Electronic Supporting Information Materials

Two telomerase-targeting Pt(II) complexes of jatrorrhizine and berberine derivatives induce apoptosis in human bladder tumor cells

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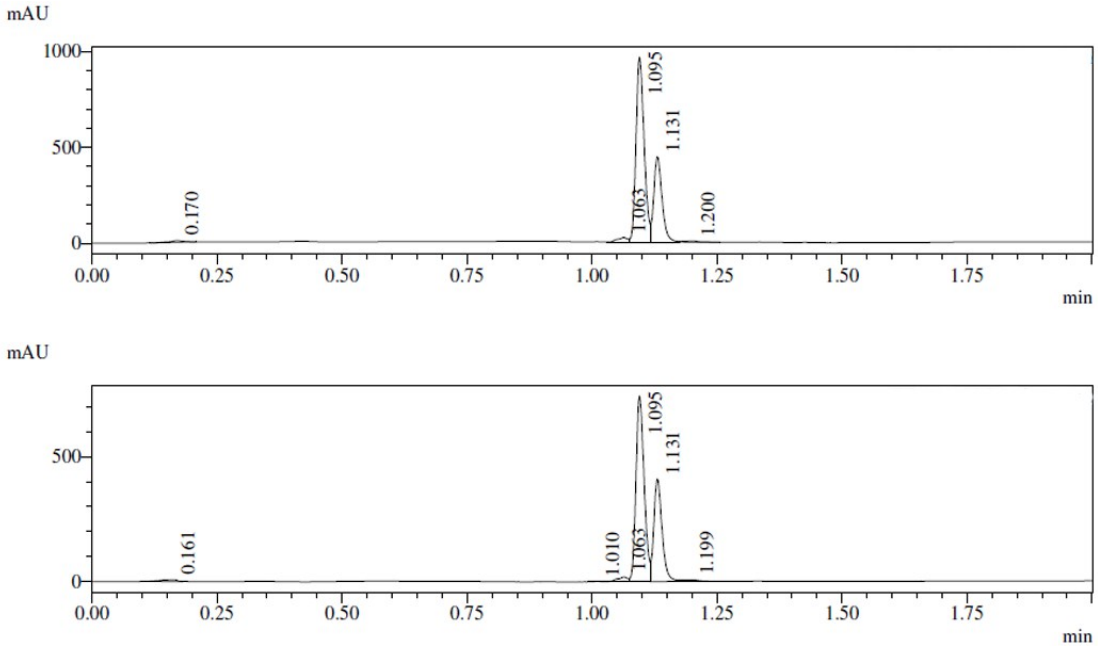


Fig. S1. LC-MS spectra for compound **2** (2.0×10^{-5} M) in TBS (Tris-HCl buffer solution, 10 mM, pH 7.35) solution with 0 h (top) and 48 h (down), respectively (λ= 250 nm). Column: Inertsustain C18 column (LC-20AT, SPD-20A HPLC COLUMN, 150mm×5.0 μm I.D.). Column temperature: 40ºC. Mobile phase: methol/H_2O containing 0.01% TFA (90:10 methol/H_2O). Flow rate: 1.0 mL/min. Injection volume: 2.0×10^{-5} M.

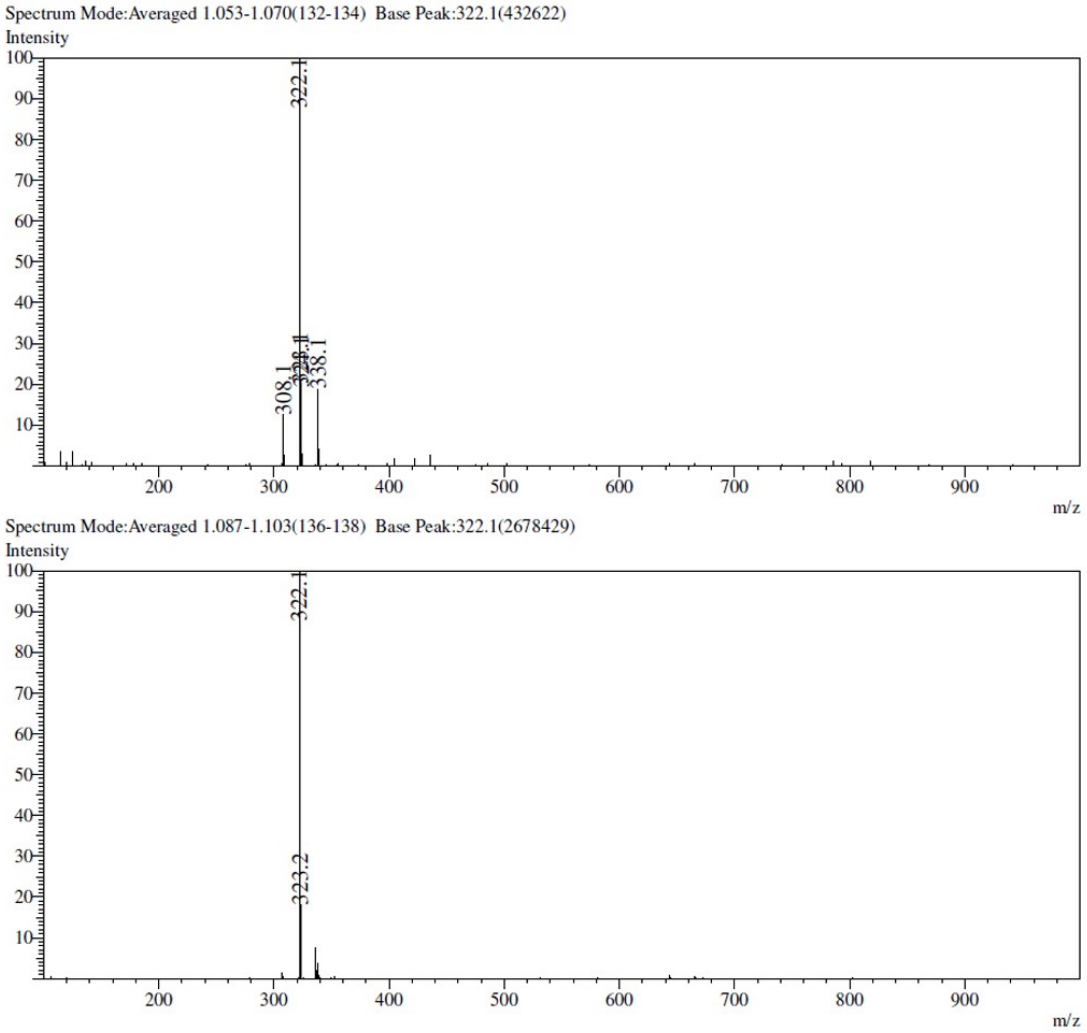


Fig. S2. The mass spectra of compound **2** (2.0×10^{-5} M) in Tris-HCl buffer solution (containing 5% DMSO) for 0 h (up) and 48 h (down), respectively.

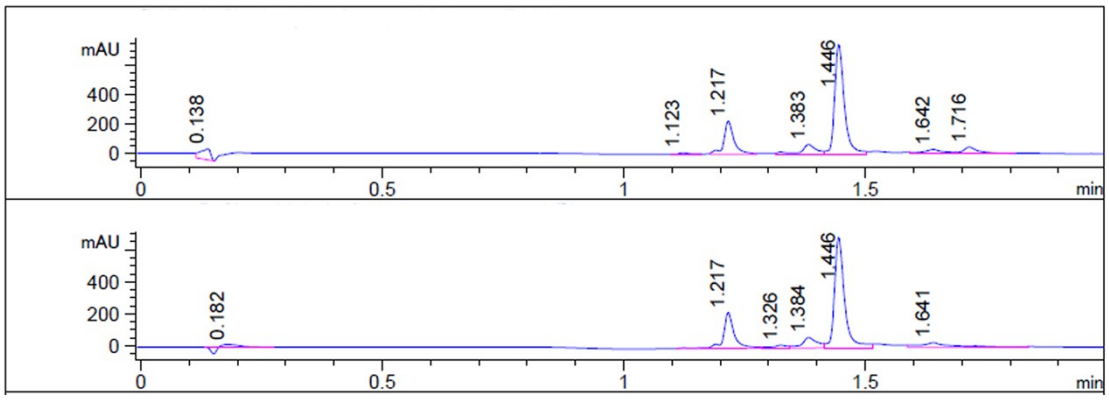


Fig. S3. LC-MS spectra for compound **4** (2.0×10^{-5} M) in TBS (Tris-HCl buffer solution, 10 mM, pH 7.35) solution with 0 h (top) and 48 h (down), respectively (λ= 250 nm). Column: Inertsustain C18 column (LC-20AT, SPD-20A HPLC COLUMN,

150mm×5.0 μm I.D.). Column temperature: 40ºC. Mobile phase: methol/H_2O containing 0.01% TFA (90:10 methol/H_2O). Flow rate: 1.0 mL/min. Injection volume: 2.0×10^{-5} M.

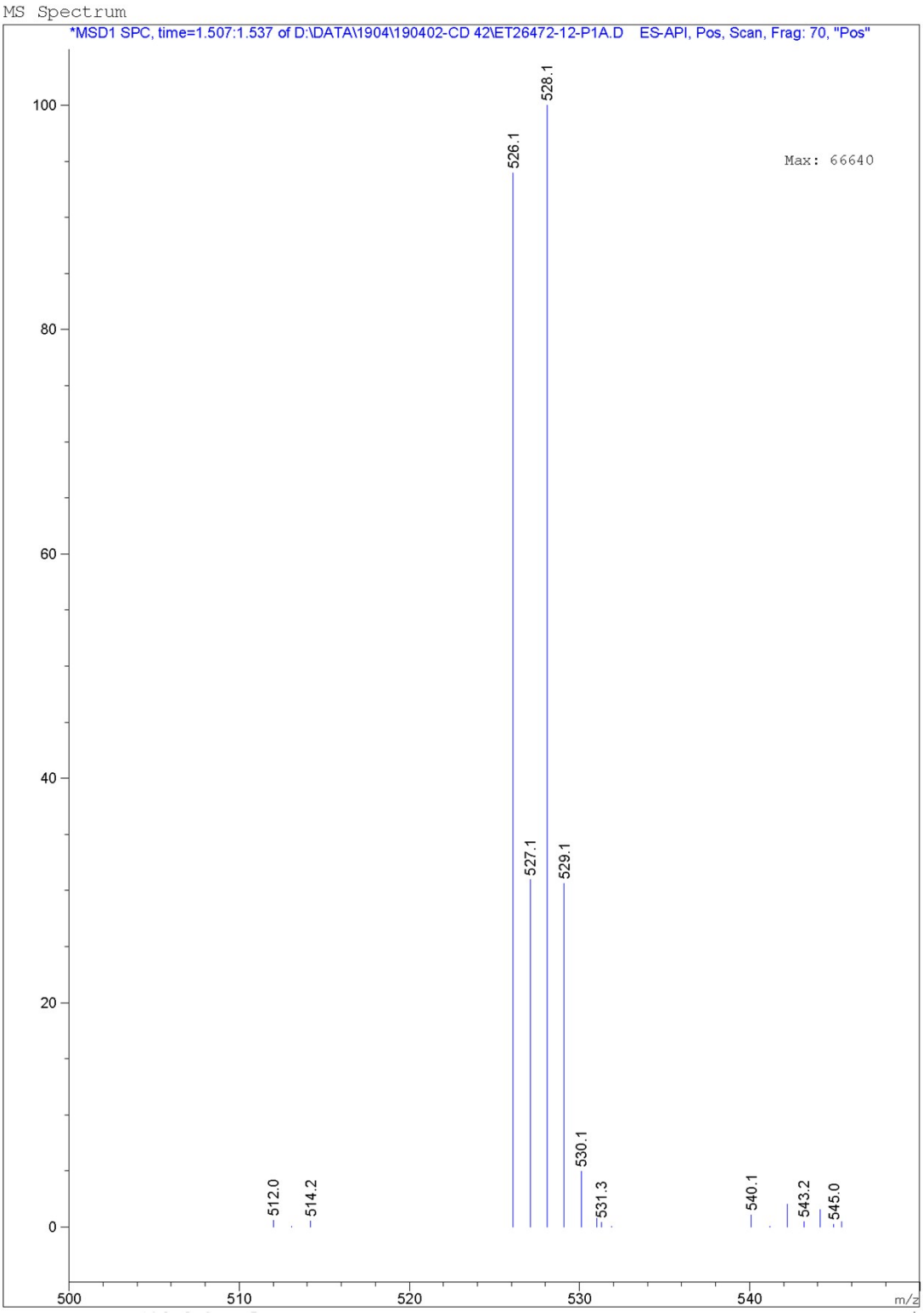


Fig. S4. The mass spectra of compound **4** (2.0×10^{-5} M) in Tris-HCl buffer solution

(containing 5% DMSO) for 0 h.

