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Synthesis, DNA-Binding, Cleavage, and Cytotoxic Activity of New 1,7-Dioxa-4,10-diazacyclododecane Artificial Receptors Containing Bisguanidinoethyl or Diaminoethyl Double Side Arms

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Abstract: Novel 1,7-dioxa-4,10-diazacyclododecane artificial receptors with two pendant aminoethyl (**3**) or guanidinoethyl (**4**) side arms have been synthesized. Spectroscopy, including fluorescence and CD spectroscopy, of the interactions of **3**, **4**, and their copper(II) complexes with calf thymus DNA indicated that the DNA binding affinity of these compounds follows the order Cu^{2+}–**4** > Cu^{2+}–**3** > **4** > **3**, and the binding constants of Cu^{2+}–**3** are Cu^{2+}–**4** are 7.2×10^{4} and $8.7\times10^{4}\,M^{-1}$, respectively. Assessment by agarose gel electrophoresis of the plasmid pUC19 DNA cleavage activity in the presence of the receptors showed that the complexes Cu^{2+}–**3** and Cu^{2+}–**4** exhibit powerful supercoiled DNA cleavage efficiency. Kinetic data of DNA cleavage promoted by Cu^{2+}–**3** and Cu^{2+}–**4** under physiological conditions fit to a saturation kinetic profile with k_{max} values of 0.865 and 0.596 h^{-1}, respectively, which give about 10^{8}-fold rate acceleration over uncatalyzed supercoiled DNA. This acceleration is due to efficient cooperative catalysis of the copper(II) center and the functional (diamino or bisguanidinium) groups. In-vitro cytotoxic activities toward murine melanoma B16 cells and human leukemia HL-60 cells were also examined: Cu^{2+}–**4** shows the highest activity with IC_{50} values of 1.62×10^{-4} and $1.19\times10^{-5}\,M$, respectively.

Keywords: copper · crown ethers · DNA cleavage · metalloenzymes · reaction mechanisms · receptors

Introduction

The interactions of small molecules with DNA through recognition, binding, modification, and cleavage have attracted extensive attention because of their potential applications in molecular biological technology and drug development.[1] Metal complexes and some small organic molecules have been widely investigated as binding and cleavage agents for phosphodiester bonds of nucleic acids,[2] and in many cases have been found to be efficient. In particular, the transition-metal complexes of macrocyclic polyamine derivatives such as 1,4,7,10-tetraazacyclododecane (cyclen) and 1,4,7-triazacyclononane (TACN) exhibit excellent ability to cleave nucleic acids, phosphodiesters, dipeptides, and proteins[3] and also show anti-tumor or anti-HIV virus activities.[4]

In nature, the cleavage rate of the phosphodiesters of DNA and RNA by a hydrolytic pathway is accelerated 10^{15}–10^{16}-fold by metalloenzymes such as staphylococcal nuclease (SNase) from *Staphylococcus aureus*[5] and nuclease S1 from *Aspergillus oryzae*.[6] At the active site of SNase, a calcium(II) ion and two of the guanidinium groups of arginine residues (Arg-35 and Arg-87) cooperate to activate the substrate and stabilize phosphorate-like transition states by electrostatic interaction, hydrogen bonding, and/or proton transfer.[5,7] In the nuclease S1, it was suggested that the cooperativity of zinc(II) ion and the amino group of a lysine residue facilitates the attack of a nucleophile Glu-carboxylate group.[6] Therefore, one of the well-established basic principles for enhanced catalytic efficiency for phosphodiester cleavage of DNA and RNA by natural nucleases is the ef-

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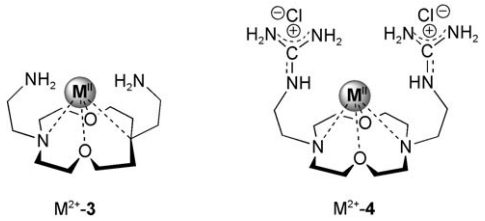
ficient cooperation of metal ions and such functional groups as amino or guanidinium groups at the active site.

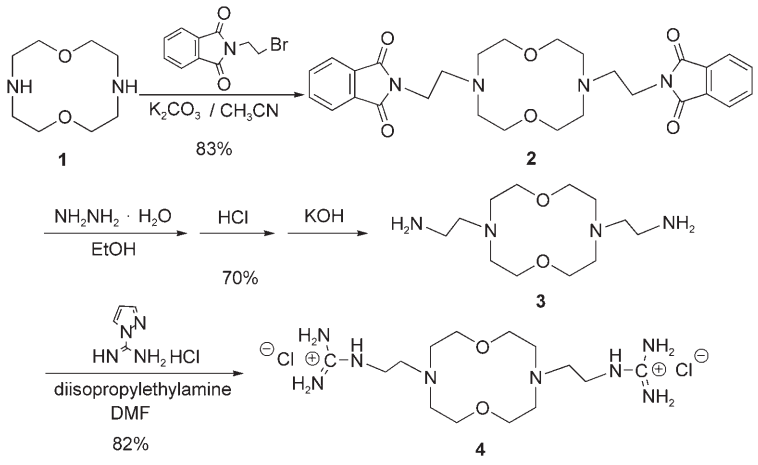
Recently, synthetic small molecules have been applied increasingly to imitate the active site of natural nucleases.[8–10] For example, several metal complexes with amino (or ammonium) or guanidine (or guanidinium) functional groups were found to accelerate phosphodiester cleavage reactions greatly. The copper(II)-binding bipy unit with two ammonium groups designed by Krämer and Kövari[8] increases the rate of phosphodiester hydrolysis of bis(*p*-nitrophenyl) phosphate (BNPP). Similarly, Anslyn and co-workers designed the zinc(II) complex of a rigid cleft with two guanidinium groups proximal to the metal ion,[9] which leads to a 3.3×10^3 rate enhancement of hydrolysis of the RNA dimer adenylyl phosphoadenine (ApA) relative to a corresponding complex lacking the guanidinium groups. Quite recently, Williams and co-workers[10a] designed the zinc(II) complex of a tetradentate tripodal ligand with aminopyridyl hydrogen bond donors, which was found to be 1.5×10^3 times more effective in promoting the cleavage of phosphodiesters of 2-hydroxypropyl-4-nitrophenyl phosphate (HPNPP) by intramolecular transesterification than the corresponding structure without the two amino groups. These authors have successfully modeled the efficient catalytic cooperativity of a metal-ion center and functional groups proximal to the metal center in a complex that results in a considerable increase in the cleavage rate of active phosphodiesters (for example, BNPP, HPNPP, and RNA dimer ApA).

