Indian Journal of Chemistry
Vol. 62, September 2023, pp. 950-955
DOI: 10.56042/ijc.v62i9.5359

Synthesis, characterization and *in vitro* antitumor activities of a biimidazole-chelated Ir(III) complex

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Received 05 February 2022; accepted(revised) 17 July 2023

In this paper, a new cyclometalated iridium(III) complex [Ir(ppy)2(mbiim)]Cl (Ir3) (in which mbiim = 1-Methyl-2,2'-biimidazole, ppy = 2-phenylpyridine) has been successfully synthesized and characterized by ESI-MS, UV-Vis spectroscopy and ^{1}H NMR. The fluorescence properties of Ir3 have been studied in different *p*H value conditions. The fluorescence properties show that the fluorescence intensity of Ir3 decreases with the increase of *p*H in the experiment range, which is due to the protonation/deprotonation of active hydrogen of the imidazole ring on the imidazole ligand. Moreover, the photodynamic antitumor activities *in vitro* of three Ir(III) complexes have been investigated by MTT assay against four cell lines (HeLa, A549, A549R and LO2). These complexes exhibit low dark toxicity and high phototoxicity in three tumor cells. To our excitement, Ir3 has achieved satisfactory photodynamic therapy effects on cisplatin-resistant tumor cell lines A549R, while its toxicity towards normal cells is relatively lower. The results indicate that Ir3 is a relatively ideal photosensitizer, which may be developed into a new potential photodynamic therapy reagent against tumor.

Keywords: Cyclometalated iridium(III) complex, 1-Methyl-2,2'-biimidazole, PDT, Photosensitizer

The imidazole has specific proton acceptance and donating properties, and the two imidazole rings of 2,2'-biimidazole are connected together by carbon-carbon bond, thus the imidazole also has strong proton acceptance and donating properties. Biimidazole is a multi-proton donating ligand and it can exist with transition metals in various states such as neutral H_2biim molecules, dehydrogenated Hbiim- and $Biim^{2-}$ ions. Moreover, the ligand Hbiim- can interact with metal cation through intermolecular hydrogen bond to form molecular self-assembly structure in multi-dimensional direction. So its coordination mode is very rich, which leads to a variety of complexes with imidazoles monodentate coordination, bidentate coordination and bridge base coordination and so on.

Derivatives of biimidazole can be made into drugs. For example, its nitro derivatives can be used as antibacterial drugs. Therefore, biimidazole is often used as raw materials and important intermediates in organic synthesis. The research of cyclometalated iridium (III) complexes has been started for a long time, but studies related to its functional properties in the past are very rare. With the continuous development of scientific research, it was found that cyclometalated iridium (III) complexes have excellent photophysical properties, such as high phosphorescent quantum yield, convenient fluorescent color tunable ability and relatively long lifetime, which make them a hot research topic in biological application and light-emitting electrochemical cells (LECs).

Photodynamic therapy (PDT) is a new method of using photosensitive drugs and laser activation to treat tumor diseases. It is a non-invasive cancer treatment method. Studies have shown that cyclometalated iridium(III) complexes can be developed into anticancer drugs, and some of them can be used as photodynamic therapy agents because they can produce fluorescence and singlet oxygen under light[1]. In recent years, there are abundant literature reports related to cyclometalated iridium(III) complexes acting as an effective sensitizer of photodynamic therapy reagent (PDT)[2-12]. Cyclometalated iridium(III) complexes gradually aroused people's great interest in the studies acting as PDT reagent due to their excellent photophysical properties. Alkyl groups are usually employed to regulate the lipophilicity and other properties of the photosensitizer and further

heighten the performance of the complexes in terms of photophysics or PDT[13].

