Supplementary Information

A new pyrazolate osmium(VI) nitrido complex exhibits anticancer activity through modulating protein homeostasis

Wan-Qiong Huang[1,+], Peng-Chao Ji[1,+], Fu-Ling Song[1], Meiyang Li[2], Hongzhi Guo[2], Yong-Liang Huang[1], Cuicui Yu[2], Chuan-Xian Wang[1], Tao Liu[1], Chengyang Huang[2,4*] and Wen-Xiu Ni[1,3*]

Experimental Section

Analysis of Mitochondrial membrane potential (MMP)

Flow cytometry. 2 ml HepG2 cells with the density of 2 x 10^5 cells / ml was counted and added into the 6-well plate. After being cultured for 24 h, Os complex at the indicated concentrations was added and the cells were further cultured for 12 h. The cells were collected and resuspended in 1ml JC-1 dyestuff and put at cell incubator for 20 min. Subsequently, the cells were rinsed with pre-cooling JC-1 staining buffer twice and measured by flow cytometry 10000 cells were acquired for each sample.

Confocal microscopy. HepG2 cells were seeded in the Glass Bottom Dish (35mm Dish with 10mm Bottom Well, 4×10^5 cells/well). After being cultured for 24 h, Os complex at the indicated concentrations was added and the cells were further cultured for 12 h. After removal of culture medium, cells were washed with PBS. Add 1mL fresh medium and 1mL JC-1 dyestuff in each dish and put in a cell incubator for 20min. Cells were washed by pre-cooling JC-1 staining buffer twice and incubated with 2ml fresh medium. LSM 880 confocal laser scanning microscope was adopted for observation and photo taking.

DNA agarose gel electrophoresis assay

The reaction of complex with DNA was conducted using plasmid DNA (pBR322, 4361 base pair (bp) and 54% GC, Thermo) and analyzed by agarose gel electrophoresis. **Os** complex at the indicated concentrations(8,16,32,64 μg/ml) and positive control cisplatin(10 μg/ml) was incubated with DNA (0.55μg) for 24h at room temperature. Prepare a 1% agarose gel and electrophoresis buffer (TAE). Then, 5μl of DNA sample mixed by 1μl loading buffer (6x) was added as running sample. The DNA Ladder and the samples were run for 30 min at 40-50 V through 1% agarose gel supports emerged in 1 x TAE buffer solution. The gels were finally stained with GENRED 10000X in water (G001) for 30 min and photographed with Gel imaging system (Tanon 2500R).

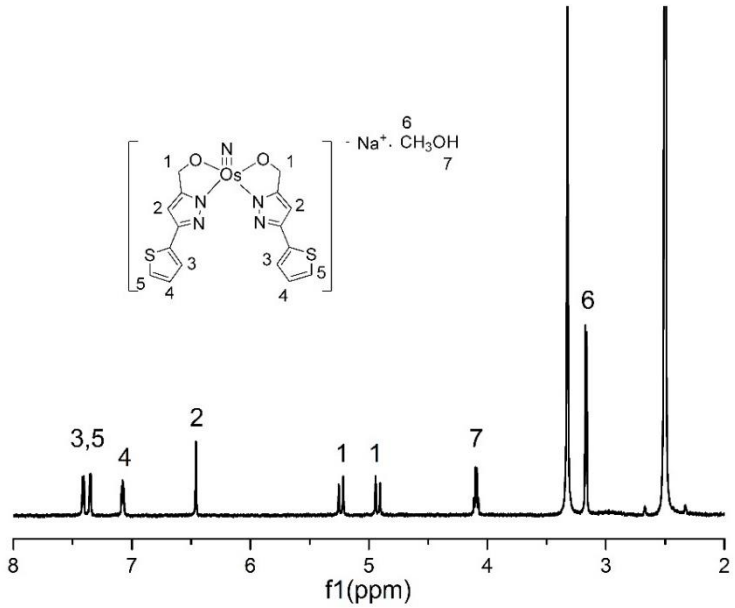
Apoptosis evaluation. HepG2 cells were rinsed with PBS and digested with 0.25% trypsin-EDTA (Gibco). Then cells with the density of 1 x 10^5 cells / ml were counted and added 2 ml into the 6-well plate. After being cultured for 24 h, Os complex at the indicated concentrations was added and reacted for 24 h. HepG2 cells were digested with EDTA-free trypsin (Gibco) and rinsed twice with cold PBS. Then resuspend cells in 1X Binding Buffer. Add 5μl FITC Annexin V and 5 μl PI to 100 μl of the solution (1 x 10^5 cells). Gently vortex the cells and stain for 15 min at RT in the dark. Add 400 μl of 1X Binding Buffer to each sample. Analyze by flow cytometry with 1 h. Flow cytometric analysis was performed with a flow Cytometer (BD Accuri™ C6, USA).

