Synthesis and characterization of the ligand based on benzoxazole and its transition metal complexes: DNA-binding and antitumor activity

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Abstract

A new ligand 2-((2-((benzo[d]oxazol-2-yl)methoxy)phenoxy)methyl)benzoxazole (L) and its four transition metal complexes M(NO3)2 (M = Cu, Co, Ni, Zn), have been synthesized and investigated. The single crystal structures of the complexes show that all of them have similar molecular structure and the ligand exhibits good coplanarity after coordination with the metal ions. Further investigation of DNA binding indicates that both the ligand L and the complexes can bond to DNA by intercalation mode, and the latter possesses much stronger binding affinity. Antitumor activity of these compounds tested on the four cancer cell lines, follows the order: Cu-L > Ni-L > Co-L > Zn-L > L, which are thought to be related with their DNA-binding affinity.

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1. Introduction

Despite many therapeutic successes, cancer is still the second-most-frequent cause of death [1], and may become the most common disease in the near future. Thus, to discover and develop novel therapeutic agents has a vital importance. Since the introduction of cisplatin ([cis-[PtCl2(NH3)2]]) into the clinic in 1979, intensive efforts have been spent on the search for cytotoxic compounds with more acceptable side effects but retentive or even expansive activity [2].

Drug researches suggest that many anticancer agents, antiviral agents and antiseptic agents take action through binding to DNA [3–5], because the interaction between small molecules and DNA can often cause DNA damage in cancer cells, blocking the division of cancer cells and resulting in cell death [6–8]. Many studies indicate that transition metal complexes can interact non-covalently with DNA by intercalation, groove binding, or external electrostatic binding [9]. Many important applications of these complexes require that they could bind to DNA in an intercalative mode [10]. Therefore, the interaction of transition metal complexes, especially containing planar aromatic heterocyclic functional groups ligands which can insert and stack into the base pairs of DNA duplex [11–13], has attracted considerable attention [14–19].

As one of the most common planar aromatic heterocyclic compounds in medicinal chemistry [20,21], benzoxazole and its ana-

logues display a remarkable array of biological and pharmacological activities involving antibiotic [22,23], antimicrobial [24–26], antiviral [27,28], topoisomerase I and II inhibitors [29,30], and antitumor activity [31]. What’s more, benzoxazole is reported to be effective complexing agents with various transition metal ions via nitrogen donor atoms [32,33]. Based on our earlier research on the activity of transition metal complexes [34,35], we are interested to explore DNA-binding and antitumor activity of the transition metal complexes derived from benzoxazole ligands.

Herein, a new kind of benzoxazole-based ligand (L) and its transition metal complexes (Co-L, Ni-L, Cu-L, Zn-L) have been synthesized. The crystals of the complexes with good coplanarity have been obtained. Furthermore, comparative study of the interactions of the complexes and the ligand with calf thymus DNA (CT-DNA) as well as the related antitumor activities against four different cancer cell lines was experimentally explored. The remarkable DNA-binding affinity and antitumor activity suggested that the compounds above would have potential application for developing new drugs for cancers.

2. Experimental

2.1. Materials and methods

All reagents and solvents were obtained commercially and used without further purification unless otherwise noted. CT-DNA and ethidium bromide (EB) were purchased from Sigma Chemicals

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2.2. Preparation of the ligand (L)

The synthesis route for the ligand is shown in Fig. 1. 2-(Chloromethyl)benzoxazole (designated as 1) was prepared according to the literature [37]. To a solution of 0.55 g (5 mmol) pyrocatechol in 50 mL of acetone was added anhydrous K$_2$CO$_3$ 3.44 g (25 mmol).

