Electronic Supplementary Information (ESI)

Manganese(II) complexes stimulate antitumor immunity via aggravating DNA damage and activating cGAS-STING pathway

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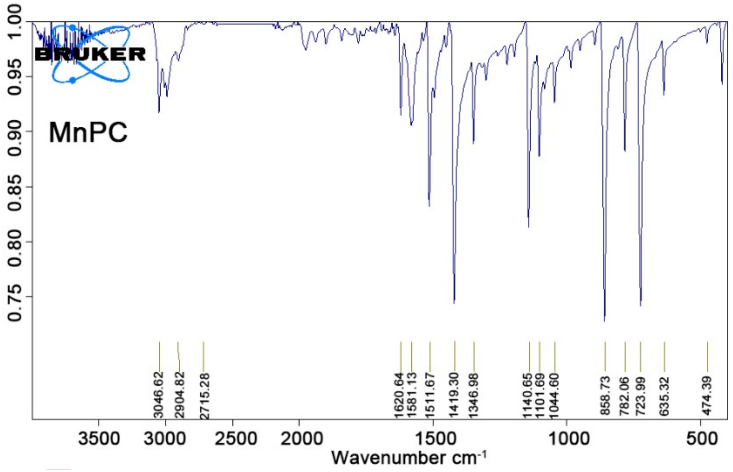
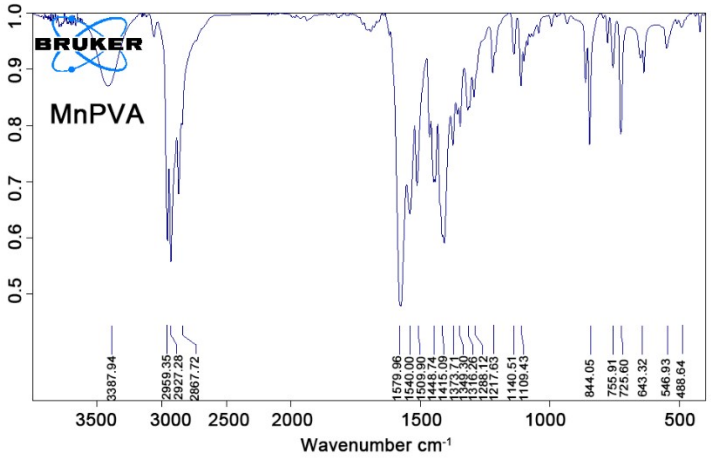
Supplementary figures and tables



A



B

Fig. S1 IR spectra of MnPC (A) and MnPVA (B).

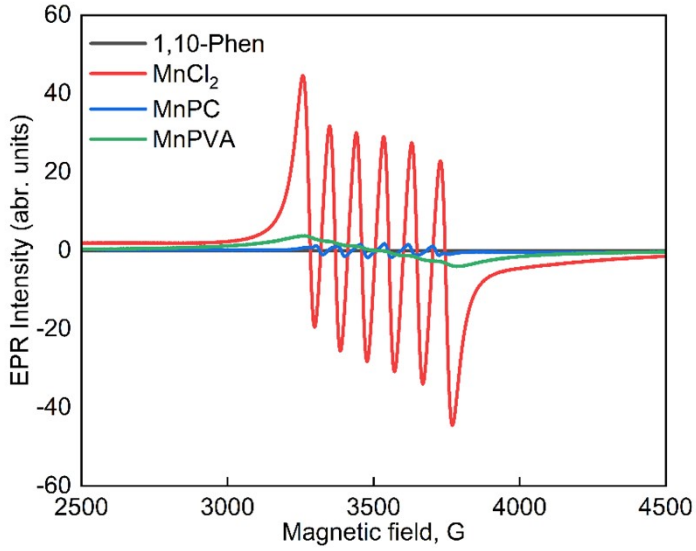


Fig. S2 EPR spectra of 1,10-phenanthroline, $MnCl_2$, MnPC, and MnPVA at 25 °C.

