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Folate-targeted iridium complexes amplify photodynamic therapy efficacy through ferroptosis

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Materials and general measurements

The reagents used in this experiment were obtained commercially and do not require further purification for use. The sources of these reagents are as follows:
Amino-PEG-folic acid (Mw=2000) was purchased from Bide Pharmaceutical Technology Co., Ltd (Shanghai, China), Triethylamine, p-toluenesulfonyl chloride, Iridium trichloride, 2-ethoxy-ether,1,10-phenanthroline 5,6-dione were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. 1-bromo-3-isothiocyanopropane was purchased from Xi'An Rhea-Chem Technology Co., Ltd. 3-(4,5-dimethyl-2-thiazole)-2,5-diphenyl tetraazolthiazolium bromide blue (MTT), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), 9,10-anthracene dimethyl-bis(methylene) dimalonic acid (ABDA), 5,5'-dithiobide (2-nitrobenzoic acid) (DTNB), Calcein AM Cell Viability Assay Kit, Lyso-Tracker Green, and Mito-Tracker Green, GSH and GSSG Assay Kit were purchased from Biyuntian Biotechnology (Shanghai, China). C11 BODIPY581/591 were purchased MedChemExpress.

The following experiments were conducted to obtain analytical data for the research:

^{1}HNMR spectra were performed on a nuclear magnetic resonance spectrometer (Bruker, 600MHz). The absorption spectra were recorded using a Shimadzu UV2700 UV/Vis spectrometer. The emission spectra were recorded using a Shimadzu RF6000 luminescence spectrometer. TEM images were recorded using a 120 kV transmission electron microscopy (Thermo Fisher Scientific, Talos F200X, American). Confocal image was obtained on Olympus FV3000. Ex vivo images were recorded on an IVIS Lumina XRMS Series III imaging system (PerkinElmer). Flow cytometry experiments were obtained on FACS Calibur (BD).

Synthesis of 6-phenylpyridine-3-yl-4-methylbenzene sulfonate

The experiment was conducted in a nitrogen atmosphere. Firstly, 1.8 g of 6-phenyl pyridine-3-alcohol was added to a Schlenk bottle. Subsequently, 50 mL of dichloromethane was added, and the solution was stirred for 0.5 h at 0 °C. 8 mL of triethylamine was added and stirred for an additional 0.5 h. 3.3 g of p-toluenesulfonyl chloride was dissolved in 10 mL of dichloromethane and gradually added to the reaction mixture using a syringe. One milliliter of pyridine was also added, and the reaction was allowed to continue for 1 h at 0 °C before being transferred to room temperature and left overnight. The reaction mixture was washed with saturated sodium bicarbonate solution, and the organic phase was extracted with methylene chloride. The resulting solution was dried with anhydrous magnesium sulfate, and the crude product was purified using column chromatography with a mixture of petroleum ether and ethyl acetate in a 10:1 ratio. Finally, a white solid was obtained. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.11 (d, *J* = 2.7 Hz, 1H), 7.85 (dd, *J* = 5.3, 3.4 Hz, 2H), 7.67 (d, *J* = 8.3 Hz, 2H), 7.63 (d, *J* = 8.7 Hz, 1H), 7.45 (dd, *J* = 8.7, 2.7 Hz, 1H), 7.40 – 7.37 (m, 2H), 7.34 (ddd, *J* = 7.3, 3.6, 1.2 Hz, 1H), 7.26 (d, *J* = 8.1 Hz, 2H), 2.38 (s, 3H).

Synthesis of Dimer

1.47 g 6-phenylpy ridine-3-yl-4-methylbenzene sulfonate and 0.65 g iridium trichloride hydrate were added to the Solanum-shaped reaction bottle, and 30 mL 2-ethoxy-ethyl ether was added. The mixture reacted overnight at 120 °C. After the reaction, cool to room temperature. The crude product is washed with deionized water and ethanol (30 mL), and the next reaction can be carried out without purification. ^{1}H NMR (600 MHz, CDCl3) δ 8.35 (d, J = 2.5 Hz, 4H), 7.82 (d, J = 9.0 Hz, 5H), 7.70 (dd, J = 8.9, 2.5 Hz, 6H), 7.53 (d, J = 8.2 Hz, 10H), 7.47 (d, J = 7.6 Hz, 5H), 6.96 (d, J = 8.1 Hz, 11H), 6.85 (t, J = 7.4 Hz, 5H), 6.61 (t, J = 7.5 Hz, 5H), 5.61 (d, J = 7.9 Hz, 5H), 2.10 (s, 12H).

Synthesis of ligand:

0.36 g of carbazoles and 0.18 g of 1,10-phenanthroline-5,6-dione were added to a Schlenk bottle. Following this, 30 mL of acetic acid was added and the reaction was conducted at 100 °C under a nitrogen atmosphere for a period of 4 h. After the reaction, the mixture was cooled to ambient temperature and transferred into 100 mL ice water. The pH of the resultant mixture was adjusted to 7 using dilute ammonia water. The solid product was subsequently collected via filtration and dried under vacuum and can be utilized for further reactions.

