Inorg. Chem. **2003**, *42*, 1420–1429

Solution Chemistry of Copper(II)–Gentamicin Complexes: Relevance to Metal-Related Aminoglycoside Toxicity

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Received August 21, 2002

The adverse effect to the inner ear of aminoglycosides, drugs widely administered for the treatment of serious infections, appears to result from the interaction of these drugs with Cu(II) or Fe(II)/Fe(III) ions. To understand more completely the metal-induced side effects of one such antibiotic, gentamicin, we studied copper(II) coordination to gentamicin C1a by potentiometry, UV–vis, CD, and EPR spectroscopies, and ESI mass spectrometry. Only monomeric complexes of the CuH_nL stoichiometry, with n ranging from 3 to −2, were detected over the pH range of 4–12. CuH_3L and CuH_2L complexes exhibit the same coordination mode, binding copper(II) through the amino nitrogen atom and a deprotonated alcoholic oxygen atom of the garosamine ring. In the CuHL and CuL complexes a second amino nitrogen atom of the purpurosamine ring participates in central ion coordination. Finally, the additional axial binding of the deprotonated oxygen of the hydroxyl group of the 2-deoxystreptamine moiety occurs in the $CuH_{-1}L$ and $CuH_{-2}L$ complexes. Interactions of the Cu(II)–gentamicin–H_2O_2 system at pH 7.4 with *N*,*N*-dimethyl-*p*-nitrosoaniline, arachidonic acid, and plasmid DNA confirmed that gentamicin complexes facilitate oxidative reactions leading to peroxidation of arachidonic acid and scission of double-stranded DNA mediated by copper-bound reactive oxygen species. However, the stability constants of Cu(II)–gentamicin complexes are inferior to the binding constants of copper(II) complexes with other components of human serum or cells. Computer simulations of copper(II) distribution in the human blood plasma showed that the concentration of gentamicin would have to be at impossible levels (100 M) before a significant fraction of Cu(II) ions would be bound to gentamicin. Further, once introduced into aqueous solution, histidine replaces gentamicin in Cu(II)–gentamicin complexes. Therefore, Cu(II)–gentamicin complexes might not exist under physiological conditions.

Introduction

Gentamicin C (Figure 1) is one of the aminoglycoside antibiotics that is widely used for treatment of serious infection caused mainly by Gram-negative and some Gram-positive organisms.[1] Commercial gentamicin C contains three closely related analogues, which differ in the degree of methylation of the purpurosamine unit. Aminoglycosides kill bacteria primarily by inhibiting the translation step in microbial protein synthesis.[2] A major problem in therapy with aminoglycosides is their relatively high toxicity to the kidney and the inner ear. Nevertheless, gentamicin is currently the first choice antibiotic in developing countries and is still widely used in industrialized countries for the treatment of serious bacterial infections.[1]

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10.1021/ic025965t CCC: $25.00
Published on Web 02/07/2003

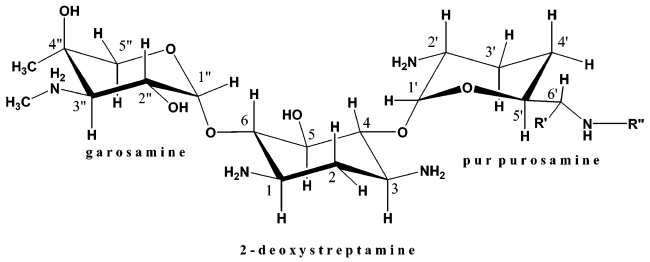

Figure 1. Structure of the three main derivatives of gentamicin sulfate: gentamicin C1, R′=R″=CH_3; gentamicin C2, R′=CH_3 and R″=H; gentamicin C1a, R′= R″= H.

The adverse affects of aminoglycosides may result from interaction with transition metal ions and oxidative reactions promoted by forming metal complexes.[3−5] Coadministration of transition metal chelators and free radical scavengers, as well as overexpression of superoxide dismutase in model animals, suppress aminoglycoside-induced ototoxicity.[6−8] Systematic in vitro studies of iron interactions with gentamicin have led to a postulated mechanism of toxicity involving free radical formation by Fe(II)/Fe(III)−gentamicin complexes.[3−5]

Another body of evidence[9−19] has suggested that both pharmacological activity and toxicity of aminoglycoside antibiotics could be related to copper(II)−aminoglycosides complexes. Jezowska-Bojczuk et al. extensively investigated chelation of copper(II) ions by gentamicin-related aminoglycoside antibiotics using potentiometry and a variety of spectroscopic techniques.[9−12] Kanamicin B, tobramicin, geneticin, and amikacin strongly bind Cu(II) ions, forming monomeric complexes over a wide pH range. In naturally occurring aminoglycosides, the amino nitrogens and deprotonated alcoholic oxygens of the terminal aminosugar rings are involved in the coordination, forming five- and six-membered chelate rings about central ions.[9−11] Amikacin, a semisynthetic derivative of kanamicin A, having the 1-amino group on the 2-deoxystreptamine moiety modified by acylation with 4-amino-2-hydroxybutyric acid, exhibits different binding modes by involving the amidated nitrogen in coordination.[12] Further, Cu(II)−amikacin complexes catalyze hydrogen peroxide disproportionation at pH 7.4 mediated by hydroxyl radicals and involving Cu(I)/Cu(II) and Cu(II)/Cu(III) redox pairs.[13] These complexes mediate oxidation of 2′-deoxyguanosine to 7,8-dihydro-8-oxo-2′-deoxyguanosine, double-stranded DNA cleavage, and both hydrolytic and oxidative t-RNA^{Phe} strand scission at a specific site in the anticodon loop.[14] Under these circumstances, copper(II) ions are proposed to be involved in aminoglycoside toxicity.

Cowan and co-workers studied the interaction of other aminoglycosides, such as kanamicin A and neomycin copper(II) complexes, with a variety of nucleic acids.[15−19] In the presence of H_2O_2 and ascorbic acid these complexes promote highly specific double-stranded DNA scission by binding to the minor groove of DNA and abstracting the 4′-C hydrogen of the deoxyribose moiety.[17] Cu(II)−kanamicin A complexes are also more effective than free kanamicin A in cleaving RNA targets within bacterial cells, suggesting a potential application for copper(II)−aminoglycoside complexes as new metal-based drugs.[19]

In this paper we present potentiometric and spectroscopic studies of Cu(II)−gentamicin complexes, an examination of the redox activity of the resulting complexes, investigation of the competition between gentamicin and bioligands using a computer simulation of copper(II) ion distribution in human blood plasma, and direct measurements of the competition between gentamicin and L-histidine for binding copper(II) at physiological pH.

Materials and Experimental Section

Abbreviations used: AA, ascorbic acid; G, gentamicin; GC*x*, gentamicin C*x*; Cu(II)−GCx, cupric complexes of gentamicin C*x* (*x* = 1, 1a, 2, or 2a).

Gentamicin C complex, containing GC1, GC1a, GC2, and GC2a, was obtained as the sulfate salts from Spectrum Chemicals. Acetonitrile and potassium nitrate were purchased from Fisher Scientific. Trifluoroacetic acid (TFA), copper(II) nitrate, zinc nitrate, *N*,*N*-dimethyl-*p*-nitrosoaniline (NDMA), nitro blue tetrazolium (NBT), arachidonic acid, Chelex 100, and the strong basic anion exchanger Amberlite IRA-400 were obtained from Sigma Chemical Co. pBR322 plasmid DNA was obtained from New England Biolabs. All stock solutions were prepared using deionized, doubly distilled water.