Table S1. Crystal data and structure refinement details of MnPC and MnPVA

Code name	MnPC	MnPVA
Moiety formula	$C_{24}H_{16}Cl_2MnN_4$	C_{28} H_{40} Mn N_2O_5
Formula weight	486.25	539.56
Temperature/K	193 K	296 (2) K
Wavelength/Å	1.34139	0.71073
Crystal system	monoclinic	monoclinic
Space group	$P2_1/c$	C2/c
a/Å	9.4276 (15)	19.6103 (5)
b/Å	15.165 (2)	18.7980 (4)
c/Å	14.397 (2)	16.0459 (5)
α/°	90	90
β/°	98.778 (8)	105.9310 (10)
γ/°	90	90
Volume/ $Å^3$	2034.2 (6)	5687.9 (3)
Z	4	8

Density (calculated) g/cm^3	1.588	1.260
Absorption coefficient /mm^{-1}	5.338	0.502
F (000)	988.0	2296
Crystal size/mm^3	0.12 × 0.1 × 0.1	/
Theta range for data collection/°	7.412 to 121.514	2.162 to 27.499
Index ranges	–12 ≤ h ≤ 12, –19 ≤ k ≤ 19, –17 ≤ l ≤ 18	–25 ≤ h ≤25, –24 ≤ k ≤ 21, –20 ≤ l ≤ 20
Reflections collected	14686	25669
Independent reflections	4517 R_{int} = 0.0795	6512 R(int) = 0.0403
Data/restraints/parameters	4517/0/280	6512/25/331
Goodness-of-fit on F2	1.037	1.455
Final R indexes [I>=2σ (I)]	R_1 = 0.0662, wR_2 = 0.1771	R1 = 0.0627, wR2 = 0.1993
R indexes [all data]	R_1 = 0.0932, wR_2 = 0.2172	R1 = 0.0773, wR2 = 0.2146
Extinction coefficient	n/a	n/a
Largest diff. peak and hole	0.59 and –0.80 e.Å^{-3}	1.042 and –0.544 e.Å^{-3}

Table S2. Selected bond distances (Å) and bond angles (°) for MnPC and MnPVA

MnPC			
Distances (Å)		Angles (°)	
Mn(1)-Cl(1)	2.4375(11)	N(1)-Mn(1)-N(2)	75.15(11)
Mn(1)-Cl(2)	2.4489(12)	N(1)-Mn(1)-Cl(1)	86.99(8)
Mn(1)-N(2)	2.281(3)	N(1)-Mn(1)-Cl(2)	160.68(9)
Mn(1)-N(3)	2.341(3)	N(2)-Mn(1)-N(4)	158.98(12)
Mn(1)-N(1)	2.369(4)	N(2)-Mn(1)-N(3)	89.00(11)
Mn(1)-N(4)	2.287(3)	N(4)-Mn(1)-Cl(2)	97.10(9)

MnPVA			
Distances (Å)		Angles (°)	
Mn(1)-O(3)	2.0651(19)	N(1)-Mn(1)-N(2)	72.73(8)
Mn(1)-O(5)	2.1494(18)	O(1)-Mn(1)-N(1)	94.30(8)
Mn(1)-O(2)	2.248(2)	O(2)-Mn(1)-N(1)	145.77(7)
Mn(1)-O(1)	2.262(2)	O(3)-Mn(1)-N(2)	161.83(9)
Mn(1)-N(1)	2.266 (4)	O(5)-Mn(1)-N(2)	88.47(7)
Mn(1)-N(2)	2.296(2)	O(3)-Mn(1)-O(2)	111.25(8)

