HCBP1 peptide conjugated ruthenium complex for targeting hepatoma therapy

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Fig. S16. The ICP-MS analysis of concentrations were determined in mitochondria, nucleus, membrane and whole cells of Hep-G2 cells treated with 15 μM Ru-β-Ala-FQHPSFI for 48 h.

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Experimental Section

Materials

$RuCl_3 \cdot nH_2O$, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and cisplatin were purchased from Alfa Aesar. Human ovarian carcinoma (A2780), cisplatin-resistant human ovarian carcinoma (A2780 cis), human oesophageal carcinoma (OE19), human colorectal carcinoma (HCT116), prostate carcinoma (PC-3), liver hepatocellular carcinoma (Hep-G2, Hepa1-G) and liver normal (HL-7702) cell lines were purchased from Sigma-Aldrich. Roswell Park Memorial Institute medium (RPMI-1640), fetal calf serum (FCS), glutamine and penicillin/streptomycin were purchased from Sigma-Aldrich. All solvents and reagents used for solid phase peptide synthesis were purchased from commercial suppliers including GL Biochem (Shanghai) Ltd., Shanghai Hanhong Chemical Co., J&K Co. Ltd., Shenzhen Tenglong Logistics Co., or Energy Chemical Co., and were used without further purification unless otherwise stated.

Instruments

UV-visible absorption spectra were recorded on a Varian Cary 300UV-vis spectrophotometer fitted with an external Varian Cary temperature controller. The fluorescence spectra were recorded on a JASCO FP-6500 Fluorimeter. Peptide was analyzed and purified by HPLC (SHIMAZU Prominence LC-20AT) with a C18 analytic column (Agilent ZORBAX SB-Aq, 4.6 × 250 mm, 5 μm, flow rate 1.0 mL/min). LC-MS data was carried out on SHIMAZU LC-MS 8030. The resonance Raman images were measured by confocal Raman microspectroscopy (inVia, Renishaw).

Synthesis

Initially, the FQHPSFI peptide was synthesized on Rink amide MBHA resin via Fmoc/tBu SPPS technique, followed by coupling reactions with HCTU (2-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and DIPEA (N, N-diisopropylethylamine) during N_2 bubbling. Successively, the FQHPSFI peptide was linked with Fmoc-β-Ala amino acid (β-Ala-FQHPSFI) for enhancing the interaction with the Ru complex. After that, β- Ala-FQHPSFI (10 eq) was blended with HoBt (1-hydroxybenzotriazole, 3 eq), PyBop (Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, 3 eq) and DIPEA (6 eq). In the subsequent stage, this solution reacted with the ruthenium complex in DMF overnight under N_2. Technically, condensation reaction occurred between carboxyl group in ligand of the ruthenium complex and amino group located at terminal of FQHPSFI. Finally, the crude product is purified by HPLC and

analyzed by mass spectrometry.

HPLC and Mass Spectrometry

Crude peptides and Ru-peptide conjugates were purified by RP-HPLC (SHIMAZU Prominence LC-20AT) with a C18 analytic column (Agilent Zorbax SB-Aq, 4.6 × 250 mm, 5 μm, flow rate 1.0 mL/min). Pure water (containing 0.1% Trifluoroacetic acid) and acetonitrile (Sigma-Aldrich, for HPLC, gradient grade, ≥99.9%) were used as mobile phase. The HPLC traces were detected by two-channel of 220 nm and 254 nm. The gradient grade of acetonitrile began from 20% and increased to 70% in 25 minutes. Mass analysis of products was detected by LC-MS 8030 (SHIMAZU LC-MS 2020 mass-spectrometer). The purity of peptides and Ru-peptide conjugates were identified by analytic HPLC traces and mass spectrometry. The final peptide and Ru-peptide conjugates were confirmed ≥ 95% purity (Supporting Information, Fig. S1-S5.).

Cell culture

The cells were grown in RPMI-1640 with or without phenol red (for photoactivable experiment). All media were supplemented with 10% v/v of fetal calf serum (FCS),1% v/v penicillin/streptomycin. All cells were grown as adherent monolayers at 37 °C in a 5% CO_2 humidified incubator and passaged regularly at approx.

