Synthesis, antitumor activity and G-quadruplex DNA/ct-DNA binding property of a cationic platinum(II) complex of 2-(4-nitro)-imidazo-[5,6-f][1,10]-phenanthroline

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A cationic platinum(II) complex (1), [Pt(4-NOIP)(en)\textsubscript{2}]\textsubscript{2} (4-NOIP = 2-(4-nitro)-imidazo-[5,6-f][1,10]-phenanthroline, en = 1,2-ethylenediamine), has been synthesized and characterized by IR, UV-vis, ESI-MS and element analysis. The interaction of complex (1) with ct-DNA and human telomeric G-quadruplex DNA [Htel-21] has been investigated by circular dichroism absorption and UV–vis absorption titration as well as fluorescent intercalator displacement assay. The circular dichroism absorption data shows that complex (1) can induce linear human telomeric DNA to transform into antiparallel G-quadruplex structure. The binding constants for complex (1) bound to Htel-21 and ct-DNA are 6.89×10\textsuperscript{5} and 7.53×10\textsuperscript{4} M\textsuperscript{-1}, respectively, indicating that complex (1) interacts with Htel-21 more intensively than ct-DNA. The FID assay suggests that complex (1) intercalates into ct-DNA and Htel-21 quite strongly. CD and UV–vis data together with FID experiments have shown that the complex can intercalate more intensely into Htel-21 and ct-DNA and induce formation and stabilization of antiparallel G-quadruplex. The anticancer activity of the complex have been tested against four different cancer cell lines and normal cell line HL-7702; the complex shows a higher activity than cisplatin and only weak cytotoxicity to HL-7702.

Keywords: Bioinorganic chemistry, Platinum, G-quadruplex DNA, Telomeric DNA, Antitumor activity, DNA binding

In the past two decades, DNA has been regarded as the primary molecular target for an array of antitumor and antimicrobial drugs\textsuperscript{10}. The remarkable success achieved with platinum-based antitumor agents, such as cisplatin, carboplatin and oxaliplatin, have promoted the development of metal complexes with significantly anticancer activities. However, all the Pt-based drugs are associated with severe side effects and the evolution of drug resistance during therapy, which has stimulated the inorganic chemists to develop less-toxic, more-effective, and target-specific metal-based anticancer drugs\textsuperscript{3}. As a routine chemotherapeutic agent for a broad range of solid malignancies, cisplatin functions by cross-linking DNA strands via the coordination of nucleic acid bases, which can subsequently induce apoptosis in cancer cells\textsuperscript{4,5}. Thus, designing and developing of new drugs targeting specific DNA secondary structures or sequences, such as the G-quadruplex DNA, continues to be a challenge. The G-quadruplexes are found to exist in the important regulatory regions of human genome, such as telomeric ends, immunoglobulin switch regions, mutational hot spots, and regulatory elements in oncogene promoters\textsuperscript{6}. Since the specific structure of G-quadruplex can be potentially targeted by small molecules bearing aromatic planar groups, as well as its close relationship with telomerase, several studies have focused on G-quadruplex DNA for developing new targeting anticancer agents\textsuperscript{7–8}.

Over the past few decades, the interaction of polypyridyl metal complexes with DNA has been a hot topic due to the potential of these complexes to serve as DNA probes or other labels\textsuperscript{9–11}, as the chelate ligands partially intercalate between the adjacent base pairs of DNA. In addition, the experimental and theoretical studies of the Ru(II) polypyridyl complexes for their DNA binding properties have been previously reported\textsuperscript{12–14}. Some of these have provided significant contributions to understand the trend in DNA binding of the complexes and to guide the molecular designing. Recently, the metal complexes based on 2-(4-nitro)-imidazo-[5,6-f][1,10]-phenanthroline (4-NOIP) and its derivatives have been synthesized, and their DNA binding properties have been extensively investigated\textsuperscript{15–18}. However,
very little is known about the apoptotic mechanism of the platinum complexes of 4-NOIP in tumour cells, and requires to be fully understood.

