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Selective recognition of thymidylylthymidine (TpT) and antitumor effects of a macrocyclic dizinc(II) complex †

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A polyamino dizinc(II) complex, [(*N*-bisdien)Zn_2(II)Cl_2]$(ClO_4)_2$, (LZn), has been synthesized as a new nucleobase receptor molecule in aqueous solution at physiological pH, and shows to be highly selective in recognizing deoxythymidine (dT) and thymidylylthymidine (TpT). The strong acidic Zn(II) ions in LZn at the fifth coordination sites interact with a variety of nucleosides. The binding and recognition processes have been studied by potentiometric titration. The X-ray crystal analysis of LZn shows that the two zinc ions are out of the basal plane of the macrocycle, favoring the effective recognition of TpT on the single strand of DNA. *In vitro* antitumor investigation shows that LZn is a patent inhibitor of tumor cell growth with IC_{50} values below 10 micromolar.

Introduction

Small molecules that bind to DNA and RNA have attracted great interest[1] and continued to be a challenging theme in current bioorganic and bioinorganic chemistry.[2] These targeting molecules recognize specific DNA sequences (or base pairs), alter the local DNA structure, inhibit access of activator or repressor protein and ultimately affect the gene expression process.[3]

A number of clinically useful drugs are obtained from natural products, like bleomyein,[4] distamycin[5] and actinomycin.[6] These molecules interact with DNA at multi-interaction sites through hydrogen-bonding or π-stacking interactions. Great efforts have been made to synthesize artificial molecules that interact with DNA in a similar fashion. Macrocyclic compounds have found utility in a number of medical applications. Investigations have focused on tetraazamacrocycles owing to their proved ability in binding biologically relevant metal ions. For example, cyclen and cyclam and their derivatives have been studied as carriers of metal ions in antitumor and imaging applications.[7] Most recently, a bis(Zn–cyclen) complex (Scheme 1) shows the potential to be a HIV-1 inhibitor due to the selective recognition on a Uridine-rich sequence of RNA.[8]

Our interest in macrocycle chemistry motivates us to investigate the macrocyclic dizinc(II) complex. LZn structurally resembles bis(Zn–cyclen) due to the contained N_4Zn subunits. Other than the bridged structure of bis(Zn–cyclen), LZn is a "coupled" bis-Zn–cyclen molecule. The structural flexibility of LZn is expected to fine-tune the steric properties in recognition of the base sequence in the DNA chain. It is reasonable to envision that LZn has the ability to selectively recognize TpT or UpU rich sequences. Their binding strength and drug effect might be improved due to the synergic interaction and host–guest effects in the interaction with base sequences. Experimental results reveal that LZn can interact with TpT to form a stable 1 : 1 complex at pH = 7.4 in aqueous solution. This complex also shows significant anticancer effects in certain cell lines.

† Dr Arthur E. Martell passed away on October 15, 2003. He was a fine chemist, full of ideas, and a stimulating adviser. His reputation in the field of Coordination Chemistry is without peers. It was my privilege to work with him for three years. I shall always be indebted to him for sharing with me his scientific insights. Along with the community of Chemistry, I mourn his passing (J. G.).

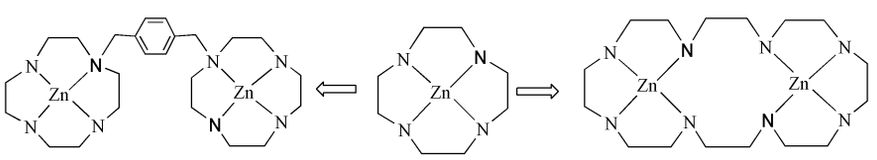
Results and discussion

Structure of [(*N*-bisdien)-Zn_2(II)Cl_2]$(ClO_4)_2$

The X-ray structure of the LZn is shown in Fig. 1a. The molecule consists of a [(*N*-bisdien)Zn_2(II)Cl_2]$^{2+}$ cation and two perchlorate anions. Both Zn(II) ions are five coordinated and each bind to four nitrogen atoms of the macrocyclic ligand and one chloride anion. Each Zn(II) sit in a square pyramidal geometry and interacts with Cl^- in the axial position. The two chloride anions bind with each Zn(II) from the same side with respect to the plane of the macrocycle (Fig. 1b). N(2)–H, N(3)–H, N(6)–H and N(7)–H directed to the same side as with Zn(II)–Cl(1) and Zn(2)–Cl(2) bonds. The eight Zn(II)–N bond lengths are significantly different and fall in the range of 2.045–2.411 Å, indicating that the pyramidal structure is highly distorted. The whole molecule geometry can be better described as a coupled bis-pyramid.

