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Copper Complex of Hydroxyl-Substituted Triazamacrocyclic Ligand and Its Antitumor Activity

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Abstract—The protonation constants and the stability constants for the formation of copper (II) complex of the ligand [1,4,7] Triazecan-9-ol (**L**) were presented. Antitumor activity of Cu**L** complex was reported. Preliminary pharmacological tests showed that it had antitumor activity against HXO-RB44 and BEL-7402 cell lines in vitro. Nuclei of [Cu**L**]-stimulated BEL-7402 cells clearly exhibited condensation and break down into chromatin clumps typical of apoptosis. Also it exhibited perturbation effects to BEL-7402 cell lines cycle and further studies showed that it could cleave supercoiled DNA (pBR 322) to nicked and linear DNA.

Increasing knowledge of the crucial role of natural polyamines in cell biology has been stimulating versatile basic and applied research interests.[1–4] It has been reported that macrocyclic complexes with tetraazamacrocyclic ligands exhibit antitumor or anti-HIV virus activity.[5–9] Recently studies have been focused upon copper(II) complexes of cyclic triamines which cleaving phosphodieaster,[10,11] RNA,[12] DNA,[13] dipeptides and proteins.[14] However, the complexes of triazacyclic ligand that exhibit antitumor activities have not been reported. Here we would like to describe briefly the preliminary results of the copper(II) complex with hydroxyl-substituted triazamacrocyclic ligand against HXO-RB44 (retinoblastoma) and BEL-7402 (human hepatocellular carcinoma) cell lines in vitro, and this is to our knowledge the first cell apoptosis induced by triazacyclic complex reported.

Ligand **L** (Fig. 1) was prepared as described earlier.[15,16] Hydroxyl macrocyclic polyamines were versatile chelating agents and could form stable complexes with transition metal ions.[17,18] The metal complex for our study was obtained by adding an appropriate amount of a solution of metal ion, to a solution of the ligand. The M:**L** molar ratio was 1:1 for the system M-**L**. The protonation constants and the stability constants for the formation of copper (II) complex of the ligand [1,4,7]Triazecan-9-ol (**L**) determined at 298.0 K in 0.1 mol·L^{-1} KNO_3 were shown in Table 1. Some relevant distribution diagrams were shown in Figure 2.

Potentiometric Measurements

Potentiometric equilibrium measurements of the ligand **L** in the absence and in the presence of Cu(II) ion were

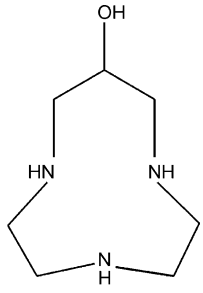

Figure 1. Structure of the ligand **L**.

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carried out with a Beckman pH meter Model ϕ71 equipped with a type 39841 combination electrode. Redistilled (quartzware equipment) H_2O was used for preparing all the solutions. KNO_3 and $Cu(NO_3)_2$ were recrystallized with redistilled H_2O before use. The concentration of Cu(II) ion in the stock solution was determined by coordination titration methods. The ionic strength was adjusted to 0.1 mol·L^{-1} with KNO_3. Computation of equilibrium constants was made using the program[19] based on the improved TITFIT[20] technique. Species distribution curves were calculated and plotted with the DP[19] program. For each system at least three titrations were performed; each titration contained ca. 50 experimental points.

Table 1. Protonation constants for ligand **L** and stability constants for the complex of Cu(II) with ligand **L** (Aqueous 0.1 mol·L^{-1} KNO_3 at 298.0±0.1 K)

Reaction	Log β
L + H = H**L**[a]	8.92 (1)
L + 2H = H_2**L**	17.51 (1)
L + 3H = H_3**L**	23.22 (2)
Cu + **L** = Cu**L**	15.29 (2)
Cu + **L** + H = Cu**L**H	20.69 (3)
Cu + **L** = Cu**L**H$_{-1}$ + H	7.76 (3)

[a]Charges omitted for clarity.

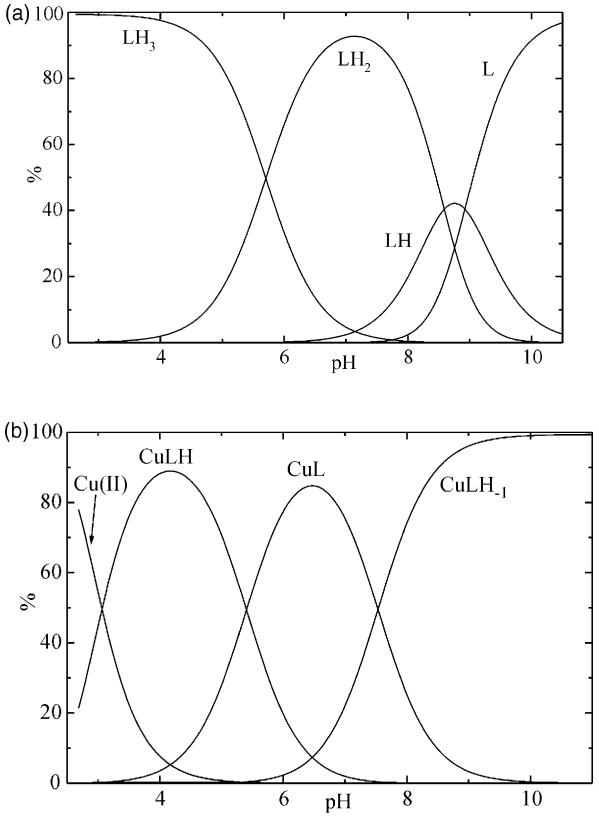

Figure 2. (a) Distribution diagram for the ligand **L**; (b) distribution diagram for Cu(II)-**L** system.

