Electronic Supplementary Information

Stable High-Oxidation-State Complex in-situ Mn(V)-Mn(III) Transition to Achieve Highly Efficient Cervical Cancer Therapy

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Experimental section

Materials. Salicylaldehyde, Ethylenediamine and Acetate Tetrahydrate were purchased from Shanghai Aladin Reagent Company. All the chemicals and solvents were analytically pure. Thiazolyl blue tetrazolium bromide (MTT), propidium iodide (PI) and 1,3-Diphenylisobenzofuran (DPBF) were purchased from Sigma-Aldrich. The water used in cellular experiments was ultrapure, supplied by a Milli-Q water purification system from Millipore.

Instruments. Detection of mass spectrometry was performed on an Agilent 1290 LC-6545 QTOF mass spectrometer (Agilent, USA). Detection of single crystal structure of complexes were carried out on XtaLAB PRO X-ray single crystal diffractometer (Rigaku, Japan). The UV–Vis spectra of the compounds were obtained using the UH4150 UV–Visible (Hitachi, Japan). Cell Viability analyzed by Cell Imaging Multi-Mode Reader (Bio Tek, USA). Cell cycle analysis was detected by Cyto FLEX Flow Cytometer (Beckman Coulter, USA).

Synthesis of complexes

Synthesis of salen ligand.

Salicylaldehyde and ethylenediamine are added to methanol in a ratio of 1:4 (n:n). After stirring at room temperature for 1 h, the yellow precipitate precipitated out. The bright yellow salen ligand was obtained after washing the precipitate with a mixed solution of ether and methanol three times.

Synthesis of Mn(III) salen. Based on the synthesis method of reference, we synthesize Mn(III) complex by a simple method.[1] 4 mM potassium hydroxide was added after 2 mM salen ligand was dissolved in methanol solution, and then 2 mM manganese acetate was added with stirring. Stir at room temperature for 1 hour before heat to reflux for 4 hours. A brownish-yellow precipitate precipitated. The precipitate was washed with water and acetone and dried to obtain Mn(III) complex.

Synthesis of Mn(V)(N) salen. Based on the synthesis method of reference, we synthesize Mn (V) complex by a simple method.[2] Salen ligand and manganese acetate tetrahydrate 1:1 (m:m) were dissolved in methanol solution, stirred and refluxed for 1h. Cooling to room temperature, and slowly add ammonia water. Then add clorox bleach and stir for 1 hour,

cool in ice water bath and add dichloromethane. Stir at room temperature for 15 minutes, extract and separate, collect the organic layer, and dry to obtain dark green solid Mn (V) complex. ^{1}H NMR (600 MHz, DMSO) δ 8.41 (s, 2H), 7.61 – 7.21 (m, 4H), 6.83 (d, J = 8.4 Hz, 2H), 6.68 (t, J = 7.2 Hz, 2H), 4.07 – 3.89 (m, 2H), 3.84 – 3.59 (m, 2H).

X-ray Crystallography. The complex is dissolved in methanol, crystals of Mn^{V}(N)salen were gained by solvent evaporation method. The crystals were conserved in the mother-liquor before taken to a loop for single-crystal diffraction. The data were recorded on a Bruker APEXII instrument (Cu Kα, λ = 1.5406 Å), and the structure was solved by direct methods. Then, the data were refined using the full-matrix least-squares method on F2 through the SHELXTL program package.

Stability of Mn complexes. UV-vis spectrum was used to determine the stability of Mn complexes in PBS.

Catalytic activity of Mn(V) and Mn(III) complexes on hydrogen peroxide. 0.05 mM complexes and 0.2 mM hydrogen peroxide were added to a phosphate buffer solution (pH= 7.4) or the mixture of methanol and water (1:1; v:v). In the mode of kinetics, the spectrometer was set up for monitoring at the wavelength between 800-200 nm to acquire spectra automatically 1 scan per 1 min in 1 h.

Determination of dissolved oxygen. Dissolved oxygen level of 4 ml phosphate buffer solution (pH= 7.4) with 0.25 mM Mn complexes and 0.2 mM H_2O_2 were detected by dissolved oxygen meter.