Cytotoxicity test

The cells were plated in flat-bottomed 96-well plates and incubated at 37 °C for 24 h. The blank medium was replaced by medium containing serial concentrations of the tested agents. The plates were incubated for 44 h. At the end of the drug-exposure period, 20 μL of MTT solution was added to each well and the plates were further incubated for 4 h. The medium was removed from the wells, and the purple formazan crystals were dissolved in 150 μL of DMSO per well. The absorbance at 490 nm was measured using a microplate reader (Bio-Rad iMark, USA). The mean absorbance reading from the wells without drug treatment was used as the control (100% viability).

ICP-MS analysis

Exponentially growing Hep-G2 cells were plated at a density of 10^5 cells/mL in 5 mL of DMEM medium. Ruthenium compounds (10 μM) were added to the culture medium and incubated for varying amounts of time at 37 °C with 5% CO_2/95% air. After digestion in trypsin−EDTA solution, Hep-G2 cells were collected and counted. Then, the membrane, nucleus and mitochondria fractions of the cells were extracted by using relative extraction kits (Promega). The cells were digested in 60% HNO_3 at room temperature overnight. Each sample was diluted with Milli-Q H_2O to obtain 2% HNO_3 solutions. The standards for calibration were freshly prepared by diluting a $RuCl_3$ stock solution with 2% HNO_3 in Milli-Q H_2O. The ruthenium concentration in each part was determined by ICP-MS (Thermo Elemental, USA).

Annexin V/PI double staining assay

After incubation with the compounds (10 μM) for 24 h, 10^6 Hep-G2 cells were trypsinized, washed twice with ice-cold PBS, and then resuspended in 100 μL bu□er (50 mmol/L HEPES/NaOH, pH 7.4, 700 mmol/L NaCl, 12.5 mmol/L $CaCl_2$) containing 5 μL of Alexa Fluor 488 annexin V stock (Invitrogen, U.K.) and 1 μL of 1 mg/mL PI (Sigma, USA). After incubation for 15 min at 37 °C in a light-protected area, another 400 μL bu□er was added and the specimens were quantified by flow

cytometry on a FACSCanto II (BD Biosciences, USA).

AO/EB staining assay

AO is a vital dye and can stain both living and dead cells. EB only stains cells that have lost their membrane integrity. A monolayer of Hep-G2 cells was incubated in the absence or presence of the compounds at a concentration of 10 μM for 48 h. After 48 h, the cells were stained with AO/EB solution (100 μg mL^{-1} AO and EB) for 30 min in the incubator. The samples were observed under an inverted fluorescence microscope.

Caspase-3 activity assay

Caspase-3 activity was measured using the Caspase 3 Activity Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cells were cultured in 96-well plates and treated Ru, Ru-β-Ala-FQHPSFI and Ru-β-Ala-IFSPHQF (10 μM) for 48 h, and then 50 μL cell lysate was added to each well, followed by the addition of 50 μL Caspase-Glo® 3/7 reagent. The mixture was incubated at room temperature for 1 h and then the luminescence was measured.

Generation of MCTSs

MCTSs were cultured using the liquid overlay method.[1] Hep-G2 cells in the exponential growth phase were dissociated using a trypsin/EDTA solution to gain single-cell suspensions. A number of 5000 diluted Hep-G2 cells were transferred to 1% agarose-coated transparent 96-well plates with 200 μL of DMEM containing 10% FCS. The cells generated singlet MCTSs of approximately 400 μm in diameter at day 3-5 with 5% CO_2 in the air at 37 °C.

Cytotoxicity of 3D MCTSs

MCTSs with diameters of approximately 400 μm were treated with the tested compounds through carefully replacing 50% of the medium with a drug-supplemented standard medium using an 8-channel pipettor. In parallel to this, for the untreated MCTSs, we replaced 50% of the medium with solvent-containing or solvent-free medium. Four MCTSs were used for treatment per condition and the complexes concentration and DMSO volume were less than 0.5% (v/v). The MCTSs were then allowed to incubate for another 72 h. The cytotoxicity of the ruthenium complexes was measured using ATP concentration with a CellTiter-Glo Luminescent Cell Viability kit (Promega). After 40 minutes of incubation, the MCTSs were carefully transferred into black-sided, flat bottomed 96-well plates (Corning) and mixed using a pipette for luminescence measurements using infinite M200 PRO equipment (TECAN).

