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A potent antitumor Zn^{2+} tetraazamacrocycle complex targeting DNA: the fluorescent recognition, interaction and apoptosis studies†

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A Zn^{2+} tetraazamacrocycle complex (2) bearing three naphthalene moieties has been prepared. Complex 2 recognizes, binds and causes damage to DNA, and shows considerable cytotoxicity against human cervical (HeLa), breast (MCF-7) and lung (NCI-H157) cancer cell lines with a different apoptotic pathway from that of cisplatin.

DNA is a key biological target for many metal-based anticancer drugs,[1] and distortions of DNA structure often associate with anticancer activity.[2] However, the widely utilized antitumor drug cisplatin and its derivatives suffer from tumor resistance and side effects[3] because they primarily interact with cellular DNA through the formation of coordination bonds.[4] Thus, it is important to understand the DNA binding of complexes containing transition metal ions other than platinum and their possible relationship to cytotoxicity in tumor cell lines. These new complexes offer more intricate and elaborate interactions with DNA by H-bonds and/or intercalation between DNA base pairs.[5] Among the most accessed methods for investigating drug–DNA interactions, the procedure using fluorescent changes of the drugs themselves is attractive in nucleic acids chemistry[6] owing to the high sensitivity and good accuracy.

Thereby motivated, we herein describe a Zn^{2+}-tetraazamacrocycle complex **2**, which recognizes and hydrolytically cleaves DNA, and eventually leads to cancer cell death in a different way from that of cisplatin. The binding mode of **2** with DNA is complicated other than merely covalent bonding, which is in accordance with the apoptosis study. Although ruthenium[5b–c,7] and osmium complexes[5a,8] have been thoroughly studied in the anticancer drug design, zinc complexes that function have seldom been referred.[9] Zn^{2+}, which exists as a divalent cation in biological systems,[10] contributes widely to DNA synthesis, apoptosis, gene expression, protein stability and/or catalytic functions.[11] Thus, clarifying the effect of **2** on DNA and cancer cells is of great value.

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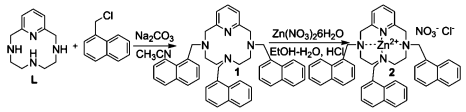

Scheme 1 Synthesis procedure of **1** and its Zn^{2+} complex **2**.

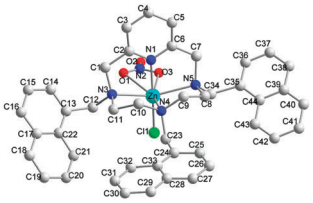

Fig. 1 The single crystal structure of **2**. All hydrogen atoms are omitted for clarity.

Ligand 3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-trimethyl naphthalene (**1**) and its Zn^{2+} complexes **2** were synthesized following the procedure shown in Scheme 1 and fully characterized (see ESI† for details). Crystallization of **2** from EtOH–H_2O mixed solution in the condition of careful pH adjustment (pH 7.4) gave pale yellow single crystals suitable for X-ray crystallography, which reveals that Zn^{2+} coordinates to four nitrogen atoms of the mother ring of **1**, and Cl^- and NO_3^- eventually fulfil the uncommon heptacoordinative mode (Fig. 1). However, the electrospray ionization mass spectrum (ESI-MS) shows that NO_3^- departs from Zn^{2+} and leaves vacant coordinative positions in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 1/999) compared to that in the crystal structure of **2**.

The fluorescent emission spectra of **2** in the neutral buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 5/995) exhibit a characteristic naphthalene monomer band[12] at 335 nm and a long-wavelength structureless band centered at 420 nm when recorded immediately (Fig. 2). However, continuous excitation (~8 min) eventually results in an approximately 1.5-fold enhancement and a blue shift (~30 nm) of the fluorescent band at 420 nm (Fig. 2). The blue shift emission can be attributed to the preformed excimer, which was justified by the observation of a red shift in the wavelength (λ 8 nm) of the excitation spectra (Fig. S3, ESI†) when monitored at the monomer (λ_{em} = 335 nm) and excimer (λ_{em} = 390 nm) emission respectively.[13] Meanwhile, the monomer emission quenches to nearly disappear at last. The crystal structure

