Mechanistic studies on DNA damage by minor groove binding copper–phenanthroline conjugates

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ABSTRACT

Copper–phenanthroline complexes oxidatively damage and cleave nucleic acids. Copper bis-phenanthroline and copper complexes of mono- and bis-phenanthroline conjugates are used as research tools for studying nucleic acid structure and binding interactions. The mechanism of DNA oxidation and cleavage by these complexes was examined using two copper–phenanthroline conjugates of the sequence-specific binding molecule, distamycin. The complexes contained either one or two phenanthroline units that were bonded to the DNA-binding domain through a linker via the 3-position of the copper ligand. A duplex containing independently generated 2-deoxyribonolactone facilitated kinetic analysis of DNA cleavage. Oxidation rate constants were highly dependent upon the ligand environment but rate constants describing elimination of the alkali-labile 2-deoxyribonolactone intermediate were not. Rate constants describing DNA cleavage induced by each molecule were 11–54 times larger than the respective oxidation rate constants. The experiments indicate that DNA cleavage resulting from β-elimination of 2-deoxyribonolactone by copper–phenanthroline complexes is a general mechanism utilized by this family of molecules. In addition, the experiments confirm that DNA damage mediated by mono- and bis-phenanthroline copper complexes proceeds through distinct species, albeit with similar outcomes.

INTRODUCTION

A large number of metal complexes mediate DNA oxidation. Natural products such as bleomycin, which oxidatively damage DNA, are therapeutically useful (1–3). Other metal complexes are useful for probing nucleic acid structure and interactions. Many of these complexes initiate strand damage by mechanisms that involve DNA radical formation. Complexes formed between EDTA (and its derivatives) and iron (e.g. Fe•EDTA), or phenanthroline and copper [Cu(OP)$_n$, $n = 1, 2$] are examples of widely used agents that damage DNA via radical processes (4–7). Tullius showed unequivocally that Fe•EDTA initiates DNA damage by producing hydroxyl radical and that strand scission is strongly influenced by the accessibility of the hydrogen atoms in the 2′-deoxyribose (4,8,9). Copper–phenanthroline complexes do not form diffusible reactive oxygen species (7,10,11). DNA oxidation is generally attributed to a copper-'oxo' or copper-hydroxyl species of uncertain structure, which abstracts hydrogen atoms rapidly, but much less so than hydroxyl radical (12). A variety of proposals have been put forth describing the reactivity of DNA with copper–phenanthroline complexes (13,14). Here, we describe kinetic experiments that address the mechanism of DNA oxidation and cleavage by conjugates of copper–phenanthroline (**1**, **2**) (Figure 1).

The major pathway for DNA damage by copper–phenanthroline complexes is believed to involve C1′-hydrogen atom abstraction, along with varying amounts of oxidation at the C4′- and/or C5′-positions in DNA (13–17). A novel aspect of copper–phenanthroline chemistry is that C1′-oxidation, a process that often gives rise to the alkali-labile 2-deoxyribonolactone (**L**) lesion, was proposed to give rise to direct (rather than alkali-labile) strand scission (15,18,19). A recent preliminary report rationalized this observation by demonstrating that the major pathway for direct strand scission by

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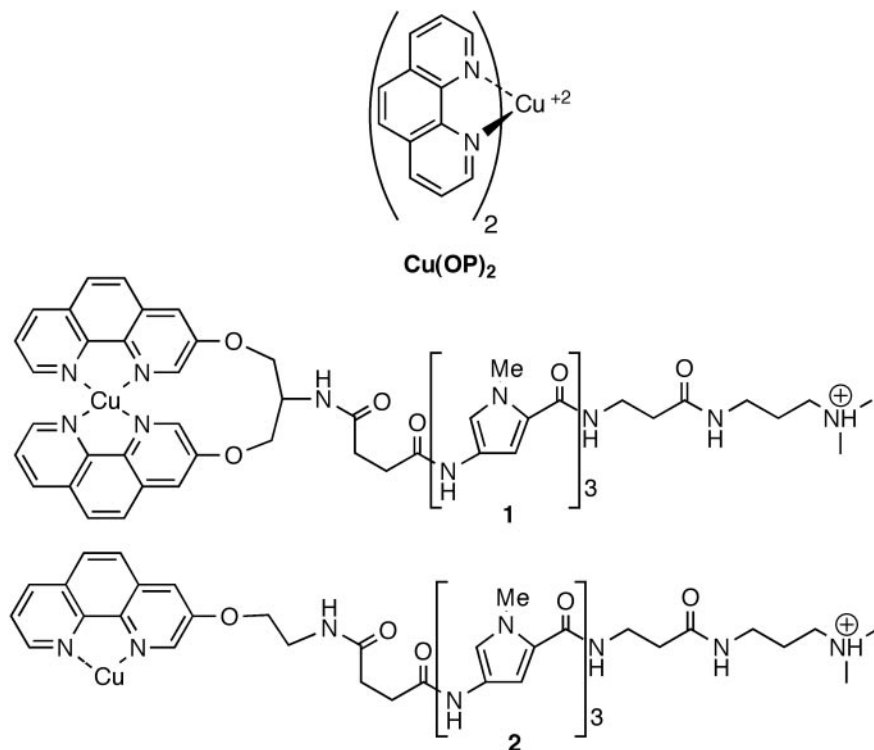
Figure 1. Structures of Cu(OP)$_2$ and distamycin conjugates **1** and **2**.
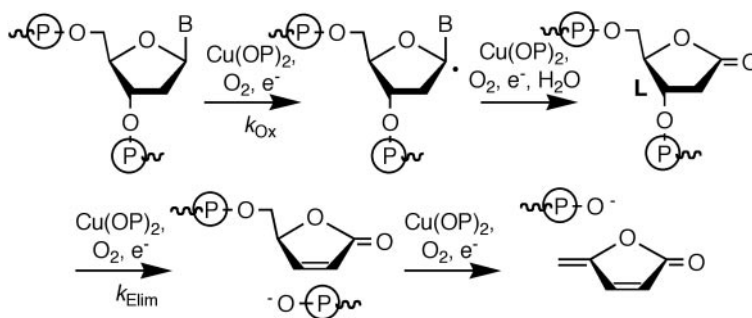
Scheme 1. Mechanism for direct strand break formation by Cu(OP)$_2$.

