Supplementary materials

Synthesis, characterization and induction of ferroptosis of iridium(III) complexes against melanoma B16 cells

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Experimental procedures

1. Materials and methods

Cell lines include human hepatocellular carcinoma cell (HepG2), mouse melanoma cells (B16), human lung carcinoma cells (A549) and NIHT 3T3 were provided by Sun Yat-Sen University (Guangzhou). 4-Hydroxy-2-methylbenzaldehyde, 2-phenylpyridine, 1-phenylisoquinoline, 1,10-phenanthroline, ammonium hexafluorophosphate and $IrCl_3 \cdot 3H_2O$ were purchased from Beijing Huawei Ruike Chemical Co. Methanol, dichloromethane and acetone were purchased from Zhiyuan Chemical Reagent Co. Fetal bovine serum (FBS), RPMI-1640 medium, DMSO and Duchenne Modified Eagle Medium (DMEM) were obtained from Gibco. MTT was purchased from Dalian Meilun Biotechnology Co., Ltd. and fluorescent dyes and related assay kits were purchased from Biyuntian Biotechnology Co. The primary and secondary antibodies for immunoprotein imprinting were purchased from Cell Signaling Technology. The HRMS spectra of the complexes were determined using a Waters Xevo G2-XS QT of mass spectrometer with direct injection. ^1H and ^{13}C NMR spectra were tested by a Varian-500 spectrometer (500 MHz). And the UV-visible spectra of the complexes were measured by UV-2550 spectrophotometer.

2. Cell culture

HepG2, A549, B16 and NIH 3T3 cells were cultured in RPMI-1640 or DMEM culture medium that contained 10% fetal bovine serum (FBS), penicillin (100 U/ml), and

streptomycin (100μg/ml). As the cells were full grown, they were ready for passaging and subsequent experiments.

3. Cytotoxicity assay

Cells were seeded evenly into 96-well plates and incubated overnight. The cells were directly treated with different concentration of **9a**-**9c** or incubated for 4 h and irradiated (white light, LED lamp) for 30 min. Then, the cells were continued to be incubated for 48 h. The control group was treated with an equal amount of DMSO, and the dark group was incubated with the complexes without light treatment. At the end of the incubation, MTT dye solution (5 mg/mL) was added into each well to replace the medium, and the incubation was carried out for another 4 h. The supernatant of the plate wells was discarded while 100 μL of DMSO was added into each well, and the absorbance at 490 nm was measured by the multifunctional enzyme counter. The IC_{50} value (half inhibitory concentration) of each complex was obtained by data calculation and analysis.

4. Cellular uptake

B16 cells were evenly spread into 12-well plates and incubated overnight. After the cells grew to 50%, they were treated with **9a-9c** containing 2 × IC_{50} concentration and incubated for 6 h. The cells were washed three times with PBS, then the cells were observed under fluorescence microscope.

5. Cell colony assay and wound healing assay

Cell colony assay is a commonly used technical method to detect the proliferative capacity of cells. Cells (5.0×10^5 cells/well) were inoculated in 12-well plates and cultured in DMEM medium. In alternate days, medium containing iridium complexes **9a-9c** (IC_{50}) was added to the plate wells. After complexes were internalized, the cells were irradiated for 30 min and continued to be incubated. Then the cells were washed twice with PBS and incubated with the new culture medium. After 7 days, added 4% neutral paraformaldehyde to the plate wells for fixation, and stained with 0.1% crystal violet to dye. Finally, the cells were washed with PBS buffer for three times and photographed under a light microscope to observe the formation of cell colonies.

In order to detect the effects of drug on cell migration, the cells were inoculated in a six-well plate and were wounded three parallel lines by a sterile 200 μL lance tip, the debris on the scratches was rinsed with PBS. Then added complex (IC_{50}) in the plate for 4 h and irradiated for 30 min and cultured to 24 h. Finally, using light microscope to observe the scratches of the cells in the labeled area at 0 h and 24 h, and the changes of scratches widths were used to evaluate the effects of the compounds on the migration and invasion ability of B16 cell.

