Efficient Inorganic Deoxyribonucleases. Greater than 50-Million-Fold Rate Enhancement in Enzyme-Like DNA Cleavage

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Abstract: Glycosylated natural products such as bleomycin, neocarzinostatin, and calicheamicin γ_1 are efficient antitumor agents that cleave ds DNA by pathways that involve redox chemistry. In this paper we demonstrate the use of metalloderivatives of natural aminoglycosides as efficient DNA cleavage agents in the absence of external reducing agents. Kinetic characterization of DNA cleavage by copper neamine under Michaelis−Menten-"type" reaction conditions revealed a maximal reaction velocity $V_{max}' = 0.031$ min^{-1}, equivalent to a greater than 50-million-fold rate enhancement in DNA cleavage, when uncorrected for catalyst concentrations. Under true Michaelis conditions, a maximal reaction velocity $V_{max} = 0.0595$ min^{-1} was obtained (with $k_{cat} = 5.95 \times 10^{-4}$ min^{-1}), corresponding to a million-fold rate enhancement using micromolar concentrations of Cu^{2+}−neamine. The specificity constants for DNA cleavage by copper neamine ($k_{cat}/K_M = 4.8 \times 10^5$ h^{-1} M^{-1}) are 2 orders of magnitude greater than those reported elsewhere for synthetic compounds, at this time. Cleavage mediated by Cu^{2+}−(kanamycin A) was found to be even more efficient. DNA cleavage was not inhibited by SOD, NaN_3, DMSO, or EtOH, nor by handling under anaerobic conditions. The results of gel electrophoretic experiments provide clear evidence for a hydrolytic cleavage pathway with generation of 5′-phosphate and 3′-hydroxyl termini.

Introduction

Enediyne antibiotics and their synthetic analogues, iron bleomycin and other metal−ligand complexes, have been successfully applied to oxidative cleavage chemistry of DNA.[1] Oxidative cleavage requires co-reactants in addition to the principal cleavage agent: for example, bleomycin forms a complex with iron and oxygen,[2] while neocarzinostatin[3] and calicheamicin γ_1[4] require thiols or light and cleave DNA by hydrogen atom abstraction and cleavage of the ribose ring. Furthermore, oxidative cleavage is mediated by reactive oxygen species (ROS) that are diffusible and can cause other severe cytotoxic effects.

To eliminate the possibility of significant cytotoxic side effect by ROS, pathways that result in DNA cleavage by hydrolysis mechanisms are preferable; however, the phosphodiester linkage is among the most inert chemical functional groups toward hydrolysis. This important feature has been utilized by Nature in the design of the structural framework for DNA and RNA.[5] Hydrolytic degradation of DNA by nuclease enzymes is an important biological reaction, and metal ions play a central role in mediating such cleavage pathways;[6,7] however, hydrolysis of DNA by small molecules is primarily hindered by the repulsive interaction of the negatively charged phosphate group toward an incoming nucleophile. Various transition metal and lanthanide cations have been shown to alleviate this repulsive interaction by direct inner-sphere binding of the metal and phosphate ester, and tuning of their hydrolytic activity has been controlled by the coordinating functional groups on the ligand. While ribozymes and their mimics, and antisense and antigene oligonucleotides, have also been evaluated for therapeutic applications, small molecules that cleave DNA and RNA in a sequence-specific manner are of potential value in the treatment of cancer and viral diseases, and for application in biotechnology.[8]

De novo approaches to DNA hydrolysis have included the design of artificial nucleases, modeled after natural hydrolytic enzymes, that incorporate Rh(III) intercalators attached to peptide moieties.[9] In addition, Co(III) complexes have been used to catalyze the hydrolysis of amides and phosphodiesters, while lanthanide cations, in millimolar concentrations, enhance DNA cleavage by factors of around 10^7 in the presence of co-reactants such as glycerol or other intercalating agents.[10,11] Similar rate enhancements by Co(III)−polyamine complexes have been reported for hydrolysis of DNA.[12,13] Noteworthy in the calculation of the rate enhancements shown by lanthanide ions and cobalt complexes is the fact that such rates were uncorrected for catalyst concentrations when using the Michaelis−Menten kinetic equations. In contrast to hydrolytic cleavage pathways

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mediated by lanthanide and Co(III) complexes, most studies of DNA cleavage with Cu^{2+} and $Fe^{2+/3+}$ complexes have focused mainly on oxidative cleavage, although some novel hydrolytic catalysts have also been reported.[14−17]

Many naturally occurring antitumor agents are capable of modifying nucleic acids only in the presence of metal ions and oxygen.[2−4] Of these antitumor agents, a vast majority are glycosylated and usually contain amino-sugars in their backbone. Myers and co-workers have demonstrated that the carbohydrate residues in neocarzinostatin both accelerate the rate and improve its efficiency in DNA cleavage, relative to its aglycon counterpart.[18] This indicates a possible role for the carbohydrate residues in nucleic acid recognition. Previous studies with basic aminoglycoside antibiotics reveal that these bind DNA at the negatively charged phosphate groups by electrostatic interactions.[19] Wong and co-workers have demonstrated hydroxyamines to be novel motifs for molecular recognition of phosphodiesters.[20] The 1,3-hydroxyamines commonly found in aminoglycoside antibiotics show molecular recognition properties for G bases and contribute significantly to protein−nucleic acid interactions.[20] We have shown that aminoglycoside ligands provide a new approach to the recognition and specific binding of anionic species through outer-sphere interactions.[21]

Given the cognitive properties of such amino-sugar moieties, it was of interest to determine if a metal complex of such a ligand could effect single- or double-stranded nicks on duplex DNA and thereby demonstrate potential antitumor activity. Simple amino-sugars can bind metal ions by inner-sphere coordination using an amine nitrogen and an appropriately positioned hydroxyl oxygen. Binding of Cu^{2+} to aminoglycosides (especially kanamycin A) has been studied using NMR[22] and potentiometric titrations.[23] We expected that a 1,2-hydroxyamine motif would chelate Cu^{2+} and form stable complexes. Such 1,2-motifs are found in ring C of kanamycin A, rings A and B of neamine, and rings A, B, and D of neomycin B (Figure 1). The anion binding properties of aminoglycosides,[21] and their capacity to coordinate metal ions,[14] encouraged us to combine these aspects and evaluate their DNA binding and nuclease activity. Preliminary results indicating efficient catalytic DNA cleavage by Cu^{2+}−(kanamycin A) were reported by us recently.[14] Herein, we report the synthesis and characterization of several copper complexes of aminoglycosides and their chemistry with plasmid DNA. Our results indicate that Cu−aminoglycosides are efficient chemical nucleases that cleave DNA in the absence of any external reducing agents and generate 5′-phosphate and 3′-hydroxyl termini under hydrolytic cleavage conditions. The kinetic characterization of DNA cleavage by Cu^{2+}−neamine under pseudo-Michaelis−Menten conditions is detailed. We also report the first example of true enzyme-like saturation kinetics for a metal complex.

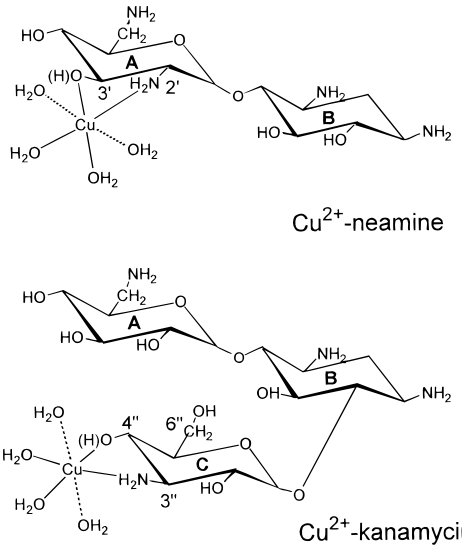

Figure 1. Schematic representation of the aminoglycosides used in this study and the Cu^{2+} binding sites based on experimental C-13 and H-1 NMR relaxation studies.

Experimental Section

1. Syntheses and Characterization. Kanamycin A (kan A) and neomycin B (neo B) were purchased from Sigma Chemical Co. Neamine hydrochloride was synthesized by following literature methods.[24] Metal salts used were of ACS grade and were purchased from Aldrich Chemical Co. HEPES and EDTA were purchased from Sigma Chemical Co and their pH adjusted with NaOH. The purity of neamine was checked by ^{1}H, ^{13}C NMR and elemental analysis. The metal complexes, Cu−(kan A), Cu−(kan A)$_2$, and Cu−(neo B), were synthesized in a similar manner.[14] Elemental analyses were performed by Oneida Research, New York. Absorption measurements were made on a Hewlett-Packard 8425A diode array spectrophotometer using the Online Instrument Systems (OLIS) 4300S operating software. EPR spectra were recorded with an X-band Bruker ESP 300 spectrometer equipped with Oxford liquid helium cryostat. Low-temperature EPR spectra were recorded using the following conditions: microwave power and frequency, 0.1 mW and 9.65 GHz; modulation amplitude and frequency, 5.054 G and 100 kHz; time constants, 10−24 ms; and temperature, 15 K.

