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Microwave-assisted Synthesis of Imidazole [4,5*f*][1,10]phenanthroline Derivatives as Apoptosis Inducers in Chemotherapy by Stabilizing *Bcl-2* G-quadruplex DNA

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Abstract: Microwave-assisted synthetic technology is widely employed in synthesis for of organic molecules has long been facilitated in organic synthesis to build versatile molecules in medicine. Moreover, this method has received widespread attention for the controlled temperature and stable condition during the reaction process. Herein, a series of imidazo [4,5*f*][1,10] phenanthroline derivatives RPIP(PIP = imidazo [4,5*f*][1,10] phenanthroline, R = NO_2, **1**; CF_3, **2**; Cl, **3**; OH, **4**) have been synthesized in yields of 82.3%–94.7% at 100°C under the irradiation of microwave. The inhibitory activity of these compounds against various tumor cells have been investigated by MTT assay. The results indicated that this class of compounds exhibited excellent inhibitory effect against different tumor cells, especially for 1 inhibited the growth of A549 cells with IC_{50} of 3.35 μM. The further study showed that this compound entered into tumor cells and distrituted at mitochondria, and then lead to tumor cells dead through induction of apoptosis. Furthermore, the DNA-binding properties were investigated by spectroscopy and CD titration suggested that **1** could bind to *bcl-2* G-quadruplex DNA, then to interfere the Bcl-2 related apoptosis pathway. In a word, **1** may act as a potential apoptosis inducer mediated-mitochondria by binding to the *bcl-2* G-quadruplex DNA in cancer chemotherapy.

Keywords: Microwave-assisted synthesis; Imidazo [4,5*f*][1,10] phenanthroline derivatives; Apoptosis inducers, *Bcl-2* G-quadruplex DNA.

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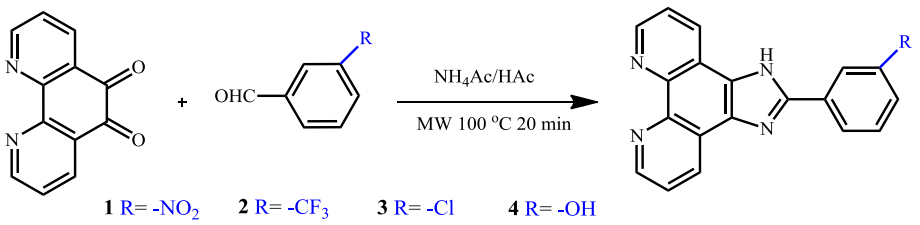
1. Introduction

1,10-Phenanthroline derivatives with an extended π-conjugated unit of fused imidazole can bind to duplex DNA and G-quadruplex DNA through π-π stacking, which exhibit great antitumor, anti-inflammatory and antiviral activity [1]. Furthermore, some researchers reported that phenanthroline-dione derivatives might increase the chemosensitivity of esophageal cancer patients to develop as a potential anticancer candidate [2]. Due to the fluorescence sensory properties towards acid, phenanthroline derivatives have also been widely used as the probes of DNA [3]. Moreover, there is clear evidence that this type of compounds can act as antitumor agents agasint

various tumors. For example, Chunying Wei and co-workers have found that the di-substituted phenanthroline derivatives can bind to human telomeric G-quadruplexes DNA and inhibit significantly the telomerase activity resulted in tumor cells apoptosis [4, 5]. In our previous work, it has been suggested that imidazo[4,5*f*][1,10]phenanthroimidazole derivatives showed excellent inbitory effect against different tumor cells, which can bind to G-quadruplex DNA in groove binding mode and block DNA replication, then induced breast cancer cells apoptosis [6, 7].