a distamycin conjugate of copper–phenanthroline (**1**) involves β-elimination of **L**, which is produced by the copper complex (Scheme 1) (20). Earlier model studies involving Cu(OP)$_2$ demonstrated the plausibility for this mechanism (21). The experiments described below further substantiate the proposed copper–phenanthroline induced elimination from 2-deoxyribonolactone. In addition, kinetic experiments provide information on the effects of the number of ligands around copper and the nature of the reductant on DNA oxidation by copper–phenanthroline complexes.

DNA cleavage by the bis-phenanthroline copper complex [Cu(OP)$_2$] was first reported by Sigman in 1979 (22). This reagent and mono-phenanthroline conjugates of a variety of molecules that bind DNA have been used as artificial nucleases from that time forward (23–30). The design of nucleases based upon copper complexes of substituted phenanthrolines and other heteroatomic ligands continues to be an active area of research (17,31–38). Despite the common use of these
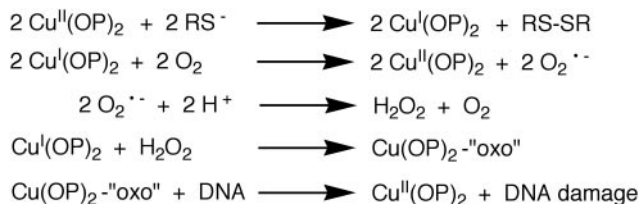
Scheme 2. Proposed mechanism for DNA damage by Cu(OP)$_2$.

reagents, questions regarding their mechanism of action remain. Sigman's pioneering research suggested a mechanism (Scheme 2) in which a Cu(I) complex reduces O$_2$ and is regenerated by the reductant (thiol, ascorbate) (11,39). The superoxide formed gives rise to H$_2$O$_2$, which reacts with Cu(I) to produce a copper-'oxo' complex of unknown structure that is responsible for nucleic acid damage. The activated complex has been drawn in different canonical forms,

including a copper-bound hydroxyl radical and a copper oxo species. Recent research on biologically based copper oxidants suggests that these and other structures are possible (40–42).

The role of the phenanthroline ligand(s) and reductants in DNA oxidation by copper complexes is one area that has received attention (7). Rill (43) showed that the structure of the copper–phenanthroline complex is affected by the nature and concentration of the reductant. Thiols can displace one of the phenanthroline ligands from $Cu(OP)_2$ and are believed to produce a complex that is incapable of DNA oxidation. The overall rate of DNA cleavage is faster with ascorbate (Asc) than 3-mercaptopropionic (MPA) acid. However, ascorbate may also accelerate DNA damage by increasing the concentration of H_2O_2 due to its autoxidation. The cleavage efficiency of $Cu(OP)_2$ is also improved by increasing the number of equivalents of phenanthroline ligand when thiol is the reductant. Examining the chemistry from the perspective of what happens (but not necessarily how fast) to the DNA, Sigman found the possibility that the species responsible for oxidation by mono- and bis-phenanthroline complexes is the same to be an attractive explanation for similarities in DNA cleavage chemistry by various phenanthroline complexes (7). Kinetic aspects of DNA damage induced by $Cu(OP)_2$ and $Cu(OP)$ are difficult to investigate due to the random cleavage patterns produced by these reagents. We have taken advantage of the availability of mono- and bis-phenanthroline distamycin conjugates to examine the kinetics of DNA oxidation and cleavage by copper–phenanthroline complexes (17,34). These studies also benefit from independent generation of 2-deoxyribonolactone at a DNA position that is oxidized by these metal complexes, which enables us to independently investigate the cleavage of the major oxidation product (44–46). These experiments reveal that the mono- and bis-phenanthroline complexes produce similar outcomes via kinetically distinguishable species.