(a) Neamine. Prepared by the acid-catalyzed hydrolysis of neomycin B, following published protocols.[24] Elemental analysis for $C_{12}H_{26}N_4O_6\cdot 4HCl\cdot 3H_2O$, calculated (observed): C, 27.58 (27.68); H, 6.90 (7.26); N, 10.73 (10.41).

(b) Synthesis of Cu−(kan A), 1. To kanamycin A sulfate (0.1455 g, 0.25 mmol), in 5 mL of water, was added $CuSO_4$ (0.0624 g, 0.25 mmol). The reaction was stirred at room temperature for 24 h, resulting in a blue solution. To this, ethanol (5 mL) was added to precipitate a blue solid, which was filtered, washed twice in EtOH by stirring for 6 h each time, dissolved in water, and EtOH-precipitated, to yield pure compound **1**. TLC was carried out with a mixed solvent system of propanol/acetone/20% aqueous NH_4OAc/NH_4OH (1:1:5:0.025) to give $R_f = 0.33$ for **1** versus $R_f = 0.60$ for kanamycin A sulfate. Elemental analysis for $[(C_{18}H_{38}N_4O_{11}Cu)(SO_4)_2]\cdot 7H_2O$, calculated (observed): C, 24.89 (24.56); H, 5.99 (5.80); N, 6.45 (6.18). UV−vis (in water): λ_{max}, nm (M^{-1} cm^{-1}), 672 (130). EPR (15 K): $g_{parallel} = 2.514$; $g_{perp} = 2.045$.

(c) Synthesis of Cu−(kan A)$_2$, 2. To kanamycin A sulfate (0.291 g, 0.5 mmol), in 5 mL of water, was added $CuSO_4$ (0.0624 g, 0.25 mmol). The reaction was stirred at 40 °C for 12 h, resulting in a green solution. A dark green complex was isolated and purified as described above for **1**. TLC was carried out with a mixed solvent system of propanol/acetone/20% aqueous NH_4OAc/NH_4OH (1:1:5:0.025), $R_f = 0.30$ for **2**. Elemental analysis for $[(C_{36}H_{76}N_8O_{22}Cu)(SO_4)_3]\cdot 8H_2O$,

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calculated (observed): C, 29.43 (29.65); H, 6.27 (6.34); N, 7.63 (7.13). UV−vis (in water): $\lambda_{max'}$, nm (M^{-1} cm^{-1}), 612 (430). EPR (15 K): $g_{parallel} = 2.459$, 2.306; $g_{perp} = 2.023$.

(d) Synthesis of Cu−(neo B), 3. To neomycin B (0.227 g, 0.25 mmol), in 5 mL of water, was added $CuSO_4$ (0.0624 g, 0.25 mmol). The reaction was stirred at 40 °C for 12 h, resulting in a pale blue solution. A pale blue complex was isolated and purified as described above for **1**. TLC was carried out with a mixed solvent system of propanol/acetone/20% aqueous NH_4OAc/NH_4OH (1:1:5:0.025), $R_f =$ 0.17 for **3** versus $R_f = 0.45$ for neomycin B. Elemental analysis for $[(C_{23}H_{50}N_6O_{13}Cu)(SO_4)_3]\cdot 8H_2O$, calculated (observed): C, 24.78 (25.30); H, 5.92 (6.05); N, 7.54 (7.43). UV−vis (in water): $\lambda_{max'}$, nm (M^{-1} cm^{-1}), 712 (65).

(e) Synthesis of Cu−Neamine, 4. To neamine hydrochloride (0.6015 g, 1.31 mmol), in 60 mL of MeOH, was added $Cu(OAc)_2$ (0.2601 g, 1.31 mmol), and the solution was brought to reflux. A light green-blue precipitate was observed immediately, and the reaction was allowed to reflux for 48 h. At this stage the solid blue product was filtered. The isolation and purification steps are essentially the same as those described above for **1**. Elemental analysis for $[(C_{12}H_{24}N_4O_6Cu)4\ H_2O\cdot 4HCl]$, calculated (observed): C, 23.93 (23.92); H, 5.98 (5.66); N, 9.30 (8.87). UV−vis (in water): $\lambda_{max'}$, nm (M^{-1} cm^{-1}), 718 (44).

2. NMR Studies. ^1H and ^{13}C NMR measurements were made on a Bruker Avance 400- or 500-MHz spectrometer at 298 K. Longitudinal relaxation times (T_1) were determined using an inversion recovery pulse sequence using various delay times ranging from 1 ms to 10 s and minimum recycle times of 10 s (at least 5 times T_1). Data were collected using a Bruker Avance 500 NMR spectrometer and processed using X-Win NMR version 2.1 software on a Silicon Graphics workstation. All NMR samples were made in 10% D_2O and appropriate buffers.

3. DNA Cleavage. (a) DNA Cleavage by Metal−Kanamycin Complexes Generated in Situ. The plasmid DNA, pT7-7, was a kind gift from Prof. D. R. Dean, with a length of 3650 base pairs.[25] The plasmid was transformed into DH5α competent cells. Pure plasmid was isolated using QIAGEN protocols and stored in HEPES, pH 7.3, at −20 °C. Supercoiled plasmid was gel purified before each experiment to eliminate contamination by other forms of plasmid DNA. For a general survey of metal ion activity, a 10 μM solution of the transition metal ion ($VOSO_4$, $FeSO_4$, $MnCl_2$, $CoSO_4$, $NiCl_2$, $CuSO_4$, and $ZnSO_4$) was treated with an equivalent volume of 10 μM kanamycin A for 12 h at 35 °C. Complex formation was monitored by thin-layer chromatography, using $PrOH/Me_2CO$/20% aqueous NH_4OAc (1:1:5:0.025) on silica gel plates. R_f values were identified as follows: kanamycin A, 0.61; V−(kan A), 0.44; Fe−(kan A), 0.38; Mn−(kan A), 0.56; Co−(kan A), 0.47; Ni−(kan A), 0.44; Cu−(kan A), 0.33. No reaction was observed with Zn^{2+} and kan A. DNA cleavage experiments were performed using 51.1 μM base pair concentration of pT7-7 and 1 μM final concentration of the metal−kanamycin A complex. The reaction mixture (10 μL) containing plasmid DNA and metal kanamycin A complex was incubated for 1 h at 37 °C, mixed with 2 μL of loading buffer (bromophenol blue, xylene cyanol, and 50% glycerol), and loaded onto a 0.8% agarose gel containing 0.3 μg/mL ethidium bromide. Control experiments were run with plasmid DNA in the presence and in the absence of 5 μM kanamycin A or $CuSO_4$. The gels were run at a constant voltage of 120 mV for 60−90 min in TAE buffer, washed with distilled water, visualized under a UV transilluminator, and photographed using a digital camera.

(b) DNA Cleavage by Isolated Cu^{2+}−Aminoglycosides. On the basis of the efficacy of Cu^{2+}−(kan A) in cleaving DNA, Cu^{2+}−(kan A)$_2$, Cu^{2+}−(neo B), and Cu^{2+}−neamine were also synthesized, isolated in the solid state, and purified. All complexes were tested for DNA cleavage activity at various concentrations and in the presence of co-reactants (H_2O_2 and ascorbic acid) and quenchers (SOD, NaN_3, DMSO, EtOH). The hydrolytic instability of Cu^{2+}−(kan A)$_2$ and Cu^{2+}−(neo B) did not allow detailed cleavage studies for these complexes.

A Poisson distribution was used to describe the cleavage reaction. The average number of strand scissions per DNA plasmid, S, is equal to $-\ln f_I$, where f_I is the fraction of form I (supercoiled plasmid).[26]

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Table 1. Efficiency of Single-Strand Scission per DNA Molecule by Various Chemical Nucleases under Hydrolytic Conditions

complex	S/M^a (μM complex)	reference
$[Cu(9aneN_3)Cl_2]$	7.7×10^{-4}	34
$[Co(cyclen)]_2[(CH_2)_6N^+(CH_3)_2]_2$	0.012	13
$[Co(cyclen)][(CH_2)_6N^+(CH_3)_2]$	5.6×10^{-3}	13
Eu^{3+} ionophore	1.84×10^{-3}	10
La^{3+} ionophore	4.46×10^{-4}	10
Eu^{3+} ionophore	9.56×10^{-4}	10
Ce^{3+} ionophore	5.13×10^{-4}	35
Pr^{3+} ionophore	3.15×10^{-4}	35
Gd^{3+} ionophore	2.75×10^{-3}	35
Dy^{3+} ionophore	3.85×10^{-3}	35
Cu−(kan A)[b]	0.40	this work
Cu−neamine	0.23	this work

[a] S = average number of single-strand scissions per DNA molecule. When only forms I and II are present, $S = -\ln f_I$, where f_I is the fraction of form I. M = concentration of the complex used to hydrolyze plasmid DNA. [b] Obtained by reacting plasmid DNA with 0.5 μM complex for 10 min.