The *bcl-2* G-quadruplex DNA is a key director to regulate the expression of Bcl-2, a crucial member of the Bcl-2 family of proteins to accelerate the apoptosis, which may act as a potential tumor chemotherapy target. Bcl-2 protein is overexpressed in various human tumor cells [8]. More recently, a number of small molecules with ligands of highly binding ability to *bcl-2* G-quadruplex DNA have been reported that can inhibit the transcription of Bcl-2 gene and induce the tumor cells apoptosis, such as pyridostatin analog [9], metal complexes [10], quindoline derivatives [11], etc. Our group has shown phenanthroline derivatives can effectively inhibit the growth of tumor cells, which attributed to the ability to induce the cancer cells apoptosis through binding and stabilizing the G-qudruplex DNA. This work might imply this kind of compounds can also interact with the *bcl*-2 G-quatruplex DNA. However, it's still not clear whether phenanthroimidazole derives can inhibit the growth of tumor cells through stabilizing the G-quadruplex structure of *bcl-2* promoter, and it's very important to design novel candidates with high activity and selectivity to tumor cells.

In this study, a series of imidazo [4,5*f*][1,10]phenanthroline derivatives have been synthesized under microwave irradiation (Scheme 1). The anti-tumor activity of these compounds against the human lung adenocarcinoma A549 cells, human hepatocarcinoma SMCC7721 cells, and human colorectal carcinoma SW620 cells were evaluated by MTT assay. The results showed this type of compounds can effectively inhibit the growth of tumor cells, especially **1** had the best inhibition activity against A549 cells. The further study displayed **1** could enter into A549 cells and be distributed at mitochondria, and inhibit the tumor cells at G1 phase then induced apoptosis. It was found that **1** exhibited some certain degree binding affinity to *bcl-2* G-quadruplex DNA, which may consider as a potential regulated role in the mechanism of this kind of compounds, resulting the tumor cells apoptosis.



Scheme 1. Microwave-assisted synthesis route for phenanthroimidazole derivatives.

2. Results and discussion

2.1 Microwave-assisted synthesis of the phenanthroimidazole derivatives

Microwave-assisted synthesis technology has been widely developed in the fields of chemical synthesis, materials science, and biotechnology [12, 13]. The technique observably shorten reaction time, while increasing yield and product purity over traditional synthesis methods, especially in the synthesis of organic molecules which typically require long hours of heating at reflux in high-boiling solvents to affect a reaction [14]. As shown in Figure 1, the reaction temperature rapidly reached setting value less than 60 s, and kept great stabilization and homogeneous of temperature and pressure in the whole heating process. As a result, the yields of the target compounds increased obviously in far more less times than conventional heating methods. These results suggested the application of microwave irradiation has significantly increased the yields for most of the compounds to about 90%, which were much higher than those of conventional synthesis methods [15].