To improve the anticancer efficacy, a new platinum(II) complex of 4-NOIP, [Pt(4-NOIP)(en)]Cl₂ (1), was synthesized and structurally characterized. The synthetic route for complex (I) is shown in Scheme 1. To investigate the antitumor activity of (1) and its binding property towards Htel-21 G-quadruplex and ct-DNA, the UV–vis and CD spectral analyses as well as FID assay were carried out.

**Materials and Methods**

All chemicals and reagents were obtained commercially and used without further purification. Calf thymus DNA (ct-DNA), c–kit1 (5′–G₃AG₃CGCT-G₃AG₃AG₃–3′), c–kit2 (5′–G₃T₂AG₂T₂AG₂AG₃–3′), ds26 (5′–CA₂TGC₃TG₃ATCGA₂T₂CGATC₂G–3′), H₂1T (5′–G₃T₂AG₂T₂AG₂AG₃–3′), and pu22 (5′–TGAG₂TG₃TAG₂TG₂–3′) were purchased from Sangon Biotech (Shanghai).

UV–vis spectra were recorded on a Cary100 spectrophotometer. CD spectra were recorded on a Jasco J-810-150L spectropolarimeter, and FID assay was performed on a Shimadzu RF-5301PC spectrofluorophotometer. IR spectra were recorded on a Perkin-Elmer Spectrum One FT–IR spectrometer. Elemental analysis was carried out on a Perkin-Elmer 2400II CHNS/O elemental analyzer. ESI-MS spectra were recorded on Thermo-Finnigan LCQ/AD Quadrupole Ion Trap ESI-MS.

For the spectral analyses, the calf thymus DNA and G-quadruplex DNA were both dissolved in Tris-KCl buffer (10 mM Tris, 100 mM KCl, adjusted to pH = 7.35 by HCl) to prepare the stock solution of 1.0 mM. The complex (1) was dissolved in DMSO to prepare the stock solution of 2.0 mM. These were all further diluted to the required concentrations by Tris buffer solution before use. All the stock solutions were stored at 4 °C before use.

4-NOIP (2-(4-Nitro)-imidazo-[5,6-f][1,10]-phenanthroline) as the organic ligand was synthesized based on the reported procedure as follows: A mixture of 1,10-phenanthroline-5,6-quinone (0.63 g, 3 mmol), 4-nitrobenzaldehyde (0.64 g, 4 mmol) dissolved in acetic acid (20 mL) was refluxed with stirring for 6 h. Then it was cooled to room temperature, and the yellow precipitate of 4-NOIP was achieved. 4-NOIP was re-crystallized from DMF and then dried in vacuo (yield: 82%).

Complex (1) was synthesized according to the revised literature as follows: A mixture of K₂PtCl₄ (5.0 mmol) and 4-NOIP (5.0 mmol) dissolved in DMSO (10 mL) was refluxed with stirring for 7 h. The reaction mixture was then cooled to room temperature, and the yellow precipitate of cis-[PtCl₂(4-NOIP)] was obtained by filtration. Subsequently, ethylenediamine (0.159 mL, 3 mmol) was added into the suspension of [PtCl₂(4-NOIP)] (0.30 mmol) in methanol (30 mL) and the mixture was refluxed and reacted for 3 h. The mixture was then allowed to cool down to room temperature, and a red precipitate was obtained. The red product was filtered and washed with acetone, and dried in vacuo (yield: 79%) (Scheme 1). It was characterized and structurally determined as [Pt(4-NOIP)(en)]Cl₂ (1) by IR, UV–vis, ESI-MS and elemental analysis. It was found to be soluble in methanol, ethanol, DMF, DMSO and stable in air and aqueous solution. IR (KBr, cm⁻¹): 3419(s), 3216(s), 3063(s), 1591(s), 1511(m), 1461(s), 1406(w), 1361(w), 1308(w), 1254(w), 1117(w), 1058(m), 996(w), 891(w), 811(m), 716(w). UV–vis: λ_max = 287 nm. ESI–MS m/z: 297.1 [M–2Cl]⁺, 594.2 [M–2Cl–H]⁺, 630.3 [M–Cl–H]⁺. Anal. Calc. (%) (for C₂₁H₁₉Cl₂N₇O₂Pt): C 37.78; H 2.85; N 14.69; Found: C 37.75; H 2.93; N 14.61.

