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Two New Ternary Complexes of Copper(II) with Tetracycline or Doxycycline and 1,10-Phenanthroline and Their Potential as Antitumoral: Cytotoxicity and DNA Cleavage

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Supporting Information

ABSTRACT: This paper reports on the synthesis and characterization of two new ternary copper(II) complexes: [Cu(doxycycline)(1,10-phenanthroline)(H_2O)(ClO_4)](ClO_4) (**1**) and [Cu(tetracycline)(1,10-phenanthroline)(H_2O)(ClO_4)](ClO_4) (**2**). These compounds exhibit a distorted tetragonal geometry around copper, which is coordinated to two bidentate ligands, 1,10-phenanthroline and tetracycline or doxycyline, a water molecule, and a perchlorate ion weakly bonded in the axial positions. In both compounds, copper(II) binds to tetracyclines via the oxygen of the hydroxyl group and oxygen of the amide group at ring A and to 1,10-phenanthroline via its two heterocyclic nitrogens. We have evaluated the binding of the new complexes to DNA, their capacity to cleave it, their cytotoxic activity, and uptake in tumoral cells. The complexes bind to DNA preferentially by the major groove, and then cleave its strands by an oxidative mechanism involving the generation of ROS. The cleavage of DNA was inhibited by radical inhibitors and/or trappers such as superoxide dismutase, DMSO, and the copper(I) chelator bathocuproine. The enzyme T4 DNA ligase was not able to relegate the products of DNA cleavage, which indicates that the cleavage does not occur via a hydrolytic mechanism. Both complexes present an expressive plasmid DNA cleavage activity generating single- and double-strand breaks, under mild reaction conditions, and even in the absence of any additional oxidant or reducing agent. In the same experimental conditions, $[Cu(phen)_2]^{2+}$ is approximately 100-fold less active than our complexes. These complexes are among the most potent DNA cleavage agents reported so far. Both complexes inhibit the growth of K562 cells with the IC_{50} values of 1.93 and 2.59 μmol L^{-1} for compounds **1** and **2**, respectively. The complexes are more active than the free ligands, and their cytotoxic activity correlates with intracellular copper concentration and the number of Cu-DNA adducts formed inside cells.

INTRODUCTION

The central role of DNA in replication, transcription, and regulation of genes has prompted the search for artificial nucleases, catalysts able to cleave the DNA molecule. The development of small molecules capable of catalyzing DNA hydrolysis at physiological conditions is of great importance for biotechnological applications and the development of novel therapeutic agents. DNA is an important target of antitumoral drugs, and different types of interactions are possible, such as intercalation between base-pairs (such as doxorubicin or daunorubicin), minor (such as netropsin, berenil and pyrrole−imidazole polyamides) or major groove binding (such as triplex-forming oligonucleotides and cisplatin), or catalysis of DNA cleavage through hydrolytic or oxidative reactions. Transition metal complexes (especially of essential iron and copper) in their reduced oxidation state can promote the formation of free radicals through Haber−Weiss or Fenton reactions that can

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oxidize several biomolecules.[1] An example is the antitumor agent bleomycin which generates radical species that abstract a hydrogen from the sugar moiety and cleave the DNA molecule.[2]

Several copper complexes were also described as cleaving DNA, and the best studied example is $[Cu(phen)_2]^{2+}$ (phen =1, 10-phenanthroline), whose nuclease activity was first reported by Sigman in 1979.[3] $[Cu(phen)_2]^{2+}$ is reduced in situ to $[Cu(phen)_2]^{+}$, which subsequently binds to the minor groove of DNA, combines with molecular oxygen, generates a nondiffusible oxidant, and finally induces strand scission by oxidation of the ribose backbone.[4] Much effort has been undertaken by several research groups to design nucleases based upon copper complexes of phenanthrolines and other heteroatomic ligands.[5−7]

Certain copper complexes that break DNA strands by an oxidative pathway were reported to be cytotoxic against tumor cells.[8−13] Recently, we have described the cytotoxic activity of some dinuclear copper compounds that hydrolytically cleave double-strand plasmid DNA.[14,15]

Tetracyclines in combination with copper(II) ions exhibit significant DNA damage potential.[16] Our research group also showed that platinum and palladium(II) complexes of tetracyclines have antitumor activity.[17,18]

Metal synthetic nucleases can be useful in the treatment of cancer because they can block genetic expression. An inconvenience is that usually the cleavage of DNA requires the concomitant addition of an external reagent, such as thiols and hydrogen peroxide. In this work, we report the synthesis, characterization both in solution and in the solid state, cellular uptake, DNA binding and cleavage, and cytotoxic activity of two novel copper(II) complexes of 1,10-phenanthroline and doxycycline or tetracycline. The compounds present an expressive cleavage of DNA in mild conditions and in the absence of additional agents.

EXPERIMENTAL SECTION

Materials and Methods. Doxycycline hydrochloride (dox) and tetracycline hydrochloride (tc) were purchased from Sigma Co. Stock solutions were prepared just before use to avoid ligand degradation caused by oxygen and light. Copper(II) perchlorate hexahydrate and 1,10-phenanthroline monohydrate were purchased from Aldrich and Synth, respectively. All other chemicals were reagent-grade and were used without further purification.

Calf thymus DNA sodium salt (CT DNA) was used as obtained from Sigma Co. DNA was extracted from K562 cells by using a kit from Sigma (Sigma's GenElute Mammalian Genomic DNA Miniprep Kit).

The plasmid pBSK II (2961 bp), used for DNA cleavage assays, was purchased from Stratagene, transformed into DH5α *Escherichia coli* competent cells, and amplified as previously described.[19] The plasmid DNA was extracted from *E. coli* and purified using Qiagen Plasmid Maxi Kit protocol.[20]

Syntheses of the Complexes. Two novel complexes of copper(II) with tetracycline or doxycycline and 1,10-phenathroline were synthesized using the same general procedure, and below only the synthesis of the complex of doxycycline and phenanthroline, complex **1**, is described.

Complex **1** was prepared by the reaction of $Cu(ClO_4)_2 \cdot 6H_2O$ (0.1853 g, 0.5 mmol) with doxycycline (0.2405 g, 0.5 mmol) in water (10 mL). The mixture was stirred for 40 min, followed by the addition of a methanolic solution (5 mL) of 1,10-phenanthroline monohydrate (0.0996 g, 0.5 mmol), and the mixture was stirred for more three hours. The green precipitate was filtered, washed with water, and dried under vacuum.

$[Cu(dox)(phen)(H_2O)(ClO_4)](ClO_4)$ (**1**). IR (KBr) ν_{max}/cm^{-1}: 3372, 3074, 2928, 1615, 1580, 1519, 1455, 1430, 1324, 1244, 1216, 1140, 1121, 1108, 1087, 1037, 946, 850, 826, 721, 626. Yield: 0.2655 mg, 59%. Anal. Calcd for $[Cu(C_{22}H_{24}N_2O_8)(C_{12}H_8N_2)(H_2O)(ClO_4)](ClO_4)$ (MM = 905.11 g mol^{-1}): C, 45.12; H, 3.79; N, 6.19; Cu, 7.02%. Found: C, 45.00; H, 3.82; N, 6.19; Cu, 6.98%. Color: Green.

$[Cu(tc)(phen)(H_2O)(ClO_4)](ClO_4)$ (**2**). IR (KBr) ν_{max}/cm^{-1}: 3401, 3081, 2926, 1618, 1580, 1520, 1455, 1430, 1346, 1281, 1227, 1144, 1121, 1108, 1087,1038, 948, 850, 827, 722, 626. Yield: 0.2761 mg, 61%. Anal. Calcd for $[Cu(C_{22}H_{24}N_2O_8)(C_{12}H_8N_2)(H_2O)(ClO_4)](ClO_4)$ (MM = 905.11 g mol^{-1}): C, 45.12; H, 3.79; N, 6.19; Cu, 7.02%. Found: C, 45.63; H, 4.01; N, 6.49; Cu, 7.12%. Color: Green.