When only forms I and II (open circular) are observed, S equals the average number of single-stranded cuts per molecule of plasmid DNA. The average number of strand breaks made in each plasmid per micromole of metal complex per hour at 37 °C was calculated under hydrolytic conditions. This ratio, S/M (where M is the micromolar concentration of complex used to observe strand scission), is a measure of the efficiency of various complexes to cleave DNA (Table 1).

(c) Kinetic Measurements for DNA Cleavage by Cu^{2+}−Neamine. DNA cleavage rates at various catalyst concentrations were carried out in 10 mM HEPES, pH 7.3, at 37 °C for different intervals of time. After incubation of the DNA and Cu^{2+}−neamine for a defined time, 2 μL of loading buffer (bromophenol blue, xylene cyanol, 50% glycerol, and 2 mM EDTA) was added and stored at 0 °C. The samples were then loaded directly onto a 1% agarose gel (containing ethidium bromide) and electrophoresed at a constant voltage of 120 mV for 120 min. The gels were viewed in a Bio-Rad GelDoc 1000 multianalyzer and photographed using a digital camera. Densitometric calculations were made using the software provided by Bio-Rad. The intensities of supercoiled DNA were corrected by a factor of 1.47 as a result of its lower staining capacity by ethidium bromide.[27] The decrease in the intensities of form I, or the increase in the intensities of form II, were then plotted against catalyst concentrations, and these were fitted well with a single-exponential decay curve (pseudo-first-order kinetics) by use of eq 1a or 1b, respectively, where y_o is the initial percentage of a form of DNA, y is the percentage of a specific form of DNA at time t, const is the percentage of uncleaved DNA, k_{obs} is the hydrolysis rate, or apparent rate constant, and V_{max} is the maximal reaction velocity.

$$y = (y_0 - \text{const}) \exp(-k_{obs}x) + \text{const} \quad (1a)$$

$$y = (100 - y_0)(1 - \exp(-k_{obs}x)) \quad (1b)$$

$$k_{obs}' = V_{max}'[\text{catalyst}]/(K_M + [\text{catalyst}]) \quad (2a)$$

$$k_{obs} = V_{max}[\text{substrate}]/(K_M + [\text{substrate}]) \quad (2b)$$

k_{obs}' versus [Cu^{2+}−neamine] was plotted and fit using eq 2a, which allows determination of both rate constants and Michaelis−Menten-"type" kinetic values. Similar experiments with constant catalyst concentration (100 μM) and varying substrate concentrations (20−200 μM DNA bp) were performed, and the intensities were plotted against substrate concentrations by use of eq 2b. Careful optimization of electrophoretic and densitometric techniques led to clean pseudo-first-order kinetics and allowed the determination of true Michaelis−Menten kinetic parameters by use of eq 2; k_{cat} is calculated as $V_{max}/[E_o]$, where $[E_o]$ = concentration of Cu−neamine = 100 μM.

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(d) Hydroxyl Radical Assay by Rhodamine B Degradation. Hydroxyl radicals were quantitated by following the degradation of the dye, rhodamine B[28a] (19.3 μM), in the presence of Cu−(kan A) (10 nM) and either ascorbic acid (10 μM) or H_2O_2 (10 μM). The reaction was monitored at 552 nm in phosphate buffer (10 mM, pH 7.0) under aerobic and anaerobic conditions. The change in concentration of rhodamine B ($\epsilon = 10.7 \times 10^4$ M^{-1} cm^{-1}) at varying times is a direct measure of the concentration of hydroxyl radicals produced in the reaction mixture. Similar experiments were performed in the presence of Cu−neamine. Hydroxyl radical formation in the presence of the metalloaminoglycosides, and in the absence of ascorbic acid and peroxide under aerobic conditions, was followed by similar methods. Spectra of 15 μM rhodamine B in the presence of 1 and 10 μM Cu−(kan A) or Cu−neamine, respectively, were identical to those obtained for the dye in the absence of the metal complex. This result indicates that there is no direct inner-sphere coordination between the dye and the Cu complexes.

(e) T4 DNA Ligase Assay and Transformation Efficiencies. An enzymatic assay was performed using T4 DNA ligase to determine whether the cleaved products were consistent with hydrolysis of the phosphodiester linkages in DNA.[28b] pT7-7 (50 μM, bp) was treated with metal derivatives of aminoglycosides at 37 °C for 1 h. The DNA was passed through a spin column, and the following reaction protocol was followed for religation. A mixture of 1 μL of BSA (10 mg/mL), 1 μL of 10× ligation buffer, 2 units of T4 DNA ligase, and DNA was incubated at 16 °C for 14 h. Similarly, pT7-7 was linearized using *Bam*H1 and treated with T4 DNA ligase. Samples of Cu−(kan A) cut plasmid (with and without treatment with T4 DNA ligase) and BamH1 linearized plasmid (with and without treatment with T4 DNA ligase) were each transformed into electrocompetent DH5α cells and plated on agarose plates (LB/amp).

(f) [^{32}P] End-Labeling of DNA. Reactions of plasmid DNA, pT7-7 (50 μM, bp), with Cu−(kan A) or Cu−neamine (200 μM) were carried out in HEPES, pH 7.3, at 37 °C for 120 min. The DNA was ethanol precipitated, lyophilized, dissolved in commercial React Buffer 3 (Gibco-BRL) [50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl], and treated with *Bam*H1 for 3 h at 37 °C. The DNA was extracted by phenol/$CHCl_3$/isoamyl alcohol, isolated by ethanol precipitation, dephosphorylated using 5 units of calf intestinal phosphatase (CIAP) in 50 mM Tris, pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT for 60 min at 37 °C, extracted, and isolated as described above. The DNA was 5′-end-labeled with [γ-^{32}P] ATP (6000 Ci/mmol) using T4 polynucleotide kinase (10 units), 5× forward reaction buffer (350 mM Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 500 mM KCl, 5 mM 2-mercaptoethanol) and incubated at 37 °C for 10 min. The sample was heated at 65 °C for 10 min to inactivate the kinase. All samples were cooled on ice for 10 min and reacted with either *Nde*I or *Hin*dIII, to generate a 23-base-pair or 27-base-pair fragment, respectively. DNA samples were lyophilized and extracted using $CHCl_3$/phenol/isoamyl alcohol and then back-extracted with $CHCl_3$, and the aqueous layer was collected and lyophilized. To the lyophilized DNA sample was added loading buffer (9 M urea, 0.06% bromophenol blue and xylene cyanol, 50% glycerol), and the solution heated at 95 °C for 2 min and cooled on ice before loading on a 19% acrylamide gel.

Oligo(dT)$_{4-22}$ (Gibco-BRL) was phosphorylated with T4 kinase and [γ-^{32}P] ATP and used as a standard ladder for products possessing authentic 5′-phosphate and 3′-OH groups, indicative of hydrolysis products. The radiolabeled samples were loaded on 19% acrylamide gels and run at a constant voltage of 1500 V for 7 h in 1× TBE (Tris−borate, 90 mM, 2 mM EDTA, pH 8.0).

(g) PNK Forward Versus Exchange Reactions. Supercoiled plasmid pT7-7 was treated with Cu−neamine and isolated as described above. The isolated DNA was treated with T4 PNK using buffer conditions that promoted either forward or exchange reactions in the presence of [γ-^{32}P] ATP. (i) Forward reaction: 50 pmol of Cu−neamine-treated DNA was incubated in a reaction mixture containing 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 50 pmol of [γ-^{32}P] ATP, and 20 units of T4 PNK for 30 min at 37 °C. The reaction was quenched by adding loading buffer containing 50 mM EDTA. (ii) Exchange reaction: 50 pmol of Cu−neamine-treated DNA was incubated in a reaction mixture containing 50 mM imidazole, pH 6.5, 12 mM $MgCl_2$, 1 mM β-mercaptoethanol, 70 μM ADP, 50 pmol of [γ-^{32}P] ATP, and 20 units of T4 PNK for 30 min at 37 °C. This method results in lower specific activity labeling.[29] (iii) Dephosphorylation followed by forward reaction: 300 pmol of Cu−neamine-treated DNA was dephosphorylated using 5 units of CIAP in 50 mM Tris, pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT for 60 min at 37 °C. The DNA was isolated by a standard phenol/chloroform extraction/ethanol precipitation method. The dephosphorylated DNA was treated with T4 PNK as described in section (i) above. (iv) Terminal deoxynucleotide transferase (TdT) reaction: Cu−neamine-treated pT7-7 was incubated in 200 mM potassium cacodylate, 25 mM Tris-HCl, pH 7.2, 250 mg/mL bovine serum albumin, 1.5 mM $CoCl_2$, 25 units of TdT, and [α-^{32}P] dGTP at 37 °C for 30 min. TdT is a template-independent polymerase that catalyzes the addition of deoxynucleotides to the 3′-OH end of DNA.