Synthesis of Ir-Ots:

In a nitrogen atmosphere, a reaction mixture was prepared by adding 70 mg of dimer and 48 mg of ligand into a reaction vessel, followed by the addition of 20 mL of methanol. The reaction was carried out at 50 °C for a duration of 12 h. The resulting solution was subjected to vacuum concentration to remove the solvent, followed by purification using column chromatography with dichloromethane: methanol mixture of 50:1. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.09 (s, 3H), 8.14 (d, *J* = 1.3 Hz, 2H), 7.82 (s, 8H), 7.67 (s, 3H), 7.49 (dt, *J* = 8.7, 5.2 Hz, 6H), 7.15 (dd, *J* = 21.5, 14.1 Hz, 8H), 7.05 (t, *J* = 7.5 Hz, 3H), 6.30 (s, 3H), 5.30 (s, 2H), 3.69 – 3.61 (m, 1H), 2.39 (s, 6H), 1.48 (s, 18H).

Synthesis of Ir-OH:

76.5 mg of Ir-Ots and 6-fold equivalent sodium hydroxide were introduced into a round-bottomed flask containing 10:1 methanol and dichloromethane as solvents. The mixture was stirred at room temperature for a duration of 4 h. Upon completion of the reaction, 10 mL of water was added to the reaction mixture, followed by pH adjustment to 6 using 1 N HCl. This led to the formation of solid precipitates. To obtain the desired product, organic solvents were removed through vacuum concentration, and the solid precipitates were filtered, and washed with water and ethyl acetate (10 mL). ^{1}H NMR (600 MHz, DMSO) δ 10.27 (s, 2H), 9.21 (s, 2H), 8.59 (d, *J* = 7.2 Hz, 2H), 8.33 (s, 2H), 8.18

– 8.01 (m, 6H), 7.89 (s, 2H), 7.77 (dd, *J* = 13.5, 7.9 Hz, 3H), 7.54 – 7.46 (m, 5H), 7.32 (d, *J* = 8.6 Hz, 1H), 7.11 (d, *J* = 7.6 Hz, 1H), 7.05 – 6.99 (m, 3H), 6.93 – 6.87 (m, 2H), 6.31 (d, *J* = 6.8 Hz, 1H), 3.70 (s, 1H), 1.44 (s, 18H).

Synthesis of Ir-NCS:

In a Schlenk flask with a nitrogen atmosphere, a mixture was prepared by adding 33 mg of Ir-OH, 10.93 mg of 1-bromo-3-isothiocyanopropane, and 12 mg of anhydrous potassium carbonate in 10 mL of N, N-dimethylformamide (DMF). The reaction mixture was heated to 80 °C and kept overnight. After the reaction, the mixture turned into an orange-yellow color. The solvent was removed using a rotary evaporator and the crude product was subjected to washing with ethyl acetate. ^{1}H NMR (600 MHz, DMSO) δ 9.08 (d, *J* = 7.4 Hz, 2H), 8.63 (d, *J* = 7.6 Hz, 2H), 8.31 (s, 2H), 8.20 (d, *J* = 8.9 Hz, 2H), 7.98 (s, 2H), 7.89 (s, 2H), 7.84 (d, *J* = 7.6 Hz, 2H), 7.66 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 8.8 Hz, 2H), 7.52 (d, *J* = 8.7 Hz, 2H), 7.44 (d, *J* = 8.6 Hz, 2H), 7.17 (s, 1H), 7.04 – 7.01 (m, 2H), 6.98 (s, 1H), 6.92 (d, *J* = 7.2 Hz, 2H), 6.34 (d, *J* = 6.9 Hz, 1H), 2.94 (s, 12H), 1.44 (s, 18H).

Synthesis of Ir-PEG-Fn

A DMSO solution containing 37 mg of Ir-NCS and 110 mg of NH_2-PEG_{2000}-Fn was subjected to stirring at room temperature for a duration of 12 h. The resulting reaction solution was subsequently introduced into a 50 mL water solution, and the removal of unreacted raw materials and DMSO was carried out using an ultrafiltration centrifuge tube via centrifugation.

Characterization of Ir-PEG-Fn

Characterization of Ir-PEG-Fn involved determining the morphology and particle size of Ir-PEG-Fn using transmission electron microscopy (TEM). Additionally, the absorption and emission spectra of Ir-PEG-Fn were determined using a UV spectrophotometer and Fluorescence spectrometer. Ir-PEG-Fn was dissolved in an aqueous solution for further analysis.