Live/dead viability assay

The live/dead assays of the MCTSs were performed using a LIVE/DEAD Viability Kit for mammalian cells (Life Technologies). Live cells were distinguished by the presence of ubiquitous intracellular esterase activity, as determined through the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein (λ_{ex} = 488 nm, λ_{em} = 520 nm). After treatment with the complexes, the MCTSs were incubated with calcein AM (2 μM) solutions for 1 h and observed directly using an inverted fluorescence microscope (Zeiss Axio Observer D1, Germany).

Figures

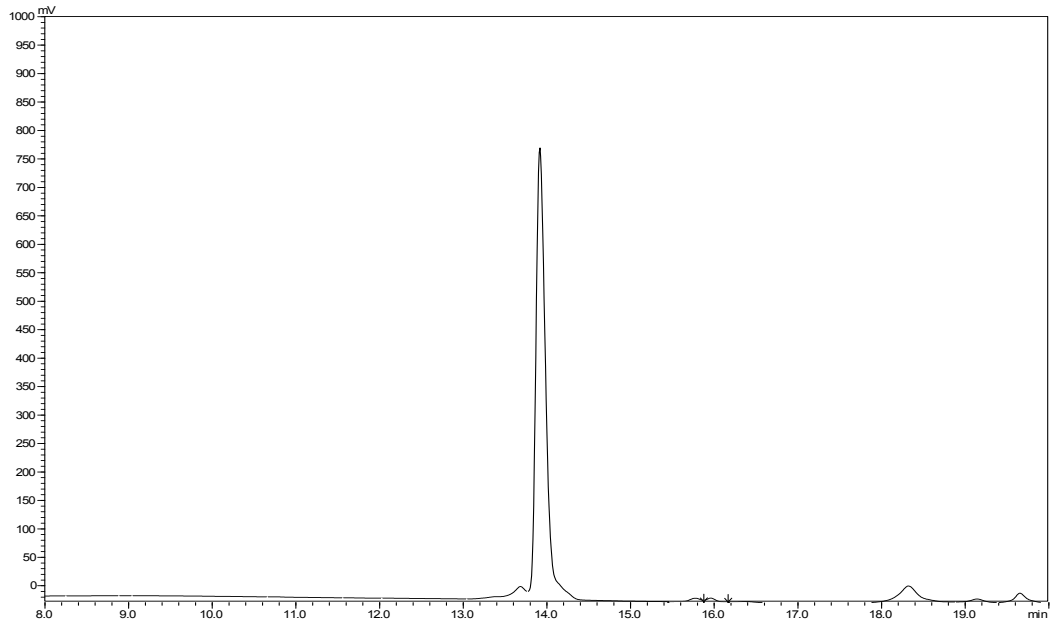

Fig. S1. The HPLC of traces β-Ala-FQHPSFI of 220 nm. Pure water (containing 0.1% Trifluoroacetic acid) and acetonitrile were used as mobile phase. The gradient grade of acetonitrile began from 20% and increased to 70% in 25 minutes.