![Synthetic route for complex (1), [Pt(4-NOIP)(en)]Cl₂](image-url)
Results and Discussion

Cytotoxicity assay of complex (1)

The cytotoxicity of complex (1) in comparison with cisplatin against SPC-A-2, MGC80-3, BEL-7404 and HeLa229 human tumor cell lines as well as HL-7702 normal cell line was evaluated by MTT method. As shown in Table 1, complex (1) showed significant cytotoxicities on these four tumor cell lines except MGC80-3, with IC_{50} values in the range 9.78~16.12 µM against SPC-A-2, BEL-7404 and HeLa229, while against MGC80-3, a much higher IC_{50} value of 51.58 µM was obtained, indicating that the gastric adenocarcinoma cell line MGC80-3 is not as sensitive to complex (1) as the other three tumor cell lines. Furthermore, complex (1) showed only low cytotoxicity on the normal cell line HL-7702. These results suggest its potent cytotoxic selectivity on tumor cell lines. On the other hand, towards all these four tumor cell lines, complex (1) showed higher cytotoxicities as compared with cisplatin. However, towards the HL-7702 cell line, cisplatin is more toxic than complex (1), suggesting that complex (1) showed appreciable selectivity on these tumor cell lines against HL-7702 normal cell line, and some advantages over cisplatin based on this in vitro cytotoxicity assay.

Fluorescent intercalator displacement assay (FID)

The MTT assay showed the significantly cytotoxic selectivity of complex (1) on different human tumor cell lines compared with human normal liver cell line. Considering that the G-quadruplex DNA is regarded as the molecular target regarding telomerase for the tumor-targeting agents, the G-quadruplex binding affinity of complex (1) was investigated by UV-vis, CD and fluorescence spectroscopy, in comparison with the normal double-stranded DNA.

The selectivity of complex (1) on different forms of G-quadruplex DNA (c-kit1, c-kit2, H21T, pu22) versus double-stranded DNA (ds26) was primarily evaluated by fluorescent intercalator displacement (FID) assay, a fast and direct method to evaluate the binding selectivity of small molecules on G-quadruplex DNA, is based on the loss of fluorescence of thiazole orange (TO) as DNA probe under the competitive binding on the DNA binding sites by other small molecules. The selectivity of a compound for DNA can be evaluated by the DC_{50} value, which is the required concentration of the compound to induce a 50% fluorescence decrease of TO.

As depicted in Fig. 1, in the presence of K^+, complex (1) exhibited much better efficiency to displace TO from c-kit2, Htel-21, pu22 G-quadruplex DNA with respective DC_{50} values of 0.46, 0.59 and 0.70 µM than from ds26 DNA with DC_{50} value of 1.25 µM. The c-kit1 G-quadruplex DNA, with DC_{50} value of complex (1) is 1.19 µM, does not show convincing better efficiency than ds26 DNA, and needs to be further studied. However, at the concentrations of complex (1) lower than 1.25 µM, it showed better efficiency to displace TO from c-kit1 G-quadruplex DNA than from ds26 DNA. FID assay further demonstrated that the complex (1) had higher binding affinity to G-quadruplex DNA than to double-stranded DNA, which may be ascribed to the stronger π–π interactions between complex (1) and G-quadruplex DNA, based on its enlarged planar structure.