Detection of intracellular reactive oxygen species (ROS) by DCFH-DA

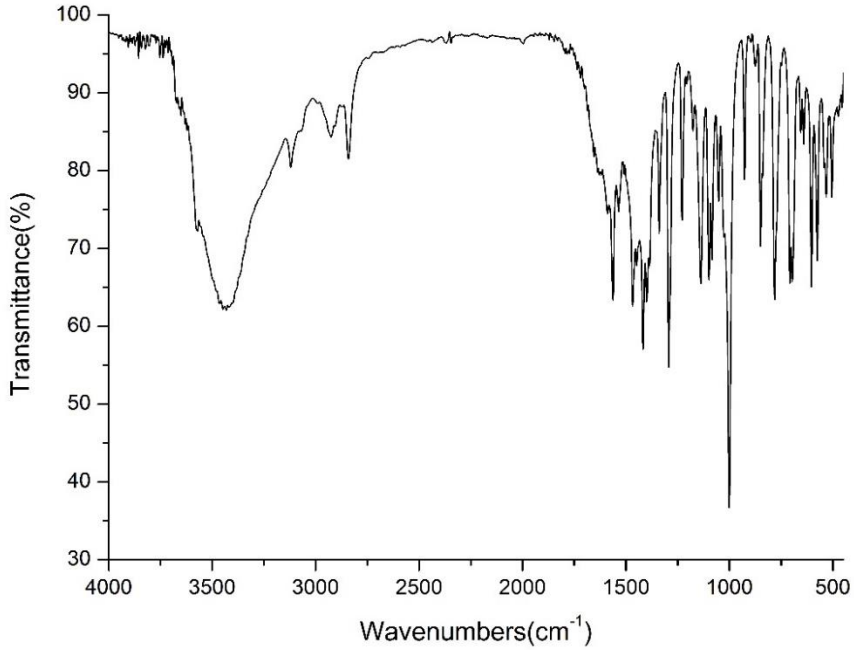
HepG2 cells rinsed with PBS and digested with 0.25% trypsin-EDTA . Then cells with the density of 1 x 10^5 cells / ml were counted and added 2 ml into the 6-well plate. After being cultured for 24 h, different concentrations of drugs (6.5、13、26μmol/L, respectively) were added. Then, the cells were placed in an incubator for further 3 hours. Replace the old liquid with the diluted DCFH-DA probe (10μmol/L). The volume is 1 ml per well. After 20 minutes, the cells were rinsed three times with serum-free cell culture medium. The green fluorescence of each sample was then observed by an inverted fluorescence microscope.