MATERIALS AND METHODS

General methods

Oligonucleotides were synthesized via standard automated DNA synthesis techniques using an Applied Biosystems model 394 instrument as described previously (44–46). DNA synthesis reagents were purchased from Glen Research (Sterling, VA). DNA purification and analysis was carried out using 20% polyacrylamide denaturing gel electrophoresis [5% cross-link, 45% urea (by weight)]. DNA manipulation, including enzymatic labeling, was carried out using standard procedures. T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Radiolabeling was carried out according to the standard protocols (47). Quantitation of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Storm 840 Phosphorimager equipped with ImageQuant Version 5.1 software. Photoreactions of oligonucleotides were carried out in Pyrex tubes in a Rayonet photoreactor fitted with 16 lamps having an output maximum at 350 nm (Fluence: 1.4 mW/cm^2).

Oxidation reaction kinetics

Solutions of **1** or **2** were prepared immediately before use by incubating the distamycin conjugate (1 mM, 2 μl) with one equivalent of $CuCl_2$ for 1 h. The 1 mM solution (2 μl) was then diluted to 10 μl with H_2O. The copper complex (**1**, **2** = 200 μM) was added to a solution (8 μl) unlabeled **7** and 5′-^{32}P-**7** in Tris (pH 7.0) and NaCl, and incubated at room temperature for 5 min. Reaction was then initiated by the addition of 1 μl of freshly prepared 3-mercaptopropionic acid (MPA, 20 mM) or ascorbate (Asc, 10 mM). The final concentration of reagents were DNA, 1 μM; copper complex, 20 μM; Tris (pH 7.0), 10 mM; NaCl, 50 mM; MPA, 2 mM or Asc, 1 mM. Aliquots (1 μl) were removed from reactions incubated at 37°C and frozen in dry ice ethanol bath at −78°C. At the conclusion of the reaction each aliquot was treated with 0.2 M NaOH (1 μl), incubated at 37°C for 20 min, and neutralized with 0.2 M HCl (1 μl). Formamide loading buffer (7 μl) was added to each sample and then subjected to 20% denaturing PAGE. Each experiment was carried out with three separate reactions. The data presented in the paper represent the average of three such experiments.

β-Elimination of 2-deoxyribonolactone

Copper complexes of the distamycin conjugates (**1, 2**) were prepared as described above. These were incubated with 5′-^{32}P-**8** and unlabeled **8** under the concentration and buffer conditions described above. Aliquots (1 μl) were removed as a function of time, quenched with formamide loading buffer (4 μl) and stored at −78°C until analyzed by PAGE. Each experiment was carried out with three separate reactions. The data presented in the paper represent the average of three such experiments.

2-Amino, 1-(1′,10′-phenanthrolin-3′-yloxy)propane (4)

To a solution of 3-bromo-1,10-phenanthroline (**3**) (404 mg, 1.55 mmol) in dry DMF (6 ml) at 4°C was added 2-aminotrityl-1-hydroxyethane (479 mg, 1.58 mmol) and NaH at 60% in mineral oil (126 mg, 3.15 mmol) (48). After stirring for 2 h at 4°C, followed by 2 h at room temperature, the mixture was heated for 15 h at 50°C. After cooling to room temperature, CH_3OH (0.5 ml) and H_2O (20 ml) were added, the crude product was extracted with CH_2Cl_2 (3 × 20 ml), and evaporated. The mixture was dissolved in CH_2Cl_2 (5 ml) and trifluoroacetic acid (5 ml) was added. After stirring for 90 min at room temperature, the solution was concentrated, H_2O (50 ml) was added, the pH was adjusted to 9 by adding concentrated aqueous ammonia, and the crude products were extracted with CH_2Cl_2. Following removal of the solvent, the product was purified on silica gel [CH_2Cl_2/CH_3OH/concentrated ammonia (89/10/1, v/v/v)] to give **4** as a yellow powder (190 mg, 52%). ^{1}H NMR (CD_2Cl_2) δ 9.09 (dd, $J = 4.5, 2.0$ Hz, 1 H), 8.88 (d, $J = 3.0$ Hz, 1 H), 8.25 (dd, $J = 8.0, 2.0$ Hz, 1 H), 7.82 and 7.76 (AB, $J = 9.0$ Hz, 2 × 1 H), 7.59 (d, $J = 3.0$ Hz, 1 H), 7.58 (dd, $J = 8.0, 4.5$ Hz, 1 H), 4.19 (t, $J = 5.0$ Hz, 2 H), 3.17 (t, $J = 5.0$ Hz, 2 H). MS (CDI, NH$_3$): m/z (%) = 240 [(M + 1)$^+$, 100]. UV-vis (CH_3OH): λ_{max} nm (ε: M^{-1} cm^{-1}) = 238 (44,500), 272 (29,700), 294 (15,000, sh), 328 (3,400), 342 (2,300).

Anal. calcd for $C_{14}H_{13}N_3O$, 0.5 CH_2Cl_2: C, 61.82; H, 5.01; N, 14.91. Found C 61.47, H 5.05, N 14.35.