Table 2. Selected C-13 T_1 Relaxation Data for Kanamycin A in the Presence of Cu^{2+} [a]

C atom, ppm	T_1, ms (kan A)[b]	T_1, ms (kan A + Cu^{2+}) pH 5.40	pH 7.90	pH 9.75
C-1″, 100.8	416.6	402.5	223.1	321.8
C-2″, 72.7	435.0	455.9	393.9	392.7
C-3″, 55.0	412.9	339.9	15.1	186.8
C-4″, 70.1	361.3	408.9	96.5	190.0
C-5″, 72.9	390.8	385.8	339.6	336.8
C-6″, 61.1	221.5	222.8	150.9	225.2

[a] Solutions contained 100 mM kanamycin A in the presence of 60 μM Cu^{2+}. [b] Data for free base kan A was obtained at pH 7.90. Similar values were obtained for the metal-free ligand at other pH values.

Table 3. Representative C-13 T_1 Relaxation Data of Neamine in the Presence of Cu^{2+} [a]

C atom, ppm	T_1, ms neamine	neamine + Cu^{2+}
C-1′, 98.4	415.4	130.8
C-2′, 54.9	403.8	10.5
C-3′, 71.8	419.9	201.2
C-1, 50.9	473.6	218.8
C-3, 49.1	672.6	176.6
C-4, 83.0	635.3	625.9

[a] Solutions contained 100 mM neamine in the presence of 60 μM Cu^{2+} at pH 7.50.

Results

Characterization of Copper−Aminoglycosides. Paramagnetic enhancement of spin−lattice relaxation rates provides a useful tool for determining the binding sites for metal ions on complex ligands. Extensive C-13 and H-1 NMR relaxation studies have supported the binding of Cu^{2+} to ring C in kanamycin A and ring A in neamine.[30] However, these studies were carried out at basic pH (9.5 or 10.2), while we have performed DNA cleavage studies at near neutral pH (7.3). To determine the binding sites under the pH conditions used in our studies, we have carried out C-13 relaxation measurements of aminoglycosides in the presence of Cu^{2+}. Tables 2 and 3 summarize C-13 relaxation data for resonances that showed significant changes in relaxation times in kanamycin A and neamine, respectively, after addition of Cu^{2+}(aq). Various solutions of Cu^{2+} (10−100 μM) were titrated into a constant (100 mM) concentration of either kanamycin A or neamine. Optimal changes in the T_1 measurements of select carbon atoms

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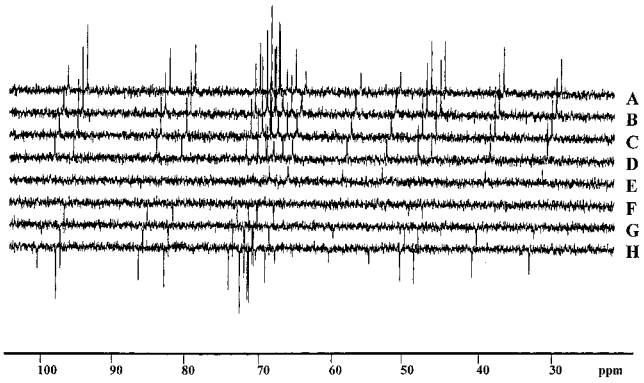

Figure 2. Stack plot of C-13 NMR inversion recovery spectra of 100 mM kanamycin A and 60 μM $CuSO_4$ in 10 mM HEPES (pH 7.90). The delay times are A, 5 s; B, 2 s; C, 1 s; D, 0.5 s; E, 0.2 s; F, 0.1 s; G, 0.05 s; and H, 0.01 s.

were obtained with 60 μM Cu^{2+}. At pH 7.90, the $C_{3''}$-NH_2 carbon of kanamycin A is influenced to a maximum extent (30-fold decrease in T_1), while considerable changes in the T_1 of $C_{4''}$-OH (~4-fold decrease in T_1) were also seen (Figure 2 and Table 2). Usually a 2-fold decrease in T_1 relaxation times is accepted as an indicator of the proximity of the paramagnetic ion to the nucleus of interest. Surprisingly, the other carbon atoms in the skeleton of kanamycin A neither were strongly influenced nor showed a significant change in their T_1 relaxation times. On the basis of these studies, the coordination of Cu^{2+} to kanamycin A is best depicted in the manner shown in Figure 1 (bottom). An approximately 2-fold decrease in T_1 values for the $C_{6''}$-OH carbon centers was seen, most likely arising from the close proximity to the metal binding site ($C_{3''}$-NH_2 and $C_{4''}$-OH). Relaxation studies were also performed at pH 5.40 and 9.75 in the presence of 60 μM Cu^{2+}. While a 5-fold decrease in T_1 of $C_{3''}$-NH_2 was observed at pH 9.75, the $C_{4''}$-OH resonance was affected by about 3-fold. However, at pH 5.40 (wherein all amines in kanamycin A are protonated) neither the $C_{3''}$-NH_2 nor $C_{4''}$-OH was greatly influenced.

While C-13 relaxation measurements of neamine at pH 7.50 show maximum effects on the T_1 of $C_{2'}$-NH_2 (30-fold decrease in T_1) upon Cu^{2+} binding, substantial effects on the T_1 of carbon atoms on $C_{3'}$-OH (~3-fold decrease), $C_{1'}$-O (3-fold decrease), and C_3-NH_2 (4-fold decrease) are also seen.

A variety of first row transition metal ions and Zn^{2+} were incubated with kanamycin A, and TLC analysis showed that most of the transition metal ions reacted with kanamycin A. The in situ generated complexes were tested for their DNA cleavage efficiency; however, only a mixture of Cu^{2+} and kanamycin A was found capable of effecting rapid (<1 h) degradation of plasmid DNA at low catalyst concentrations. Accordingly, the Cu−(kan A) complex, as well as Cu−(kan $A)_{2'}$ Cu−(neo B), and Cu−neamine, were synthesized, isolated, purified, and tested for their DNA cleaving ability. UV−visible spectra obtained for copper−aminoglycosides were characteristic of a Cu^{2+} ion in a square planar N/O environment with weakly bound axial ligands, while the measured EPR parameters are suggestive of a Cu^{2+} ion with a $d(x^2-y^2)^1$ configuration and are within the range observed for other square planar Cu^{2+} complexes.[31]

DNA Cleavage by Cu^{2+}−(kan A). A mixture of Cu^{2+} and kanamycin A at 1 μM concentrations was found to rapidly degrade supercoiled (form I) plasmid DNA (Supporting Information). A systematic reactivity profile of the isolated and purified Cu−(kan A) complex with plasmid allowed observation of nicked DNA after a brief (5 min) exposure to 0.5 μM complex. DNA treated with 1 μM Cu^{2+}(aq) or 5 μM aminoglycoside (kanamycin A or neamine) did not show evidence of cleavage after incubation at 37 °C for 60−120 min. In contrast, both Cu−$(kan A)_2$ and Cu−(neo B) are capable of mediating DNA cleavage over similar concentration ranges; however, as a result of the instability of aqueous solutions of these complexes over a period of several hours, further DNA scission studies were focused on Cu−(kan A) alone. DNA cleavage by Cu−(kan A) was completely inhibited when the solution was saturated with metal-free kanamycin A (5 μM) or NaCl (150 mM). UV−vis and EPR spectroscopic characterization was carried out under the ionic conditions employed in cleavage experiments (150 mM NaCl). These complexes were found to be stable over the time frame of cleavage studies (4−24 h). Inhibition of DNA cleavage in the presence of metal-free kanamycin or at elevated [NaCl] indicates that electrostatic interactions play an important role in the binding of the drug to DNA. A salt dependence for anion binding affinity has also been observed for the binding of anions and RNA by aminoglycosides.[21]

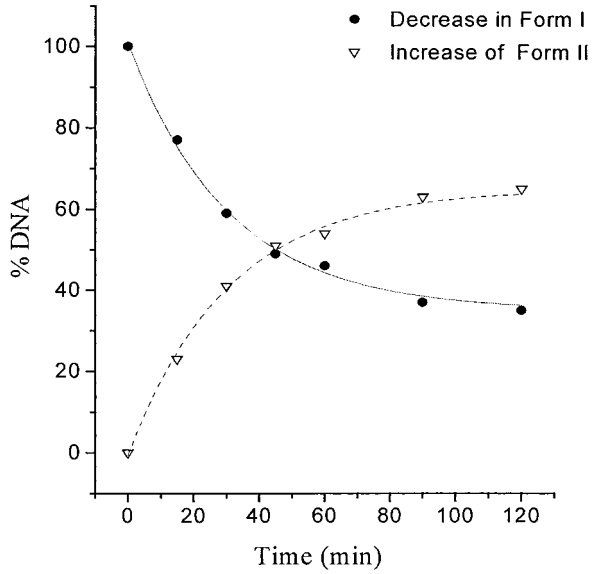

Figure 3. Time course of DNA cleavage at pH 7.3 (10 mM HEPES buffer) and 37 °C in the presence of 50 μM (base pair) pT7-7 DNA and 50μM Cu^{2+}−neamine. ● and ▽ correspond to the experimental data, while lines connecting them are single-exponential curve-fits.