6. Cell cycle arrest analysis

B16 cells were evenly spread into six-well plates (5.0×10^5 cells/well) and incubated 4 h with the IC_{50} concentration of complexes **9a-9c** and irradiated for 30 min, the

cells were continuously cultured for 24 h. After the cells were washed twice with PBS, 300 μl of trypsin was added to each well for digestion. Collecting the cells into 1.5 mL EP tubes for centrifugation. Then, the cells were washed with PBS, the supernatant was discarded and fixed with 75% alcohol, and the cells were stained with Tritanx-100, PI staining solution and RnaseA in a dark environment. The cells were detected with flow cytometry.

7. Co-localization and mitochondrial membrane potential measurement

B16 cells were evenly spread into 6-well plates, and metal compounds were added when the cells grew to a certain amount. After 4 h of incubation, the cells were irradiated for 30 min and continued to be incubated for 24 h. Then the cells were washed with PBS for two times and added with MitoTracker ® Deep Red FM to stain in the dark for 30 min at 37 °C. Finally, these cells were observed and analyzed under a fluorescence microscope. For the measurement of mitochondrial membrane potential, cells were treated with complexes for 4 h and then were irradiated for 30 min and continuously cultured for 24 h. All the samples were stained with JC-1 to avoid light for 30 min, and then washed with PBS for two times, the cells were observed under a fluorescence microscope.

8. Microtubule morphology

B16 cells were seeded in a 12-well plate overnight, the cells were treated with an IC_{50} dose of 9a, 9b and 9c for 4 h and irradiated for 30 min and continuously cultured

for 24 h. Then the cells were washed and fixed in 75% alcohol. At the end of the fixation the cells were washed using an immunostaining wash and then sealed with an immunostained solution for 1 h. The cells were washed again three times, the cells were incubated with an α-tubulin primary antibody for 12 h, followed by a FITC-labeled secondary antibody for 3 h. Microtubule morphology was observed under a microscope.

9. DNA damage detection by γ-H2AX

B16 cells were inoculated into 12-well plates, when the cells grew to about 50% fusion, added complexes to incubate for 4 h. The cells were irradiated for 30 min and continuously cultured for 24 h. Then PBS was used to wash the cells for 3 times, 75% ethanol was added and fixed for 20 min at 4 °C. Finally, the primary antibody of γ-H2AX was added overnight. Next day, removing the primary antibody and the secondary antibody was added and incubated for 70 min in the dark at 4 °C. The cells were detected under a fluorescence microscope.

10. Determination of GSH and MDA content

B16 cells were spread into six-well plates and the complexes **9a-9c** (IC_{50}) was added to incubate for 4 h and then irradiated for 30 min. After 24 h, the cells were collected into EP tubes, the intracellular GSH content was determined according to the instructions of the GSH assay kit, the GSH content was calculated by measuring the absorbance at 412 nm. For the determination of MDA content, after cells were

treated with compounds, RIPA lysate was added, and the supernatant was obtained by centrifugation. The protein concentration of the sample was measured by BCA method, then followed the MDA assay kit instructions to mix the sample and prepare MDA working solution and measured the MDA level in the cells.

11. C11-BODIPY 581/591 detection of iron death

B16 cells were evenly spread into 12-well plates, complexes **9a-9c** were added to incubate for 4 h and the cells were illuminated for 30 min. After 24 h, 300 μL of BODIPY 581/591 (2.5 μM) working solution was added into each well according to the instructions of the C11-BODIPY 581/591 kit. Finally, the cells were washed twice with PBS and observed under a fluorescence microscope.

12. LDH release measurement

B16 cells were evenly spread to 96-well plate overnight, the cells were incubated with IC_{50} concentration of 9a, 9b and 9c for 4 h, then irradiated for 30 min and continued to incubate for 24 h, the control group was exposed to the LDH releasing solution for 0.5 h while all the wells supernatant was mixed with the LDH detecting solution in a dark environment for 30 min. Finally, the absorbance at 490 nm was determined.