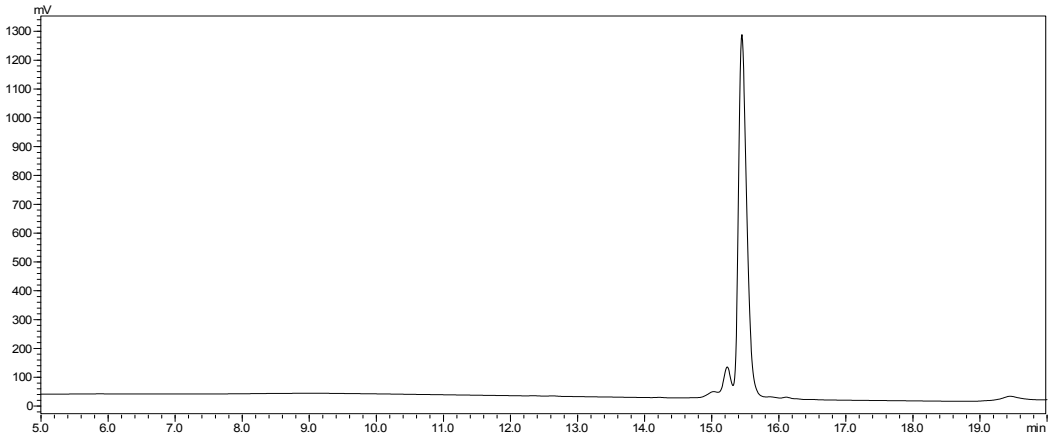

Fig. S2. The HPLC traces of Ru-β-Ala-FQHPSFI of 220 nm. Pure water (containing 0.1% Trifluoroacetic acid) and acetonitrile were used as mobile phase. The gradient grade of acetonitrile began from 20% and increased to 70% in 25 minutes.

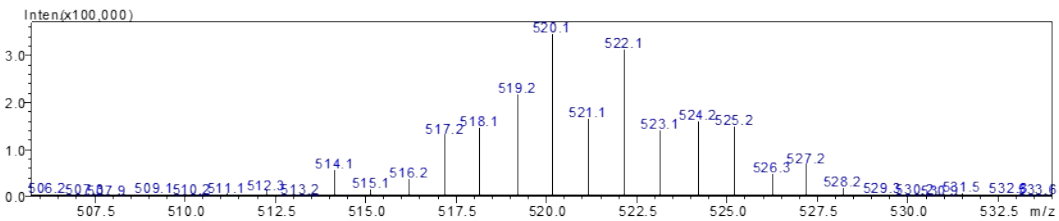

Fig. S3. The ESI-MS spectrum of Ru complex in 50% (v/v) CH_3CN/H_2O.

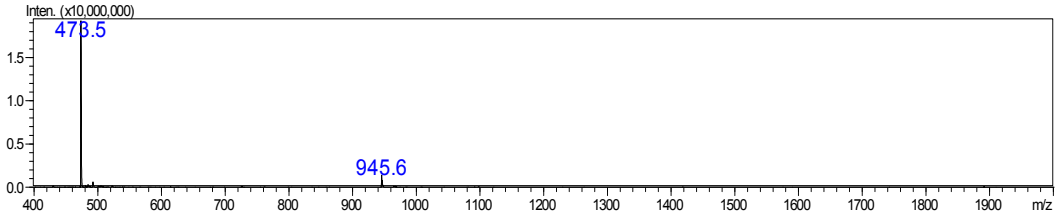

Fig. S4. The ESI-MS spectrum of β-Ala-FQHPSFI in 50% (v/v) CH_3CN/H_2O.

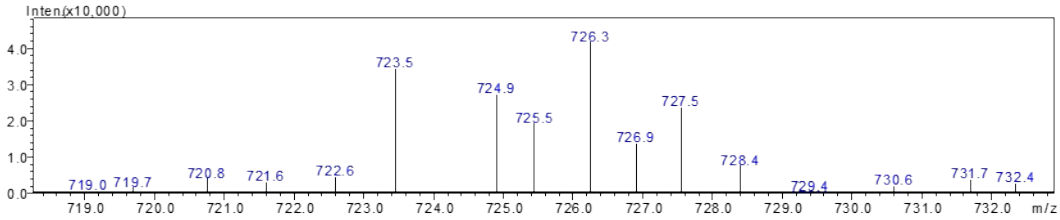

Fig. S5. The ESI-MS spectrum of Ru-β-Ala-FQHPSFI in 50% (v/v) CH_3CN/H_2O.

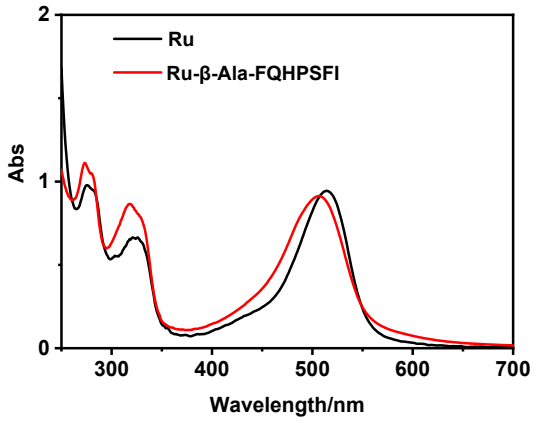

Fig. S6. UV-vis spectra of Ru complex and Ru-β-Ala-FQHPSFI (20 μM) in the PBS solution.