Stability constants and species distribution of the 1 : 2 bisdien–Zn(II) complex system

The titration curve for the *N*-bisdien·8HBr (Fig. 2) indicates a buffered region between *a* values, $a = 0$ to 4 and $a = 4$ to 8 by a break with the remainder of the curve buffered at high pH. The first buffer region corresponds to the deprotonation of *N*-bisdien·8HBr to the tetra-protonated state *N*-bisdien·4HBr. The following buffer region is the formation of totally deprotonated states of the free ligand. The protonation constants of *N*-bisdien have been reported previously.[9] They were



Bis(Zn-Cyclen) Zn-Cyclen LZn

Scheme 1

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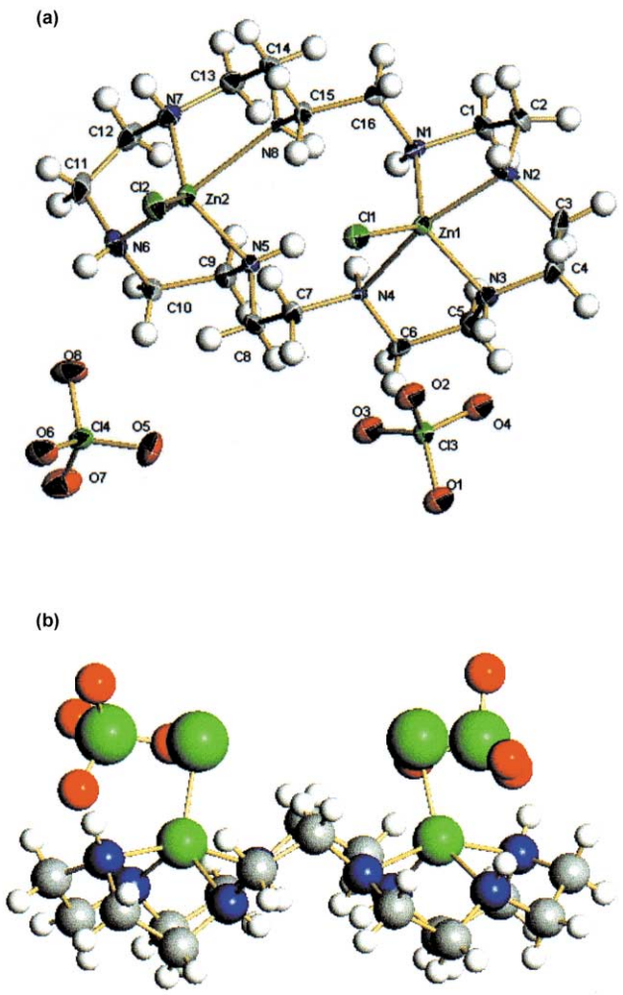


Fig. 1 (a) Perspective view of $[(N\text{-Bisdien})Zn_2(II)Cl_2](ClO_4)_2$. Selected bond lengths (Å): Zn(1)–N(3) 2.045(4), Zn(1)–N(1) 2.070(4), Zn(1)–N(2) 2.128(4), Zn(1)–N(4) 2.411(3), Zn(1)–Cl(1) 2.2034(13), Zn(2)–N(5) 2.068(4), Zn(2)–N(7) 2.071(5), Zn(2)–N(6) 2.157(4), Zn(2)–N(8) 2.388(4), Zn(2)–Cl(2) 2.2169(14). Selected bond angles (°) N(3)–Zn(1)–N(1) 114.19(17), N(3)–Zn(1)–N(2) 86.36(17), N(3)–Zn(1)–Cl(1) 121.62(13), N(1)–Zn(1)–Cl(1) 123.58(12), N(5)–Zn(2)–N(7) 114.87(17), N(5)–Zn(2)–N(6) 83.77(17), N(5)–Zn(2)–Cl(2) 118.31(13), N(7)–Zn(2)–Cl(2) 126.24(13). (b) Side view of LZn showing the different N–H bond directions.

