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Determination of IC50 value of anticancer drug on cell by D_2O-single cell Raman spectroscopy

Wanxue lv,[a,b] Boqiang Fu, [b*] Manli Li, [b] Yu Kang,[a] Shouli Bai [a] and Chao Lu[a*]

Experimental Section

Chemicals and Materials.

DMEM/F12 -Dulbecco's Modified Eagle Medium, fetal bovine serum (FBS), 0.25% trypsin-EDTA (1×), phosphate buffered saline (PBS), DMEM/F-12 power and pencillin-streptomycin were purchased from Gibco; cisplatin and deuterium oxide(D_2O) were purchased from Sigma-Aldrich; Pacltaxel(Taxol) was purchased from Aladdin; Cell Counting Kit-8 (CCK-8) was purchased from GlpBio; 4% paraformaldehyde fix (PFA) solution was purchased from BOSTER; CellTiter-Glo® Luminescent Cell Viability Assay was purchased from Promega. A549 cells (Human Non-Small Cell Lung Cancer Cells), Code 1101HUM-PUMC000002, obtained from Bio-Medical Cell Resource Bank, China.

Instruments.

The CO_2 incubator (Model 3543) and Biosafety cabinet（Type A2, Model 1379）were obtained from Thermo (USA). The centrifuge, was purchased from Sartorius AG (Germany). CKX41SF Inverted microscope was obtained from Olympus Corporation (Japan); Centro LB 960 Microplate Luminometer and SPECTROstar Nano High flux ULTRAVIOLET spectrophotometer were obtained from BMG LABTECH(Germany). InVia Reflex Laser Micro-Raman spectrometer was purchased from Renishaw (United Kingdom).

Cell Culture.

A549 cells grew in DMEM-F12 culture medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in cell culture flask (25 cm^2) in a humidified 5% CO_2 atmosphere at 37 °C. When cells confluency reached to 80%-90%, the culture medium was removed from

culture flask. The cells were rinsed with PBS buffer twice to remove any floating cells, then added 1 mL of 0.25% trypsin-EDTA. After 2 minutes, the adherent cells were observed by inverted microscope to deform slowly from shuttle shape to round shape and to detach from bottom of the culture flask, then adding 1 mL medium to stop the enzymatic hydrolysis. Cell suspension was collected and centrifuged for 5 min at 1500 rpm. Cells were passaged every 3 -4 days until reaching 90% confluence.

Prepare Cell Culture Medium with D_2O.

Weigh 1.56 g DMEM/F-12 power and resolve in 10 mL sterile water. Add 1.2 mg/mL sodium bicarbonate ($NaHCO_3$) solution to the medium to adjust the pH value to 7.0-7.2. The medium solution was filtered immediately into a sterile container through membrane filtration with 0.2-μm pore size to get the 10× medium.

Different volumes of D_2O, 0, 2, 3, 5 and 7 mL were added to 1mL medium (10×), respectively. The final volumes were all adjusted to 10 mL with sterile water. Then the medium with 0%, 20%, 30%, 50% and 70% D_2O concentration were obtained.

D_2O Cytotoxicity Assay.

After growing overnight, old medium without D_2O was removed and replaced by new prepared medium with different content of D_2O. Cells were incubated for 48 and 72 hours in a humidified 5% CO_2 atmosphere at 37 °C. CellTiter-Glo® Luminescent Cell Viability Assay and CCK-8 assay were used to detect the cytotoxicity of D_2O, respectively.

Cell Counting Kit-8 (CCK-8) containing tetrazolium salt determines cell viability by measuring the absorbance of soluble orange formazan product generated when WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) is bioreduced by cellular dehydrogenases in mitochondria. The CellTiter-Glo® Luminescent Cell Viability Assay measures the number of viable cells in culture based on detecting the luminescent signal produced when mono-oxygenation of luciferin is catalyzed by luciferase in

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the presence of Mg^{2+}, ATP released from cells and molecular oxygen. The luminescence is proportional to the amount of ATP in cells and signals the presence of metabolically active cells.

For the CellTiter-Glo® Luminescent Cell Viability assay, transfer appropriate volume of CellTiter-Glo® Buffer into an amber bottle containing CellTiter-Glo® substrate, to reconstitute the lyophilized enzyme/substrate mixture to get the CellTiter-Glo Reagent. Mix by gently vortexing, swirling or inverting the contents to obtain a homogeneous solution in less than 1 minute. Mix CellTiter-Glo® solution and cells for 2 minutes on an orbital shaker to induce cell lysis. Incubate the plate at room temperature for 10 minutes to stabilize the luminescence signal and record the luminescence by Centro LB 960 Microplate Luminometer.