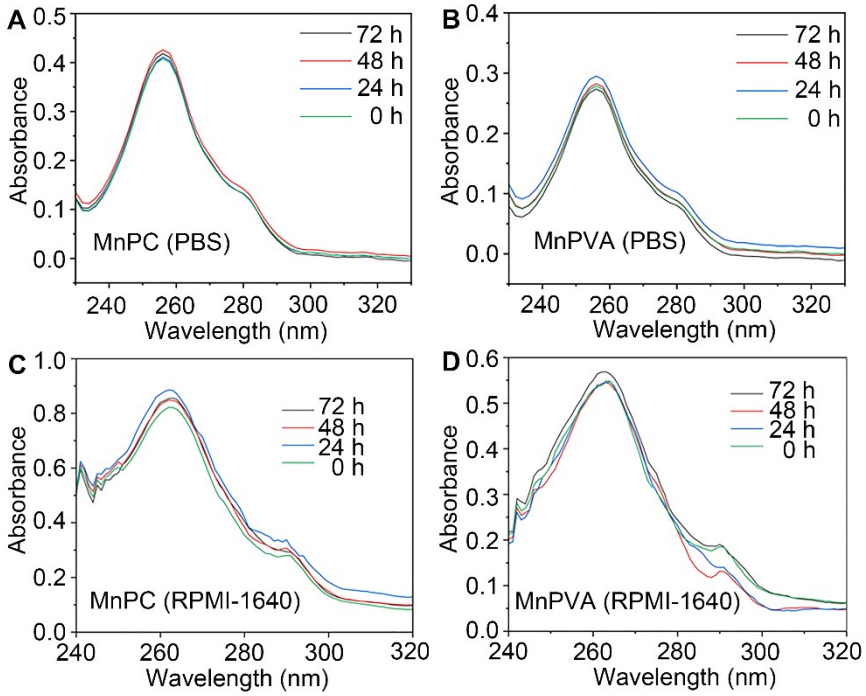


Fig. S3 UV-Vis spectra of MnPC (A, C) and MnPVA (B, D) in phosphate buffered saline (PBS) with 5% DMSO and in RPMI-1640 cell culture media (containing 10% FBS) under physiological conditions (pH 7.4, 37 ℃) at 0, 24, 48, 72 h.

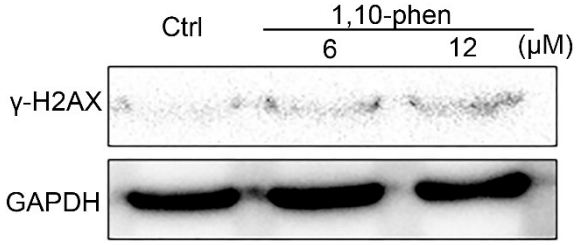


Fig. S4 Expression of DNA damage marker γ-H2AX determined by western blotting after MDA-MB-231 cells were treated with 1,10-phen for 24 h.

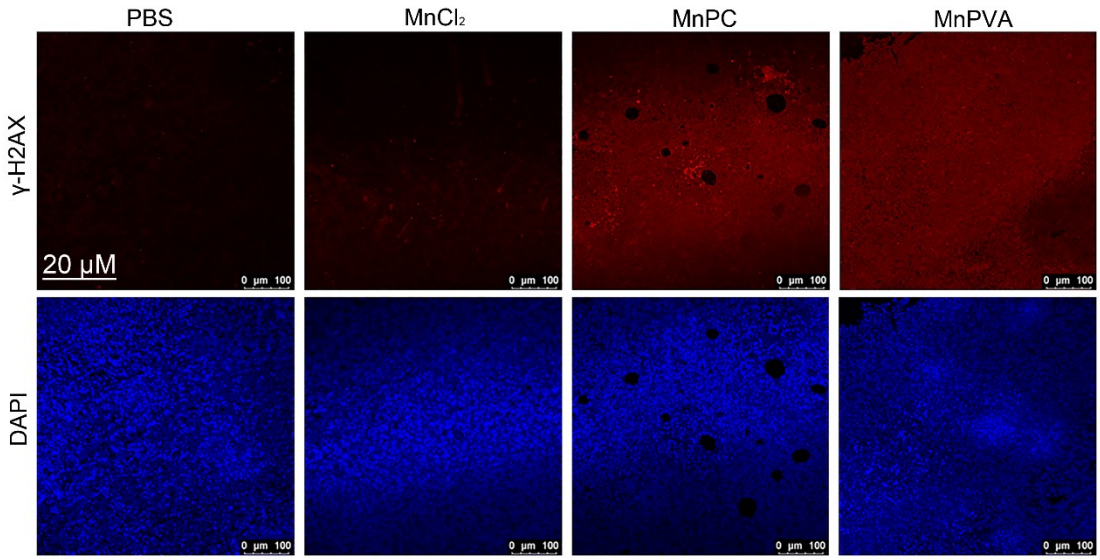


Fig. S5 Immunofluorescence images of γ-H2AX expression in 4T1 tumor tissue of mouse after treatment with each compound (1.3 mg Mn·kg^{-1}) once every 2 days for 16 days.