Test of Singlet oxygen

A 1 mM methanol solution of 9,10-Anthracenediyl-bis(methylene) di malonic Acid(ABDA) was prepared and then diluted to 40 uM using water. A 100 μM aqueous solution of Ir-PEG-Fn was also prepared. The two solutions were mixed in equal volumes in a colorimetric dish. The fluorescence intensity of ABDA was then measured by exposing the mixture to light(420 nm, 50 mW) for 10 S. The excitation wavelength of ABDA was 360 nm, and the emission wavelength was between 375-550 nm.

Glutathione oxidation experiment

Ir-PEG-Fn (10 μM) was mixed with GSH (200 uL) at room temperature. After passing the 420 nm light, 50 μL of this solution was added into 450 uL PBS, and then 2 μL 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (10 mg/mL) was

added. Then the absorbance spectra of the mixture were measured by UV-vis spectroscopy

The cells that are being examined in this study were conserved by the previous laboratory personnel. To grow these cells, they were cultured in an environment with 5% humidity and a carbon dioxide atmosphere, and the temperature was maintained at a constant 37°C. The cell lines that were specifically cultured for this study consisted of Hela cells, which were grown in DMEM medium that contained 10% fetal bovine serum, and A549 cells, which were cultured in Roswell Park Memorial Institute 1640 medium.

Cell uptake assay

The study involved culturing both Hela and A549 cells in confocal culture dishes at appropriate concentrations, followed by adding 10 μM of Ir-PEG-Fn for a co-incubation period of 8 h. Confocal Laser Scanning Microscopy (CLSM) was used to capture images of the drug using an excitation wavelength of 420 nm and an emission wavelength of 620 nm. The resulting images were then analyzed to gain insights into the behavior and distribution of the drug within the cells.

Colocalization experiment

Firstly, the cells were inoculated in confocal culture dishes and incubated for at least 24 h. Next, the cells were treated with Ir-PEG-Fn at a concentration of 10 μM for 8 h. After the treatment, the cells were washed with PBS and exposed to either Lyso-Tracker Green (LTG) or MitoTracker Green (MTG) at a concentration of 50 nM. The treated cells were then incubated for 30 min. Finally, CLSM co-location images were acquired after three washes with fresh PBS.

Mitochondrial membrane potential experiment

Hela cells were incubated in 6-well plates for a duration of 24 h and subsequently cultured in a medium comprising Ir-PEG-Fn under two different conditions: darkness and light. Following the supplier's guidelines, JC-1 dye was employed to stain cells, and the resulting experimental data were analyzed using a flow cytometer.

Monitoring of Ir-PEG-Fn uptake behavior in Hela cells

To investigate the cellular uptake behavior of Ir-PEG-Fn, HeLa cells were subjected to incubation at a temperature of 37 °C for a duration of 4 h in the presence of folic acid and entotic inhibitors, separately. Subsequently, following an additional incubation period of 8 hours with Ir-PEG-Fn, the intracellular fluorescence of Ir-PEG-Fn was observed and monitored using confocal microscopy.

Intracellular phototoxicity test

Hela cells were cultured in 96-well plates and incubated for 24 h. Subsequently, varying concentrations of Ir-PEG-Fn were added to the wells (80, 60, 40, 20, 10, and 5 μM) and the cells were exposed to 420 nm light (50 mW, 120 s) or kept in darkness for another 24 h. Then, MTT (5 mg/mL,.20 μL) was added to

each well and incubated for 4 h. The waste medium was then replaced with 100 μL of DMSO and the absorbance at 570 nm was measured using a microplate reader. The results are presented as mean standard deviation (n=3).

For Hela cells, the cell viability in the presence of different cell death inhibitors was also determined. Therefore Hela cells were respectively pre-incubated with z-VAD-fmk (50 μM) and ferrostatin (50 μM), and then co-incubated Ir-PEG-Fn(10 μM). After 24 h co-incubation, irradiation was imposed with a laser of 420 nm (120 s 50 mW/cm^2), and the cell viability was then determined.

Live-dead cell assay

Hela cells were seeded into six-well plates and allowed to culture for 24 h. Following this, the cells were subjected to various drug treatments, including 1) PBS, 2) Cisplatin, 3) Ir-PEG-Fn+Dark, and 4) Ir-PEG-Fn+Light. After incubation with the drugs for 24 h, the cells were stained using an AM/PI double staining kit, following the manufacturer's instructions. The stained cells were then digested using pancreatic enzyme without Ethylene Diamine Tetraacetic Acid (EDTA), washed twice with PBS after centrifugation, and finally re-suspended in 500 μL of PBS. The resulting cell suspension was visualized under a CLSM.