Table 1—IC_{50} values of complex (1) and cisplatin against four human tumor cell lines and the normal liver cell line HL-7702 in vitro

<table>
<thead>
<tr>
<th>Comp.</th>
<th>IC_{50} (µM)</th>
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<tbody>
<tr>
<td></td>
<td>SPC-A-2</td>
</tr>
<tr>
<td>Complex (1)</td>
<td>9.78±0.80</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>&gt;100</td>
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*Values are ± standard deviations of three repeated experiments.
UV-vis spectral analysis

From the FID assay, it can be found that complex (1) replaced TO more effectively from the above human telomeric DNA than the double-stranded DNA. Hence, the Htel-21 G-quadruplex DNA was selected as representative to confirm the binding preferential of complex (1) to G-quadruplex DNA. The UV–vis absorption spectra of complex (1) titrated with ct-DNA and Htel-21 G-quadruplex DNA are shown in Fig. 2(a) and (b), respectively. As shown in Fig. 2(a), complex (1) alone at 20 µM showed maximum absorption peak at 278 nm. Upon the addition of ct-DNA with increasing amounts ([ct-DNA]/[1] = 0 - 0.8 every 0.1 interval), a gradient hypochromicity of the peak absorption can be observed with total hypochromicity ratio of 40%. Along with the isosbestic absorption point at 340 nm which suggests the conjugation of complex (1) and ct-DNA, it may be concluded that complex (1) intercalates between the neighboring base pairs of ct-DNA via π–π stacking interaction.

The UV–vis absorption spectra of complex (1) with Htel-21 G-quadruplex DNA is given in Fig. 2(b). For Htel-21, the concentration of complex (1) was set at 40 µM. On addition of Htel-21 G-quadruplex DNA with increasing amounts of Htel-21 ([Htel-21]/[1] = 0-0.5 every 0.05 interval in ratio), obvious hypochromicity was observed along with a 4 nm bathochromic shift.

This is characteristic of the intercalative binding mode of complex (1) to Htel-21 G-quadruplex DNA, which is also supported by the isosbestic point at ca. 330 nm. A total of 27% hypochromicity at 278 nm was achieved when the [Htel-21]/[1] ratio only reached 0.05. These results suggest that complex (1) shows higher binding affinity to Htel-21 G-quadruplex DNA than to double-stranded ct-DNA.

To quantitatively compare the binding property of complex (1) with different types of DNA, the intrinsic binding constant, $K_b$, for complex (1) was determined using the classic linear fitting equation,

$$\frac{[\text{DNA}]}{(\varepsilon_a - \varepsilon_f)} = \frac{[\text{DNA}]}{(\varepsilon_b - \varepsilon_f)} + \frac{K_b(\varepsilon_b - \varepsilon_f)}{[\text{DNA}]} \ldots (1)$$

where $\varepsilon_a$, $\varepsilon_f$, and $\varepsilon_b$ correspond to the extinction coefficients of complex (1) at a given DNA concentration, the free complex in solution, and the complex fully bound with DNA, respectively. By plotting the $[\text{DNA}]/(\varepsilon_a - \varepsilon_f)$ versus $[\text{DNA}]$, $K_b$ can be obtained as the ratio of the slope to the intercept of the equation. The calculated intrinsic binding constant, $K_b$, of complex (1) for ct-DNA and Htel-21 G-quadruplex DNA is $7.53 \times 10^4$ and $6.88 \times 10^5 \text{ M}^{-1}$, respectively, which confirmed that complex (1) preferentially binds with Htel-21 G-quadruplex DNA than with double-stranded ct-DNA.