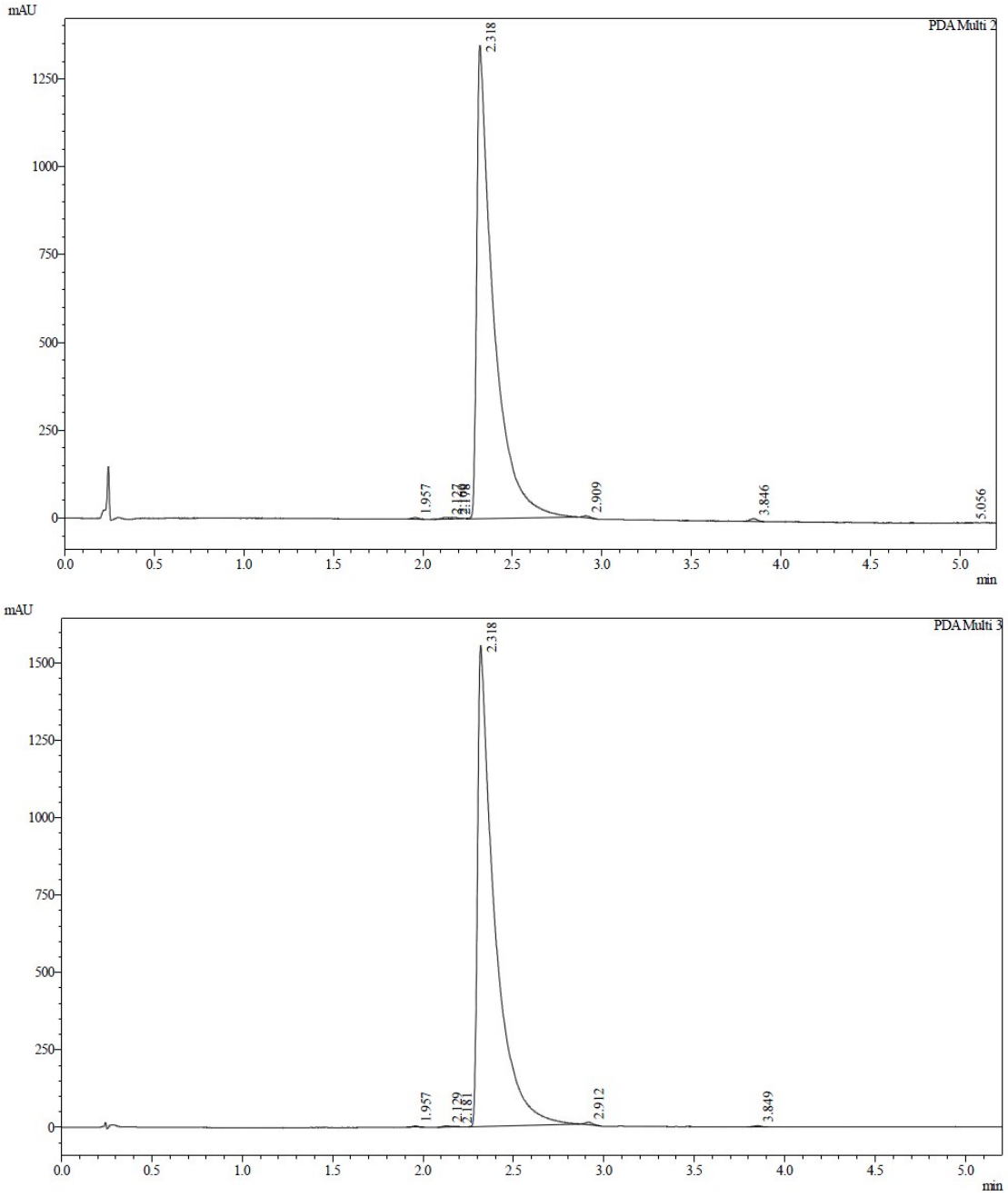


Fig. S5. LC-MS spectra for B-TFA (2.0×10^{-5} M) in TBS (Tris-HCl buffer solution, 10 mM, pH 7.35) solution with 0 h (top) and 48 h (down), respectively (λ= 250 nm). Column: Inertsustain C18 column (LC-20AT, SPD-20A HPLC COLUMN, 150mm×5.0 μm I.D.). Column temperature: 40ºC. Mobile phase: methol/H_2O containing 0.01% TFA (90:10 methol/H_2O). Flow rate: 1.0 mL/min. Injection volume: 2.0×10^{-5} M.

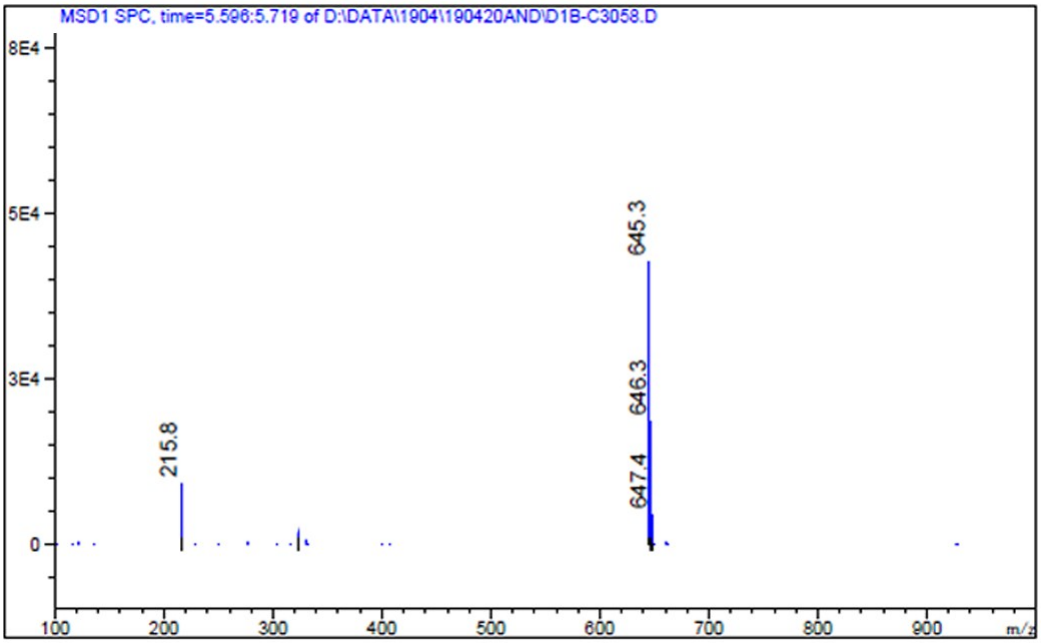


Fig. S6. The mass spectra of B-TFA (2.0×10^{-5} M) in Tris-HCl buffer solution (containing 5% DMSO) for 0 h.

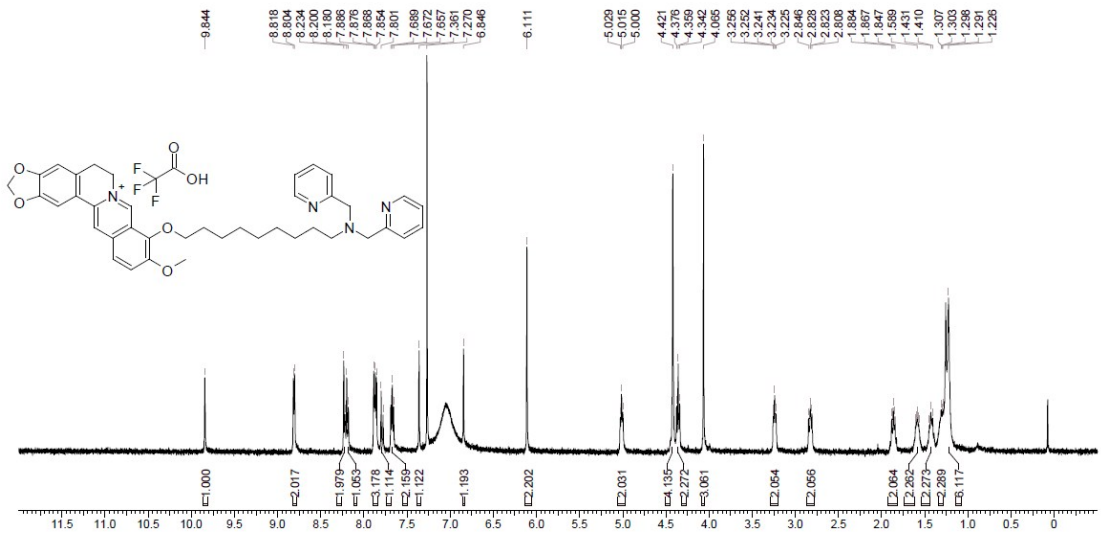


Fig. S7. ^{1}H NMR (400MHz, $CHCl_3$-d) for B-TFA.

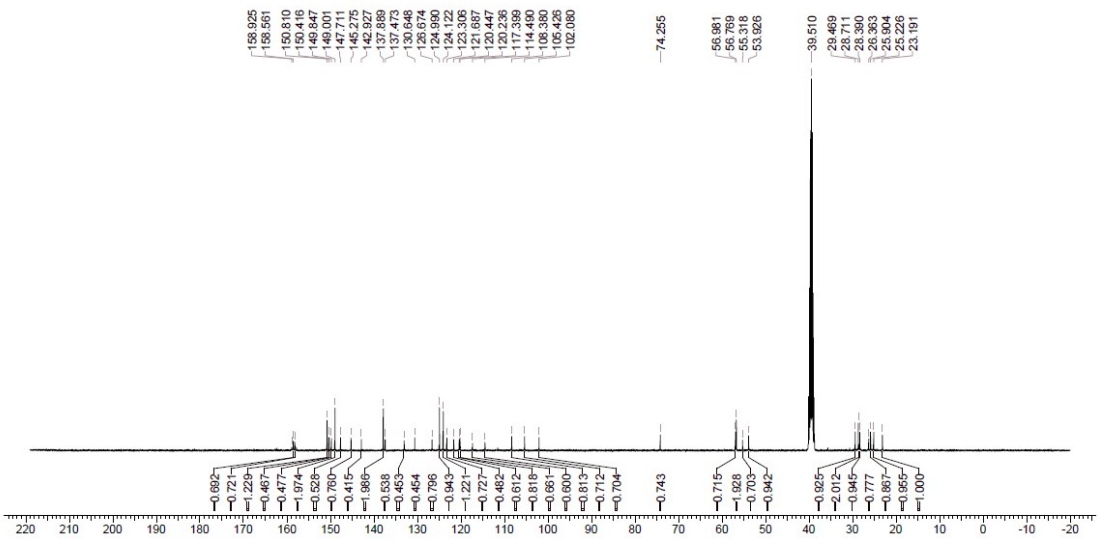


Fig. S8. ^{13}C NMR (400MHz, DMSO-d6) for B-TFA.

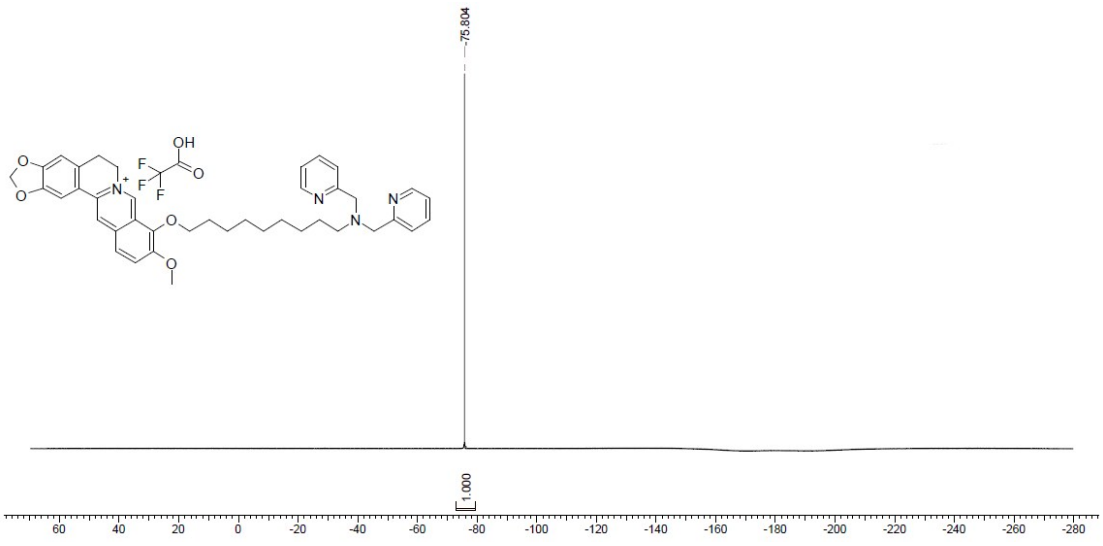


Fig. S9. ^{19}F NMR (471 MHz, $CHCl_3$-d) for B-TFA.