Cell morphology was observed by Inverted microscope. HepG2 cells rinsed with PBS and digested with 0.25% trypsin-EDTA (Gibco). Then cells with the density of 1 x 10^5 cells / ml were counted and added 2 ml into the 6-well plate. After being cultured for 24 h, different concentrations of drugs (6.5、13、26μmol/l, respectively) were added. Then, the cells were placed in an incubator

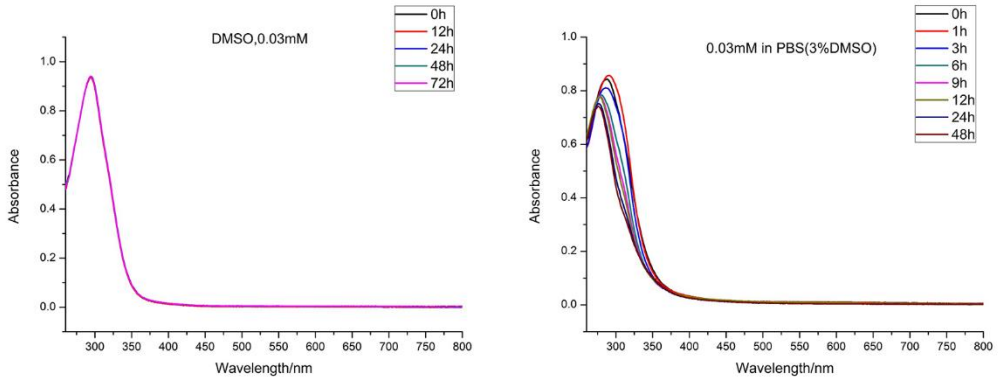
for further 3 hours, and the cell morphology of each group was directly observed with an inverted microscope.



Supplementary Figure S1. The ^{1}H NMR spectrogram of the Os complex.



Supplementary Figure S2. The IR spectrogram of the Os complex.



Supplementary Figure S3. The stability of the Os complex in DMSO and in PBS (3% DMSO).

Supplementary Table S1 Crystal data and structure refinements for **Os**

	Os
Formula	$C_{17}H_{16}N_5NaO_3OsS_2$
Mr.	615.66
Crystal system	Orthorhombic
Space group	$P\,2_12_12_1$
Temp (K)	293(2)
a (Å)	7.52480(10)
b (Å)	13.9934(3)
c (Å)	18.9699(3)
α (°)	90
β (°)	90
γ (°)	90
V (Å^3)	1997.48(6)
Z	4
D_c (g·cm^{-3})	2.047
no. of reflns	4617
no. of unique	3330
data collected	3.93 to 74.54
unique refl.(R_{int})	0.0283
GOF on F^2	1.086
$R_1[I\geq2\sigma(I)]^a$	0.0374
$wR_2[I\geq2\sigma(I)]^b$	0.1018
R_1[all data]	0.0381
wR_2[all data]	0.1022
Flack parameter	0.33(2)

$^{a}R_1 = \Sigma \,||F_o| - |F_c||/\Sigma\,|F_o|$. $^{b}\,wR_2 = [\Sigma[w(F_o^2 - F_c^2)^2]/\Sigma w(F_o^2)^2]^{1/2}$, where $w = 1/[\,w^2(F_o)^2 + (aP)^2 + bP]$ and $P = (F_o^2 + 2F_c^2)/3$.

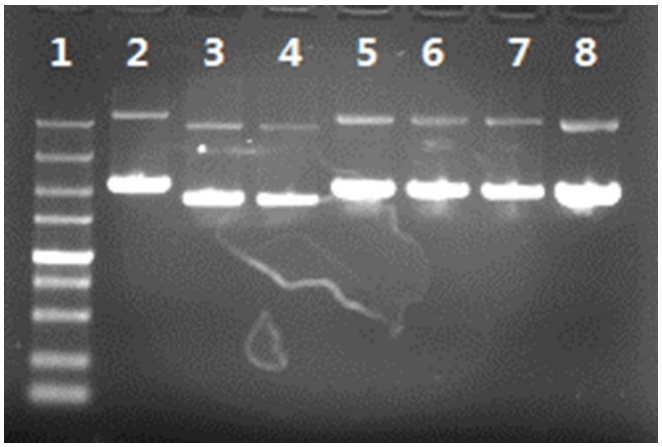
Supplementary Table S2 Selected bond lengths (Å) and angles (º) for **Os**.