The phosphodiester bonds of DNA are known to be very stable under uncatalyzed physiological conditions and the half-life of DNA in hydrolysis is estimated to be about 200 million years.[11] Therefore, DNA cleavage by a hydrolytic pathway by artificial nucleases is a challenging topic. The strategy of introducing functional groups proximal to the metal ion in the side chains of the ligand may open a way to cleave a DNA phosphodiester backbone effectively. To mimic efficient DNA nuclease models, the metal complexes (M^{2+}–**3**, M^{2+}–**4**) of 1,7-dioxa-4,10-diazacyclododecane with double aminoethyl (**3**) or guanidinioethyl (**4**) side arms, below) were designed as the "bifunctional catalyst" to promote DNA cleavage. Aza-crown ethers are known to be good complexing agents for transition-metal ions.[3a–c,12–14] The guanidinium groups can recognize, bind, and electrophilically activate the anionic phosphodiester through hydrogen bonding and electrostatic interaction.[15] Guanidinium compounds acting as nuclease mimics for cleavage of active phosphodiesters have been reported by several laboratories (for example, by Anslyn,[1k,16] Göbel,[17] Hamilton,[18] and their co-workers), and a few of them were identified as powerful cleavers of RNA. Therefore, the combination of the Lewis acid metal center coordinated by the aza-crown ether and two functional groups (guanidinium or amino) in close proximity might construct a "bifunctional catalytic" model to promote DNA cleavage efficiently. In this paper, we report the synthesis of the novel receptors 1,7-dioxa-4,10-diazacyclododecane derivatives 4,10-bis(*N*,*N*′-(2-ethyl)amino)-1,7-dioxa-4,10-diazacyclododecane (**3**) and 4,10-bis[*N*,*N*′-(2-ethyl)guanidino]-1,7-dioxa-4,10-diazacyclododecane hydrochloride (**4**). We also report the investigation by spectroscopic techniques including fluorescence and circular dichroism (CD) spectroscopy of the interactions of **3**, **4**, and their copper(II) complexes with calf thymus DNA. Agarose gel electrophoresis was used to assess the plasmid pUC 19 DNA cleavage activities in the presence of the receptors and their complexes Cu^{2+}–**3** and Cu^{2+}–**4**. In-vitro cytotoxic activities were also tested toward murine melanoma B16 cells and human leukemia HL-60 cells.



Results and Discussion

Synthesis: Synthesis of the target compound **4** was achieved by a reaction sequence of nucleophilic substitution, hydrazinolysis, and guanylation, starting from a known compound, 1,7-dioxa-4,10-diazacyclododecane (**1**) (Scheme 1). The primary amino group was guanylated with 1*H*-pyrazole-1-carboxamidine hydrochloride.[19] The crude product was purified by column chromatography on strong base anion ex-



Scheme 1. Synthesis of **3** and **4**.

change resin (no. 717) followed by neutralization with 10% hydrochloric acid to give **4** in good yield. All new compounds were characterized by ^{1}H NMR and ^{13}C NMR spectroscopy, and ESI mass spectrometry. In ^{13}C NMR spectrum of **4**, the signal at $\delta = 157.98$ ppm comprises the chemical shift of the guanidinium group and in the ESI mass spectrum the signals at m/z 173.2 and 345.4 correspond to $[M-2Cl]^{2+}$ (calcd 173.1) and $[M-2Cl+H]^{+}$ (calcd 345.3), respectively (see Supporting Information page S8).

DNA binding assays: DNA binding is the critical step for DNA cleavage in most cases. Therefore, the binding of the ligands **3** and **4** and their complexes Cu^{2+}–**3** and Cu^{2+}–**4** to CT-DNA were studied by fluorescence and CD spectroscopy. UV/Vis absorption spectroscopy is also a convenient tool for examining the interaction between small molecules and nucleic acids, but these complexes with calf thymus (CT-DNA) have very similar UV absorbances at 260 nm, so DNA binding was not studied by using UV/Vis absorption in this case.

Fluorescence spectroscopy: The binding of the compounds to CT-DNA was studied by evaluating the fluorescence emission intensity of the ethidium bromide (EB)–DNA system upon addition of the four compounds. If these compounds added to the EB–DNA system replace the bound EB, the emission intensity will be reduced. The fluorescence quenching of EB bound to DNA by **3**, **4**, Cu^{2+}–**3**, and Cu^{2+}–**4** are shown in Figure S1, in which the fluorescence intensity at 600 nm ($\lambda_{ex} = 518$ nm) of EB in the bound form is plotted against the compound concentration. The relative binding propensity of the complexes to CT-DNA was determined by the classical Stern–Volmer equation $I_0/I = 1 + Kr$[20] in which I_0 and I are the fluorescence intensities in the absence and the presence of the quencher, respectively; K is the linear Stern–Volmer quenching constant, dependent on r_{bE} (the ratio of the bound concentration of EB to the concentration of DNA), and r is the ratio of total concentration of the quencher to that of DNA.[20] The quenching constants K obtained for **3**, **4**, Cu^{2+}–**3**, and Cu^{2+}–**4** are given by the slopes of the plots in Figure 1; they are 0.045 ± 0.001, 0.164 ± 0.015, 0.344 ± 0.017, and 0.400 ± 0.015, respectively. These results

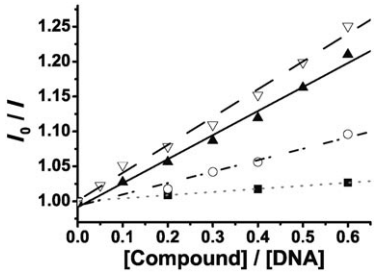


Figure 1. The Stern–Volmer quenching plots of EB bound to DNA by **3** (■), **4** (○), Cu^{2+}–**3** (▲), and Cu^{2+}–**4** (▽), which give the quenching constants K. They were obtained by adding **3**, **4**, Cu^{2+}–**3**, or Cu^{2+}–**4** (0–60 μM) to the EB-bound CT-DNA solution in Tris–HCl (5 mM) buffer (pH 7.5).

demonstrate that complexes Cu^{2+}–**3** and Cu^{2+}–**4** have a stronger affinity for DNA than the corresponding ligands **3** and **4**. Moreover, Cu^{2+}–**4** possesses higher DNA binding ability than Cu^{2+}–**3**. The apparent binding constant K_{app} was also calculated from the equation K_{EB}·[EB] = K_{app}·[complex], in which the complex concentration was the value at a 50% reduction of the fluorescence intensity of EB and $K_{EB} = 1.0 \times 10^{7}\,M^{-1}$ ([EB] = 1.3 μM).[21] The K_{app} values are $7.2 \times 10^{4}\,M^{-1}$ for Cu^{2+}–**3** and $8.7 \times 10^{4}\,M^{-1}$ for Cu^{2+}–**4** (Figure S2). The binding constants can be used to evaluate the three main DNA-binding modes; a value above $10^{6}\,M^{-1}$ is an indication of intercalation (ethidium and daunomycin bind DNA with an affinity over $10^{6}\,M^{-1}$), while values in the range 10^{4}–$10^{5}\,M^{-1}$ imply the groove binding mode.[22,23] Therefore the DNA binding of the complexes Cu^{2+}–**3** and Cu^{2+}–**4** might be by the groove binding mode. This is perhaps because these compounds contain no fused aromatic ring to facilitate intercalation.

