Synthesis, DNA-cleaving activities and cytotoxicities of $C_2$-symmetrical dipyrrole-polyamide dimer-based Cu(II) complexes: A comparative study

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ABSTRACT

Two $C_2$-symmetrical dipyrrole-polyamide dimers 2 and 3 that were tethered with triethylenetetramine and spermine, respectively, and their corresponding Cu(II) complexes $2@Cu^{2+}$ and $3@Cu^{2+}$, were synthesized and fully characterized. Agarose gel electrophoresis studies on pBR322 DNA cleavage indicated that both Cu(II) complexes exhibited potent DNA-cleaving activities under physiological conditions, most probably via an oxidative mechanism. Kinetic assay indicate that $2@Cu^{2+}$ and $3@Cu^{2+}$ exhibited comparable catalytic efficiency with the Cu(II) complex of their 2,2’-(ethane-1,2-diylbis(oxy))diethanamine-tethered analog 1. The finding that compounds 2 and 3 showed higher Cu(II) ion-complexing abilities than compound 1, suggests that strong metal complexation does not necessarily lead to an enhancement in the catalytic efficiency of a DNA-cleaving agent. In addition, three Cu(II) complexes displayed moderate inhibitory activities toward three tumor cell lines.

1. Introduction

Cancer is one of modern diseases that are increasingly threatening the public health, therefore finding efficient therapy for it has been attracting considerable interest from medicinal chemists. It is widely recognized that some antitumor drugs exert their activities by binding to, modifying and cleaving DNA [1]. On the other hand, DNA has sufficient stability under physiological conditions. These have inspired considerable efforts to identify agents that are capable of efficiently cleaving DNA. Such so-called DNA-cleaving agents offer a multitude of applications, for example, as DNA structural and conformational probes, and as potential chemotherapeutic agents for clinical use [2,3].

As DNA-cleaving molecules are generally constructed by tethering catalytically active centers with DNA-recognizing units [4–8], DNA-binding units and their linkers are thus two structural factors that should be taken into consideration in creating of effective DNA-cleaving agents. DNA-binding units that are frequently used include polypyrroles, anthraquinones, acridines and naphthalenes, whereas polyethers, polyamines, azacrown and EDTA are often used as the tethers [9–15]. As part of our projects to create potent DNA cleavers that are active under physiological conditions [16–20], we have keenly become interested in polypyrrrole-based polyamides as DNA-recognizing units. Thus, in earlier studies we have shown that the Cu(II) complexes of polymer-tethered pyrrole-polyamide dimers 1 and 1 (Fig. 1), are capable of efficiently cleaving pBR322 DNA under physiological conditions. Interestingly, the Cu(II) complex of compound 1 ($1@Cu(II)$ hereafter) exhibits at least 30-fold higher catalytic activity ($k_{max}/K_M$) than the Cu(II) complex of compound 1, which we assume is a likely consequence of its higher DNA-binding affinity [19,20]. These results highlight the fact that $1@Cu(II)$ is exploitable as a potent DNA-cleaving agent with promising properties.

It is widely recognized that, however, polyether exhibits low affinity toward Cu(II) ion. This is evidenced from the overwhelming literature that report the use of ligands containing other atoms, for example, nitrogen atom instead of oxygen atom [21–24]. Thus, one immediate concern we had was whether a DNA-cleaving agent that is based on a nitrogen atom-containing ligand would exhibit higher activity than an analog prepared from the corresponding oxygen-containing ligand. To test this, herein we synthesize two polyanine-tethered symmetrical dipyrrole-polyamide dimers 2 and 3 (Fig. 1), and conduct a comparative study on the DNA-binding, cleaving and antitumor activities of the Cu(II) complexes of compounds 1–3. Structurally, compounds 1–3 have two dipyrrole sub-units, whereas compound 1 has a polyether linker and compound 2...
has a shorter polyamine linker than compound 3. Thus, comparison of the Cu(II) complexes of compounds 1, 2 and 3 will provide a means for judging the effect of the linkers on the Cu(II) coordination ability, DNA-binding and cleaving and anticancer activities.