Os(1)-O(1)	1.965(7)	Os(1)-O(2)	1.956(8)	Os(1)-N(1)	2.000(8)
Os(1)-N(3)	2.006(8)	Os(1)-N(5)	1.649(9)	Na(1)-O(1)#2	2.493(9)
Na(1)-O(2)#2	2.388(9)	Na(1)-O(3)	2.116(7)	Na(1)-N(2)	2.463(9)
Na(1)-N(4)	2.511(10)				
O(2)-Os(1)-O(1)	82.3(3)	O(2)-Os(1)-N(1)	143.5(4)	O(2)-Os(1)-N(3)	80.0(4)
O(1)-Os(1)-N(1)	79.5(3)	O(1)-Os(1)-N(3)	144.0(3)	N(1)-Os(1)-N(3)	96.8(3)
N(5)-Os(1)-O(2)	109.9(5)	N(5)-Os(1)-O(1)	109.3(4)	N(5)-Os(1)-N(1)	106.0(4)
N(5)-Os(1)-N(3)	106.1(4)	O(2)#2-Na(1)-O(1)#2	63.8(3)	O(2)#2-Na(1)-N(4)	109.2(3)
O(2)#2-Na(1)-N(2)	168.9(4)	O(1)#2-Na(1)-N(4)	171.3(3)	N(2)-Na(1)-O(1)#2	106.7(3)
N(2)-Na(1)-N(4)	79.7(3)	O(3)-Na(1)-O(2)#2	114.4(3)	O(3)-Na(1)-O(1)#2	115.9(3)
O(3)-Na(1)-N(4)	71.2(3)	O(3)-Na(1)-N(2)	74.4(3)		
Os(1)-O(2)-Na(1)#1	109.1(4)	Os(1)-O(1)-Na(1)#1	104.8(3)		

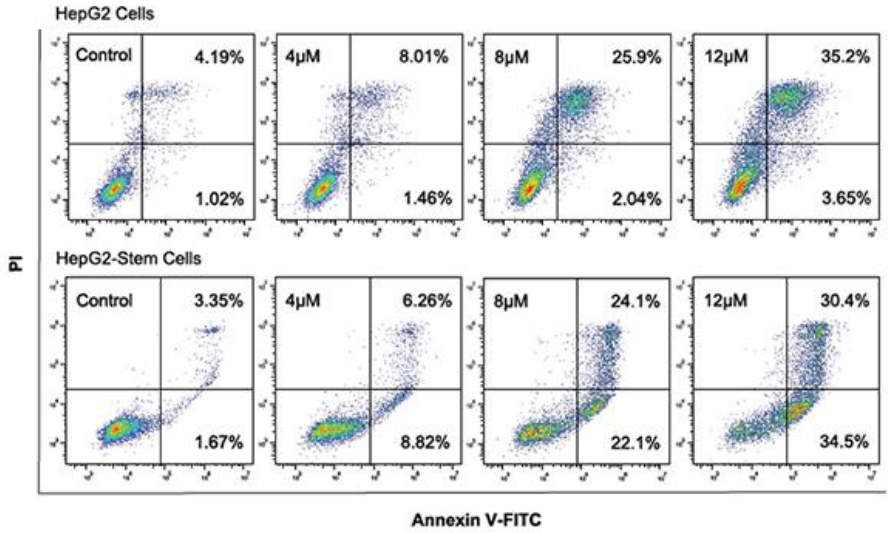
Symmetry transformations used to generate equivalent atoms: #1 x-1, y, z #2 x+1, y, z

Supplementary Table S1 The Na content of Os complex detected by ICP-MS.