2-Succinoyl-amino-1-(1′,10′-phenanthrolin-2′-yloxy)ethane (5)

To a solution of **4** (150 mg, 0.63 mmol) in dry $CHCl_3$ (20 ml), succinic anhydride (190 mg, 1.90 mmol) was added and the mixture was heated at reflux for 90 min. The product was precipitated after cooling by addition of diethyl ether (65 ml), filtered, and washed with diethyl ether. The precipitate was crystallized from hot CH_3OH (8 ml), centrifuged, and washed with diethyl ether, and dried under vacuum to give **5** as a white powder (121 mg, 57%). ^{1}H NMR (DMSO-d_6) δ 11.76 (bs, 1H), 9.04 (dd, $J = 4.0, 2.0$ Hz, 1 H), 8.80 (d, $J = 3.0$ Hz, 1 H), 8.44 (dd, $J = 8.0, 2.0$ Hz, 1 H), 8.26 (t, $J = 11.0$ Hz, 1 H), 7.99 (d, $J = 3.0$ Hz, 2 H), 7.96 and 7.92 (AB, $J = 9.0$ Hz, 2×1 H), 7.69 (dd, $J = 8.0, 4.0$ Hz, 2 H), 4.23 (t, $J = 4.5$ Hz, 2 H), 3.54 (m, 2 H), 2.40 (m, 4 H). MS (ES, positive mode) $m/z = 340$ $(M + H)^+$. UV-vis (CH_3OH): λ_{max} nm (ε: M^{-1} cm^{-1}) = 240 (49,400), 272 (33,500), 296 (16,700, sh), 328 (4,100, sh), 344 (2,700). Anal. calcd for $C_{18}H_{17}N_3O_4$, 0.3 H_2O: C, 62.71; H, 5.15; N, 12.19. Found: C, 62.74; H, 4.63; N, 12.08.

3-Phen-Py-Py-Py-β-Dp (6)

The succinylated phenanthroline (**5**) (34 mg, 0.10 mmol) was activated with (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (136 mg, 0.31 mmol) and 1-hydroxybenzotriazole (56 mg, 0.41 mmol) in DMF/N-methylmorpholine (1.5 ml, 2/1, v/v) for 15 min. The mixture was added to Py-Py-Py-β-Pam-resin (~0.04 mmol) and agitated for 3 h at room temperature. DMF (5 ml) was added and the suspension was agitated for an additional 15 h. The resin was then isolated by filtration and washed sequentially with an excess of DMF (1×) and CH_2Cl_2 (2×) before heating in 3-dimethylamino-1-propylamine (0.5 ml) for 15 h at 55°C. The reaction was filtered to remove the resin, and the crude product was precipitated with diethyl ether and centrifuged. The pellet was dissolved in water, acidified with TFA and purified by high-performance liquid chromatography (HPLC) on a reverse-phase Nucleosil C18 10 μ

semi-preparative column (250 × 10 mm) in 0.1% TFA and varying the acetonitrile from 14 to 56% in 40 min using a linear gradient at 3 ml/min. Retention time (HPLC): 19 min. The chromatograms were monitored at 260 nm. Conjugate **6** was obtained after lyophilization as a yellow powder (3.6 mg, 8.3% of recovery). ^{1}H NMR (D_2O) δ 8.58 (d, $J = 5.5$ Hz, 1 H), 8.58 (d, $J = 8.0$ Hz, 1 H), 8.37 (d, $J = 2.5$ Hz, 1 H), 7.76 and 7.67 (AB, $J = 9.0$ Hz, 2×1 H), 7.67 (dd, $J = 8.0, 5.5$ Hz, 1 H), 7.39 (d, $J = 2.5$ Hz, 1 H), 6.95 (d, $J = 1.5$ Hz, 1 H), 6.82 (d, $J = 1.5$ Hz, 1 H), 6.58 (d, $J = 1.5$ Hz, 1 H), 6.15 (d, $J = 1.5$ Hz, 1 H), 5.87 (d, $J = 1.5$ Hz, 1 H), 5.72 (d, $J = 1.5$ Hz, 1 H), 3.86 (m, 2 H), 3.70 (s, 3 H), 3.70 (m, 2 H), 3.54 (s, 3 H), 3.52 (t, $J = 7.0$ Hz, 2 H), 3.43 (s, 3 H) 3.25 (t, $J = 6.0$ Hz, 2 H), 3.06 (t, $J = 8.0$ Hz, 2 H), 2.79 (s, 6H), 2.53 (m, 4 H), 2.49 (t, $J = 7.0$ Hz, 2 H), 1.88 (m, 2 H). MS (ES, positive mode, in CH_3CN/H_2O [1/1, v/v] with 0.1% TFA) $m/z = 861.5$ $(M + H)^+$, 431.3 $(M + 2 H)^{2+}$. UV-vis (H_2O): λ_{max} nm (ε: M^{-1} cm^{-1}) = 238 (49,800), 280 (32,400), 298 nm (35,500, sh).