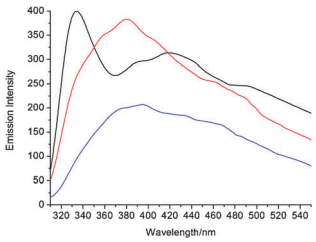

Fig. 2 Fluorescent spectra of **2** by immediate recording (—) and continuous exciting (—), and **2** with the molar ratio of [DNA]/[**2**] = 0.8 (—). λ_{ex} = 280 nm.

of **2** shows that the formation of the excimer is unexpected because such a process was reported to be feasible only in systems having an interplanar distance of less than 3.5 Å between two naphthyl groups.[14] This unusual behavior of **2** in the aqueous medium may be ascribed to the existence of a highly ordered conformation owing to hydrophobic interactions, which bring two naphthalene subunits in a face-to-face position favouring the formation of an intramolecular excimer by π–π interaction. Upon adding calf thymus (CT)-DNA to the solution containing **2** whose fluorescence has already become steady, the excimer emission of **2** is "switched off" to 50% of its original intensity when the molar ratio of [DNA]/[**2**] attains 0.8 (Fig. 2). On the contrary, adding a large amount of DNA to the solution containing **1** only induces negligible changes (Fig. S4, ESI†). Therefore, **2** is predisposed to form an intramolecular excimer capable of recognizing DNA, and the fluorescent change of **2** is attributed to the fact that binding of **2** to DNA leads to the quenched excimer emission through the PET process.[15] This process takes place from the nitrogen atoms of the macrocycle moiety to naphthalene groups. The intrinsic binding constant of **2**–DNA is determined from Scatchard plots utilizing the McGhee and von Hippel model[16] as $K_b = 1.1 \times 10^6$ M^{-1} (Fig. S5, ESI†) with the binding sites $n = 2.7$.

The destruction of DNA by **2** functions once **2** recognizes DNA. CD studies show that both positive and negative bands of CT-DNA display a decrease in the ellipticity with the molar ratio ([**2**]/[DNA]) of 0.5 (Fig. S6, ESI†), suggesting that **2** can effectively unwind the DNA helix and result in the loss of helicity.[17] Besides, **2** perturbs the base stacking and helicity bands more considerably than **1** at the same concentration, indicating **2** is more effective than **1** in perturbing the secondary structure of DNA. Complex **2** shows potent DNA cleavage activity, which totally converts supercoiled pUC19 DNA (Form I) to its nicked (Form II) and linear (Form III) forms at a concentration of 10 μM (Fig. S7, ESI†). However, the presence of **1** (10 μM) alone can hardly cleave the supercoiled DNA (Fig. S8, ESI†). Redox inactive Zn^{2+} complexes were known to generally cleave DNA by the hydrolytic pathway,[18] and **2** was successfully applied to catalyze the 2-hydroxypropyl-*p*-nitrophenyl phosphate (HPNP) hydrolysis reaction (Fig. S9, ESI†) by coordinating with H_2O molecules or deprotonated OH^- with the vacant coordinative positions of Zn^{2+} in the buffer. Hence, the DNA cleavage activity and fluorescent recognition behavior of **2** show promise for targeted DNA hydrolytic damage.

Hydrolysis of the phosphodiester bond of DNA is crucial at several stages in the cell cycle, including DNA repair and excision, integration and signal transduction.[19] Meanwhile, the hydrolytic damage of the DNA backbone has been reported to be related to antitumor potential,[20] which promotes us to evaluate the cytotoxicity of **2** towards different cancer cell lines by the MTT assay. HeLa cells were exposed to different concentrations of **2** for 24 and 48 h. As shown in Fig. S10 (ESI†), **2** causes dose-dependent cytotoxicity in the range of 2.5–7.6 μM with approximately 50% of the cells having lost viability after treating with **2** at the concentration of 6.7 μM in 24 h. Besides, the antitumor performance of **1** in 48 h was also investigated, and it did not induce apparent changes compared to that of **2**. Zn^{2+} ions were cytotoxic to cells at 10^{-3} M. The IC_{50} values of **2** toward MCF-7 and NCI-H157 cells in 24 h were also investigated and obtained as 4.8 μM and 7.0 μM, respectively. The cytotoxicity of **2** is comparable with the reported antitumor drugs utilizing complexes containing transition metal ions.[7–9]