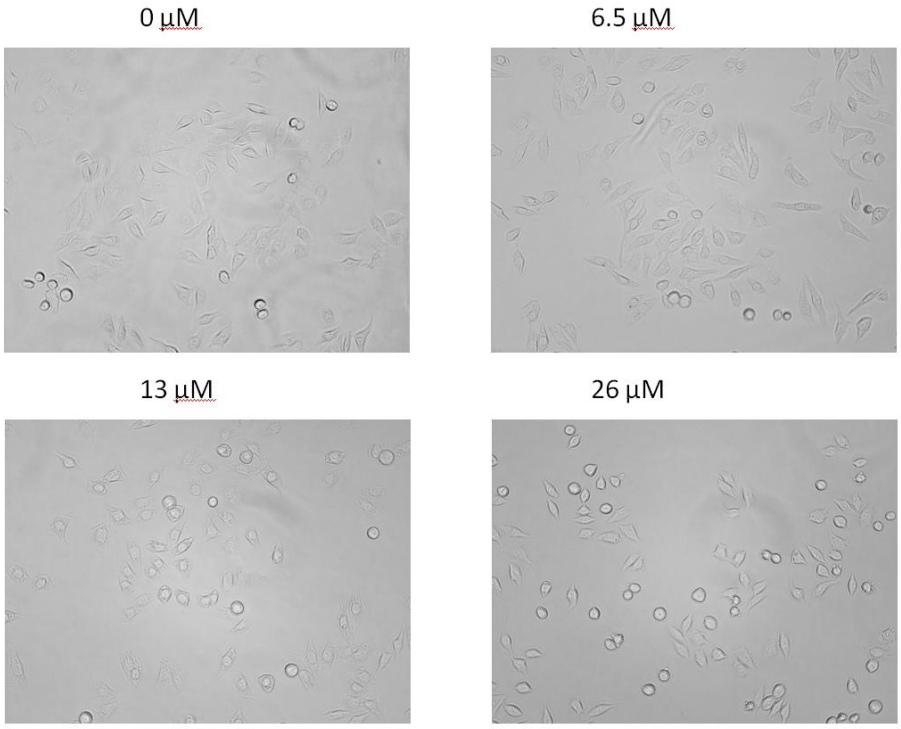
Sample weight	Calculated content of Na （mg）	Measured content of Na (mg)
2.52mg	0.0993	0.09725
4.86mg	0.1915	0.16975



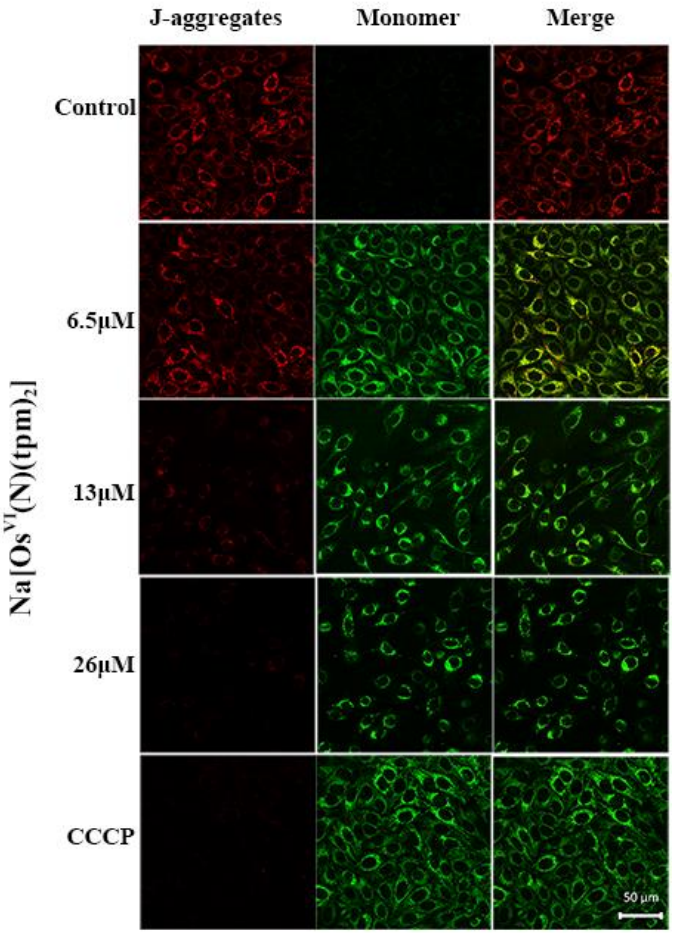
Supplementary Figure S4. Study on the interaction between different concentration Os complex and 0.55μg DNA. (1) marker; (2) Plasmid DNA;(3,4)10μg/ml CDDP;(5-8)8,16,32,64 μg/ml Os complex.