The triamine **L** formed the mononuclear complexes $[CuLH]^{3+}$, $[CuL]^{2+}$ and $[CuLH_{-1}]^{+}$ while no binuclear complexes have been detected in this system. $[CuL]^{2+}$ was the dominant complex species in the pH range 6.0–7.5. The stability constant for the species $[CuL]^{2+}$ compared well with those constants obtained for related triaza macrocycles like [11]aneN_3 ($\log\beta_{[CuL]} = 14.44$), [10]ane$N_3$ ($\log\beta_{[CuL]} = 15.48$) or [9]ane$N_3$ ($\log\beta_{[CuL]} = 15.52$).[21] All experiments below were performed in the pH range 6.0–7.5.

Antitumor Activities of Ligand L, Cu(II) and CuL Complex against HXO-RB44 and BEL-7402 Cell Lines In Vitro

The antitumor activities of ligand **L**, Cu(II) and Cu**L** complex were all measured against HXO-RB44 and BEL-7402 cell lines using MTT assay.[22,23] The inhibition percentages were listed in Table 2. It could be observed that Cu**L** complex showed the highest antitumor activity. Heavy metal ions have cytotoxicity to cells at 10^{-3} mol·L^{-1}, so Cu(II) exhibited antitumor activity at this level; but when Cu(II) was diluted to 10^{-4} mol·L^{-1}, the percentage inhibition decreased sharply to 3 or 5 respectively. On the contrary, Cu**L** complex still kept its antitumor activity even at 10^{-5} mol·L^{-1}.

Determination of the Fraction of Apoptotic Cells by Hoechst 33258 Staining[24,25]

The BEL-7402 cells were stained with Hoechst 33258 fluorescence dye. The morphology of the cell nuclei was viewed and photographed with an Olympus BH2-RFCA fluorescence microscope. Apoptotic cells were recognized by a condensed or fragmented chromatin (Fig. 3).

Figure 3a shows the morphology of nuclei of control BEL-7402 cells, while Figure 3b shows nuclear morphology of BEL-7402 cells treated with Cu**L** complex for 48 h. After stained with Hoechst 33258, the apoptotic cells showed nuclear condensation and fragmentation. Simultaneously the dispersion of nucleus and appearance of apoptotic body were observed.

Table 2. The percentage of growth inhibition data. The cells were treated with ligand **L**, Cu(II) or Cu**L** complex for 48 h and incubated with 5 g·L^{-1} MTT for 4 h. The amount of MTT formazan produced was determined by measuring absorbance at 490 nm. All data are represented as the means±SD values obtained from five separate cultures

Compd	Cell lines					
	BEL-7402			HXO-RB44		
(mol·L^{-1})	10^{-3}	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-6}
L	34	16	[a]	3	3	1
Cu (II)	81	3	2	5	3	0
Cu**L**	97	47	11	64	13	2

[a]It was not measured.

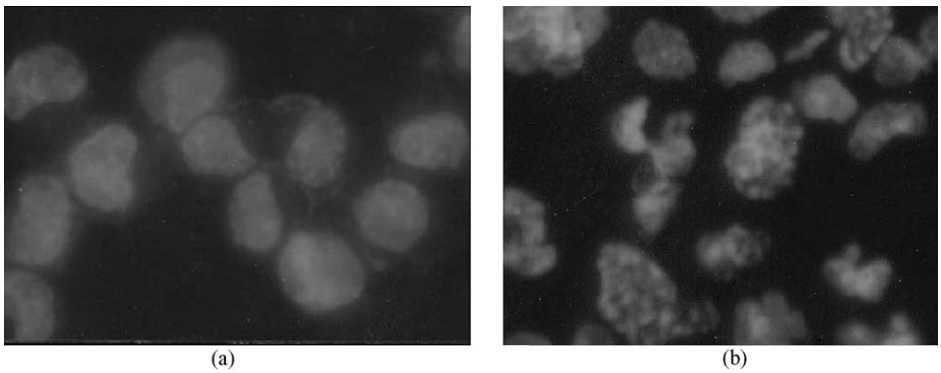

Figure 3. Hoechst 33258 staining of BEL-7402 cells. (×400): (a) normal cells after 48 h of cultivation; (b) cells treated with 3.60×10^{-4} mol·L^{-1} Cu**L** complex after 48 h of cultivation, cells undergoing apoptosis. The cells were stained with 10 mg·L^{-1} Hoechst 33258 fluorescence dye.