In our previous work, we have developed a series of cyclometalated IrIII complexes based on 2,2'-biimidazole with different alkyl substitutions which exibited prominently high photoinduced cytotoxic activity[12]. As a continuous part of our works in this field, we have designed and obtained a monomethyl-substituted derivative of 2,2′-biimidazole, 1-Methyl-2,2'-biim- idazole, in which only one methyl group replaces the hydrogen on just one nitrogen atom. Then, cyclometalated iridium(III) complexes with 1-Methyl-2,2'- biimidazole as the main ligand was prepared as a potential PDT reagent. And its (photo)cytotoxicity was studied by MTT assay. Meanwhile, the initial unsubstituted biimidazole complex $[Ir(ppy)_2(biim)]Cl$ (Ir1) and dimethyl substituted biimidazole complex $[Ir(ppy)_2(dmbiim)]Cl$ (Ir2) (in which biim = 2,2'-biimidazole, dmbiim = 2,2'-dimethyl- 2,2'- biimidazole) were employed for comparison. The results indicate that the complex Ir3 is an ideal photosensitizer and a potential PDT reagent, which lays a foundation for the follow-up research and providing valuable experience.

Experimental Section

Materials and Instruments

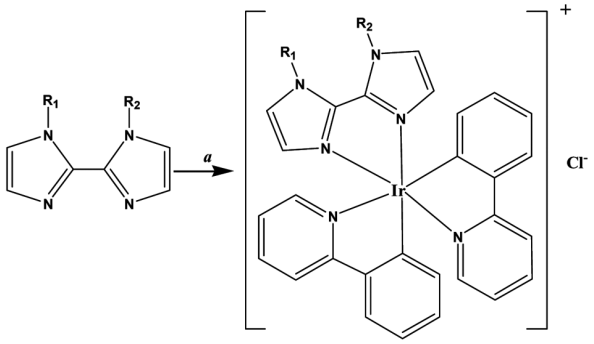
The solvents and inorganic salts used in the study (unless otherwise specified) were purchased commercially and used without further purification. All buffers were of biological grade and were used directly. 1-Methyl-2,2'-biimidazole was synthesized according to the methods reported in the literature[14] with a slight adjustment in molar ratio of biimidazole and CH_3I to 1:1.2. $[(ppy)_2Ir(\mu\text{-}Cl)]_2$ was synthesized and purified according to the literature method[15].

Physical Measurements

UV-Vis spectra were recorded on a Perkin-Elmer Lambda 850 spectrophotometer. Electrospray ionization mass spectra (ESI-MS) were recorded by LCQ system (Finnigan MAT, USA). ^{1}H NMR spectra were performed with a Mercury Plus 300 Nuclear Magnetic Resonance Spectrometer (AVANCE III 400 MHz) at 278 K. Fluorescence spectra were performed with a Perkin-Elmer LS 55 Luminescence Spectrometer at 298 K.

Synthesis of $[Ir(ppy)_2(mbiim)]Cl$

The synthesis of the complexes Ir1-Ir3 is shown as Scheme I.



Ir1: R_1=R_2=H; **Ir2:** R_1=R_2=CH_3; **Ir3:** R_1=H, R_2=CH_3

(*a*) $[(ppy)_2Ir(\mu\text{-}Cl)]_2$, 150°C, 12 h, glycol, N_2

Scheme I — Synthesis of the complexes

Under N_2 protection, $[(ppy)_2Ir(\mu\text{-}Cl)]_2$ (0.2 mmol) and mbiim (0.5 mmol) were mixed in 15 ml glycol, then the mixture was heated to 150°C and refluxed for 12 h under the constant temperature. After the reaction, the solution was cooled to room temperature, and appropriate amount of water was added to it, and dichloromethane was used for extraction twice, and then the extract was washed twice by water. Then the organic phase was separated, the solvent was dried by rotary evaporator, and the solid product was placed in a vacuum drying oven at 50°C for 24 hours. The desired pure product $Ir(ppy)_2(mbiim)Cl$ (Ir3) was obtained by column chromatography on silica gel and recrystallization from the mixed solvent CH_2Cl_2/CH_3OH(1:10), with a yield of 43.7%. The complex was characterized by mass spectrometry, ^{1}H NMR and UV-Vis. ESI-MS (m/z) (Figure 1): 649.0 [$\{M\text{-}Cl\}^+$] (calcd 649.2). 1H NMR (400 MHz, CD_3CN) (Figure 2): δ 7.98 – 7.90 (m, 3H), 7.78 – 7.63 (m, 5H), 7.77 – 7.63 (m, 2H), 7.03 (dd, J = 13.0, 5.9 Hz, 2H), 6.97 (s, 1H), 6.94 – 6.86 (m, 3H), 6.79 (t, J = 7.8 Hz, 2H), 6.40 (d, J = 7.5 Hz, 1H), 6.35 (d, J = 7.3 Hz, 1H), 6.29 (s, 1H), 6.20 (s, 1H), 4.12 (s, 3H). The results indicate that the title complex was in good agreement with the expected structure. The structure of the title complex $Ir(ppy)_2(mbiim)Cl$ is shown as Scheme I.