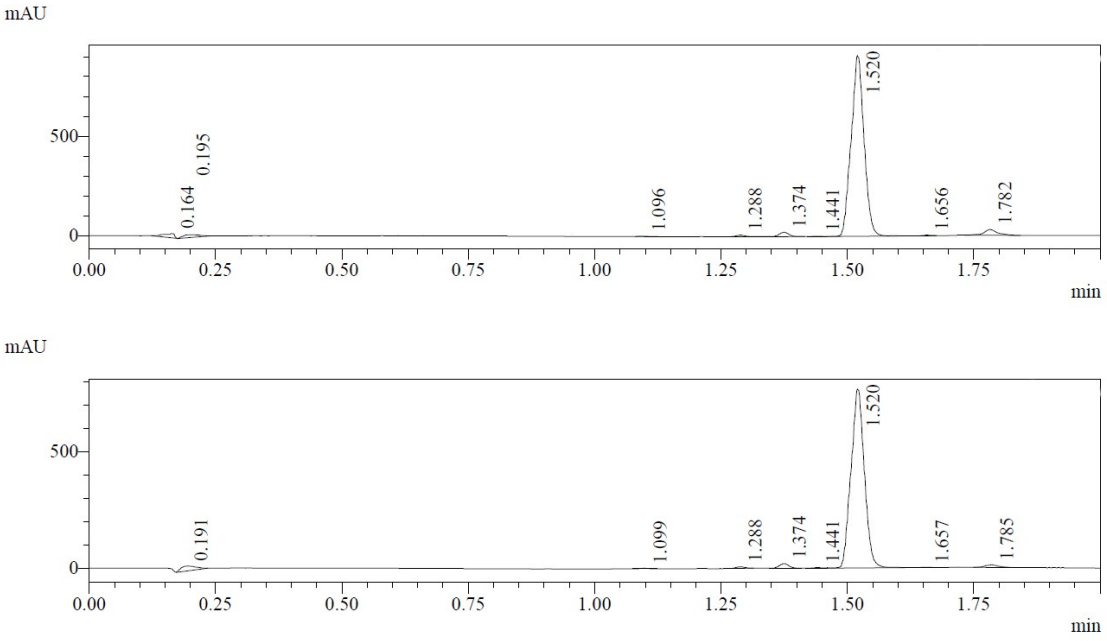


Fig. S10. LC-MS spectra for compound **8** (2.0×10^{-5} M) in TBS (Tris-HCl buffer solution, 10 mM, pH 7.35) solution with 0 h (top) and 48 h (down), respectively (λ= 250 nm). Column: Inertsustain C18 column (LC-20AT, SPD-20A HPLC COLUMN, 150mm×5.0 μm I.D.). Column temperature: 40ºC. Mobile phase: methol/H_2O containing 0.01% TFA (90:10 methol/H_2O). Flow rate: 1.0 mL/min. Injection volume: 2.0×10^{-5} M.

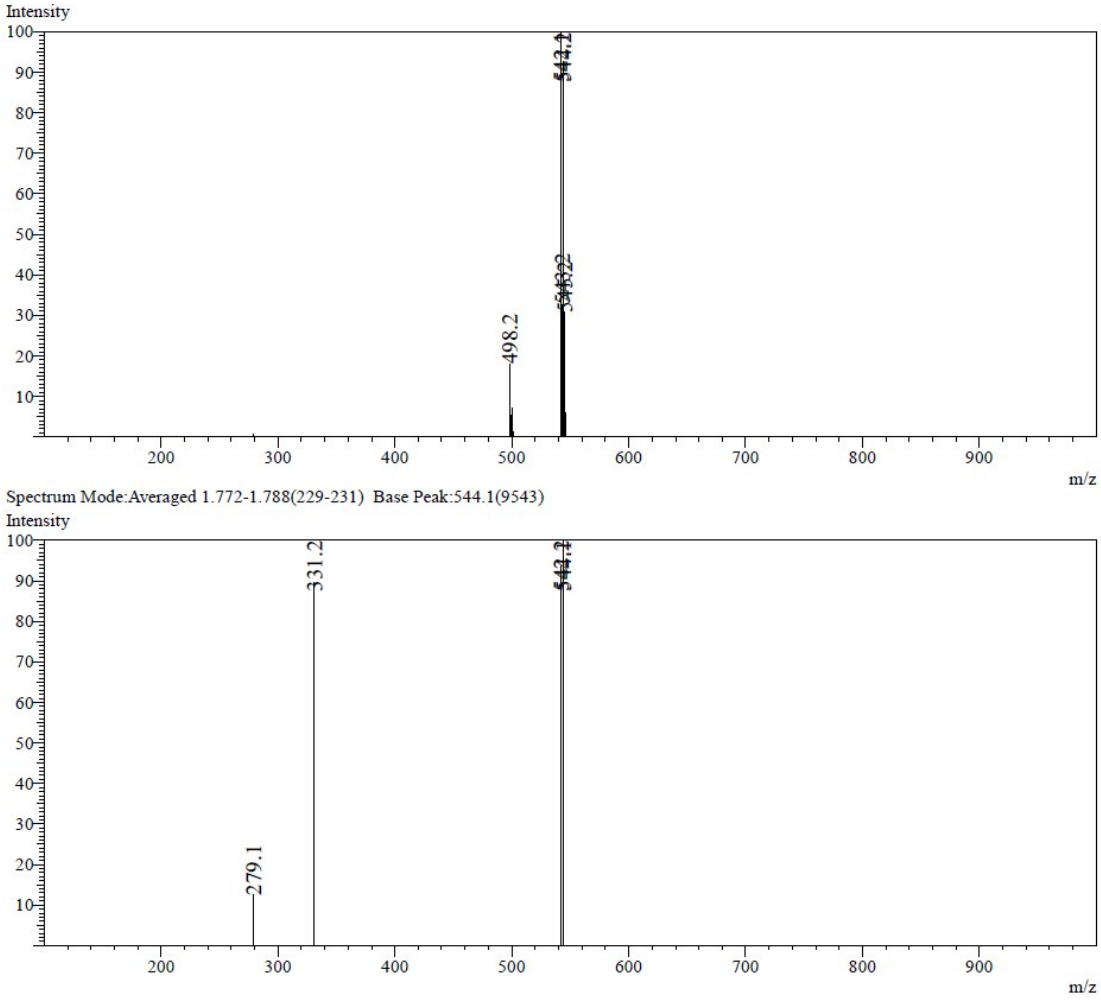


Fig. S11. The mass spectra of compound **8** (2.0×10^{-5} M) in Tris-HCl buffer solution (containing 5% DMSO) for 0 h (up) and 48 h (down), respectively.

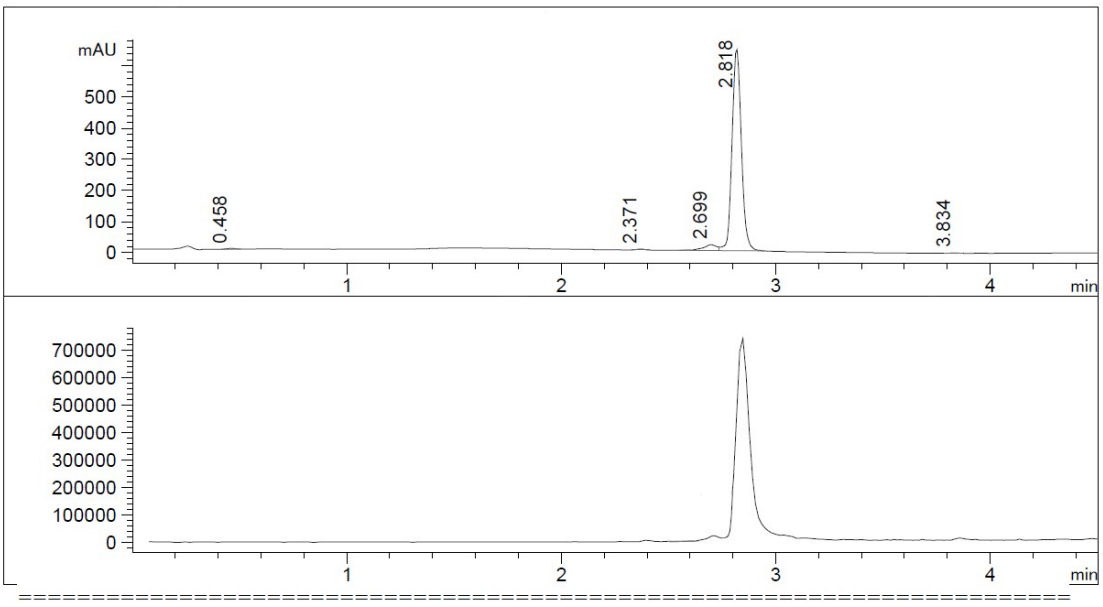


Fig. S12. LC-MS spectra for compound J-TFA (2.0×10^{-5} M) in TBS (Tris-HCl buffer solution, 10 mM, pH 7.35) solution with 0 h (top) and 48 h (down), respectively (λ= 250 nm). Column: Inertsustain C18 column (LC-20AT, SPD-20A HPLC COLUMN, 150mm×5.0 μm I.D.). Column temperature: 40ºC. Mobile phase: methol/H_2O containing 0.01% TFA (90:10 methol/H_2O). Flow rate: 1.0 mL/min. Injection volume: 2.0×10^{-5} M.

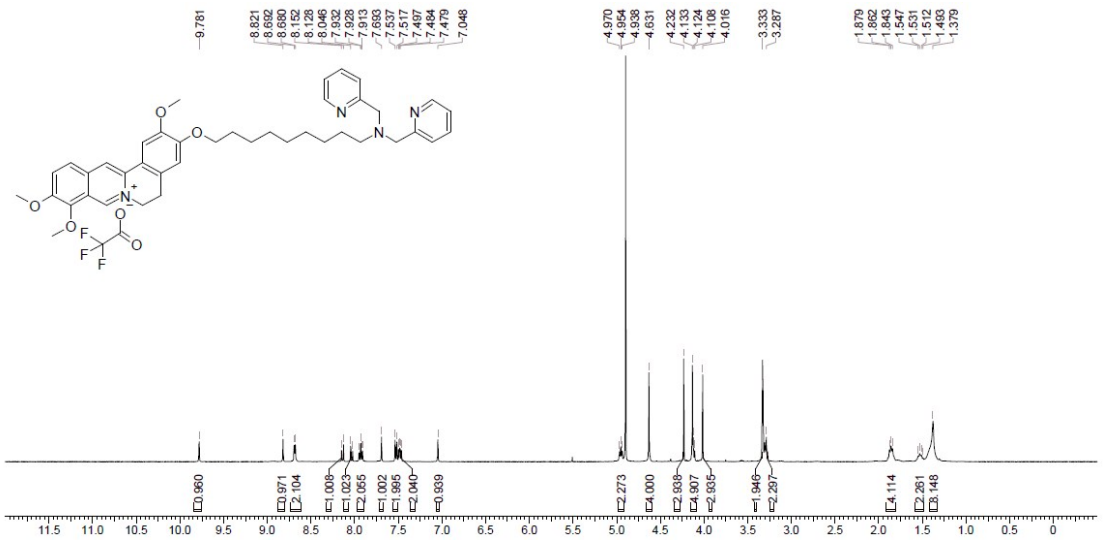


Fig. S13. ^{1}H NMR (400MHz, CH_3OH-d_4) for J-TFA.

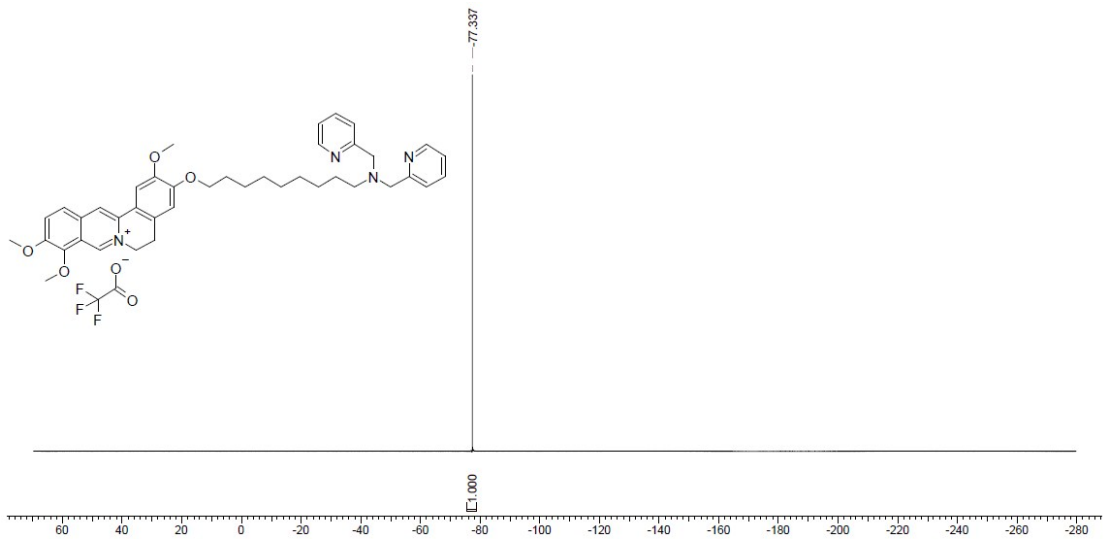
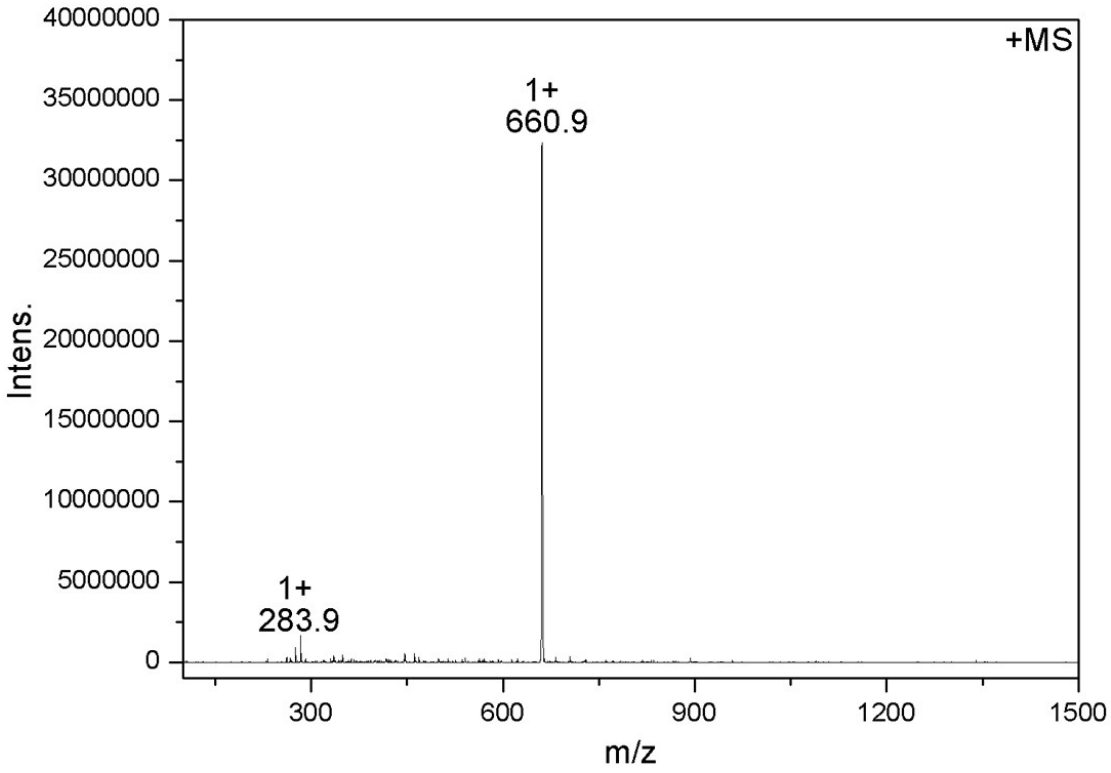


Fig. S14. ^{19}F NMR (471 MHz, CH_3OH-d_4) for J-TFA.