Circular dichroism studies: Circular dichroism (CD) is a useful technique to assess whether nucleic acids undergo conformational changes as a result of complex formation or changes in the environment. In the CD spectra of CT-DNA treated with **3**, **4**, Cu^{2+}–**3**, and Cu^{2+}–**4** ([compound]/[DNA] = 0.5:1) (Figure 2), the positive (≈275 nm) band

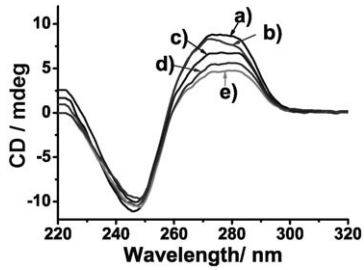


Figure 2. CD spectra of a) CT-DNA (1.2×10^{-4} M), and the interaction with b) **3** ,c) **4** ,d) Cu^{2+}–**3**, and e) Cu^{2+}–**4** with [compound]/[CT-DNA] = 0.5. All the spectra were recorded in Tris-HCl buffer, pH 7.5.

decreased in intensity with an increase in the compound concentration except for **3**, while the negative (≈245 nm) band had no significant change. This suggests that **4**, Cu^{2+}–**3**, and Cu^{2+}–**4** can unwind the DNA helix and lead to loss of helicity.[24,25] The larger decrease in the CD band intensity caused by Cu^{2+}–**4** than the other compounds at the same concentration implies that Cu^{2+}–**4** is more effective than **3**, **4**, and Cu^{2+}–**3** in perturbing the secondary structure of DNA.

From the results of these fluorescence and CD spectroscopic studies, it is concluded that the binding interaction ability of the compounds to CT-DNA follows the order Cu^{2+}–**4** > Cu^{2+}–**3** > **4** > **3**, and the DNA binding constants of Cu^{2+}–**3** and Cu^{2+}–**4** indicate that these copper(II) complexes might bind with DNA through the groove binding mode.

DNA cleavage activity: To assess the DNA cleavage activity of **3**, **4**, Cu^{2+}–**3**, and Cu^{2+}–**4**, the interaction with pUC 19 plasmid DNA was studied under physiological conditions. Agarose gel electrophoresis (1% agarose gel) was used to visualize the effects. In the results obtained at pH 7.5 (50 mM Tris-HCl/10 mM NaCl) at 37 °C for 3 h (Figure 3) we cannot

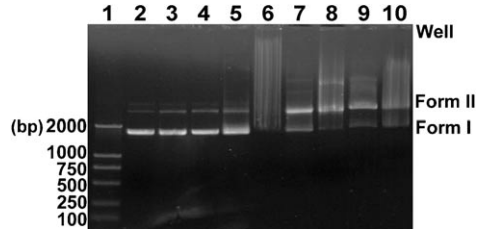


Figure 3. Agarose gel (1%) electrophoretograms of pUC 19 DNA (0.05 mM bp) cleavage promoted by **3**, **4**, Cu^{2+}–**3** and Cu^{2+}–**4** (incubated for 3 h at 37 °C in pH 7.5 buffer). Lane 1, DNA markers; lane 2, DNA control; lane 3, DNA + 0.10 mM **3**; lane 4, DNA + 0.20 mM **3**; lane 5, DNA + 0.10 mM **4**; lane 6, DNA + 0.20 mM **4**; lane 7, DNA + 0.10 mM Cu^{2+}–**3**; lane 8, DNA + 0.20 mM Cu^{2+}–**3**; lane 9, DNA + 0.10 mM Cu^{2+}–**4**; lane 10, DNA + 0.20 mM Cu^{2+}–**4**.

see any form II DNA fragments from lanes 3–5, which indicates that compound **3** or **4** alone does not show efficient cleavage ability. When the concentration of **4** is increased to 0.20 mM the gel electrophoresis bands become smeared (Figure 3, lane 6), but **3** causes no smearing at the same concentration (Figure 3, lane 4). By referring to the DNA size markers, we can conclude that these smeared bands are created not by small cleaved fragments but by the DNA–**4** conjugation, and there is binding only in the presence of **4**. We can also conclude that **4**, containing bisguanidinium groups, has greater DNA binding ability than **3**, containing diamino groups. Lanes 7–10 in Figure 3 show that Cu^{2+}–**3** and Cu^{2+}–**4** can efficiently cleave supercoiled (form I) to nicked (form II) DNA, which suggests that adding copper(II) ion to the 1,7-dioxa-4,10-diazacyclododecane containing diamino or bisguanidinium groups is quite an efficient strategy for supercoiled DNA cleavage. When the concentrations of Cu^{2+}–**3** and Cu^{2+}–**4** are increased to 0.20 mM, band smearing appears again (lanes 8 and 10), but this smearing is different from that in lane 6 and is due to the DNA binding after nicking caused by copper(II) complexes.

Figure 4 shows the results of plasmid pUC 19 DNA cleavage promoted by **3** and **4** (0.05 mM) in the presence of different added metal(II) ions (0.05 mM). After incubation for 1.5 h at 37 °C with metal–-**3** (or **4**) complexes, conversion of form I to form II DNA is apparent and the efficiency of DNA cleavage with added metal(II) decreases in the order $Cu^{II} > Zn^{II} > Co^{II} > Mn^{II} > Ni^{II}$ (Table 1), which is in accord with the results reported by Morrow.[26] The control assays were carried out under the same conditions but with the metal ions alone (Figure S3 and Table S1) in the absence of **3** (or **4**). Little cleavage was found; the same result was reported by Barton.[27] Therefore, we have further investigated the DNA cleavage activities of Cu^{2+}–**3** and Cu^{2+}–**4** in detail.

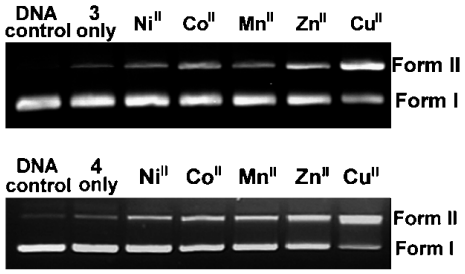


Figure 4. Agarose gel (1%) electrophoretograms of pUC 19 DNA (0.05 mM bp) cleavage promoted by **3** (0.05 mM; top) and **4** (0.05 mM; bottom) in the presence of added metal ion (0.05 mM) incubated for 1.5 h at 37 °C in pH 7.5 buffer. From left to right: DNA control, compound only, Ni^{II}, Co^{II}, Mn^{II}, Zn^{II}, and Cu^{II}.

Table 1. DNA cleavage promoted by **3** and **4** in the presence of metal ion.