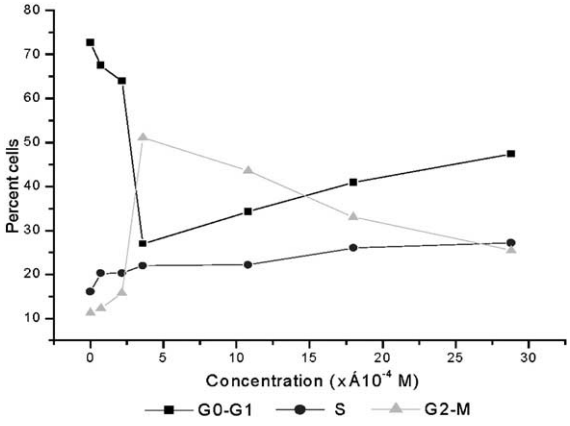

Figure 4. The perturbation of Cu**L** complex to cell cycle. BEL-7402 cells were treated with 0.721, 2.16, 3.60, 10.8, 18.0, 28.8×10^{-4} mol·L^{-1} Cu**L** complex for 48 h. Cells were then fixed with 70% ethanol and stained with 0.1 g·L^{-1} propidium iodide solution. Cell suspensions were then incubated at 37 °C for 30 min, and stained nuclei were analyzed with a Becton Dickinson FACSort.

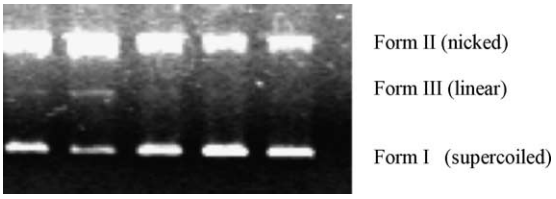

Figure 5. Electrophoresis gel demonstrating cleavage of double stranded DNA (pBR322; concentration 6.1 μmol·L^{-1}) after 2 h at 37 °C and pH 6.5. Lane 1: DNA alone; Lane 2: 10 μmol·L^{-1} Cu(II) complex + DNA; Lane 3: 50 μmol·L^{-1} Cu(II) complex + DNA; Lane 4: 100 μmol·L^{-1} Cu(II) complex + DNA; Lane 5: 100 μmol·L^{-1} Cu(II) complex + DNA + DMSO.

Perturbation of Cell Cycle Progression

The BEL-7402 cells treated with Cu**L** complex at different concentrations for 48 h were collected and examined by a Becton Dickinson FACSort after DNA staining with propidium iodide (PI) as described method.[26] In our experiment no induction of tumor cell apoptosis was observed. The changes in cell cycle were shown in Figure 4. Cu**L** complex caused changes in the proportion of cells in G_1 phase (from 72.30% to 27.10% to 44.02%) accompanied by changes in G_2-M phase (from 11.70% to 50.88% to 16.86%), and changes in S phase (from 16.00% to 39.12%).

Measurements on the Interaction to pBR 322 DNA

Chemotherapy continues to be a mainstay of cancer treatment, with DNA-damaging agents being among the most effective classes of compounds in clinical use.[27] To investigate whether the Cu**L** complex has direct interaction with DNA, we treated it with pBR 322 DNA. The preliminary electrophoresis results were shown in Figure 5.

No significant cleavage was found under comparable conditions by the metal ion[28, 29] or ligand **L**[29] alone. It can be observed that the circular supercoiled DNA converted to nicked DNA via single-strand cleavage (lane 2 to 4) in Figure 5. With the increase of complex concentration, the supercoiled DNA decreased and converted to nicked and linear DNA at the same time.

In order to clarify the possible mechanism of this cleavage, DMSO was added to the reaction mixture as the radical scavenger in lane 5.[13] In this case, no linear DNA was observed, and nicked DNA decreased. This action was similar to that of copper (II) complex with tetraazamacrocyclic ligand,[6] but is different from that of copper (II) complex of [9]aneN_3.[13] In our case, the experimental conditions were different, which means two different cleaving mechanisms may co-exist.

In conclusion, we have demonstrated the copper (II) complex formation in solution of the ligand 3-hydroxyl-1,5,8-triazacyclicamine (**L**). $[Cu\mathbf{L}]^{2+}$ was the dominant complex species in the pH range 6.0–7.5. Cu**L** complex showed antitumor activity against HXO-RB44 and BEL-7402 cell lines in vitro. Nuclei of [Cu**L**]-stimulated BEL-7402 cells clearly exhibited condensation and break down into chromatin clumps typical of apoptosis. Also it exhibited perturbation effects to BEL-7402 cell lines cycle. These results implied that antitumor activity of Cu**L** complex maybe resulted from its ability to damage DNA in the cell cycle. Further work is in progress to elucidate the detailed mechanisms of antitumor activity of this complex.

Acknowledgements

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