Caution! *Perchlorate salts with organic ligands are potentially explosive and should be handled in small quantities with care.*

Spectroscopic Measurements. Infrared spectra were recorded over the region 400−4000 cm^{-1} with a Perkin−Elmer 283 B spectrometer. The samples were examined in KBr pellets.

A Cary100 Varian spectrometer was used for UV and visible absorption measurements. The ligand and complex concentrations used were 2.0×10^{-5} mol L^{-1}. 1,10-Phenanthroline monohydrate was dissolved in a minimum amount of methanol and diluted in water to obtain a final concentration of 2.0×10^{-5} mol L^{-1}. For the interactions with CT DNA, the complex concentration used was 2.0×10^{-5} mol L^{-1} and the DNA concentration varied from 0 to 3×10^{-4} mol L^{-1}. The DNA concentration per nucleotide was determined by the $\varepsilon = 6600$ L mol^{-1} cm^{-1} at 260 nm. The ionic strength was maintained constant with 1×10^{-3} mol L^{-1} NaCl, and the pH was fixed at 7.3 with 20 mmol L^{-1} HEPES buffer. The absorbance of DNA itself was subtracted by adding an equal quantity of DNA to both the complex solution and the reference solution.

Diffuse reflectance spectra were obtained on a Shimadzu UV-2401 PC spectrophotometer. The samples were examined in $BaSO_4$ pellets.

Full scan mass spectra (MS mode) were obtained on a MicroTOF LC Bruker Daltonics spectrometer equipped with an electrospray source operating in positive ion mode. Samples were dissolved in a MeOH/H_2O (50/50) solution and were injected in the apparatus by direct infusion.

EPR spectra were registered on a Bruker EMX instrument, operating at the X-band frequency (9.49 GHz), 100 kHz modulation frequency, and 20 mW power, with standard Wilmad quartz tubes, at 77 K. DPPH (α,α′-diphenyl-β-picrylhydrazyl) was used as the frequency calibrant (g = 2.0036). Spectra were registered for the complexes dissolved in (4:1, v/v) MeOH/H_2O, using 15G modulation amplitude.

Conductivity Measurements. Conductivity measurements were carried out with a Digimed DM 31 conductivity meter using a cell of constant 1.013 cm^{-1}. The solvent used was spectroscopic grade nitromethane (Merck) (Λ_M = 8.80 μS cm^{-1}), and tetramethylammonium bromide (Λ_M = 102.20 μS cm^{-1}) was used as standard.

Elemental Analyses. Carbon, nitrogen, and hydrogen were determined on a Perkin-Elmer 2400 CHN. Copper content was determined by atomic absorption on a Hitachi spectrophotometer model 8200.

Cells and Culture. The K562 cell line was purchased from the Rio de Janeiro Cell Bank (number CR083 of the RJCB collection). This cell line was established from pleural effusion of a 53 year-old female with chronic myelogenous leukemia in terminal blast crisis. Cells were cultured in RPMI 1640 (Sigma Chemical Co.) medium supplemented with 10% fetal calf serum (CULTILAB, São Paulo, Brazil) at 37 °C in a humidified 5% CO_2 atmosphere. Cultures grow exponentially from 10^5 cells mL^{-1} to about 8×10^5 cells mL^{-1} in three days. Cell viability was checked by Trypan Blue exclusion. The cell number was determined by Coulter counter analysis.

For cytotoxicity assessment, 1×10^5 cells mL^{-1} were cultured for 72 h in the absence and the presence of a range of concentrations of tested compounds. The sensitivity to compound was evaluated by the

concentration that inhibits cell growth by 50%, IC_{50}. Stock solutions of the compounds were prepared in water.

Cellular Accumulation. K562 cells (1×10^5 cells mL^{-1}) were incubated for 72 h in the absence and presence of a range of concentrations of the copper compound. After incubation, an aliquot was taken and washed three times with ice-cold isotonic buffer, and the pellet was resuspended in 33% HNO_3. Copper concentration was determined by GFAAS in a Varian model Zeeman 220 spectrophotometer equipped with a graphite tube atomizer and an autosampler. The total copper concentration in cells determined in the absence of added compound was subtracted from the samples incubated with copper(II) compounds.

DNA Extraction. K562 cells (3×10^6) were incubated with different concentrations of the complexes ranging from 2 to 12 μM for 2 h. After this period, DNA was extracted from cells by using a kit from Sigma (Sigma's GenElute Mammalian Genomic DNA Miniprep Kit), as previously reported.[15] DNA concentration per nucleotide was determined by spectrophotometric analysis (ε = 6600 L mol $^{-1}$ cm^{-1} at 260 nm). The ratio of absorbance at 260 to 280 nm was between 1.6 and 1.9. Copper concentration was determined by GFAAS.

Plasmid DNA Cleavage. The DNA cleavage ability of the complexes **1** and **2** was examined following the conversion of pBSK II supercoiled DNA (F I) to the open circular (F II) and linear DNA (F III) using agarose gel electrophoresis to separate the cleavage products.[21,22] In general, 400 ng of pBSK II DNA (~30 μmol L^{-1} bp) in 10 mmol L^{-1} Tris-HCl pH 7.4 buffer were treated with different concentrations of complex **1** and **2** for 4 h at 37 °C. All the assays were conducted including a reaction control (without complex) to serve as a reference of spontaneous plasmid DNA fragmentation. Thereafter, each reaction was quenched by adding 5 μL of a loading buffer solution (50 mmol L^{-1} Tris-HCl pH 7.5, 0.01% bromophenol blue, 50% glycerol, and 250 mmol L^{-1} EDTA) and then subjected to electrophoresis on a 0.8% agarose gel containing 0.3 μg mL^{-1} of ethidium bromide in 0.5 × TBE buffer (44.5 mmol L^{-1} Tris pH 8.0, 44.5 mmol L^{-1} boric acid, and 1 mmol L^{-1} EDTA) at 90 V for 1.5 h. The resulting gels were visualized and digitized using a DigiDoc-It gel documentation system (UVP). The proportion of plasmid DNA in each band was quantified using Kodak Molecular Imaging Software 5.0 (Carestream Health). The quantification of supercoiled DNA (F I) was corrected by a factor of 1.47, since the ability of ethidium bromide to intercalate into this DNA topoisomeric form is decreased relative to open circular and linear DNA.[23]

In order to investigate the contribution of electrostatic interactions in the plasmid DNA cleavage promoted by complexes **1** and **2**, assays were conducted as described above but with an increase in the ionic strength of the reaction media by the addition of NaCl (from 50 to 300 mmol L^{-1}). To elucidate the plasmid DNA cleavage mechanism performed by **1** and **2**, different cleavage interfering agents were added to the reaction media prior to the complex. The reactive oxygen species (ROS) scavengers DMSO (10%), KI (10 mmol L^{-1}), superoxide-dismutase (SOD, 15 units), and NaN_3 (10 mmol L^{-1}) were used in order to identify the involvement of ROS in the plasmid DNA scission event promoted by **1** and **2**. In addition, assays in the presence of a copper(I) stabilizer, bathocuproine (BC, 0.5 mmol L^{-1}), were performed. Assays in the presence of the DNA minor groove binder, distamycin[24] (50 μmol L^{-1}), and the DNA major groove binder, methyl green[25] (50 μmol L^{-1}), were also performed to clarify the DNA groove binding preference of the complexes. The plasmid DNA was pretreated with distamycin and methyl green for 30 min and then treated with the complex as described above.

The number of single-strand breaks (n_1) and double-strand breaks (n_2) per plasmid molecule promoted by **1** and **2** were determined from gel quantification based on the Freifelder–Trumbo relationship.[26,27] The value of n_1 was obtained quantifying the amount of the supercoiled DNA expressed in $f_I = \exp[-(n_1 + n_2)]$, where f_I is the fraction of supercoiled DNA and n_1 and n_2 are the number of single- and double-strand breaks, respectively. The value of n_2 was obtained quantifying the amount of the linear DNA expressed in $f_{III} = n_2 \exp(-n_2)$, where f_{III} is the fraction of DNA in the linear form and n_2 is the number of single- and double-strand breaks. Fifteen plasmid DNA cleavage full data sets containing simultaneously the three forms of the fragmented plasmid were selected for calculations.