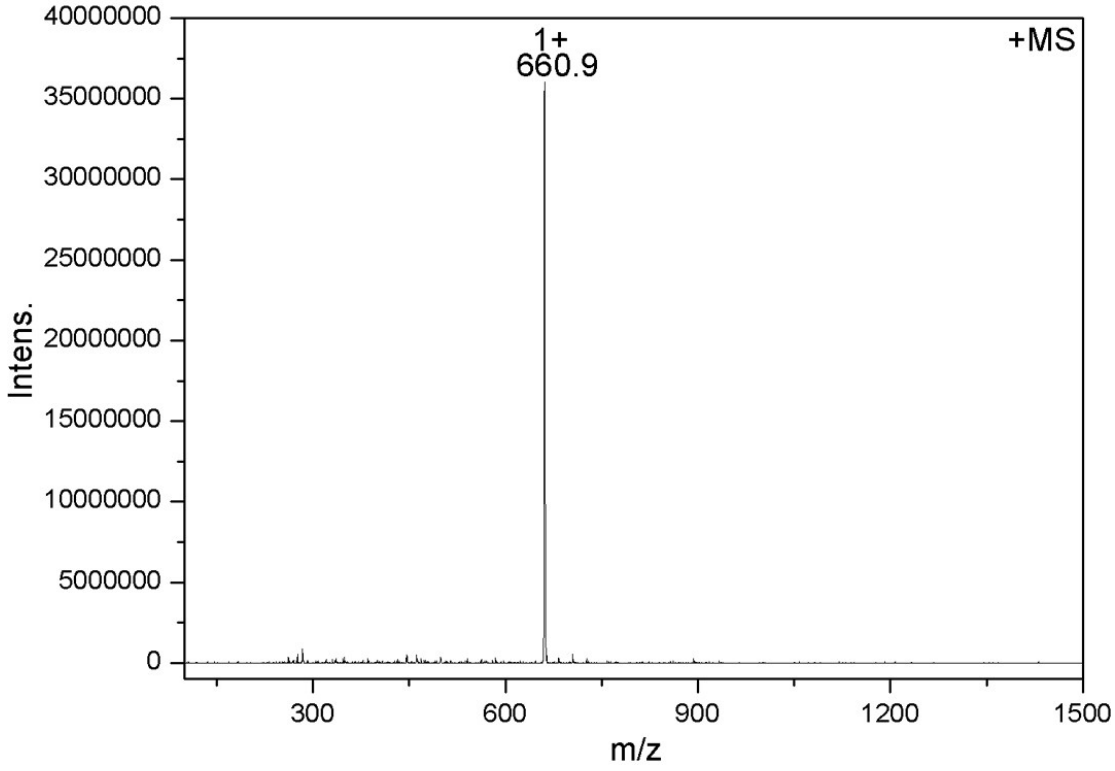
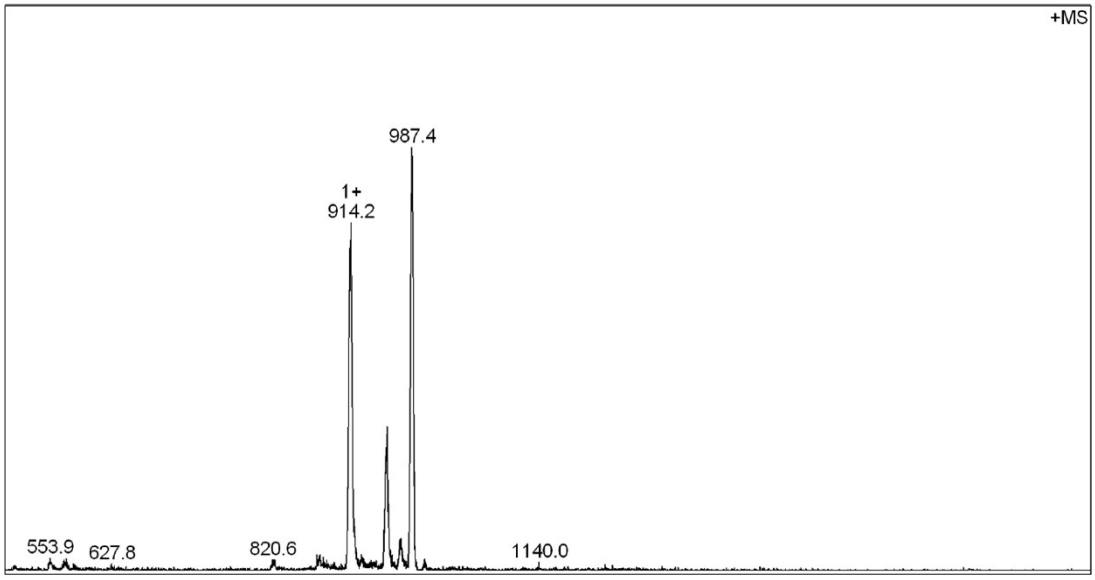


Fig. S15. The mass spectra of J-TFA (2.0×10^{-5} M) in Tris-HCl buffer solution (containing 5% DMSO) for 0 h (top) and 48 h (down), respectively.



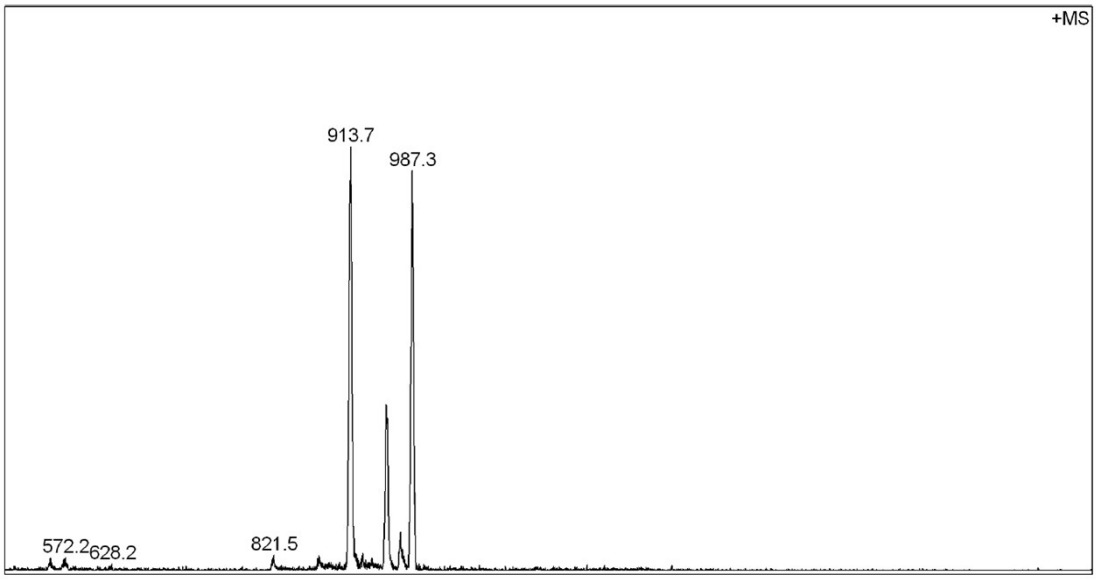
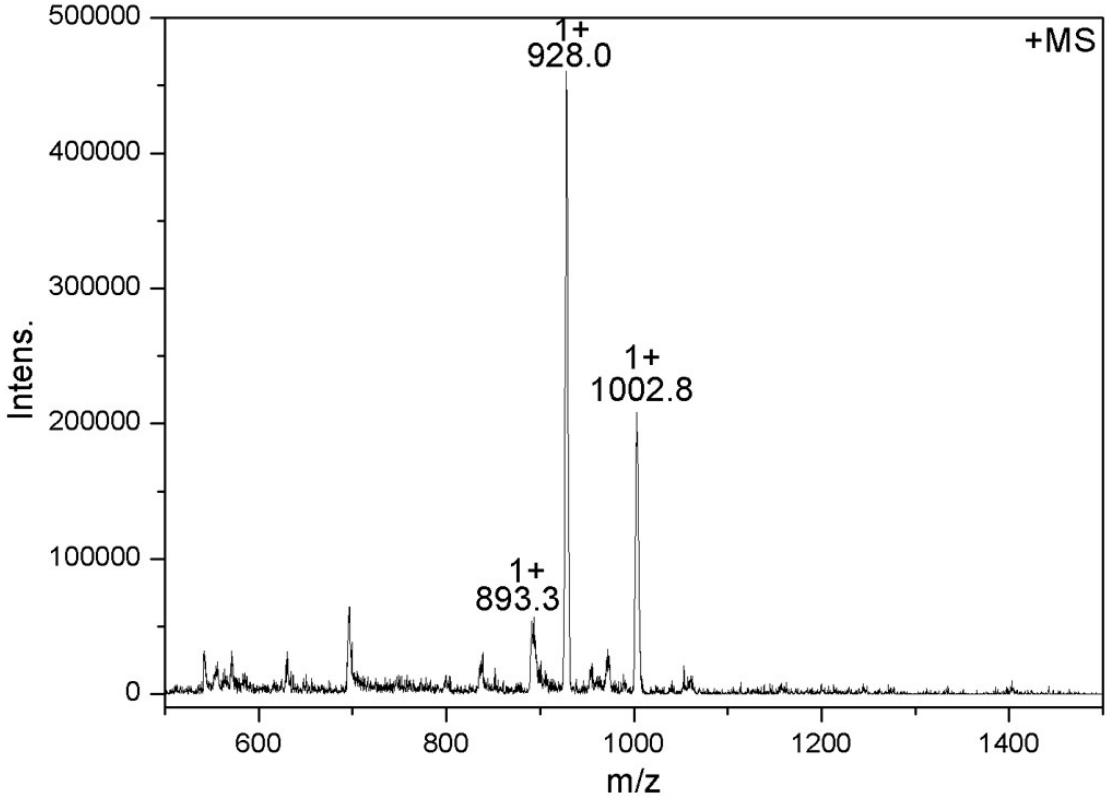


Fig. S16. The mass spectra of **Pt1** (2.0×10^{-5} M) in Tris-HCl buffer solution (containing 5% DMSO) for 0 h (top) and 48 h (down), respectively.



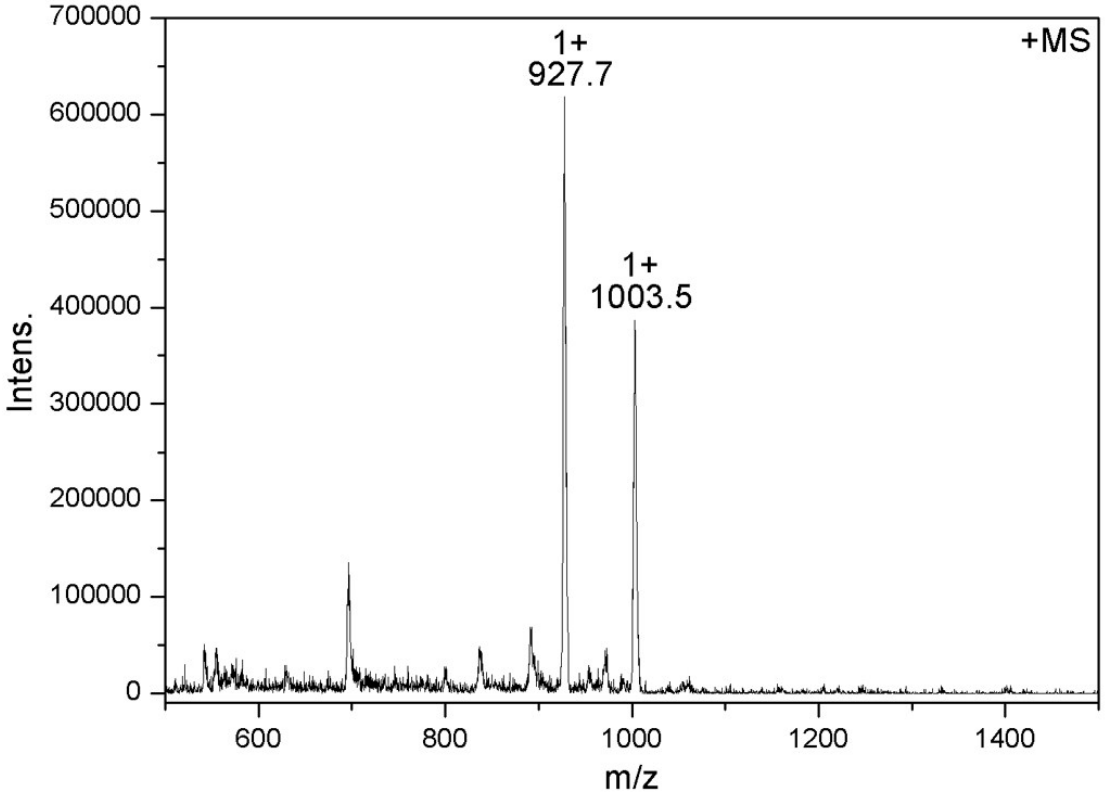


Fig. S17. The mass spectra of **Pt2** (2.0×10^{-5} M) in Tris-HCl buffer solution (containing 5% DMSO) for 0 h (top) and 48 h (down), respectively.