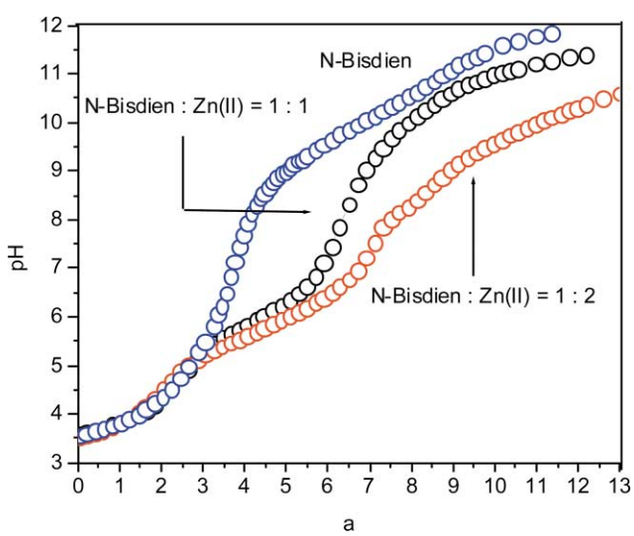


Fig. 2 Potentiometric equilibrium curves for *N*-bisdien, and *N*-bisdien–Zn(II) in 1 : 1 and 1 : 2 ratios. ([*N*-bisdien] = 0.10 M, μ = 0.100 M KCl, t = 25 °C).

Table 1 Logarithms of the protonation constants of *N*-bisdien and the stability constants of its dinuclear Zn(II) complexes (t = 25 °C, μ = 0.100 M KCl, L = *N*-bisdien, M = Zn)

Protonation constants		
Symbol	Equilibrium quotient	LogK^H_i
K^H_8	$[H_8L]/[H_7L][H]$	1.98
K^H_7	$[H_7L]/[H_6L][H]$	2.87
K^H_6	$[H_6L]/[H_5L][H]$	3.84
K^H_5	$[H_5L]/[H_4L][H]$	5.03
K^H_4	$[H_4L]/[H_3L][H]$	8.35
K^H_3	$[H_3L]/[H_2L][H]$	9.03
K^H_2	$[H_2L]/[HL][H]$	9.58
K^H_1	$[HL]/[L][H]$	10.02
ΣK^H_i	$[H_8]/[L][H]^8$	50.70
Stability constants		
Symbol	Equilibrium quotient	LogK^H_i
K_1	$[LM_2H_2]/[H]^2[M_2L]$	13.74
K_2	$[M_2L]/[M][ML]$	17.34
K_3	$[M_2(OH)L][H]/[M_2L]$	−1.08

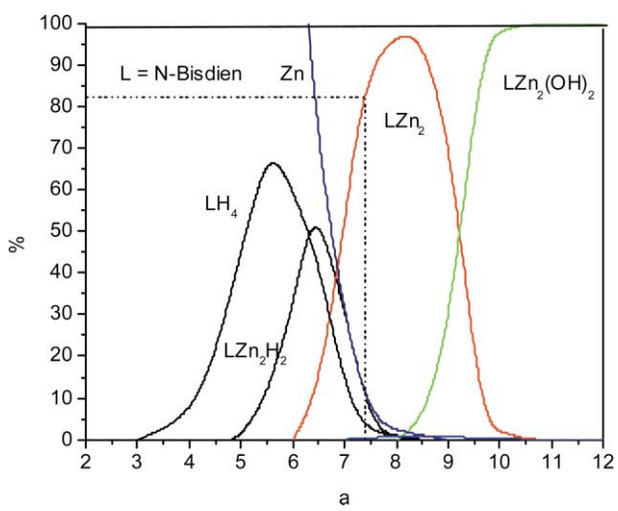


Fig. 3 Species distribution diagram for *N*-Bisdien–Zn(II) system in 1 : 2 ratio. (μ = 0.100 M KCl, t = 25 °C, T_L = 0.001 M, T_{Zn} = 0.002 M).

re-determined using the experimental conditions employed in this work (Table 1). The potentiometric titrations for 1 : 1 and 2 : 1 ratios of Zn(II) to *N*-bisdien were conducted and the species distribution curves for the 2 : 1 system are shown in Fig. 3. It was found that the di-hydrolyzed species exist only in the basic region pH 8.0–12.0. The tetra-coordinated dizinc species reaches 82.0 % at physiological pH 7.40.

