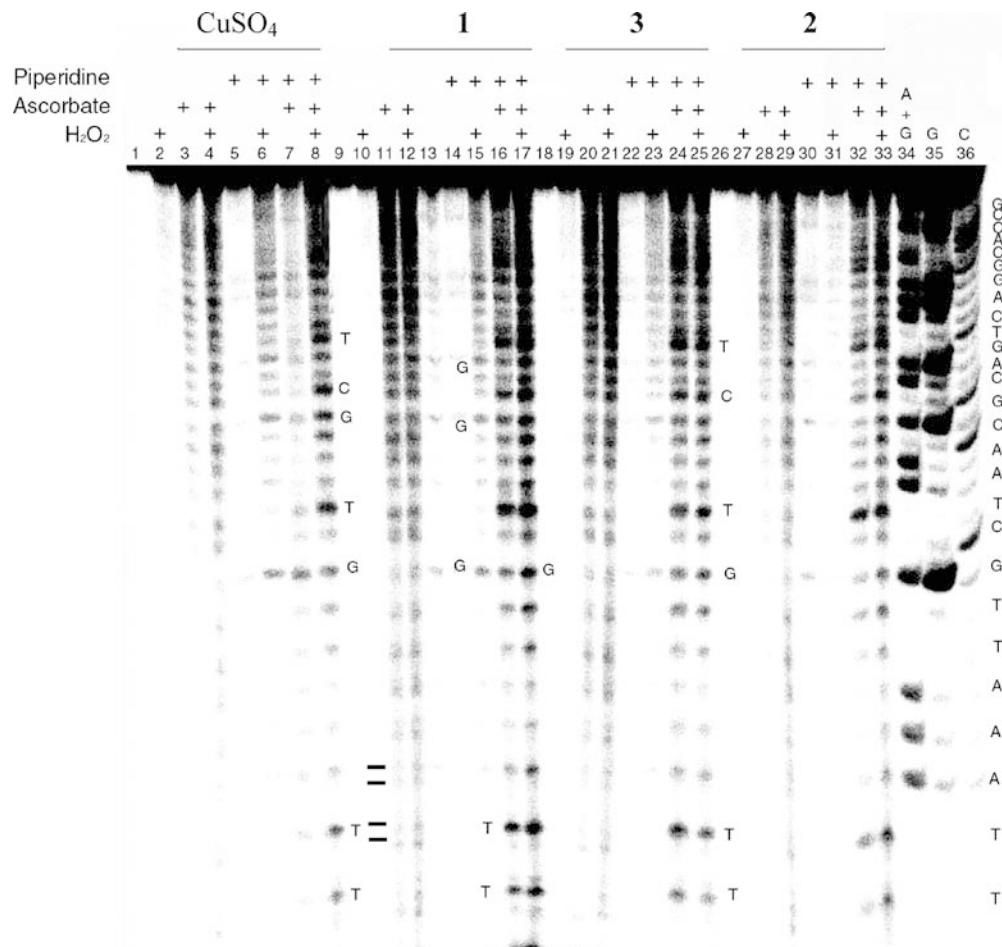
the presence of the H$_2$O$_2$/ascorbate tandem. As can be deduced from Fig. 4, DNA degradation by complexes **2** and **3** is slightly higher than that obtained with complex **1**. All copper complexes are much more active than the copper salt itself. The presence of important quantities of 5-MF shows that the cleavage mechanism of complexes **1**, **2** and **3** is the result of the oxidation at C1' of 2-deoxyribose rings. Moreover, the small among of furfural (Fig. 4) indicates that oxidation at C5' by these complexes is a minor route of the oxidative DNA degradation process.

It is remarkable that the same types of DNA oxidation were observed in the case of the activation of copper complexes by ascorbate alone (without hydrogen peroxide, but air being present). However, only 9%, 26% and 25% of the quantity of markers was released for **1**, **2** and **3**, respectively, in comparison to ascorbate/H$_2$O$_2$ activation. On the other hand, CuSO$_4$ was inactive when activated by ascorbate alone and no markers could be detected when H$_2$O$_2$ alone was used as activator for all these copper complexes (see Supplementary material).

Evidence for C4' oxidation of DNA deoxyribose

The reaction of thiobarbituric acid (TBA) with calf thymus DNA after being oxidized by the copper complexes **1**, **2** or **3** in the presence of a reductant and air

gives a maximum at 531 nm, indicating the presence of malondialdehyde, a marker for C4' oxidation. This type of DNA oxidation was associated with the release of base propenals in the case of DNA degradation by iron-bleomycin or neocarzinostatin. However, different attempts to characterize the presence of base propenals on HPLC profiles failed [18]. However, similar results have been observed on γ-radiolysis of DNA aqueous solutions (known to produce C4' oxidation with MPA-reactive malondialdehyde formation associated with the formation of DNA fragments with 3′-phosphoglycolate ends but without base propenals) [28].