DNA Cleavage by Cu−Neamine. Neamine also shows favorable positioning of amino and hydroxyl groups for Cu^{2+} chelation by ring A (demonstrated by NMR studies).[30] The isolated Cu−neamine complex was tested for DNA cleavage under hydrolytic conditions, and a concentration-dependent cleavage was observed. Reaction that leads to formation of open circular DNA (form II) from the supercoiled form I over various concentrations of complex (5−200 μM) and constant DNA concentration (50 μM, bp) was followed for up to 120 min at 37 °C (Supporting Information). Under these conditions, form II is observed even with low complex concentrations, and no other forms are observed. Plots of the appearance of form II or the disappearance of form I versus time followed pseudo-first-order kinetic profiles and were fit well to a single-exponential decay curve (Figure 3). DNA cleavage was not promoted by either Cu^{2+}(aq) or kanamycin A alone (Supporting Information). Rates of cleavage were calculated, and kinetic parameters were

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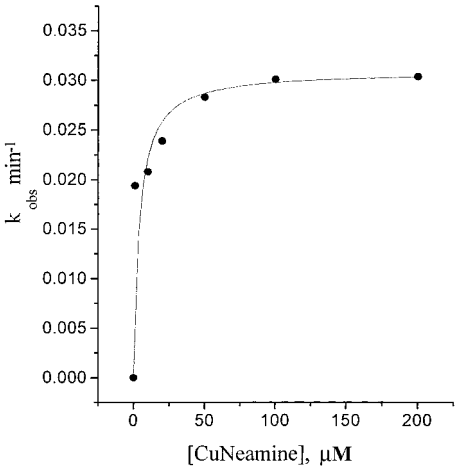

Figure 4. Saturation kinetics for the cleavage of plasmid DNA, pT7-7, with 1−200 μM Cu^{2+}−neamine under hydrolytic conditions at 37 °C, 10 mM HEPES, pH 7.30. DNA and the complex were incubated for 120 min, and the samples were run on a 1% agarose gel and stained with ethidium bromide.

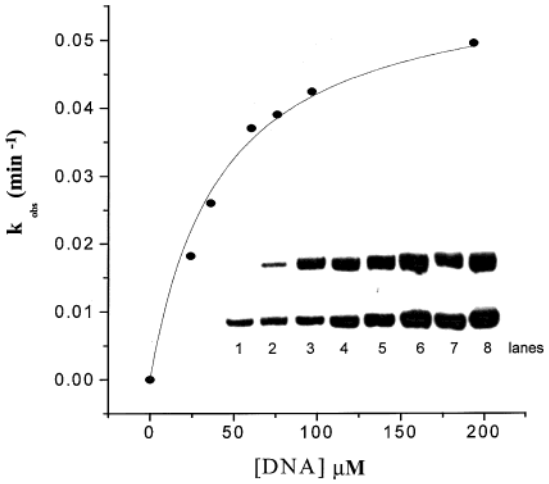

Figure 5. Saturation kinetics of the cleavage of plasmid DNA using 100 μM Cu^{2+}−neamine. Varying concentrations of plasmid were reacted with a constant concentration of the drug for 120 min at 37 °C in 10 mM HEPES, pH 7.30 (see inset). Samples were run on a 1% agarose gel and stained with ethidium bromide (see inset). Inset: Cleavage profile for varying concentrations of plasmid DNA showing nicked (upper band) and supercoiled (lower band). Lanes: 1, DNA (20 μM); 2, 20 μM DNA; 3, 36.3 μM DNA; 4, 48.4 μM DNA; 5, 72.6 μM DNA; 6, 84.7 μM DNA; 7, 96.8 μM DNA; 8, 193.6 μM DNA. Samples in lanes 2−8 were incubated in the presence of 100 μM Cu^{2+}−neamine, as described above.

derived from a plot of k_{obs} versus concentration of Cu−neamine. Under these experimental conditions, values of $V_{max}' = 0.031$ min^{-1} and $K_M = 3.9$ μM were obtained, where these values represent pseudo-Michaelis−Menten conditions (Figure 4).

DNA cleavage reactions were also monitored under "true" Michaelis−Menten kinetic conditions using a constant catalyst concentration (100 μM) and varying substrate concentration (20−200 μM) (Figure 5 and Supporting Information). Under these conditions, V_{max} (0.0595 min^{-1}), k_{cat} (5.95×10^{-4} min^{-1}), and K_M (41.6 μM) values (Table 4, Figure 5) were determined, and so it is likely that the complex binds to the plasmid in an enzyme-like manner.

Hydrolytic Versus Oxidative Cleavage. DNA cleavage reactions under aerobic conditions were performed in the presence of singlet oxygen scavengers, including (NaN_3), a superoxide scavenger (superoxide dismutase) (SOD), and hydroxyl radical scavengers (DMSO and EtOH). Similar studies were also performed under anaerobic conditions (under argon) to evaluate the role of oxygen in mediating DNA cleavage, and the experimental results show that DNA cleavage by Cu−(kan A) and Cu−neamine was not inhibited under any of the above experimental conditions (Supporting Information). Such scavengers were found to completely inhibit the DNA cleavage activity promoted by low concentrations of Cu−(kan A) in the presence of ascorbic acid (Supporting Information). Thus, DNA cleavage by Cu−(kan A), in the absence of a reducing agent, proceeds via a hydrolytic degradative pathway at the higher concentrations used in these studies.

The possibility of hydroxyl radical formation in the presence of Cu−(kan A) was also evaluated by monitoring the changes in absorbance at 552 nm for rhodamine B dye. Degradation of the dye provides a direct measure of the concentration of hydroxyl radicals formed. No radicals were detected over the time of the experiment, in the presence of either the complex alone or the complex with added DNA, indicating that such reactive oxygen species (ROS) are not formed under these conditions. However, as a positive control, we were able to trap hydroxyl radicals in the presence of Cu−(kan A) plus ascorbic acid or H_2O_2, indicating that under redox conditions, DNA cleavage by Cu−(kan A) can be mediated by ROS. A similar radical-trapping experiment was performed in the presence of Cu−neamine and ascorbic acid or peroxide (Figure 6). A more detailed account of the reactivity of these complexes under oxidative conditions will be described elsewhere.

Additional evidence for a hydrolytic mechanism was obtained by use of a T4 DNA ligase enzymatic assay. Plasmid DNA was nicked using Cu−(kan A), and nicked-form DNA was gel extracted and purified using QIAGEN spin columns. Ligation was performed using T4 DNA ligase at 16 °C for 14 h. The reaction mixture was aliquoted into two eppendorfs, one of which was used for gel electrophoresis and the other for transformation into DH5α competent cells by electroporation. As positive controls, plasmid pT7-7 was linearized with *Bam*Hl, which has one unique site, and the linearized plasmid was religated using T4 ligase as described above. Further gel electrophoresis and transformations were carried out with pT7-7 treated with Cu−(kan A) and H_2O_2 (both untreated and treated with T4 ligase) and with regular supercoiled pT7-7. Transformation efficiencies are provided in the Supporting Information. Nicked plasmid DNA was readily circularized by T4 DNA ligase and demonstrated significantly higher transformation efficiencies than the nicked form.

Analysis of DNA Cleavage by 5′-[^{32}P] End-Labeling. The nature of the cleavage products generated from DNA nicking by Cu^{2+}−aminoglycosides was examined by running high-resolution polyacrylamide gels. Plasmid DNA (pT7-7) was reacted with Cu^{2+}−(kanA) or Cu^{2+}−neamine in the absence of any reducing agents ("hydrolytic conditions") at 37 °C for 120 min. Random cleavage events on the plasmid DNA should generate a ladder with nonspecific cleavage evidenced at all sites. This hypothesis was tested by generating either a 23 or 27 bp fragment following a double restriction digestion in the presence of *Bam*H1 and either *Nde*I or *Hin*dIII, respectively, after treatment of the plasmid with copper−aminoglycosides. Results from Cu^{2+}−neamine cleavage experiments are shown in Figure 7 and reveal no significant sequence selectivity in cleavage under our experimental conditions. Results from primer extension reactions also support the conclusion of random cleavage of pT7-7 in the presence of Cu−aminoglycosides

Table 4. Pseudo-Michaelis−Menten Kinetic Analysis for Hydrolytic Cleavage of DNA

complex	K_M (M)	k_{cat}' (h^{-1})[a]	enhancement[b]	k_{cat}'/K_M (h^{-1} M^{-1})[c]	reference
(Pr_2^{3+}) ionophore	3.0×10^{-4}	0.90	2.50×10^7	3.0×10^3	42
Cu(9aneN_3)	nd[d]	~0.04	~1.1×10^6	nd	34
Eu^{3+}	3.9×10^{-5}	0.25	7.00×10^6	6.4×10^3	10
(Eu^{3+}) ionophore	5.7×10^{-4}	2.10	5.83×10^7	3.7×10^3	10
Co^{3+}-cyclen	9.8×10^{-4}	0.79	2.00×10^7	8.1×10^2	13
Co^{3+}-tamen	nd	0.18	5.00×10^6	nd	43
Rh−P−Zn	nd	0.09	2.50×10^6	nd	9
Cu−neamine	3.9×10^{-6}	1.86	5.17×10^7	4.8×10^5	this work
Cu−neamine*	41.6×10^{-6}	3.57	9.99×10^7	8.6×10^4	this work

[a] These calculated values are not k_{cat} per se, but rather k_{obs}' at saturation levels (i.e., V_{max}). Under true Michaelis−Menten conditions (with constant enzyme concentration), $k_{cat} = V_{max}/[E]_o$. In all the above cases, other than our Cu−neamine* results (which were obtained under "true" Michaelis−Menten conditions), the complex (catalyst) concentration was varied during kinetic characterization. [b] Rate enhancement over unhydrolyzed double-stranded DNA where $k = 3.6 \times 10^{-8}$ h^{-1}. [c] The specificity constants for individual complexes, and thus a measure of the efficiency of DNA hydrolysis. [d] nd = not determined.