13. Intracellular ROS generation

B16 cells were seeded in 12-well culture plates with a density of 4×10^4 each well

and allowed to grow overnight at 37 °C in a 5% CO_2 incubator. Then the IC_{50} concentrations of the complexes were added to the wells and co-incubated for 4 h, the cells were irradiated for 30 min and continued to incubate until 24 h. After cells were washed with PBS twice, 2',7'-dichlorodihydro-fluorescein diacetate (DCHF-DA, 10 μM) was added to the medium to stain the cells. After 0.5 h, the cells were stained with Hoechst for 10 min and photographed under a fluorescence microscope.

14. Ferroptosis inhibitor assay

Ferroptosis inhibitor assay can intuitively reflect whether the cell has undergone ferroptosis. B16 cells were evenly spread in 96-well plates, cultured overnight, and treated with IC_{50} complexes and 10 nM ferroptosis inhibitor for 4 h, the cells were irradiated for 30 min and continuously cultured for 24 h. Next day, the culture medium was discarded, and the cells were incubated with 10% MTT solution for 4 h. Then, the cell was dissolved in 100 μL DMSO. After shaking the culture plate for one minute, the absorbance was detected at 490 nm with microplate reader, and then the survival rate of cells was calculated.

Fig. S1 Purity determination using methanol and H_2O as mobile phase, $V_{methanol}$: V_{H2O} = 75:25 for **9a**, 85:15 for **9b**, 90:10 for **9c**

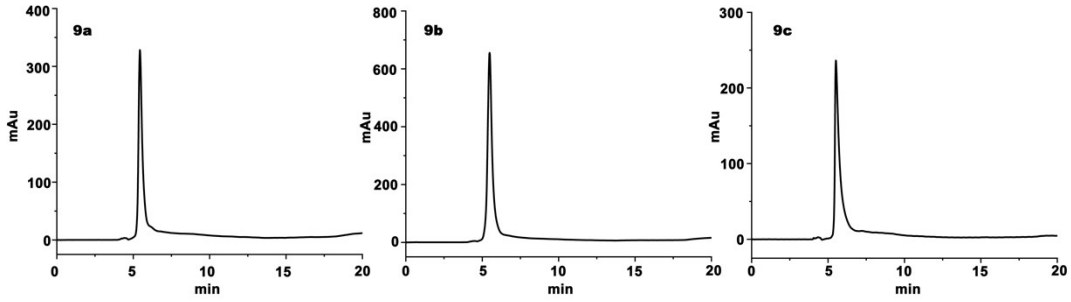

Fig. S2 (a) UV-Vis of 9a-9c (20 μM) and (b) fluorescence spectra of complexes 9a-9c (IC_{50} concentration) in PBS, (c) Stability determination of complexes 9a, 9b and 9c in PBS at 0, 24 and 48 h.