2. Results and discussion

2.1. Chemistry

The synthetic route that was used to prepare compounds 2 and 3, and their Cu(II) complexes is shown in Scheme 1. Thus, activation of 1-methyl-4-[1-methyl-4-nitro-1H-pyrrole-2-carboxylic acid 4 with N-hydroxysuccinimide (NHS) in the presence of DCC and subsequent reaction with \( \text{NH}_2\text{-bis(}N\text{-aminoethyl})\text{-N}_2\text{-bis(tert-butoxycarbonyl)} \) ethylenediamine 5 or \( \text{N}_4\text{N}_2\text{-bis(tert-butoxycarbonyl)} \) spermine 6, respectively, afforded compounds 7 and 8 in 55% and 50% yields, respectively. Boc-deprotection of compounds 7 and 8 with TFA gave compounds 2 and 3 in 95% and 94% yields, respectively. The Cu(II) complexes of compounds 2 and 3 were prepared in 70% and 60% yields from their reaction with CuCl\(_2\) in aqueous DMF, respectively. Compound 1 and its Cu(II) complex were prepared according to our reported procedures [20].

2.2. Characterization of \( \text{Cu}^{2+} \) and \( \text{Cu}^{2+} \) complexes

Compounds 2 and 3 were fully characterized by MS (ESI and HR), NMR (\( ^1\text{H} \) and \( ^{13}\text{C} \)) and IR (see experimental section and Supporting information). The structures of the Cu(II) complexes of compounds 2 and 3 were established by means of ESI-MS, UV–Vis, \( ^1\text{H} \) NMR, IR and molar conductivity. In the ESI MS spectrum of the Cu(II) complex of compound 2 (Fig. 2a), the ion peaks at \( m/z \) 756.5 and 792.7 were assignable to \([\text{Cu}^{2+}+\text{H}^+]+\) and \([\text{Cu}+\text{H}^+]+\), respectively. Similarly, the ion peaks at \( m/z \) 830.4 and 848.6 in the ESI MS spectrum of the Cu(II) complex of compound 3, were assignable to \([3+\text{Cu}+\text{H}_2\text{O}+\text{H}^+]+\) and \([3+\text{Cu}+\text{Cl}]+\), respectively (Fig. 2b). These results suggest that compounds 2 and 3 formed 1:1 complexes with CuCl\(_2\) in aqueous solution. This was further supported by spectrophotometric titration experiments of compounds 2 and 3 with CuCl\(_2\) in aqueous solution (Fig. 3). The absorbance at 285 nm decreased with the concentration ratios of Cu(II) ion to compound 2 or 3 up to the \([\text{Cu}^{2+}]\)[compound 2 or 3] ratio being 1. Above this ratio, there was no significant change in the absorptivity. These spectroscopic variations are indicative of the strong interaction of Cu(II) ion with compounds 2 and 3, and support their 1:1 stoichiometries [17,19,20,25]. This was in accordance with our previous finding that compound 1 formed a 1:1 complex with Cu(II) ion [20].

The coordination of Cu(II) ion to compounds 2 and 3 was convincingly established on the basis of \( ^1\text{H} \) NMR (Table 1) and IR. As shown in literature [10,17,26–28], NMR techniques can be used to characterize a copper complex. Thus, in the \( ^1\text{H} \) NMR spectrum of the Cu(II) complex of compound 2, almost all the protons showed chemical shift changes. For the Cu(II) complex of compound 3, H15, H16 and H20 exhibited significant downfield shifts (\( \Delta \delta \approx 0.07, 0.14 \) and 0.17 ppm, respectively), and H17 and H19 showed downfield shifts. These results indicate the coordination of Cu(II) ion to the two N atoms of the polyamine linkers. On the other hand, in the IR spectra, the bands at 1595.0 and 1595.5 cm\(^{-1}\) that were assigned to \( \nu\text{C}–\text{N} \) of the C13-amido groups in compounds 2 and 3, respectively, were shifted to 1579.0 and 1580.4 cm\(^{-1}\) for their Cu(II) complexes, respectively. This result, together with the above observation that H15 exhibited significant downfield shifts, suggests that Cu(II) ion coordinates to the N atoms of the C13-amido groups.
In addition, the molar conductivities ($\Lambda_{\text{M}}$'s) of the Cu(II) complexes of compounds 2 and 3 were measured to be 102.5 and 110.5 S cm$^2$ mol$^{-1}$ in DMF/H$_2$O (1/4, v/v) at room temperature, respectively, whereas that of CuCl$_2$ was 209.5 S cm$^2$ mol$^{-1}$ under similar conditions. These values reveal that the Cu(II) complexes of compounds 2 and 3 existed as 1:1 electrolytes under our measuring conditions [10,29].

Taken together, these results convincingly suggest that compounds 2 and 3 formed 1:1 complexes with Cu(II) ion ($2@\text{Cu}^{2+}$ and $3@\text{Cu}^{2+}$ hereafter). Specifically, the Cu(II) ions coordinated to the N atoms of both the polyamine linkers and the C$_{13}$-amido groups, and one chloride (Scheme 1).