After refluxed for 0.5 h under N$_2$ atmosphere, the mixture was treated dropwise with 1 (1.675 g, 10 mmol) in acetone (10 mL) and then allowed to refluxed for another 5 h under N$_2$ atmosphere. Upon pouring into 200 mL cold water, the white solid was precipitated out from the mixture solution, which was collected by filtration, washed several times with water and then dried in vacuo to give the desired product 2 as white powder. Yield: 1.26 g (68%). m.p. 116–117°C. ESI-MS: $m/z = 373.2 (M + 1)$, calcld 372.1 (M).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ (ppm): 7.74–7.71 (m, 2H, $H_a$), 7.53–7.50 (m, 2H, $H_b$), 7.37–7.32 (m, 4H, $H_c$), 7.14–7.10 (m, 2H, $H_d$), 6.99–6.95 (m, 2H, $H_e$). 5.40 (s, 4H, $-$OCH$_2$–). UV–vis (in CH$_3$CN): $\lambda_{\text{max}}$ (nm) [$\epsilon_{\text{max}}/10^4$ M$^{-1}$ cm$^{-1}$]) 201 (4.7), 234 (3.0), 270 (1.2).

2.2.2. Preparation of the complexes

The reaction of L (0.1 mmol) with Cu(II), Co(II), Ni(II) and Zn(II) nitrates (0.1 mmol) in acetonitrile (10 mL) for a few minutes afforded solid. After the mixture was continually stirred for 2 h at room temperature, the precipitate was collected by filtration, washed with acetoniitrite/methanol (5:1) three times, and dried in a vacuum.

All crystals of the complexes suitable for an X-ray crystallographic analysis were obtained from acetoniitrite by slow evaporation of the solvent after several days. The formulae of the complexes were M(NO$_3$)$_2$$\cdot$L (M = Co, Ni, Cu, Zn). All the complexes along with their characteristics were recorded in Table 1, their IR spectra in Table 2.

2.3. X-ray crystallography

The single-crystal X-ray diffraction measurements for all the complexes were determined on a SMART APEX II CCD diffractometer equipped with a graphite crystal monochromatized Mo Kα radiation ($\lambda = 0.71073$ Å) at 298(2) K. The structures were solved by direct methods and completed by iterative cycles of least-squares refinement. The H-atoms were placed in their geometrically calculated positions and treated as riding on the atoms to which they were attached. Absorption correction was employed using semi-empirical methods from equivalents [38]. All details of the crystal parameters, data collection and refinements were listed in Table 3, representative bond lengths (Å) and angles (°) were presented in Table 4.

2.4. DNA-binding experiment

In these studies, all the compounds were dissolved in a mixed solvent of 1% CH$_3$CN and 99% Tris–HCl buffer (5 mM Tris–HCl, 50 mM NaCl, pH 7.1) at a concentration of $1.0 \times 10^{-5}$ M. All the experiments involving the interaction of complexes with CT-DNA were carried out in Tris–HCl buffer.

2.4.1. Electronic absorption spectra

Absorption titration experiment was performed with fixed concentrations of the drugs (10 μM), while gradually increasing concentration of DNA. When measuring the absorption spectra, an equal amount of DNA was added to both compound solution and the reference solution to eliminate the absorbance of DNA itself. The solutions were allowed to incubate for 2 min before the absorption spectra was recorded. The titration processes were repeated until there was no change in the spectra, indicating binding saturation had been achieved.

2.4.2. Fluorescence spectra

The excitation wavelength was fixed and the emission range was adjusted before measurements. Excitation wavelength of the samples were 500 nm, scan speed = 240 nm/min, slit width 10/10 nm. CT-DNA and EB at a fixed concentration (15 μM, 1 μM respectively) were titrated with increasing amounts of the compounds.

2.4.3. CD spectra study

Circular dichroism spectra was obtained on JASCO J-810 spectropolarimeter at increasing compound/DNA ratio ($r = 0.0, 0.5$) in buffer at room temperature. Each sample was scanned in the range of 220–320 nm using a 1 cm path quartz cell. The concentration of CT-DNA was $1.0 \times 10^{-4}$ M. Each test solution was scanned at a
speed of 50 nm min$^{-1}$ for three repetitions, from which the buffer background had been subtracted, and the average spectra was used. High-frequency noise was filtered out using JASCO Spectra Manager software.