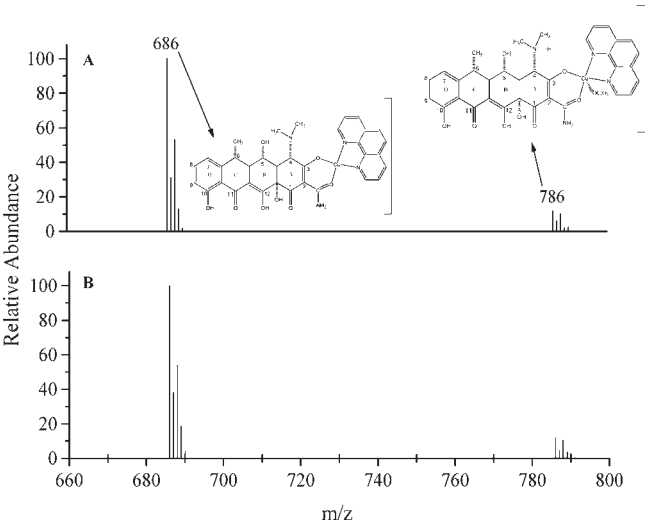

Figure 1. ESI spectrum of complex **1** in a MeOH/H_2O (50/50) solution (A). Isotopic distribution calculated for the species $[Cu(dox)(phen)\text{-}H^+]^+$ and $[Cu(dox)(phen)(ClO_4)]^+$ (B) (Qual Browser version 2.0.7, Thermo Fischer Scientific Inc. 1998–2007).

The kinetics of plasmid DNA cleavage performed by **1** and **2** were evaluated following the loss of supercoiled DNA fraction along the reaction time under pseudo-first-order conditions. The apparent plasmid DNA cleavage rates (k_{obs}) were obtained from the plot of ln-[supercoiled DNA (%)] versus time. The reaction conditions were identical to those described above.

DNA Ligation by T4 DNA Ligase. DNA (1 μg) was treated with the complexes (100 μM) for 3 h in Tris-HCl (10 mM, pH 7.4) and submitted to agarose gel electrophoresis as described previously. The DNA in linear form (F III) was excised from the gel and purified using Wizard SV Gel and PCR Clean-Up Kit (Promega). Then, 200 ng of linearized DNA was treated with 2 units of T4 DNA ligase (Promega) for 16 h at 15 °C in a final volume of 20 μL containing 2 μL of ligation buffer. After treatment, the samples were again submitted to agarose gel electrophoresis. Control assays were conducted with the restriction enzyme *Bam*HI (Promega) using manufacturer procedures.

RESULTS AND DISCUSSION

Two novel copper(II) complexes of 1,10-phenanthroline and doxycycline or tetracycline were synthesized and characterized by elemental analysis, conductivity measurements, ESI-MS, EPR, and IR and UV–vis spectroscopies. The results of the elemental analyses are in accordance with the proposed formula: $[Cu(dox)(phen)(H_2O)(ClO_4)](ClO_4)$ (**1**) and $[Cu(tc)(phen)(H_2O)(ClO_4)](ClO_4)$ (**2**), in which tc and dox correspond to the same minimal formula, $C_{22}H_{24}O_8N_2$, and phen to $C_{12}H_8N_2$.

The molar conductivity values of 10^{-3} mol L^{-1} solutions of complexes **1** and **2** in nitromethane, at 25 °C, are Λ_M= 173.9 μS cm^{-1} and Λ_M= 187.9 μS cm^{-1}, respectively, indicating that they are 2:1 electrolytes.[28] This result indicates that, in solution, both perchlorates are acting as counterions.

The ESI-MS studies confirmed the formula proposed for complexes **1** and **2**. The ESI-MS spectra of both compounds in

positive mode give a main peak at m/z 686, in which the distribution of isotopologue ions is characteristic of a $^{63}Cu/^{65}Cu$-containing ion (2.2%), assigned to the singly charged form of either $[Cu(dox)(phen)\text{-}H^+]^+$ (Figure 1A) or $[Cu(dtc)(phen)\text{-}H^+]^+$ (Figure 2A) (calculated masses 686.14). In the spectrum of complex **2**, a peak at m/z 668 is also present and corresponds to the species m/z 686 minus a water molecule (calculated value 668.13) (Figure 2A). In both complexes, a minor peak, at m/z 785.9, exhibiting the isotopic envelopes of copper and chlorine, should correspond to the $[Cu(tc)(phen)(ClO_4)]^+$ or $[Cu(dox)\text{-}(phen)(ClO_4)]^+$ species (calculated masses 786.1). The isotopic distribution for the proposed species was calculated with the program Qual Browser (version 2.0.7, from the Thermo Fischer Scientific Inc.), and there is a good accordance with the experimental spectra (Figures 1B and 2B).

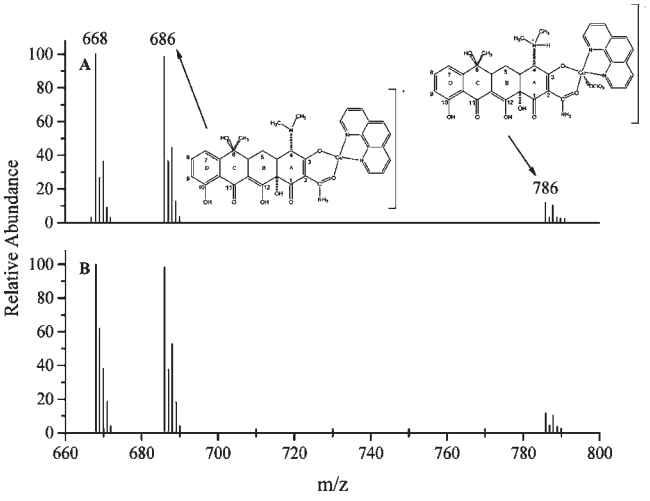

Figure 2. ESI spectrum of complex **2** in a $MeOH/H_2O$ (50/50) solution (A). Isotopic distribution calculated for the species $[Cu(tc)(phen)\text{-}H^+]^+$ and $[Cu(tc)(phen)(ClO_4)]^+$ (B) (Qual Browser version 2.0.7, Thermo Fischer Scientific Inc. 1998−2007).

In the solid state, the spectra of both complexes (diffuse reflectance) exhibit only one broad d−d band in the range 500−800 nm, centered at approximately 680 nm, which is typical of tetragonally distorted octahedral geometry (Figure S1). In aqueous solutions, this band shifts to higher energies appearing at 642 nm (ε = 51.9 L mol^{-1} cm^{-1}) for complex **1**, and at 637 nm (ε = 61.0 L mol^{-1} cm^{-1}) for complex **2** (Figure 3A,B), which suggests that the weakly bonded axial ligands are displaced by solvent molecules.

EPR spectra measured in frozen methanolic solutions of the complexes (Figure S2) are in agreement with a tetragonal or distorted octahedral geometry. Both complexes exhibit quite similar spectroscopic parameters, as expected, since the differences in environment around the copper ion are not remarkable (Table 1). The order $g_{\|} > g_{\perp}$ is consistent with axial symmetry typical of a d^9 configuration in fundamental state, with the unpaired electron in the $d_{x^2-y^2}$ orbital.[29] The region around 1500G does not exhibit copper(II) hyperfine coupling, attesting to the absence of magnetic interactions between copper atoms, which would be characteristic of a dimer or binuclear species. For copper(II) complexes, the empirical ratio $g_{\|}/A_{\|}$ is frequently

Table 1. EPR Parameters for Complexes 1 and 2

complex	$g_{\perp}$	$g_{\|}$	g_{iso}	$A_{\|}$ (G)	$A_{\|}$ (10^{-4} cm^{-1})	$g_{\|}/A_{\|}$ (cm)
1	2.063	2.271	2.132	172	183	124
2	2.063	2.274	2.132	171	183	124

Both complexes exhibited quite similar spectroscopic parameters, as expected, since the differences in environment around the copper ion are not remarkable.