For CCK-8 assay, incubate the 96-well microtiter plates (5×10^4 cells/well) for an appropriate time (48 and 72 hours) in the incubator. Add 10 μL of CCK-8 solution to each well, and incubate the plate for 3 hours. The absorbance at 450 nm was determined by SPECTROstar Nano High flux ULTRAVIOLET spectrophotometer.

Anticancer Drug Inhibitory Activity by Cell Viability Assay.

For the interaction with anticancer drugs, a series of concentrations of anti-cancer drug were added into the medium with 30% D_2O in different wells. The final concentrations of anti-cancer drugs in cell wells were 0, 13, 17, 27, 33 and 53 μmol/L for cisplatin and 0, 0.005, 0.05, 0.1 and 0.5 μmol/L for taxol, respectively. Cells were incubated for 48 hours in a humidified 5% CO_2 atmosphere at 37 °C.

CellTiter-Glo® Luminescent Cell Viability Assay and CCK-8 assay were used to detect cell viability at the end-point after interaction with cisplatin and taxol. The experimental methods were the same as the above.

Anticancer Drug Inhibitory Activity by D_2O-Single Cell Raman Measurement.

After cultivation with anti-cancer drugs cisplatin and taxol for 48 hours in the medium with 30% D_2O, cells were washed with PBS for three times to remove medium and then fixed in 4% vol/vol paraformaldehyde for 30 min and were washed for three times with PBS to remove unbound paraformaldehyde. The fixed cells were dropped onto an aluminum foil and were air dried in room temperature.

Single cell Raman Spectroscopy was obtained using a confocal Raman microscope (Renishaw inVia Reflex, UK) equipped with a grating with 2400 grooves/cm. Routine instrument calibration was performed prior to sample analysis using the silicon peak. Raman spectrum were acquired using Ar laser operating at λ = 514 nm and a 50× air objective lens. For each Raman spectrum, the laser output power was 12 mW, the collecting time was 10 s, the spot size is 1 μm.

In order to study if the focusing laser spot positions on a cell have effect on C-D and C-H bond Raman intensity measurement, peak area (C-D) / peak area (C-D+C-H) ratio of five subcellular localities in each cell were measured.

For each concentration of anti-cancer drugs cisplatin and taxol, 6 cells were measured and to calculate the average Raman intensity ratio of C-D/(C-D+C-H) and their relative standard deviation (RSD).

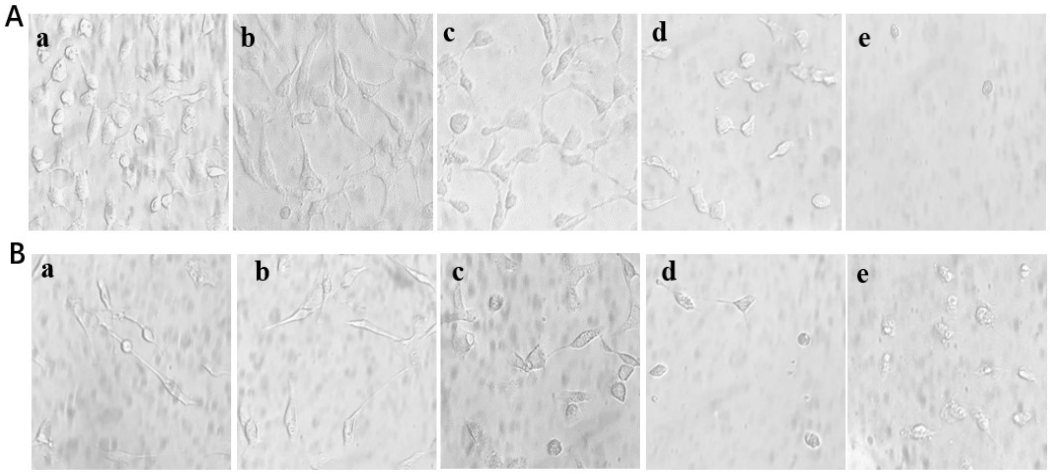


Figure S1. Bright field image of A549 cells growing for 48 hours(A) and 72 hours (B) in medium with D_2O(a-e): 0%,20%,30%,50% and 70% D_2O.