![Fig. 2](image-url) — UV-vis absorption spectra of complex (1) with (a) ct-DNA and (b) Htel-21 G-quadruplex with increasing concentration of DNA. ([ct-DNA]/[complex] ranged from 0–1.0; [Htel-21]/[complex] ranged from 0–0.1).
Circular dichroism spectra
The CD spectra of ct-DNA in the absence (dashed line) and presence (solid lines) of complex (1) with increasing concentrations at the ratios of [complex (1)]/[ct-DNA] in the range from 0.1 - 0.8 are shown in Fig. 3. The CD spectrum of ct-DNA alone shows the positive absorption peak at ca. 280 nm and the negative absorption peak at ca. 245 nm, due to the $\pi-\pi$ base stacking and right-hand helicity, respectively. This is consistent with the characteristic B conformation of DNA. It can be seen that the gradual addition of complex (1) caused a dramatic increase on the positive absorption intensity at ca. 280 nm of ct-DNA, which may be partly ascribed to the $\pi-\pi$ stacking interactions between complex (1) and DNA base pairs. However, the additive effect of the Pt(II) complex (1) should be also involved, since the strong positive absorbance of complex (1) at ca. 280 nm is also suggested. Simultaneously, the negative absorption intensity decreased stepwise, till it disappeared and a weak positive absorption peak at ca. 237 nm emerged. This suggests that the double-stranded DNA was partly relaxed to lose the B-form character. We also noticed a positive absorption band at 320 nm, which was regarded as the induced CD signal (ICD). All the CD spectral changes above are generally attributed to the intercalative binding of complex (1) between the neighboring base pairs of DNA. These results mainly suggest an intercalative binding mode of complex (1) to ct-DNA.

The CD spectra of Htel-21 telomeric DNA in the absence and presence of K$^+$ were also studied (Fig. 4(a) and 4(b)). The CD of Htel-21 in 10 mM Tris buffer (pH = 7.34) in the absence of K$^+$ shows three positive absorption bands of Htel-21 at 255, 294 and 328 nm and a negative absorption band at 238 nm, indicating that DNA was in a disordered state and did not fully transform to the G-quadruplex structure. After the addition of complex (1) at 5 µM, obvious changes in the CD absorption of Htel-21 were found. The positive absorption peak at 255 nm shifted to 248 nm and diminished to some extent, while the positive absorption at 294 nm enhanced more than double, suggesting the Htel-21 was induced to antiparallel G-quadruplex structure on the addition of complex (1).
In the presence of K⁺, Htél-21 transformed to the antiparallel G-quadruplex structure, which shows two positive absorption bands at 290 and 250 nm, respectively. On addition of increasing concentration of complex (1) ([I] = 0, 1, 2, 3, 4 μM) into the antiparallel G-quadruplex DNA from Htél-21, regular changes on the CD spectra were observed (Fig. 4(b)). The positive absorption at 290 nm enhanced stepwisely, along with a slight decrease at 250 nm. These CD spectral changes demonstrate that complex (1) can induce the disordered oligonucleotide Htél-21 to form antiparallel G-quadruplex DNA and stabilize this structure. It can be deduced that the intercalative binding of the planar aromatic structure of the 4-NOIP ligand as well as the electrostatic interaction of the cationic [Pt(4-NOIP)]²⁺ facilitated its stabilization on G-quadruplex DNA. These results agree with the results of FID assay and UV-vis spectral analyses.

Conclusions

In this study, a cationic platinum(II) complex bearing aromatic planar group of imidazo-[5,6-f]-[1,10]-phenanthroline was synthesized and characterized. Compared with cisplatin, this complex exhibited higher cytotoxicity towards a series of human tumor cell lines, but lower cytotoxicity towards human normal liver cell line in vitro. This suggests that such platinum(II) complexes have some selectivity towards tumor cells, and potential for tumor targeting. As a specific intracellular target for most of the tumor cells, human telomeric G-quadruplex DNA was primarily considered. The results of the spectroscopic analyses showed that this complex induced parallel G-quadruplex DNA to transform into antiparallel G-quadruplex and intercalatively bound G-quadruplex DNA with higher binding affinity than ct-DNA, which can be ascribed to the large aromatic plane stacked between the terminal G-tetrads of G-quadruplex. This provides convincing proof to explain the cytotoxic selectivity of this complex on the tested human tumor cell lines.

Acknowledgement

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References

19. The human telomeric sequence used in this work: AGGGTTAGGGTTAGGGTTAGGG. The telomeric sequence was dissolved in MilliQ water and annealed by heating to 95 °C for 5 min then by cooling to room temperature, the human telomeric G-quadruplex (Htél-21) was obtained.