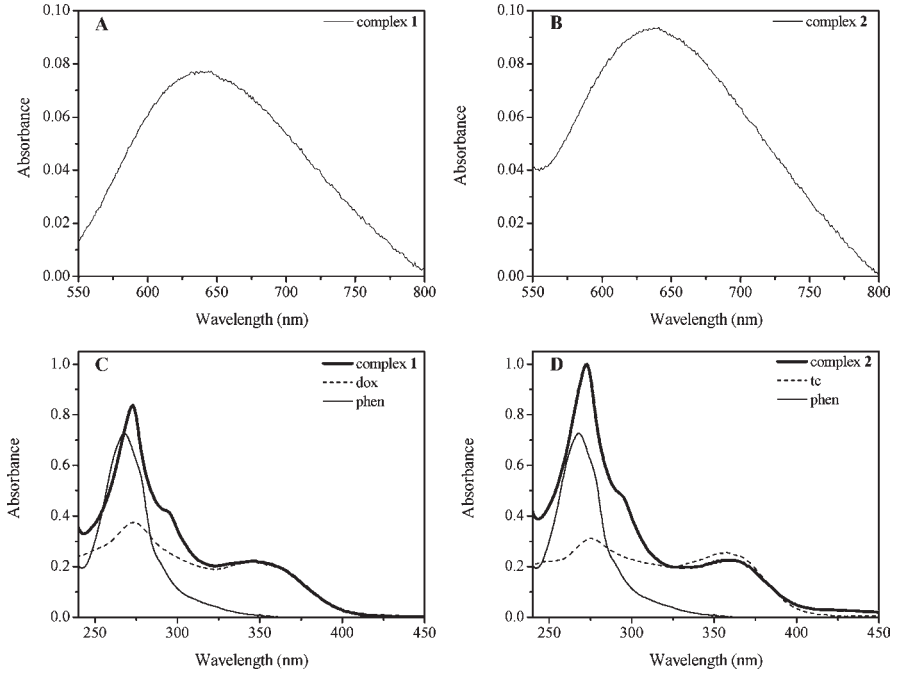

Figure 3. Electronic spectra of aqueous solutions of complex **1** (A) and complex **2** (B) at 1.5×10^{-3} mol L^{-1}; phen, dox and complex **1** (C), phen, tc, complex **2** (D) at 2×10^{-5} mol L^{-1}; l = 1 cm.

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used to evaluate tetrahedral distortions in tetragonal structures of Cu^{2+} compounds or active sites in copper proteins. Values between 105 and 135 cm are usually indicative of a quadratic geometry, while higher values point to more tetrahedrically distorted environments.[30] Both complexes show the ratio $g_{\|}/A_{\|}$ typical of square-based geometries (see Table 1).

Tetracycline and doxycycline molecules exhibit several potential metal-binding sites: oxygens at the C10−C12 phenolic β-diketone system, enolic oxygens, and the nitrogens at C4 and at the carboxamide group in ring A.[31,32] Copper(II) interactions with tetracycline have attracted the attention of many researchers, and there is no general agreement about the structure of the complexes formed.[33−39] Jezowska-Bojczuk et al.[33] studied copper(II)-tetracycline systems in aqueous solution by UV−vis and circular dichroism spectroscopies and proposed that three different sites can participate in the binding, depending on the pH: O3, O10−O12 system, and N4. According to these authors, in acidic medium, coordination takes place via O3 atom and at higher pH values, via the O10−O12 system. In the same work, participation of alternate bonds involving N4 and O10−O12 was proposed to explain the coordination mode of tetracycline in ML_2 complexes.

In order to assign the metal binding sites to tetracycline and doxycycline, we have analyzed the spectra in the UV−vis and IR regions.

The assignments of the infrared spectra were made on the basis of a detailed study of IR and vibrational assignments for tetracyclines and analogues made by Dziegielewski et al.[40]

The modifications observed in the spectra of both complexes are very similar, and only the spectrum of the copper complex of doxycycline and phenanthroline, complex **1**, will be discussed.

The amide I band, ν(C═O), which appears in the spectrum of doxycycline at 1670 cm^{-1}, cannot be distinguished in the spectrum of the complex, suggesting that the oxygen of the amide group is involved in the coordination sphere. Two very strong bands at 1615 and 1580 cm^{-1}, assigned to carbonyl stretching ν(C═O) on rings A and C, respectively, appear at the same wavenumber, ruling out the participation of carbonyl oxygens in the coordination. The behavior of the weakly coordinating perchlorate ion can be distinguished by the Cl−O stretching frequencies. The lowering of symmetry from T_d to C_{3v} upon coordination induces the splitting of ν_3 into two bands. Three intense bands at 1121, 1108, and 1087 cm^{-1} appear in the spectra of the complexes, which can be explained by the presence of one free and one unidentate perchlorate ion. The species containing one coordinated perchlorate have appeared in the mass spectra of the complexes.

The UV−vis absorption spectra of neutral aqueous solutions of doxycycline and tetracycline exhibit two main bands centered at 270 and 355 nm for doxycycline and 270 and 362 nm for tetracycline. According to McCormick et al.,[41] $\pi-\pi^*$ transitions associated to the tricarbonyl system on ring A contribute only to the band near 270 nm, while $\pi-\pi^*$ transitions of the BCD chromophore contribute to both the 270 and the 362 nm bands.[42] Phenanthroline also exhibits a band centered at about 265 nm. In Figure 3C,D, the spectra of aqueous solutions of the ligands and their respective copper(II) complexes are shown. In the complexes, the absorptions around 265 nm undergo a bathochromic shift, as a consequence of ligand coordination. The fact that the band around 360 nm, which originates only from the transitions of tetracycline BCD chromophore, remains at the same wavelength excludes the participation of BCD sites in the coordination sphere and points to copper(II) coordination at ring A sites. One possible bidentate mode is the oxygen at C_1 and the amide oxygen. The shift of the ν(C═O) mode associated with the amide group indicates that the oxygen of the amide group is involved in the coordination sphere. Nevertheless, the strong band at 1615 cm^{-1}, assigned to ν(C═O) mode of the carbonyl group at C_1, does not change upon Cu(II) coordination.

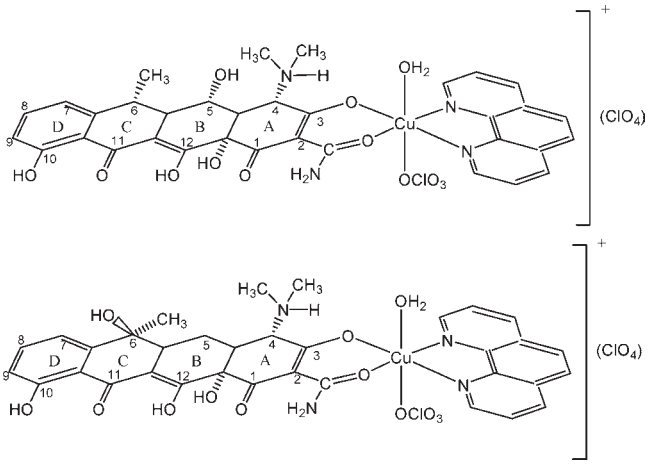

Figure 4. Proposed structures for complexes **1** (top) and **2** (bottom).

These results led us to propose that the compounds exhibit a distorted tetragonal geometry with two bidentate ligands, 1,10-phenanthroline and tetracycline or doxycyline, coordinated in the equatorial plane, a water molecule, and a perchlorate ion in the axial positions (Figure 4). In both compounds, copper(II) binds to tetracyclines via the oxygen of the hydroxyl group and oxygen of the amide group at ring A and with the 1,10-phenanthroline via its two heterocyclic nitrogens.