Mass Spectrometry Characterization. Methanol and water (1:1; v:v) were used as solvents, and 0.05 mM Mn complex was detected by ESI-MS before and after mixed with 0.2 mM H_2O_2 or 0.1 mM GSH for 2 h.

Reaction activity of Mn(V) and Mn(III) complexes with glutathione. 0.05 mM Mn complex and 0.1 mM GSH were added to a phosphate buffered saline solution with a pH of 7.4. In the mode of kinetics, the spectrometer was set up for monitoring at the wavelength between 800-200 nm to acquire spectra automatically 1 scan per 1 min in 1h.

Cell culture, MTT assay and determination of growth inhibition. Cervical cancer cells (Hela, SiHa, Caski, SiHaCIS-R, HelaCIS-R) and others (Ect1/E6E7, NIH-3T3, MCF-7,

4T1, MiHa) were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in DMEM with 10% FBS, penicillin (100 units/ml) and streptomycin (50 units/ml) at 37 ℃ in CO_2 incubator (95% relative humidity, 5 % CO_2). The cell viability with and without radiation was examined by MTT assay.[2] Cells were seeded in 96-wells plate for 24 h (the density of cells are 2×10^4), cultured with various doses of complexes for 72 h. After incubation, 30 μL per well of MTT solution (5 mg/ml) was added and then incubated for 4 further hours. Then, the medium was replaced with 150 μl DMSO per well. The color intensity of the solution, which reflects the cell growth condition, was measured at 570 nm by microplate spectrophotometer (Versamax).

Cell growth inhibition (%) = $(OD_{control} - OD_{treatment} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\%$.

The half maximal inhibitory concentration (IC_{50}) was obtained from the plot of growth (%) vs. complex concentrations.

Measurement of intracellular reactive oxygen species (ROS) generation. The DCFH-DA probe was used to evaluate the antioxidant capacity of the drug. The cell density in the 96-well plate was 2×10^4 cells per well. After 24 hours of incubation, a final concentration of 10 μM DPBF probe is added to incubate for 30 minutes. Different concentrations of complexes were added before the fluorescence measured using cytation multifunctional fluorescence microplate reader. We use the same method to test the superoxide anion and singlet oxygen levels using Dihydroethidium (DHE) and 1,3-diphenylisobenzofuran (DPBF) probes.

Morphological changes of mitochondria. Mitochondrial Membrane Potential Probe (JC-1) staining assay was employed to monitor the morphological changes of mitochondria. SiHa cells were exposed to Mn complexes (20 μM) for different times and then incubated with JC-1 (2 μM) for 30 min. After incubation, the excess probe and complexes were washed away with PBS for three times. Then the morphological changes of mitochondria were observed under a laser scanning confocal microscope (LSM 800).

Cell cycle analysis. Effects of the Mn complexes on SiHa cells cycle distribution were analyzed by flow cytometry, as previously described.[3]

Induction of apoptosis. SiHa cells (4×10^5 cells/mL) were preincubated in a 6 cm Petri dish for 24 h and then treated with Mn complexes at concentrations of 20 μM for 72 h. The cells were gathered and washed with PBS, and resuspended and stained with a mixture of 200 μL of annexin-V and 10 μL of PI at room temperature in the dark for 15 min. The specimens were analyzed by Beckman flow cytometer.

Statistical Analysis. All the tests in this essay were duplicated at least three times, as well as the experimental results were displayed as mean ± standard deviation (mean ± SD). Using double-tailed Student's t-test and one-way analysis of variance to analyze the differences of two groups or three or more groups respectively. There are statistically significant requirements $P < 0.05$ (*) and $P < 0.01$ (**).

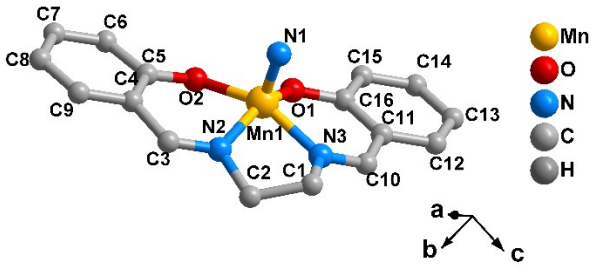

Figure S1 Structural representations of Mn^{V}(N) salen.