LogPo/w Measurement

The lipophilicity (LogPo/w) of the complexes is experimentally determined by the "shake-flask" method. The specific experimental procedures were carried out in strict accordance with the methods described in the literature[15].

Cell Fluorescence Imaging Experiment

To preliminarily study the entry and distribution of complex Ir3 in cells, confocal luminescence imaging

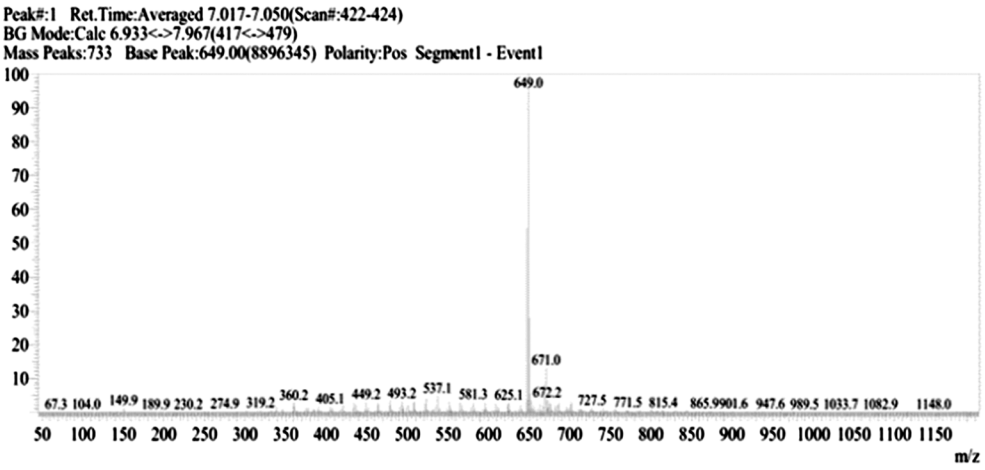


Figure 1 — Mass spectrometry of Ir3

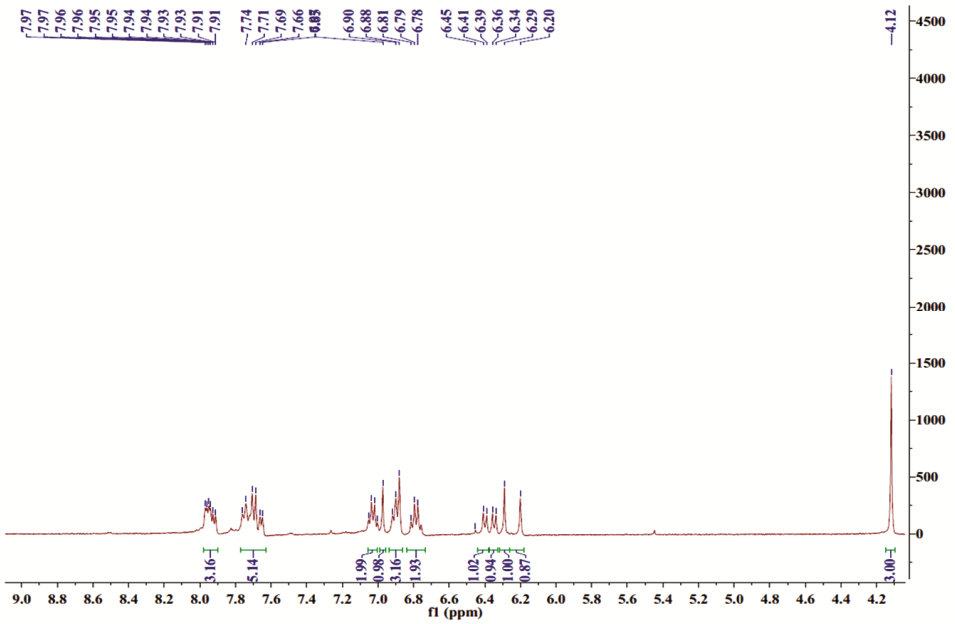


Figure 2 — ^{1}H NMR spectra of Ir3

was performed. HeLa cells were seeded in 3.5 cm dishes at a density of 1×104 cells per mL and were incubated for 24 h at 37°C under 5% CO_2. Then, the cells were treated with 5 μM Ir3 in DMSO/PBS (*p*H = 7.4, 1:99, v/v) for 2 h at 37°C. After treatment, the cells were washed twice with a PBS solution and then were used for confocal luminescence imaging.

(Photo-)Cytotoxicity Assays

The *in vitro* (photo-)cytotoxicity of the Ir(III) complexes was evaluated by the MTT assay. The HeLa cells were harvested and seeded into 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h. Then the culture medium was removed, and the cells were incubated with different concentrations (40 μM, 20 μM, 10 μM, 5 μM, 2.5 μM) of the Ir(III) complexes or cisplatin for 2 h at 37°C under 5% CO_2 in the dark. In the end, the culture medium was replaced quickly and further incubated under the same conditions for another 42 h. For the photocytotoxicity experiment, after 2 h incubation of the cells treated with the title complex or cisplatin in the dark, the culture medium was renewed with fresh medium. An LED area light source (20 mW cm^{-2}) was used to irradiate the treated cells for 5 min. Then the cells were allowed to incubate for another 42 h in the dark. Finally, 10 μL