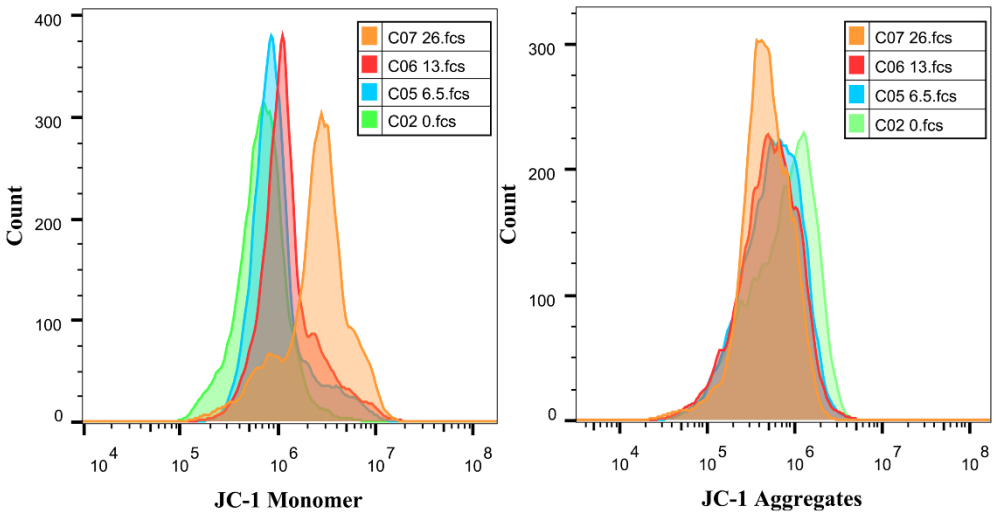
Supplementary Figure S5. Apoptosis analysis of HepG2 cancer cells and HepG2-stem cancer stem cells after 24 h of exposure to the $Na[Os^{VI}(N)(tpm)_2]$ complex as determined by flow cytometry using Annexin V-FITC/PI staining.



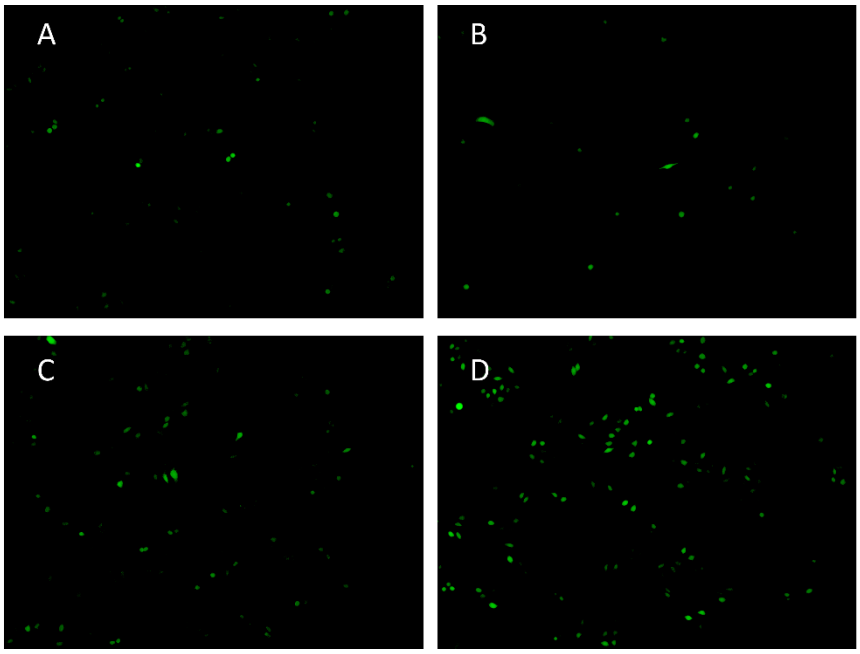
Supplementary Figure S6. Effects of different concentrations of Os complex on the morphology of HepG2 cells at 3 h.