Interaction of LZn with dT and dG groups in dinucleotides

We initially tested the pH change of ZnL upon interaction with 2′-deoxynucleosides (Fig. 4a). To an unbuffered solution (pH = 7.41) of 0.50 mM LZn at 25 °C with I = 0.10 (KCl), was added two equimolar amounts of dT. The pH drops to 7.27. In this process, the recognition occurs through the coordination bonds between the deprotonated imide N(3)H groups of two dT with LZn to form a LZn–$(dT^-)_2$ complex (Scheme 2). The complementary hydrogen bonds between the imide oxygens and NH groups of the macrocycle may also be involved.

When 2 equivalents of 2′-deoxyguanosine (dG) were added to 0.50 mM of LZn, the pH increased to 7.46. On interacting with dG (Scheme 3), no proton was released and the suggested binding model of the guanine groups (G) with the Zn(II) complex was derived from the X-ray crystal analysis of a cyclen–Zn(II) complex.[10] On the other hand, 2′ deoxycytidine (dC) and 2′-deoxyadenosine (dA) showed negligible pH changes (drop to

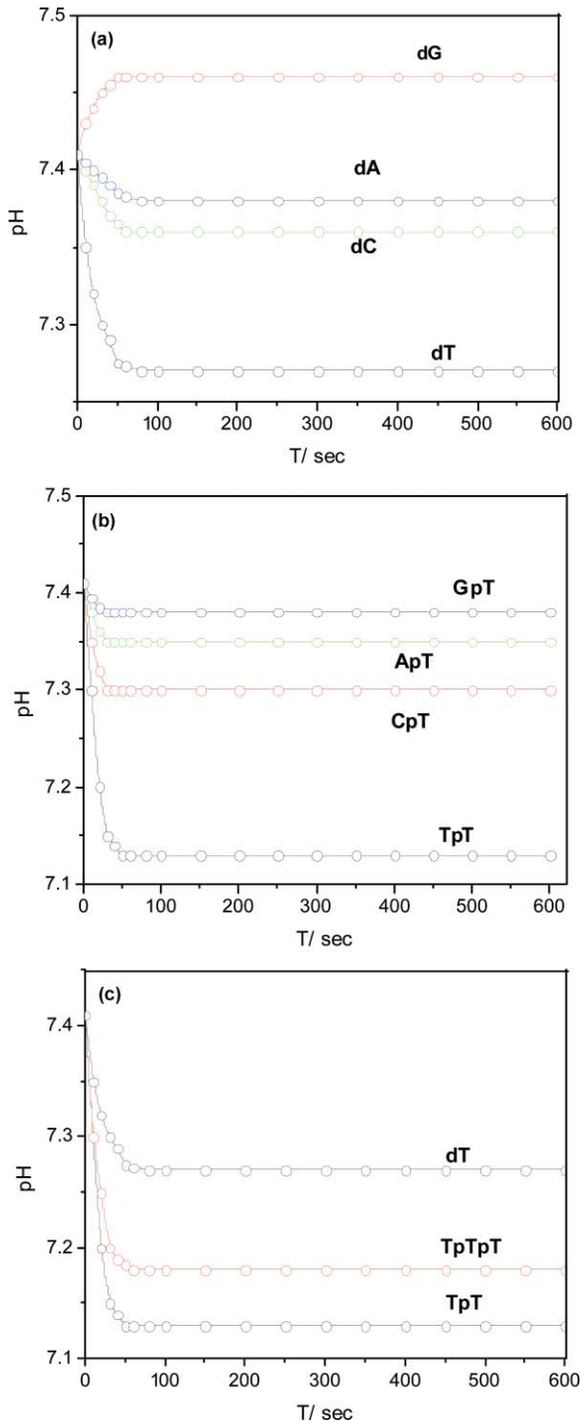


Fig. 4 pH changes of the unbuffered solution containing LZn (0.5 mM, pH = 7.40 at 25 °C with I = 0.10 (KCl)) upon addition of 2′-deoxynucleosides (1.0 mM) and oligonucleotides (all containing 1.0 mM dT or T).

7.36 and 7.38 respectively), reflecting their weaker association with LZn.