Determination of intracellular glutathione content

Hela cells were subjected to implantation in six-well plates that had been previously treated with Ir-PEG-Fn at concentrations of 5 and 10 μM. The implantation was carried out under both light and dark conditions for 24 h. Following this, the cells were subjected to thawing through continuous freezing using liquid N_2 for 30 S, and then to lysis at 37°C for 2 min. The process of lysis was continued until no significant cells were observed under the microscope. The supernatant was subsequently collected through centrifugation at 10000g and 4 °C for 10 min. The concentration of GSH was analyzed immediately using a GSH and GSSG Assay Kit..

Western Blot Analysis for Hela cells treated by Ir-PEG-Fn

Hela cells were co-incubated with Ir-PEG-Fn for a duration of 8 h. Subsequently, the 6-well plates were placed on ice following 24 h of incubation under dark and light conditions, respectively. The cells were washed thrice with pre-cooled PBS and then cleaved using 100 μL of lysate for 5 min. A cell scraper was employed to scrape the cells, which were then transferred to a 1.5 mL centrifuge tube. The obtained samples were subjected to ultrasonic treatment using a cell crusher. The ultrasonic process involved 3 S of treatment followed by an intermittent time of 10 S, repeated three times for each sample. The resulting supernatant was collected and centrifuged at 12000 rpm and 4 for 15 minutes. The protein concentration was determined using a BCA protein kit. Equal amounts of sample protein were mixed with 5 SDS-PAGE loading buffers, heated at 100 °C for 10 min, and loaded onto an SDS-PAGE spacer gel. Electrophoresis was conducted at 120 V for 1 hour, followed by the transfer of the separated proteins to a PVDF membrane at 100 V for 1 h. The membrane was subsequently blocked with blocking buffer (5% skim dry milk) at room temperature for a period of 7 h. The primary antibody was diluted in TBST containing 2% buttermilk and incubated overnight at 4 °C. Following primary antibody incubation, the membrane was washed with TBST for 15 min, repeated three times, and subsequently treated with the corresponding enzyme-labeled secondary antibody. The secondary antibody was also diluted in TBST containing 2% skim milk powder and incubated at room temperature

for 4 h. Following secondary antibody treatment, the membrane was washed with TBST for 15 min, repeated three times, and visualized using the Omega Lum C imaging system (Aplegen, USA).

Evaluation of intracellular reactive oxygen species(ROS) and lipid peroxidation(LPO) levels

The research investigated the capacity of Ir-PEG-Fn to induce the production of ROS in cells using CLSM. Hela cells were cultured in confocal dishes for 24 h, followed by the addition of Ir-PEG-Fn and incubation for 8 h. The medium was then replaced with fresh medium, and the cells were further incubated with 20 μM DCFH-DA for 30 min. The cell's CLSM images were obtained, and luminescence intensity was analyzed using Image J. (*Ex*=488 nm, *Em*=515-535 nm)

LPO was examined using C11-BODIPY, a fluorophore that produces green fluorescence upon oxidation by intracellular LPO. Hela cells were treated similarly to the previous experiment, and C11-BODIPY was added to the cells for 30 min following incubation with Ir-PEG-Fn. After replacing the medium with fresh DMEM, the cells were exposed to light (420 nm, 50 mW, 120 s), and imaging was performed under confocal microscopy with excitation and emission wavelengths of 488 nm and 520-540 nm, respectively.

Animal model

The animal experiments conducted in this study utilized female BALB/c-Nc mice, which were between 6-8 weeks of age and weighed between 19-25 g. All animal experiments adhered to the principles outlined in the Guidelines for the Care and Use of Laboratory Animals and efforts were made to minimize any pain or discomfort experienced by the mice. Before tumor heterotransplantation, the mice were acclimatized for 7 d upon arrival in the laboratory. Tumor models were established by collecting and re-suspending Hela cells in PBS and subsequently inoculating the tumors subcutaneously in the right axilla, except in the light group.

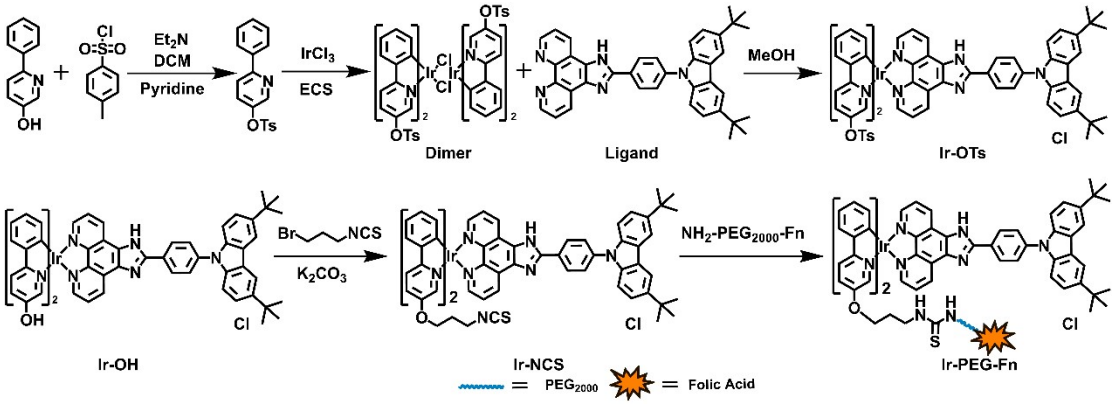
To perform in vitro imaging, mice bearing Hela and a549 tumors were administered Ir-PEG-Fn intravenously at a dose of 5mg/kg. At 4, 6, 8, and 12 h post-administration, the mice were sacrificed and their tumors and major organs (heart, liver, spleen, lung, and kidney) were removed for imaging examination. In vitro, fluorescence images were obtained using the IVIS Lumina XRMS Series III imaging system.