High-Pressure Liquid Chromatography (HPLC). Three closely related analogues of gentamicin, C1a, C1, and C2, were separated from commercial gentamicin sulfate by HPLC using a Waters Prep LC 2000 system equipped with a Hamilton PRP1 reversed phase column (250 × 21.50 mm, particle size 12−20 μm), a Waters variable wavelength absorption detector set at 213 nm, a Rheodyne injector with a 2 mL loop, and an HP 3395 integrator. The mobile phase consisted of 0.1 M TFA and 0.5 M acetonitrile. The flow rate was 9 mL/min. The method has been described elsewhere.[20]

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Potentiometry. Solutions of copper(II) with the purified, individual gentamicin components (GC1a, GC1, and GC2) as chloride salts and zinc(II) with gentamicin C1a were titrated with carbonate-free KOH in a jacketed cell maintained at 25 °C by an external circulating water bath. All sample solutions were adjusted to 0.1 M ionic strength with KNO_3 and were protected from atmospheric carbon dioxide by continuously purging the cell with CO_2-free argon. The titrations were controlled by a custom-built autotitrator running within Labwindows v 2.1 on a PC. The autotitrator included a Dosimat model 655 autoburet equipped with a 1 mL cylinder so that the titrant could be delivered in 1 μL increments. The pH values were measured by a Fisher model 25 pH meter equipped with a Hamilton combination electrode that was calibrated daily to read the hydrogen ion concentration by titrations of nitric acid. The autotitrator prompted the addition of an aliquot of titrant, monitored the pH as a function of time until the drift fell below a preset maximum value, recorded the volume and pH, and then made the next addition of titrant. The sample volume was 2 mL. Concentrations of ligands were 2×10^{-3} M, and metal:ligand molar ratios, 1:1, 1:2, and 1:4. Data were analyzed using the computer program Superquad.[21]

Circular Dichroism Spectroscopy (CD). The CD spectra were recorded using an Aviv circular dichroism model G2DS spectropolarometer over the range of 190−800 nm, using 1 and 0.1 cm cuvettes. Gentamicin C1a and copper(II) concentrations were 6×10^{-3} and 3×10^{-3} M, respectively. Studies of the competition of gentamicin and histidine toward Cu(II) binding were performed in Tris buffer (1×10^{-1} M, pH 7.4). Three different samples were investigated: (1) Cu(II):gentamicin sulfate 1:2 molar ratio; (2) Cu(II):histidine 1:2 molar ratio; (3) Cu(II):gentamicin sulfate:histidine 1:2:2 molar ratio with Cu(II) concentration at 1×10^{-3} M. Spectra are expressed in terms of $\Delta\epsilon = \epsilon_l - \epsilon_r$, where ϵ_l and ϵ_r are molar absorption coefficients for left and right circularly polarized light, respectively.

Electronic Absorption (UV−Vis) Spectroscopy. Spectra were obtained using a Perkin-Elmer Lambda 9 spectrophotometer over the spectral range of 190−1000 nm in 1 and 0.1 cm cuvettes. The same samples were investigated as in CD measurements. The peroxidation of arachidonic acid was monitored by measuring the conjugated diene absorption at 235 nm. Incubations were carried out at 37 °C for 60 min. All reaction mixtures (200 μL) contained 0.25% arachidonic acid (v/v), sodium phosphate buffer (5×10^{-3} M, pH 7.4), and additional components as listed: (1) H_2O_2 (5×10^{-4} M); (2) gentamicin sulfate (1×10^{-4} M) + H_2O_2 (5×10^{-4} M); (3) Cu(II) (5×10^{-5} M) + H_2O_2 (5×10^{-4} M); (4) Cu(II) (5×10^{-5} M) + gentamicin sulfate (1×10^{-4} M) + H_2O_2 (5×10^{-4} M); (5) Cu(II) (5×10^{-5} M) + gentamicin sulfate (1×10^{-4} M). Reactions were quenched by the addition of 1 mL of chloroform−methanol (2:1). Following vortexing and centrifugation, the organic phase was removed and dried at 45 °C under a stream of nitrogen. Cyclohexane (200 μL) was added to solubilize the residue of the sample, and spectra over the range of 200−600 nm were recorded in 1 cm cuvettes. A molar extinction coefficient of 2.52×10^4 M^{-1} cm^{-1} for the conjugated diene was used in calculations.

In the study of reactive oxygen species (ROS) formation, we used *N*,*N*-dimethyl-*p*-nitrosoaniline (NDMA), which is a reporter molecule for ROS such as singlet oxygen and hydroxyl radicals, and nitro blue tetrazolium (NBT), which is a reporter molecule for superoxide anion. The same samples were investigated as in the study of lipid peroxidation, but arachidonic acid was substituted by 1.72×10^{-5} M NDMA or 2×10^{-5} M NBT. Since NDMA is not specific for hydroxyl radical, we carried out the following experiment[13] to confirm that we were in fact detecting that species: 2 M ethanol was added to a solution of Cu(II) (5×10^{-5} M), gentamicin sulfate (1×10^{-4} M), H_2O_2 (5×10^{-4} M), phosphate buffer (5×10^{-3} M, pH 7.4), and NDMA (1.72×10^{-5} M). While in the absence of ethanol, the observed NDMA band at 440 nm decayed over time, indicating reaction with some ROS; in the presence of ethanol, the NDMA band did not decay. Since ethanol is a hydroxyl radical scavenger, this indicates that the ROS involved is indeed hydroxyl radical. An additional sample, containing ascorbic acid instead of H_2O_2, was also analyzed. The time courses of the reactions were monitored by periodically recording spectra over the entire wavelength range to find whether any absorbing reaction products would interfere with the detection of hydroxyl radicals. No interference was found. The reactions were monitored at 440 nm, the characteristic wavelength of NDMA. The studies using NBT showed no scavengable $O_2^{\bullet -}$ formation in both Cu(II)−gentamicin−H_2O_2 and Cu(II)−gentamicin−ascorbic acid systems.

In both the lipid peroxidation and the ROS formation studies all stock solutions except Cu(II), H_2O_2, and arachidonic acid were purified with Chelex 100 prior to use, to remove trace metal ions.

Electron Paramagnetic Resonance (EPR). The EPR spectra were recorded at 120 K using a Bruker EMX spectrometer at the X-band frequency (9.3 GHz). An ethylene glycol−water (1:2) solution was used as a solvent to obtain homogeneity of the frozen samples. The sample concentrations were the same as those reported for the CD and UV−vis experiments.

Electrospray Ionization Mass Spectrometry (ESI-MS). Mass spectra were obtained using a Micromass VG Platform instrument, equipped with a single quadrupole mass analyzer using the ESI technique, with nitrogen as a nebulizing gas at a flow rate of 350 L/h. The 10 μL samples were injected into a solvent stream of 50% acetonitrile and 0.1% formic acid. A scan range of m/z 50−2000 was used. Six different samples were analyzed at pH 7.4, after adjusting the pH with ammonium carbonate: (1) gentamicin C1a; (2) Cu(II)−gentamicin C1a (1:2 molar ratio); (3) histidine; (4) Cu(II)−histidine (1:2 molar ratio); (5) Cu(II)−gentamicin C1a−histidine (1:1:1 molar ratio); (6) Cu(II)−gentamicin C1a−histidine (1:2:2 molar ratio). All samples were prepared in water solutions, and acetonitrile was added to a final concentration of 50% to decrease surface tension. In samples 1 and 3, the final concentrations were 5×10^{-4} M gentamicin C1a and histidine, respectively. In all other samples the final concentration were relative to 5×10^{-4} M Cu(II).

Molecular Modeling. The energy-minimized model structures of Cu(II)−gentamicin C1a complexes were obtained using the commercial software Insight II 3.0.0 Molecular Modeling System. Energy minimizations were carried out using the esff force field in the Discover 3 module and entailed 300 steps of minimization to remove initial strain, followed by a 1 ps dynamics simulation with a 1 fs time step and finally a maximum of 1000 steps of energy minimization to obtain the final structure. All calculations were carried out with a distance dependent dielectric field.