It should be noted that the above-mentioned results, in combination with our previous finding that complexation of compound 1 with Cu(II) ion did not cause any considerable change in the $^1$H NMR spectrum, suggest that compounds 2 and 3 have higher affinities toward Cu(II) ion than compound 1. To further demonstrate this, we carried out ESI MS competitive experiment of compounds 1 and 3 with CuCl$_2$ in the equimolar ratios (Fig. 2c). Such an experiment allows the easy determination of which one of compounds 1 and 3 coordinates to CuCl$_2$ preferentially, because preferential coordination usually leads to greater relative abundance in the ESI-MS spectrum. The ion peaks at $m/z$ 697.3, 830.4 and 886.5 were assignable to $[1+\text{H}]^+$, $[3+\text{Cu}+\text{H}_2\text{O}+\text{H}]^+$ and $[3+\text{Cu}+2\text{Cl}+\text{H}]^+$, respectively.

Fig. 2. ESI-MS spectra of (a) $2@\text{Cu}^{2+}$, (b) $3@\text{Cu}^{2+}$ and (c) an equimolar mixture of compounds 1, 3 and CuCl$_2$ in aqueous MeOH solution.

Fig. 3. Plots of the absorbance at 285 nm of (a) compounds 2 ($2.0 \times 10^{-5} \text{ M}$) and (b) 3 ($2.0 \times 10^{-5} \text{ M}$) vs the [Cu(II) ion]/[2 or 3] ratios in 5 mM Tris–HCl buffer (5 mM NaCl, pH 7.0).
respectively. No ion peaks from compound 3 or the Cu(II) complex of compound 1 were observed. These results suggest that compound 3 had much higher affinity with Cu(II) ion than compound 1.

2.3. DNA-cleaving activities of 2@Cu^{2+} and 3@Cu^{2+}

The cleaving activities toward pBR322 DNA of compounds 2 and 3 in the presence of six biologically important metal ions, including Co(II), Mg(II), Ni(II), Cu(II), Zn(II), and Mn(II), were screened with agarose gel electrophoresis (GE). It can be seen from Fig. 4 that the supercoiled form of pBR322 DNA (CCC) relaxed to generate open circular (OC) and linear forms in the presence of compounds 2 and 3 with the six metal ions. Among them, all the CCC forms were converted into the OC and linear forms in the presence of Cu(II). These results suggested that compounds 2 and 3 were capable of cleaving pBR322 DNA in the presence of these metal ions. The highest activity was observed in the presence of Cu(II) ion.

Detailed examination of the cleaving activities of 2@Cu^{2+} and 3@Cu^{2+} were then carried out. Firstly, we investigated the concentration dependences. It is clear that the cleaving efficiency increased with the concentrations of 2@Cu^{2+} (Fig. 5a) and 3@Cu^{2+} (Fig. 5b). When their concentrations reached 0.05 mM, all the CCC forms were converted into the OC and linear forms. It should be noted that compound 2 (Lane 7, Fig. 5a), compound 3 (Lane 7, Fig. 5b) and CuCl_{2} (Lane 8, Fig. 5a and b) showed negligible activities under the similar conditions. These findings demonstrated that the Cu(II) complexes acted as the cleaving agents.

Secondly, we measured the kinetics of pBR322 DNA degradation. Fig. 6a indicates that the extent of the relaxation of supercoiled DNA by 2@Cu^{2+} (0.05 mM) varies exponentially with the reaction time, giving pseudo-first-order kinetics with an apparent initial first-order rate constant (k_{obs}) of (0.48 ± 0.06) h^{-1} [19,20,30,31]. The maximal first-order rate constant (k_{max}) and Michaelis constants (K_{M})s obtained from the saturation kinetics profile of the supercoiled DNA cleavage (Fig. 6b and Table 2) were (1.49 ± 0.16) h^{-1} and (101 ± 22) μM for 2@Cu^{2+}, and (1.41 ± 0.10) h^{-1} and (72 ± 13) μM for 3@Cu^{2+}, respectively. Thus, 2@Cu^{2+} and 3@Cu^{2+} can catalyze the cleavage at a rate acceleration of ca. 10^{3}-fold over uncatalyzed supercoiled DNA cleavage (k = 3.6 × 10^{-8} h^{-1} at 37 °C) [32]. In an earlier study we have shown that the maximal first-order rate constant k_{max} and Michaelis constant K_{M} of 1@Cu^{2+} are 14.3 h^{-1} and 600 μM, respectively [20]. Thus, 1@Cu^{2+} showed ca. 10-fold higher catalytic ability than 2@Cu^{2+} and 3@Cu^{2+}, suggesting that a weak Cu(II) ion-complexing ligand can be equally used to construct a potent DNA-cleaving agent. This finding may provide useful guidance for future rational design of effective DNA-cleaving agents. That is, the construction of an effective DNA-cleaving agent is not necessarily restricted to a ligand having strong metal ion-complexing ability.