However, because of the drastic conditions used for the TBA reaction, the known formation of malondialdehyde from DNA oxidation products other than ones resulting from C4' oxidation cannot be totally discarded in the DNA oxidation by complexes **1**–**3** [9].

PAGE analysis of DNA breaks resulting from oxidation of ODN I-ODN II by complexes 1, 2 and 3

While the use of plasmid DNA is helpful to assess the general reactivity of the different copper complexes, the DNA oxidation sites are better characterized by a well-defined system based on deoxyoligonucleotides. Radiolabeling of one strand of double-helical DNA with [32]P
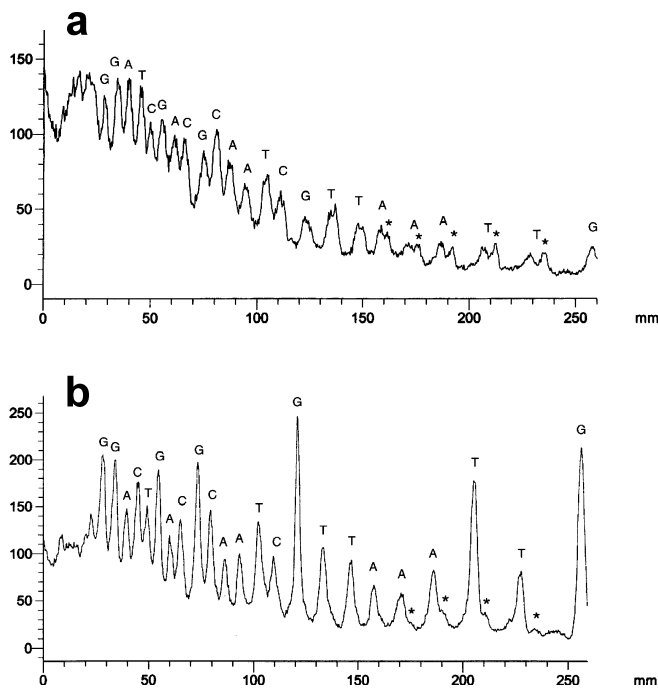
Fig. 6a, b Analysis of cleavage of the ODN I-ODN II duplex (1 μM), 5'-end-labeled on ODN I, by complex **1** (6 μM) during 1 h at 25 °C in the presence of 100 μM ascorbate/H_2O_2 in 0.1 M cacodylate, pH 6.0. **a** Densitometer trace of direct cleavage products. **b** As **a** + heat treatment (30 min at 90 °C) in 1 M piperidine. *Unmarked signals* represent scission products containing a 3'-phosphate, and *marked* (*) *signals* represent 3'-phosphoglycolate. It is possible to observe that 3-phosphoglycolate bands appeared to be stable to further heat treatment under alkaline conditions
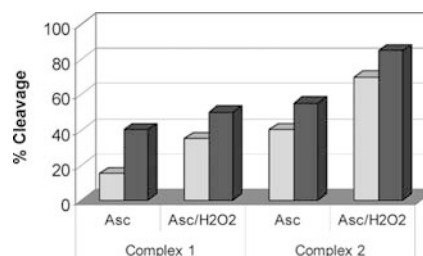
Fig. 7 Histogram demonstrating the influence of activating agent on DNA strand cleavage before and after piperidine treatment. ODN I-ODN II duplex (1 μM) was oxidized by 6 μM of copper complex in the presence of ascorbate (100 μM) or the ascorbate/H_2O_2 tandem (100 μM/100 μM) during 1 h at 25 °C. The percentage of cleavage was calculated by subtracting the percentage of ^{32}P-parent strand in each lane remaining after reaction from the percentage of ^{32}P-parent strand in the control lane. The *light shading* denotes direct strand scission prior to piperidine treatment. Strand scission due to both base and sugar oxidation following piperidine treatment is represented by the *dark shading*

allows for comparisons between major base versus sugar oxidation. The synthetic oligonucleotide duplex ODN I-ODN II selected for investigation corresponds to a 35 base-pair fragment of pBR322. When we consider that C1', C4' and C5' oxidations previously observed were performed in the minor groove of double-stranded DNA, the presently used duplex presents as an important feature a rich zone in adenine and thymine which is easily recognized by different minor binders, including complexes [4].