When adding dinucleotides TpT (0.50 mM), GpT (0.50 mM), CpT (0.50 mM) or ApT (0.50 mM) to an unbuffered solution of LZn (0.50 mM) under the same conditions as used for 2′-deoxynucleosides, the extent of the pH decrease by TpT (to 7.13) was larger than that by dT (7.27) (Fig. 4b), suggesting a cooperative binding of the two thymidine N(3)H groups to LZn. With CpT and ApT, the pH values decreased less than that by dT and TpT, further indicating that LZn bound to a thymine group stronger than to dC or dA in CpT or ApT respectively. With 0.50 mM GpT, the pH decrease (to 7.35) was less extensive, suggesting that one Zn(II) site bound with G.

Table 2 Complexation constants for *N*-bisdien–Zn(II)–dT and *N*-bisdien–Zn(II)–TpT systems (μ = 0.10 M KCl, 25 °C, [LZn] = [dT]/2 = [TpT] = 0.001 M, L = *N*-bisdien)

Stoichiometry					
L	Zn	dT	TpT	Quotient K	LogK_{com}
1	2	2		$[LZn(dT^-)_2]/[LZn][dT]^2$	29.01
1	2	1		$[LZn(dT^-)]/[LZn][dT]$	24.00
1	2		1	$[LZn(T^-pT^-)]/[LZn][TpT]$	32.80
1	2		1	$[LZn(TpT^-)]/[LZn][TpT]$	26.10

Among thymine nucleosides and nucleotides, dT (1.00 mM), TpT (0.50 mM) and TpTpT (0.33 mM) (*i.e.*, $[T]_{total}$ = 1.00 mM), TpT brought the biggest pH drop (Fig. 4c), leading to a suggestion that LZn most favorably bound to the two consecutive thymidine bases in TpT, due to the synergic effect in forming a LZn–T^-pT^- complex. The multipoint binding model was proposed as shown in Scheme 4, in which the assigned H-bonding with the N(3)H and N(7)H groups were suggested by the crystal structure analysis of LZn.

Potentiometric titration of L–Zn with dT and TpT

The synergistic effect in the interaction of TpT with LZn was quantitatively assessed by potentiometric titration of the ternary systems (*N*-bisdien : Zn(II) : TpT = 1 : 2 : 1). In this study, the deprotonation constant being used for dT is 9.45.[11] The two imide deprotonation constants for TpT are 9.55 and 10.25.[12] For comparison, the titration curves for *N*-bisdien–Zn(II)–dT systems in 1 : 2 : 1 and 1 : 2 : 2 ratios were also shown in Fig. 5. The buffer pH region of the LZn system dropped significantly in the presence of TpT, suggesting the successive deprotonation from two thymidylimide N(3)H on complexation with LZn. The neutralization break at a = 8 was sharper, indicating that TpT formed a stable complex with LZn. The stability constants obtained by analyzing the complexation equilibria are listed in Table 2. The complexation constant for log$K_{[LZn(T^-pT^-)]/[LZn][TpT]}$ was estimated to be 32.80, which is higher than that of log$K_{[LZn(dT^-)_2]/[LZn][dT]}$ (29.01), indicating the strong cooperative binding with TpT.