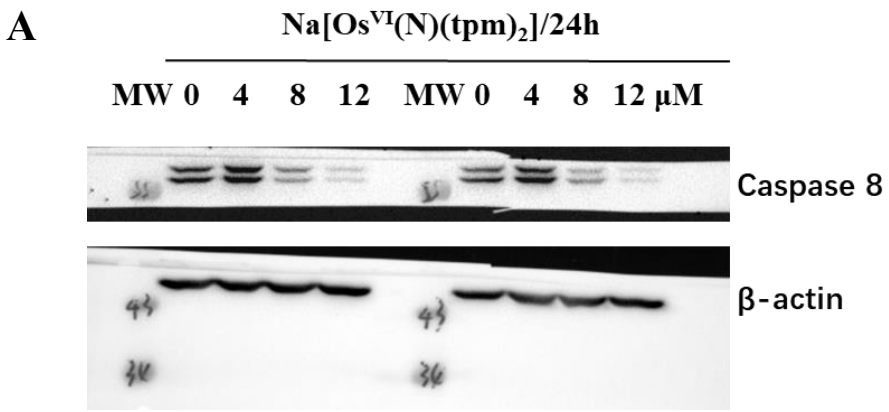
Supplementary Figure S7. Effects of the $Na[Os^{VI}(N)(tpm)_2]$ complex (6.5, 13, or 26 μM) incubated for 12 h, a blank control and the positive control carbonyl cyanide m-chlorophenylhydrazone (CCCP) (10 μM) incubated for 20 min on the mitochondrial membrane potential of HepG2 cells by confocal microscopy.