For in vivo tumor treatment, tumor-bearing mice were divided into 4 groups, each consisting of 3 mice. The drug was administered through the caudal vein on day 1 and day 3, and light was given 8 hours after administration. Group 1 received an intravenous saline injection, Group 2 was injected with cisplatin, Group 3 received an intravenous dose of Ir-PEG-Fn (5mg/kg), and Group 4 was injected with Ir-PEG-Fn (5mg/kg) followed by exposure to a 420 nm light (50 mW, 300 s) 8 hours later. Tumor volume and mouse body weight were recorded every 3 days, and the experiment ended on day 24, at which point the tumors were removed and weighed. Tumor volume was calculated using the formula $v = ab^2 \times 0.52$, where a represented the longest tumor diameter and b represented the shortest tumor diameter. Mean tumor weight results were expressed as mean ± standard deviation.

Tumor tissues analysis

The experiment involved dividing the mice into four groups, each receiving a different treatment. One group was injected with normal saline, the second group received Cisplatin (5 mg/kg), the third group received Ir-PEG-Fn (5 mg/kg), and the fourth group received Ir-PEG-Fn (5 mg/kg) in addition to light exposure. The light exposure was provided by a 420 nm laser with a power of 50 mW for 300 seconds.

On day 24, all mice were sacrificed, and their tumors were collected for staining. The staining methods used were hematoxylin and eosin (H&E), and immunofluorescence staining with TUNEL and Ki67. H&E staining is a standard method used to visualize tissue structure, while immunofluorescence staining with TUNEL and Ki67 is used to detect cell death and proliferation, respectively.



Scheme S1. Synthesis route of Ir-PEG-Fn

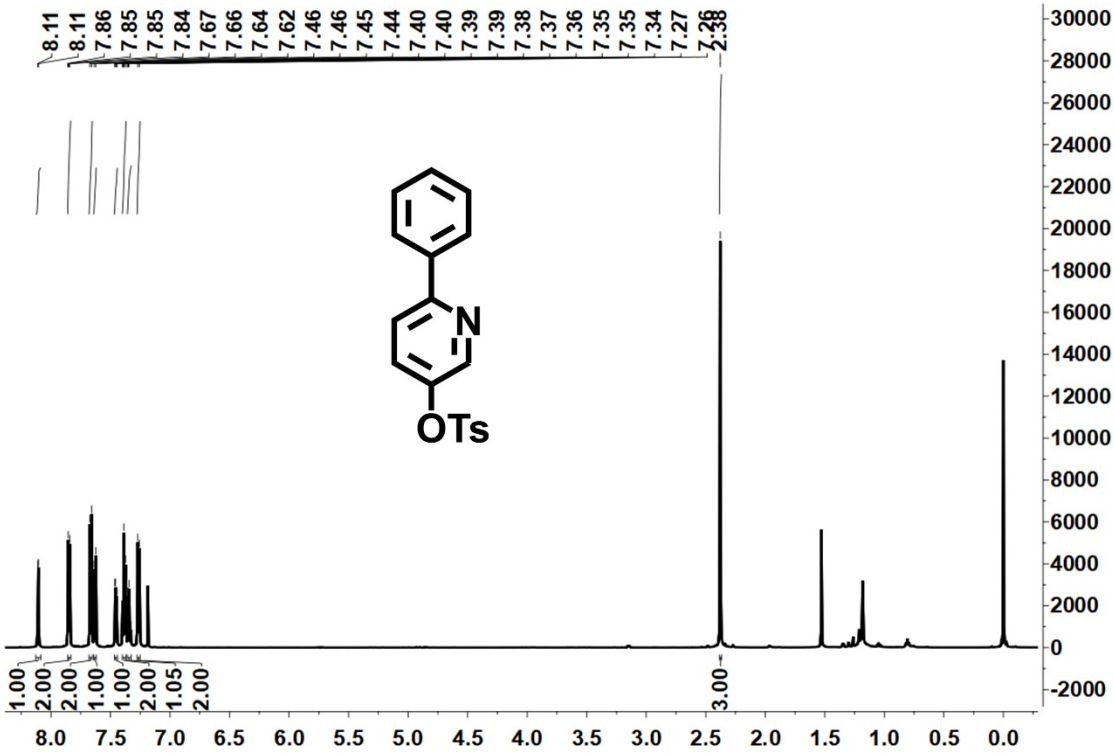


Figure S1. [1]H NMR (600 MHz) spectrum of6-phenylpyridine-3-yl-4-methylbenzene sulfonate.