It is interesting to compare the DNA-cleaving efficiency of 2@Cu^{2+} and 3@Cu^{2+} with that of 1@Cu^{2+}. As described above, 2@Cu^{2+} and 3@Cu^{2+} had ca. 6- and 9-fold greater Michaelis constants than 1@Cu^{2+}. Therefore, these three complexes showed comparable overall catalytic activities (k_{max}/K_{M}), that is, 23.83 h^{-1} mM^{-1} for 1@Cu^{2+}, 14.76 h^{-1} mM^{-1} for 2@Cu^{2+}, and 19.63 h^{-1} mM^{-1} for 3@Cu^{2+}. It is known that Michaelis constant K_{M} reflects the association of a substrate with its enzyme and the dissociation of the resulting product from the enzyme. Thus, to better understand the DNA-cleaving efficiency of 1@Cu^{2+}, 2@Cu^{2+} and 3@Cu^{2+}, we firstly measured the binding constants (K_{S})s of compounds 1, 2, 3 and their Cu^{2+} complexes toward calf-thymus (CT) DNA by means of ethidium bromide (EB) displacement experiments (Table 2). It can be seen that compounds 2 and 3 exhibited slightly higher DNA-binding affinities than compound 1. This is a likely consequence of the stronger interaction of the polyamine chains with DNA through hydrogen-bonding and electrostatic forces [33]. The presence of Cu(II) ion increased the DNA-binding affinities of compounds 1, 2 and 3 by ca. 10-fold. However, all these three Cu(II) complexes showed comparable DNA-binding affinities. This finding strongly suggests that the difference in the Michaelis constants of 1@Cu^{2+}, 2@Cu^{2+} and 3@Cu^{2+} might be due to their dissociation from the cleaved pBR322 DNA rather than their association with pBR322 DNA.

Secondly, we investigated the probable mechanism of action by monitoring the pBR322 DNA cleavage by 2@Cu^{2+} and 3@Cu^{2+} in the presence of singlet oxygen scavenger 2, 2, 6, 6-tetramethyl-4-piperidone (TMP), superoxide scavenger KI, and hydroxyl radical scavengers DMSO and t-BuOH (Fig. 7) [17,20,34,35]. As a result, the addition of TMP (Lane 4), DMSO (Lane 6) or t-BuOH (Lane 7) led to no or slight inhibition of the cleavage, suggesting that neither singlet oxygen nor hydroxyl radical is involved in the reaction. The obvious inhibition of the cleavage reaction in the presence of KI (Lane 4) suggests that superoxide radicals may participate in the DNA cleavage by 2@Cu^{2+} and 3@Cu^{2+}.

In addition, we monitored the hydrolysis of bis(4-nitrophenyl) phosphate (BNPP) in the presence of excess 2@Cu^{2+} and 3@Cu^{2+} at physiological pH and 25 °C. If BNPP can be hydrolyzed by 2@Cu^{2+} or 3@Cu^{2+}, this is detected by an increase in the absorbance at 400 nm of the released 4-nitrophenolate [36]. As a result, no change in the absorbance was observed over 18 h, ruling out the possibility that 2@Cu^{2+} and 3@Cu^{2+} acted as a hydrolytic agent [17,20].

These results suggest that 2@Cu^{2+} and 3@Cu^{2+} efficiently converted pBR322 DNA into OC and linear forms under physiological conditions, most probably via an oxidative mechanism. Thus, it is proposed that the copper centers strongly bind O_{2} to form reactive oxygen species (ROS) and catalyze the cleavage.
oxygen species, such as superoxide, which can further activate the cleavage of supercoiled DNA to form nicked DNA. Compounds 2 and 3 have higher affinities toward Cu(II) ion than compound 1, which in turn may reduce the affinity of their Cu(II) centers toward O₂ and thereby decrease the ability to form reactive oxygen species. Therefore, 1@Cu²⁺ showed higher catalytic ability than 2@Cu²⁺ and 3@Cu²⁺. Because 2@Cu²⁺ and 3@Cu²⁺ have lower affinities toward O₂, they may more readily dissociate from the cleaved pBR322 DNA so as to enter the next catalytic cycle. These results imply that DNA cleavage is involved in a multi-step process including association, catalysis and dissociation. Depending on the nature of the DNA-cleaving molecule that is to be used, any one of these steps may become rate-limiting. These considerations should weigh heavily in optimizing the design of DNA-cleaving agents, in particular based on metal complexes.