Complexes **1**, **2** and **3** and $CuSO_4$ have been assayed. Figure 5 shows typical results obtained for the three complexes reacting with 0.1 μM of oligonucleotide and different reducing agents in the presence of air. Complexes **1–3** and $CuSO_4$ do not produce DNA degradation without a reducing agent (lanes 1, 9, 18 and 26). There is a very little degradation with H_2O_2 (lanes 2, 10, 19 and 27). The three complexes and $CuSO_4$ produce significant oligonucleotide degradation in the presence of ascorbate and H_2O_2/ascorbate (lanes 3–4, 11–12, 20–21 and 28–29). Cleavage patterns were non-specific and roughly the same ones with both activating systems. The similarities in the cleavage patterns suggest that common intermediates mediated the DNA oxidation under the different used conditions for complex activation.

It was possible to estimate that the complexes in the presence of ascorbate or ascorbate/H_2O_2 degrade more than 90% of the oligonucleotide while $CuSO_4$ degrades about 50% of the ODN at the same conditions. Degradation was lower than 10% with H_2O_2 alone for all three complexes and the copper salt.

Comparison with Maxam and Gilbert sequencing lanes (lanes 34, 35 and 36) that yield 3'-phosphate ends indicated that the major reaction products with ascorbate/air or ascorbate/H_2O_2 appear to contain 3'-phosphate termini. It was possible to observe small amounts of fragments that most likely presented 3'-phosphoglycolate termini. As previously described, they migrated slightly faster than the corresponding 3'-phosphate derivatives, giving together the appearance of a doublet pattern [6]. The proportion of 3'-phosphate to 3'-phosphoglycolate products varied between experiments. In some cases, 3'-phosphoglycolate termini resulting from oxidation at C4' of 2-deoxyribose were only produced in low yield (lanes 20–21). Similar results were observed for DNA oxidation promoted by a 1,10-phenantroline-copper complex [25].

The direct strand scission of DNA is typically indicative of the oxidation of deoxyriboses of the DNA backbone. A moderate heating step in HEPES buffer at pH 8 is highly useful to reveal other aspects of the oxidative DNA cleavage because of favored β-eliminations such as of a lactone intermediate. This type of lesion is relatively stable at pH 7 or pH 8 [29, 30]. Under these conditions, DNA cleavage by complexes **1–3** increased, but the cleavage patterns were unchanged. A typical example is shown in the Supplementary material.

In contrast, oxidative modification of the nucleobases does not generally produce strand scission unless the modified DNA is treated with heat and/or alkali under more drastic conditions [9, 13]. In order to detect eventual nucleobase oxidations, a heating step in 1 M piperidine was performed [7]. An enhancement in strand scission that results with piperidine indicates that these copper complexes produce strand scission and also oxidize selective positions. After piperidine treatment,

two cleavage patterns can be observed: (1) a preference for guanine oxidation (lanes 6, 7, 15, 23 and 31) at low levels of ODN degradation, and (2) a preference to oxidize mainly thymine (lanes 8, 16–17, 24–25, 32–33) when the degradation conditions are more drastic. The oxidation pattern was T > G > C > > A, which is typical for copper(II) complexes that act through OH· radicals [6]. This result was also confirmed from experiments conducted at higher duplex concentration (1 μM instead of 0.1 μM; for this latter value, see Fig. 5). A weaker duplex degradation percentage was observed and was also related to a weaker thymine oxidation (not shown).

The minor cleavage products attributed to 3′-phosphoglycolate ends appeared stable to further heat/piperidine treatment. Figure 6 shows a typical example of densitometer traces obtained in the case of 1 μM of duplex I-II oxidized by copper complex **1** in the presence of H_2O_2/ascorbate. There were no significant differences after a heating step at pH 8.0. Complexes **2** and **3** have the same behavior (data not shown). Since most of the intense cleavage fragments co-migrated with the Maxam-Gilbert lane and were sensitive to T4 polynucleotide kinase, it can be assumed that these fragments have 3-phosphate ends.

The quantification of the 1 μM duplex degradation from electrophoretic assays is shown in Fig. 7. It can be deduced that (1) the degradation produced by the dinuclear complexes is higher than that due to the mononuclear one, (2) an enhancement of the degradation process takes place after piperidine treatment, and (3) degradation of ODN is more effective in the presence of ascorbate/H_2O_2 than in the presence of ascorbate/air.

Conclusions

Complexes **1, 2** and **3** are able to perform efficient cleavages of DNA because of the structural characteristics of the sulfonamide ligand L (the presence of aromatic rings) and the favorable geometry of the complexes. Dinuclear complexes (**2** and **3**) are much more active than a mononuclear complex. This result indicates that nuclearity is a very important factor in the oxidative cleavage of DNA. The effectiveness of the complexes as chemical nucleases depend on the reducing agent used for activation, the order being ascorbate/H_2O_2 > ascorbate/air > MPA/air > H_2O_2 alone. The oxidative cleavage is probably produced by formation of reactive Cu(I) complexes with DNA, with subsequent generation of reactive oxygen species (OH·, O_2·).