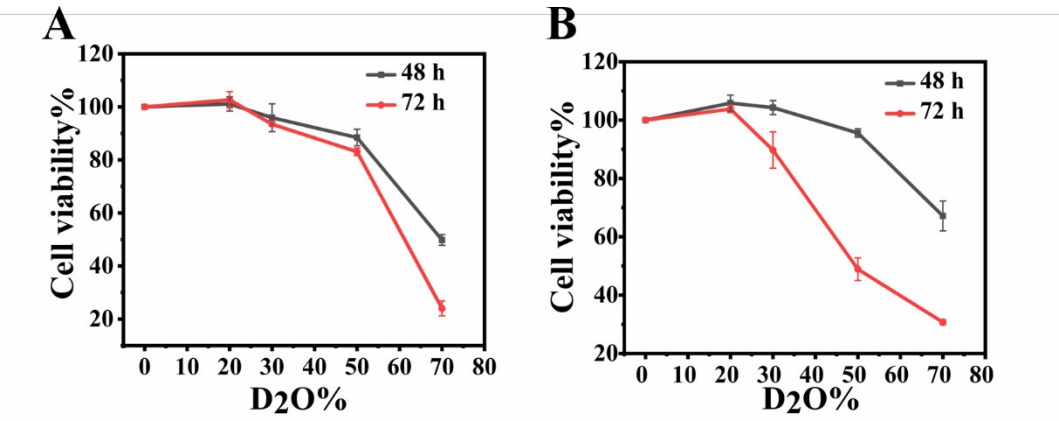


Figure S2. Cytotoxicity assay of different concentrations of D_2O on cells for 48 hours and 72 hours for A549 cells by CCK-8 assay (A) and CellTiter-Glo® Luminescent cell viability assay (B)

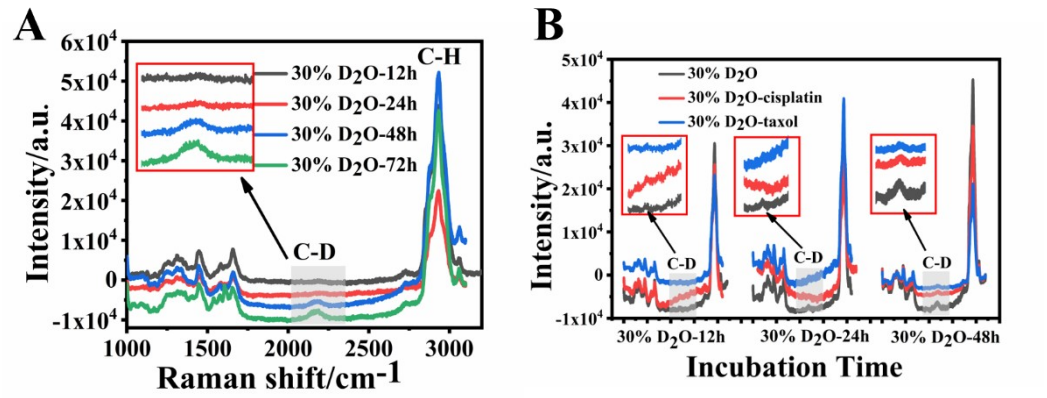


Figure S3. D_2O-single cell Raman spectrum of A549 cells incubation in medium containing 30% D_2O for different time: (A) 12, 24, 48 and 72 hours without anticancer drug and (B) 12, 24 and 48 hours in medium without drug, with 17 μmol/L cisplatin and with 0.1 μmol/L taxol.