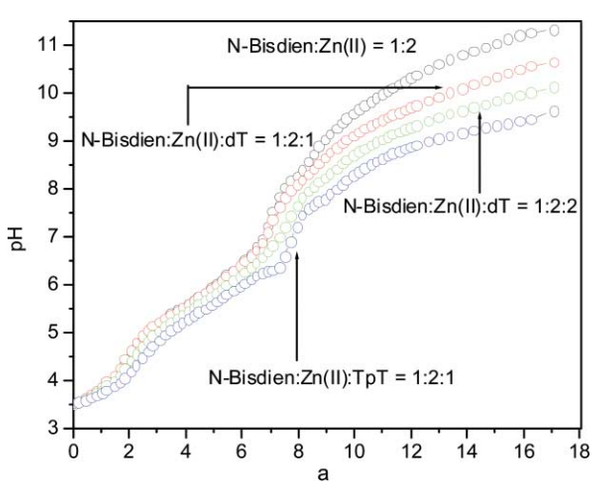
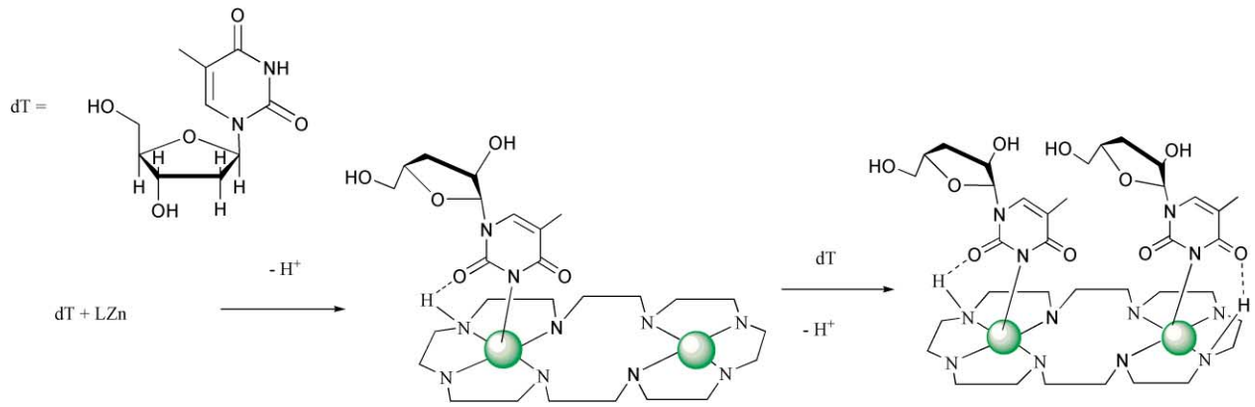
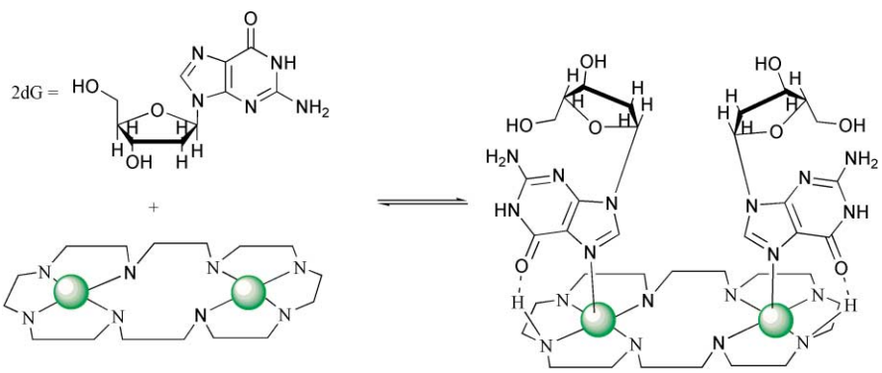


Fig. 5 Potentiometric equilibrium curves for *N*-bisdien–Zn(II)–TpT (1 : 2 : 1) and *N*-bisdien–Zn(II)–dT (1 : 2 : 1 and 1 : 2 : 2) systems ([*N*-bisdien] = 0.10 M, μ = 0.100 M KCl, t = 25 °C).

The species distribution curves for 1 : 2 : 2 *N*-bisdien–Zn(II)–dT and 1 : 2 : 1 *N*-bisdien–Zn(II)–TpT systems are shown in Fig. 6 and Fig. 7. The synergistic binding of LZn to TpT was well illustrated by analyzing the species distribution diagrams. The stable ternary complex LZn(T^-pT^-) formed between pH 7.0–11.0, reaching 50.2% at pH = 7.4. However only 12.0% $LZn(dT^-)_2$ complex formed at physiological pH.



Scheme 2



Scheme 3

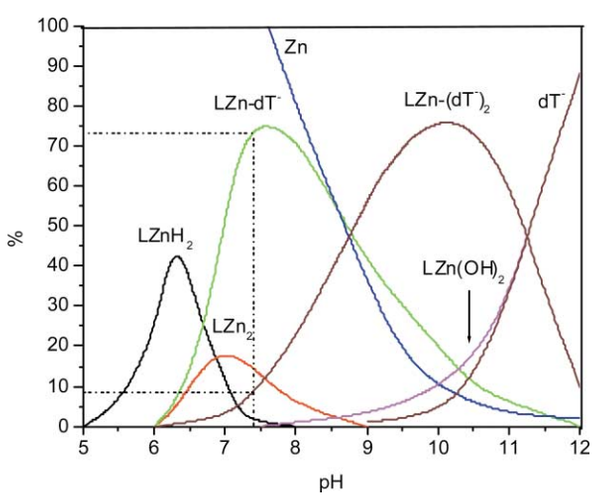


Fig. 6 Species distribution diagram for *N*-bisdien–Zn(II)–dT in 1 : 2 : 2 ratio. (μ = 0.100 M KCl, t = 25 °C, T_{LZn} = 0.001 M, T_{dT} = 0.002 M).