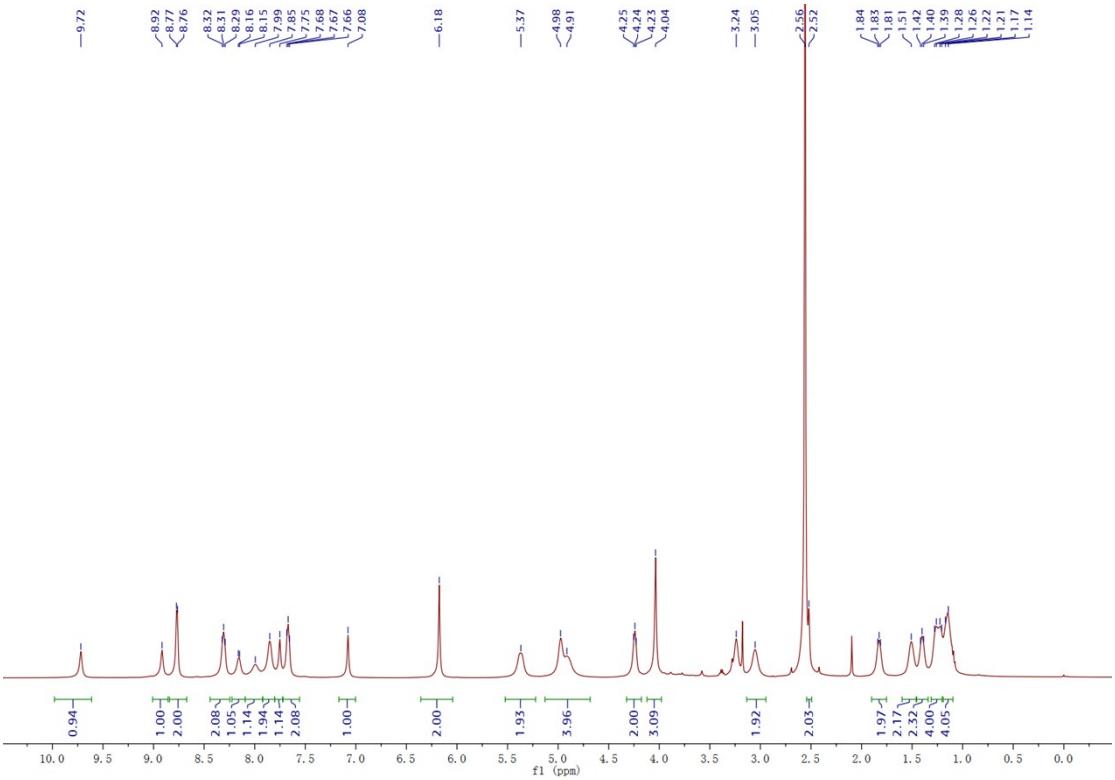


Fig. S18. ^{1}H NMR (500MHz, DMSO-d_6) for **Pt1.**

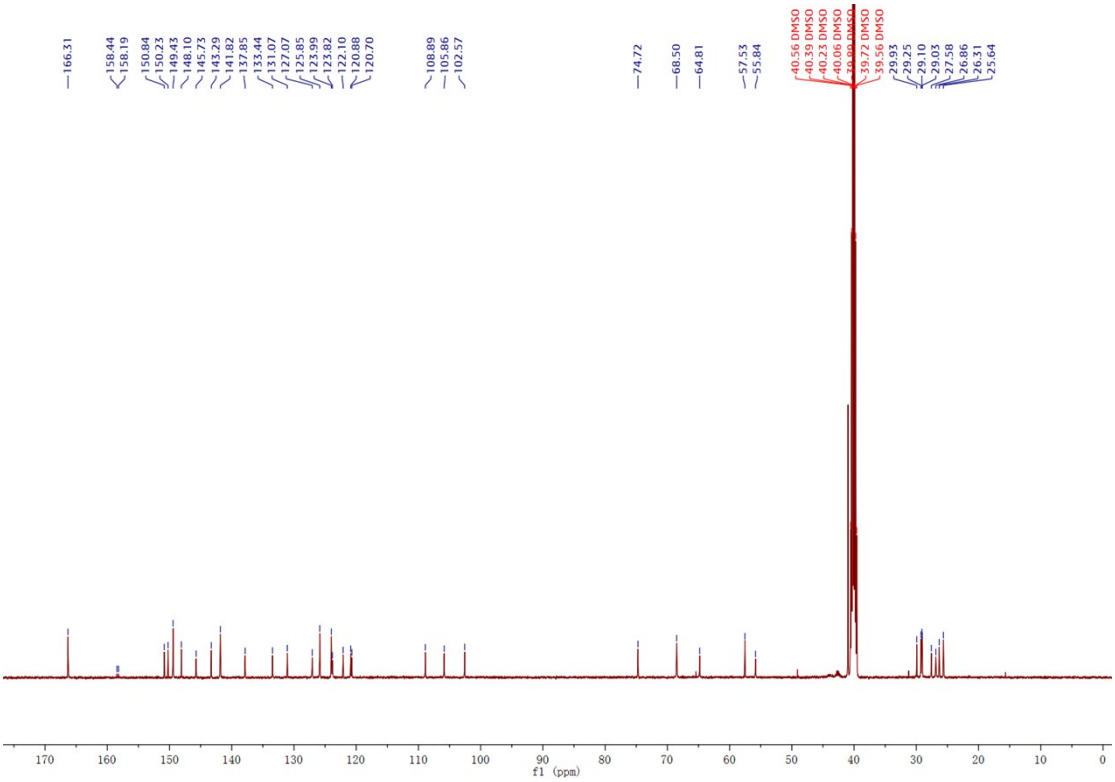


Fig. S19. ^{13}C NMR (500MHz, DMSO-d_6) for **Pt1.**

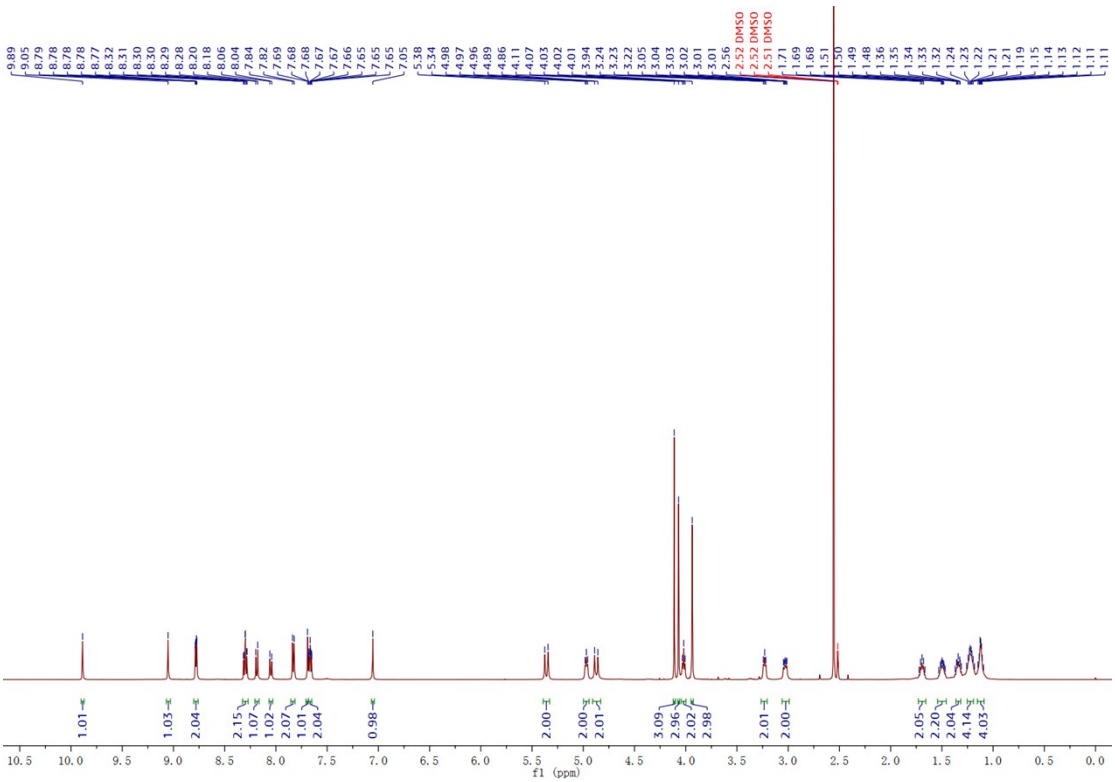


Fig. S20. ^{1}H NMR (500MHz, DMSO-d_6) for **Pt2.**

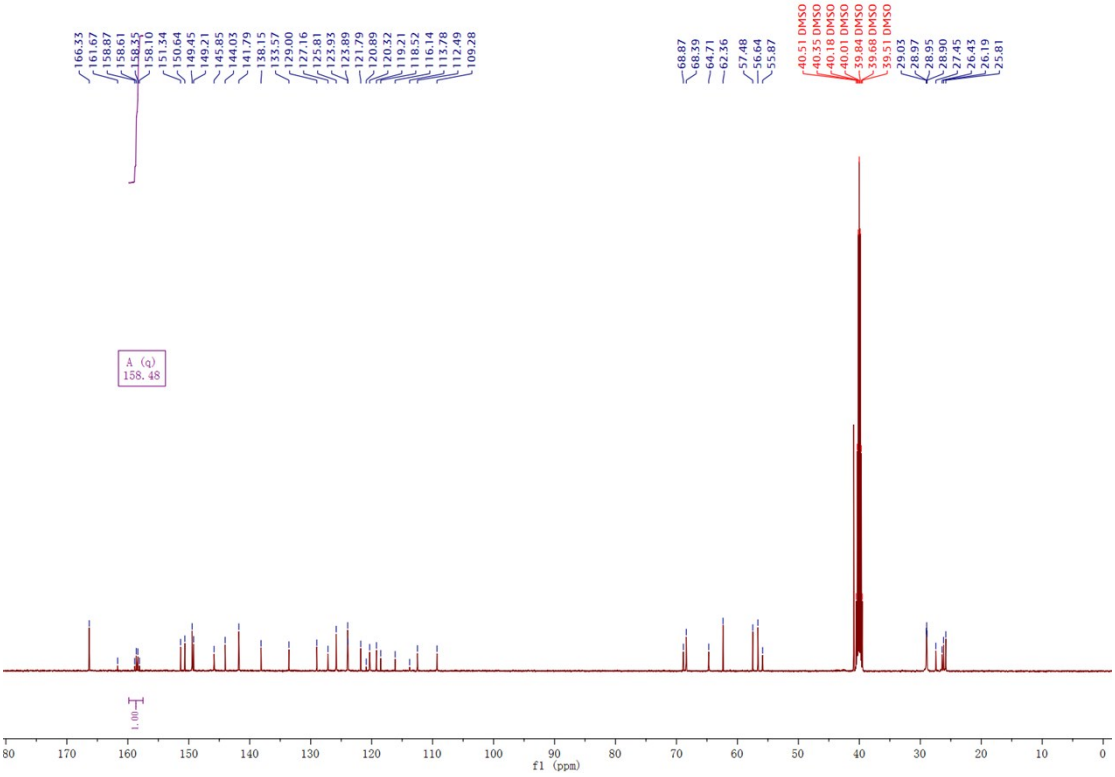


Fig. S21. ^{13}C NMR (500MHz, DMSO-d_6) for **Pt2.**

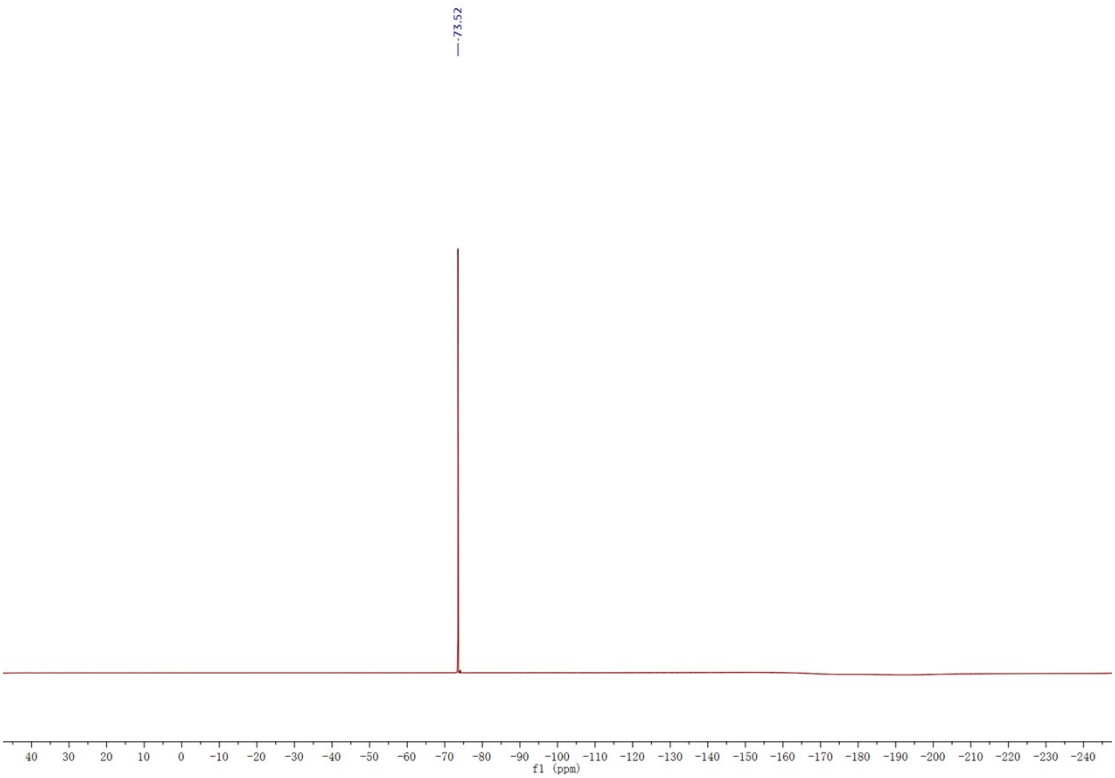