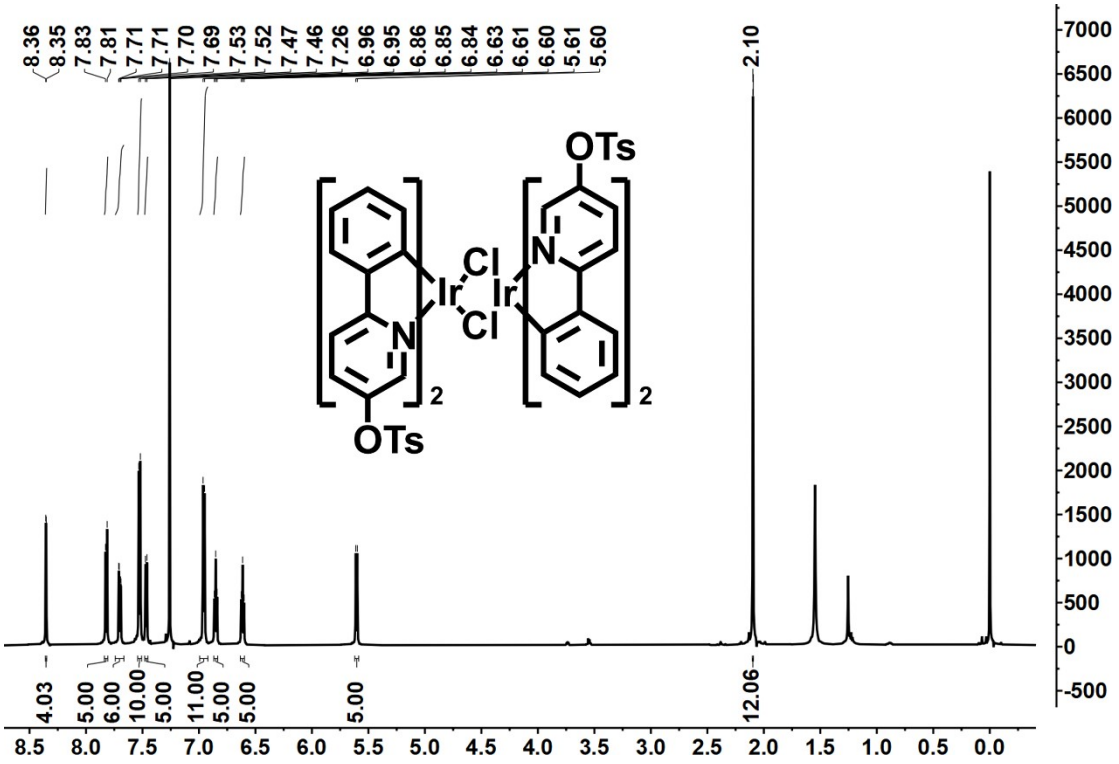


Figure S2. [1]H NMR (600 MHz) spectrum of Dimer.