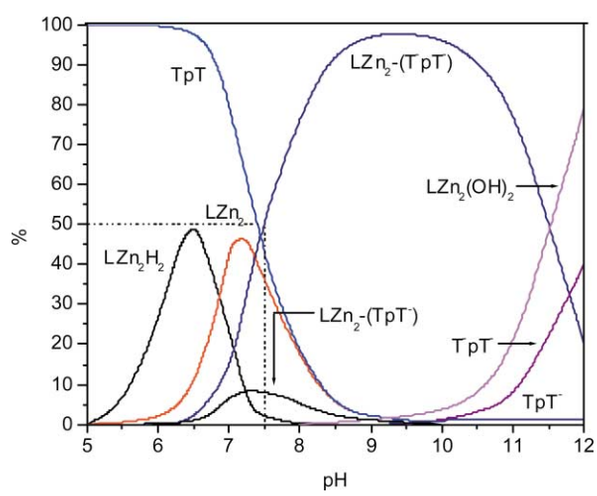


Fig. 7 Species distribution diagram for *N*-bisdien–Zn(II)–TpT in 1 : 2 : 1 ratio. (μ = 0.100 M KCl, t = 25 °C, T_{LZn} = T_{TpT} = 0.001 M).

Table 3 Percentage of growth inhibition against three cancer cell lines. (The 1 : 2 *N*-bisdien–Zn(II) complex utilized was buffered at pH = 7.4)

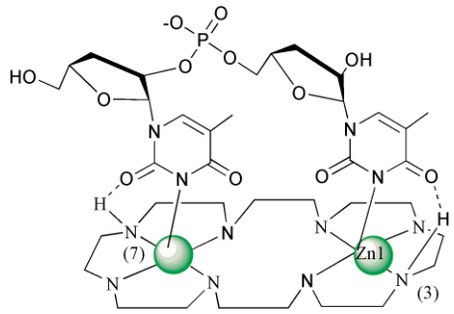
Drug Conc. /μM	10.0	8.0	6.0	4.0	2.0	1.0	0.1
P388	98.0	83.4	57.3	46.2	38.5	29.6	1.6
A549/ATCC	54.0	44.2	37.2	28.6	17.1	10.1	0
MCF7	78.7	67.2	45.6	35.7	24.0	12.6	0

Table 4 IC_{50} (μM) of LZn and cyclen–Zn on growth of three cancer cell lines. (IC_{50} is defined as the concentration of drug where the growth of the cell is one-half that of the control experiment)

Compound	P388	A549/ATCC	MCF7
LZn	5.2	8.8	6.7
Cyclen-Zn	>20	>20	>20

In vitro antitumor activity

The antitumor activities of the cationic dinuclear complex were measured on P388, A549/ATCC and MCF7 cell lines. As shown in Table 3, the complex possessed significant activity against the p388 leukemia cell line when administered in water. The percentage inhibition was 98.0% and 29.6% at 10^{-5} and 10^{-6} M respectively, with an IC_{50} value of 5.2 μM observed (Table 4). Relatively low activities were displayed when the complex was treated to A549/ATCC and MCF7 cell lines, with IC_{50} values of 8.8 and 6.7 μM, respectively. In comparison to the dinuclear complex, cyclen–Zn had no effect on the tested three cell lines at lower than 20 μM. In contrast to the majority of antitumor azacrown metal complexes[7a–c] LZn is the most effective with the potential to become a tumor cell growth inhibitor. The promised antitumor effects might be ascribed to the selective recognition of the TpT-rich element on cell-DNA molecules. Further biological applications of these compounds are currently under investigation.



Scheme 4

Experimental

Materials

All of the metal stock solutions for potentiometric studies are reagent grade chloride salts prepared with doubly distilled water and standardized by EDTA. CO_2-free dilute-it ampules of KOH were obtained from J. T. Baker Inc. KOH solutions (about 0.1 M) were prepared with doubly distilled water and standardized. The extent of carbonate accumulation (<1.8%) was checked periodically by titration with a standard HCl solution. Thymidylylthymidine (TpT), 2′-deoxyguanylylthymidine (GpT), 2-deoxyadenylylthymidine (ApT), 2′-deoxycytidylylthymidine (CpT) and thymidylylthymidylylthymidine (TpTpT) were prepared as their sodium salts by the literature methods[13] and were characterized by elemental analysis and ^{1}H NMR.