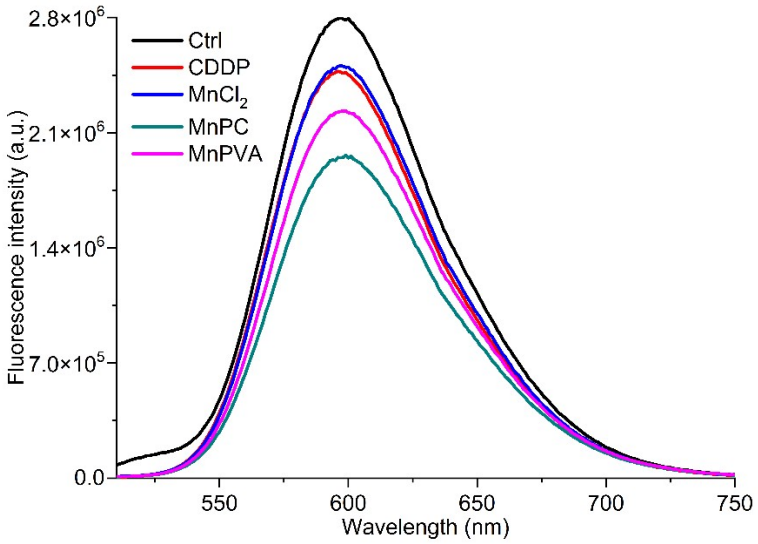


Fig. S6 Fluorescence spectra of CT-DNA-EB system (CT-DNA: 50 μM; EB: 5 μM) in the absence and presence of CDDP, $MnCl_2$, MnPC, and MnPVA (100 μM), respectively.

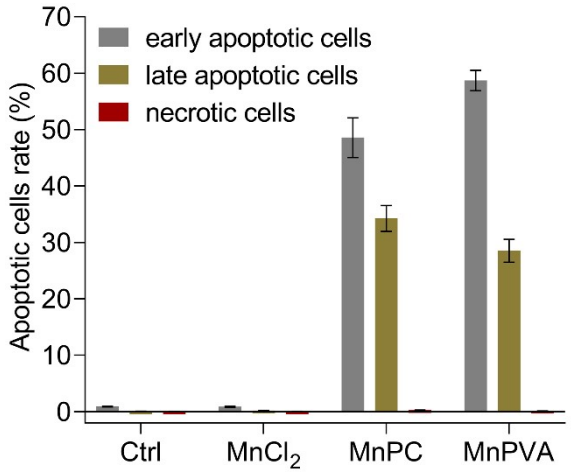


Fig. S7 Histograms showing the apoptotic rate of PI-stained MDA-MB-231 cells after incubation with different compounds (6 μM) for 72 h determined by flow cytometry.

Table S3. IC_{50} values (μM) of MnPC and MnPVA against THP-1 cells at 72 h, with $MnCl_2$, VA, and CDDP as references. Data are shown as mean ± standard deviation (SD, n = 3).

Complex	MnPC	MnPVA	$MnCl_2$	VA	CDDP	1,10-Phen
THP-1	4.33 ± 0.55	4.61 ± 0.57	> 64	> 64	3.66 ± 0.48	> 64

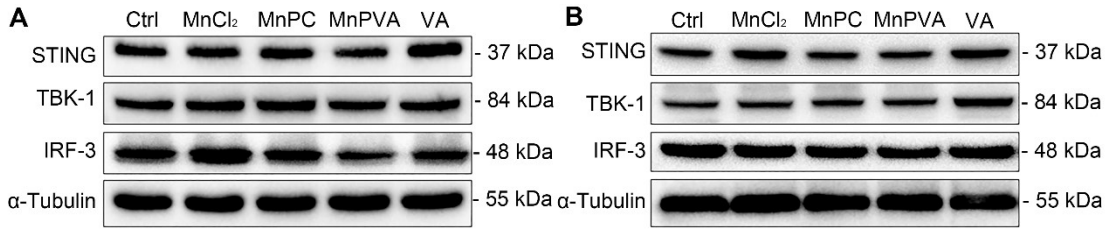


Fig. S8 Expressions of total proteins involved in the cGAS-STING pathway determined by western blotting after MDA-MB-231 (A) and THP-1 (B) cells were treated with 6 and 3 μM of different compounds for 24 h, respectively.