2.4.4. Viscosity experiment

Viscosity measurements were carried out using an Ubbelodhe viscometer, immersed in a thermostatic water-bath that maintained at a constant temperature at 25.0 ± 0.1 °C. The compounds (1–10 μM) were titrated into the CT-DNA solution (10 μM) which presented in the viscometer. The flow time of each sample was measured by a digital stopwatch for three times, and an average one was calculated. Data are presented as $(\eta/\eta_0)^{1/3}$ vs. binding ratio [39], where $\eta$ and $\eta_0$ are the viscosity of DNA in the presence and absence of complex, respectively.

Viscosity values were calculated from the observed flow time of CT-DNA containing solutions corrected from the flow time of buffer alone ($t_0$), $\eta = t/t_0$ [39–41].

2.5. Antitumor activity test

Antitumor activity in vitro was evaluated by using a system based on the tetrazolium salt (MTT). Four kinds of cells line (A549, Hep G2, K562, K562/ADM) were cultured at 37 °C under a humidified atmosphere of 5% CO$_2$ in RPMI 1640 medium supplemented with 10% fetal serum and dispersed in replicate 96-well plates with $1 \times 10^4$ cells per well. Compounds were then
added. After 72 h exposure to the toxins, cell viability was determined by measuring the absorbance at 570 nm with an enzyme-linked immunosorbent assay (ESILA) reader. Each test was performed in triplicate. IC₅₀ values were calculated from curves constructed by plotting suppression ratios (%) vs. compound concentration.

3. Results and discussion

3.1. Infrared spectra

The main infrared bands of the compounds are illustrated in Table 2. The absorption at 1613, 1454 and 1500 cm⁻¹ can be attrib-

Table 4
Selected bond lengths (Å) and angles (°) for metal environments of the complexes.

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<thead>
<tr>
<th>Co-L</th>
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<th>Cu-L</th>
<th>Zn-L</th>
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<td>Zn(1)-O(5)</td>
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<td>O(3)-Cu(1)-O(3a)</td>
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<td>2.053(5)</td>
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<td>O(3)-Cu(1)-O(3a)</td>
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<td>88.4(2)</td>
<td>89.2(2)</td>
<td>1.948(3)</td>
<td>85.3(2)</td>
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Note: R = \sum |F_o - |F_c||\sum F_o, wR_B = \sum w(F_o^2 - F_c^2)^2/\sum w(F_o^2)^2].
uted to the skeletal vibration of aromatic benzene ring and benzoxazole ring [42]. The bands of free ligand at 1575 and 1386 cm–1 are assigned to ν(C=N) and ν(==C–N), respectively. And in the IR spectra of all the complexes, these bands shift about 5 and 37 cm–1, indicating that the nitrogen of the benzoxazole ring have formed a coordination bond with the metal ions. The vibration (−C−O−C−) of the free ligand is presented at 1159 cm–1, for the complexes, the bands shift about 7 cm–1 toward higher wave numbers, which show that there exist weak interactions between the ether oxygen atoms and the metal ions.

As shown in Table 2, the absorption bands assigned to the coordinated nitrates are observed for the complexes. The separations of the two highest frequency bands |ν4−ν1| in Co-L, Ni-L and Zn-L are more than 180 cm–1, implying that coordinated nitrate groups are bidentate ligands. In contrast, they are monodentate ligands for Cu-L [43].