of MTT solution (5 μM) in PBS was added to each well, followed by incubation for another 4 h at 37°C. The absorbance value was then measured at a wavelength of 595 nm on a microplate spectrophotometer. Meanwhile, the widely used clinical chemotherapeutic drug cisplatin is employed as a positive contrast. Three independent tests were set up in all the experiments, and all data are presented as the mean ± standard deviation (SD).

Results and Discussion

UV-Vis Spectrum of Ir3

The ultraviolet-visible spectrum of compound Ir3 is shown in Figure 3. The strong absorption bands in the range of 250-350 nm are assigned to spin-allowed intra ligand electron transitions (π-π^*), and while above 350 nm relatively weak absorption that extend to the visible light region are attributed to $^3\pi$-π^*, ^{1}MLCT (metal-to-ligand charge transfer), ^{1}LLCT, ^{3}MLCT, ^{3}LLCT and LC (ligand to center) electron transitions[2].

Fluorescence Spectra of Ir3

Studies have shown that Ir3 with active hydrogen will disturb the electronic properties of the compounds in the process of protonation/ deprotonation, resulting in the *p*H-sensitive fluorescence phosphorescence properties of the compounds[16]. As can be seen from Figure 4, *p*H-sensitive phosphorescence was observed in Britton Robinson (BR) buffer solution of the complex Ir3, which was due to the protonation/deprotonation of active hydrogen of the imidazole ring on the imidazole ligand. In the *p*H titration experiment of BR buffer, the fluorescence of the complex Ir3 decreases with the increase of *p*H in the range of 5-9, which is similar to that has been reported in the literature[17]. Under strong base conditions (*p*H=9.0), the fluorescence of the complex Ir3 becomes very weak and even "turned-off". With the increase of *p*H value, the solubility in water and the fluorescence intensity of complex Ir3 decreases, indicating that the complex has aggregation-caused quenching (ACQ) characteristics.