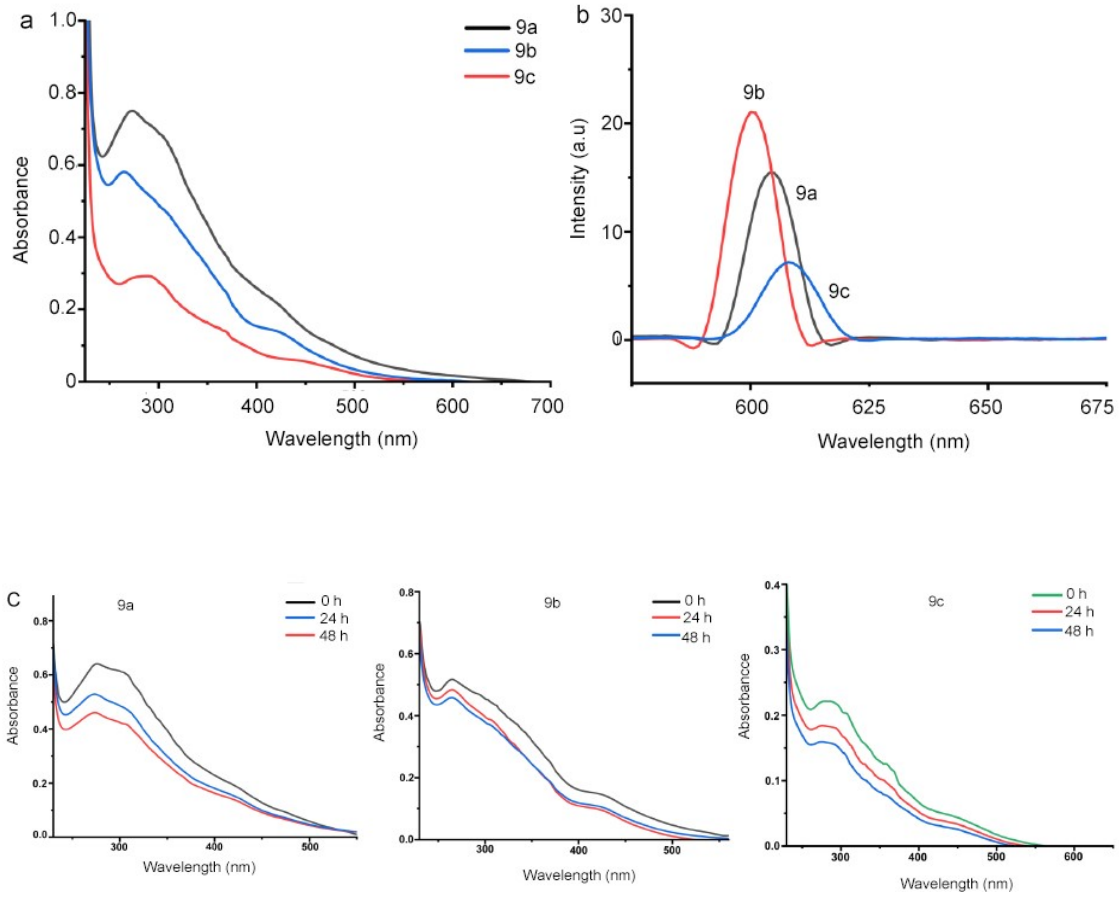

Fig. S3 ^{1}H NMR spectra for **9a**

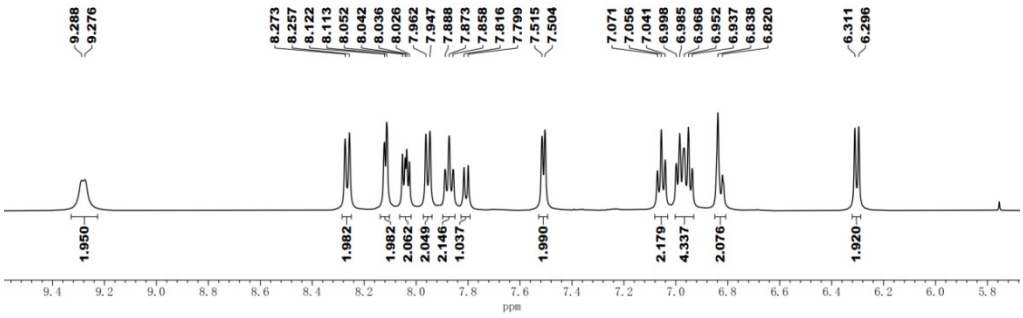

Fig. S4 ^{13}C NMR spectra for **9a**