RESULTS AND DISCUSSION

Synthesis of mono-phenanthroline distamycin conjugate 2

The synthesis of bis-phenanthroline conjugate **1** has been described previously (17). The mono-phenanthroline conjugate **2** was synthesized in a similar manner (Scheme 3). Ethanolamine was used in order to have the same functional groups and linker length between the phenanthroline and distamycin components of conjugates **1** and **2**. Briefly, 3-bromophenanthroline **3** (48) was condensed with N-triphenylmethyl protected ethanolamine. After acid hydrolysis of the trityl group, the resulting phenanthroline-amine derivative **4** was transformed into carboxylic acid **5** by reaction with succinic anhydride. Compound **5** was then activated by (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate/1-hydroxybenzotriazole in DMF/N-methylmorpholine and conjugated with Py-Py-Py-β-Pam-resin. Aminolysis of the resin ester linkage in 3-(dimethylamino)-1-propylamine allowed to obtain

Key: a) NaH, DMF b) Trifluoroacetic acid, CH_2Cl_2 c) Succinic anhydride, $CHCl_3$ d) Benzotriazol-1-yloxy tris(dimethylamino)phosphonium hexafluorophosphate, 1-hydroxybenzotriazole, DMF, N-methylmorpholine then Py-Py-Py-β-Pam-resin e) 3-Dimethylamino-1-propylamine f) $CuCl_2$

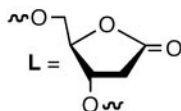**Scheme 3.** Synthesis of mono-phenanthroline distamycin conjugate **2**.

5'-d(GTA GTT TAT CAC A₁₃GT TAA ATT GC₂₃T₂₄ AAC₂₇ GCA GTC AG)
(CAT CAA ATA GTG T CA ATT TAA C G   A TT G   CGT CAG TC)

7

5'-d(GTA GTT TAT CAC **L**GT TAA ATT GCT AAC GCA GTC AG)
(CAT CAA ATA GTG TCA ATT TAA CGA TTG CGT CAG TC)

8

Figure 2. Structures of DNA duplex substrates.

Table 1. Rate constants for oxidation (k_{Ox}) at A_{13} of **7** and 2-deoxyribonolactone elimination (k_{Elim}) from **8** by 3-substituted phenanthroline distamycin conjugates (**1, 2**)[a]

Conjugate	Reductant	k_{Ox} (s⁻¹)	k_{Elim} (s⁻¹)
1	MPA	$4.9 \pm 0.9 \times 10^{-5}$	$5.6 \pm 0.7 \times 10^{-4}$
1	Asc	$4.1 \pm 0.6 \times 10^{-5}$	$4.9 \pm 1.2 \times 10^{-4}$
2	MPA	$2.6 \pm 1.5 \times 10^{-6}$	$1.4 \pm 0.3 \times 10^{-4}$
2	Asc	$1.1 \pm 0.4 \times 10^{-5}$	$1.9 \pm 0.8 \times 10^{-4}$

[a]Reactions were carried out using MPA (2 mM) or Asc (1 mM), **7** or **8** (1 μM), and **1** or **2** (20 μM).

phenanthroline distamycin conjugate **6** as a trifluroacetate salt due to its HPLC purification. Conjugate **6** was then incubated with one equivalent of $CuCl_2$ immediately prior to its reaction with DNA to give **2** (Scheme 3).

A comparison of DNA oxidation between mono- (2) and bis-phenanthroline conjugates (1)