DNA Strand Break Analysis. The ability of Cu(II)−gentamicin C1a complexes to induce single- and double-stranded breaks in plasmid DNA in the presence and absence of hydrogen peroxide and ascorbic acid was tested using pBR322 plasmid DNA. All samples contained 1×10^{-6} M DNA in 5×10^{-3} M sodium phosphate buffer (pH 7.4) and combinations of gentamicin C1a, Cu(II), H_2O_2, and ascorbic acid. The concentrations of these reagents were as follows: gentamicin C1a, 0 or 5×10^{-6} M; Cu(II), 0 or 5

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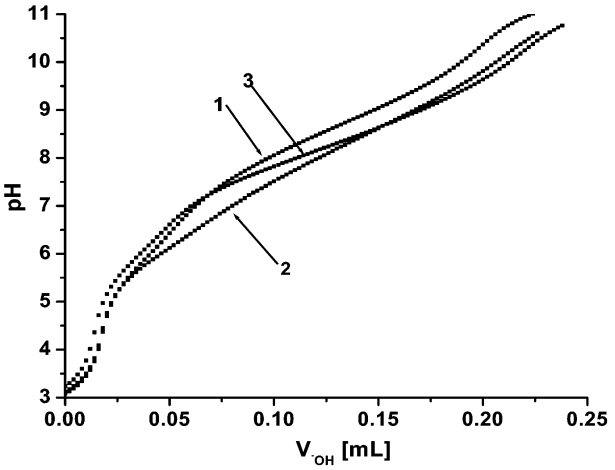

Figure 2. Potentiometric titration curves of (1) gentamicin C1a, (2) Cu(II)−gentamicin C1a 1:2 (metal to ligand molar ratio), and (3) Zn(II)−gentamicin C1a 1:2 (metal to ligand molar ratio).

$\times 10^{-6}$ M; H_2O_2, 0 or 5×10^{-5} M; ascorbic acid, 0 or 5×10^{-5} M. Systems containing ascorbic acid were incubated under both aerobic and anaerobic conditions. After a 1 h incubation at 37 °C, 20 μL reaction mixtures were mixed with 4 μL of loading buffer (bromophenol blue in 30% glycerol) and loaded on 1% agarose gels containing ethidium bromide prepared in TBE buffer. Separations were done at a constant voltage of 4 V/cm for 45 min. As the control for double-stranded breaks, pBR322 plasmid DNA was linearized with XhoI endonuclease. The gels were photographed using a Fotodyne digital image system. Except for the solutions of H_2O_2, DNA, and ascorbic acid, all stock solutions used in these studies were purified with Chelex 100 prior to use.

Results

Potentiometric Studies of Copper(II)− and Zinc(II)−Gentamicin Systems. Coordination of Cu(II) and Zn(II) ions was studied by potentiometric titrations at 25 °C over the pH range of 3−12. Four different samples were analyzed: Cu(II)−GC1a; Cu(II)−GC1; Cu(II)−GC2; Zn(II)−gentamicin C1a. The gentamicin concentrations were 2×10^{-3} M, and metal:ligand molar ratios were 1:1, 1:2, and 1:4. Figure 2 shows the titration curves of Cu(II)−gentamicin C1a 1:2 and Zn(II)−gentamicin C1a 1:2 systems. As can be clearly seen, coordination of Cu(II) and Zn(II) ions by gentamicin C1a starts at pH 5 and 7, respectively. Analysis of the titration curves using the computer program Superquad[21] yielded only monomeric species of the MH_nL stoichiometry with n ranging from 3 to −2 for copper(II) and 2 to −2 for zinc(II) compounds. Table 1 presents the stability constants of the resulting complexes. In these calculations the protonation constants of specific gentamicin components (collected in Table 2), obtained using the same potentiometric approach and ^{1}H NMR spectroscopy, were taken into account.[20] The values of the stability constants for Cu(II)−GC1a, Cu(II)−GC1, and Cu(II)−GC2 are in good agreement with those previously reported for other gentamicin related aminoglycosides.[9−12] The species distribution diagrams for Cu(II) and Zn(II) as a function of pH are shown in Figure 3A,B, respectively. These diagrams were derived for the concentrations used in the spectroscopic studies of copper(II) complexation by gentamicin C1a and using the formation constants presented in Tables 1 and 2. One can see that Cu(II)−GC1a complexes are much more stable than the corresponding species formed by Zn(II) with stability constants higher by ∼3 log units. Therefore, it seems unlikely that zinc ions contribute to the biological activity of aminoglycosides. For that reason, the coordination modes of Zn(II)−GC1a complexes were not studied in detail; however, stability constants of these species were used in computer simulation of Zn(II) and Cu(II) ions in human blood plasma (see section Computer Simulation of the Influence of Gentamicin C1a on Cu(II) and Zn(II) Distribution In Human Blood Plasma).

Table 1. Stability Constants (log β and pK_a Values) of Gentamicin C1a, C2, and C1 Complexes with Copper(II) and Gentamicin C1a with Zinc(II) [25 °C; $I = 0.1$ M (KNO_3)][a]

	gentamicin C1a		gentamicin C2		gentamicin C1	
species	log β	pKa	log β	pK_a	log β	pK_a
CuH_3L	30.68 ± 1		30.92 ± 1		30.73 ± 1	
CuH_2L	24.25 ± 1	6.43	24.34 ± 1	6.58	24.15 ± 1	6.58
CuHL	17.03 ± 1	7.22	17.15 ± 1	7.19	16.77 ± 1	7.38
ZnHL	14.05 ± 1					
CuL	9.25 ± 1	7.78	9.45 ± 1	7.7	8.93 ± 1	7.84
ZnL	5.57 ± 1	8.48				
CuH_{-1}	0.48 ± 1	8.77	0.56 ± 1	8.89	−0.13 ± 1	9.06
$ZnH_{-1}L$	−3.19 ± 1	8.76				
$CuH_{-2}L$	−9.51 ± 1	9.99	−9.50 ± 1	10.60	−10.15 ± 1	10.28
$ZnH_{-2}L$	−13.22 ± 1	10.03				

[a] $\beta(MH_nL) = [MH_nL]/[M][L][H^+]^n$.

Table 2. Protonation Constants (log β and pK_a Values) of Gentamicin C1a, C2, and C1[20 a]

	gentamicin C1a		gentamicin C2		gentamicin C1		
species	log β	pK_a	log β	pK_a	log β	pK_a	group
H_5L	39.689(1)	5.768	39.85(1)	5.83	39.801(1)	5.686	3
H_4L	33.921(1)	7.389	34.021(1)	7.421	34.115(1)	7.317	2′
H_3L	26.532(1)	8.181	26.600(1)	8.211	26.798(1)	8.112	1
H_2L	18.351(1)	8.87	18.389(1)	8.793	18.677(1)	8.877	3″
HL	9.491(1)	9.491	9.596(1)	9.596	9.86(1)	9.86	6′

[a] H_5L = fully protonated gentamicin.

Table 1 shows close agreement within copper(II)−gentamicin C1a, C2, and C1 complexes, confirming that methylation of the purpurosamine unit does not change the coordination properties within the gentamicin species studied. Because of these results, only gentamicin C1a was chosen for further studies of copper(II) coordination.

Spectroscopic Study of Cu(II)−Gentamicin C1a System. Table 3 lists spectroscopic parameters of the Cu(II)−GC1a complexes obtained from EPR, CD, and UV−vis techniques. The assignment of these parameters was done on the basis of previous studies of copper(II) chelation by simple aminosugars and gentamicin-related aminoglycosides.[11−13,22−28]

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Table 3. Spectroscopic Parameters of Cu(II)−Gentamicin C1a Complexes[a]

species and coordn mode	UV−vis		CD		EPR	
	λ	ϵ	λ	$\Delta\epsilon$	$g_{\|\|}$	$A_{\|\|}$
CuH_3L	640	41[b]	690	−0.02[b]	2.28	168
CuH_2L			298	−0.07[c]		
{N, O$^-$}	258	963[d]	251	+0.21[d]		
CuHL	581	95[b]	593	+0.31[b]	2.25	179
CuL			457	−0.25[b]		
{2N, O$^-$}			290	−3.05[c]		
	258	1511[d]	248	+2.43[d]		
$CuH_{-1}L$	581	70[b]	552	+0.28[b]	2.23	189
$CuH_{-2}L$			470	−0.06[b]		
{2N, 2O$^-$}			283	−4.73[c]		
	258	1511[d]	247	+2.54[d]		

[a] λ units are nm; ϵ and $\Delta\epsilon$ units are in dm^3 mol^{-1} cm^{-1}. A_{II} units are G. [b] d−d electronic transitions of Cu(II) in tetragonal complexes. [c] NH_2 → Cu(II) charge-transfer band. [d] O^- → Cu(II) charge-transfer band.