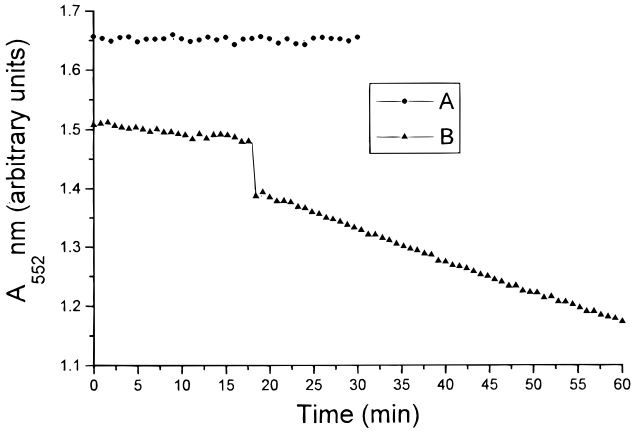

Figure 6. Quantitation of hydroxyl radicals by following the degradation of rhodamine B at 552 nm in 10 mM phosphate buffer, pH 7.0, under aerobic conditions. (A) Reaction of 1 μM Cu^{2+}−neamine with 15.1 μM rhodamine. (B) Reaction of 1 μM Cu^{2+}−neamine with 14 μM rhodamine was followed for 20 min, after which 10 μM H_2O_2 was added to the reaction mixture and the resultant absorbance was recorded.

(Supporting Information). The migration of authentic ^{32}P-end-labeled $(dT)_{4-22}$ ladders was examined in comparison with that of the DNA fragments generated from Cu^{2+}−aminoglycoside treatment. 5′-End-labeled (with γ-^{32}P ATP) oligonucleotides $(dT)_{4-22}$ possess 5′-phosphate and 3′-hydroxyl groups and comigrate with the products generated by Cu^{2+}−neamine cleavage. These experiments also indicate the absence of base release, which also supports a hydrolytic cleavage pathway. To further characterize the termini generated following hydrolytic cleavage, Cu−neamine-treated DNA was subjected to enzymatic reactions in the presence of either [γ-^{32}P] ATP and T4 PNK, or [α-^{32}P] dGTP and TdT (Figure 8). These results indicate 5′-phosphorylation of DNA by T4 PNK to be allowed only after treatment with CIAP, but direct incorporation of [α-^{32}P] dGTP into the 3′-ends.

Discussion

Synthesis and Characterization. Aminoglycosides comprise a large class of polycationic antibiotics that interact with a wide variety of specific RNA structures in the major groove. Complexes of this class of antibiotics with both cations and anions have previously been reported.[21,22,30] By virtue of their vicinal and nonvicinal hydroxyl and amino groups, aminoglycoside antibiotics can form a variety of transition metal ion complexes, which affords a strategy for selective protection of these functional groups.[22] Binding of Cu^{2+} has been the subject of several studies with kanamycin A and neamine. Coordinating

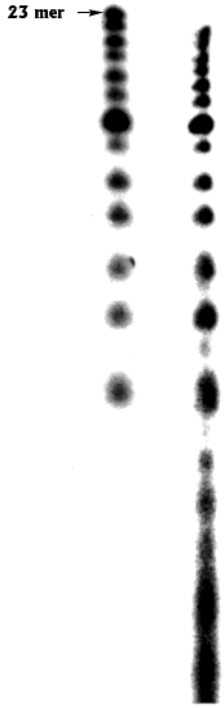

Figure 7. Autoradiograph of a denaturing 10% polyacrylamide electrophoretic gel revealing fragments generated from Cu^{2+}−neamine cleavage of pT7-7 after treatment with restriction enzymes *Bam*H1 and *Nde*I (see Experimental Section). (Left) Cu^{2+}−neamine, 200 μM, 37 °C, 120 min, HEPES, pH 7.3; (right) oligo $(dT)_{4-22}$ ladder.

modes similar to those of simple amino-sugars, but not involving deprotonation of the hydroxyl oxygen, were proposed.[32] Simple amino-sugars bind Cu^{2+} in the weakly acidic to neutral pH range, primarily through the amino nitrogen.[32] The stability of metal coordination requires a five- or six-membered chelate ring to be formed with an appropriately positioned hydroxyl oxygen. For example, Cu^{2+} binds to the 3″-NH_2 and 4″-OH functional groups of ring C of kanamycin A and protects the amino group from acylation (Figure 1).

The copper coordination site has been deduced from extensive C-13 NMR studies[22] and proton T_1 relaxation measurements.[30] Longitudinal relaxation studies of various metal ions, including copper, have been a useful tool in determining metal binding sites and for understanding metal ion−nucleotide and −nucleic acid interactions.[33] From our C-13 relaxation studies on kanamycin A, unambiguous assignment of the Cu^{2+} binding site to the 3″-NH_2 and 4″-OH groups of kanamycin A is indicated at

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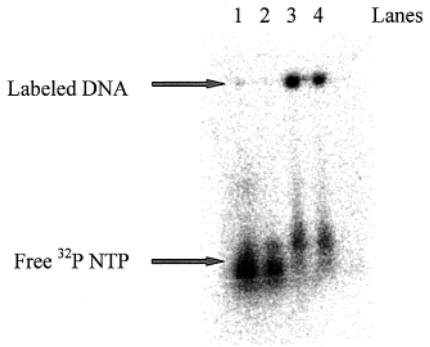

Figure 8. Agarose gel electrophoresis of Cu−neamine-treated pT7-7 after incubation with T4 PNK and [γ-^{32} P] ATP (1) under conditions favoring the exchange reaction, (2) under conditions favoring the forward reaction, and (3) after CIAP treatment. Lane 4 shows Cu−neamine-treated pT7-7 following incubation with TdT and [α-^{32} P] dGTP.

pH 7.9. At this pH, the metal center lies closer to the amino functional group than to the alkoxy oxygen (where the latter coordinates to Cu^{2+} as a protonated hydroxyl). This influences the relaxation rates of the carbon bearing the amino group to a larger extent than the carbon atom with the alkoxy oxygen. Under more basic conditions (pH 9.75), we found that the relaxation times of C_3''-NH_2 and C_4''-OH are influenced to similar extents, indicative of the fact that the metal ion is equidistant from both binding sites. This would be possible only if the hydroxyl oxygen atom were deprotonated and bound to the Cu^{2+} center as an alkoxide. The latter conclusions are supported by C-13 relaxation measurements carried out at pH 5.40, wherein all the amino groups in kanamycin A are protonated. Weaker binding, or rapid exchange, is expected for Cu^{2+} binding to kanamycin A and the relaxation rates are not influenced to the extent observed at pH 7.90 and 9.75.

Neamine shows a more complex pattern of relaxation behavior than kanamycin A; however, coordination of Cu^{2+} to ring A is supported by our C-13 T_1 relaxation measurements. Carbon-13 relaxation data obtained for neamine in the presence of Cu^{2+} indicate that Cu^{2+} binds to neamine through coordination to $C_{2'}$-NH_2, $C_{1'}$-O, and C_3-NH_2. This structure was refuted on the basis of H-1 NMR relaxation studies,[30] since the paramagnetic contribution to T_1 (T_1^e) were negligible for $C_{1'}$-O and C_3-NH_2; however, we find that the relaxation time of the C_4 carbon is not influenced by Cu^{2+}, and so this supports the view that the glycosidic oxygen of neamine is not involved in copper binding. Similar to kanamycin A, the other carbon atoms in the skeleton of neamine are not influenced or show a negligible change in their T_1 relaxation times. The only reasonable structure for the Cu^{2+} chelate of neamine that is consistent both with our C-13 relaxation studies, and with those of H-1 relaxation measurements by Mobashery and co-workers,[30] is depicted in Figure 1 (top), where Cu^{2+} binds to the C_2'-NH_2 and C_3'-OH of ring A. Further support for this structure is provided when neamine is treated with BOC anhydride in the presence of $Cu(OAc)_2$, yielding a C_6' and C_3 amino-protected neamine derivative, clearly indicating that C_3-NH_2 is not bound to Cu^{2+}. Additionally, the signals corresponding to C_2'-NH_2 broaden and disappear upon increasing the concentration of Cu^{2+} (100 μM), indicating a stronger paramagnetic effect at this center.[24]