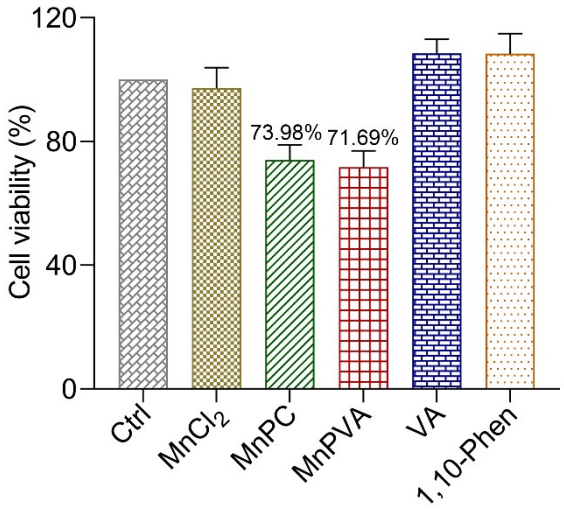


Fig. S9 Average viability (%) of PBMCs after incubation with different compounds (6 μM) for 48 h. Data are shown as mean ± SD (n = 3).

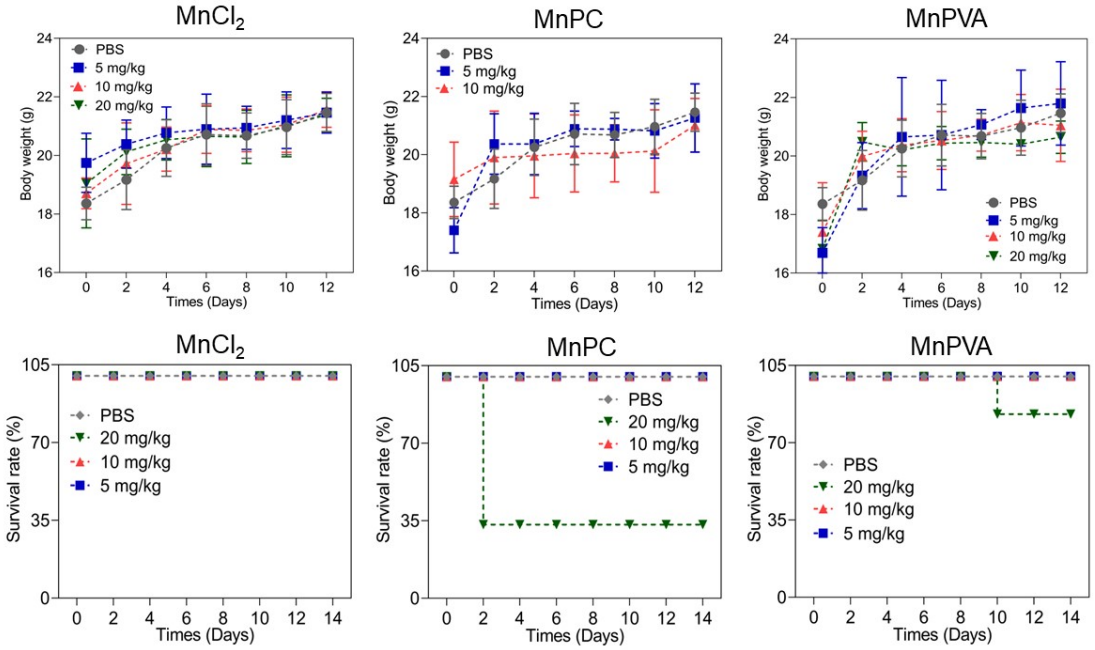


Fig. S10 Body weight and survival rate of Balb/c mice treated intravenously with different doses of $MnCl_2$, MnPC, and MnPVA, respectively, every 2 days. All mice died at 40 mg kg^{-1}.