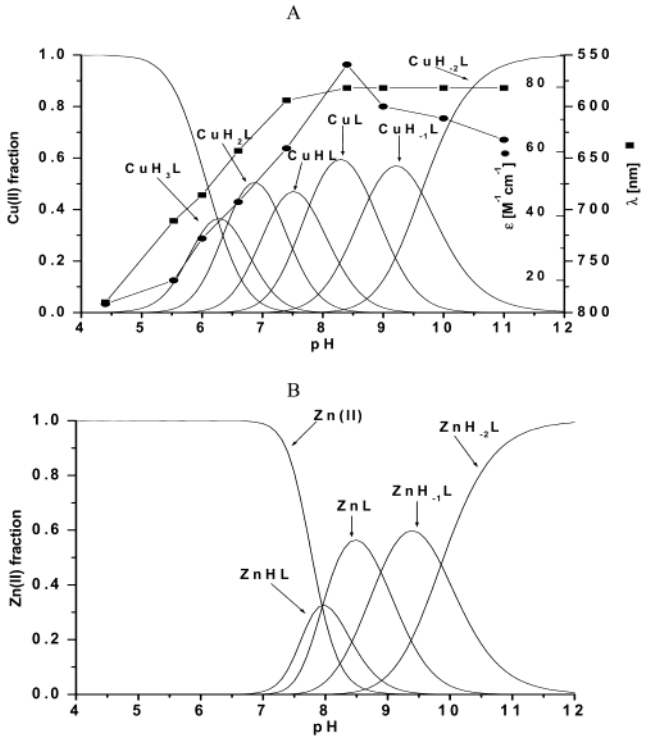

Figure 3. (A) Species distribution diagram of Cu(II)−gentamicin C1a complexes. Data calculated for concentrations used in UV−vis, CD, and EPR spectroscopic study and stability constants presented in Table 1. Dependences of λ_{max} (squares) and ϵ (cycles) on the d−d transition are overlaid. [Cu(II)] = 3×10^{-3} M; [gentamicin] = 6×10^{-3} M. (B) Species distribution diagram of Zn(II)−gentamicin C1a complexes with [Zn(II)] = 3×10^{-3} M and [gentamicin C1] = 6×10^{-3} M.

Figure 4 presents the parallel part of the EPR spectra of the Cu(II)−GC1a system recorded for frozen solutions at 120 K, in the pH range between 4.5 and 9.5. The pH values are approximate, due to the presence of 33% v/v of ethanediol and low temperature. However, these results are in good agreement with potentiometric speciation showing the presence of three distinct coordination modes {NH_2, O^-},

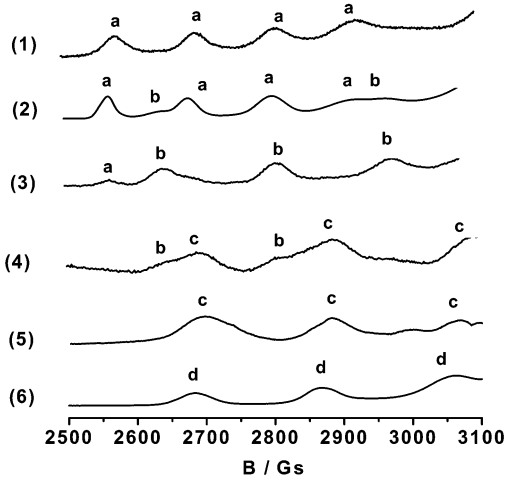

Figure 4. pH dependence of the parallel parts of EPR spectra of the frozen solution (120 K) of Cu(II)−gentamicin C1a system: (1) pH 4.5; (2) pH 5.5; (3) pH 6.5; (4) pH 7.5; (5) pH 8.5; (6) pH 9.5; (a) $Cu(H_2O)_6$; (b) CuH_3L, CuH_2L; (c) CuHL, CuL; (d) $CuH_{-1}L$, $CuH_{-2}L$. The pH values are approximate due to the presence of 33% v/v of ethanediol and low temperature.

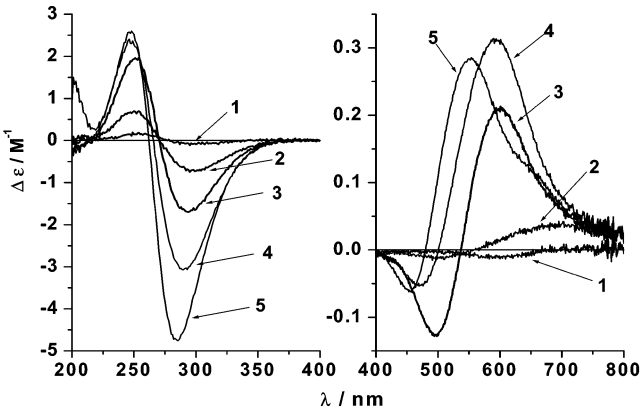

Figure 5. pH dependence of the CD spectra of the Cu(II)−gentamicin C1a system. Spectra were recorded at pH values as follows: (1) pH 5.5; (2) pH 6; (3) pH 7; (4) pH 8; (5) pH 10.

{$2NH_2$, O^-}, and {$2NH_2$, $2O^-$} for Cu(II)−GC1a complexes. Formation of the CuH_3L and CuH_2L complexes can be clearly seen in the spectra recorded at pH 5.5 and 6.5, where both the typical four line pattern of Cu(II) aqua ion and the resonances for Cu(II)−GC1a complexes were detected. The values of A_{II} and g_{II} of the CuH_3L and CuH_2L species indicate involvement of one nitrogen and three oxygens in the coordination of copper(II). The values of the EPR parameters obtained from the spectra recorded at pH 7.5 and 8.5, the region where the CuHL and CuL complexes are predominant, suggest formation of a species exhibiting the coordination mode with copper(II) bound to two nitrogen and two oxygen donors in equatorial positions. Further changes in values of A_{II} and g_{II} upon increasing the pH to 9.5, where $CuH_{-1}L$ and $CuH_{-2}L$ complexes are present, suggest distortion of the structure of these species, most likely due to an additional oxygen donor binding axially from the gentamicin molecule.

The Cu(II)−GC1a system was further studied using CD spectroscopy (Figure 5). The two charge transfer (CT) bands at 251 and 298 nm in the spectrum recorded at pH 5.5 could be easily assigned as O^- → Cu(II) and NH_2 → Cu(II)

(26) Jezowska-Bojczuk, M.; Chruscinska, E.; Trnka, T.; Micera, G. *J. Inorg. Biochem.* **1996**, *63*, 231−239.
(27) Jezowska-Bojczuk, M.; Lesniak, W. *J. Inorg. Biochem.* **2001**, *85*, 99−105.
(28) Jezowska-Bojczuk, M.; Kozlowski, H.; Trnka, T.; Cerny, M. *Carbohydr. Res.* **1994**, *253*, 19−28.