3.2. Description of crystal structure

A summary of the crystallographic data is given in Table 3. Selected bond lengths and angels for the coordination of M(II) (M = Co, Ni, Zn, Cu) are listed in Table 4, and the ORTEP drawings of the complexes are shown in Fig. 2a–d. X-ray analysis of the crystals indicates that three of the complexes (Co-L, Ni-L, Zn-L) adopt similar distorted octahedral structures. They all consist of a neutral ML(NO3)2 without any solvent molecule or counter anion. Take Ni-L as an example, the metal coordination sphere consists of a slightly distorted octahedron where two bidentate nitrate groups

![Fig. 3. Electronic spectra of the ligand (a), Co-L (b), Ni-L (c), Cu-L (d), Zn-L (e) upon addition of CT-DNA. [Compound] = 10 μM, [DNA] = 0–10 μM. Arrow shows the absorbance changes upon increasing DNA concentration.](#)
and two N-donor atoms of \( L \) complete the sixfold coordination. In the complex, the central benzene ring and the two benzoazole rings of the ligand are almost coplanar. The mean plane of the ligand is almost vertical to the plane built through coordinated nitrate groups \((O(5), O(7), O(8) \text{ and } O(10))\), and the dihedral angles are 89.206°, 89.137°, 89.048° for Co-\( L \), Ni-\( L \), Zn-\( L \), respectively.

Although the stoichiometry of the copper complex Cu-\( L \) is the same as that of the other complexes, the structure gets its own characteristics. As shown in Fig. 2c, the complex crystallized in the monoclinic space group \( C_{2h} \). The ligand arms adopt cis-arrangement with respect to the central benzene ring. The copper atom enjoys a square planar geometry, being coordinated by the nitrogen atoms of two adjacent ligand arms and the oxygen atoms of two monodentate nitrate anions. In addition, there are several weak interactions such as \( \text{Cu}(1)–O(2) (2.692(3) \text{ Å}), \text{Cu}(1)–O(2\text{a}) (2.692(3) \text{ Å}), \text{Cu}(1)–O(5) (2.700(4) \text{ Å}), \text{and Cu}(1)–O(5\text{a}) (2.700(4) \text{ Å}) \), being consistent with the IR spectrum. The \( \text{Cu}(1)–O(3) \) and \( \text{Cu}(1)–N(1) \) distances are typical at 1.948 Å and 2.041(3) Å, respectively. Similar to the complexes above, the ligand in Cu-\( L \) is almost coplanar. The mean-planes of the benzoazole rings are inclined at angles of 5.482° and 5.077° to the central benzene ring and lie at an angle of 7.478° to each other.

**Fig. 4.** Fluorescence spectra of EB bound to CT-DNA in the presence of the ligand (a) \( L \) and the complexes Co-\( L \) (b), Ni-\( L \) (c), Cu-\( L \) (d), Zn-\( L \) (e) with different concentrations. Stern–Volmer plots of the fluorescence quenching of EB bound to CT-DNA in the presence of the compounds in different concentrations (f). \( [\text{EB}] = 1 \mu\text{M}, [\text{DNA}] = 15 \mu\text{M}, [\text{compound}] = 0–20 \mu\text{M}, \lambda_{ex} = 500 \text{ nm} \).
As shown above, the flexible ligand was assembled into good planar structure with the aid of M(II), which would be one of the advantage factors for DNA intercalation of the complexes [44–46].

3.3. DNA-binding experiment

3.3.1. Electronic absorption spectra

The application of electronic absorption spectroscopy in DNA-binding studies is one of the most effective techniques [47]. The absorption spectra of the ligand, and the complexes in the absence and presence of CT-DNA are given in Fig. 3. Upon the addition of CT-DNA, notable hypochromic effect was observed. The absorption bands of the L and complexes (Co-L, Ni-L, Cu-L, Zn-L) at about 270 nm exhibited hypochromism of about 43.05%, 53.22%, 54.89%, 62.26%, 49.10%, respectively; while the absorption bands at about 234 nm exhibited hypochromism of about 34.59%, 64.46%, 65.16%, 63.30%, 61.84%, respectively. The hypochromic effect, characteristic of intercalation has been usually attributed to the interaction between the electronic states of the compound chromophores and those of the DNA bases [48]. Thus, the spectroscopic changes suggested that all of the compounds, especially the complexes had strong interaction with DNA.