Spectrophotometric Studies of DNA Binding. We have registered the spectra of solutions of the complexes in the absence and in the presence of increasing concentrations of CT DNA. The addition of DNA induces a hypochromic effect and a minor hypsochromic shift (of approximately 1 to 5 nm), indicating that both Cu^{II} complexes form a ternary complex with calf thymus DNA. A representative experiment obtained with complex **2** at 2×10^{-5} mol L^{-1} and [DNA] varying from 0 to 3×10^{-4} mol L^{-1} is shown in Figure 5. In order to evaluate the binding strengths of the complexes, the binding constant, K, was calculated accordingly to the equation

$$[DNA]/(\varepsilon_a-\varepsilon_f) = [DNA]/(\varepsilon_0-\varepsilon_f) + 1/K(\varepsilon_0-\varepsilon_f)$$

in which [DNA] is the concentration of DNA in base pairs, ε_a is the ratio of the absorbance/[Cu], ε_f is the extinction coefficient of the free Cu^{II} complex, and ε_0 is the extinction coefficient of the complex in the fully bound form.[43] The ratio of slope to intercept in the plot of $[DNA]/(\varepsilon_a - \varepsilon_f)$ versus [DNA] gives the value of K (inset Figure 5). Complex **1** has a slightly lower affinity to DNA than complex **2** with K values of 2.02×10^4 and 2.95×10^4, respectively. These values are comparable to those reported to other copper(II) complexes with a 1,10-phenanthroline ligand (ranging from 10^2 to 10^5 M^{-1}).[44−51] Binding of a complex to DNA through intercalation usually results in hypochromism and strong bathochromic shift due to the intercalative binding involving a strong stacking interaction between the planar aromatic chromophore and the base pairs of DNA.[52] The changes in the spectra of complexes **1** and **2** caused by the

addition of DNA are quite different from this latter description, suggesting that the binding mode of the title complexes may be other than intercalative, i.e., by the DNA groove binding or by electrostatic interactions.

DNA Cleavage. The intact double-stranded plasmid DNA exists in a compact circular closed conformation, the supercoiled DNA. If only one DNA strand is cleaved, the supercoiled DNA form assumes a more relaxed conformation known as "open circular DNA", which may be converted into linear DNA, following a cleavage event on the complementary strand near the first cleavage site. These three plasmid DNA conformations are distinguishable when subjected to agarose gel electrorophoresis. Relatively fast migration is observed for supercoiled DNA (F I), while open circular DNA (F II) migrates slowly and the linear DNA (F III) migrates between F I and F III. Hence, the DNA cleavage can be defined by the conversion of the supercoiled DNA (F I, intact plasmid) into open circular and linear DNA (F II and F III, cleaved plasmid).[21,22]

Complexes **1** and **2** exhibit extensive plasmid DNA cleavage activity at 37 °C, pH 7.4 after 4 h of reaction, converting the plasmid supercoiled DNA into its cleaved forms (open circular and linear DNA). The DNA cleavage is clearly concentration-dependent with a similar pattern for both complexes (Figure 6.). Under this reaction condition, the plasmid DNA alone had a very small spontaneous cleavage (lane 1). However, in the presence of the complexes at 5 and 10 μmol L^{-1} (lanes 2 and 3), a significant amount of supercoiled DNA was converted to open circular DNA, as a result of single-strand breaks over the plasmid molecule. At 20 μmol L^{-1} (lane 4) a small amount of linear DNA becomes present in the gel, originated by DNA double-strand breaks. At the highest concentration assayed (40 μmol L^{-1}, lane 5), the supercoiled DNA was fully depleted, and finally, above 40 μmol L^{-1}, the plasmid DNA was converted into indistinguishable small fragments resulting in a smear in the gel (data not shown). Control experiments, in equal reaction conditions but replacing the complexes by $CuCl_2$, were carried out in parallel and no DNA cleavage was observed (Supporting Information Figure 3)

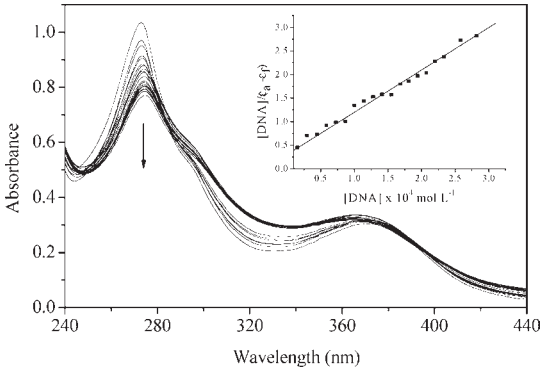

Figure 5. Spectra of solutions containing complex **2** (2×10^{-5} mol L^{-1}) and increasing concentrations of DNA from 0 to 3×10^{-4} mol L^{-1} in HEPES buffer pH 7.3, $I = 1 \times 10^{-3}$ mol L^{-1}. Inset: $[DNA]/(\varepsilon_a - \varepsilon_f)$ versus [DNA].

The influence of electrostatic properties of complexes **1** and **2** on the plasmid DNA cleavage was analyzed by the addition of increasing amounts of NaCl in a range 50−300 mmol L^{-1} (Figure 7) to the reaction medium. The spontaneous plasmid cleavage in the presence of NaCl was similar or even lower than the control reaction (lane 1). When NaCl is added, the proportion of supercoiled DNA increases and the cleaved DNA falls down, indicating a proportional decrease of the plasmid DNA cleavage promoted by the complexes following the gradual increase in ionic strength (compare lane 2 with lanes 3−6). This effect was somehow expected, because **1** and **2** behave as cations in solution, and the increase in salt concentration neutralizes negative charges in DNA, which decreases the electrostatic attraction between the complexes and DNA. This result suggests that electrostatic interactions may contribute for the DNA binding event.

The plasmid DNA cleavage assays in the presence of ROS scavengers showed similar results for both complexes and revealed that the DNA cleavage mechanism of **1** and **2** may be may be linked to formation of ROS (Figure 8). In the presence of DMSO (10%) and SOD (15 U), the plasmid DNA cleavage was fully inhibited (compare lane 2 with lanes 3 and 5). This suggests the participation of hydroxyl ($^{\bullet}OH$) and superoxide radicals ($O_2^{\bullet-}$) on the DNA breakage. The addition of NaN_3 (10 mmol L^{-1}), a singlet-oxygen ($^1O_2$) scavenger, did not affect the plasmid DNA cleavage promoted by **1** and **2** (compare lane 2 with 6). Surprisingly, the presence of KI (10 mmol L^{-1}) protects the plasmid DNA from scission by **2**, suggesting the participation of a copper-peroxide-type species [copper(I)-OOH] in the cleavage mechanism at least for this complex (compare lane 2

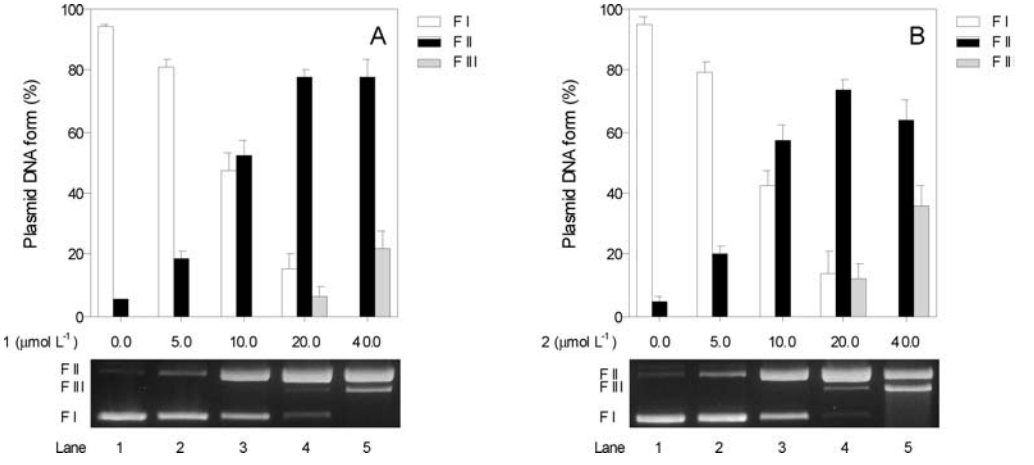

Figure 6. Cleavage of supercoiled DNA by **1** (A) and **2** (B) for 4 h at 37 °C in 10 mmol L^{-1} Tris-HCl (pH 7.4) and different concentrations of complex (5−40 μmol L^{-1}).