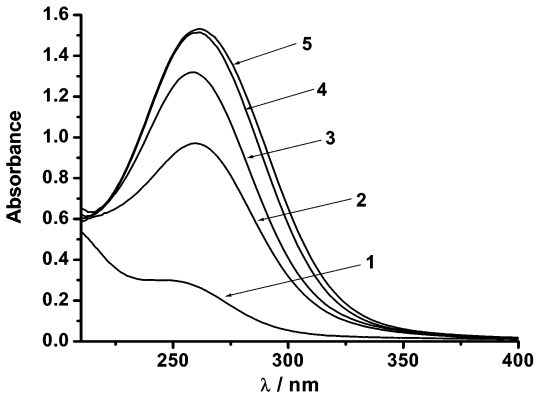

Figure 6. pH dependence of the UV part of absorption spectra of the Cu(II)−gentamicin C1a system. Spectra were recorded at pH values as follows: (1) pH 5.5; (2) pH 6.5; (3) pH 7.5; (4) pH 8.5; (5) pH 9.5.

transitions, respectively. This indicates that coordination of copper(II) by gentamicin C1a in the CuH_3L complex occurs through oxygen and nitrogen. The coordination of a $\{NH_2, O^-\}$ donor set in the CuH_2L complex is well supported by an increase of both CT bands and the d−d transition below 700 nm and the value of $\Delta\epsilon$ above 0.1. Formation of CuHL and CuL complexes resulted in further intensification of both CT and d−d bands, suggesting equatorial binding of the next nitrogen donor, which is in good agreement with EPR spectroscopy. Upon transition to $CuH_{-1}L$ species, an increase in intensity of the band at 290 nm and a shift of the d−d transition toward higher energy were observed. These observations are most likely due to the additional binding of an oxygen donor in an axial position.

Complexation of copper(II) by gentamicin C1a was also studied by UV−vis spectroscopy. The dependence of λ_{max} and ϵ of the d−d transitions are in excellent agreement with speciation obtained from potentiometry (see Figure 3). The values for the ϵ of d−d bands obtained for CuH_3L and CuH_2L fall in the characteristic range for copper(II) complexes with an $\{NH_2, O^-\}$ donor set. The presence of an $O^- \rightarrow$ Cu(II) transition at 258 nm (Figure 6) corresponds to bands in the CD spectrum at 251 nm, which clearly supports the binding of a deprotonated oxygen ligating group. The shift and increase in intensity of the d−d transitions upon conversion to the CuHL and CuL species are consistent with formation of the $\{2N, O^-\}$ coordination mode. Formation of $CuH_{-1}L$ and $CuH_{-2}L$ resulted in a decrease of intensity of the d−d transitions, suggesting a slight distortion of the structure of these complexes.

Cu(II)−Gentamicin C1a−H_2O_2 and −Ascorbic Acid Interaction with pBR322 Plasmid. Figure 7 presents the agarose gel electrophoresis of the products of pBR322 plasmid DNA incubated with various combinations of gentamicin C1a, copper(II), hydrogen peroxide, and ascorbic acid. Depending on the reaction mixture, either the nicked/relaxed (I), linearized (II), or native supercoiled (III) forms of the plasmid were observed.

In the first experiment (part A), pBR322 plasmid (1×10^{-6} M) was incubated for 1 h at 37 °C with 5×10^{-6} M gentamicin C1a in the presence and absence of either H_2O_2 or AA. Samples were prepared in sodium phosphate buffer

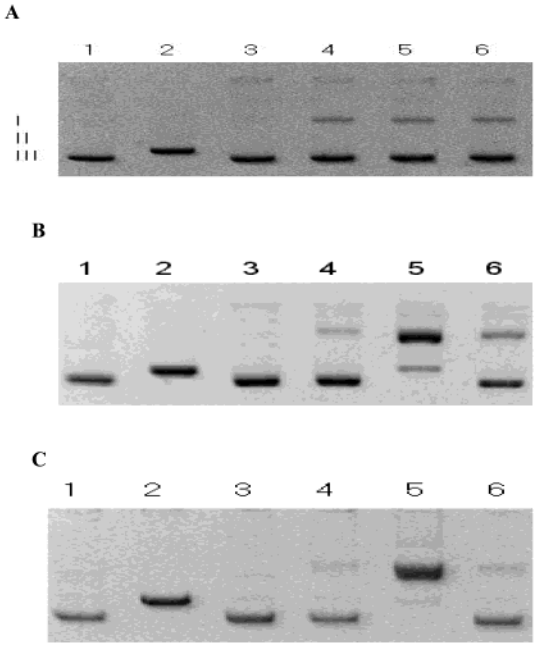

Figure 7. Agarose gel electrophoresis of pBR322 plasmid cleavage by gentamicin C1a and its copper(II) complexes in either absence or presence of H_2O_2 or ascorbic acid. The samples were processed on agarose gel as described in the Experimental Section. I−III denote nicked/relaxed, linear, and supercoiled forms, respectively. (A) Plasmid cleavage by gentamicin C1a in the presence or absence of H_2O_2 and ascorbic acid (AA): (1) plasmid; (2) plasmid linearized with XhoI endonuclease; (3) plasmid + 5×10^{-5} M H_2O_2; (4) plasmid + 5×10^{-5} M H_2O_2 + 5×10^{-6} M GC1a; (5) plasmid + 5×10^{-5} H_2O_2 + 5×10^{-6} M GC1a + 5×10^{-5} M ascorbic acid; (6) plasmid + 5×10^{-6} M GC1a. (B) Plasmid cleavage by Cu(II)−gentamicin C1a complexes in the presence or absence of H_2O_2: (1) plasmid; (2) plasmid linearized with XhoI endonuclease; (3) plasmid + 5×10^{-5} M H_2O_2; (4) plasmid + 5×10^{-5} M H_2O_2 + 5×10^{-6} M Cu(II); (5) plasmid + 5×10^{-5} M H_2O_2 + 5×10^{-6} M GC1a + 5×10^{-6} M Cu(II); (6) plasmid + 5×10^{-6} M GC1a + 5×10^{-6} M Cu(II). (C) Plasmid cleavage by Cu(II)−gentamicin C1a in the presence or absence of ascorbic acid: (1) plasmid; (2) plasmid linearized with XhoI endonuclease; (3) plasmid + 5×10^{-5} M AA; (4) plasmid + 5×10^{-5} M AA + 5×10^{-6} M Cu(II); (5) plasmid + 5×10^{-5} M AA + 5×10^{-6} M GC1a + 5×10^{-6} M Cu(II); (6) plasmid + 5×10^{-6} M GC1a + 5×10^{-6} M Cu(II).

(5×10^{-3} M, pH 7.4). All samples containing gentamicin C1a showed the same extent of form I of the plasmid. Experiments B and C present the interaction of Cu(II)−GC1a−H_2O_2 and Cu(II)−GC1a−AA systems with plasmid DNA. Samples were prepared in phosphate buffer at pH 7.4 and contained combinations of Cu(II) (5×10^{-6} M), gentamicin C1a (5×10^{-6} M), hydrogen peroxide (5×10^{-5} M), and ascorbic acid (5×10^{-5} M). Potentiometric data indicate that, at these conditions, 5% of the Cu(II) was present as CuH_3L, 25% as CuH_2L, 48% CuHL, and 22% CuL complexes. Cu(II), H_2O_2, and AA individually or in combination did not promote the formation of the relaxed or linear forms of the plasmid. Similarly, free gentamicin C1a and its copper(II) complexes in the absence of hydrogen peroxide and ascorbic acid caused little plasmid DNA cleavage.

The presence of H_2O_2 or ascorbic acid/O_2 greatly increased the DNA cleaving abilities of the copper(II)−GC1a complexes. Double-stranded DNA scission was promoted, which is in good agreement with previously reported results for copper(II) complexes of other gentamicin related aminoglycoside antibiotics.[14−17]

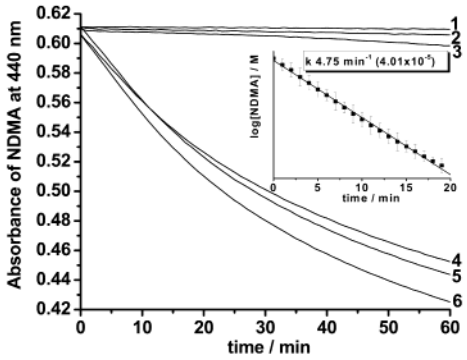

Figure 8. Experimental curves of the kinetic of measurements of NDMA (the OH• reporter molecule) oxidation by the Cu(II)−gentamicin−H_2O_2 system: (1) NDMA; (2) NDMA, H_2O_2, gentamicin; (3) NDMA, H_2O_2, Cu(II); (4−6) NDMA, H_2O_2, gentamicin, Cu(II). Insert: determination of the first-order reaction rate constant obtained from the 20 initial points of lines 4−6 exhibiting linear dependence of log $c\bullet_{OH}$ versus time.