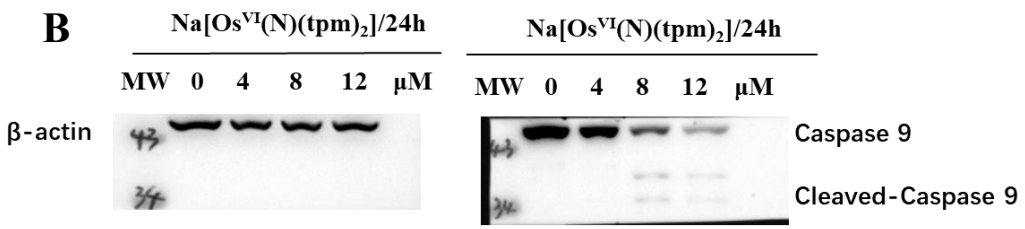


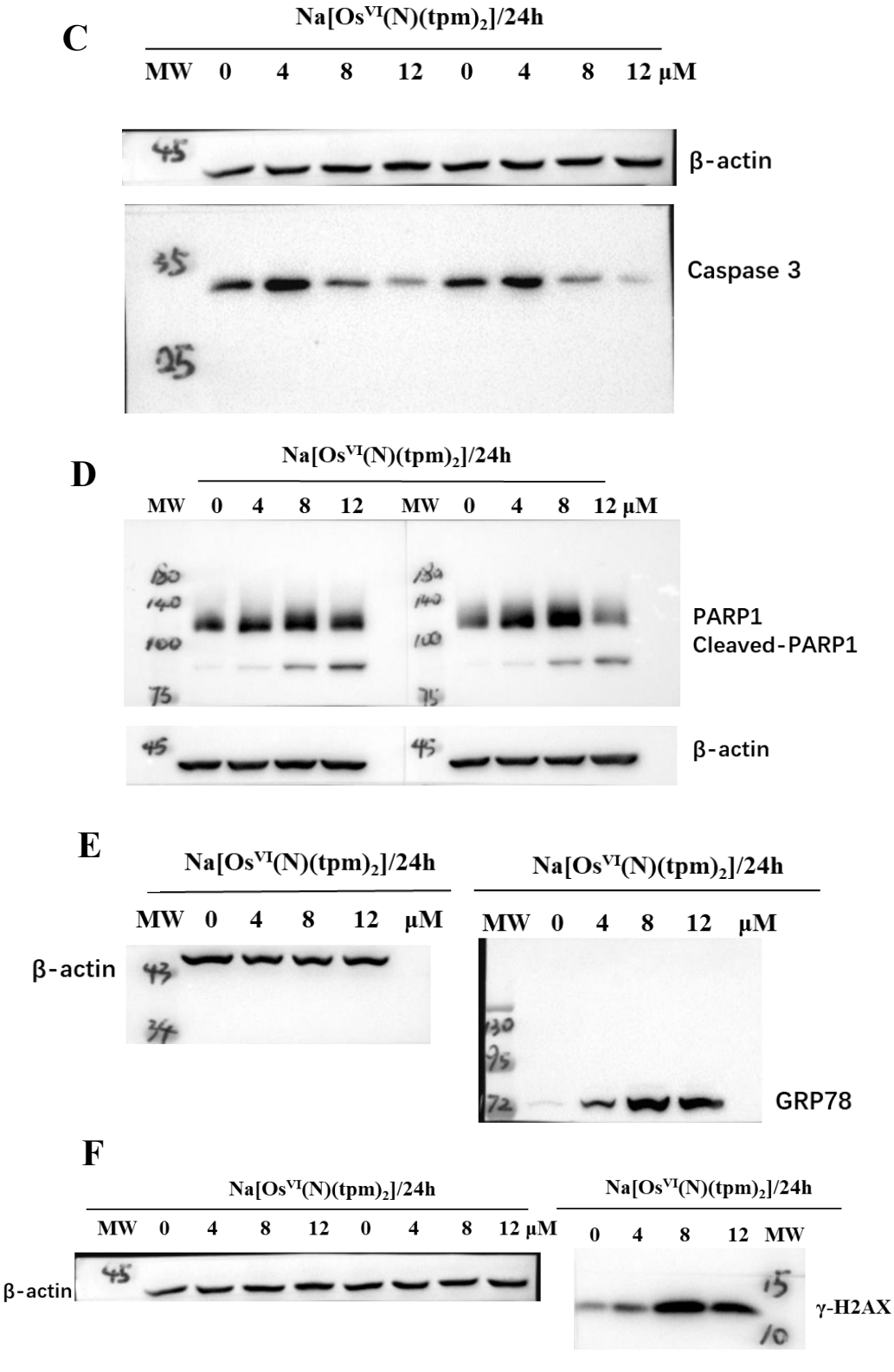
Supplementary Figure S8. Effect of complex Na[OsVI(N)(tpm)2] (6.5,13,26μM) incubated for 12h and black control on mitochondrial membrane potential of HepG2 cells by flow cytometry.



Supplementary Figure S9. Fluorescent microscopic photographs of DCFH-DA staining in HepG2 cells treated with Os complex for 3 h. (A) Control (B)3.25μM (C) 6.5 μM and（D）13μM







Supplementary Figure S10. Unprocessed original membranes of Western blotting shown in Figure 3. (A) Caspase 8, (B) Caspase 9 and Cleaved caspase 9, (C) Caspase 3, (D) PARP1 and Cleaved PARP1, (E) GRP78 and (F) γ-H2AX protein expression levels. MW: molecular weight ladder. According to the molecular weight of protein, the shown blots are cropped from different parts of the same gel, as explicit by using clear delineation with dividing lines and white space.