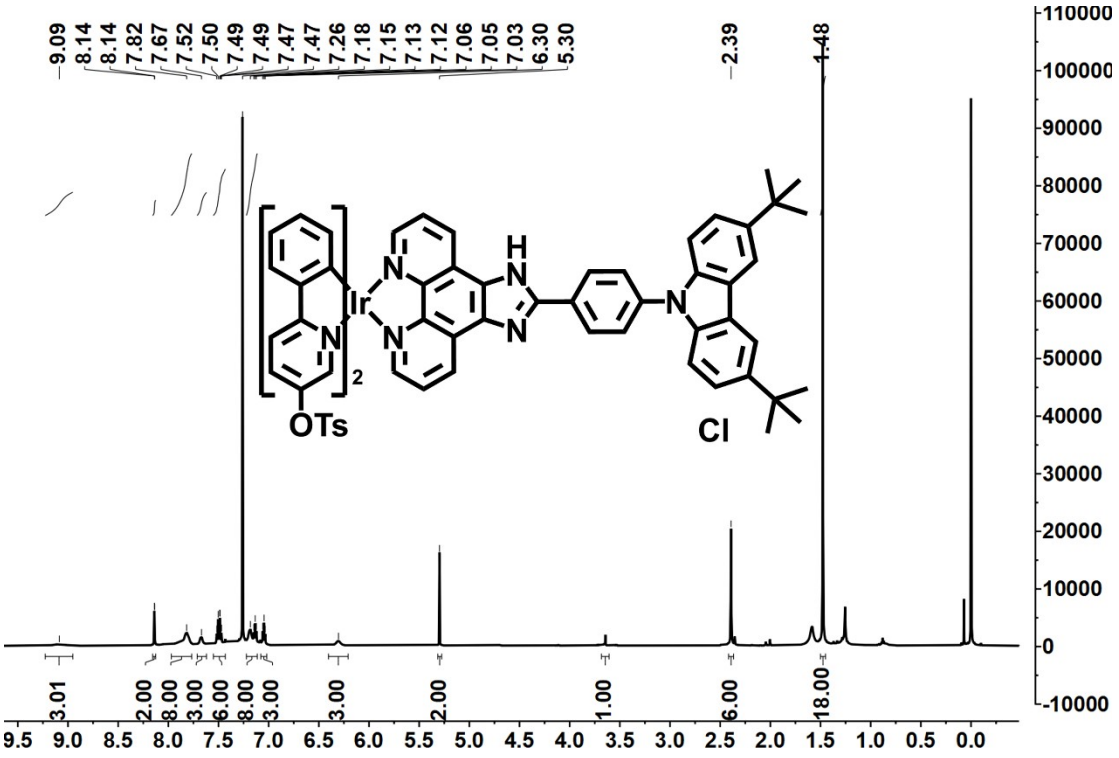


Figure S3. ^{1}H NMR (600 MHz) spectrum of Ir-OTs.

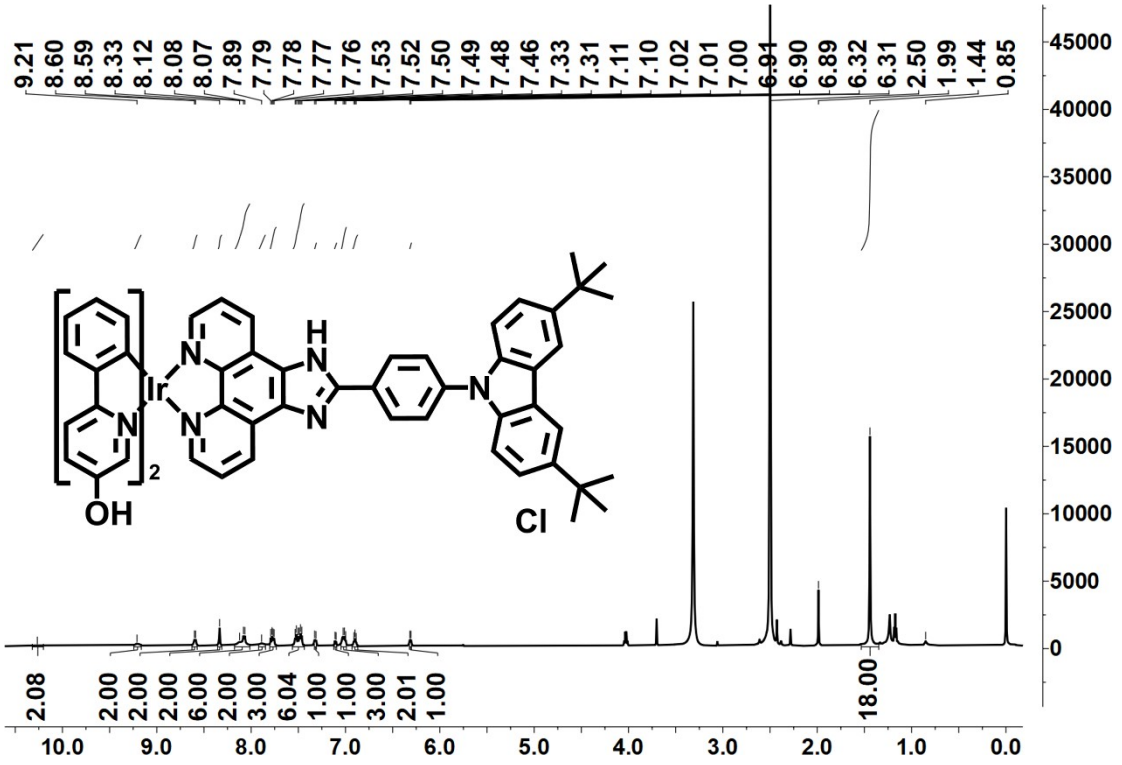


Figure S4. ^{1}H NMR (600 MHz) spectrum of Ir-OH.