3.3.2. Fluorescence spectra

Upon excitation at π–π∗ transitions either in CH3CN or in the presence of CT-DNA, Cu-L cannot emit strong luminescence. Similar results have been reported before [10,48–50]. Therefore, steady state competitive binding studies of the compounds were monitored by a fluorescent EB displacement assay, which could provide rich information regarding DNA-binding nature and relative DNA-binding affinities. EB, a planar aromatic heterocyclic dye intercalates non-specifically into the DNA which causes it to fluorescence strongly [50].

EB (weak fluorescent) + DNA (non-fluorescent) → EB-DNA (strong fluorescent).

It was previously reported that this enhanced fluorescence could be quenched, at least partly, by the addition of a second molecule [51]. Such a characteristic change is often observed in intercalative binding modes [48]. The emission spectra of EB bound to CT-DNA in the absence and presence of the compounds with different concentrations are given in Fig. 4. With the addition of the samples into DNA, pretreated with EB, an appreciable decrease in the emission intensity (λ = 594 nm) and an isobestic point at 540–560 nm were observed. These changes showed that all the compounds could replace EB from the DNA-EB system, and a complex-DNA system was formed. The decreased emission of the DNA-EB system was caused by EB being expelled from the hydrophobic environment into the water solution [52].

According to the classical Stern–Volmer Eq. (1) [46]:

$$F_0/F = 1 + K_q [Q]$$

where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of samples respectively, $K_q$ is the linear Stern–Volmer quenching constant, $[Q]$ is the concentration of the compounds. The fluorescence quenching curves (shown in Fig. 4) illustrate that the quenching of EB bound to DNA by these samples are in good agreement with the linear Stern–Volmer equation, which also show that DNA is influenced by complexes with only one kind of quenching process, static quenching or dynamic quenching, taking the intercalation binding mode [53].

To compare binding affinities of these samples to DNA quantitatively, the apparent binding constants were calculated from Eq. (2) [51]:

$$K_{app} = K_{EB} [EB] / C_{50}$$

where $K_{EB}$ is the binding constant of EB to CT-DNA (1.0 × 10⁷ L/mol) [2,51], $C_{EB}$ is the concentration of EB in buffer solution, $C_{50}$ is the concentration necessary to reduce fluorescence intensity to 50% of the initial value. The Kapp values were estimated as 7.02 × 10⁴, 2.99 × 10⁴, 6.10 × 10⁴, 7.37 × 10⁵, 1.07 × 10⁵, for L, Co-L, Ni-L, Cu-L, Zn-L, respectively. The binding affinity followed the order of Cu-L > Ni-L > Co-L > Zn-L > L. The increased binding affinity of the complexes may be attributed to the chelation and the planar structure of the ligand. While the different intercalative capabilities among the complexes with DNA could be due to the type of central metal elements, which are responsible for the geometry of the complexes and have significant influence on the intercalative ability [10,54,55].

3.3.3. CD spectra study

CD spectroscopy is useful in diagnosing changes in DNA morphology during drug–DNA interactions, since the positive band due to base stacking (273 nm) and the negative one due to right-handed helicity (246 nm) are quite sensitive to the mode of DNA interactions with small molecules. The changes in CD signals of DNA observed on interaction with drugs may often be assigned to the corresponding changes in DNA structure [56]. CD spectra of CT-DNA in presence and absence of the complexes are shown in Fig. 5. The calculated percents of increase in ellipticity are 33.52%, 12.98%, 8.08%, 5.41% at 273 nm and decrease in ellipticity are 43.42%, 38.32%, 34.41%, 20.00% at 245 nm for Cu-L, Ni-L, Co-L, Zn-L, respectively. Thus, it can be concluded that the interactions of all complexes towards CT-DNA follow the same order as the former experiments. Those significant changes indicate conformational changes and unwinding of DNA base pairs with

![Fig. 6. CD spectra of CT-DNA (1 × 10⁻⁶ M) in the absence and presence of the compounds (0.5 × 10⁻⁶ M).](image-url)
destabilization of the DNA double helix, which is consistent with DNA intercalation binding mode suggested above [57]. The interaction of CT-DNA with the ligand has also been checked by CD spectroscopy (Fig. 5). The nature of CD spectra of free CT-DNA and that of CT-DNA complexed with the ligand are very similar, and also the changes are less notable than the complexes.