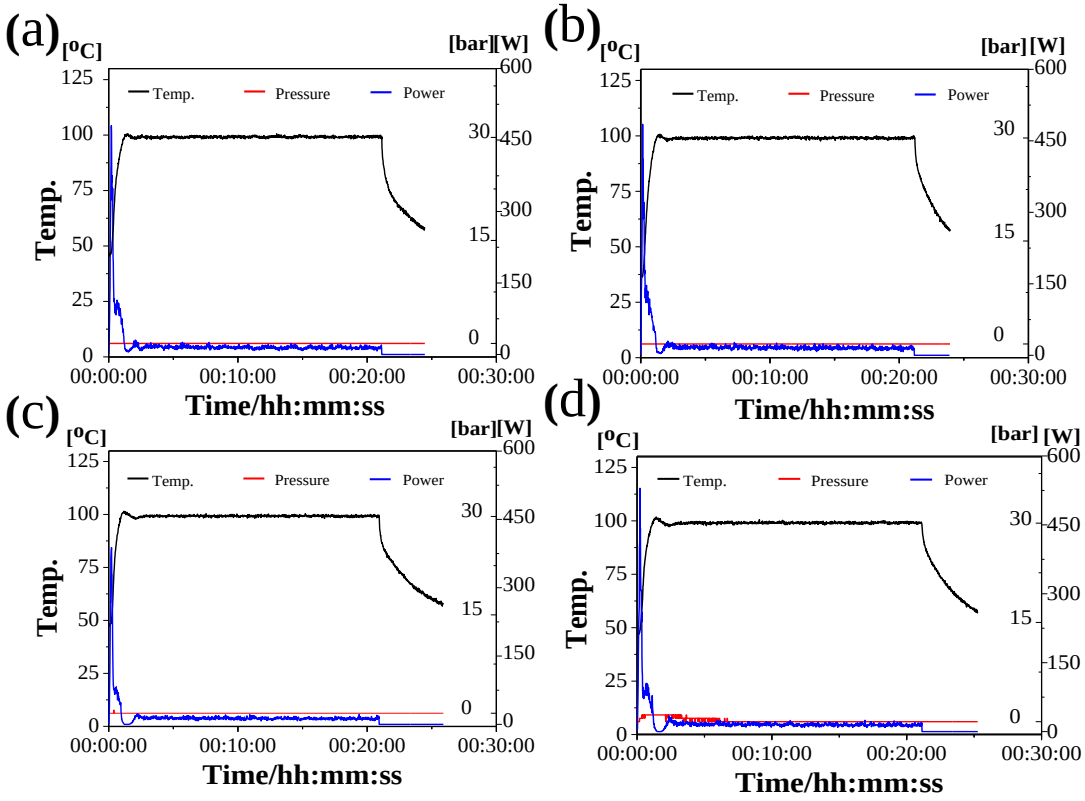


Figure 1. Reaction profile of the target complexes in glacial acetic acid irradiated by microwave for 20 min at 100°C, **(a) 1**; **(b) 2**; **(c) 3**; **(d) 4**.

Table 1. The yields of the target complexes by microwave-assisted synthesis method.

Compd.	Microwave-Assisted		
	Temperature/°C	Time/min	Yield%
1	100	20	91.3%
2	100	20	82.3%
3	100	20	94.7%
4	100	20	89.7%

2.2 Biological activity

The synthetic phenanthroimidazole derivatives can effectively inhibit the human lung adenocarcinoma A549 cells, human hepatocarcinoma SMCC7721 cells, and human colorectal carcinoma SW620 cells growth and proliferation, which were confirmed by MTT assay. *Cis*-platin was used as a positive control, and the inhibitory activities (IC_{50}) of these complexes were listed in Table 2. It was observed that these compounds displayed great growth inhibition against various tumor cells after 72 h treatment, especially the inhibitory activity (IC_{50}) of **1** against A549 cells was about 3.35 μM, which was far more less to that of cis-platin (16.54 μM). The data suggested that phenanthroimidazole derivatives blocked the growth of tumors cells, and those derivatives with electron-withdrawing meta-substituents, the complex with $–NO_2$ substitution exhibited higher anti-tumor activity against A549 cells than that of the complex with $–CF_3$ substituent, as to electron-donating meta-substituents, the complex with –Cl substitution had higher inhibitory activities of A549 tumors cells than that of the complex with –OH substituent.

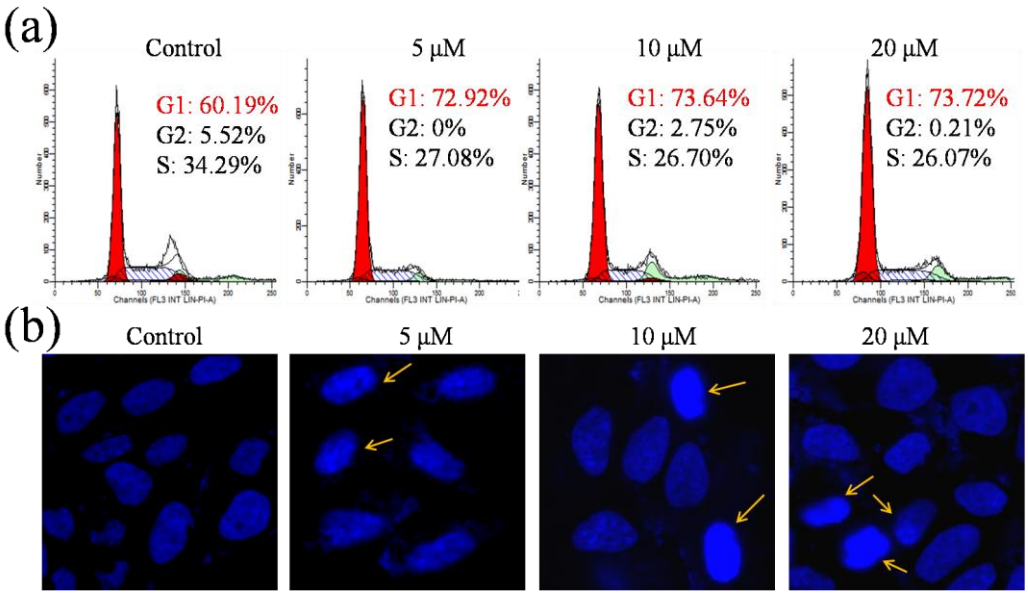
Table 2. The inhibitory effect IC_{50} (μM) of the target complexes and *cis*-platin on human cancer cells