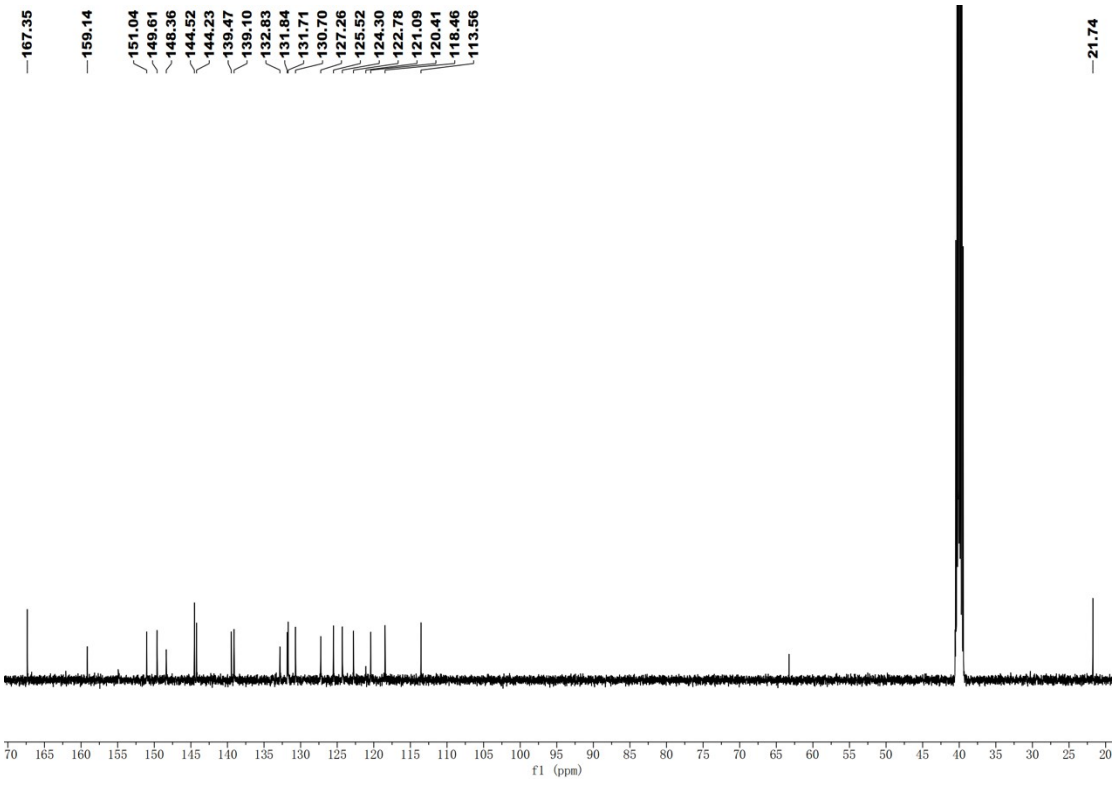

Fig. S5 ^{1}H NMR spectra for **9b**

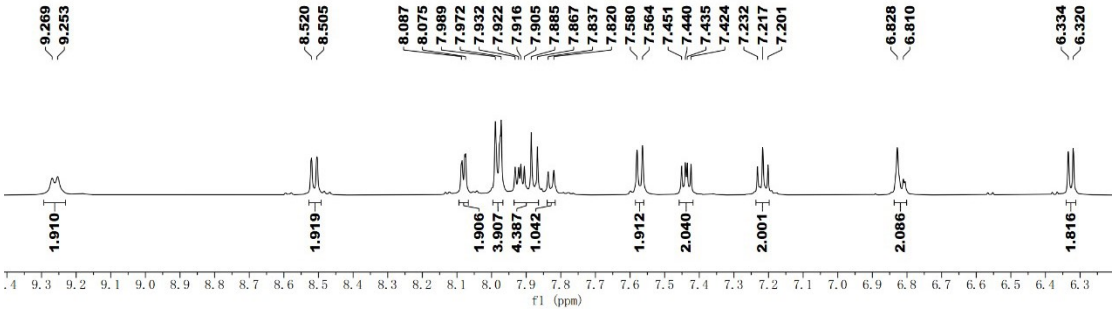

Fig. S6 ^{13}C NMR spectra for **9b**