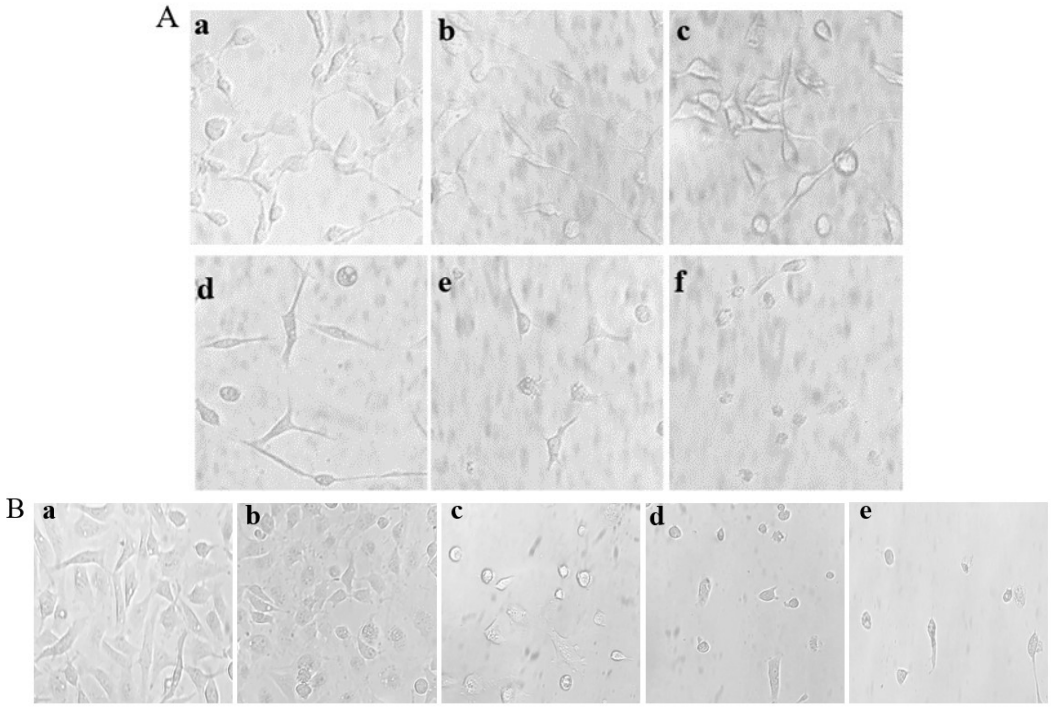


Figure S4. (A) Bright field image of A549 cells grown on 48 hours in medium with 30% D_2O and anti-cancer drug (A) a-f: 0, 13,17,27,33 and 53 μmol/L for cisplatin and (B) (a-e): 0, 0.005,0.05,0.1 and 0.5μmol/L for taxol.

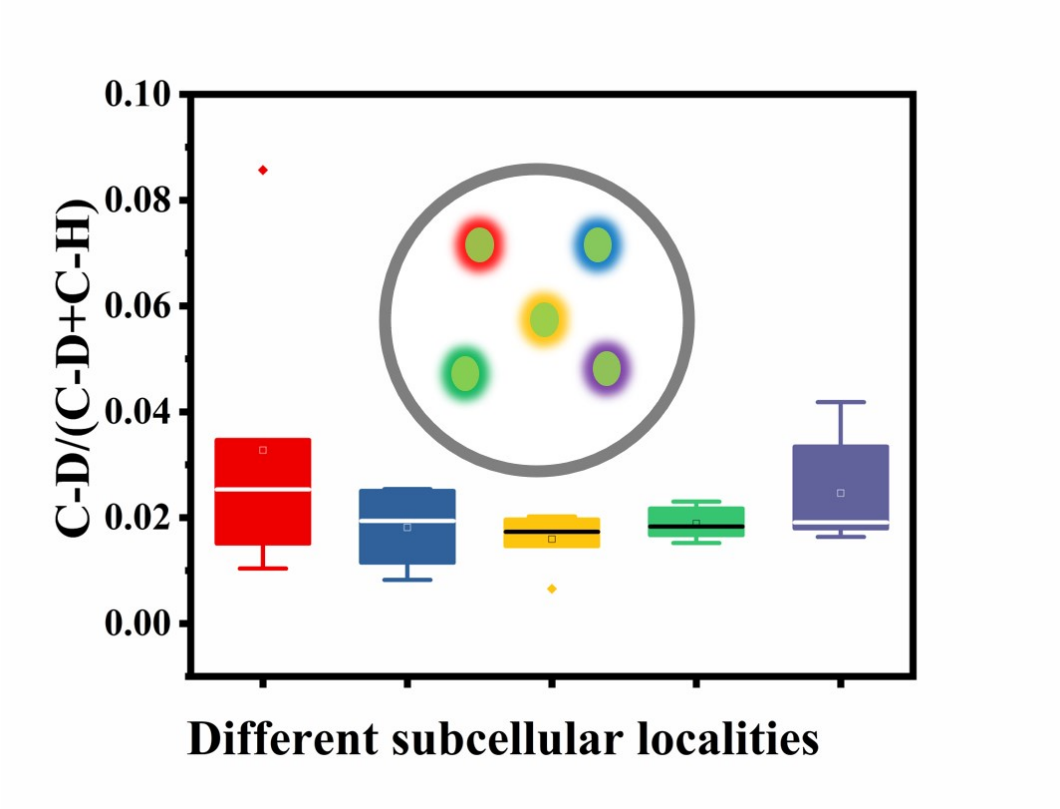


Figure S5. Peak area (C-D)/ Peak area (C-D+C-H) ratio of six cells for growing for 48 hours in medium with 30% D_2O from 5 subcellular localities within the cell.