2.4. Cytotoxicities

Since DNA cleavage is considered essential for some anticancer agents to exert their activities, the in vitro antitumor activities of 1@Cu²⁺, 2@Cu²⁺ and 3@Cu²⁺ toward HepG2, A549 and NCI-N87 cells, were evaluated by a cell proliferation assay using MTT. The obtained EC₅₀ values, together with those of compounds 1–3 for comparison, are listed in Table 3. It can be seen that compounds 1, 2 and 3 were inactive under our assay conditions, whereas their Cu(II) complexes exhibited moderate inhibitory activities in the order of 2@Cu²⁺ > 1@Cu²⁺ > 3@Cu²⁺. In particular, 2@Cu²⁺ exhibited potent activity toward NCI-N87 cells with the EC₅₀ Value being 6.6 µM. This result suggests that the antitumor activities of the Cu(II) complexes of dipyrrole-polyamide dimers might be modulated by fine-tuning the structures of the linkers.

3. Conclusions

In summary, two new polyamine-tethered symmetric dipyrrole-polyamide dimers have been successfully synthesized and fully characterized on the basis of NMR (¹H- and ¹³C-), MS (ESI and HR) and IR data. Their Cu(II) complexes were capable of efficiently converting pBR322 DNA into OC and linear forms under physiological conditions, most probably via an oxidative mechanism and at a rate acceleration of ca 10³-fold over uncatalyzed cleavage of supercoiled DNA. These findings suggest a synergistic effect from the strong binding of the dipyrrole subunits and the effective cleavage of polyamine-coordinated Cu(II) ion. Complex 2@Cu²⁺ exhibited the most effective inhibitory activity with the EC₅₀ value being 6.6 µM against NCI-N87 cells. These properties, together with the high potential DNA-binding specificity of dipyrrole-polyamides, make this type of dimers and their metal complexes exploitable in search for potential chemotherapeutic agents, for example, for cancers.

4. Experimental section

4.1. Methods and materials

¹H and ¹³C NMR spectra were recorded in DMSO-d₆ or its mixture with D₂O on Varian Mercury 400 spectrometers. ESI-MS and HR-ESI-MS spectra were measured on Waters UPLC/Quattro Premier XE and Agilent 6460 Triple Quadrupole mass spectrometers, respectively. IR spectra were recorded on a Brucker Tensor IR instrument using KBr discs in the range of 400–4000 cm⁻¹. Conductivity was measured on a DDS-12A conductivity meter. Silica gel 60 A (reagent pure, Qingdao Haiyang Chemical Co. Ltd) was used for column chromatography. Analytical thin-layer chromatography was performed on silica gel plates 60 GF254 (chemical pure, Qingdao Haiyang Chemical Co. Ltd). Detection on TLC was made by use of iodine and UV (254 or 365 nm). UV–Vis and fluorescence spectra were measured on a TU-1901 spectrophotometer and a Shimadzu RF-5301PC spectrofluorimeter, respectively. Agarose GE was carried out on a DYY-8C electrophoresis apparatus and DYCP-31DN electrophoresis capillary (Beijing Liuyi Instrument Factory, Beijing, China). DNA cleavage was analyzed with Alpha Hp 3400 fluorometric and visible light digitized image analyzer.

CT DNA, EB and plasmid pBR322 DNA were purchased from Sigma Chemical Co. (St Louis, USA). The concentration of CT DNA

![Fig. 5. Agarose GE patterns for the pBR322 DNA cleavage by (a) 2@Cu²⁺ and (b) 3@Cu²⁺ of varying concentrations in 5 mM Tris–HCl buffer (5 mM NaCl, pH 7.0) and at 37 °C for 4 h. Lanes 1–6: 0, 0.01, 0.02, 0.035, 0.04 and 0.05 mM for 2@Cu²⁺ or 3@Cu²⁺, respectively; Lane 7: compound 2 or 3 (0.05 mM); and Lane 8: CuCl₂ (0.05 mM).](image)

![Fig. 6. (a) Time course of pBR322 DNA cleavage by 2@Cu²⁺ (0.05 mM). Inset: agarose GE patterns of the time—variable reaction products. Lanes 1–8, reaction time 0, 1, 1.5, 2, 2.5, 3, 3.5 and 4 h, respectively. (b) Saturation kinetics of k₄ versus the concentrations of 2@Cu²⁺ (□) and 3@Cu²⁺ (▲).](image)
was determined spectrophotometrically using the molar extinction coefficient of 6600 M⁻¹ cm⁻¹/base at 260 nm. Compounds 4, 5 and 6 were prepared according to the reported procedures [37,38]. All the other chemicals were of analytical grade and used without further purification.