Arachidonic Acid Peroxidation by the Cu(II)−Gentamicin−H_2O_2 System. Conjugated dien formation from arachidonic acid promoted by the Cu(II)−gentamicin−H_2O_2 system was also analyzed. Arachidonic acid (final concentration 0.25% emulsified in phosphate buffer of concentration 5×10^{-3} M at pH 7.4) was incubated for 1 h at 37 °C with combinations of 5×10^{-5} M Cu(II), 1×10^{-4} M commercial gentamicin sulfate, and 5×10^{-4} M hydrogen peroxide. Neither free gentamicin nor Cu(II) ions promote lipid peroxidation. In contrast, Cu(II)−gentamicin complexes are effective mediators of arachidonic acid oxidation, promoting formation of 87 ± 2 μM of the conjugated dien, similar to the action that was previously demonstrated for the Fe(II)/Fe(III)−gentamicin system.[3]

Detection of Reactive Oxygen Species (ROS) in Cu(II)−Gentamicin C1−H_2O_2 System. The oxidative scission of double-stranded DNA promoted by Cu(II)−GC1a complexes in the presence of hydrogen peroxide or ascorbic acid and the lipid peroxidation by Cu(II)−gentamicin−H_2O_2 confirmed that reactive oxygen species may be produced in these systems. To identify intermediate free radicals in these reactions, we used NDMA, a hydroxyl radical reporter, and NBT, a superoxide anion reporter.[13] Figure S1 displays the decay of NDMA observed for the sample consisting of 5×10^{-5} M Cu(II), 1×10^{-4} M commercial gentamicin sulfate, 5×10^{-4} M H_2O_2, and 5×10^{-3} M sodium phosphate buffer at pH 7.4. In light of the previously mentioned ethanol experiment (see Experimental Section), this result confirms hydroxyl radical formation during H_2O_2 activation by Cu(II)−gentamicin complexes. These results also indicate that none of the reaction products interfere with OH• radical detection. The kinetics of hydroxyl radical formation for combinations of Cu(II), gentamicin, and H_2O_2 were followed for 1 h at 25 °C by monitoring the decrease of the NDMA band at 440 nm, as shown in Figure 8. Slow destruction of the reporter molecule was observed for Cu(II) in the presence of H_2O_2 due to a Fenton-type reaction that produces low levels of hydroxyl radicals.[29] However, complexation of Cu(II) ions by gentamicin resulted in a burst of OH• radicals. The reaction initially followed pseudo-first-order kinetics with $k = 4.75$ min^{-1} as obtained from linear dependence of log $c\bullet_{OH}$ versus time (Figure 8 insert). When the concentration of the hydrogen peroxide decreased, the reaction slowed and higher order kinetics were observed. A similar reactivity pattern has been previously shown for Cu(II)−amikacin complexes.[13] No trace of scavengable $O_2^{\bullet-}$ radicals could be detected using NBT as monitor for the Cu(II)−gentamicin−H_2O_2 system.

Neither hydroxyl nor superoxide radicals were detected in the Cu(II)−gentamicin−AA mixtures. These results (and the oxidative double-stranded DNA scission promoted by Cu(II)−GC1a complexes in the presence of AA/O_2) indicate formation of metal bound hydroxo and peroxo species which would not be reactive toward the reporter molecules.[29]

Computer Simulation of the Influence of Gentamicin C1a on Cu(II) and Zn(II) Distribution in Human Blood Plasma. Relatively high stability constants for Cu(II)−gentamicin and Zn(II)−gentamicin complexes (Table 1), coupled with the ability of Cu(II)−gentamicin to promote oxidative reactions, inspired us to investigate the possibility of labile Cu(II) ions binding to gentamicin in human blood plasma (HBP). Computer simulations of Cu(II) and Zn(II) ion distributions in HBP were performed as a function of gentamicin C1a concentration. Table S1 presents the concentrations of the ligands and metal ions used in calculation. Using the components listed in Table S1, a model consisting of 235 complexes was developed (Table S2), and the distributions of Cu(II) and Zn(II) ions at pH 7.4 were computed. At a gentamicin C1a concentration of 2.2×10^{-5} M, this ligand cannot compete with serum ligands for either Cu(II) or Zn(II). The calculated concentrations of the Cu(II)− and Zn(II)−gentamicin C1a complexes in serum are on the order of 10^{-15}−10^{-16} M. Further simulations showed that the concentration of gentamicin C1a would have to reach outrageous levels (100 M) before one could observe a significant fraction of copper(II) bound to that antibiotic. Under these conditions 98.80% of copper(II) would be in the form of Cu(II)−albumin, with only 0.30% Cu(II)−HGC1a, 0.20% Cu(II)−H_2GC1a, 0.10% Cu^{II}(histidine)-(threonine), 0.10% Cu^{II}(histidine)(glutamine), 0.10% Cu(II)−GC1a, and 0.10% Cu^{II}(histidine)$_2$.

Competition between Gentamicin and Histidine toward Copper(II) Binding. The results obtained from the computer simulation of copper(II) distribution in human serum prompted us to investigate the competition between gentamicin and L-histidine toward Cu(II) ion binding. The addition of histidine to solutions of Cu(II)−gentamicin complexes and the addition of gentamicin into Cu(II)−histidine solutions were followed by UV−vis, CD, and EPR spectroscopies. In these studies, commercial gentamicin sulfate was used. Three different samples prepared in TRIS buffer of pH 7.4 and concentration 1×10^{-1} M were investigated: (A) Cu(II)−gentamicin complex 1:2 molar ratio; (B) Cu(II)−histidine 1:2 molar ratio; (C) Cu(II)−gentamicin−histidine 1:2:2 molar ratio with metal ion concentration fixed at 1×10^{-3} M. Figure S2 shows the CD spectra of these samples recorded just after mixing of reagents. Addition of 2 equiv of histidine

(29) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 3rd ed.; Oxford University Press: Oxford, U.K., 1999.

Table 4. Spectroscopic Parameters of Cu(II)−Histidine Complexes[a]

	UV−vis		CD		EPR	
components of soln	λ	ϵ	λ	$\Delta\epsilon$	$g_{\|\|}$	$A_{\|\|}$
Cu(II) + his 1:2	630	84.1	662	+0.23		
	253	1554	316	−0.10	2.24	178.22
Cu(II) + GC1a + his 1:2:2	217	7371	274	+0.11		
			243	−1.15		

[a] The same spectra were obtained for Cu(II)−histidine 1:2 and Cu(II)−gentamicin C1−histidine 1:2:2 systems so the data are presented only once. sh = shoulder. λ units are nm; ϵ and $\Delta\epsilon$ units are dm^3 mol^{-1} cm^{-1}. $A_{||}$ units are G.