DNA oxidation by distamycin conjugates **1** and **2** was examined using a synthetic duplex (**7**, Figure 2), which was previously employed to characterize the chemistry of **1** (17,20). Rate constants for the overall oxidation of **7** (pH 7.0) at individual nucleotides by **1** or **2** were determined by removing aliquots as a function of time. The aliquots were treated with NaOH (0.1 M, 37°C, 20 min) prior to separating the products by denaturing gel electrophoresis (PAGE). These conditions are known to completely cleave DNA containing abasic sites, as well as C1′- and C4′-oxidized abasic sites (49). Nucleotide A_{13} is the major cleavage site, as previously reported (Figure 3) (17,20). C_{27} is the second most frequently damaged nucleotide in **7** by **1**. However, the rate constant for oxidation (k_{Ox}) at A_{13} ($4.9 \pm 0.9 \times 10^{-5}$ s⁻¹, Table 1) by **1** in the presence of MPA is more than 30 times greater than that at C_{27} ($1.4 \pm 0.6 \times 10^{-6}$ s⁻¹). In addition, the rate constant for oxidation by **1** is not strongly affected by the choice of reductant and the cleavage pattern is the same (Table 1 and Figure 3).
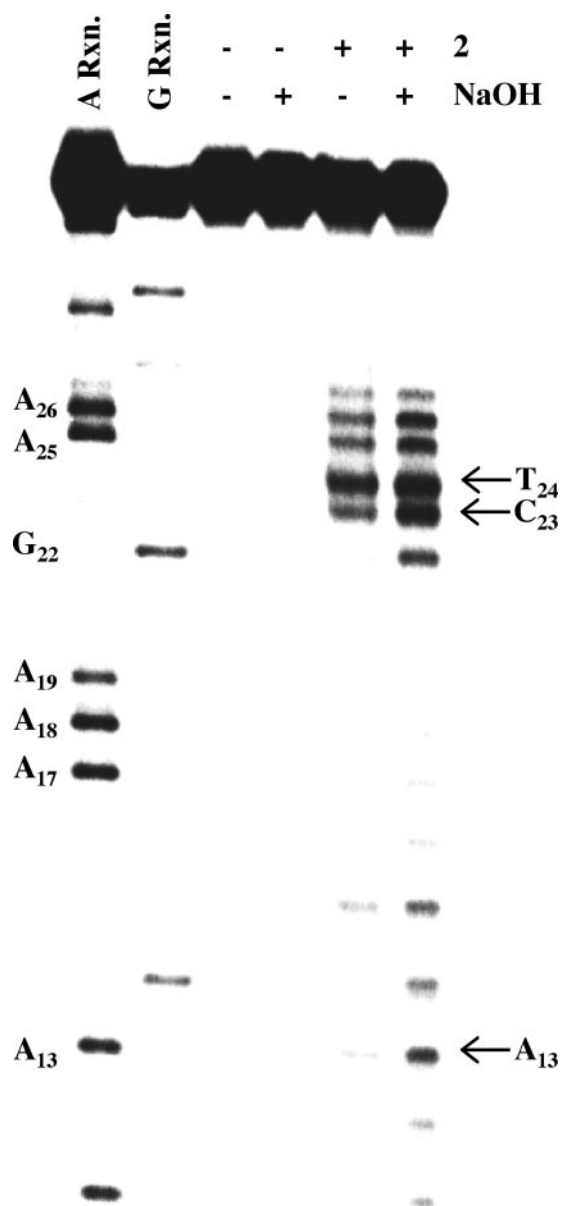
Oxidation of **7** by mono-phenanthroline conjugate **2** is qualitatively and quantitatively distinct from that induced by **1**. Distamycin conjugate **2** is far less selective or efficient than bis-phenanthroline conjugate **1** at oxidatively cleaving substrate **7**, and background cleavage is significantly higher when **2** is used (Figure 4). Nucleotide A_{13} is still a major site of damage by **2**, but the rate constant for oxidative cleavage at this position when MPA is employed is ~20-fold slower
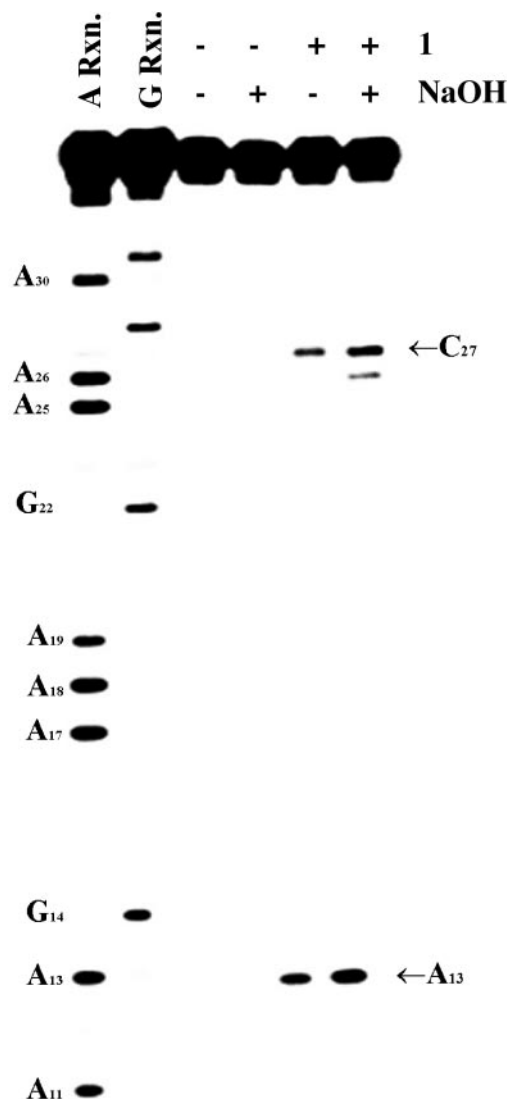
Figure 3. Autoradiogram of **7** treated with **1**. Sequencing reactions: A Rxn. and G Rxn. (47,58). Reactions were carried out using **1** (20 μM), **7** (1 μM), MPA (2 mM) in Tris buffer (pH 7.0, 10 mM), NaCl (50 mM) at 37°C for 30 min. NaOH (0.1 M) was carried out at 37°C for 20 min. Note: band intensities are presented in nonlinear mode.

than by **1** (Table 1). Furthermore, the rate constants describing oxidation at C_{23} and T_{24} are comparable with that at A_{13} (Table 2). Conjugates **1** and **2** respond differently to changes in reductants as well. Although the cleavage pattern is the same, oxidation of **7** by the mono-phenanthroline conjugate (**2**) is ~3- to 4-fold faster when ascorbate is used instead MPA. However, under these conditions the rate constant for oxidation by **2** is still ~4-fold slower than that by the bis-phenanthroline conjugate (**1**) at their common site of cleavage (A_{13}).