Compd.	IC_{50} (μM)		
	A549	**SMMC-7721**	**SW620**
1	3.35	> 100	4.53
2	11.23	22.34	10.32
3	4.16	15.39	10.52
4	8.36	11.28	7.52
***cis*-platin**	16.54	2.92	5.74

105

106 *2.3 Apoptosis induction*

107 According to the above results of the MTT assay, **1** showed great inhibitory activity aginst A549
108 cells comparing with the other compounds. Apoptosis and cell cycle arrest, or a combined action of
109 both can result in growth inhibition or death of cells [16]. Thus, to further understand the
110 underlying mechanisms of **1**, flow cytometric analysis was carried out. The A549 cells were exposed
111 to different concentrations of **1** (0, 5, 10, and 20 μM) and showed a significant increase G1-phase
112 arrest for 24 h (Figure 2a), upon the increasing concentration of **1,** the ratio of cell G1-phase arrest
113 increased markedly, even reached about 73.72% for the 20 μM target compound, which was
114 approximately a quarter higher than that of the control (60.19%). Owing to G1 phase arrest is more
115 related cell apoptosis [17, 18], Hoechst 33258 dye cell nucleus was used to observe cell apoptosis. It
116 was found that, with the increasing of **1**, the cell nucleus shrink obviously and highlight markedly,
117 which is the marker of apoptosis (Figure 2b). The observations indicated that **1** may induce A549
118 cells G1-phase arrest then resulted in apoptosis.



119

120 **Figure 2**. **(a)** G1-phase arrest of A549 cells induced by **1**. A549 cells were treated with **1** (0, 5, 10, and
121 20 μM) for 24 h, almost 73% cycling cells were in the G1-phase and the sharp peak suggested that some
122 cells were experiencing G1-phase delay or arrest . **(b)** Induction of cells apoptosis of **1** by Hoechst 33258
123 dye. A549 cells were dyed with Hoechst 33258 at different concentrations of **1** (0, 5, 10, and 20 μM) for 24
124 h.

125 *2.4 Drug distribution and location*

126 Cellular localization of **1** in A549 cells was further investigated, as shown in Figure 3. It was
127 observed that **1** can be uptook by A549 cells and glow weak green fluorescence, a possible reason is

mainly due to the absence of hypochrome nitro-group. Here, the mitochondria were marked in red by Mito-tracker, and the nuclei of A549 cells were stained blue by Hoechst 33258. Upon the addition of **1**, an increasing green fluorescence was great merged with red fluorescence in the cell mitochondria, but little overlay in cell nuncleus. These results indicated this compound entered into cells majorly accumulated in the mitochondria of A549 cells, which may induce the cell apoptosis through mitochondria-mediated pathway.