Fig. S22. ^{19}F NMR (471 MHz, DMSO-d_6) for **Pt1.**

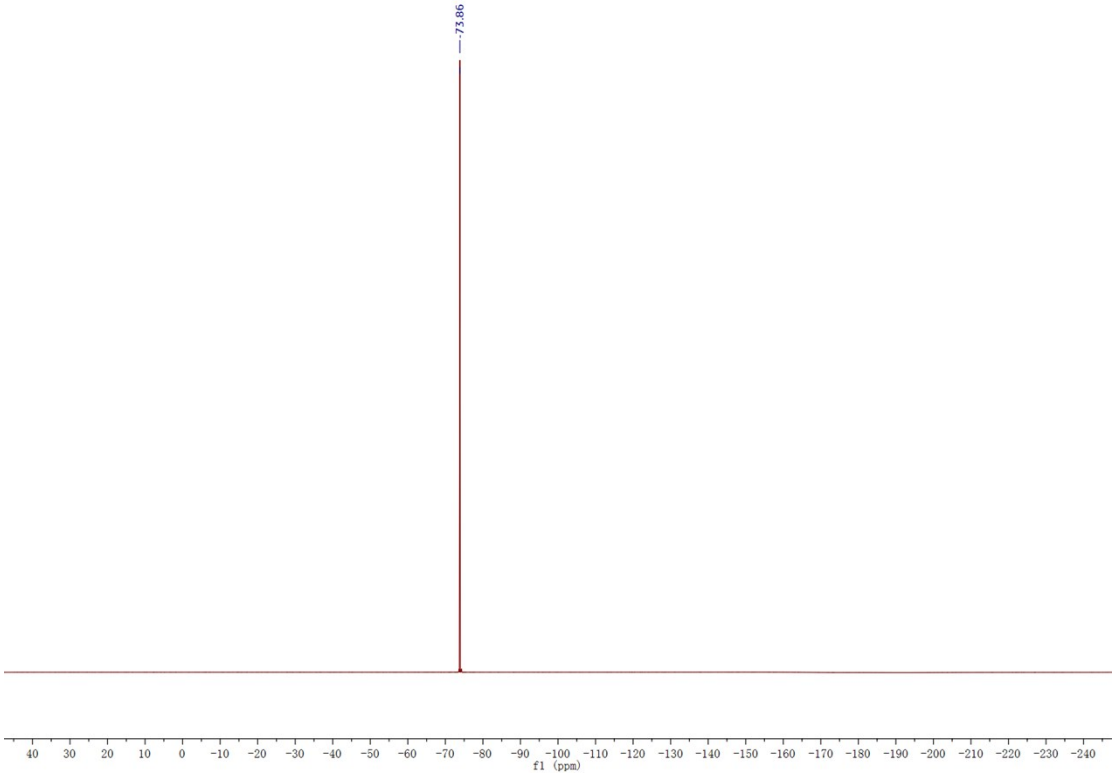


Fig. S23. ^{19}F NMR (471 MHz, DMSO-d_6) for **Pt2.**

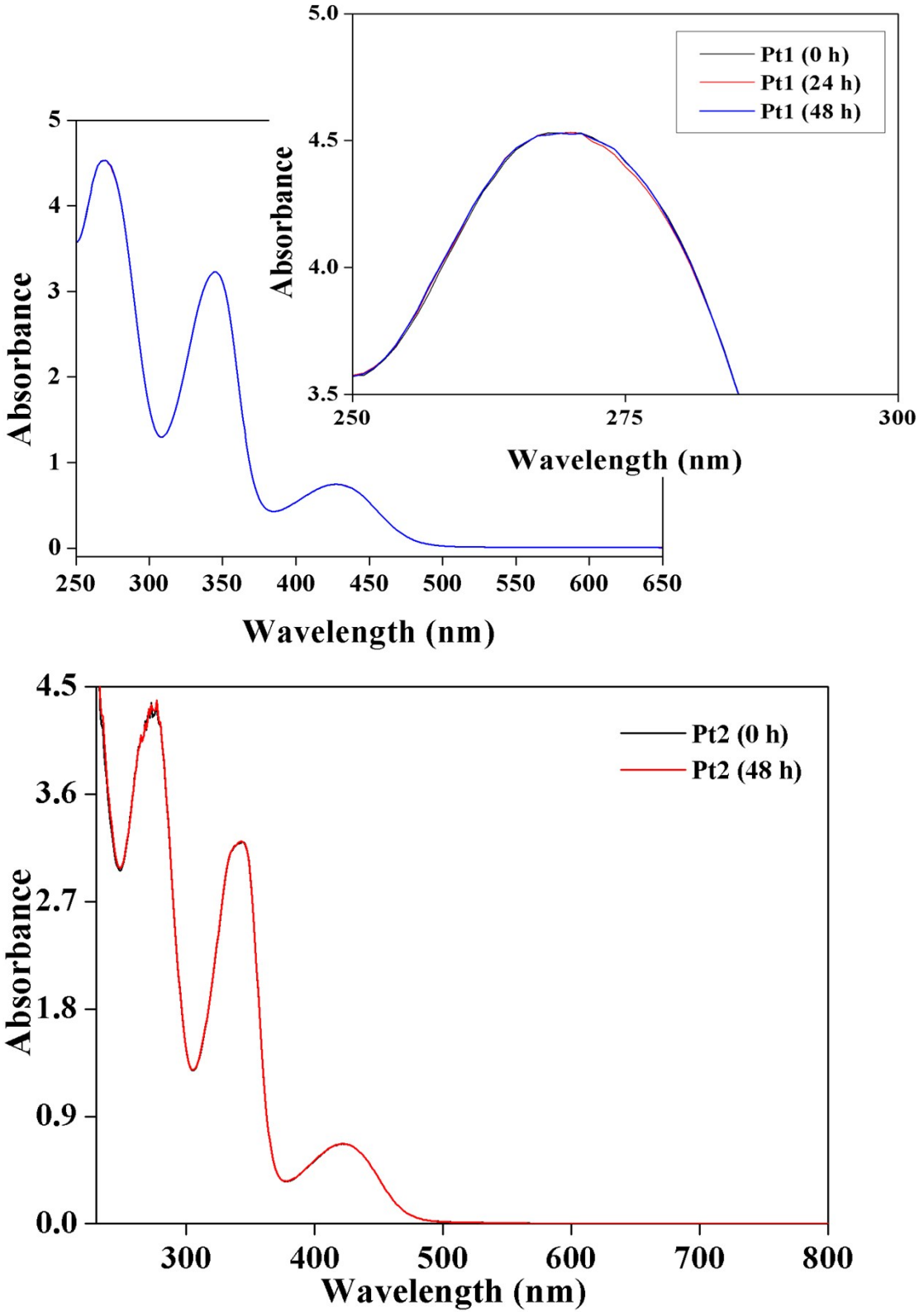
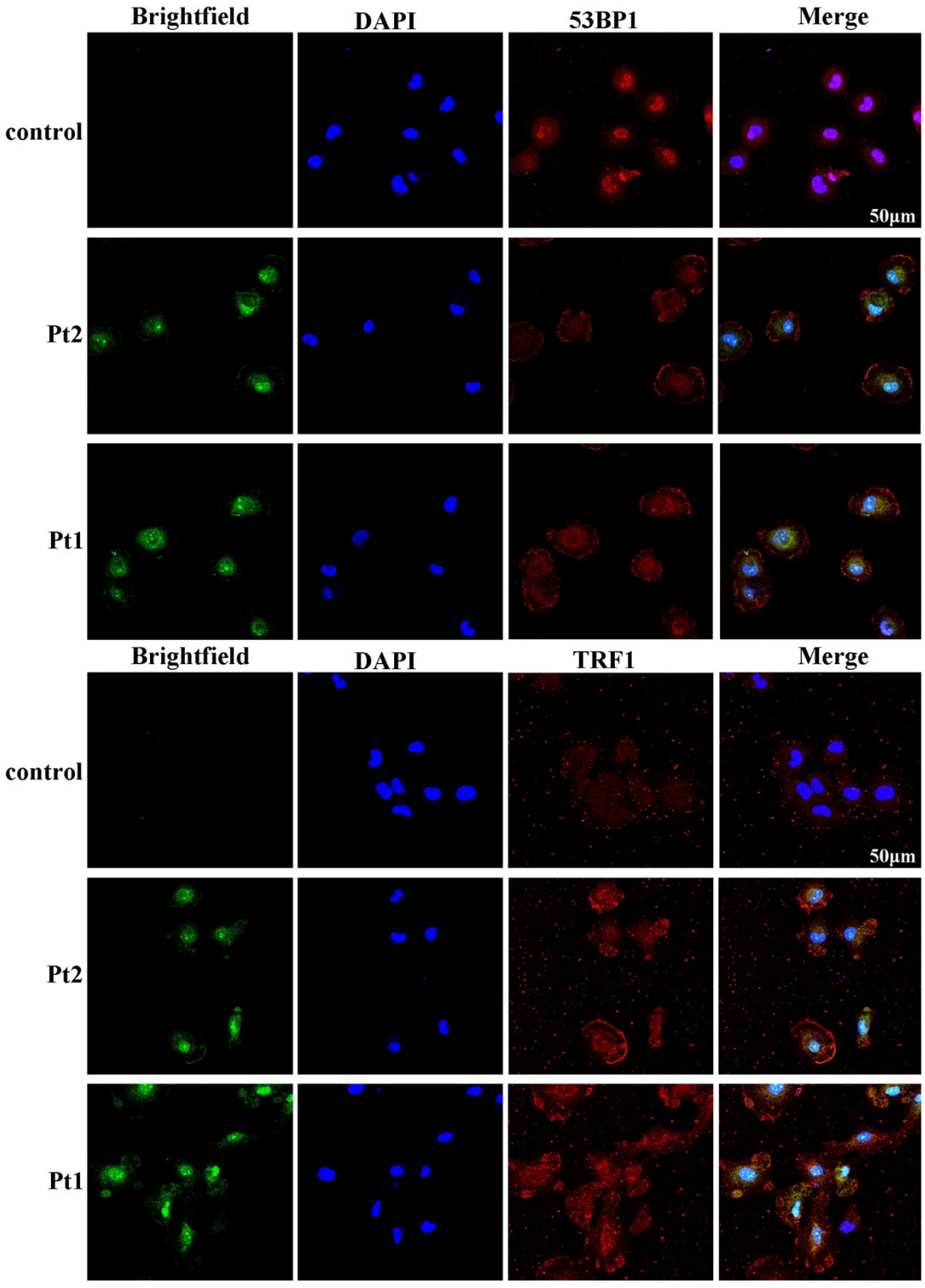


Fig. S24. UV-Vis absorption spectra of **Pt1** and **Pt2** (2.0×10^{-5} M) in TBS (Tris-HCl buffer solution, 10 mM, pH 7.35) solution in the time course 0, 24 and 48 h.