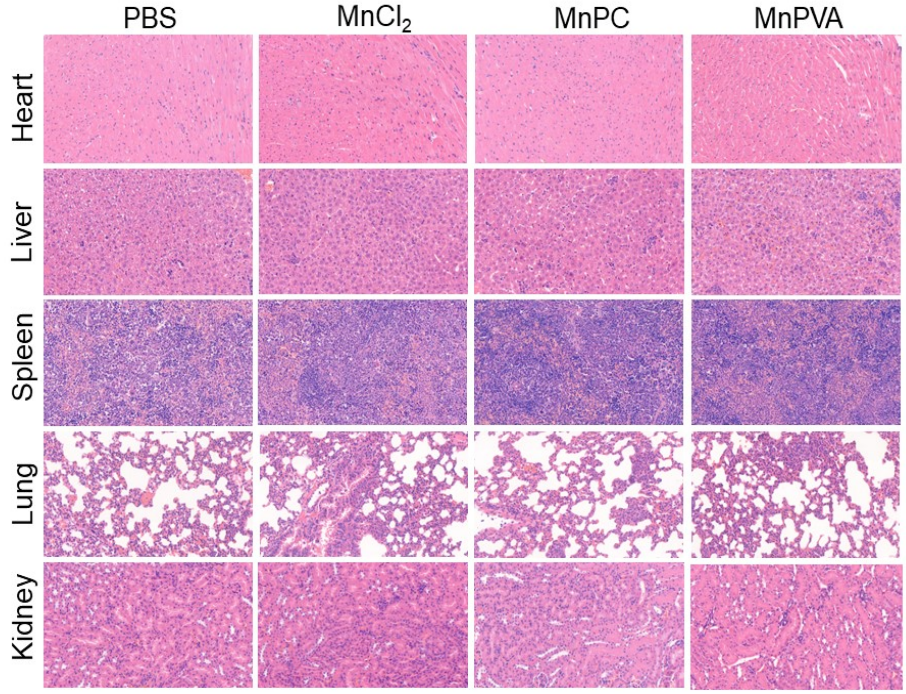


Fig. S11 Images of H&E-stained heart, liver, spleen, lung, and kidney sections collected from Balb/c mice after treatment with PBS, $MnCl_2$ (2.98 mg kg^{-1}), MnPC (11.75 mg kg^{-1}) and MnPVA (12.78 mg kg^{-1}), respectively.