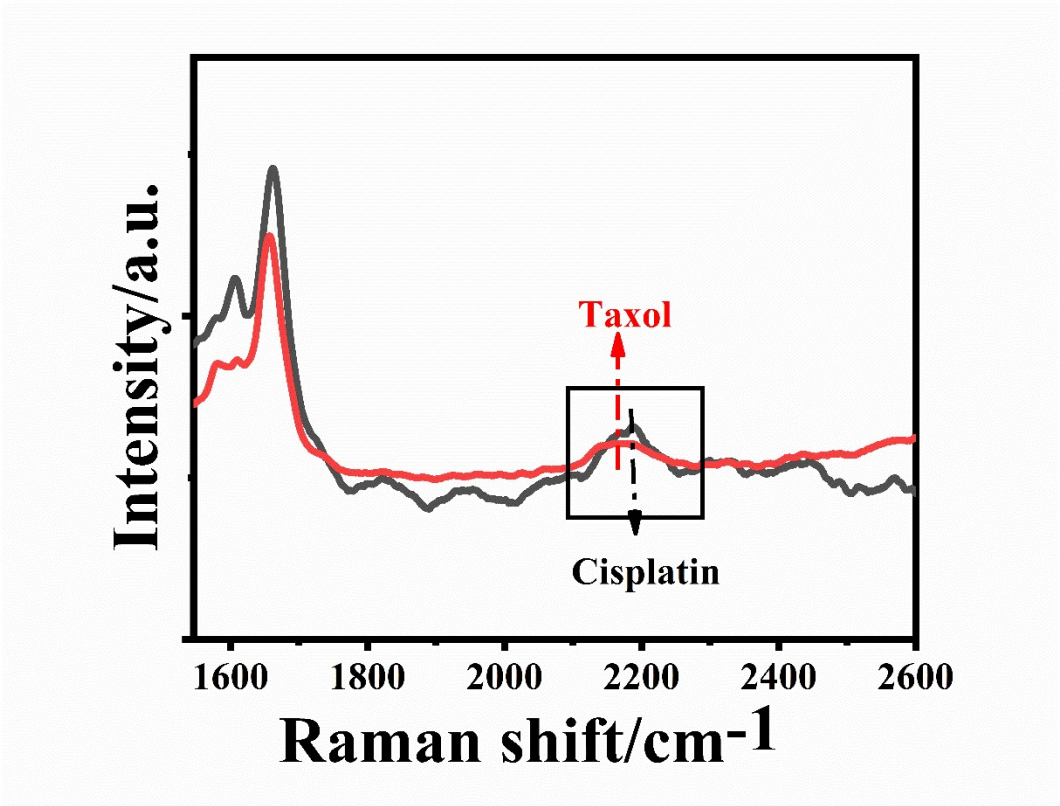
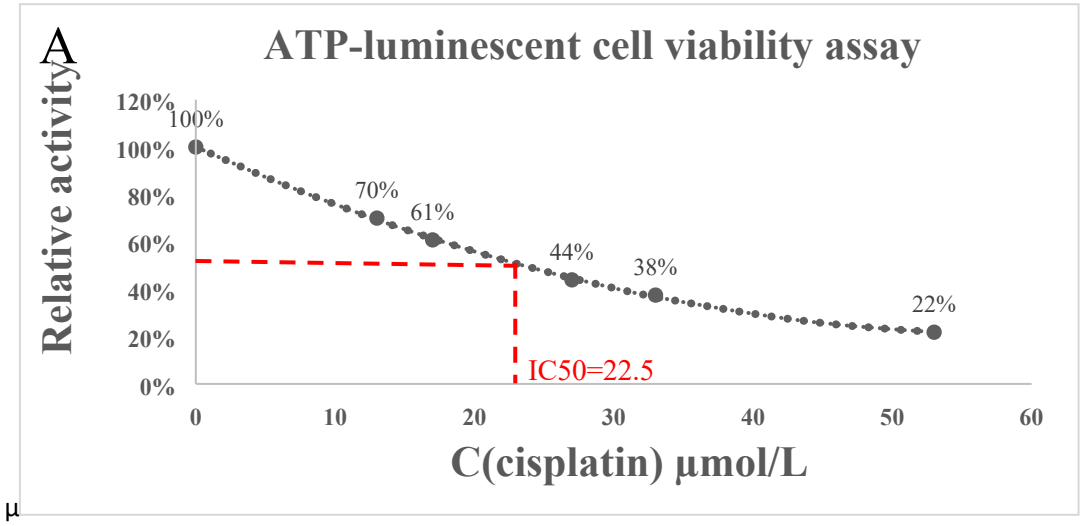


Figure S6. C-D Raman peaks of A549 cells growing for 48 hours in 30% D_2O-medium with anticancer drugs: cisplatin (black line) and taxol (red line).