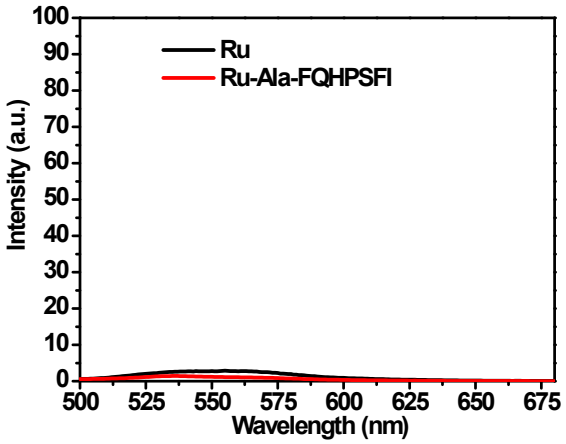

Fig. S7. Emission of Ru complex and Ru-β-Ala-FQHPSFI in the PBS solution at 298 K. The wavelength of excitation was 460 nm.

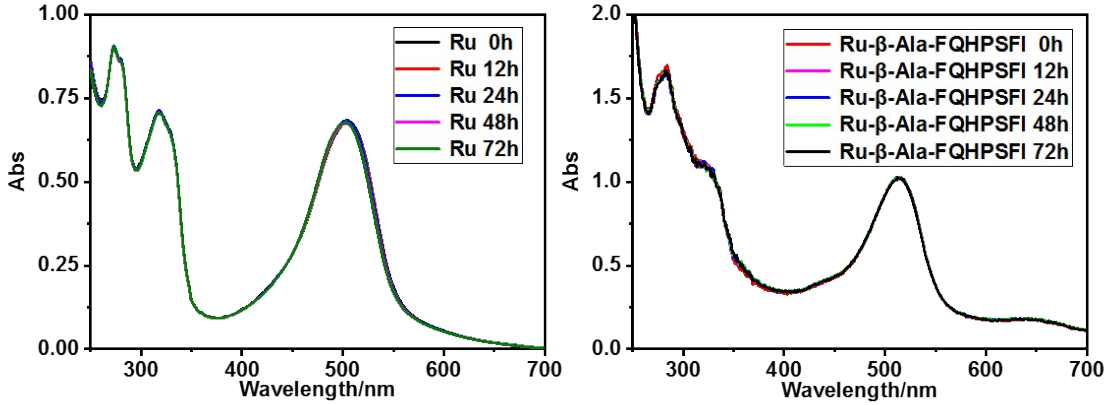

Fig. S8. The stabilities of Ru and Ru-β-Ala-FQHPSFI in the cell culture medium (RPMI-1640) for 0~72 h at 37°C.