These data are consistent with independent observations reported by Rill and Sigman (7,43). We propose that the lower rate constant for oxidation by the mono-phenanthroline complex (**2**) in the presence of MPA compared to when Asc is used is due to a change in the thermodynamics for oxygen reduction when the thiol carboxylic acid coordinates to copper. A sulfur ligand(s) stabilizes the cuprous

Figure 4. Autoradiogram of **7** treated with **2**. Sequencing reactions: A Rxn. and G Rxn. (47,58). Reactions were carried out using **2** (20 μM), **7** (1 μM), MPA (2 mM) in Tris buffer (pH 7.0, 10 mM), NaCl (50 mM) at 37°C for 30 min. NaOH (0.1 M) was carried out at 37°C for 20 min. Note: band intensities are presented in nonlinear mode.

Table 2. Rate constants for oxidation (k_{Ox}) of **7** by mono-phenanthroline distamycin conjugate (**2**)[a]

	Observed rate constant (s^{-1})	
Position	MPA	Asc
A_{13}	$2.6 \pm 1.5 \times 10^{-6}$	$1.1 \pm 0.4 \times 10^{-5}$
C_{23}	$3.8 \pm 1.4 \times 10^{-6}$	$1.3 \pm 0.1 \times 10^{-5}$
T_{24}	$5.3 \pm 2.2 \times 10^{-6}$	$1.5 \pm 0.3 \times 10^{-5}$

[a]Reactions were carried out using MPA (2 mM) or Asc (1 mM), **7** (1 μM), and **2** (20 μM).

mono-phenanthroline complex (**2**), even when ascorbate is used. Although we attribute the kinetic differences primarily to varying redox potentials of the copper complexes, we cannot rule out any contribution due to structural differences between **1** and **2**. For instance, ligand variation may give rise to differences in the effective molarity of the oxidants in the two complexes when they are delivered to the minor groove as their distamycin conjugates. However, the rate constants for elimination of 2-deoxyribonolactone (**L**, Scheme 1) described below show that accessibility of the DNA cannot be a major factor.

In addition, although we cannot rule out that oxidation is further complicated due to dimeric binding by **1** and/or **2**, this is deemed unlikely. Distamycin molecules bind in an anti-parallel manner (52). Hence, dimeric binding of **2** does not enable it to form a bis-phenanthroline copper complex. Formation of a bis-phenanthroline complex requires a head-to-head orientation of the distamycin conjugates (**2**). This is unlikely, as it requires the distamycin molecules to bind in the minor groove of **7** with its phenanthroline components oriented toward the center of the extended A-T region, where oxidation by **2** is not observed (Figure 4).

Experiments using exogenous H_2O_2 also argue against steric effects as a major cause for reactivity differences between **1** and **2**. DNA oxidation by **2** in the presence of MPA increased linearly with H_2O_2 concentration (Figure 5). Furthermore, the observed first order rate constant for oxidation by **2** when MPA (2 mM) was used as reductant increased more than 5-fold in the presence of 0.1 mM H_2O_2 (Table 3). The rate constant is slightly greater than that measured for oxidation of **7** by mono-phenanthroline **2** when ascorbate is used as reductant in the absence of exogenous H_2O_2. Exogenous H_2O_2 has a similar effect on DNA oxidation by **2** when ascorbate is the reductant (data not shown). These observations are consistent with the mechanism outlined in Scheme 2 and suggest that the formation of the activated copper species responsible for DNA oxidation is the rate-determining step in the process (12).

Ligand effects on DNA cleavage

Distamycin conjugates **1** and **2** differ predominantly by the number of phenanthroline ligands bonded to the tripeptide's N-terminus. As described above, the conjugates exhibit qualitatively distinct cleavage patterns, with A_{13} of **7** being a major oxidation site for both conjugates. Duplex DNA (**8**, Figure 2) containing 2-deoxyribonolactone (**L**) at the original location of A_{13} was synthesized in order to determine the rate constant for copper–phenanthroline induced cleavage (k_{Elim}, Scheme 1) via this lesion (20,44,45). The rate constants for cleavage of

complex more than does a second phenanthroline ligand (50). Consequently, it is more difficult for O_2 to oxidize the Cu^I complex and the concomitant production of superoxide is slower (51). Superoxide is necessary for the formation of hydrogen peroxide, which reacts with the recycled Cu^I complex to ultimately produce the DNA oxidant (Scheme 2). The slower rate of superoxide, and consequently H_2O_2 formation, results in the lower observed k_{Ox} for **2** compared with **1**. Much of this difference is overcome by changing the reductant to ascorbate, although it is not known how copper is complexed under these conditions. As noted, the observed rate constant for oxidation at A_{13} by **1** is ~4-fold greater than that by the

Figure 5. Effect of H_2O_2 concentration on DNA oxidation by mono-phenanthroline distamycin conjugate **2** at position A_{13} in **7**. Reactions were carried out using **2** (20 µM), **7** (1 µM), MPA (2 mM) in Tris buffer (pH 7.0, 10 mM), NaCl (50 mM) at 37°C for 30 min, followed by NaOH (0.1 M) at 37°C for 20 min.

Table 3. Effect of H_2O_2 on oxidation of **7** at A_{13} (k_{Ox}) by mono-phenanthroline conjugate **2**[a]

	Observed rate constant (s^{-1})	
Reductant	$[H_2O_2]$ = 0.0 mM	$[H_2O_2]$ = 0.1 mM
MPA	$2.6 \pm 1.5 \times 10^{-6}$	$1.5 \pm 0.2 \times 10^{-5}$
Asc	$1.1 \pm 0.4 \times 10^{-5}$	$3.7 \pm 0.4 \times 10^{-5}$

[a]Reactions were carried out using MPA (2 mM) or Asc (1 mM), **7** (1 µM), and **2** (20 µM).