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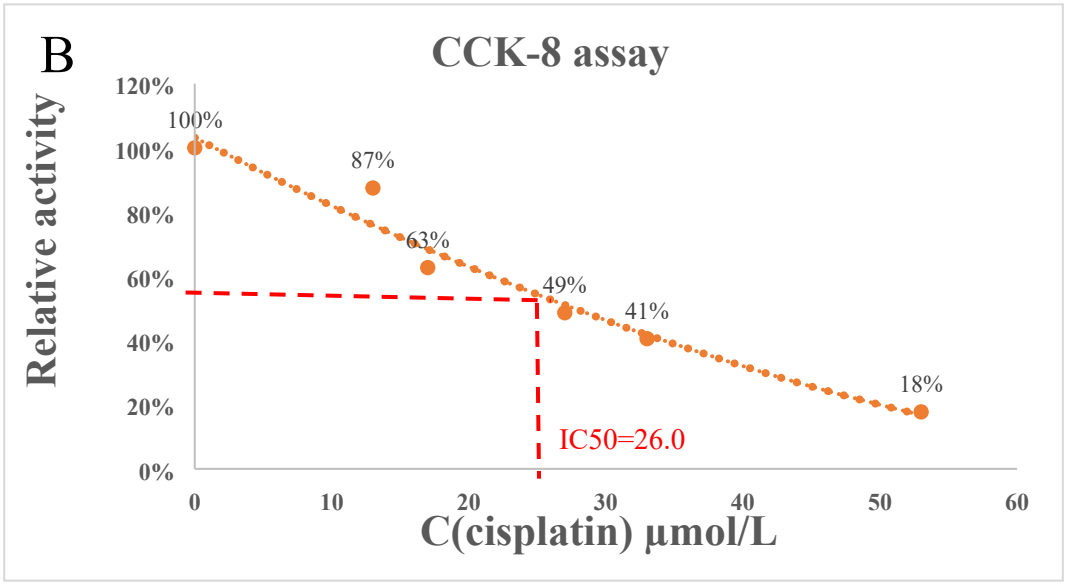