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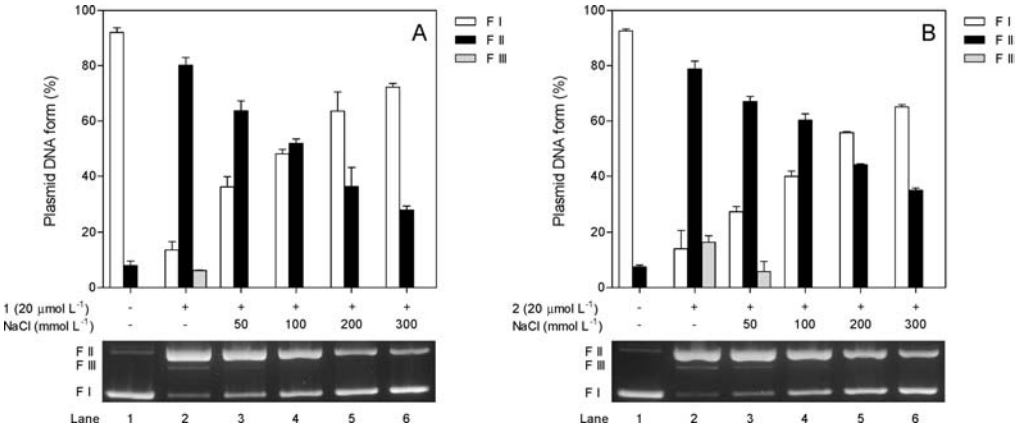

Figure 7. Effect of ionic strength on cleavage of supercoiled DNA by **1** (A) and **2** (B) at 20 μmol L^{-1} for 4 h at 37 °C in 10 mmol L^{-1} Tris-HCl (pH 7.4). Before the addition of the complexes, different concentrations of NaCl (0−300 mmol L^{-1}) were added to the reaction medium.

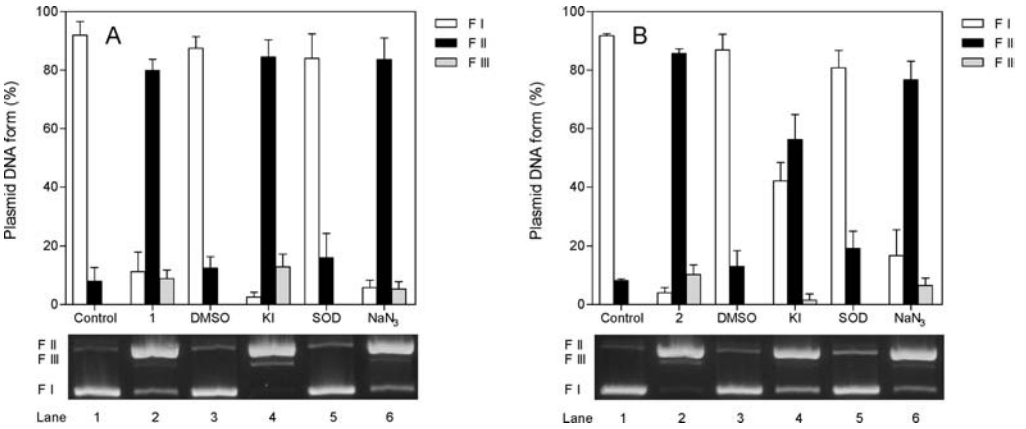

Figure 8. Effect of ROS scavengers on the cleavage of supercoiled DNA by **1** (A) and **2** (B) at 20 μmol L^{-1} for 4 h at 37 °C in 10 mmol L^{-1} Tris-HCl (pH 7.4). The ROS scavengers used were: DMSO (10%), KI (10 mmol L^{-1}), SOD (15 units), and NaN_3 (10 mmol L^{-1}). The controls (reaction without complex) of each inhibitor show a DNA cleavage similar to or even lower than the control presented in the figure (data not shown).

with 4 in Figure 8A). These results are also in agreement with previous reports which suggested the participation of ROS in the DNA breakage by tetracyclines and copper(II)-tetracyclines upon light irradiation[53,54] or in the dark in the absence of an external reagent.[16]

A previous report showed the cleavage of ϕX-174 supercoiled DNA by tetracycline and doxycycline under high-pressure mercury lamps irradiation.[53] Furthermore, tetracycline in the presence of copper(II) cleaves the DNA under visible light (fluorescent lamps) irradiation[54] and also in the dark in the absence of an external reagent.[16] These three studies also demonstrated that tetracyclines and derivatives can efficiently cleave DNA by generation of ROS.[16,53,54] Recently, plasmid DNA cleavage activity of a ternary copper(II)-antibiotic complex of ciprofloxacin and 1,10-phenanthroline was reported, but only in the presence of ascorbate, a mild reducing agent.[55] Considering these observations, the present work may be considered the first report of two copper(II) complexes containing an antibiotic and 1,10-phenanthroline as ligands with an expressive plasmid DNA cleavage by an oxidative mechanism even in the absence of any oxidant or reducing agent. Interestingly, there are few examples of copper(II) complexes with oxidative DNA cleavage activity in the absence of a reducing agent,[16,56−63] and the complexes **1** and **2** are clearly new examples of this interesting property.

Since the oxidative mechanism of plasmid DNA cleavage by many metal complexes involves a metal center reduction, assays in the presence of bathocuproine (0.5 mmol L^{-1}), a copper(I) chelator, were performed (Figure 9). Upon addition of bathocuproine, the plasmid DNA cleavage promoted by **1** and **2** was completely inhibited (compare lane 3 with 4), which suggests the participation of copper(I) on the cleavage event.

To additionally prove the evidence of an oxidative mechanism on a DNA cleavage event, the effect of the enzyme T4 DNA ligase on the products of DNA cleavage was investigated. The T4 DNA ligase enzyme is able to ligate fragments of DNA containing 5′-PO_4^{2-} and 3′-OH terminals. The hydrolysis of phosphodiester linkage usually forms these terminal groups while the DNA fragmentation by an oxidative mechanism does not. The treatment of the linearized DNA by restriction enzyme *Bam*HI with T4 DNA ligase produces different fragments of DNA with increasing sizes (Figure 10, lane 5, bands 1−4) which may be related to ligation of several plasmid molecules to each other. In addition, part of the linearized DNA was converted to F II and also to original F I plasmid DNA form (Figure 10, lanes 2 and 5). None of these effects could be observed in the T4 DNA ligase treatment of DNA linearized by **1** or **2** (Figure 10, lanes 3−4 and 6−7). These findings suggest that the cleavage process related to linearization of DNA may involve oxidative pathways rather than

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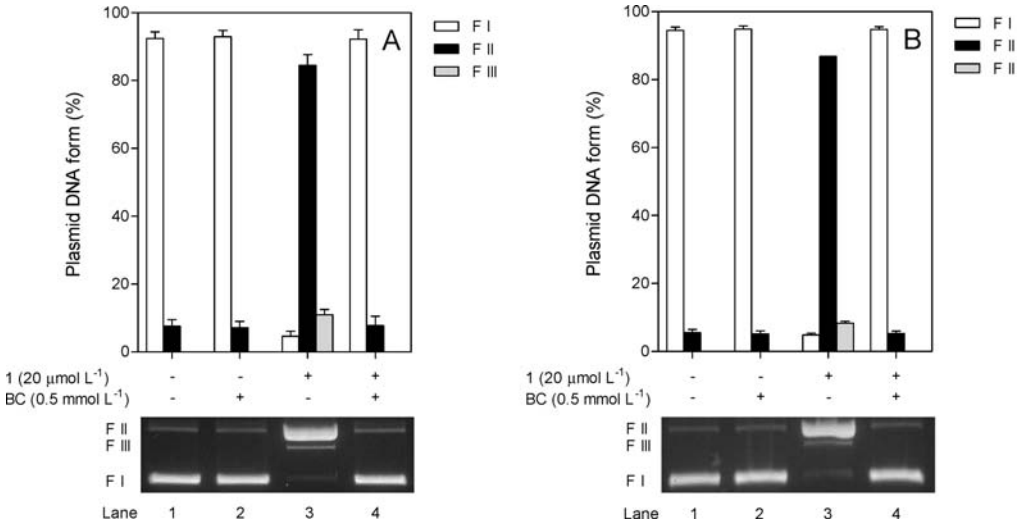

Figure 9. Effect of bathocuproine (BC, 0.5 mmol L^{-1}) on the cleavage of supercoiled DNA by **1** (A) and **2** (B) at 20 μmol L^{-1} for 4 h at 37 °C in 10 mmol L^{-1} Tris-HCl (pH 7.4).