Table 5. Summary of the Observed ESI-MS Ions (*m*/*z*) of the Cu(II)−Gentamicin C1a−Histidine System

components of the soln	*m*/*z*	assgnt
gentamicin C1a	899	gentamicin C1a dimer
	488.5	K(I)−gentamicin C1a
	449.5	gentamicin C1a
	321.3	gentamicin C1a−purpurosamine
	160.0	2-deoxystreptamine or garosamine
	128.1	purpurosamine
Cu(II) + gentamicin C1a 1:2	548.2	K(I)−Cu(II)−gentamicin C1a
	510.3	Cu(II)−gentamicin C1a
		and all peaks seen for gentamicin C1a
histidine	349.2	K(I)−histidine dimer
	311.3	histidine dimer
	194.1	K(I)−histidine
	156.1	histidine
	110.1	histidine−COO$^-$
	83	imidazole ring + CH$_3$
Cu(II) + gentamicin C1a + histidine 1:1:1	668.4	Cu(II)−gentamicin C1a−histidine
	435.1	Cu(II)−histidine dimer
	390.2	Cu(II)−histidine dimer−COO$^-$
	256.1	K(I)−Cu(II)−histidine
	218.1	Cu(II)−histidine
	194.2	K(I)−histidine
	173	Cu(II)−histidine−COO$^-$
		and all peaks seen for histidine, gentamicin C1a, and Cu(II)−gentamicin C1a
Cu(II) + gentamicin C1a + histidine 1:1:2	410.1	K(I)−Cu(II)−histidine$_2$
	372.1	Cu(II)−histidine$_2$
		and all peaks seen for gentamicin C1a and histidine

to solution A resulted in the characteristic spectrum of the Cu(II)−His species, indicating release of free gentamicin and formation of the Cu(II)−histidine complex. These results are in good agreement with UV−vis and EPR (recorded at 120 K using a ethylene glycol−water 1:2 solution as a solvent) experiments, where typical spectra of Cu(II)−histidine were obtained after introduction of histidine into the solution of Cu(II)−gentamicin complexes. Spectroscopic parameters of copper(II)−His species presented in Table 4 suggest Cu(II)−His$_2$ complex formation in both the presence and absence of gentamicin.[30−34]

As presented in Table 5, analysis of the solutions using mass spectrometry confirmed that Cu(II) was removed from complexes with gentamicin upon the addition of histidine.[35]

Discussion

The combined results of the potentiometric and spectroscopic studies indicate that gentamicin C1a is a relatively strong chelator of Cu(II) ions. The analysis of potentiometric titration curves identified the formation of six monomeric complexes with stoichiometries ranging from CuH$_3$L to CuH$_{-2}$L over the pH range of 3−12 (Table 2). The spectroscopic parameters determined for these species (Table 3) confirmed the presence of three distinct coordination modes {NH$_2$, O$^-$}, {2NH$_2$, O$^-$}, and {2NH$_2$, 2O$^-$}, a finding which is in good agreement with the results previously reported for copper(II) complexes with other gentamicin-related aminoglycosides.[9−12] All spectroscopic parameters of the CuH$_3$L and CuH$_2$L complexes, especially two bands in the UV range of the CD spectrum at 251 nm (258 nm corresponding band in the electronic spectrum) and 298 nm were assigned as O$^-$ → Cu(II) and NH$_2$ → Cu(II) charge transfer (CT) transitions, respectively (Figures 5 and 6). This strongly suggests the presence of the {NH$_2$, O$^-$} coordination mode in these species. Simultaneous binding of the Cu(II) ion to nitrogen and oxygen donors in the case of gentamicin C1a can only occur through the 3″-amino and 4″-, 2″-hydroxyl groups (belonging to the garosamine unit) located in vicinal positions to each other. However, due to steric restrictions, only binding to 3″-NH$_2$ and 4″-OH allows formation of the complexes existing at higher pH values. A complex with a protonated 4″-alcoholic oxygen was not detected because deprotonation of that group is simultaneously induced by the high overall positive charge of the Cu(II)−GC1a molecule.

The energy-minimized model of the CuH$_3$L complex built using the above considerations is presented in Figure 9A. That binding mode of Cu(II) to gentamicin is favored by the formation of the thermodynamically stable five-membered chelate ring and electrostatic repulsion of copper(II) by the protonated amino group present in the 2-deoxystreptamine and purpurosamine rings. The CuH$_3$L and CuH$_2$L complexes share the same coordination pattern, the only

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(31) Cocetta, P.; Deiana, S.; Erre, L.; Micera, G.; Piu, P. *J. Coord. Chem.* **1983**, *12*, 213−217.
(32) Casella, L.; Gullotti, M. *J. Inorg. Biochem.* **1983**, *18*, 19−31.
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(34) Szabo-Planka, T.; Peintler, G.; Rockenbauer, A.; Gyor, M.; Varga-Farbian, M.; Institorisz, L.; Balazspiri,L. *J. Chem. Soc., Dalton. Trans.* **1989**, 1925−239.

(35) According to the data presented in Table 5, a solution containing gentamicin C1a alone can be characterized by signals at *m*/*z* 449.5, 321.3, 160.0, and 128.1 corresponding to monoprotonated gentamicin C1a, gentamicin C1a minus purpurosamine (ring′), garosamine (ring″), or 2-deoxystreptamine (central ring) and purpurosamine, respectively. Gentamicin C1a dimer (*m*/*z* 889) and complex with potassium (*m*/*z* 488.5) resulted from ionization process. Detection of the signal at *m*/*z* 510.3 (Cu(II)−gentamicin C1a) and 548.2 (Cu−gentamicin C1a−K(I)) in a sample consisting of copper(II) and gentamicin C1a (pH 7.4) confirmed the presence of equimolar monomeric complexes in solution which is in good agreement with potentiometric and spectroscopic studies. For each copper(II) complex, two peaks were detected due to existence of ^{63}Cu and ^{65}Cu isotopes. A peak corresponding to the Cu(II)−GC1a−K(I) adduct is an artifact of ionization process. Addition of 1 equiv of histidine to the Cu(II)−gentamicin C1a 1:1 molar ratio system (pH 7.4) resulted in detection of several novel peaks e.g. at *m*/*z* 156.1, 218.1, and 668.4, which correspond to histidine, Cu(II)−His species, and Cu(II)−GC1a−His ternary complex. Several new, low-intensity peaks were detected due to fragmentation of histidine and formation adducts with potassium during ionization. The sample that consisted of Cu(II)−GC1a−His (1:2:2 molar ratio) gave no signals corresponding to copper(II)−gentamicin C1a complexes, while formation of Cu(II)−His$_2$ was confirmed, consistent with prior results obtained through the use of potentiometry and spectroscopic methodologies.[30−34] It also confirmed the higher affinity of histidine than gentamicin C1a toward Cu(II) ions.

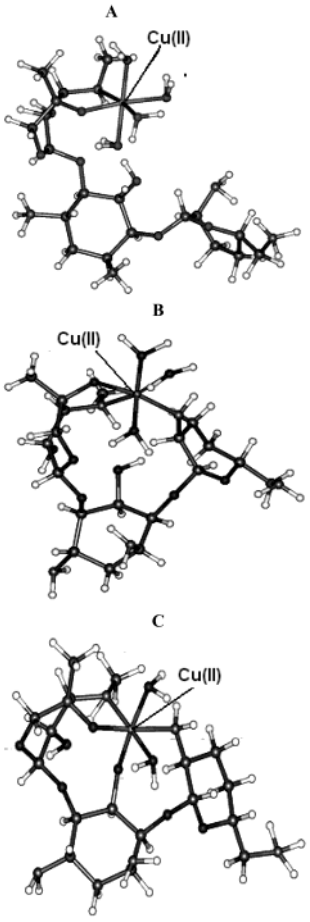

Figure 9. Energy minimization models of (A) CuH_3L complex of gentamicin C1a, (B) CuHL complex of gentamicin C1a, and (C) $CuH_{-1}L$ complex of gentamicin C1a.

difference between these species being the dissociation of the 3-amino group. Despite its basicity, the 3-amino group cannot participate in metal binding because of steric restrictions.[9−12] Changes in spectroscopic parameters upon transition to the next species suggest equatorial coordination of the second nitrogen. Inspection of the energy-minimized models of the Cu(II)−gentamicin C1a suggests that the 2′-amino group is in the best location to participate in this process. Figure 9B presents the model of the CuHL complex. Formation of the CuL species is consistent with deprotonation of the remaining amino group of the central ring, without affecting the coordination of the central ion.