Hoechst 33258 staining experiments were then conducted to illuminate the **2**-induced apoptotic procedure. After 24 h of incubation of HeLa cells with 7.6 μM **2**, the staining of Hoechst 33258 shows that **2** results in typical morphological changes of apoptosis such as condensation of chromatin, nuclear fragmentations, and apoptotic bodies (Fig. S11, ESI†). In the annexin V-FITC/propidium iodide (PI) assay, after incubation for 48 h, 6.7 μM **2** induces the major population of HeLa cells into the early apoptotic stage (annexin V +/PI-, 81.70%) (Fig. 3), indicating that **2** is significantly effective in triggering membrane phospholipids PS exposure and plasma membrane integrity loss. Moreover, cisplatin has been demonstrated to induce apoptosis associated with increased caspase-3 activity.[21] However, treatment with close to IC_{50} concentrations of **2** in HeLa cells over a period of 24 h did not significantly enhance the enzyme activity compared to the untreated cells at any incubation time tested. Western blot analysis also shows that **2** triggers caspase-3-independent apoptosis in HeLa cell lines (Fig. 4). Therefore, the cell death caused by **2** does not depend on the activation of the caspase-3 pathway, which appears different from cisplatin.

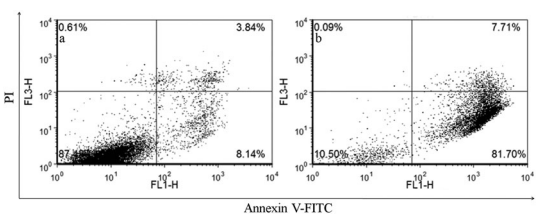

Fig. 3 Annexin V/PI assay of HeLa cells treated by **2** measured by flow cytometry at the concentration of 0 μM (a) and 6.7 μM (b) for 48 h.

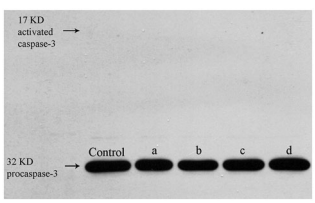

Fig. 4 Western blotting of caspase-3 activation in HeLa cells after treatment without (control) and with 5.1 μM (a), 5.9 μM (b), 6.7 μM (c), 7.6 μM (d) **2** for 24 h.

The absence of caspase-3 activity promotes us to clarify the binding mode of **2** with DNA. The interaction of **2** with DNA was first evaluated by the EB–DNA system, which can be used to distinguish intercalating and nonintercalating compounds.[22] Competitive binding of other intercalators leads to a loss of fluorescence because of depletion of the EtBr–DNA complex. As shown in Fig. S12 (ESI†), the fluorescent intensity of EB in the bound form is remarkably quenched upon adding **2**. The DNA apparent binding constant K_{app} value of **2** was calculated to be $7.8 \times 10^5\ M^{-1}$, which is about 8-fold greater than that of **1**. The DNA binding and cleavage activity of **2** is equivalent to or even better than that of other reported transitional-metal antitumor complexes.[8c,9b,20,23]

Then, UV spectra assist to further clarify the interaction of **2** with DNA in the ground state. The absorbance of **2** exhibits a [DNA]-dependent two-stage process upon adding CT-DNA. Hypo- and then hyperchromism are observed in both bands centered at 222 nm and 285 nm (Fig. S13, ESI†). Meanwhile, a blue-shift of 3 nm of the band at 285 nm is also discerned with the appearance of one distinct isosbestic point at 298 nm upon adding CT-DNA. The initial decrease in absorbance followed by the marked hyperchromism reveals more than one DNA binding mode for **2**, which is attributed to binding of **2** in the interior of the DNA at higher DNA concentration and more bases embedding in DNA are thus exposed. The possibility of intercalation cannot be ruled out either. However, adding DNA to the solution containing **1** only increases its absorbance at both bands without any shift or isosbestic point, suggesting that the chromophore environment is not altered after the interaction with DNA. The continuous hyperchromism can be ascribed to the external contact (electrostatic binding).[24]

In short, the results presented herein support the conclusion that DNA is the target for the Zn^{2+} complex **2**. The destruction of DNA by **2** leads to the satisfactory cytotoxicity. The lack of caspase-3 activation after treatment with **2** in cancer cells combined with an intricate DNA binding mode indicate that the mechanism of apoptotic induction may differ from that of cisplatin, which deserves further scrutiny and may provide grounds for establishing new structure–pharmacological activity relationships for DNA-targeting complexes as novel anti-tumor drugs.

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