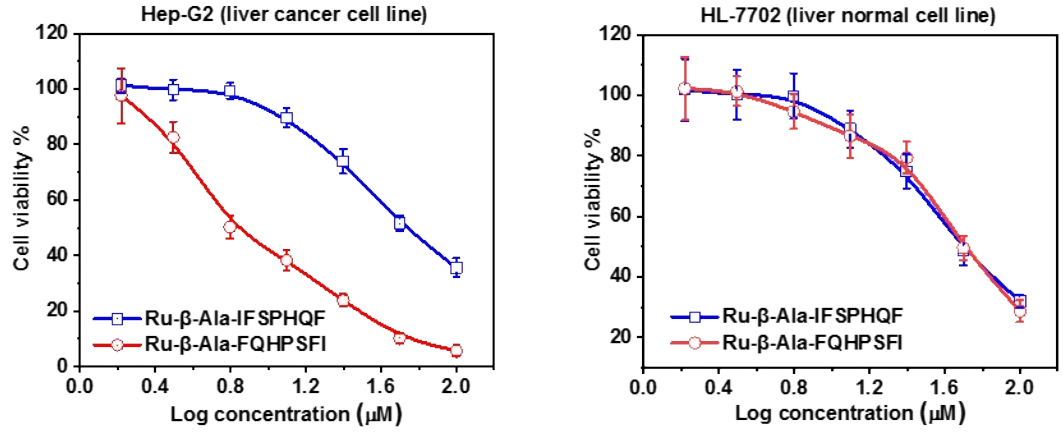

Fig. S9. The cell viabilities for Hep-G2 cells and HL-7702 cells treated with different concentrations of Ru-β-Ala-IFSPHQF and Ru-β-Ala-FQHPSFI conjugate for 48 h.

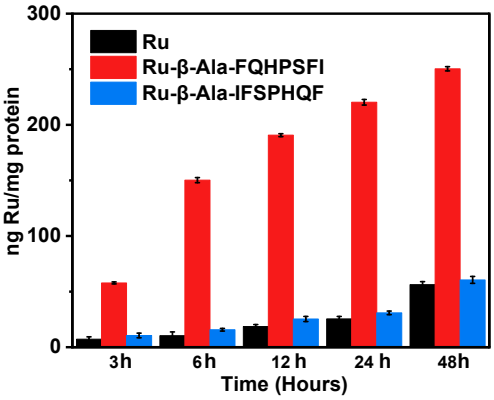

Fig. S10. The ruthenium concentrations in Hep-G2 cells after exposure to 15 μM various compounds for 3-48 h were determined by ICP-MS.

Table 2. Ruthenium concentrations were determined in whole cells exposure to 15 μM complexes for 48h by ICP-MS

	Hep-G2	**Hep-G2 DDP**	**A549**
Ru	56.27±2.7	54.22±2.4	50.13±5.2
Ru-β-Ala-FQHPSFI	250.4±1.9	128.51±2.9	109.26±4.6
Ru-β-Ala-IFSPHQF	60.52±3.1	51.22±3.5	57.45±1.3

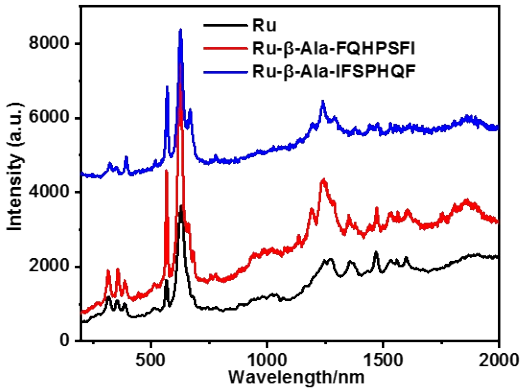

Fig. S11. The resonance Raman spectra of Ru, Ru-β-Ala-FQHPSFI and Ru-β-Ala-IFSPHQF in PBS buffer after excitation at 458 nm.

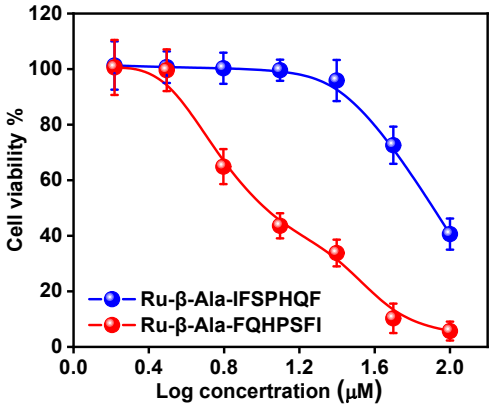

Fig. S12. The cell viabilities for 3D Hep-G2 tumor spheroids treated with different concentrations of Ru-β-Ala-IFSPHQF and Ru-β-Ala-FQHPSFI conjugate for 72 h.