4.2. Synthesis and characterization

4.2.1. Synthesis of compounds 7 and 8

4.2.1.1. Compound 7. To a solution of compound 4 (175 mg, 0.6 mmol) and NHS (104 mg, 1.8 mmol) in anhydrous DMF (3 mL) was added DCC (619 mg, 3 mmol). The resulting mixture was stirred at room temperature for 2 h and filtered to remove the insoluble urea. The filtrate was then added, drop wise, to a solution of Boc-protected polyamine 5 (104 mg, 0.3 mmol) and DMAP (147 mg, 1.2 mmol) in anhydrous DMF (2 mL). The resulting mixture was stirred at room temperature for another 12 h, and then concentrated under reduced pressure. The obtained residue was purified by chromatography on a silica gel column, eluting with CHCl₃/MeOH (80:1, v/v), to afford compound 7 (148 mg, 55%) as a yellowish solid, having ¹H NMR (400 MHz, DMSO-d₆) δ 1.34 (s, 9H), 1.35 (s, 9H), 3.26 (m, 12H), 3.80 (s, 6H), 3.94 (s, 6H), 6.38 (s, 1H), 6.88 (s, 1H), 7.18 (s, 1H), 7.20 (s, 1H), 7.55 (d, 2H), 8.13 (s, 1H), 8.14 (s, 1H), 10.23 (s, 2H); ¹³C NMR (100 MHz, DMSO-d₆) δ 27.92, 35.74, 35.96, 37.41, 107.49, 118.10, 121.32, 123.05, 126.26, 128.13, 133.75, 156.84, 161.27; ESI-MS m/z: 917.6 [M + Na⁺] and HR-ESI-MS for C₉₆H₃₅N₂O₁₂ ([M + H⁺]⁺) calcld: 985.4062, found 985.4044.

4.2.1.2. Compound 8. Similar procedures as described for compound 7; from compound 4 (175 mg, 0.6 mmol) and Boc-protected spermine 6 (121 mg, 0.3 mmol). Yield: 143 mg (50%). ¹H NMR (400 MHz, DMSO-d₆) δ 1.35 (s, 18H), 1.40 (m, 4H), 1.66 (m, 4H), 3.13 (m, 12H), 3.79 (s, 6H), 3.93 (s, 6H), 6.83 (s, 2H), 7.18 (s, 2H), 7.54 (s, 2H), 7.93 (s, 2H), 8.12 (d, 2H), 10.23 (s, 2H); ¹³C NMR (100 MHz, DMSO-d₆) δ 27.97, 30.76, 35.79, 35.90, 37.39, 78.36, 103.90, 107.47, 117.95, 121.16, 123.15, 126.20, 128.11, 133.75, 156.76, 162.42; ESI-MS m/z: 973.7 [M + Na⁺] and HR-ESI-MS for C₉₆H₃₅N₂O₁₂ ([M + H⁺]⁺) calcld: 951.4688, found 951.4670.

4.2.2. Synthesis of compounds 2 and 3

4.2.2.1. Compound 2. To a solution of compound 7 (40 mg, 0.045 mmol) in CH₂Cl₂ (4 mL) was slowly added trifluoroacetic acid (4 mL), and the solution was stirred at room temperature for 2 h. Then, the reaction mixture was concentrated under reduced pressure, dissolved in DMF (1 mL) and added to 5% ammonia solution (30 mL). The resulting mixture was stirred at room temperature for 10 min and filtered to afford compound 2 (30 mg, 95%) as a yellowish solid having ¹H NMR (400 MHz, DMSO-d₆) δ 2.62 (br, 4H), 2.66 (br, 2H), 2.67 (br, 2H), 3.25 (br, 2H), 3.27 (br, 2H), 3.81 (s, 6H), 3.95 (s, 6H), 6.86 (s, 2H), 7.20 (s, 2H), 7.57 (s, 1H), 7.58 (s, 1H), 7.99 (s, 2H), 8.17 (s, 2H), 10.23 (s, 2H); ¹³C NMR (100 MHz, DMSO-d₆) δ 36.00, 37.42, 48.66, 104.02, 107.51, 117.96, 121.31, 123.17, 126.26, 128.13, 133.75, 156.83, 161.22; ESI-MS m/z: 695.7 [M + H⁺] and HR-ESI-MS for C₉₆H₃₅N₂O₂ ([M + Na⁺]⁻) calcld: 717.2833, found 717.2813; and main IR bands (Kbr, cm⁻¹) ν 3412.3 (m), 3280.0 (w), 3142.3 (w), 3115.5 (w), 2928.9 (w), 2850.0 (w), 1644.7 (s), 1595.2 (s), 1539.8 (s), 1522.4 (s), 1497.6 (s), 1437.1 (m), 1420.7 (m), 1404.7 (m), 1311.6 (s), 1257.9 (m), 1210.3 (m), 1115.5 (m), 814.1 (m), 753.2 (w).