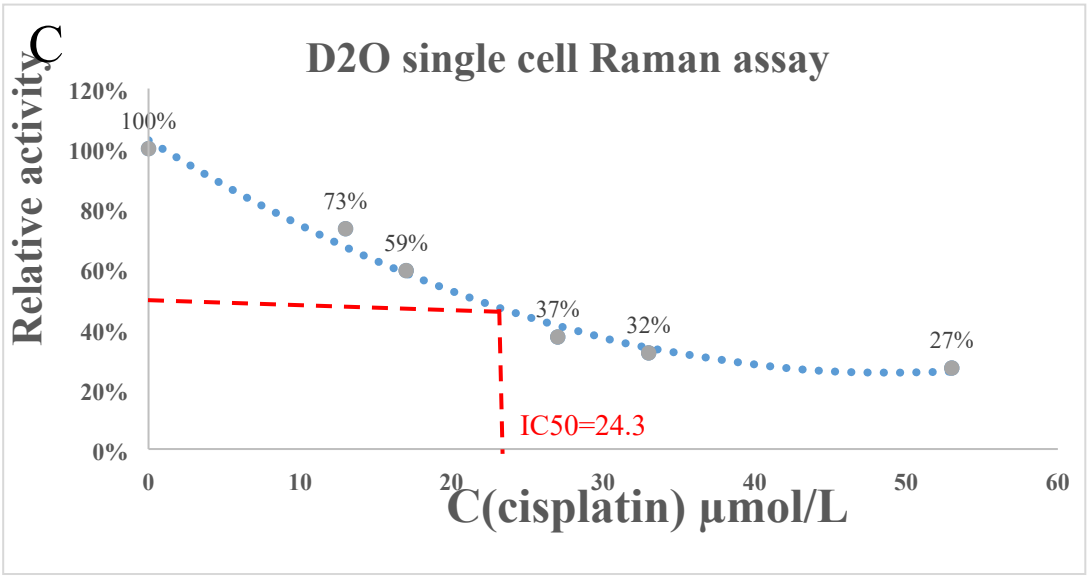
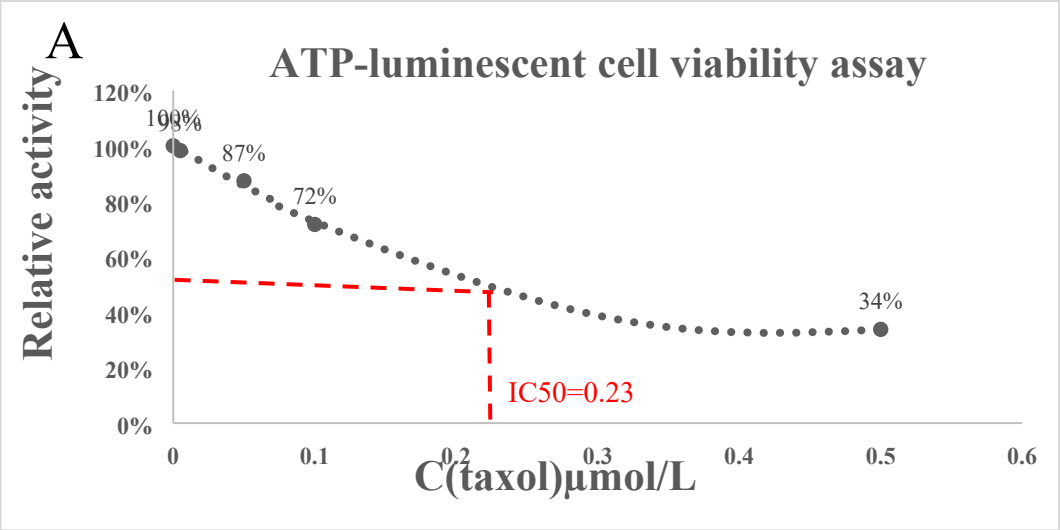
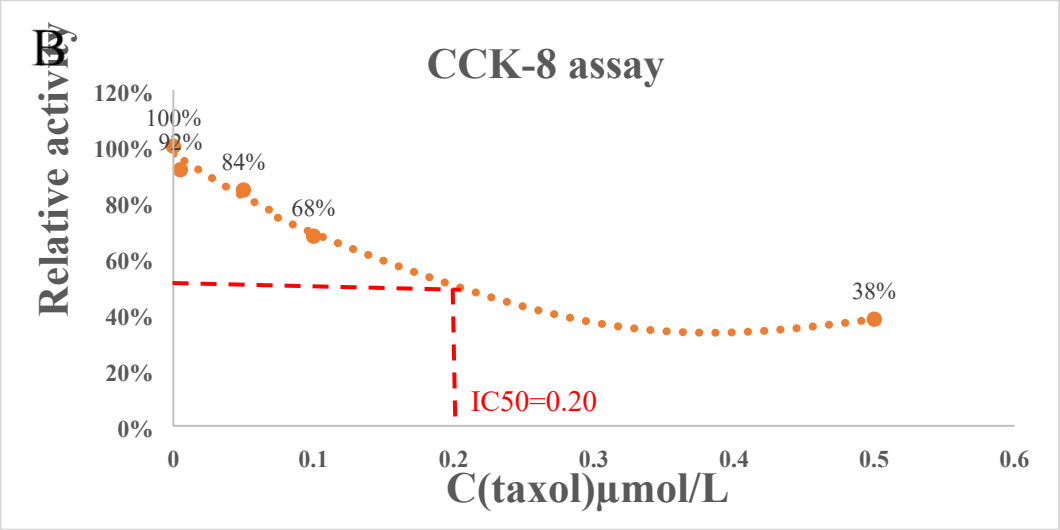


Figure S7. Relative activity of A549 cells treated by anti-cancer drug cisplatin to A549 cells control without

drug measured by luminescent cell viability assay (A), CCK-8 assay(B) and D_2O-single cell Raman assay(C).