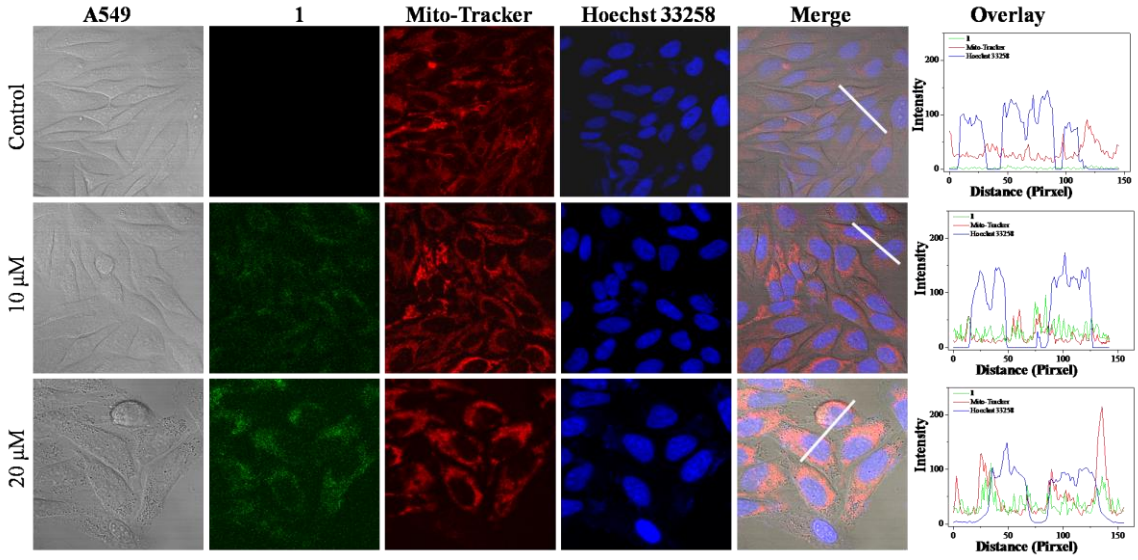


Figure 3. Cellular localization of **1** in A549 cells. Cells were treated with the **1** for 6 h at 37 °C: blue, Hoechst 33258; red, Mito-Tracker . [**1**] = 0, 10 and 20 μM. The overlay data were analyzed using Image Pro Plus.

2.5 The interaction of Bcl-2 G-quadruplex DNA with phenanthroimidazole derivatives

2.5.1 Electronic titrations

UV absorption was carried out to monitor the interaction of **1** with *bcl-2* G-quadruplex DNA. As shown in Figure 4a, the electronic spectra of **1** in Tris-HCl buffer (pH = 7.4) solution exhibited the characteristic IL (intraligand charge transfer) absorption in the range of 250–300 nm with the maximum at about 273 nm. When the DNA was gradually added to the solution of the complex, a hyperchromic effect and a bathochromic effect were observed. The hyperchromism value of **1** at the IL absorption band was about 14.1% ($\Delta\lambda$ = 2.1 nm), which may be attributed to bases of the DNA exposed, when the ligand binding to DNA, induced external contact or to partial uncoiling of the DNA structure [19, 20]. Furthermore, the value of the intrinsic binding constant (K_b) for **1** was about 8.7×10^7 M^{-1}, calculated according to the decay of IL absorption. These data implied that **1** bound to *bcl-2* G-quadruplex DNA with high affinity in intercalating mode [21]. The interaction was further confirmed by the following fluorescence quenching experiment.

2.5.2 Fluorescence quenching

Because of the low fluorescence of **1** in the Tris-HCl KCl buffer (PH = 7.4), fluorescence quenching of ethidium bromide (EB) and DNA was carried out. As shown in Figure 4b, when excited at 350 nm, the EB-DNA (*bcl-2* G4 DNA) solution emited a strong fluorescence in the range of 500 to 700 nm with the maximum at about 597 nm [22]. Upon the **1** added into solution, it was observed the fluorescence intensity of the solution decreased graduatly, which suggested that **1** can competitive association with *bcl-2* G-quadruplex DNA. At [Compd.] = 20.89 μM, the relative intensity (I/I_0) for solution was about 0.83. The results implied that **1** exhibited a certain interaction with *bcl-2* G-quadruplex DNA.