4.2.2.2. Compound 3. Similar procedures as described for compound 2; from Boc-protected 8 (43 mg, 0.045 mmol). Yield: 32 mg (94%). ¹H NMR (400 MHz, DMSO-d₆) δ 1.51 (br, 4H), 1.67 (m, 4H), 2.60 (br, 4H), 2.62 (br, 4H), 3.23 (m, 3H), 3.81 (s, 6H), 3.95 (s, 6H), 6.86 (s, 2H), 7.20 (s, 2H), 7.58 (s, 2H), 8.15 (s, 2H), 8.17 (s, 2H), 10.25 (s, 2H); ¹³C NMR (100 MHz, DMSO-d₆) δ 26.45, 28.60, 35.98, 36.45, 37.42, 46.22, 48.43, 103.99, 105.51, 110.78, 121.32, 123.16, 126.26, 128.16, 133.75, 156.84, 161.21; ESI-MS m/z: 715.1 [M + H⁺] and HR-ESI-MS for C₉₆H₃₅N₂O₂ ([M + H⁺]⁺) calcld: 751.3640, found 751.3611; and main IR bands (Kbr, cm⁻¹) ν 3412.8 (m), 3293.1 (w), 3141.8 (m), 3110.4 (m), 2829.9 (m), 2854.1 (w), 1643.2 (s), 1595.5 (s), 1538.3 (s), 1523.0 (s), 1497.4 (s), 1437.3 (s), 1421.2 (s), 1404.9 (s), 1311.1 (s), 1257.5 (m), 1210.4 (m), 1114.8 (m), 814.1 (w), 752.2 (w).

4.2.3. Synthesis of 2@Cu²⁺ and 3@Cu²⁺

4.2.3.1. Cu(II) complex of compound 2 (2@Cu²⁺). To a solution of compound 2 (21 mg, 0.03 mmol) in DMF (5 mL) was added a solution of CuCl₂·H₂O (6.8 mg, 0.04 mmol) in water (1 mL). The
solution turned azure immediately and was allowed to stir at room temperature overnight. Water (30 mL) was added to the reaction mixture to give blue precipitates. The precipitates were collected by filtration, dissolved in methanol and filtered. The filtrate was allowed to stand in the refrigerator overnight and blue precipitates were formed. The precipitates were collected and dried in vacuum to afford 2@Cu(II) (17 mg, 70%) as a blue solid having 1H NMR (400 MHz, 1:1 DMSO-d6-D2O, v/v) δ 2.68 (br, 4H), 3.35 (br, 4H), 3.72 (s, 6H), 3.80 (s, 6H), 6.77 (s, 2H), 7.07 (s, 2H), 7.26 (s, 2H), 7.83 (s, 2H); ESI-MS m/z: 792.7 ([2 + Cu + Cl]⁺); main IR bands (KBr, cm⁻¹) ν 3396.9 (br), 3130.8 (m), 2925.6 (m), 2843.6 (w), 1645.3 (s), 1579.0 (m), 1536.6 (s), 1495.3 (s), 1437.9 (s), 1420.4 (m), 1301.7 (s), 1257.9 (m), 1209.8 (m), 1154.5 (m), 814.4 (m), 751.5 (m); and molar conductivity (DMF/H2O, 1/4, v/v): 110.5 S cm² mol⁻¹ for 2@Cu(II) and 209.5 S cm² mol⁻¹ for CuCl₂.

4.2.3.2. Cu(II) complex of compound 3 (3@Cu(II)). Similar procedures as described for 2@Cu(II); from compound 3 (23 mg, 0.03 mmol). Yield: 16 mg (60%). 1H NMR (400 MHz, 1:1 DMSO-d6-D2O, v/v) δ 1.63 (br, 4H), 1.79 (br, 4H), 2.91 (br, 4H), 3.26 (br, 4H), 3.67 (s, 6H), 3.78 (s, 6H), 6.72 (s, 2H), 7.07 (s, 2H), 7.26 (s, 2H), 7.83 (s, 2H); ESI-MS m/z: 848.6 ([3 + Cu + Cl]⁺); main IR bands (KBr, cm⁻¹) ν 3446.0 (s), 3357.0 (s), 2930.3 (w), 1646.8 (s), 1580.4 (m), 1521.4 (s), 1420.4 (m), 1311.5 (s), 1256.6 (w), 1208.1 (w), 1187.8 (m), 814.2 (w), 751.6 (w); and molar conductivity (DMF/H2O, 1/4, v/v): 110.5 S cm² mol⁻¹ for 3@Cu(II) and 209.5 S cm² mol⁻¹ for CuCl₂.