Octanol/Water Partition Coefficients

Octanol/water partition coefficients (LogPo/w) was used to assess the lipophilicity/ hydrophilicity of the complexes. The results show that their LogPo/w values are arranged in the following order[12]: Ir1(1.60)>Ir3 (1.15)>Ir2 (0.60). The LogPo/w values of Ir1-Ir3 are positive, indicating that these complexes have strong lipophilic properties. The reason why such a result occurs may be related to the active hydrogen that can be dissociated from the coordination unit. The more active hydrogen, the higher the probability of dissociating hydrogen ions, and higher the probability of obtaining neutral coordination units, the lower the water solubility, so their oil-water distribution coefficient is as shown above.

Distribution of Ir3 in Cells

To preliminarily explore the uptake and distribution of the complex Ir3 in cells, confocal luminescence imaging was carried out. As can be seen

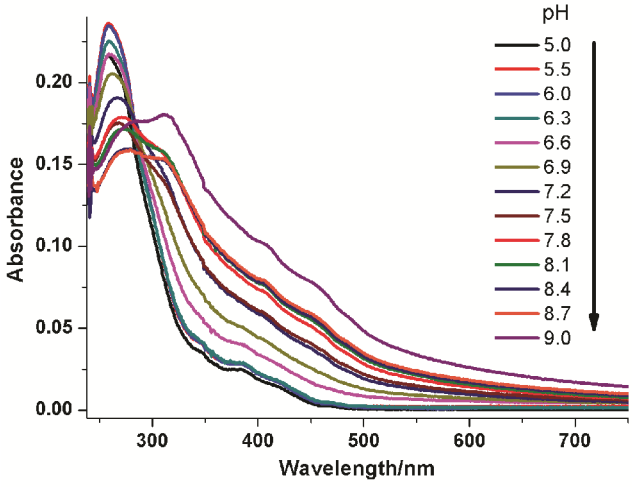


Figure 3 — UV-Vis spectra of Ir3 (5μM) measured at different *p*H value at 298 K

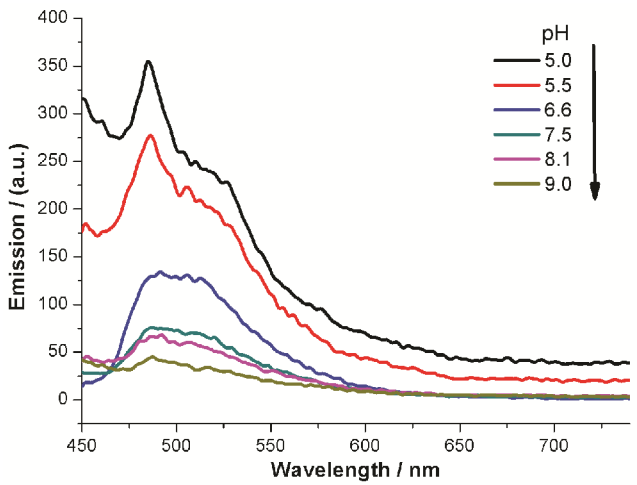


Figure 4 — Fluorescence emission spectra of Ir3 (5 μM) measured at different *p*H values

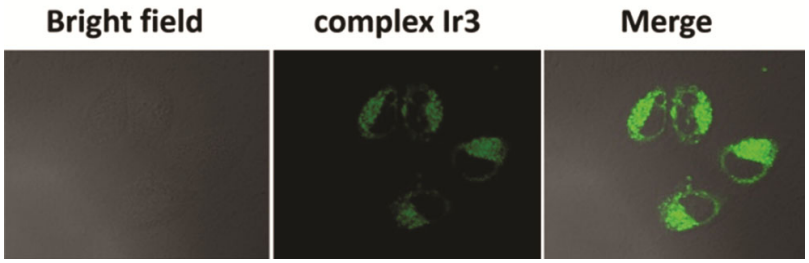


Figure 5 — Cell uptake and distribution of Ir3 in Hela cells.