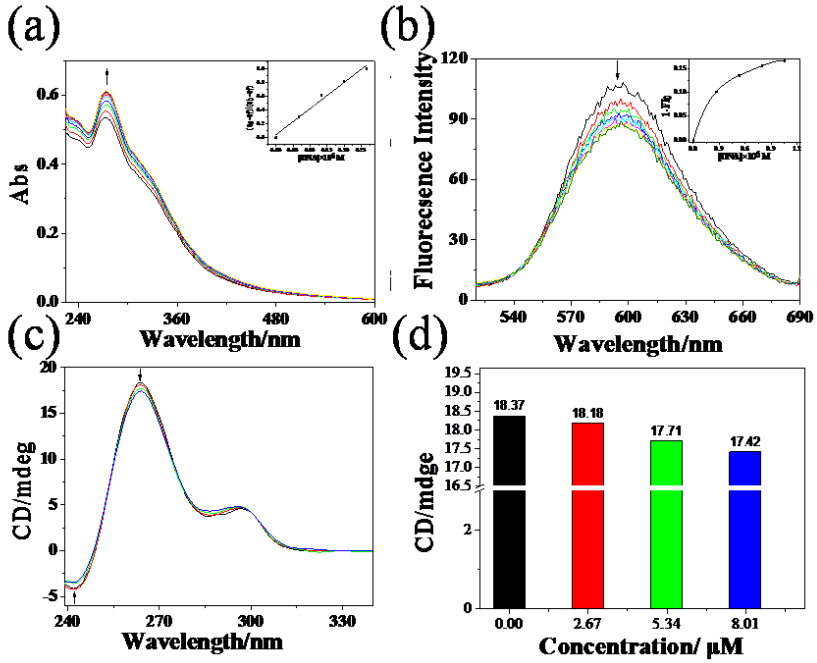


Figure 4. The study of the interaction between **1** with *bcl-2* G-quadruplex DNA by spectroscopic methods. **(a)** The electronic spectra of **1** in absence and in presence of *bcl-2* G-quadruplex DNA. [**1**] = 60 μM, [DNA] = 100 μM. **(b)** Emission spectra of EB and *bcl-2* G-quadruplex DNA in the incubation buffer in the absence and presence of **1**, [EB] = 16 μM, [DNA] = 2 μM. **(c)** CD titration spectra of *bcl-2* G-quadruplex DNA at different concentrations of **1**, ([**1**] = 0, 2.66, 5.305, 7.937 μM). **(d)** The change of CD signal at 263 nm of *bcl-2* G-quadruplex DNA with the addition of **1** in the Tris-HCl buffer.

2.5.3 Circular dichroism spectra

CD spectra is one of the most common and convenient methods for detecting DNA conformational changes. CD titration experiment was further carried out to confirm the binding of **1** with *bcl-2* G-quadruplex DNA, and the results were shown in Figure 4c. The CD spectra of *bcl-2* G-quadruplex DNA displayed a positive CD signal between 250 and 300 nm with the maximum at 264 nm, which was the characteristic CD signal of G4 DNA, in addition to a weak negative CD signal in the range of 200-250 nm with the maximum at 245 nm [23]. Upon the addition of **1**, the positive signal of *bcl-2* G-quadruplex DNA in the CD spectra were decreased, the strength of the positive CD signal (263 nm) and the negative CD signal (245 nm) decreased by 5.6% and 15.2%, respectively. These data indicated that the ligind can stabilize and bind to the *bcl-2* G-quadruplex DNA.

3. Conclusions

In summary, a series of phenanthroimidazole derivatives have been synthesized by using microwave-assisted synthesis technology, with high yields of approximately 90%, which can rapid rising to the required temperature, and kept almost no change during the whole process. The results of MTT assay showed these complexes can block the growth of tumor cells, especially **1,** exhibited excellent antitumor activity against A549 cells through inducing apoptosis of the cells. The further study displayed **1** can distribute at mitochondria, and inhibit tumor cells at G1 phase then induce apoptosis. Futhermore, the investigation by spectroscopy and CD titration demonstrated that this compound can bind to the *bcl-2* G-quadruplex DNA. Take together, this type of phenanthroimidazole derivatives can act as potential antitumor agents targeting to the *bcl-2* G-quadruplex DNA in cancer chemotherapy.

4.Experomental Section

4.1 Materials

All reagents were purchased from commercial suppliers and used without further purification. Solvents were dried and purified by conventional methods prior to use. Distilled water was used in all experiments. *bcl-2* G-quadruplex DNA (5′-CGGGCGCGGGAGGAAGGGGGCGGGAGC-3′) was purchased from Sangon Biotech and formed a G-quadruplex conformation as literature by renaturation for 24 h at 4°C, after denaturation for 5 min at 90°C [24]. All aqueous solutions were prepared with doubly distilled water.The Tris-HCl buffer consisting of Tris and KCl, and the pH value was adjusted to 7.4 by HCl solution, which was applied to UV titration and Fluorescence emission titrations.