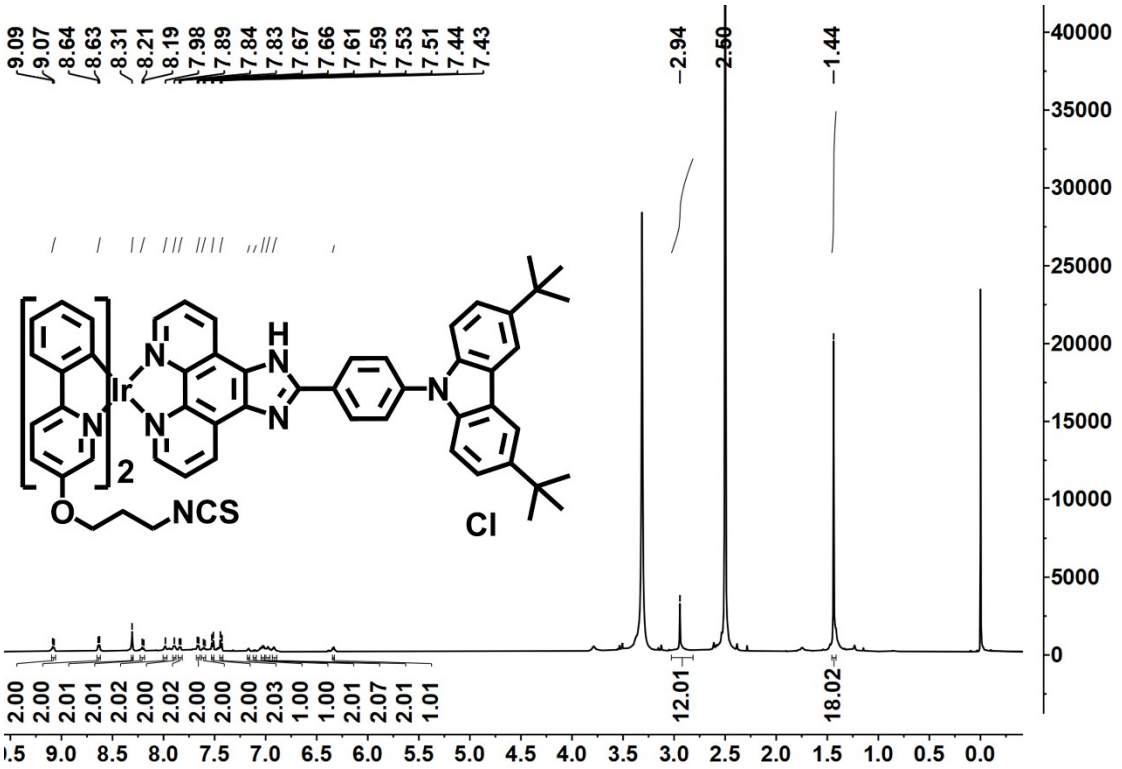


Figure S5. ^{1}H NMR (600 MHz) spectrum of Ir-NCS.

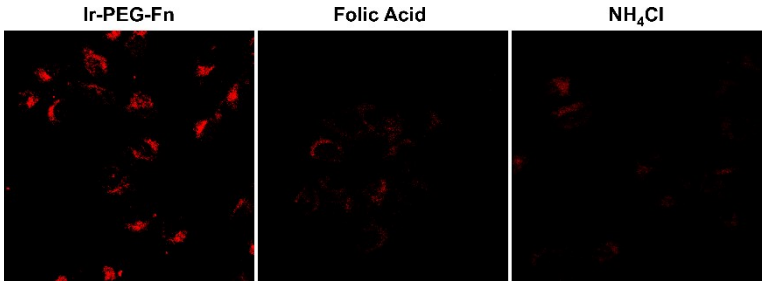


Figure S6. CLSM imaging of MCF-7 cells incubated with Ir-PEG-Fn (10 μM, 8 h) in different conditions. The cells were pre-incubated by endocytic inhibitor or folic acid for 1h.

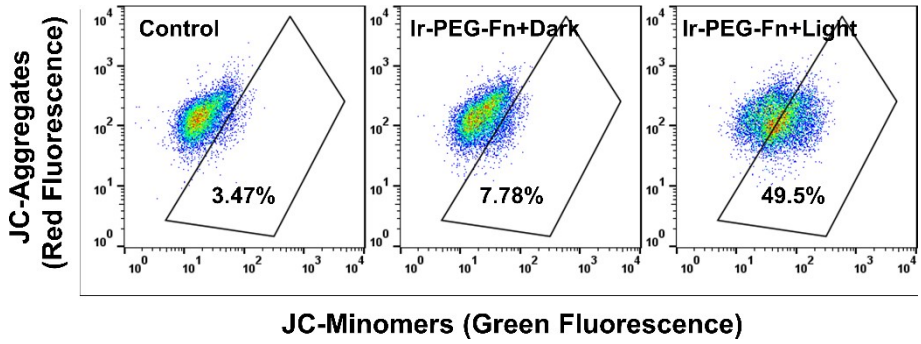


Figure S7. Flow cytometry results of MMP in Hela cells incubated with Ir-PEG-Fn (10 μM, 24 h) via subsequent JC-1 staining.