Added Metal Ion	**3**[a]		**4**[a]	
	% Form I	% Form II	% Form I	% Form II
DNA control	97.65	2.35	96.32	3.68
no metal added	93.51	6.49	92.99	7.01
Ni^{II}	87.01	12.99	81.31	18.69
Co^{II}	79.06	20.94	71.69	28.31
Mn^{II}	84.41	15.59	73.15	26.85
Zn^{II}	75.38	24.62	63.18	36.82
Cu^{II}	55.61	44.39	38.11	61.89

[a] Cleavage reactions were carried out in pH 7.5 Tris-HCl buffer for 1.5 h at 37 °C.

*pH and ionic strength dependence of DNA cleavage promoted by Cu^{2+}–**3** and Cu^{2+}–**4***: The bell-shaped pH-dependence profiles of Figure 5 indicate that 7.5 is the optimal pH for

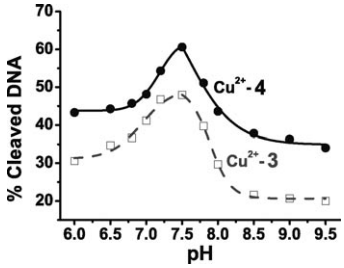


Figure 5. pH-dependent profiles for DNA cleavage promoted by Cu^{2+}–**3** and Cu^{2+}–**4** in buffers (50 mM Tris-HCl/10 mM NaCl) of different pH at 37 °C.

DNA cleavage in the presence of Cu^{2+}–**3** or Cu^{2+}–**4** (the agarose gel electrophoretogram is shown in Figure S4). Ionic strength also has a significant effect on DNA cleavage. Ionic dependence assays show that the cleavage activity decreases with an increase in the ionic strength (Figure 6), which implies that the positively charged complexes bind to the anionic sites in phosphodiesters of DNA through electrostatic interactions rather than to the DNA bases.[2g] Thus, the optimal pH value of 7.5 and a relatively low ionic strength of 10 mM were selected in all DNA cleavage assays.

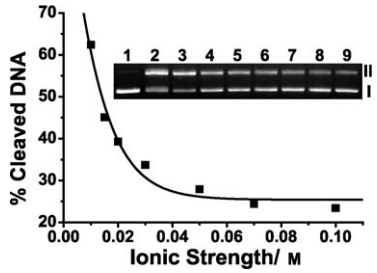


Figure 6. Ionic strength dependence of the plasmid DNA cleavage promoted by Cu^{2+}–**4** (0.05 mM). The reactions were carried out at 37 °C for 1.5 h in 50 mM Tris-HCl buffer (pH 7.5). Ionic strength was controlled by NaCl. Inset: agarose gel (1 %) electrophoretograms of the ionic strength dependence. Lane 1, DNA control; lanes 2, no NaCl; lanes 3–9, ionic strength 0.010, 0.015, 0.020, 0.030, 0.050, 0.070, 0.100 M, respectively.

*Concentration dependence assays of DNA cleavage promoted by Cu^{2+}–**3** and Cu^{2+}–**4***: The saturation profiles for the agarose gel electrophoretograms of pUC 19 DNA with different concentrations of Cu^{2+}–**3** and Cu^{2+}–**4** in Figure 7 are represented in Figure 8; they indicate that the DNA cleavage activities of Cu^{2+}–**3** and Cu^{2+}–**4** are increased with an increase in the concentration of the complexes, but this effect is close to being saturated at a complex concentration of about 0.150 mM.

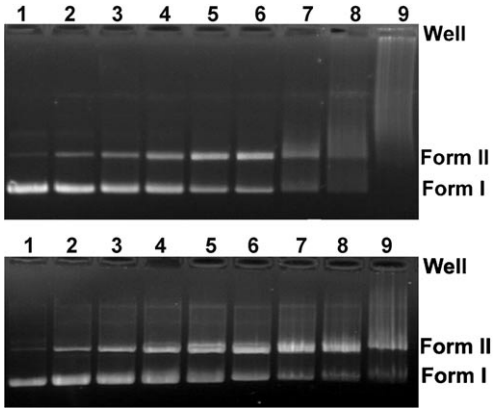


Figure 7. Agarose gel (1 %) electrophoretograms of pUC 19 DNA (0.05 mM bp) incubated for 2.0 h at 37 °C with different concentrations of Cu^{2+}–**3** (top) and Cu^{2+}–**4** (bottom) in pH 7.5 buffer (50 mM Tris-HCl/ 10 mM NaCl). a) Lanes 1–9, 0, 0.005, 0.010, 0.020, 0.050, 0.100, 0.150, 0.200 and 0.250 mM Cu^{2+}–**3**, respectively; b) lanes 1–9, 0, 0.005, 0.010, 0.020, 0.030, 0.050, 0.100, 0.150, and 0.200 mM Cu^{2+}–**4**, respectively.

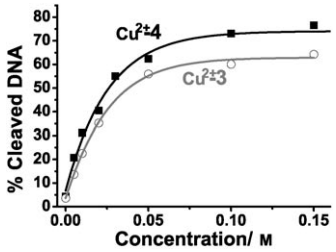


Figure 8. Percentages of cleaved DNA versus concentrations of Cu^{2+}–**3** and Cu^{2+}–**4**.

Kinetic assays: The kinetics of pUC 19 DNA degradation has been studied. Figure 9 indicates that the extent of supercoiled DNA cleavage into nicked form promoted by Cu^{2+}–**4** varies exponentially with the reaction time, giving pseudo first-order kinetics with an apparent initial first-order rate constant (k_{obs}) of $0.749 \pm 0.027\ h^{-1}$. The apparent initial first-order rate constants of DNA cleavage reactions promoted by a series of various concentrations of Cu^{2+}–**3** and Cu^{2+}–**4** under the conditions described above are summarized in (Table S2). The saturation kinetics profiles of the supercoiled DNA cleavage at various concentrations of Cu^{2+}–**3** and Cu^{2+}–**4** (Figure 10) give maximal first-order rate constants k_{max} of 0.596 ± 0.058 and $0.865 \pm 0.046\ h^{-1}$ for Cu^{2+}–**3** and Cu^{2+}–**4**, respectively.[28] For comparison, kinetic studies of Cu^{2+}-**1** without any side arms under the same conditions gave a k_{max} of Cu^{2+}-**1** of $0.087 \pm 0.011\ h^{-1}$ (Table S3), indicating a relatively low cleavage activity. The cleavage efficiency of Cu^{2+}–**4** is significantly enhanced compared with the non-hydrogen-bonding donor complex Cu^{2+}-**1**. This indicates that the cooperation of copper(II) ion with the pendant bis-guanidinium group increases the DNA cleavage activity effectively. Furthermore, Cu^{2+}–**4** has a remarkably greater rate acceleration than Cu^{2+}–**3**, because the guanidinium group is a more efficient binding and electrophilic activation

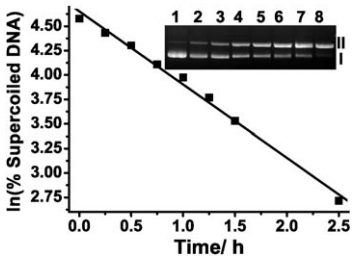


Figure 9. Time course of pUC19 DNA (0.05 mM bp) cleavage promoted by Cu^{2+}–**4** (0.10 mM) in Tris-HCl/NaCl buffer (pH 7.5) at 37 °C. Inset: agarose gel (1 %) electrophoretograms of the time-variable reaction products. Lanes 1–6, reaction time 0, 0.25, 0.50, 0.75, 1.00, 1.25, 2.50 h, respectively.