Copper ion binding to kanamycin A and neamine through their vicinal NH_2 and OH groups seems to indicate a mechanism of binding specificity based on the stereochemical orientation of these amino and hydroxyl groups. While the binding of nitrogen lone pairs to Cu^{2+} is unidirectional and provides a stable bond, a strong chelate depends on a vicinal hydroxyl group which has two lone pairs to share. Upon deprotonation, the alkoxide ligand provides a stronger bond to Cu^{2+} and thereby forms a more stable chelate. Since there are several contiguous amine/hydroxyl sites in aminoglycoside antibiotics, formation of a specific Cu^{2+} chelate is defined by the angle and orientation of the amine and hydroxyl functional groups. Such a requirement eliminates many possibilities in kanamycin A (such as C_1-NH_2; C_5-OH; C_3-NH_2; and C_2'-OH) from binding Cu^{2+} strongly. Also, this requirement eliminates C_3-NH_2; C_1-NH_2 and C_6-OH from Cu^{2+} binding to neamine (vide infra). Other aminoglycosides such as neomycin B have more than one pair of contiguous amine/hydroxyl groups for binding. However, as a result of electrostatic repulsion with neighboring protonated amines the Cu^{2+} complexes seem to be unstable, making structural characterization difficult. Tobramycin, another aminoglycoside of interest, differs from kanamycin A in two positions and has C_2'-NH_2 and C_3'-OH functional groups in ring A. However, ring C in tobramycin retains the same orientation of the amine/hydroxyl sites and would most likely bind Cu^{2+} in ring C, as found for kanamycin A. Thus, the trends that are established from published results and those discussed in this paper show the unambiguous binding of Cu^{2+} to $3''$-NH_2 of kanamycin A and $2'$- NH_2 of neamine.

DNA Cleavage and Efficiencies. Published reports of enhancements to rate constants (0.09−0.25 h^{-1}) for DNA hydrolysis by metal complexes are impressive[10−13,34,35] but are still far from the rate enhancements produced by natural enzymes. The first-order rate constants for the hydrolysis of a phosphodiester bond in single-stranded and double-stranded DNA under physiological conditions have been estimated as 6×10^{-9} and 6×10^{-10} min^{-1}, respectively,[36] while hydrolysis of an inactivated phosphodiester lies around 6×10^{-14} min^{-1}.[37] The extraordinary stability of DNA toward hydrolysis is biochemically essential for it to act as genetic material but poses a challenge for the synthesis of novel phosphodiesterases or nucleases that can promote hydrolysis in a reasonable time scale.

In our laboratory, DNA cleavage by copper−aminoglycosides has been performed under hydrolytic conditions in the absence of external reducing reagents (such as H_2O_2 or ascorbate).[14] While Cu^{2+}−(kan A) was observed to cleave DNA at micromolar concentrations, it was found that rapid DNA degradation occurred (smear observed on the gel) and proved difficult to follow by standard kinetic techniques. Cu^{2+}−neamine was also found to cleave DNA under similar conditions, but in a more controllable fashion. A concentration-dependent cleavage was observed that was followed kinetically using quantitative agarose gel electrophoresis. Under conditions of constant DNA concentration and varying catalyst concentration (pseudo Michaelis−Menten conditions), we obtained $V_{max}' = 0.031$ min^{-1} and $K_M = 3.9$ μM, providing a rate enhancement of around 5.2×10^7 for ds DNA cleavage. Under Michaelis−Menten conditions of constant catalyst concentration (100 μM) and varying substrate concentrations, we were able to extract $k_{cat} = 5.95 \times$

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10^{-4} min^{-1} (from $V_{max} = 0.0595$ min^{-1}) and $K_M = 41.6$ μM, thus enhancing DNA cleavage by about a million-fold. In previously reported work, V_{max} has been inappropriately used to calculate rate enhancements.[10−13,34,35] *Our experimental V_{max} value of 0.0595 min^{-1} at saturating concentrations of DNA and 100 μM catalyst (Cu−neamine) concentration indicates a rate enhancement of about 100 million-fold over uncatalyzed DNA cleavage!* Though dramatic, these values are uncorrected for catalyst concentrations and should not be used to calculate rate enhancements. True rate enhancements should be calculated using k_{cat} (where k_{cat} is defined as V_{max}/[catalyst]), rather than using either k_{obs}' or V_{max}' values.

To the best of our knowledge, the rate enhancements under these conditions are the largest reported yet for any metal complex (after revision of published data to eliminate differences arising from definition of concentrations) and probably reflect the large binding constants for Cu^{2+}−neamine to plasmid DNA ($\sim 2.6 \times 10^5$ M^{-1}). In fact, the rate enhancement for Cu^{2+}−(kan A) cleavage is even greater. We have shown previously that neomycin B and kanamycin A bind nucleic acid components with affinities in the range of 3.9×10^3−7.0×10^4 M^{-1}.[21] Accordingly, while the aminoglycoside ligand provides tight binding to the negatively charged phosphate backbone in DNA, the metal center mediates phosphodiester cleavage.

Having demonstrated that metal−aminoglycosides provide a greater than 50 million-fold rate enhancement in DNA cleavage under pseudo-Michaelian conditions, we directed our efforts to understanding and comparing the efficiencies of various complexes in such a reaction. Table 1 records the efficiencies (defined as *S*/*M*, where *S* is the average number of strand scissions per DNA molecule, and *M* is the concentration of complex to achieve such cleavage) of various published DNA cleavage agents. As can be seen in this table, the *S*/*M* value of Cu^{2+}−aminoglycosides is up to 2 orders of magnitude greater than those for other nucleases based on mononuclear copper, cobalt, or lanthanide complexes, or 30-fold higher than the dinuclear Co^{3+} complex. This is probably due to the higher binding affinity of the positively charged aminoglycoside ligands to DNA compared to the neutral or less positively charged ligands. This is also reflected in the K_M values, which are indicative of tight binding and relative affinity of the substrate−catalyst Michaelian complex. The prominent difference in the efficiency of Cu^{2+}−(kan A)- over Cu^{2+}−neamine-mediated cleavage can be explained by the higher positive charge in the former, resulting in higher binding constants. Comparison of literature reports indicates that rings A and B form the core elements in recognition and binding of aminoglycoside antibiotics to nucleic acids.[38] The Cu^{2+} ion is bound to ring C in kanamycin A, which makes the fewest electrostatic or hydrogen-bonding contacts with target nucleic acids.[21] Thus, the binding capacity of rings A and B is not inhibited by Cu^{2+} binding to kanamycin A. However, ring A in neamine, which also binds Cu^{2+}, provides both electrostatic and hydrogen-bonding contacts to nucleic acids.[21] Such binding to Cu^{2+} probably diminishes some electrostatic contacts between the amine groups of neamine and the phosphate backbone of DNA.

Hydrolytic Versus Oxidative DNA Cleavage. A hydrolytic degradative pathway of DNA by Cu^{2+}−(kan A) is supported by several experimental criteria. In particular, the DNA cleavage reactions were not inhibited by radical scavengers nor under anaerobic conditions. However, when a reductant (ascorbic acid or DTT) was deliberately added to the reaction mixture, DNA cleavage was inhibited in the presence of all scavengers (azide, SOD) and under anaerobic conditions. The lack of hydroxyl radicals in DNA cleavage by Cu−aminoglycosides was also verified by use of rhodamine B as the reporter molecule in an absorption assay. We have also carried out EPR experiments in the presence of a radical-trapping agent, DMPO, and copper−aminoglycosides. The Cu^{2+} complexes did not show DMPO-trapped hydroxyl radicals in the absence of ascorbate or H_2O_2. Addition of H_2O_2 to the reaction mixture containing Cu^{2+}−(kan A) and DMPO gave rise to a quartet characteristic of a DMPO−OOH adduct. This indicates that production of ROS by copper−aminoglycosides could be initiated *only* in the presence of reagents such as H_2O_2. Additionally, plasmid DNA could be religated after cleavage by Cu^{2+}−(kan A) and could be transformed back into DH5α cells with efficiencies of around 75% (Supporting Information). If a mixture of hydrolytic and oxidative cleavage had occurred on the plasmid DNA, then religation and further transformation would not have been detected. Hence, these assays of DNA cleavage by Cu^{2+}−(kan A) are indicative of a hydrolytic cleavage pathway. We have previously published gel electrophoresis data that support religation.[14]

High-resolution polyacrylamide gel analysis of products generated from DNA upon copper−aminoglycoside treatment reveals comigration with oligo $(dT)_{4-22}$ fragments possessing a 5′-phosphate and 3′-hydroxyl groups. This result demonstrates DNA cleavage by these Cu^{2+} complexes to be hydrolytic in nature. No piperidine or aniline treatment (usually required for oxidative cleavage and base loss) was required to generate fragments before analysis by gel electrophoresis experiments.[39] In fact, by use of HPLC assays we have found that base release for DNA cleavage is achieved only under oxidative conditions, where either ascorbic acid or H_2O_2 were used in concert with Cu−aminoglycosides (to be published).