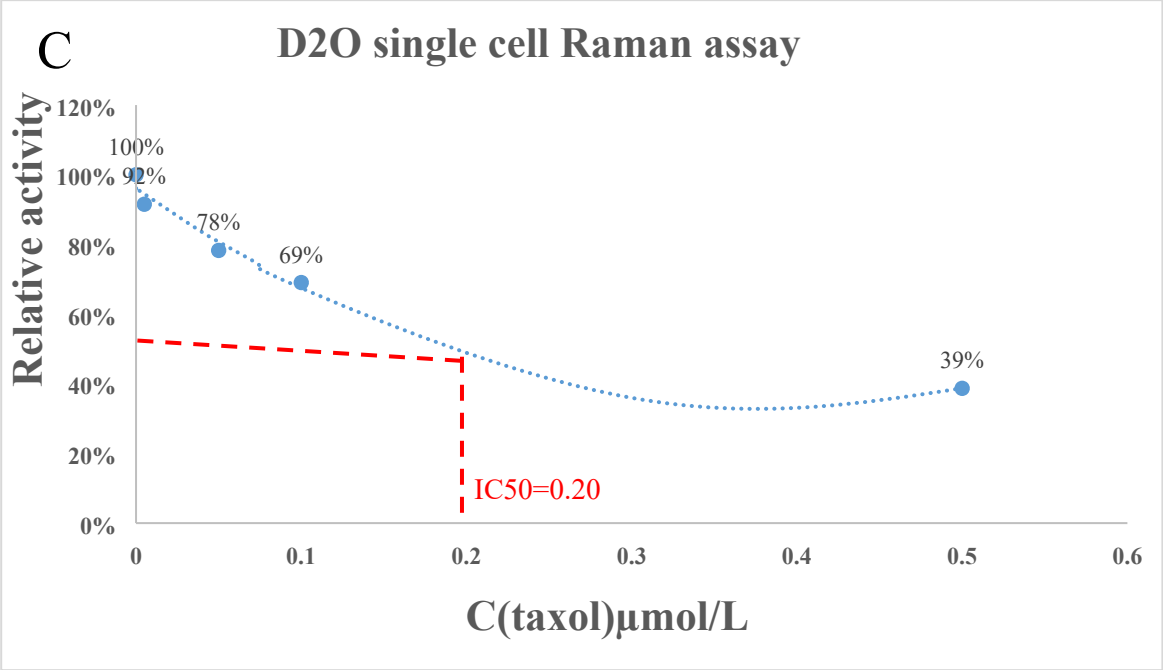


Figure S8. Relative activity of A549 cells treated by anticancer drug taxol to A549 cells control without drug measured by luminescent cell viability assay (A), CCK-8 assay(B) and D_2O-single cell Raman assay(C).

Cisplatin	C-D/(C-D+C-H) Raman peak area ratio						
（μmol/L)	1	2	3	4	5	6	RSD%
0	0.0222	0.0202	0.0240	0.0223	0.0201	0.0210	6.3
13	0.0192	0.0176	0.0202	0.0194	0.0180	0.0183	4.7
17	0.0141	0.0140	0.0146	0.0135	0.0130	0.0143	3.8
27	0.00952	0.00826	0.00952	0.00860	0.00926	0.00826	6.2
33	0.00782	0.00760	0.00690	0.00853	0.00862	0.00690	8.9
53	0.00545	0.00581	0.00491	0.00637	0.00625	0.00491	10.3

Table S1. The results of D_2O-single cell Raman spectroscopy measurements of 6 cells for cisplatin of different concentrations.

Taxol（μ mol/L)	C-D/(C-D+C-H) Raman peak area ratio						
	1	2	3	4	5	6	RSD%
0	0.0228	0.0256	0.0239	0.0260	0.0240	0.0270	6.7
0.005	0.0205	0.0209	0.0196	0.0217	0.0214	0.0204	3.7
0.05	0.0151	0.0149	0.0155	0.0171	0.0158	0.0163	3.6
0.1	0.0129	0.0134	0.0123	0.0138	0.0095	0.0128	5.9
0.5	0.00981	0.00989	0.00935	0.00808	0.00813	0.00859	7.9

Table S2. The results of D_2O-single cell Raman spectroscopy measurements of 6 cells for taxol of different concentrations.

Day	Equation of fitted curve	r	$IC50_{(Cisplatin)}$ μmol/L	RSD%
1	$y = 0.0001x^2 - 0.0212x + 0.9569$	0.993	24.3	7.0
2	$y = 0.0004x^2 - 0.032x + 1.004$	0.999	21.5	
3	$y = 0.0003x^2 - 0.0321x + 1.0309$	0.992	20.9	

Table S3. The inter-day repeatability of the D_2O-single cell Raman spectroscopy for IC50 value measurement of cisplatin.