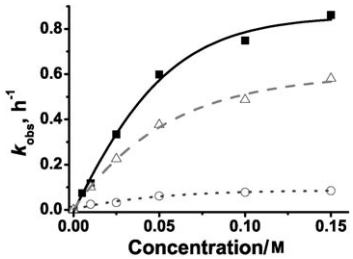


Figure 10. Saturation kinetics plots of k_{obs} versus various concentrations of Cu^{2+}–**4** (■), Cu^{2+}–**3** (▲) and Cu^{2+}-**1** (●).

group for the phosphodiester of DNA than the amino group.

DNA cleavage mechanism: Copper(II) complexes can cleave DNA by both hydrolytic[27,29] and oxidative pathways.[30] In the second case, they have been shown to react with molecular oxygen or hydrogen peroxide to produce a variety of reactive oxygen species (ROs).[31] Normally, a reducing agent (a thiol or ascorbic acid) is required to initiate and sustain the radical reaction, but particularly with the employment of DNA derived from biological sources, the presence of adventitious reducing agents is always possible.[29a] To establish whether ROs are, at least in part, responsible for the cleavage of DNA promoted by the copper(II) complexes Cu^{2+}–**3** and Cu^{2+}–**4**, reactions were carried out in the presence of typical scavengers for singlet oxygen (NaN_3), for superoxide (KI), and for hydroxyl radical (DMSO and *t*BuOH) (Figure 11). For both Cu^{2+}–**3** and Cu^{2+}–**4**, there is no significant inhibition effect on the DNA cleavage in the presence of any of these scavengers (NaN_3, DMSO, *t*BuOH, KI), which rules out the involvement of these reactive oxygen species, at least in a free and diffusible form. Yet, at the same concentrations of the complexes, all the copper(II) complexes (Cu^{2+}-**1**, Cu^{2+}–**3**, and Cu^{2+}–**4**) essentially have the same activity as free copper(II) ions. However, in this case, the DNA cleavage rate constants of the three complexes are quite different, suggesting a nonoxidative pathway.

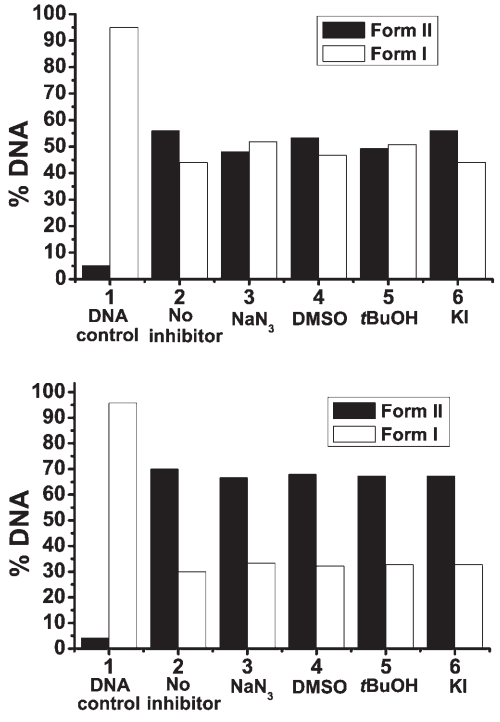
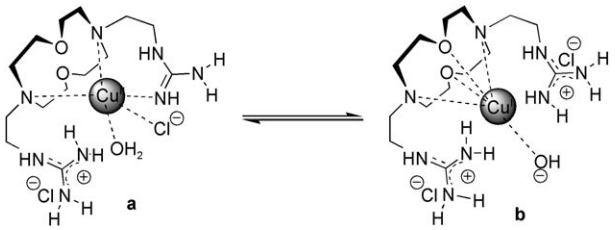


Figure 11. Histograms representing cleavage of pUC19 plasmid DNA (0.05 mM bp) by Cu^{2+}–**3** (0.05 mM; top) and Cu^{2+}–**4** (0.05 mM; bottom) in the presence of standard radical scavengers for singlet oxygen (NaN_3, 10 mM), for superoxide (KI, 10 mM), and for hydroxyl radical (1 mM DMSO and 1 mM *t*BuOH), incubated for 2 h at 37 °C in pH 7.5 buffer (50 mM Tris-HCl/10 mM NaCl).

Generally, the oxidative DNA cleavage process is due to the oxidation of the ribose or base group of DNA by the reactive oxygen species.[30] Therefore the DNA samples containing Cu^{2+}–**4** were incubated in the presence of each of the four nucleosides adenosine, uridine, guanosine, and cytidine, [nucleoside]/[DNA bp] = 1:1, followed by electrophoresis and quantification (Figure S5). No inhibition was detected in the DNA cleavage after the treatment with Cu^{2+}–**4** in the presence of each of the four nucleosides. This suggests a nonoxidative process (Table S4). On the other hand, in the DNA cleavage reactions promoted by Cu^{2+}–**4**, when the active phosphate bis(2,4-dinitrophenyl) phosphate (BDNPP) without the pentose and the pyrimidine or purine base was added, the reactions were partially inhibited (Table S4). The inhibition can be ascribed to the preferentially hydrolyzed phosphodiester of BDNPP, and implies that the hydrolysis pathway for the DNA cleavage process is possible. In addition, planar heterocyclic bases such as 1,10-phenanthroline (phen) or dipyridoquinoxaline (dpq) are necessary for oxidative DNA cleavage,[32] whereas there are no aromatic rings in the present compounds. All these results suggest that the DNA cleavage promoted by Cu^{2+}–**3** and Cu^{2+}–**4** is mainly through the hydrolysis pathway.

To verify the hydrolysis pathway further, a small dinucleotide model system, adenylyl(3′-5′)phosphoadenine (ApA), was used as the nucleic acid mimic. ApA (0.05 mM) and Cu^{2+}–**4** (0.01 mM) were dissolved in deionized water and, after 8 h of equilibration at room temperature, ESI-MS analysis was carried out. In the ESI mass spectrum (Figure S6, Supporting Information), the signals at *m*/*z* 267.94 and 348.16 show the presence of ApA cleavage products adenosine (calcd 268.10) and adenine monophosphate (AMP, calcd 348.06). The generation of the adenosine and AMP indicates that the phosphodiester bond of ApA was cleaved by Cu^{2+}–**4** through the hydrolysis pathway.[3h,7c] Therefore, the hydrolysis pathway should be a possible mechanism for DNA cleavage promoted by Cu^{2+}–**4**, similarly to the case of ApA.