Table S1 Crystallographic data and structure refinement summary for Mn^{V}(N) salen**.**

Mn^{V}(N) salen	
formula	$C_{16}H_{14}MnN_3O_2$
*M*r	335.24
crystal system	Monoclinic
space group	$P2_1/c$
a (Å)	9.4997(2)
b(Å)	12.1319(3)
c(Å)	12.8513(3)
α(°)	90
β(°)	103.814(2)
γ(°)	90
V(Å^3)	1438.27(6)
Z	4
Dc(g/cm^3)	1.548
μ(mm^{-1})	7.558
F(000)	688.0
goodness-of-fit	1.128
$R_1[I>2\sigma(I)]$[a]	0.0512(2536)
ωR_2(all data)[b]	0.1583(2853)

[a]$R_1=\sum^{II}F_0I\text{-}I{F_C}^{II}/\sum IF_0I$. [b]$\omega R_2=\{\sum[\omega({F_0}^2\text{-}{F_C}^2)^2]/\sum({F_0}^2)^2\}^{1/2}$.

Table S2 Selected bond distances(Å) and angles(°) for Mn^{V}(N)salen.

Mn^{V}(N) salen	
Mn(1)-O(2)	1.914(2)
Mn(1)-O(1)	1.912(2)
Mn(1)-N(2)	1.959(2)
Mn(1)-N(3)	*1.949(3)*
Mn(1)-N(1)	1.522(3)
O(2)-Mn(1)-N(2)	90.02(9)
O(2)-Mn(1)-N(3)	152.83(10)
O(1)-Mn(1)-O(2)	81.89(9)
O(1)-Mn(1)-N(2)	148.37(9)
O(1)-Mn(1)-N(3)	91.21(10)
N(3)-Mn(1)-N(2)	82.17(10)
N(1)-*Mn*(1)-*O*(2)	106.16(12)
N(1)-Mn(1)-O(1)	109.55(11)
N(1)-Mn(1)-N(2)	102.07(12)
N(1)-Mn(1)-N(3)	100.92(12)
C(5)-O(2)-Mn(1)	125.27(17)
C(16)-O(1)-Mn(1)	126.74(19)
C(2)-N(2)-Mn(1)	115.77(17)
C(3)-N-(2)-Mn(1)	125.00(19)
C(10)-N(3)-Mn(1)	127.0(2)
C(1)-N(3)-Mn(1)	113.01(19)

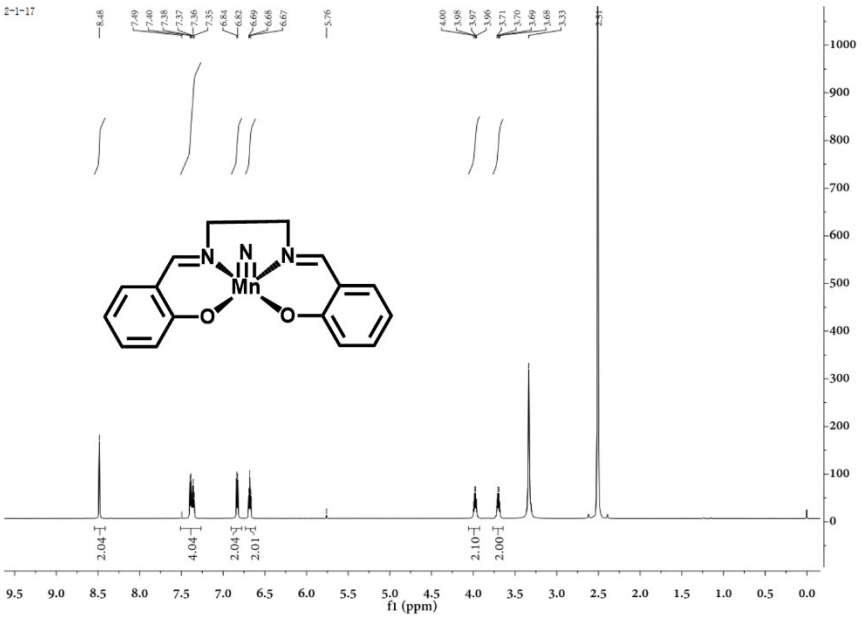

Figure S2 ^{1}H NMR spectrum of Mn^{V}(N) salen.