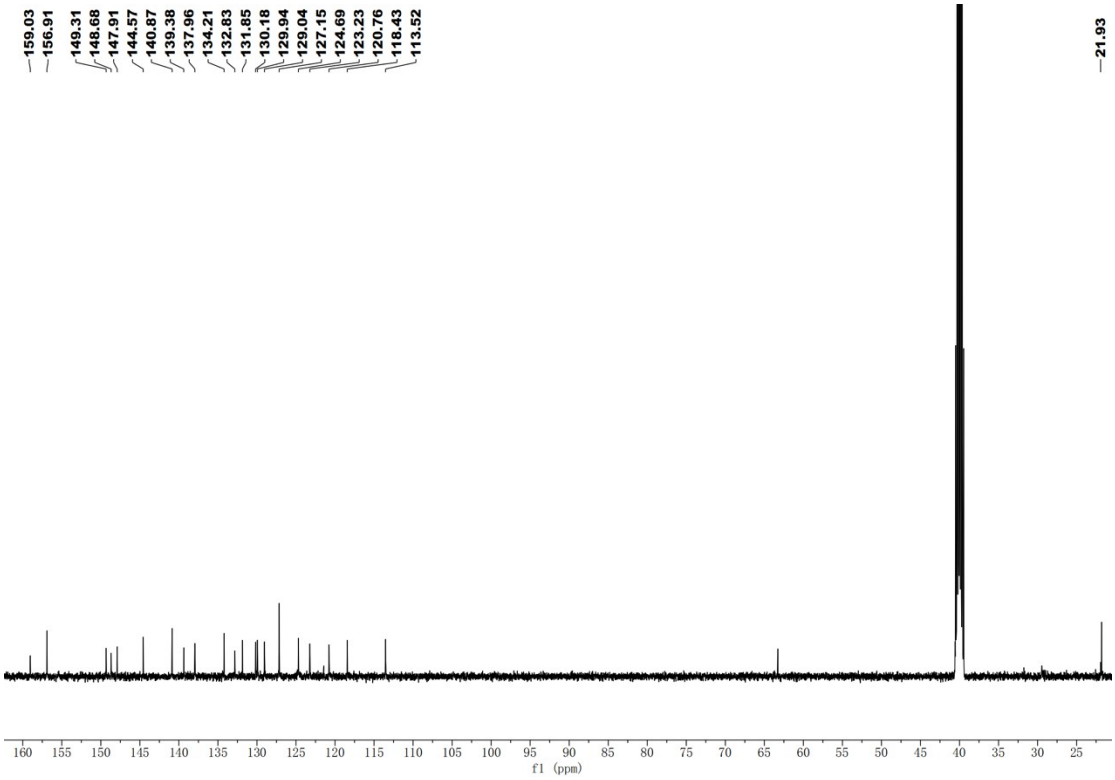

Fig. S7 ^{1}H NMR spectra for **9c**

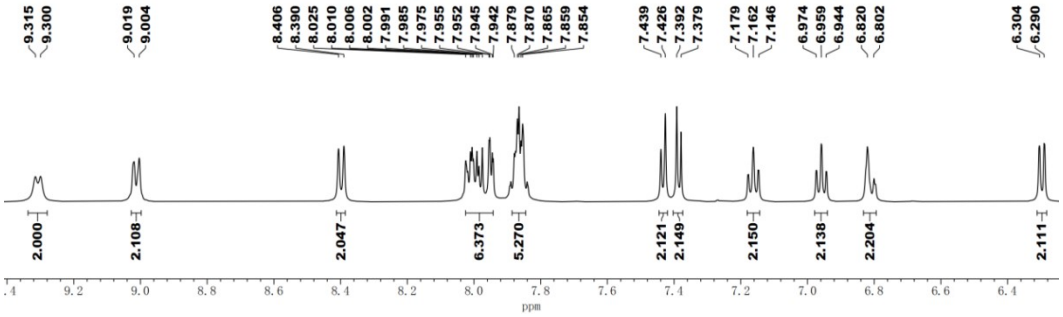

Fig. S8 ^{13}C NMR spectra for **9c**