Potentiometric equipment

A Corning 250 digital pH meter, fitted with Fisher full-range blue-glass and Fisher calomel reference electrodes, was used for potentiometric titrations. A Metrohm l0 mL capacity piston buret was used for precise delivery of standard KOH. The solution to be studied was contained in a 75 mL jacketed glass cell thermostated at 25.00 ± 0.05 °C by a circulating constant-temperature water bath.

Potentiometric determinations

All pH calibrations were performed with standardized HCl aqueous solutions to measure hydrogen ion concentrations directly (p[H] = −log [H^+]). The ionic strength was adjusted to 0.100 M with KCl. Titrations of the ligand in the presence of metal ions in aqueous solution were conducted in the manner described by Martell and Motekaitis.[14] Cell solutions (in general, 50.00 mL) were purged with a purified argon stream. Standard base was introduced into the sample solutions with a Metrohm piston buret. Experimenta1 runs were carried out by adding increments of standard base to a solution containing *N*-bisdien·8HBr plus other components such as KCl solution and metal solution. The concentrations of the sample solutions were 1 × l0^{-3} M for *N*-bisdien·8HBr. The titrations of *N*-bisdien–Zn(II) in a 1: 2 ratio were carried out to investigate the pH distribution of the complex. The pH range for accurate measurements was considered to be 2–12. The pK_w for the aqueous system, defined as −log([H][OH]) at the ionic strength employed was found to be 13.78.

Computations

Protonation constants and stability constants from the direct titrations were calculated from the potentiometric data with the program BEST. Species distribution diagrams were computed from the measured equilibrium constants with SPE and plotted with SPEPLOT.[14]

Preparation of [(*N*-bisdien)Zn_2(II)Cl_2]$(ClO_4)_2$

The macrocyclic ligand *N*-bisdien was synthesized according to a literature method.[15] To a aqueous solution containing 0.10 mmol of *N*-bisdien·8HBr was added 0.30 mmol of Et_3N (pH~9.0), 0.20 mmol of $Zn(ClO_4)_2$·$6H_2O$ and 0.6 mmol of KCl. After stirring for 2 h, colorless micro-crystals formed. Yield 78%; mp > 298 °C. Anal calc. for $C_{16}H_{40}Cl_4Zn_2N_8O_8$, C, 25.79; H, 5.41; N, 15.04. Found, C, 25.45; H, 5.52; N, 15.17%.

In vitro antitumor studies

The tumor cell lines p388, A549 and MCF7 were grown as suspension cultures in RPMJ 1640 medium containing 15% fetal calf serum and 1% glutamine.[16] The drug was dissolved in water, with a maximum solvent concentration of 0.5%. The growth inhibition was tested in log-phase cells after 48 h in the presence of drug or solvent.

Crystallography

Single crystals suitable for X-ray analysis were obtained by re-crystallization of [(*N*-bisdien)Zn_2(II)Cl_2]$(ClO_4)_2$ from H_2O–MeOH solution at pH = 7.50. The X-ray structure was solved by direct methods and subsequent Fourier difference techniques, and refined anisotropically by full matrix least-squares on F^2. Nonhydrogen atoms were refined with anisotropic thermal parameters. Hydrogen atoms were included in calculated positions refined with isotropic thermal parameters riding on those of the parent atoms.

Crystal data. Crystal dimensions: 0.3 × 0.3 × 0.03 mm^3, $C_{16}H_{40}Cl_4Zn_2N_8O_8$, M = 745.10, orthorhombic, space group *Pna*2(1), a = 25.6379(12) Å, b = 9.2441(4) Å, c = 12.2274(6) Å, α = 90.0°, β = 90.0°, γ = 90.0°, V = 2897.9(2) Å^3, Z = 4, Dc = 1.708 cm^{-3}, F(000) = 1536, T = 110(2) K, Mo–Kα radiation, λ = 0.71073 Å. A total of 6838 (R(int) = 0.0480) independent reflections. Full-matrix least-squares refinement on F^2 converged to $R1$ = 0.0498, ϖR_2 = 0.1255 ($I > 2a$ (I)), goodness of fit F^2 = 1.140. ‡

‡ CCDC reference number 209806. See http://www.rsc.org/suppdata/ob/b3/b308358f/ for crystallographic data in CIF or other electronic format.

Acknowledgements

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