In the ESI mass spectrum of Cu^{2+}–**4** in neutral aqueous solution (see the (Supporting Information page S10), the peaks at *m*/*z* 496.71 and 498.64 (isotope) show the signals of species **a** or **b** (calcd *m*/*z* 496.15 and 498.15). Kimura and co-workers have reported that the pendant guanidine of (2-guanidinyl)ethylcyclen is a good metal(II)-binding ligand at neutral pH in aqueous solution.[33] In the present work, copper(II) can be coordinated to one of the pendant guanidines of **4** to form **a** (Scheme 2) in neutral aqueous solution. Anslyn[16] and Krämer[8] have demonstrated independently that the metal-bound water is easily deprotonated to form the metal-bound OH, when the guanidine (or amino) arms are in close proximity to the metal-bound water. As a result, there is a possible equilibrium (Scheme 2) between the copper(II)-bound water (**a**) and the copper(II)-bound OH (**b**). Therefore, a plausible mechanism for DNA cleavage promoted by Cu^{2+}–**4** is depicted schematically in Scheme 3.



Scheme 2. A possible equilibrium between the copper(II)-bound water (**a**) and the copper(II)-bound OH (**b**).

First, one of the guanidinium groups recognizes and binds the phosphodiester bond of DNA through hydrogen bonding and electrostatic interaction, which assists the binding of the phosphodiester to the copper(II) center by electrostatic attraction. Meanwhile, the other guanidine group coordinates with the copper(II) center and the copper(II)-bound water is activated by the proximal guanidine group to form the copper(II)-bound OH. The activated phosphorus atom is then attacked nucleophilically by the copper(II)-bound OH in close vicinity, resulting in the formation of the trigonal bipyramidal phosphorous intermediate (Scheme 3, step **B**,**C**).[7,16,18] In succession, one of the P–O ester bonds of the phosphodiester is cleaved (Scheme 3, step **C**,**D**). Finally, the negatively charged leaving fragment accepts a proton from the guanidinium group, which serves as a potential proton donor to the leaving group for catalyst regeneration.

Cytotoxic activities: To evaluate the antitumor activity, B16 and HL-60 cells were exposed to different concentrations of the compounds for 72 h. Cell viability was determined by MTT assays.Compounds **3** and **4** do not show any inhibitory effects on B16 cells (Figure 12, top), while their copper(II)

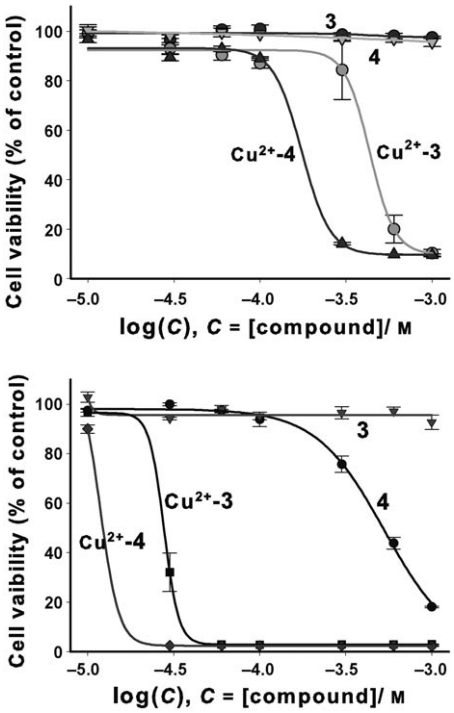
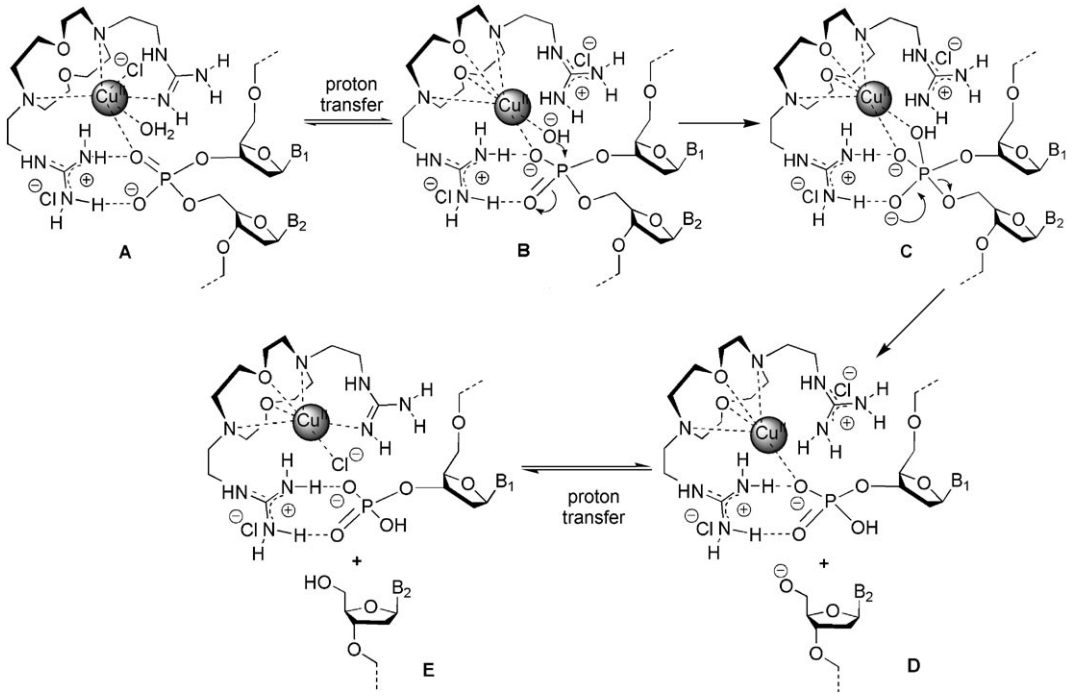


Figure 12. Inhibitory effects of **3**, **4**, Cu^{2+}–**3**, and Cu^{2+}–**4** on the proliferation of B16 (top) and HL-60 (bottom). Cells were exposed to the compounds for 72 h. The relative survival rate was determined relative to that of untreated control cells, which was set as 100%. Data were the mean ± SD of three experiments and each experiment included triplicate wells.