3.4. Viscosity experiment

To throw further light on the DNA binding mode, viscosity measurements which regarded as the least ambiguous and the most critical test of a DNA binding model in solution and provides stronger arguments for intercalative DNA binding mode, has been undertaken [38,59]. A classical intercalation model results in the lengthening of the DNA helix because base pairs become separated to accommodate the binding ligand, leading to an increase in the viscosity of CT-DNA. In contrast, a partial and/or non-classical intercalation of ligand could bend (or kink) the DNA helix, reducing its effective length and, concomitantly, its viscosity [60,61]. The effect on the CT-DNA shown in Fig. 6 reveals that the relative viscosity of DNA increased steadily following the order of Cu-L > Ni-L > Co-L > Zn-L > L, with an increasing amount of the above compounds. The increased degree of viscosity may depend on the affinity of the compounds to DNA, which is also consistent with our foregoing hypothesis.

3.5. Cytotoxic activity

The cytotoxicity assays of all the compounds against four different tumor cell lines (A549, Hep G2, K562, K562/ADM) were evaluated by MTT assay, and the IC50 values derived from the experimental data were summarized in Table 5.

It was notable that the ligand was almost inactive against all the cell lines (IC50 > 100 µM/L). However, all of the complexes exhibited considerable antitumor activity and cytotoxic specificity to the tested cancer cell lines, with the IC50 values ranged from 15.38–139.15 µM/L. Moreover, Cu-L showed much higher antitumor activity than other complexes did on all the cell lines. To our best knowledge, although the antitumor activity of copper complex is no better than cisplatin, its IC50 values in a µM range are pretty well, considering that of other complexes reported recently [52–66]. The cytotoxicity displayed by Ni-L and Co-L were comparable, with Ni-L slightly over Co-L except for A549. The antitumor activity of the compounds followed the order: Cu-L > Ni-L ≈ Co-L > Zn-L, which was similar to their DNA-binding affinities. We should conclude that their strong DNA-binding affinities may account for the good antitumor activity of these complexes [67].

4. Conclusions

The benzoxazole-based ligand (L) and its transition metal complexes (Co-L, Ni-L, Cu-L, Zn-L) have been prepared and fully characterized. In agreement with UV–vis absorption, fluorescence, CD spectra and viscosity measurements, all the compounds, especially the complexes exhibited strong intercalation binding affinity, following the order of Cu-L > Ni-L > Co-L > Zn-L > L. Significantly, further investigations on their antitumor activity revealed that such complexes possessed considerable antitumor activities, especially Cu-L had an obviously higher inhibitory rate than L and other complexes which was thought to be related with its DNA-binding affinity. The better binding properties of the complexes should be attributed to the good coplanarity of the ligand after coordination with metal ions. Meanwhile, nature of the central metal ions also affected the intercalative ability. These results indicate that DNA might also serve as the primary target of these compounds; in addition, they should have many potential practical applications, just like the promising therapeutic drug candidates. Moreover, it provides an important rationale for designing new lead anticancer compounds of such transition metal complexes derived from benzoxazole and shed some light on experiment in vivo and pharmacological assay. Further study of biological activity of complexes based on benzoxazole is ongoing in our group.

5. Abbreviations

Table 5

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<th>Table 5</th>
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<td>Hep G2 IC50 (µM/L)</td>
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<tr>
<td>K562 IC50 (µM/L)</td>
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<td>K562/ADM IC50 (µM/L)</td>
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Antitumor activity is expressed as IC50 (% inhibitory concentration) in four kinds of cells (A549, Hep G2, K562, K562/ADM). Data are average data of triplicate assay.

References