4.2.4. Determination of the stoichiometries of compounds 2 and 3 with CuCl₂

The stoichiometries of compounds 2 and 3 with CuCl₂ were measured by keeping the concentrations of compounds 2 and 3 constant, while gradually increasing the concentration of CuCl₂. Thus, to a solution of compound 2 (2.0 × 10⁻⁵ M) in 5 mM Tris–HCl buffer (5 mM NaCl, pH 7.0) were added aliquots of CuCl₂ (2.0 × 10⁻⁵ M) solution containing compound 2 (2.0 × 10⁻⁵ M) in the same Tris–HCl buffer. After the mixture was stirred for 2 min, the corresponding absorption spectra were measured. The operations repeated until saturation reached. The stoichiometries of compounds 2 and 3 with CuCl₂⁻ ion were obtained by analyzing the relationship between the absorbance at 285 nm and the concentrations of CuCl₂⁻ ion [25].

4.3. Procedures for agarose GE

The DNA-cleaving activity was measured by using the methods similar to those described previously [16–20]. Specifically, a mixture of pBR322 DNA (0.5 g/L, 0.7 μM) and each complex of varying concentrations was diluted with 5 mM Tris–HCl buffer (5 mM NaCl, 5% DMF, pH 7.0) to 16 μL. The resulting mixture was incubated at 37 °C. The reaction was quenched by adding EDTA and bromophenol blue, and then analyzed by GE. For mechanistic investigations, inhibition reaction was carried out in the presence of TMP (0.2 mM), KI (10 mM), DMSO (1.0 mM), and t-BuOH (1.0 mM), followed by addition of 2@Cu(II) and 3@Cu(II).

DNA cleavage rates of 2@Cu(II) and 3@Cu(II) at varying concentrations were measured in 5 mM Tris–HCl buffer (5 mM NaCl, 5% DMF, pH 7.0) at different intervals of time. The percentages of the supercoiled DNA form (CCC) were quantified by the software in the Alpha Hp 3400 fluorescent and visible light digitized image analyzer, and plotted against time for each concentration. The data were fitted with a single-exponential curve to give the kobs value at each concentration [19,20,30,31].

4.4. DNA-binding experiments

EB displacement experiments were conducted by keeping the concentrations of EB and CT DNA constant, while increasing the concentrations of the Cu(II) complexes. Thus, to a solution of CT DNA (1.2 × 10⁻⁶ M) and EB (1.5 × 10⁻⁶ M) in 5 mM Tris–HCl buffer (5 mM NaCl, 5% DMF, pH 7.0) were added aliquots of a solution of 2@Cu(II) containing CT DNA (1.2 × 10⁻⁶ M) and EB (1.5 × 10⁻⁶ M) in the same Tris–HCl buffer. The corresponding fluorescence spectra were measured (ex 520 nm) until saturation was observed. The EB displacement experiments of the other compounds and Cu(II) complexes were conducted in a similar fashion. The apparent binding constants were obtained by analyzing the relative fluorescence intensity (Il/Io) as a function of the concentrations of the added compound or complex [39,40].

4.5. Cell proliferation assay

Cell proliferation of compounds 1, 2, and 3 and their Cu(II) complexes was evaluated against MKN45, HepG2 and MCF-7 cells by MTT assay [41]. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). Exponentially growing cells were placed in 96-well plates (3000 cells/well/100 μL) and incubated at 37 °C for 24 h for attachment. Test compounds were prepared by dissolving in DMSO at 10 mM and diluted with the medium. Then, culture medium was changed, and cells grew in medium with the test compounds. Cells were incubated at 37 °C for 72 h. Then the medium was replaced with MTT solution (0.5 mg/mL, 100 μL) followed by incubation for another 4 h. The medium was then aspirated and formazan crystals were dissolved in DMSO (100 μL). The absorbance of the suspension at 570 nm or 490 nm (Abs) was measured on an enzyme-linked immunosorbent assay (ELISA) reader. The inhibition percentage was calculated using the following formula: % inhibition = [(Abscontrol – Abscompound)/Abscontrol] × 100%. The EC50 values of the test compounds were measured by treating cells with drugs of varying concentrations, and analyzing by use of the prism statistical package (GraphPad Software, San Diego, CA, USA).

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.06.020.

References