Scheme 3. A plausible mechanism for DNA cleavage promoted by Cu^{2+}–**4**.

complexes Cu^{2+}–**3** and Cu^{2+}–**4** both exhibit strong activities (IC_{50} 4.10×10^{-4} and 1.62×10^{-4} M, respectively). In the case of HL-60 cells (Figure 12, bottom), the IC_{50} values of **4**, Cu^{2+}–**3**, and Cu^{2+}–**4** are 5.30×10^{-4}, 2.78×10^{-5}, and 1.19×10^{-5} M, respectively. From these cytotoxic assay results, we conclude that the two copper(II) complexes (Cu^{2+}–**3** and Cu^{2+}–**4**) exhibit much stronger cytotoxic effects than the corresponding ligands (**3** and **4**, which have almost no antitumor activities), and HL-60 cells are proven to be more sensitive to these compounds than B16 cells. In the case of HL-60, the antitumor activities are shown to follow the order **3** < **4** < Cu^{2+}–**3** < Cu^{2+}–**4**, which is in agreement with the order of the DNA binding and cleavage abilities of these compounds.

Conclusion

Novel artificial receptors 1,7-dioxa-4,10-diazacyclododecanes with two pendant aminoethyl (**3**) or two guanidinoethyl (**4**) side arms were synthesized. Fluorescence and CD spectroscopy results indicate that the binding affinity of **3**, **4**, and their copper(II) complexes with calf thymus DNA follows the order Cu^{2+}–**4** > Cu^{2+}–**3** > **4** > **3**. The binding constants of Cu^{2+}–**3** and Cu^{2+}–**4** are 7.2×10^4 and 8.7×10^4 M^{-1}, respectively, which implies the groove-binding modes. Agarose gel electrophoresis assessment showed that these complexes exhibit powerful plasmid pUC 19 DNA cleavage efficiencies in the presence of the receptors. Kinetic data of DNA cleavage promoted by Cu^{2+}–**3** and Cu^{2+}–**4** under physiological conditions give maximal initial rate constants k_{max} of 0.865 and 0.596 h^{-1}, respectively, which correspond to a rate acceleration of about 10^8-fold over uncatalyzed supercoiled DNA.[11,34] The acceleration is due to the efficient cooperative catalysis of the the copper(II) cation center and the proximal functional groups (diamino or bisguanidinium). In-vitro cytotoxic activities toward murine B16 cells and human leukemia HL-60 cells were also examined: Cu^{2+}–**4** showed the highest activity, with $IC_{50}=1.62\times10^{-4}$ (B16) and 1.19×10^{-5} M (HL-60).

Experimental Section

Materials: All reagents and chemicals such as $CuCl_2$, KI, NaN_3, DMSO, and *t*BuOH were of analytical grade and used without further purifications. *N*-(2-Bromoethyl)phthalimide, 1*H*-pyrazole-1-carboxamidine hydrochloride, calf thymus DNA (CT-DNA), adenosine, uridine, guanosine, cytidine, and dinucleotide (ApA) were all purchased from Sigma Aldrich. pUC 19 plasmid DNA was purchased from TaKaRa Biotechnology, Shiga (Japan); its purity was checked by agarose gel electrophoresis, and its concentration was determined by UV spectroscopy by using the extinction coefficient appropriate for double-stranded DNA ($1.0\ OD_{260}=50\ \mu g\,mL^{-1}$). Agarose was from Oxoid, Basingstoke (UK), ethidium bromide (EB) was from Amresco, and tris(hydroxymethyl)aminomethane (Tris-Base) was from Robiot, Nanjing (P. R. China). Bromophenol blue, glycerol, and ethylenediamine tetraacetic acid (EDTA) were commercially available. Deionized water was obtained by ion exchange from double distilled water. All solvents were purified by standard procedures.

The stock solution of CT-DNA (stored at 4 °C and used for not more than two days) was prepared in 5 mM Tris-HCl/10 mM NaCl in water, pH 7.5. The CT-DNA concentration was determined from its absorption intensity at 260 nm with a known molar extinction coefficient ($6600\ M^{-1}cm^{-1}$), and the ratio of UV absorbance at 260 nm and 280 nm, $A_{260}/A_{280}=1.8$–1.9, indicating that the DNA was sufficiently protein-free.[30c,35]

Apparatus: ^{1}H NMR and ^{13}C NMR data were recorded on a Brucker AM 300 spectrometer (Germany). Mass spectra were obtained on a Finnigan LCQ electrospray mass spectrometer in positive mode. The pH value was confirmed by ORION868 pH meter with an Ag/AgCl reference electrode in saturated KCl solution at room temperature. The UV/Vis absorption spectra were acquired by a Perkin–Elmer Lambda 25 UV/Vis spectrometer; a 1.00 cm quartz cell was used. The fluorescent spectra were recorded on an AMINCO Bowman Series 2 luminescence spectrometer. A Jasco J-810 automatic recording spectropolarimeter was used for the circular dichroism (CD) spectroscopy. Agarose gel electrophoresis was performed with DYY-5 electrophoresis apparatus. Bands were visualized by UV light and photographed using DigiDoc-It gel imaging and documentation system (version 1.1.23, UVP Inc. Upland, CA). The intensity of the DNA bands was estimated by TotalLab image analysis software (version 2.01).

Fluorescence measurements: The fluorescent spectral emission intensity of ethidium bromide (EB) was measured on an AMINCO Bowman Series 2 luminescence spectrometer, by adding **3**, **4**, Cu^{2+}–**3** or Cu^{2+}–**4** (0–60 μM) to the EB-bound CT-DNA (0.1 mM) solution in Tris–HCl buffer (5 mM, pH 7.5). The measured fluorescence was normalized to 100 % relative fluorescence.

Circular dichroism measurements: All CD spectroscopic studies were carried out with a continuous flow of nitrogen purging the polarimeter, and the measurements were performed at room temperature with 1 cm pathway cells. The CD spectra were run from 320 to 220 nm at 20 nm min^{-1} and the buffer background was subtracted automatically. Data were recorded at 0.1 nm intervals. The CD spectrum of CT-DNA (120 μM) alone was recorded as the control experiment.

Gel electrophoresis: DNA cleavage experiments were performed using 50066 ng per reaction of pUC 19 derived plasmid, length 2686 bp. The supercoiled DNA in Tris-HCl (50 mM) buffer containing NaCl (10 mM) was treated with different concentrations of the compound, then diluted with the buffer to a total volume of 15 μL. The sample was incubated at 37 °C. The loading buffer (30 mM EDTA, 0.05 % (w/v) glycerol, 36 % (v/v) bromophenol blue) (3 μL) was added to end the reactions and the mixture was loaded onto agarose gel (1 %) containing EB ($1.0\ \mu g\,dm^{-3}$). The DNA fragments after cleavage assays were separated and monitored by agarose gel electrophoresis at 80 V for 1.5 h in 0.5 × Tris-acetate EDTA (TAE) buffer. Bands were visualized by UV light and photographed. The proportion of DNA in the supercoiled and nicked forms after electrophoresis was estimated quantitatively from the intensities of the bands using TotalLab analysis software. Supercoiled pUC 19 DNA values were corrected by a factor of 1.3 on the basis of average literature estimates of the lowered binding of EB to this structure.[36]

1,7-Dioxa-4,10-diazacyclododecane (1): This was prepared according to the literature method.[37] ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=2.11$ (s, 2NH), 2.81 (t, $J=4.8$ Hz, 8H; $4CH_2NH$), 3.62 ppm (t, $J=4.8$ Hz, 8H; $4CH_2O$)?