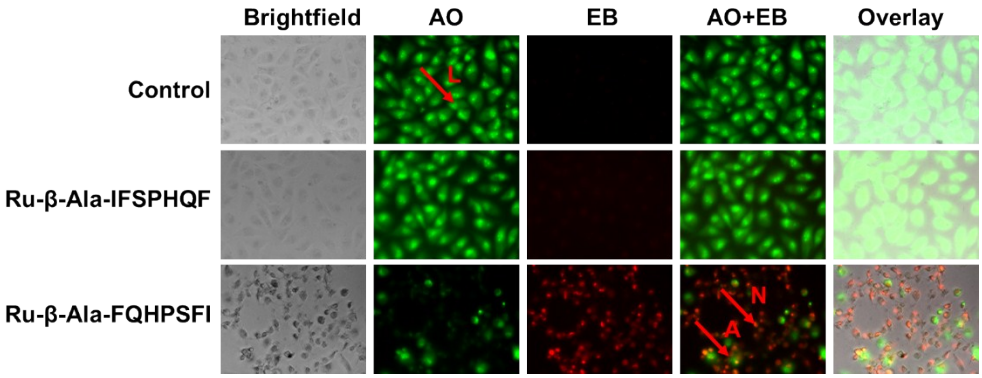

Fig. S13. Hep-G2 cells were stained by AO/EB and observed under fluorescence microscope. From up to below: Hep-G2 cells without treatment; in the presence of Ru-β-Ala-IFSPHQF and Ru-β-Ala-FQHPSFI conjugate (10 μM) incubated at 37 °C and 5% CO_2 for 48 h. Arrows pointed to the cells representing certain cell viable status: L—the live cells, A—the apoptotic cells, and N—the necrotic cells.

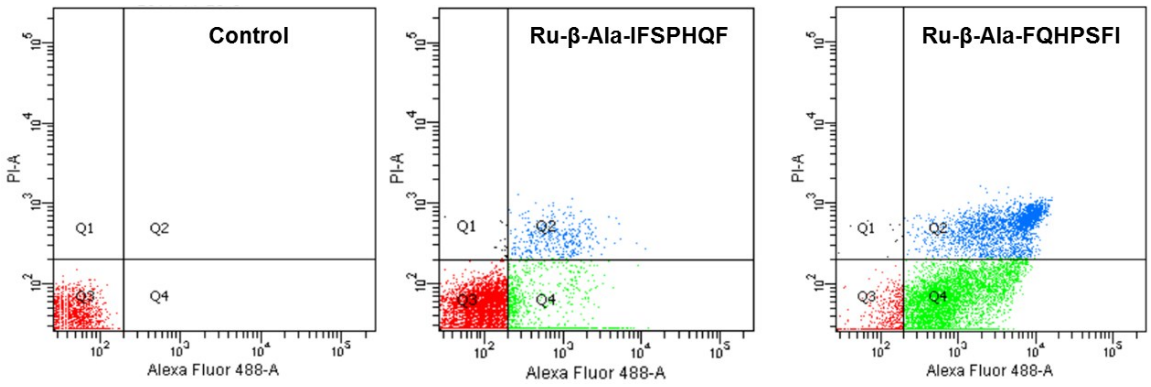

Fig. S14. Ru-β-Ala-IFSPHQF and Ru-β-Ala-FQHPSFI conjugate (10 μM) induced apoptotic cell death as examined by the annexin V-FITC/PI assay after 48 h of incubation.

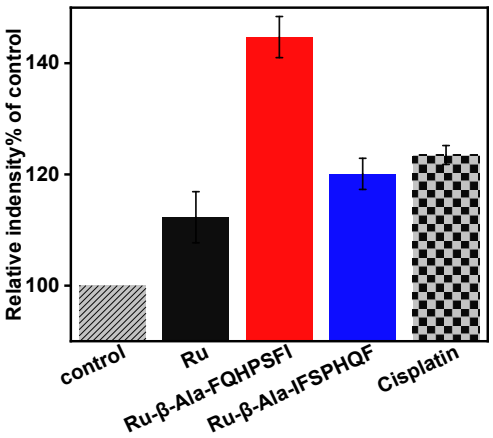

Fig. S15. The analysis of caspase-3 protein of Hep-G2 cells treated with 10 μM Ru, Ru-β-Ala-FQHPSFI, Ru-β-Ala-IFSPHQF and cisplatin for 48 h.