4.2 Instruments

The phenanthroimidazole derivatives were synthesized by using Anton Paar Monowave 300 microwave reactor. ESI-MS spectra were obtained in methanol on Agilent 1100 ESI-MS system operating at room temperature. The ^{1}H NMR and ^{13}C NMR spectra were recorded in a dimethyl-d^6 sulfoxide (DMSO-d^6) solution on a Bruker Avance Ⅲ 500 spectrometer operating at room temperature. UV-vis absorption spectra were recorded on a Shimadzu UV-2550 spectrophotometer. The steady-state emission spectra were recorded on a RF-5301 fluorescence spectrophotometer.

4.3 Synthesis of imidazole[4,5*f*][1,10] phenantholine derivatives

Phenanthroimidazole derivatives were synthesized according tothe literature procedure with some modification [8].

4.3.1 Synthesis of compound **1.**

A mixture of 1,10-phenanthroline-5,6-dione (315.06 mg, 1.50 mmol), 3-nitrobenzaldehyde (339.81 mg, 2.25 mmol), ammonium acetate (4 g, 51.9 mmol) and glacial acetic acid（20 mL) was heated at 100 °C for 20 min under microwave irradiation. Then, 20 ml of water was added and the pH value was adjusted to 7.0 at room temperature. The solution was filtered and dried in vacuum to obtain a yellow precipitate, which was collected and washed with water and small amounts of ethanol. The crude product dissolved in ethanol was purified by filtration on silicagel column (60–100 mesh). ESI-MS (in MeOH, m/z): 342.1 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO) δ 9.03 – 9.02 (m, 1H), 9.00 (dd, *J* = 4.3, 1.7 Hz, 2H), 8.84 (dd, *J* = 8.1, 1.7 Hz, 2H), 8.64 (d, *J* = 8.0 Hz, 1H), 8.27 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.83 (t, *J* = 8.0 Hz, 1H), 7.78 (dd, *J* = 8.1, 4.3 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 150.38 (s), 145.70 (s), 134.14 (s), 132.63 (s), 131.70 (s), 125.58 (s), 125.29 (s), 122.30 (s).

4.3.2 Synthesis of compound **2**.

2 was prepared using the method described above, but with 3-trifluoromethylbenzaldehyde (391.5 mg, 2.25 mmol). ESI-MS (in MeOH, m/z): 365.5 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO) δ 9.00 (dd, *J* = 4.3, 1.7 Hz, 1H), 8.84 (dd, *J* = 8.1, 1.6 Hz, 1H), 8.55 (s, 1H), 8.53 (d, *J* = 7.3 Hz, 1H), 7.85 – 7.81 (m, 1H), 7.78 (dd, *J* = 8.1, 4.3 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 150.88 (s), 149.99 (s), 145.75 (s), 133.03 (s), 132.29 (s), 131.98 (s), 131.64 (s), 125.30 (s).

4.3.3 Synthesis of compound **3**.

3 was prepared using the method described above, but with 3-chlorobenzaldehyde (315 mg, 2.25 mmol). ESI-MS (in MeOH, m/z): 331.3 $[M+H]^+$. ^{1}H NMR (500 MHz, MeOD:$CDCl_3$ = 1:1) δ 8.95 – 8.89 (m, 2H), 8.79 (s, 1H), 7.99 (t, *J* = 9.4 Hz, 2H), 7.62 (s, 2H), 7.57 (s, 1H), 7.29 (t, *J* = 8.7 Hz, 2H). ^{13}C

NMR (126 MHz, MeOD:$CDCl_3$ = 1:1) δ 149.06 (s), 144.91 (s), 141.69 (s), 131.63 – 131.46 (m), 131.08 (s), 127.96 (s), 124.76 (s).

4.3.4 Synthesis of compound **4**.

4 was prepared using the method described above, but with 3-Hydroxybenzaldehyde (274.59 mg, 2.25 mmol). ESI-MS (in MeOH, m/z): 313.1 $[M+H]^+$. ^1H NMR (500 MHz, DMSO) δ 9.02 (dd, *J* = 4.3, 1.7 Hz, 2H), 8.91 (dd, *J* = 8.1, 1.7 Hz, 2H), 7.81 (dd, *J* = 8.1, 4.3 Hz, 2H), 7.73 (dd, *J* = 6.0, 3.8 Hz, 1H), 7.72 – 7.69 (m, 1H), 7.39 (t, *J* = 7.9 Hz, 1H), 6.91 (ddd, *J* = 8.1, 2.4, 0.8 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 152.95 (s), 149.75 (s), 145.55 (s), 133.43 (s), 132.07 (s), 131.68 (s), 125.30 (s), 119.07 (s), 118.72 (s), 115.16 (s).