Table S1. Cellular distribution of **Pt2** (10.0 nM), cisplatin (10.4 μM) and **Pt1** (100.0 nM) in T-24 cancer cells after 6.0 h of incubation.

	total	nuclear fraction	mitochondrial fraction
Pt1	(15.06±0.03 ng of Pt)/10^6 cells	(5.39±0.10 ng of Pt)/10^6 cells	(6.11±0.05 ng of Pt)/10^6 cells
Pt2	(31.27±0.05 ng of Pt)/10^6 cells	(15.49±0.03 ng of Pt)/10^6 cells	(7.18±0.10 ng of Pt)/10^6 cells
cisplatin	(5.25±0.03 ng of Pt)/10^6 cells	(0.72±0.05 ng of Pt)/10^6 cells	(2.94±0.10 ng of Pt)/10^6 cells


Brightfield
DAPI
53BP1
Merge
control
50μm
Pt2
Pt1
Brightfield
DAPI
TRF1
Merge
control
50μm
Pt2
Pt1

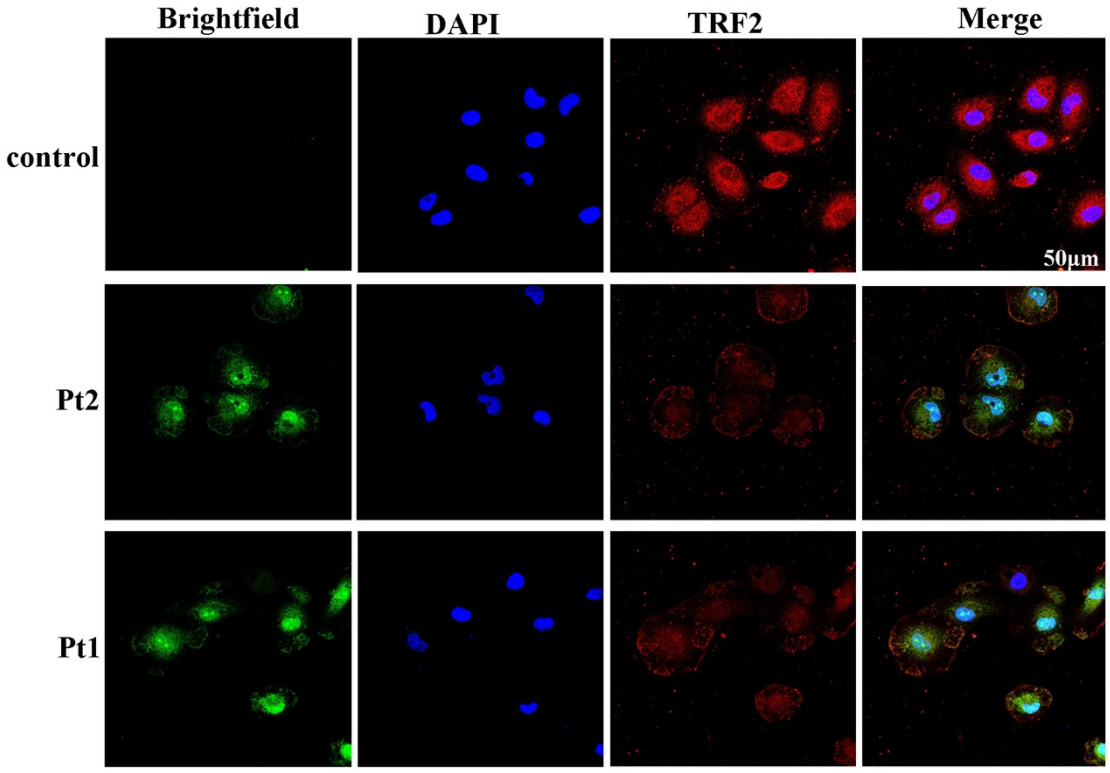


Fig. S25. Pt1 (100.0 nM) and **Pt2** (10.0 nM) induced telomere dysfunction in T-24 cells. The T-24 cells were incubated with **Pt1** (100.0 nM) and **Pt2** (10.0 nM) at 37 °C for 6 h, and then were processed for 53BP1, TRF2 and TRF1 (red). Excitation wavelength (λ_{ex}) of **Pt1** and **Pt2**: 490−495 nm; Emission filters (λ_{em}): 525−530 nm.

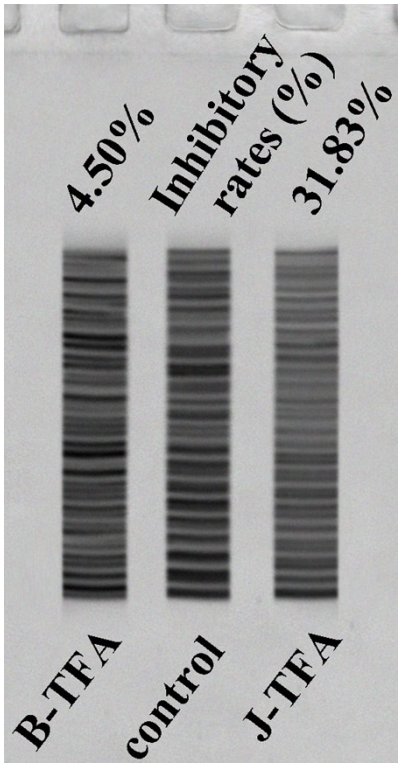


Fig. S26. B-TFA (18.7 μM) and J-TFA (13.2 μM) inhibited the expressions of telomerase in bladder T-24 tumor cells after treatment for 6.0 h.

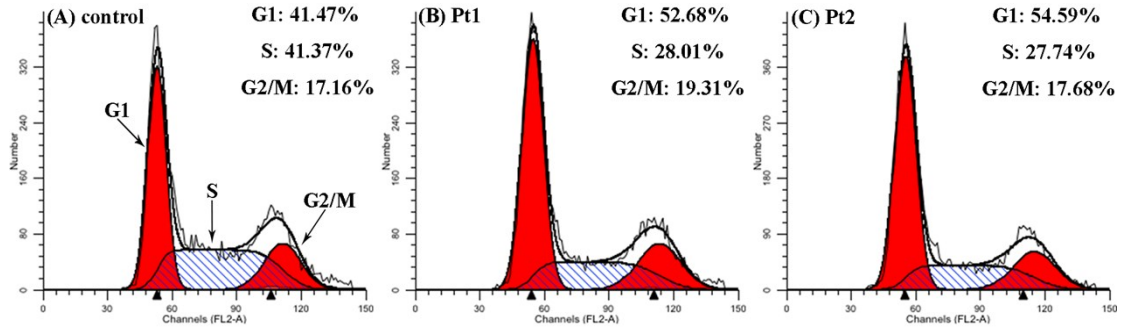


Fig. S27. Cell cycle arrest effect in T-24 cells after **Pt1** (100.0 nM) and **Pt2** (10.0 nM) treatment for 6.0 h.

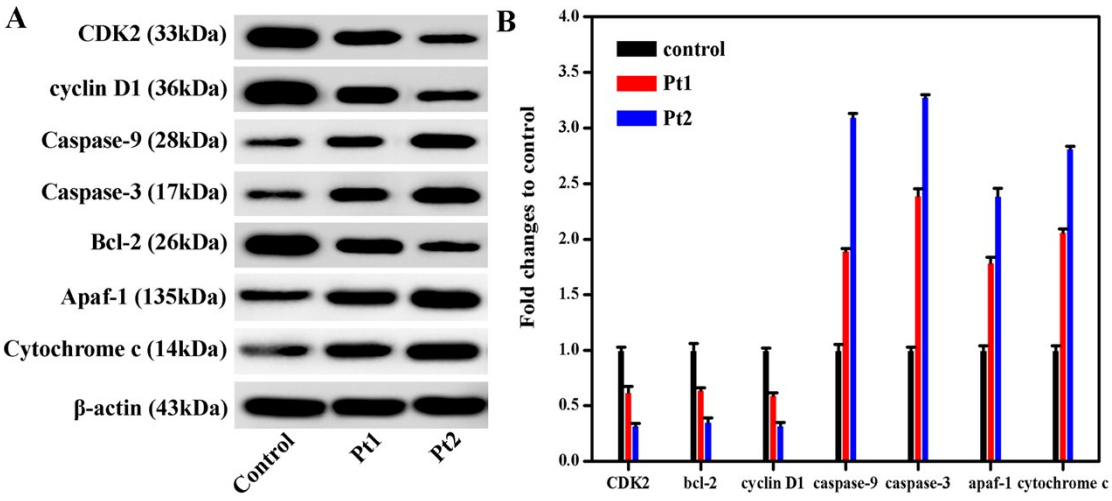


Fig. S28. (A) Western blot analysis to detect the levels of G1 phase- and apoptosis-related proteins after **Pt1** (100.0 nM) and **Pt2** (10.0 nM) treatment for 6.0 h. (B) The whole cell extracts were prepared and analyzed by Western blot analysis using antibodies against the related proteins. The same blots were stripped and reprobed with a β-actin anti-body to show equal protein loading. Western blotting bands from three independent measurements were quantified with Image J (B). The control group cells were treated with vehicle (1% DMSO).

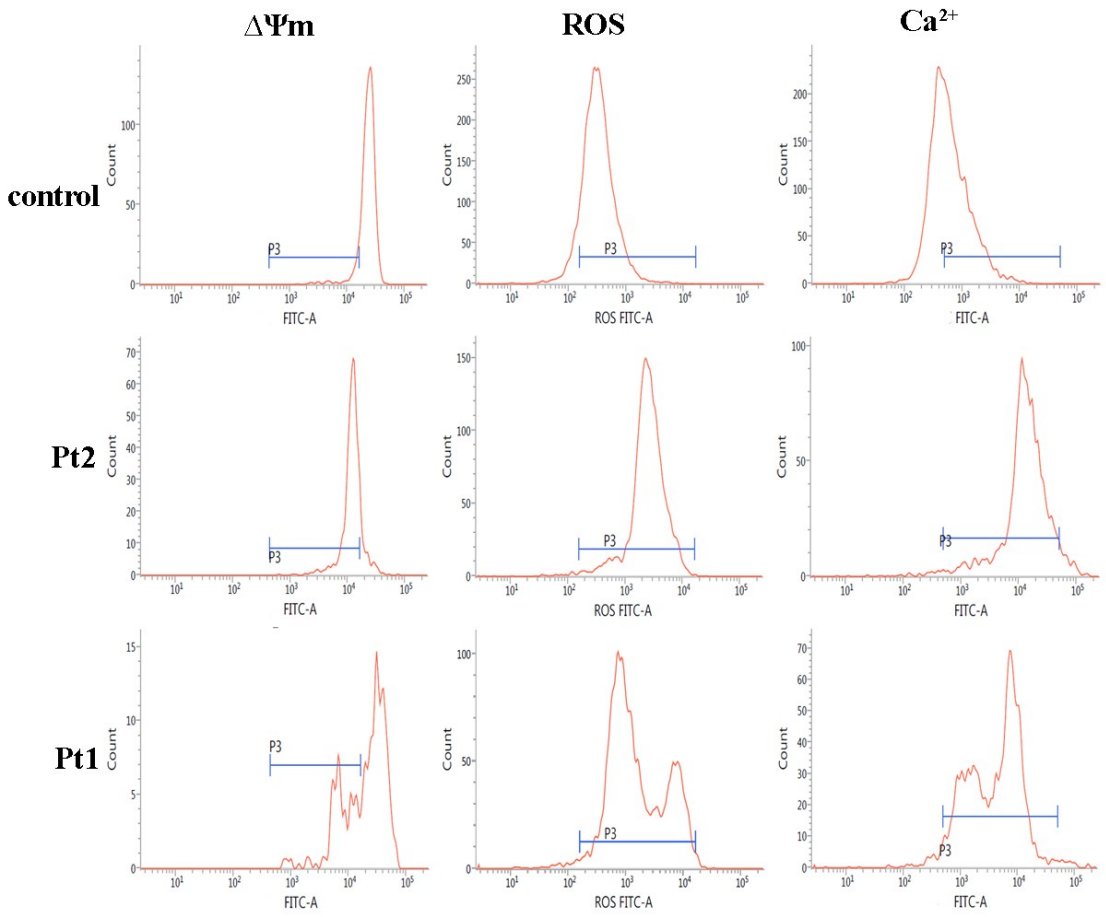


Fig. S29. Pt1 (100.0 nM) and **Pt2** (10.0 nM) decreased and increased the mitochondrial membrane potential (ΔΨm), ROS (reactive oxygen species) and [Ca^{2+}] level of T-24 cells at 6.0 h. The control group T-24 cells were treated with vehicle (1% DMSO).

Table S2. The tumor volume in treated and non-treated mice from the date of surgery to the study end point in the T-24 xenograft model.

Group	Tumor Volume (mm^3)		T/C
	(start)	(end)	(%)
control	84.0±12.4	875.0±146.0	-
cisplatin (2.0 mg/kg)	83.8±13.3	524.9±71.8	59.6[a]
Pt1 (2.0 mg/kg)	83.8±14.7	630.6±132.0	72.2[a]
Pt2 (2.0 mg/kg)	84.1±7.1	237.8±23.5	44.2[a]

[a] mean $p<0.05$, *p vs* vehicle control

Table S3. Average body weight in treated and non-treated mice from the date of surgery to the study end point in the T-24 xenogfart model.

Group	Body Weight (g)		RBW (%)
	(start)	(end)	(end)
control	19.3±0.7	20.5±0.7	106.2
cisplatin (2.0 mg/kg)	19.1±0.8	20.1±1.0	105.2
Pt1 (2.0 mg/kg)	19.1±1.3	20.2±1.5	105.8
Pt2 (2.0 mg/kg)	19.1±0.7	20.4±1.1	106.8

Table S4. In vivo anticancer activity of **Pt1** and **Pt2** toward T-24 tumor xenograft.