PAGE analyses have shown that the complexes cleave DNA without sequence selectivity. The complexes degraded DNA in a rather random fashion by oxidation at C1', C4' and C5' of the 2-deoxyribose units. The C1' oxidation, the major chemical pathway, generates nucleobase release and unstable intermediates, leading to 5-MF after a heating step. Production of DNA fragments with 3′-phosphoglycolate ends resulted from C4' oxidation, which was not associated with base propenals release but malondialdehyde was detected. The minor C5' oxidation pathway generated direct DNA cleavage and release of furfural and nucleobases after a thermal step. This reactivity reflects the ability of complexes **1**, **2** and **3** to interact at the floor of the DNA minor groove, perhaps by intercalation, leading to C1' oxidation, but also as minor pathway, at the edge of the DNA minor groove, generating C4' and C5' oxidation.

The complexes are able to oxidize specific bases of the oligonucleotide duplex. Under mild conditions, guanine was oxidized preferably by the complexes, but in more drastic degradation conditions the base/oxidation reactivity appeared to be T > G > C > > A, which is characteristic of copper(II) complexes acting via OH· radicals.

References

1. Pyle AM, Barton JK (1990) Prog Inorg Chem 38:413–475
2. Perrin DM, Mazumder A, Sigman DS (1996) Prog Nucleic Acid Chem Mol Biol 52:123–151
3. Pratviel G, Bernaudou J, Meunier B (1995) Angew Chem Int Ed Eng 34:746–769
4. Pratviel G, Bernaudou J, Meunier B (1998) Adv Inorg Chem 45:251–312
5. Chen CB, Milne L, Landgraf R, Perrin DM, Sigman DS (2001) ChemBiochem 2:735–740
6. Pogozelski WK, Tullius TD (1998) Chem Rev 98:1089–1107
7. Burrows CJ, Muller JC (1998) Chem Rev 98:1109–1151
8. Sigman DS, Mazumder A, Perrin DM (1993) Chem Rev 93:2295–2316
9. Pitie M, Burrows CJ, Meunier B (2000) Nucleic Acids Res 28:4856–4864
10. Humpreys KJ, Johnson AJ, Rokita SE, Karlin KD (2001) J Am Chem Soc 123:5588–5589
11. Humpreys KJ, Karlin KD, Rokita SE (2002) J Am Chem Soc 124:6009–6019
12. Humpreys AJ, Karlin KD, Rokita SE (2002) J Am Chem Soc 124:8055–8066
13. Humpreys KJ, Johnson AJ, Karlin KD, Rokita SE (2002) J Biol Inorg Chem 7:835–842
14. Macias B, Villa MV, Fiz E, Garcia A, Castiñeiras A, González-Álvarez M, Borrás J (2002) J Inorg Biochem 88:101–107
15. González-Álvarez M, Alzuet G, Borrás J, Macias B, del Olmo M, Liu-González M, Sanz F (2002) J Inorg Biochem 89:29–35
16. González-Álvarez M, Alzuet G, Borrás J, Garcia-Granda S, Montejo-Bernardo JM (in press) J Inorg Biochem
17. González-Álvarez M, Alzuet G, Borrás J, del Castillo Agudo L, Montejo-Bernardo JM, García Granda S (2003) J Biol Inorg Chem 8:112–120
18. Murugesan N, Xu C, Ehrenfeld GM, Sugiyama H, Kilkuskie RE, Rodriguez LO, Chang LH, Hecht SM (1985) Biochemistry 24:5735–5744
19. Fasman GD, Sober HA (eds) (1975) Handbook of biochemistry and molecular biology. CRC Press, Boca Raton
20. Sambrook J, Frtsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Maxam AM, Gilbert W (1980) Methods Enzymol 65:499–599

652

22. Chiou SH, Ohtsu N, Bensch KG (1985) In: Karlin D, Zubieta J (eds) Biological and inorganic copper chemistry. Adenine Press, New York, pp 119–123
23. Detmer CA III, Pamatong FV, Bocarsly JR (1996) Inorg Chem 35:6292–6298
24. Detmer CA III, Pamatong FV, Bocarsly JR (1997) Inorg Chem 36:3676–3682
25. Sigman DS (1986) Acc Chem Res 19:180–186
26. Frey ST, Sun HHJ, Murthy NN, Karlin KD (1996) Inorg Chim Acta 242:329–338
27. Pratviel G, Pitié M, Bernadou B, Meunier B (1991) Nucleic Acids Res 19:6283–6288
28. Rashid R, Langfinger D, Wagner R, Schuchmann H-P, von Sonntag C (1999) Int J Radiat Biol 75:101–109
29. Roupioz Y, Lhomme J, Kotera M (2002) J Am Chem Soc 124:9129–9135
30. Lenox HJ, McCoy CP, Sheppard TL (2001) Org Lett 3:2415–2418