4.4 Cell Lines, Cell Culture and MTT Assay

Human cancer cell lines, human lung adenocarcinoma A549 cells, human hepatocarcinoma SMMC7721 cells, and human colorectal carcinoma SW620 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with fetal bovine serum (10%), penicillin (100 units/mL), and streptomycin (50 units/mL) at 37 °C in a CO_2 incubator (95% relative humidity, 5% CO_2).

Cell viability was confirmed by measuring the ability of cells to transform 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan dye [25]. Cells were seeded in 96-well tissue culture plates (3×10^3 cells per well) for 24 h. The cells were then incubated with the tested compounds at different concentrations for 72h. After incubation, 20 μL per well of MTT solution (5 mg/mL in phosphate buffere[d saline, PBS) was added, followed by incubation for a further 5 h. The medium was aspirated and replaced with 150 μL/well of DMSO to dissolve the formazan salt formed. The colour intensity, which reflects the cell growth condition, was measured at 570 nm using a microplate spectrophotometer (SpectroAmaxTM250, BioTek Instruments, Inc., Winooski, VT, USA)

4.5 Flow Cytometric Analysis

The apoptosis rate was analysed by flow cytometry. Treated or untreated cells were trypsinised, washed with PBS, and then fixed in 75% ethanol overnight at -20 °C. Next, the fixed cells were washed with PBS and stained with propidium iodide (PI) for 4 h in the dark. Finally, the above-described cells were analyzed with an Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA).

4.6Cellular localization

Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2. Cells in complete growth medium at 2×10^6 cells per mL were incubated for 6h at 37°C, unless otherwise stated. Cells were washed with PBS and then treated with **1** in DMSO/PBS (pH 7.2, 1 : 99, v/v) for 2 h at 37 °C under 5% CO_2. Then, cells were stained with Mito-tracker and Hoechst 33258 for another 20 min, and finally, luminescence imaging was carried out by confocal microscopy.

4.7 Electronic absorption measurements

Electronic absorption spectra were recorded on a Shimadzu UV-2550 spectrophotometer using 1 cm path length quartz cuvettes (3 mL). The absorption titration of the target complexe in Tris-HCl buffer was performed by using a fixed complex concentration to which increments of the DNA stock solution were added. The concentration of the complex solution was 20 μM and *bcl-2* G4 DNA

was added by degrees. Complex-DNA solutions were allowed to incubate for 3 min before the absorption spectra were recorded.

4.8 Fluorescence quenching measurements

Fluorescence spectroscopy measurements were performed on a RF-5301 fluorescence spectrophotometer using a 1 cm path length quartz cell. Fluorescence quenching of the EB + *bcl-2* G4 DNA system can be used for a compound having an affinity to DNA in spite of its binding mode, and only measures the ability of the compound to affect the EB fluorescence intensities in the EB + *bcl-2* G4 DNA system. The titration processes were repeated until there was no apparent change in the spectra for at least three titrations, indicating the achievement of the binding saturation [26].

4.9 CD spectra measurements

CD spectra were recorded on a Jasco J810 circular dichroism (CD) spectrophotometer with a thermoelectrically controlled cell holder. The cell path length was 1 cm. CD spectra were recorded in the range of 230-600 nm in 0.5 nm increments with an averaging time of 0.5 s [27].

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Author Contributions: Li Li contributes to electronic spectra titration, emission spectra, CD spectra titration and writes the paper. Yu-Mei Li and Yong-Chang Zeng contributes to the synthesis of the target complexes; Cheng-Xi Wang, Qiong-Wu and Zhi-Ping Zeng contribute to the cells experiments. Fen-Yong Sun and Wen-Jie Mei contributed to designed the experiments and read the manuscript.

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