Group	average tumor weight(mean ± SD g)	inhibition of tumor growth(%)
control	0.77±0.13	-
cisplatin (2.0 mg/kg)	0.48±0.06	37.1[a]
Pt1 (2.0 mg/kg)	0.57±0.10	26.4[a]
Pt2 (2.0 mg/kg)	0.35±0.04	54.0[a]

[a] mean $p<0.05$, *p vs* control.

Experimental section

1 The stability of Pt1 and Pt2

The solution behaviour of **Pt1** and **Pt2** (2.0×10^{-5} M) in 10 mM Tris-HCl buffer (pH= 7.35) was further studied by ESI-MS spectra and UV-Vis absorption spectra. As shown in Fig. S24, the time-dependent (at 0 h, 24h and 48 h) UV−vis spectra of **Pt1** and **Pt2** (2.0×10^{-5} M) indicated that **Pt1** and **Pt2** were stable in Tris-HCl buffer for 48 h at 37 °C. At present, the ESI-MS characterization of **Pt1** and **Pt2** (2.0×10^{-5} M) in 10 mM Tris-HCl buffer revealed maximum abundance at for m/z = 987.4 for $[M\text{-}Cl]^+$ and m/z = 928.0 for $[M\text{-}(TFA\text{-}H)]^+$, respectively (Figs. S16 and S17). Taken all together, the results indicated that **Pt1** and **Pt2** (2.0×10^{-5} M) were stable in Tris-HCl buffer for 48 h at 37 °C.

2. The antitumor mechanism of Pt1 and Pt2

2.1 Cell Culture

All the human cells were grown in DMEM medium, both containing heat-inactivated fetal calf serum (FCS, Sigma, USA) (10%) and antibiotics (penicillin/streptomycin) at 37 °C and CO_2 (5%).

2.2 MTT assay

Cytotoxicity was determined using the MTT assay (MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2Htetrazolium bromide). Cells were seeded in 96-well plates as monolayers with 100 μL of cell solution (approximately 20000 cells) per well and preincubated for 24 h in medium supplemented with 10% FCS. The two jatrorrhizine and berberine derivatives B-TFA and J-TFA ligands, their Pt(II) complexes **Pt1** and **Pt2**, and cisplatin, were prepared as 0.5% DMSO solution. Then 100 μL of each compound, and cisplatin solution was added to each well and the plates were incubated for another 6.0 h. Subsequently, MTT (5.0 mg/mL solution) was added to the cells and the plates were incubated for a further 4 h. The culture medium was aspirated, and the purple formazan crystals formed by the mitochondrial dehydrogenase activity of vital cells were dissolved in DMSO. The optical density,

directly proportional to the number of surviving cells, was quantified at 540 nm using a multiwell plate reader and the fraction of surviving cells was calculated from the absorbance of untreated control cells.

2.3 Cell cycle analysis

HeLa cancer cells were seeded in 6-well plates (3×10^5 cells/well) and incubated in the presence or absence of **Pt1** (100.0 nM) and **Pt2** (10.0 nM) for 6.0 h. Then, cells were harvested by centrifugation and fixed in ice-cold 70% ethanol overnight. After the ethanol was removed the next day, the cells were resuspended in the ice-cold PBS and treated with RNase A (Keygen Biotech, China) at 37 °C for 30 min, followed by incubated with the DNA staining solution propidium iodide (PI) (Keygen Biotech, China) at 4.0 °C for 30 min. About 10,000 events were detected by flow cytometry (BECKMAN-COULTER) at 488 nm. The data regarding the number of cells in different phases of the cell cycle were analyzed by EXPO32 ADC analysis software.

2.4 Apoptosis analysis

HeLa cells were seeded in 6-well plates (3×10^5 cells/well) and incubated in the presence or absence of **Pt1** (100.0 nM) and **Pt2** (10.0 nM) for 6.0 h to induce T-24 cancer cell apoptosis. After incubation, the cancer cells were harvested and incubated with 5.0 μL of Annexin-V/FITC (Keygen Biotech, China) in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$ at pH 7.4) at room temperature for 30.0 min. PI solution was then added to the medium for another 5.0 min of incubation. Almost 10,000 events were collected for each sample and analyzed by flow cytometry (BECKMAN-COULTER). The percentage of apoptotic cells was calculated with EXPO32 ADC Analysis software.

2.5 Immunofluorescence assay

After treated with **Pt1** (100.0 nM) and **Pt2** (10.0 nM) for 6.0 h, the T-24 cells were grown on polylysine-coated coverslips, rinsed in phosphate-buffered saline, fixed in cold methanol for 20 min, permeabilized for 10 min in 0.5% Triton X-100 on ice, and

blocked in 5% BSA for 30 min at room temperature. The coverslips were incubated with rabbit monoclonal anti-53BP1, mouse monoclonal anti-TRF1 or anti-TRF2 (Abcam) primary antibodies for 3.0 h at 25 °C. The coverslips were washed and incubated with fluorescein conjugated goat anti-mouse (1:1000) and rhodamine conjugated goat anti-mouse (1:1000) secondary antibodies. Finally, the cells stained with 0.1 mg/mL DAPI. Fluorescence images were captured using confocal microscopy (CarlZeiss LSM 710, Germany). Excitation wavelength (λ_{ex}) of **Pt1** and **Pt2**: 490−495 nm; Emission filters (λ_{em}): 525−530 nm.

2.6 Mitochondrial membrane potential (MMP) assay

A lipophilic cationic dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine (JC-1, Beyotime, China) was used to monitor the level of MMP in the T-24 cells. At normal state, the MMP is high, and JC-1 appears as aggregates, which is indicated by red fluorescence. However, when apoptosis occurs, the MMP reduced and JC-1 displayed as monomers, which is indicated by green fluorescence. For flow cytometry analysis, HeLa cancer cells were plated in 6-well plates (3×10^5 cells/well) and grown for 6.0 h and treated with **Pt1** (100.0 nM) and **Pt2** (10.0 nM) for 24 h. Then the cells were harvested by centrifugation and incubated with JC-1 solution for 30 min. After briefly washing, the proportion of green and red fluorescence intensity was immediately detected and analyzed by flow cytometry.

2.7 Western blot analysis

The T-24 cancer cells seeded in 60.0 mm dishes at a density of 6×10^5 cells/well were incubated with or without **Pt1** (100.0 nM) and **Pt2** (10.0 nM) for 6.0 h. After incubation, these cells were washed twice with ice-cold PBS and then lysed in RIPA lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, and 1 mM EDTA (Beyotime,

China). The lysates were incubated at 0 °C for 30 min and vortexed every 10.0 min intermittently, and then the total protein was harvested by centrifuging at 12,500 g for 35.0 min. After the protein concentrations were determined by a BCA Protein Assay Kit (Beyotime, China), the protein extracts were reconstituted in loading buffer containing 62 mM Tris-HCl, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol (Beyotime, China) and boiled at 100 °C for 5.0 min. An equal amount of the proteins (40.0 μg) was separated by 8−12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred to nitrocellulose membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). After blocking with 5% nonfat dried milk in TBS containing 1% Tween-20 for 90 min at room temperature, the membranes were incubated overnight with specific primary antibodies (CST, USA) at 4 °C. After three washes in TBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies at room temperature for 2.5 h. The blots were developed with enhanced chemiluminescence and were detected by an imager.

2.8 ***Measurement of reactive oxygen species (ROS) generation***

DCFH-DA is a freely permeable tracer specific for ROS. DCFH-DA can be deacetylated by intracellular esterase to the non-fluorescent DCFH which is oxidized by ROS to the fluorescent compound 2',7'-dichloroflorescein (DCF). Thus, the fluorescence intensity of DCF is proportional to the amount of ROS produced by the cells. 6×10^5 cells were exposed to **Pt1** (100.0 nM) and **Pt2** (10.0 nM) for 24 h. After exposure, the T-24 cells were harvested, washed once with ice-cold PBS and incubated with DCFH-DA (100 μM in a final concentration) at 37 °C for 30.0 min in the dark. Then the cells were washed again and maintained in 1 mL PBS. The ROS generation was assessed from 10,000 cells each sample by flow cytometry with excitation and emission wavelengths of 488 and 525 nm, respectively.

2.9 Intracellular free [Ca^{2+}] detection

The level of intracellular free [Ca^{2+}] is decided by using a fluorescent dye Fluo-3 AM which can across the cell membrane and be cut into Fluo-3 by intracellular esterase. The Fluo-3 can specifically combine with the [Ca^{2+}] and has a strong fluorescence with an excitation wavelength of 488 nm. After exposed to **Pt1** (100.0 nM) and **Pt2** (10.0 nM) for 6.0 h, respectively, the T-24 cells were harvested and washed twice with PBS, then resuspended in Fluo-3 AM (5.0 mM) for 30 min in dark. Detection of intracellular [Ca^{2+}] was carried by Flow cytometer at 525 nm excitation wavelength.

2.10 TRAP assay

Telomerase extract was prepared from HeLa cells. A modified version of the TRAP assay was used. PCR was performed in a final 50 μL reaction volume composed of reaction mix (45 μL) containing Tris-HCl (20 mM, pH 8.0), deoxynucleotide triphosphates (50 mM), $MgCl_2$ (1.5 mM), KCl (63 mM), EGTA (1 mM), Tween-20 (0.005%), BSA (20 μg/mL), primer HTG21 (3.5 pmol; 5'-G3ACHTUNGTRENUNG[T2AG3]3-3'), primer TS (18 pmol; 5'-AATCCG TCGAGC AGAGTT-3'), primer CXext (22.5 pmol; 5'-GTGCCCT TACCCTT ACCCTTA CCCTAA-3'), primer NT (7.5 pmol; 5'-ATCGCT TCTCGG CCTTTT-3'), TSNT internal control (0.01 amol; 5'-ATTCCG TCGAGC AGAGTT AAAAGG CCGAGA AGCGAT-3'), Taq DNA polymerase (2.5 U), and telomerase (100 ng). **Pt1** (100.0 nM) and **Pt2** (10.0 nM) or distilled water were added (5 μL). PCR was performed in an Eppendorf Master cycler equipped with a hot lid and incubated for 30 min at 30°C, followed by 92°C 30 s, 52°C 30 s, and 72°C 30 s for 30 cycles. After amplification, loading buffer (8 μL; 5×TBE buffer, 0.2% bromophenol blue, and 0.2% xylene cyanol) was added to the reaction. An aliquot (15.0 μL) was loaded onto a nondenaturing acrylamide gel (16%; 19:1) in 1×TBE buffer and resolved at 200.0 V

for 1.0 h. Gels were fixed and then stained with $AgNO_3$.

2.11 Animale used

The male and female KM mice, 5-6 weeks old, weight 20-23 g, six-week-old athymic BALB/cA nu/nufemale mice (18-20 g) were purchased from Guangxi Medical University Laboratory Animal Centre (Guangxi, China), the Acute toxicity studies and in vivo antitumor study were carried out in there. Animals were housed at a sterile environment with conditions of constant photoperiod (12 h light/ 12 h dark at 23-24 °C and 65-85% humidity).

In addition, T-24 xenograft mouse models were purchased from Beijing HFK Bioscience Co., Ltd (Beijing, China, approval No. SCXK 2014-004). The animal procedures were approved by the Institute of Radiation Medicine Chinese Academy of Medical Sciences (Tian Jin, China, approval No. SYXK 2014-0002). And all of the experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Animal experiments were approved by the Animal Care and Use Committee of Institute of Radiation Medicine Chinese Academy of Medical Sciences. In addition, statistical analysis and abbreviations used have been reported.

2.12 Anti-cancer activity toward T-24 in vivo

The T-24 cells were harvested and injected subcutaneously into the right flank of nude mice with 5×10^6 cells in 200 μL of serum-free medium. When the xenograft tumor growth to the volume about 1000 mm^3, the mice were killed and the tumor tissue were cut into about 1.5 mm^3 small pieces, and then transplanted into the right flank of female nude mice, When tumors reach a volume of 80-300 mm^3 on all mice, the mice were randomized into vehicle control and treatment groups (n=6/group), received the following treatments: (a) control, 5.0% v/v DMSO/saline vehicle, (b) **Pt1** at dose 2.0 mg/kg every two day (5.0% v/v DMSO/saline), (c) **Pt2** at dose 2.0 mg/kg every two

day (5.0% v/v DMSO/saline), (d) cisplatin at dose 2.0 mg/kg every two day (5.0% v/v DMSO/saline). The tumor volumes were determined every three days by measuring length (*l*) and width (*w*) and calculating volume, tumor volume and inhibition of tumor growth were calculated using formulas 1−3:

Tumor volume: $V = (w^2 \times l)/2$ (1)

The tumor relative increment rate: $T/C\ (\%) = T_{RTV}/C_{RTV} \times 100\%$ (2)

inhibition of tumor growth: $IR(\%) = (W_c - W_t)/W_c \times 100\%$ (3)

Where w and l mean the shorter and the longer diameter of the tumor respectively; T_{RTV} and C_{RTV} was the RTV of treated group and control group respectively. (RTV: relative tumor volume, $RTV = V_t / V_0$); W_t and W_c mean the average tumor weight of complex-treated and vehicle controlled group respectively.

2.13 Statistical analysis

The experiments have been repeated from three to five times, and the results obtained are presented as means ± standard deviation (SD). Significant changes were assesses by using Student's *t* test for unpaired data, and p values of <0.05 were considered significant.