4,10-bis[*N,N'*-(2-ethyl)phthalimido]-1,7-dioxa-4,10-diazacyclododecane (2): A stirred solution of 1,7-dioxa-4,10-diazacyclododecane (0.26 g, 1.5 mmol), *N*-(2-bromoethyl) phthalimide (0.80 g, 3.1 mmol), and potassium carbonate (0.50 g) in anhydrous $CHCl_3$ (20 mL) was heated at 50 °C for 8 h. The mixture was then allowed to cool to room temperature and filtered. The filtrate was evaporated under reduced pressure to give a yellow solid. The crude product was purified on a silica gel chromatographic column (chloroform/methanol, 5:1 then 1:1) and washed with acetone (2 × 2 mL) to obtain **2** (0.65 mg, 1.2 mmol) as a colorless needle-like crystal. Yield: 83 %; m.p. 181–184 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=2.58$ (s, 8H; $4NCH_2$), 2.73 (t, $J=4.8$ Hz, 4H; $2NCH_2$), 3.44 (s, 8H; $4OCH_2$), 3.72 (t, 4H; $J=4.8$ Hz, $2CONCH_2$), 7.68–7.72 (m, 4H; 4ArH), 7.80–7.84 ppm (m, 4H; 4ArH); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=36.18$ (NCH_2), 53.82 (NCH_2), 55.33 (NCH_2), 69.65 (OCH_2), 123.50 (Ar C),

132.6 (Ar C), 134.24 (Ar C), 168.79 ppm (C=O); ESI-MS: *m*/*z* calcd for $[M+H]^+$: 521.23, found 521.03; *m*/*z* calcd for $[M+Na]^+$: 543.22, found 542.92.

4,10-Bis[*N*,*N*′-(2-ethyl)amino]-1,7-dioxa-4,10-diazacyclododecane (3): A stirred solution of **2** (0.26 g, 0.50 mmol) and 80% hydrazine hydrate (0.3 mL) in anhydrous ethanol (5 mL) was heated at 50 °C for 3 h. The resulting solution was cooled to the room temperature and then 37% hydrochloric acid (0.5 mL) was added. After being heated to reflux and stirred for 15 min, the mixture was cooled to room temperature and filtered. The filtrate was evaporated under reduced pressure to remove ethanol. The residue was neutralized with potassium hydroxide (40% aqueous solution) to pH 10 and extracted with $CHCl_3$ (5×30 mL). The organic layer was dried and evaporated in vacuum to afford **3** as a yellow oil (0.09 g, 0.35 mmol). Yield: 70%; ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.58 (t, J=5.7 Hz, 4H; $2NCH_2$), 2.67 (t, J=4.5 Hz, 8H; $4NCH_2$), 2.76 (t, J=5.7 Hz, 4H; $2NH_2CH_2$), 3.00 (br s, $2NH_2$), 3.57 ppm (t, J=4.5 Hz, 8H; $4OCH_2$); ^{13}C NMR (75 MHz, $CDCl_3$): δ=38.36 (CH_2NH_2), 47.42 (NCH_2), 54.56 (NCH_2), 68.25 ppm (OCH_2); ESI-MS: *m*/*z* calcd for $[M+H]^+$: 261.2, found 261.3.

4,10-Bis[*N*,*N*′-(2-ethyl)guanidine]-1,7-dioxa-4,10-diazacyclododecane (4): A mixture of **3** (0.11 g, 0.38 mmol), 1*H*-pyrazole-1-carboxamidine hydrochloride (0.11 g, 0.75 mmol), and $((CH_3)_2CH)_2NC_2H_5$ (DIEA) (0.10 g, 0.77 mmol) in DMF (7 mL) was stirred for 10 h at room temperature under a dry nitrogen atmosphere. Immediately after anhydrous ether (15 mL) was added, a brown oil was deposited. This deposit was then dissolved in deionized water and subjected to chromatography on a no. 717 strong base anion exchange resin column (eluent deionized water). Subsequently, the eluent was evaporated under reduced pressure to remove water and the residue was washed with diethyl ether (3×30 mL) to eliminate the unreacted reactant **3** and other organic impurities. The residue was then dissolved in deionized water (15 mL) and neutralized to pH 7.0 by adding 10% hydrochloric acid. Water was removed in vacuo to give **4** as a strongly hygroscopic brown solid (0.13 g, 0.31 mmol). Yield: 82%; ^{1}H NMR (300 MHz, D_2O): δ=3.53 (t, J=4.5 Hz, 4H; $2CNHCH_2$), 3.45 (t, J=3.6 Hz, 8H; $4OCH_2$), 3.19 (t, J=4.5 Hz, 4H; $2NCH_2CH_2NH$), 2.63 ppm (t, J=3.6 Hz, 8H; $4NCH_2CH_2O$); ^{13}C NMR (75 MHz, D_2O): δ=39.16 (CH_2NH_2), 54.04 (NCH_2), 55.60 (NCH_2), 59.14 (OCH_2), 157.98 (guanidine C); ESI-MS: *m*/*z* calcd for $[M-2Cl]^{2+}$: 173.1, found 173.2; *m*/*z* calcd for $[M-2Cl+H]^+$: 345.3, found 345.4.

Preparation of copper(II) complex stock solutions: A mixture of $CuCl_2 \cdot 2H_2O$ (0.015 g, 0.10 mmol) and **3** (0.026 g, 0.10 mmol) or **4** (0.042 g, 0.10 mmol) in methanol (10 mL) was stirred for 1 h at room temperature and then evaporated to remove the solvent. The residue was dissolved in deionized water (5 mL) as the copper(II) complex stock solution (20 mM).

Cytotoxic assays

Cell lines: Murine B16 melanoma cell line and human HL-60 promyelocytic leukemia cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and maintained in DMEM (Life Technologies Inc., Grand Island, NY) and RPMI 1640 (Gibco, Scotland, UK) supplemented with penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), and fetal bovine serum (10%) (Life Technologies), respectively, at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

MTT assays for cell viability: Cells were cultured in 96-well plates for 72 h with various compounds. Cell viability was evaluated with a modified MTT assay.[38] Briefly, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (20 μL, 4 mg mL^{-1}) (MTT, Sigma) in medium was added for a further 4 h of incubation. After removal of the supernatant, DMSO (200 μL) was added to dissolve the formazan crystals. The absorbance was read on an ELISA reader (Tecan, Austria) at 540 nm. The relative survival rate was determined in relation to that of untreated cells, which was set as 100%.

Acknowledgements

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