Further characterization of the phosphorylation state of the ends generated following hydrolysis by Cu−aminoglycoside treatment was obtained from experiments using the specific reactivity of T4 PNK and TdT. T4 PNK specifically requires a free 5′-OH group to add the γ-phosphate of ATP. Should the 5′-end be phosphorylated, labeling is possible only after prior treatment with CIAP to generate a free 5′-OH groups. In turn, TdT adds an α-phosphate from dNTPs only to the 3′-hydroxyl groups of DNA. Following hydrolytic cleavage by Cu−neamine, the plasmid can have either a 5′-phosphate or a 5′-hydroxyl group. Reaction of the Cu−neamine-treated plasmid with T4 PNK under "forward reaction" conditions did not yield a signal when the sample was analyzed by autoradiography following gel electrophoresis (Figure 8). However, when we treated with CIAP first (to dephosphorylate the 5′-end to generate 5′-OH) and then carried out the forward T4 PNK reaction, we could quantitatively measure γ-^{32}P incorporation into the product DNA fragments (Figure 8). This indicates that the DNA possesses a 5′-phosphate following treatment with Cu−neamine. Further support of such a hydrolytic mechanism, yielding 5′-phosphate and 3′-OH termini, was provided when we treated the DNA with [α-^{32}P] dGTP and TdT. TdT is a template-independent polymerase that catalyzes the incorporation of [α-^{32}P]-dG only when the 3′-end of DNA has a free hydroxyl group.[40] As shown in Figure 8, [α-^{32}P] was readily incorporated into the Cu−

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neamine-treated DNA. The necessity to dephosphorylate before incorporation of 5′-phosphate using PNK, and facile incorporation of [α-^{32}P]-dG, clearly demonstrate the generation of both 5′-phosphate and 3′-hydroxyl groups in pT7-7 upon treatment with copper−aminoglycosides.

Comparison of Kinetic Analyses. Recently it has been demonstrated that DNA hydrolysis rate enhancements on the order of 10^7 are achievable by binuclear Co^{3+} and lanthanide metal ion complexes, though the concentrations used are not of biological relevance. The characterization of such reactions has typically utilized a pseudo Michaelis−Menten kinetic profile, and the maximal rate of hydrolysis with varying catalyst concentration has been reported as a "k_{cat}" value. This model represents a deviation from regular Michaelis−Menten enzyme kinetics, wherein the true characterization depends on varying the substrate concentration while keeping a constant catalyst concentration. In various literature examples reporting kinetics of DNA hydrolysis, the modified model has been applied and Michaelis−Menten-"type" reaction kinetics have been reported to give rate enhancements.[8,10−13,35] The data are valid at maximum concentrations of the catalyst employed, but this method does not represent an accurate characterization of the catalytic behavior using the Michaelis−Menten equations. Herein we report measurement of the efficiency of Cu^{2+}−neamine under both pseudo-Michaelis−Menten conditions, and also under "true" Michaelis−Menten reaction conditions. The data are presented in Table 4 for various complexes reported in the literature. Cu^{2+}−neamine showed k_{obs}' of around 0.031 min^{-1} at the highest concentration employed, thus enhancing the rate of DNA hydrolysis by 5.2×10^7 times. The rate is approximately twice as efficient as the binuclear Co^{3+} complex reported by Schneider's group, although the plasmid employed, the catalyst concentration, and the pH are not necessarily the same. Table 4 also shows the specificity constants, "k_{cat}'/K_M" values for various complexes, which give the hydrolysis rate enhancement per micromolar catalyst concentration. The specificity constants for Cu^{2+}−neamine under pseudo-Michaelis−Menten conditions reach a value of 4.8×10^5 h^{-1} M^{-1}, which is 2 orders of magnitude greater than those seen for various metal ions, including lanthanides. However, under Michaelis−Menten conditions we obtained $V_{max} = 0.0595$ min^{-1}, $k_{cat} = 5.95 \times 10^{-4}$ min^{-1}, and $K_M = 41.6$ μM after fitting the data to the Michaelis−Menten equations. The k_{cat} values obtained under such conditions gave a hundred-million-fold rate enhancement over uncatalyzed DNA hydrolysis and are accurate rate enhancements. Since the values reported in the literature are uncorrected for the concentration terms of the catalysts or substrates, they represent rate enhancements at a particular substrate concentration and are not good indicators for comparison with enzyme catalysts. Discrepancies in K_M values between pseudo and true Michaelis−Menten conditions most likely reflect the distinct binding equations (2a and 2b) used. This suggests that caution should be taken when interpreting K_M values from pseudo-Michaelian conditions.

Comparison with Literature Results. Given the stability of DNA toward hydrolysis (half-life is approximately 130 000 years at neutral pH and 25 °C), it is not surprising that agents that promote efficient DNA hydrolysis are rare. Both copper complexes[34] (for example, $[Cu(tach)(OH)(OH_2)]^{2+}$, $[Cu(9)aneN_3]^{2+}$), and cobalt complexes of cyclen, tamen, and trpn,[12,13] have been demonstrated to hydrolyze DNA.

Schneider's group has provided evidence for hydrolysis of phosphodiester bonds in DNA and activated esters.[10−13,35] They also demonstrate that increasing the charge by addition of alkylammonium side chains to the Co(III)-binding ligands increased the rate of hydrolysis of form I DNA due to enhanced binding. They reported that, by use of pseudo-Michaelis−Menten conditions, the rate constant remained fairly constant, while the dissociation constant, K_M, increased with increasing charge on the ligands. Similar saturation kinetic studies were also carried out for lanthanide ions and their macrocyclic complexes. Additionally, it was found that dinuclear complexes enhance the rate of hydrolysis, indicating cooperativity between the two Lewis acid centers.

Novel copper-containing nucleases that work by "self-activating" mechanisms have been reported recently.[39] Most of these systems undergo a $Cu^{2+} \rightarrow Cu^{+}$ reduction in the absence of an externally added reducing agent. Such electron transfers are suggested to be from ligands coordinated to Cu^{2+} (alkyl resorcinols or tambjamine).[39] In a typical DNA cleavage experiment, Cu^{2+} reduction to Cu^{+} follows activation of molecular O_2 or H_2O_2, producing ROS. Addition of neocuproine (specific for Cu^{+}) inhibits cleavage by alkyl resorcinols, while anaerobic conditions depleted the nuclease activity of a tambjamine−Cu system.[39] These experiments suggest that DNA cleavage under "self-activating" conditions proceeds via an oxidative pathway. Similar oxidative cleavage of DNA by metallo-(hydroxy-salen) complexes by production of free radicals and a Cu^{2+}/Cu^{3+} redox cycle has been reported.[39c,d] $[Cu(9)aneN_3]^{2+}$ cleaves DNA in both an O_2-dependent and an O_2-independent manner.[34] This makes it one of the few complexes that are able to cleave DNA under anaerobic conditions, presumably by a hydrolytic mechanism.

In our hands, DNA cleavage by Cu^{2+}−aminoglycosides was not inhibited by free radical quenchers (e.g., NaN_3, DMSO, EtOH, SOD), nor under anaerobic conditions. High-resolution PAGE experiments indicate that the products generated do, indeed, comigrate with hydrolysis products. Enzymatic reactions with T4 PNK and TdT demonstrate exclusive generation of 5′-phosphate and 3′-hydroxyl termini. These experiments support the idea that Cu^{2+}−aminoglycosides mediate DNA cleavage in micromolar concentrations via a hydrolytic pathway. In the presence of micromolar concentrations of ascorbate, nanomolar levels of Cu^{2+}−(kan A) yielded linear DNA,[14] indicating nonrandom cleavage by an oxidative mechanism. Additionally, when ascorbic acid was added externally to facilitate a $Cu^{2+} \rightarrow Cu^{+}$ reduction, DNA cleavage reactions were inhibited by all of the above-mentioned quenching agents. Under anaerobic conditions, and in the presence of ascorbic acid, Cu^{2+}−(kan A) did not cleave DNA, indicating the importance of molecular oxygen in such an oxidative mechanism. However, under similar conditions, reduction of $Cu^{2+} \rightarrow Cu^{+}$ still takes place and is supported by EPR studies. These experiments indicate that while one-electron reduction by ascorbic acid generates an active Cu^{+} complex that activates molecular oxygen and/or H_2O_2, such a reduced Cu^{+} species is not mandatory for activating a nonoxidative or hydrolytic DNA cleavage mechanism by Cu−aminoglycosides. Accordingly, DNA cleavage by Cu^{2+}−aminoglycosides proceeds via two distinct mechanisms: one is dependent on O_2 and an external reducing agent, thereby causing oxidative damage to DNA at extremely low (nanomolar) concentrations of Cu^{2+}−(kan A), and the other is O_2 independent and does not require activation by reducing agents at micromolar levels of Cu^{2+}−(kan A). Our identification of Cu^{2+}−aminoglycoside complexes as effective DNA cleavage agents,[14,41] with excellent rate enhancements, significantly adds to the repertoire of available artificial nuclease reagents.

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Supporting Information Available: Table of transformation efficiencies from a T4 DNA ligase assay, gel data showing the influence of NaN_3, DMSO, EtOH, and SOD on the cleavage of DNA by Cu−neamine, the cleavage of plasmid by various transition metal−kanamycin complexes, the time course of DNA cleavage, and interactions of kanamycin A and Cu^{2+}(aq) on plasmid DNA (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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