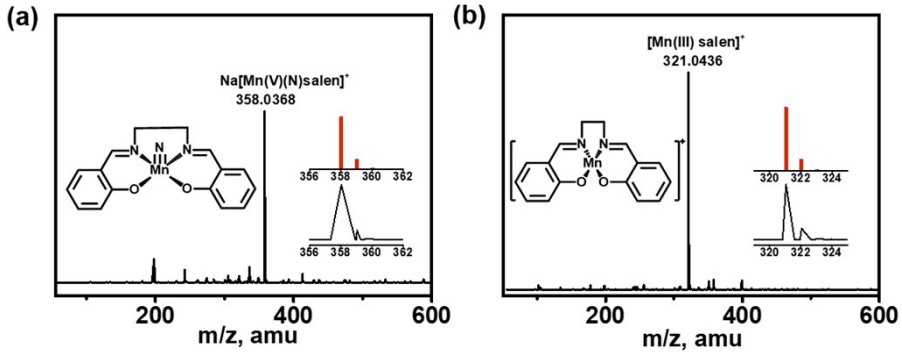

Figure S3 ESI-MS spectrum of Mn(V) and Mn(III) complexes.

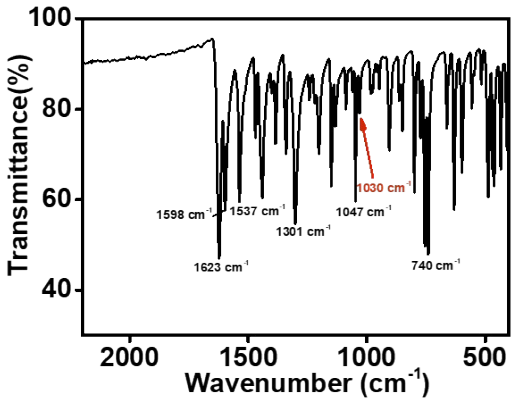

Figure S4 IR spectra of Mn^{V}(N) salen.

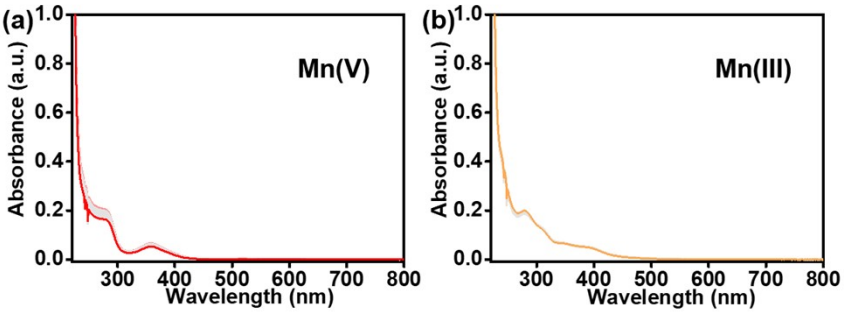

Figure S5 UV-Vis spectrum of 0.01 mM Mn(V) (a) and Mn(III) (b) complexes in PBS for 24 h.

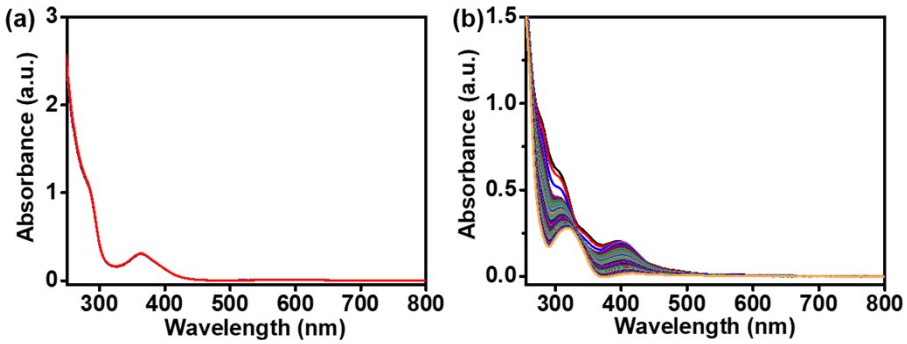

Figure S6 UV-Vis spectrum of 0.05 mM complexes interact with 0.2 mM H_2O_2 (Mn(V) (a) and Mn(III) (b) complexes) in a mixed solution of methanol and water (1:1, v:v).