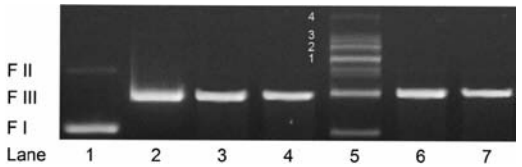

Figure 10. Plasmid DNA linearization by *Bam*HI and the complexes **1** and **2** without T4 DNA ligase treatment (lanes 2, 3, and 4, respectively) and with ligase treatment (lanes 5, 6, and 7, respectively). Untreated DNA is shown in lane 1.

a hydrolytic mechanism, even in the absence of a coreagent, which is in agreement with other examples in the literature.[9,61,62]

Since no reducing agent was used in the assays described above, the reduction of the copper(II) site to copper(I) in **1** and **2** should be mediated by the DNA itself[58] or by the complex ligands. This last suggestion is supported by the work of Buschfort and Witte which postulates that in the absence of any reducing agent the tetracycline molecule undergoes oxidation in the presence of oxygen, reducing the copper(II) ion to copper(I) and triggering the ROS formation by the reoxidation of copper(I) ion.[16]

The presence of specific DNA groove binders revealed similar results for both complexes (Figure 11). No apparent inhibition of plasmid DNA cleavage activity was observed pretreating the plasmid with the minor groove binding agent, distamycin (compare lane 2 with 4), while methyl green completely inhibits the plasmid DNA cleavage (compare lane 2 with 6). These results suggest that the DNA cleavage by the **1** and **2** preferentially occurs by the DNA major groove, which matches with the proposed DNA binding behavior by absorption spectra titration results.

According to all these observations we propose a mechanism in which the complexes first bind to the plasmid DNA through the major groove. Then the reduced form of the complexes, $[Cu(dox)(phen)]^+$ or $[Cu(tc)(phen)]^+$, reacts with dissolved oxygen giving rise to superoxide anion ($O_2^{\bullet-}$) in reaction a:

$$\text{copper(I)} + O_2 \rightarrow \text{copper(II)} + O_2^{\bullet-} \qquad \text{(a)}$$

In parallel, the superoxide anion dismutates into hydrogen peroxide in reaction b:

$$2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2 \qquad \text{(b)}$$

The formation of hydroxyl radicals may follow a Fenton-type reaction c:

$$\text{copper(I)} + H_2O_2 \rightarrow \text{copper(II)} + OH^- + OH^{\bullet} \qquad \text{(c)}$$

Exclusively for **2**, the reaction of the copper(I) complex with hydrogen peroxide could also form a copper-peroxide-type radical, reaction d:

$$\text{copper(I)} + H_2O_2 \rightarrow \text{copper(I)-OOH} + H^+ \qquad \text{(d)}$$

Finally, the ROS attacks the plasmid DNA leading to single- and double-strand breaks.

During the DNA cleavage assays presented here, both complexes were able to linearize the supercoiled DNA. The mechanism used for plasmid DNA linearization was investigated analyzing the number of single-strand (n_1) and double-strand (n_2) breaks per plasmid molecule induced by **1** and **2**. The Freifelder−Trumbo relationship postulates that if the ratio of single to double-strand breaks (n_1/n_2) is approximately 120, the double-strand breaks result from the accumulation of single-strand breaks randomly distributed over the plasmid molecule.[64] On the other hand, a low n_1/n_2 means that the linear DNA form is generated by a nonrandom mechanism of double-strand cleavage as, the result of two nearly subsequent single-strand breaks, one on each DNA strand within 16 base pairs of each other. Fifteen plasmid DNA cleavage reactions were monitored for **1** and **2**, and the calculated n_1/n_2 ranged from 20 to 32 and 22 to 36, respectively. These results are significant lower than 120, indicating that the linearization of plasmid DNA induced by **1** and **2** may follow a nonrandom double-strand cleavage mechanism and are in accordance with other copper(II) complexes with a similar cleavage mechanism.[65−67]

The kinetic analysis of plasmid DNA cleavage shows typical pseudo-first-order reaction behavior, since the plot of ln-[supercoiled DNA (%)] versus the reaction time is linear. At 100 μmol L^{-1}, the observed DNA cleavage rates (k_{obs}) in the

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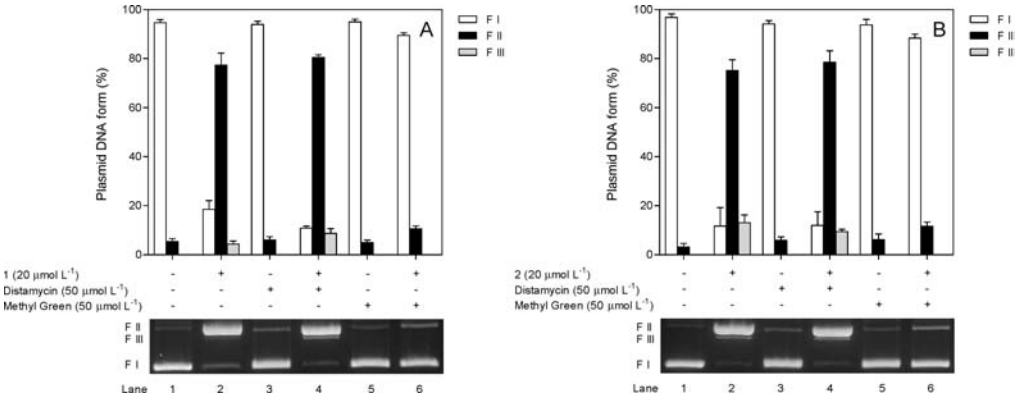

Figure 11. Effect of DNA groove binders distamycin (50 μmol L^{-1}) and methyl green (50 μmol L^{-1}) on the cleavage of supercoiled DNA by **1** (A) and **2** (B) at 20 μmol L^{-1} for 4 h at 37 °C in 10 mmol L^{-1} Tris-HCl (pH 7.4).

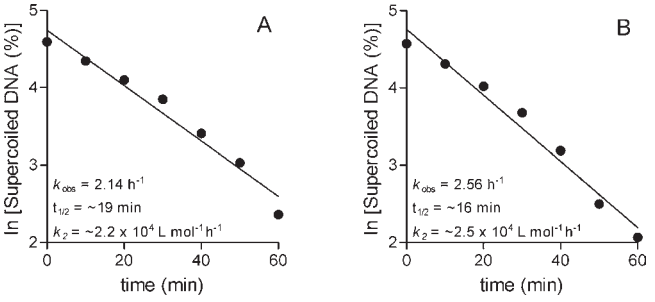

Figure 12. Kinetics of supercoiled DNA cleavage by **1** (A) and **2** (B) at 100 μmol L^{-1} at 37 °C in 10 mmol L^{-1} Tris-HCl (pH 7.4).

presence of **1** and **2** were 2.19 and 2.56 h^{-1}, respectively. These kinetic rates represent a supercoiled DNA half-life time ($t_{1/2}$) about ~19 (**1**) and ~16 (**2**) min, indicating that complexes can cleave DNA rapidly under these experimental conditions. Previous studies have considered that an oxidative process for the conversion of supercoiled DNA form into its cleaved forms follows a second-order kinetic profile.[65,68] Taking this as a precedent, we have used the following rate law:

$$\frac{\delta[\mathrm{DNA_I}]}{\delta t} = -k_2[\mathrm{complex}][\mathrm{DNA_I}]$$