The analysis of potentiometric curves yielded the formation of the next complex with $CuH_{-1}L$ stoichiometry. The pK_a value of the formation of that species is too low to correspond to the dissociation of the 6′-amino group. The $CuH_{-1}L$ species can be formed by the deprotonation of either a Cu(II)-coordinated water molecule or a hydroxyl group of gentamicin. The first process can be excluded because it would only promote a slight shift in the d−d band position in the CD spectrum.[34] On the other hand, apical coordination of the deprotonated alcoholic oxygen is well-known for aminosugars and results in the appearance of CT bands in the near UV.[26,27]

Alterations in both d−d and CT transitions in the CD spectrum (Figure 5) upon conversion to $CuH_{-1}L$ suggest the

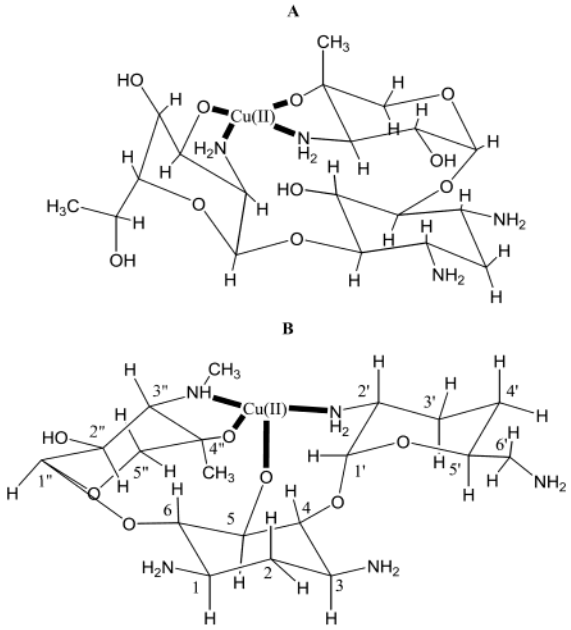

Figure 10. Comparison of the binding mode of Cu(II) complexes with geneticin and gentamicin. The schematic depiction of the Cu(II)−geneticin complex proposed previously by Jezowska-Bojczuk et al.[10] The schematic depiction of Cu(II)−gentamicin C1a complex is redrawn from Figure 9C.

axial coordination of a ligand oxygen rather than a water molecule. Contrary to previously studied aminoglycosides, the second amionosugar ring (purpurosamine) of gentamicin C1a is devoid of hydroxyl groups which could participate in Cu(II) binding to form five- or six-membered chelate rings as previously proposed for geneticin[10] (Figure 10). For that reason, the 5-alcoholic group of the inosotol ring seems to be the most likely to participate in this process as shown in Figure 9C. Finally, dissociation of the 6′-amino group leads to the formation of the $CuH_{-2}L$ species, which exhibits the same copper(II) coordination mode.

To investigate the oxidative properties of Cu(II)−gentamicin complexes their interactions with plasmid DNA were studied. Reaction of gentamicin C1a with plasmid DNA in the presence and absence of hydrogen peroxide and ascorbic acid generates small amounts of form I of plasmid. This form of the DNA can be obtained through either superhelix unwinding or hydrolytic phosphodiester bond cleavage. Unfortunately, these two processes cannot be distinguished with this experiment. Interestingly, Cu(II)−GC1a complexes, at pH 7.4, in the absence of H_2O_2 and in the presence of ascorbic acid under anaerobic conditions exhibit the same activities as free gentamicin C1a. Cu(II)−GC1a complexes in the presence of hydrogen peroxide and ascorbate under aerobic conditions are very effective mediators of DNA destruction. While complete conversion of the plasmid DNA to forms I and II was observed, complete DNA degradation did not occur. Lack of shorter DNA fragments suggests nonrandom double-stranded DNA scission promoted by nondiffusable copper(II)-bound hydroxo and oxo species.

Oxidation of NDMA by the Cu(II)−GC1a−H_2O_2 system in the absence of ethanol proved that diffusible hydroxyl radicals were generated during the reaction of Cu(II)−gentamicin C1a with hydrogen peroxide. However, interac-

tion of Cu(II)−gentamicin complexes with plasmid DNA suggested that major DNA cleavage agents are metal-bound rather then a free reactive oxygen species. This issue is also well supported by the lack of scavengable superoxide anions in the Cu(II)−GC1a−H_2O_2 system.

Further, interaction of Cu(II)−GC1a complexes with plasmid DNA in the presence of ascorbic acid indicated that these complexes could be easily reduced to Cu(I)−GC1a and then interact with molecular oxygen to mediate the oxidative damage of duplex DNA. NDMA and NBT failed to detect $OH^{\bullet}$ or $O_2^{\bullet -}$ in the Cu(II)−GC1a−AA system, supporting a copper-bound reactive species responsible for DNA damage. The possibility of a copper-bound species, either Cu(I) and Cu(II), rather then free oxygen radical species has been previously proposed for copper(II) complexes with gentamicin-related aminoglycosides and pepetides.[14−19,36−38]

Our results are in good agreement with those previously reported for copper(II)−kanamicin A (a related aminoglycoside antibiotic) complex, which was shown to bind to the minor groove of DNA and promote highly specific proton abstraction from the C-4′ carbon of deoxyribose under similar conditions.[18] Proton abstraction from deoxyribose carbons initializes the series of elimination reactions that lead to double-stranded DNA scission.[18]

We have also demonstrated that Cu(II)−gentamicin complexes existing at physiological pH facilitate peroxidation of arachidonic acid in the presence of hydrogen peroxide to a similar extent as previously studied iron complexes of gentamicin.[3] Under the same conditions, Cu(II) aqua ion and uncomplexed gentamicin C1a do not promote that reaction.

Oxidative damage of DNA and lipid peroxidation mediated by copper(II) complexes of gentamicin C1a could contribute to the cytotoxic activities of that drug. The important question is whether the affinity of gentamicin C1a toward Cu(II) is high enough to effectively compete with biological ligands. To address this issue, the possibility of labile Cu(II) ions binding to gentamicin C1a in human blood plasma was investigated by computer simulations and studies of direct interaction of Cu(II)−gentamicin complexes with L-histidine. Although gentamicin C1a, like other previously studied aminoglycosides, is a relatively strong chelator of copper(II) ions and forms stable complexes in vitro over a wide pH range, it cannot compete for Cu(II) with naturally occurring bioligands. The computer simulations showed that in solution, at neutral pH the concentration of gentamicin C1a has to be at an impossibly high level (100 M) to obtain a significant fraction of Cu(II) ions bound to gentamicin C1a. In agreement with this conclusion is the fact that L-histidine, a major low molecular weight carrier of Cu(II) ions in human blood plasma, can extract copper(II) ions from Cu(II)−gentamicin complexes once introduced into aqueous solution.

In view of these results, copper(II) ions do not appear to play a major role in the toxicity of aminoglycosides. However, a small pool of nonchelated copper may be locally available for xenobiotic ligands under specific conditions such as inflammation and via degradation or damage of respiratory chain enzymes.[39] But under these conditions only a minute concentration of Cu(II)−gentamicin complexes can be present to promote oxidation of DNA, fatty acids, and other biomolecules. At best, Cu(II)−gentamicin complexes may be marginal elements of the cellular toxicity of gentamicin. That scenario is supported by the fact that histidine will attenuate aminoglycoside-induced hearing loss in animals.[6]

Conclusion

Three major components of gentamicin C form relatively stable complexes with Cu(II) ions (with a stoichiometry from CuH_3L to $CuH_{-2}L$) in aqueous solution over a wide pH range. The resulting complexes promote in vitro lipid peroxidation and oxidative damage of plasmid DNA mediated by metal-bound reactive oxygen species. Since aminoglycosides share similar coordination properties toward copper(II) as other potential chelators present in human blood plasma, but with a lower binding affinity, they do not affect the distribution of Cu(II) ions in human blood plasma. Further, Cu(II)−gentamicin complexes cannot withstand the presence of histidine. In view of these results, we conclude that Cu(II) ions can play only a marginal role in the biological activity of aminoglycosides.

Acknowledgment. This work was supported by Grant DC-03685 from the National Institute on Deafness and Other Communicational Disorders, National Institute of Health.

Supporting Information Available: Additional figures and tables for experimental and computer simulation results. This material is available free of charge via the Internet at http://pubs.acs.org.

IC025965T

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