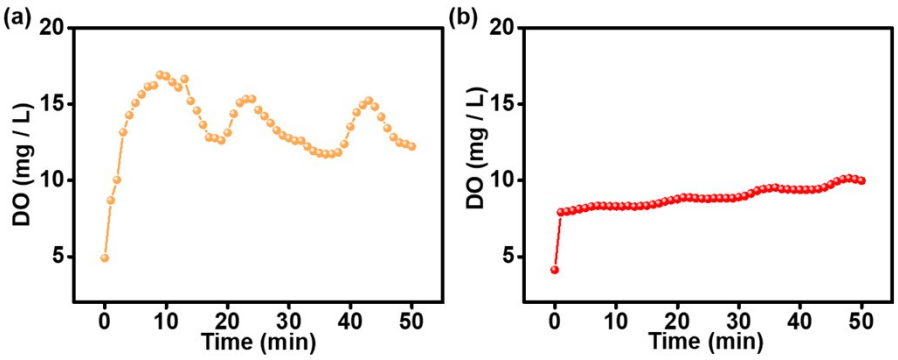

Figure S7 Levels of dissolved oxygen (DO) in phosphate buffer solution after 0.25 mM complexes (Mn(III) (a) and Mn(V) (b) complexes) interacts with 0.2 mM H_2O_2.

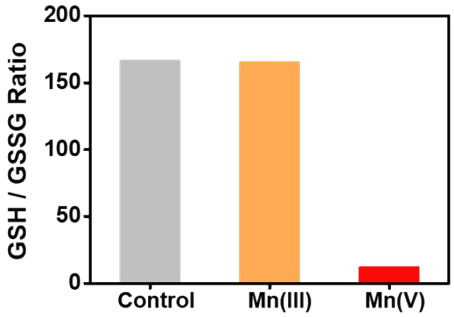

Figure S8 Intracellular GSH/GSSG ratio changes after inducing by 20 μM Mn complex for 12 h in SiHa cells.

Table S3 Anti-tumor activity of the manganese acetate, Mn(III) complex and Mn(V)

complex.

	IC50 (μM)		
	Mn (Ⅴ)	Mn (Ⅲ)	Mn $(CH_3COO)_2$
SiHa	6.8 ± 0.8	123.5 ± 6.6	309.7 ± 41.2
HeLa	22.8 ± 0.9	156.9 ± 8.6	226.7 ± 5.9
Caski	5.7 ± 1.4	76.1 ± 8.9	80.2 ± 8.2
SiHa CIS-R	105.2 ± 6.5	350.9 ± 5.8	> 400.0
Hela CIS-R	164.8 ± 23.2	211.7 ± 25.4	220.2 ± 20.4
MCF-7	59.9 ± 7.2	125.7 ± 9.9	148.3 ± 30.1
4T1	< 6.3	79.6 ± 29.1	112.4 ± 13.2
Ect1/E6E7	90.8 ± 1.0	256.0 ± 22.8	820.4 ± 76.9
MiHa	207.5 ± 9.6	249.3 ± 3.1	> 400.0
NIH-3T3	83.4 ± 7.3	129.6 ± 7.1	307.3 ± 27.5

Safe Index = IC_{50} (Ect1/E6E7) / IC_{50} (SiHa) ($Mn(COOH)_2$: 2.65, Mn(III) complex: 2.07, Mn(V) complex: 13.34, Cisplatin: 1.95)

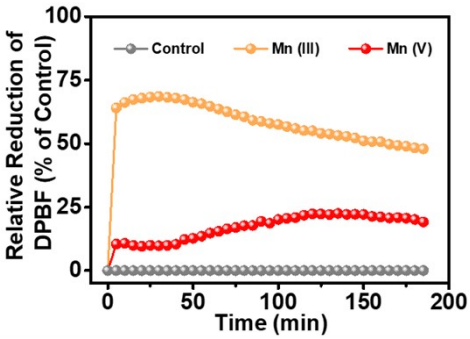

Figure S9 $^1O_2$ level in SiHa cells after treatment with 10 μM complex.

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