Here, [DNA$_I$] represents the portion of supercoiled DNA form, t is the reaction time, and k_2 is the second-order kinetic constant. From this equation, the obtained values of the second-order kinetic constant (k_2) of the reaction performed by **1** and **2** were ~2.2 × 10^4 and ~2.5 × 10^4 L mol^{-1} h^{-1}, respectively (Figure 12). These second-order kinetic constants are in agreement with those reported for some other copper(II) complexes that exhibit oxidative DNA cleavage in the presence of ascorbate or $KHSO_5$ (k_2 ~10^3 to 10^5 L mol^{-1} h^{-1}), but even in absence of a coreagent.[65,66,69,70]

In addition, the kinetic data obtained to **1** and **2** were compared to those found for the classic metal compound [Cu(phen)$_2$]$^{2+}$ under the same conditions used in this work, i.e., without reducing or oxidant agents. The cleavage rate of DNA by [Cu(phen)$_2$]$^{2+}$ at 100 μmol L^{-1} in Tris-HCl buffer corresponds to a k_{obs} of 0.023 h^{-1} which indicates a supercoiled DNA half-life of about ~31 h. In a similar way, considering that the conversion of supercoiled DNA form into its cleaved forms follows a second-order kinetic profile, the k_2 was estimated to be only ~3.8 L mol^{-1} min^{-1}. From these results the complexes **1** and **2** are approximately 100-fold more active toward DNA cleavage than [Cu(phen)$_2$]$^{2+}$.

Table 2. Growth Inhibition of K562 Cells by Compounds 1 and 2

compound	IC$_{50}$[a] (μmol L^{-1} ± s.d.)
dox	17.70 ± 0.90
tc	52.37 ± 3.10
phen	3.17 ± 0.25
[Cu(phen)$_2$](ClO$_4$)$_2$	3.44 ± 0.30
complex 1	1.93 ± 0.20
complex 2	2.59 ± 0.26

[a] IC$_{50}$ is the concentration required to inhibit 50% of cell growth, after 3 days of incubation. The values are the mean of triplicate determinations.

It is difficult to compare the kinetic behaviors toward DNA cleavage of metal complexes presenting an oxidative reaction mechanism in the absence of coreagents, because of the lack of reports in the literature. An example is the complex [Cu$_2$BMXD] (BMXD = 3,6,9,17,20,23-hexaazatricyclo[23.3.1.1(11,15)]triaconta-1(29),11(30),12,14,25,27-hexaene) reported by our group.[59] Fitting the kinetic data from DNA cleavage reaction by Cu$_2$BMXD, the calculated k_2 is about ~3.5 × 10^2 L mol^{-1} h^{-1}, i.e., at least 2 orders of magnitude lower when compared with complexes **1** and **2** (~10^4 L mol^{-1} h^{-1}). This result clearly shows the impressive DNA cleavage ability of **1** and **2**, since their activity is similar to complexes with oxidative DNA cleavage mechanism in the presence of reducing agents rather than in the absence, as in our case.

Cytotoxic Studies and Cellular Accumulation. Both complexes inhibit the growth of K562 cells with the IC$_{50}$ values of 1.93 and 2.59 μmol L^{-1} for compounds **1** and **2**, respectively (Table 2). Values obtained for the free ligands and the complex [Cu(Phen)$_2$](ClO$_4$)$_2$ are also shown for the sake of comparison. Complex **1** is more active than **2**, and in all cases, the activity of the complexes is higher than those of the corresponding free ligands. While complex **2** is only slightly more cytotoxic than [Cu(phen)$_2$](ClO$_4$)$_2$, the activity of complex **1** is 2-fold higher.

We have also determined the cellular concentration of the complex after treating cells with equitoxic complex concentrations (Figure 13). There is a good correlation between the

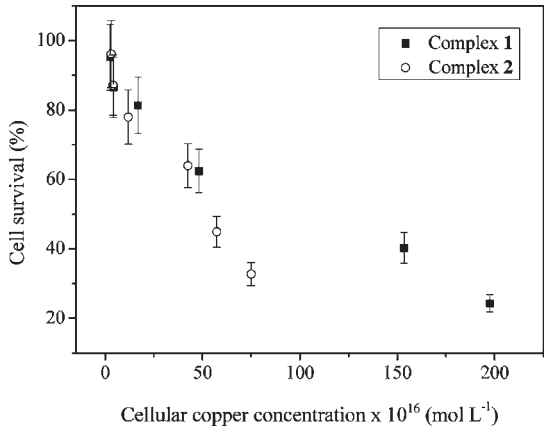

Figure 13. Correlation between cell survival and intracellular copper concentration. K562 cells were incubated for 72 h with different complex concentrations.

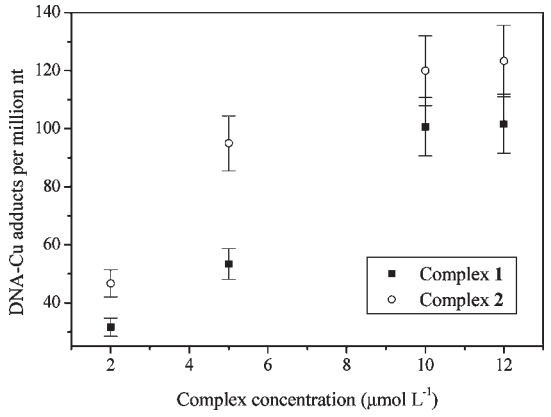

Figure 14. Amount of copper bound to DNA as a function of the concentration of complex added to the incubation medium. 3×10^6 K562 cells were incubated with different concentrations of complexes ranging from 2 to 12 μmol L^{-1} for 2 h. The values are the average of three separate measurements.

cytotoxic activity and cellular accumulation; i.e., the inhibition of cellular growth increases with the increase of intracellular complex concentration. The effect of both compounds follows the same pattern, and the intracellular concentration required to inhibit 50% of cell growth is approximately 55×10^{-16} mol $cell^{-1}$.

In order to verify if the compounds reach DNA inside cells, we have extracted DNA from cells after a 2 h-incubation in the presence of increasing concentrations of complexes **1** and **2**. In Figure 14 one can observe that the number of Cu-DNA adducts increases as the concentration of complex added to the cells increases. Similar experiments were performed after a 72-h-incubation, i.e., in the same conditions of the cytotoxic studies, and once again, the number of Cu-DNA adducts increases with the increase of the concentration of complex added to the cells, which points to a relation between cytotoxic activity and DNA binding.

CONCLUSIONS

Complexes **1** and **2** present an expressive plasmid DNA cleavage activity generating single- and double-strand breaks, under mild reaction conditions and even in the absence of any additional oxidant or reducing agent. The complexes bind to the DNA preferentially by the major groove and then cleave the DNA strands by an oxidative mechanism involving the generation of ROS. The kinetic profile of promoted plasmid DNA cleavage indicates a rapid DNA cleavage where the intact DNA half-life was lower than 20 min. These complexes are among the most potent DNA cleavage agents reported so far.

The sensitivity of chronic myelogenous leukemia cells to the complexes is higher than to the free ligands. The cytotoxic effect increases with the increase of the intracellular copper concentration. Once inside cells, the complexes reach DNA, and the number of adducts formed is proportional to the concentration of complex externally added. These results suggest that interactions with DNA are involved in the mechanism of the cytotoxic action.

ASSOCIATED CONTENT

Supporting Information. Figures S1 [Diffuse reflectance spectra of doxycycline and complex **1** (A) and tetracycline and complex **2** (B)], S2 (experimental EPR spectra of complexes **1** and **2**), and S3 (comparison of cleavage of supercoiled DNA by **1** and **2** with $CuCl_2$) in PDF format. This material is available free of charge via the Internet at http://pubs.acs.org.

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