2-deoxyribonolactone by **1** or **2** (Figure 6) show little if any dependence upon reductant (mercaptopropionic acid, ascorbate) (Table 1). However, in the presence of the same reductant bis-phenanthroline conjugate **1** induces cleavage ~2–4 times faster than **2**. This is a much smaller difference than observed for the respective rate constants for DNA oxidation (k_{Ox}, Scheme 1) by **1** and **2**, supporting the proposal (above) that the differences in k_{Ox} are not due to steric or binding effects.

How do copper–phenanthroline conjugates induce cleavage of DNA containing 2-deoxyribonolactone?

The rate constants for oxidation by the copper–phenanthroline distamycin conjugates vary widely, and for **2** the reductant plays a significant role. In contrast, the rate constants describing elimination at 2-deoxyribonolactone (**L**) by **1** and **2** span only a 4-fold range, and they are ~100-fold greater than in the absence of the copper complexes. The orientation of the copper complex upon conjugate binding to DNA is one parameter that could explain such a modest modulation in the rate constant for cleavage, but this does not address how the copper complexes induce DNA cleavage at **L**.
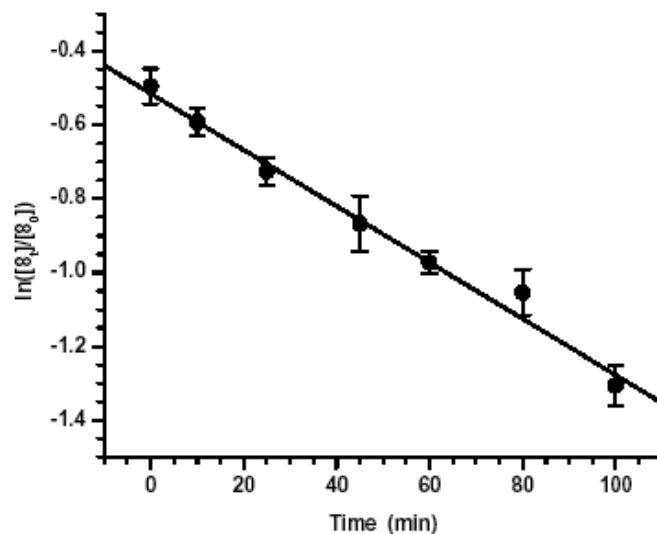
Figure 6. Disappearance of **8** as a function of time in the presence of **2** (20 µM), **8** (1 µM), MPA (2 mM), in Tris buffer (pH 7.0, 10 mM), NaCl (50 mM).

Model studies showed that Cu(OP)$_2$ catalyzes β-elimination from a 2-deoxyribonolactone (**L**) molecule containing a phosphate diester at the C3-position (21). These data do not allow us to determine whether the activated copper oxo (hydroxyl) complex that effects initial oxidation is also responsible for cleavage. However, related experiments of others lead us to favor this explanation. Using gas phase measurement of α-proton acidity in butyrolactone as a guide, we estimate that the pK_a of **L** is ~22 (53). Hence, regardless of the structure of the copper species responsible for elimination, it (they) must significantly lower the pK_a of the lactone. The pK_a of a copper-bound hydroxyl is typically between 7 and 8 when the metal is coordinated to phenanthroline or related nitrogenous ligands (54,55). We suggest that the metal plays an active role in reducing the pK_a of 2-deoxyribonolactone such that the elimination can proceed at pH 7. Recent studies indicate that copper complexes facilitate 1,3-diketone enolate formation by coordinating to the carbonyl oxygen (56). Mononuclear copper complexes reduced the pK_as of these ketones by as much as 12 units in acetonitrile. Although we cannot rule out an interaction between the copper complex and the phosphate leaving group, model studies showed that Cu(OP)$_2$ did not facilitate solvolysis of an allylic phosphate (21,57). Consequently, we believe that oxygen coordination, as demonstrated by Anslyn, is the most likely explanation for the β-elimination from 2-deoxyribonolactone (**L**) in DNA by copper–phenanthroline conjugates acting as general base catalysts.

CONCLUSIONS

Independent kinetic analysis of DNA oxidation and cleavage steps by mono- and bis-phenanthroline copper conjugates of distamycin reveals that the different ligand environments produce copper complexes capable of effecting similar transformations, but the reactive species are not identical. To our knowledge, this is the first kinetic comparison of mono- and bis-phenanthroline complexes. In each case, the major pathway for strand scission by these DNA-binding molecules

is believed to involve formation of the alkali-labile lesion, 2-deoxyribonolactone (**L**), as an intermediate. The rate constants for decomposition of this intermediate are considerably greater than the rate constants that describe their formation. The copper center in these complexes is proposed to induce β-elimination from 2-deoxyribonolactone despite the high pK_a of the α-proton, by coordinating to the carbonyl oxygen of the substrate.

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