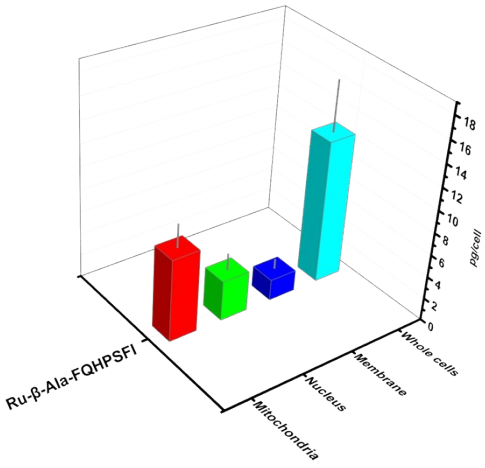

Fig. S16. The ICP-MS analysis of concentrations determined in mitochondria, nucleus, membrane and whole cells were treated of Hep-G2 cells with 15 μM Ru-β-Ala-FQHPSFI for 48 h.

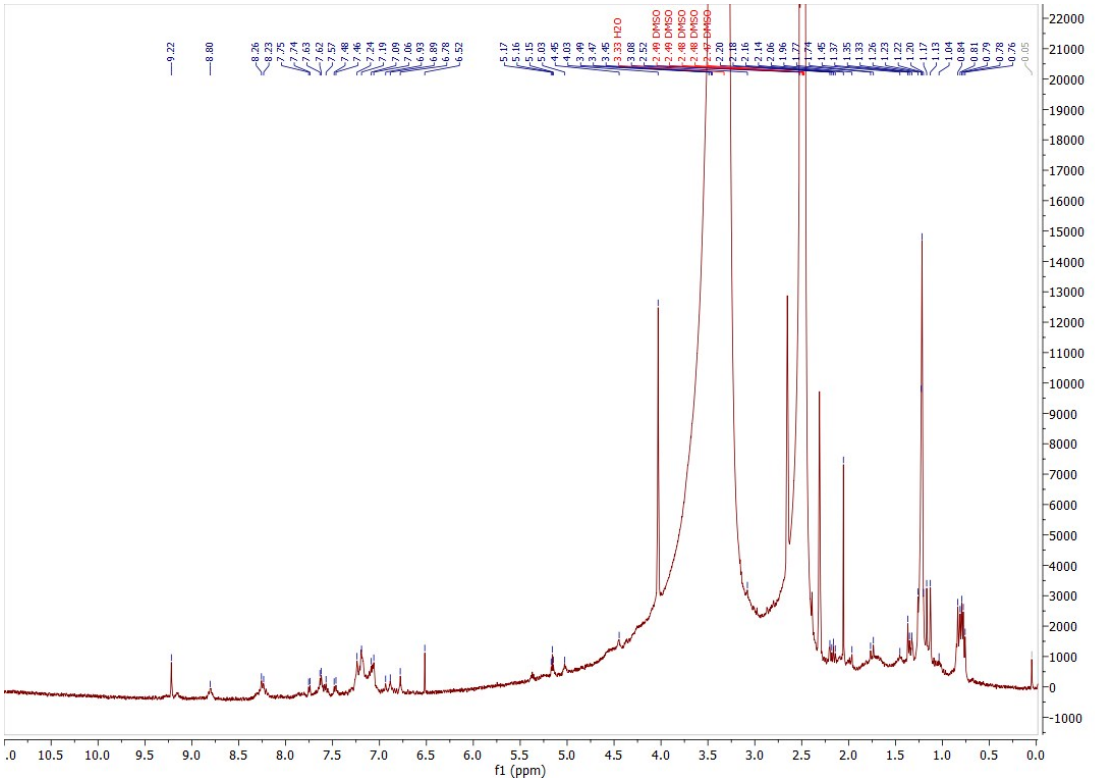

Fig. S17. The ^{1}H-NMR spectra of the Ru-β-Ala-FQHPSFI at 400 MHz (Bruker Avance III 400MHz, 298K) in DMSO-d^{6}. The NMR data were processed with TopSpin®, and further analyzed using NMR View J software.

Reference

1. H. Y. Huang, P. Y. Zhang, Y. Chen, L. N. Ji and H. Chao, *Dalton Trans.*, **2015**, *44*, 15602–15610.