Table I — Cytotoxicity [IC_{50}[a]/μM] of the compounds in the absence and presence of 405 nm light towards different cell line

Complexes	Hela	PI[c]	A549	PI	A549R	PI	LO2	PI
	Dark[a] (light[b])		Dark(light)		Dark(light)		Dark(light)	
$Ir(ppy)_2(biim)Cl$（Ir1）	16.5± 0.5 (0.45± 0.02)	36.7	28.5 ± 1.2 (6.1 ± 0.3)	4.7	＞40 (1.8 ± 0.04)	n.a.[d]	25.3 ± 1.1 (6.4 ± 0.3)	4.0
$Ir(ppy)_2(dmbiim)Cl$（Ir2）	＞40 (0.85 ± 0.03)	n.a.[d]	29.6 ± 1.3 (5.8 ± 0.2)	5.1	＞40 (1.5 ± 0.03)	n.a.[d]	26.5 ± 1.2 (5.3 ± 0.4)	5.0
$Ir(ppy)_2(mbiim)Cl$（Ir3）	35.89±1.1 (0.50±0.03)	71.8	29.0 (4.5 ± 0.2)	6.4	＞40 (1.7 ± 0.05)	n.a.[d]	36.5 ± 1.3 (8.3 ± 0.5)	4.4
cisplatin	18.5±0.09 (18.1±0.08)	1.0	20.3 ± 0.8 (19.5± 0.9)	1.0	114.1 ± 5.3 (113.8 ± 5.5)	1.0	35.6 ± 2.8 (29.8 ± 1.9)	1.2

from Figure 5, the complex Ir3 can quickly enter the cell and distribute in the cytoplasmic region, presenting bright green fluorescence, which lays a foundation for further research on the mechanism.

(Photo-)Cytotoxicity of the Complexes

The three Ir(III) complexes were used to evaluate whether they could cause (photo-)cytotoxicity to four cell lines (HeLa, A549, A549R, LO2) under two different situations, light avoidance or light radiation at 405 nm. Cisplatin was used as the positive control. The IC_{50} and PI value of Ir1-Ir3 toward various cells are summarized in Table I. As can be seen from the Table, the complexes in the cancer cells exhibit low dark toxicity and high light toxicity, especially complex Ir3, displaying excellent photodynamic therapy effect towards HeLa cell lines with PI=71.8. To our excitement, the title complex Ir3 has achieved satisfactory photodynamic therapy effects on cisplatin-resistant tumor cell lines (A549R), while its toxicity to normal cells is relatively lower. The results indicate that Ir3 is an ideal photosensitizer, which may be developed into a new potential photodynamic therapy reagent for tumor.

[a]The cells were incubated in the dark with the specified complex for 2 hours, then the medium was dissolved again and incubated in the dark or exposed to 405 nm light for 5 minutes. The total incubation time was 48 hours. PI = phototoxicity index; PI is the ratio of the dark IC_{50} value to the light IC_{50} value. [d]Not applicable.

Conclusion

In summary, a novel cyclometalated Ir(III) complex has been designed and prepared. The complex was characterized by mass spectrometry, ^{1}H NMR spectra and UV-Vis spectra. The results showed that the title complex Ir3 exhibited low dark toxicity and high phototoxicity in HeLa cells. It indicates that the complex Ir3 is a relatively ideal photosensitizer, which may be used as a new photodynamic therapy reagent against tumor.

Acknowledgment

The authors would like to sincerely thank the Doctoral Scientific Research Initiation Project of Hanshan Normal University (QD20181120), the Research Platform of Hanshan Normal University (419004), the Guangdong Basic and Applied Basic Research Foundation (2019A1515011974), the Special Projects in Key Fields from Department of Education of Guangdong Province (2021ZDZX2065), Guangdong Provincial Key Laboratory of Functional Substances in Medicinal Edible Resources and Healthcare Products (2021B1212040015), Chaozhou Science and Technology Project (2019ZC08), Characteristic Innovation Project of Universities in Guangdong Province (2019KTSCX100).

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