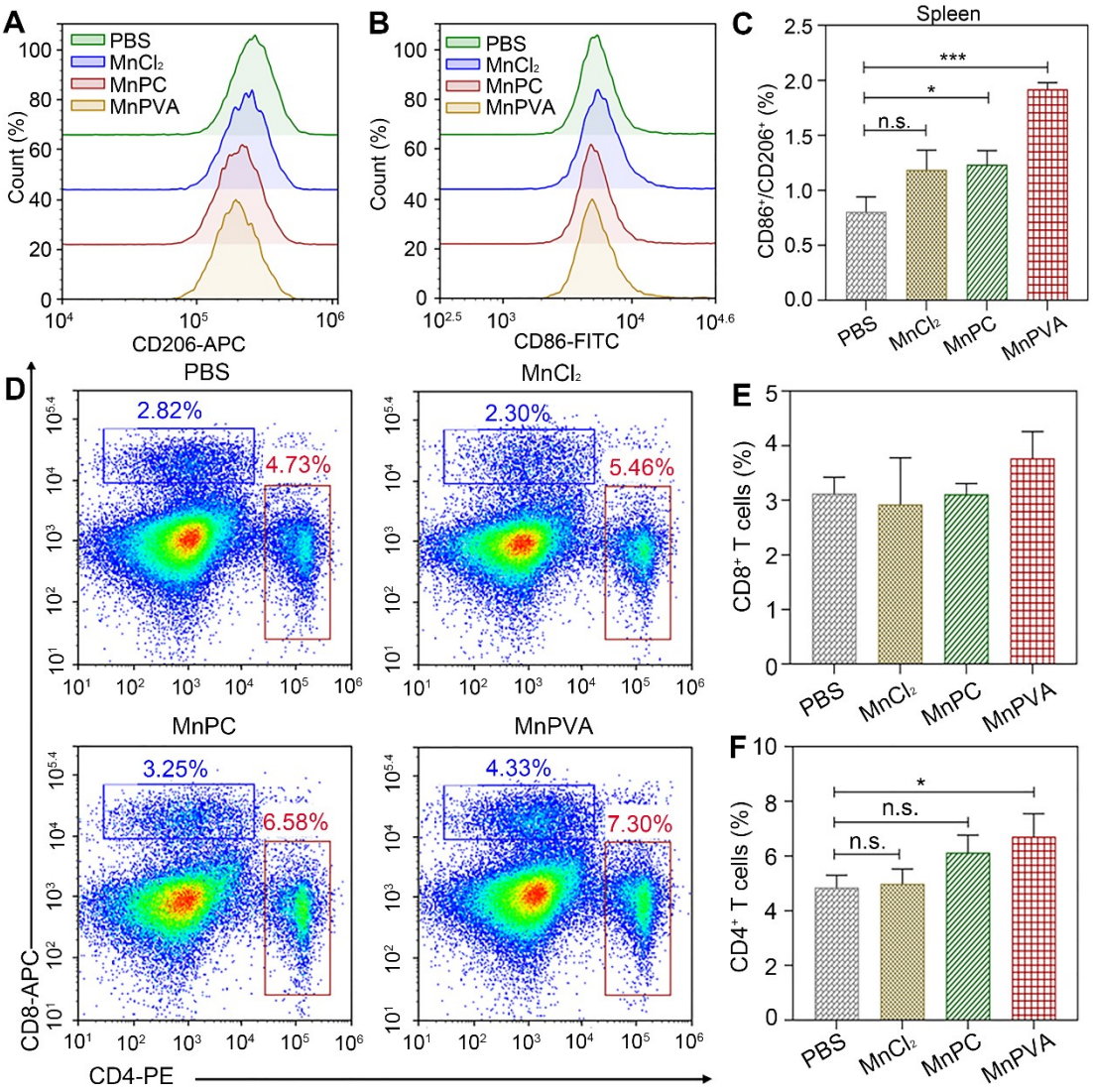


Fig. S12 In vivo antitumor immune responses. Flow cytometric and quantitative analyses of macrophage polarization ($CD206^+CD86^+$ gated on $CD11b^+$) (A, B, C), and $CD4^+$ and $CD8^+$ T cells (D, E, F) in the spleen of 4T1 tumor tissue of mouse after treatment with each compound (1.3 mg $Mn \cdot kg^{-1}$) once every 2 days for 16 days determined by flow cytometry. Data are presented as mean ± SD (n = 3). *P < 0.05; ***P < 0.001.

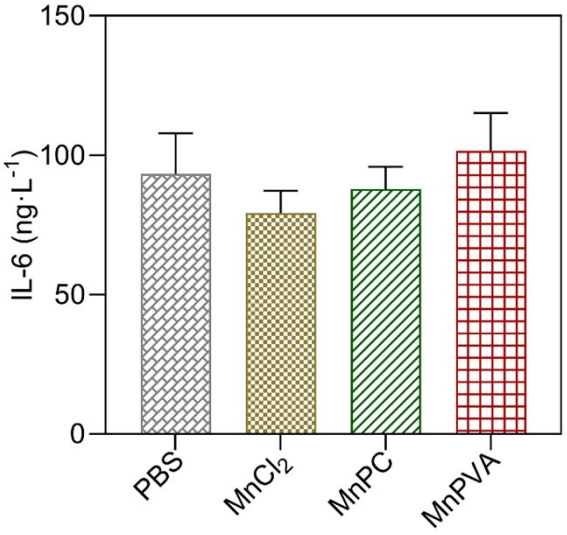


Fig. S13. IL-6 level in serum from mice after treatment with each compound (1.3 mg Mn·kg^{-1}) once every 2 days for 16 days determined by ELISA. Data are presented as mean ± SD (n = 3).

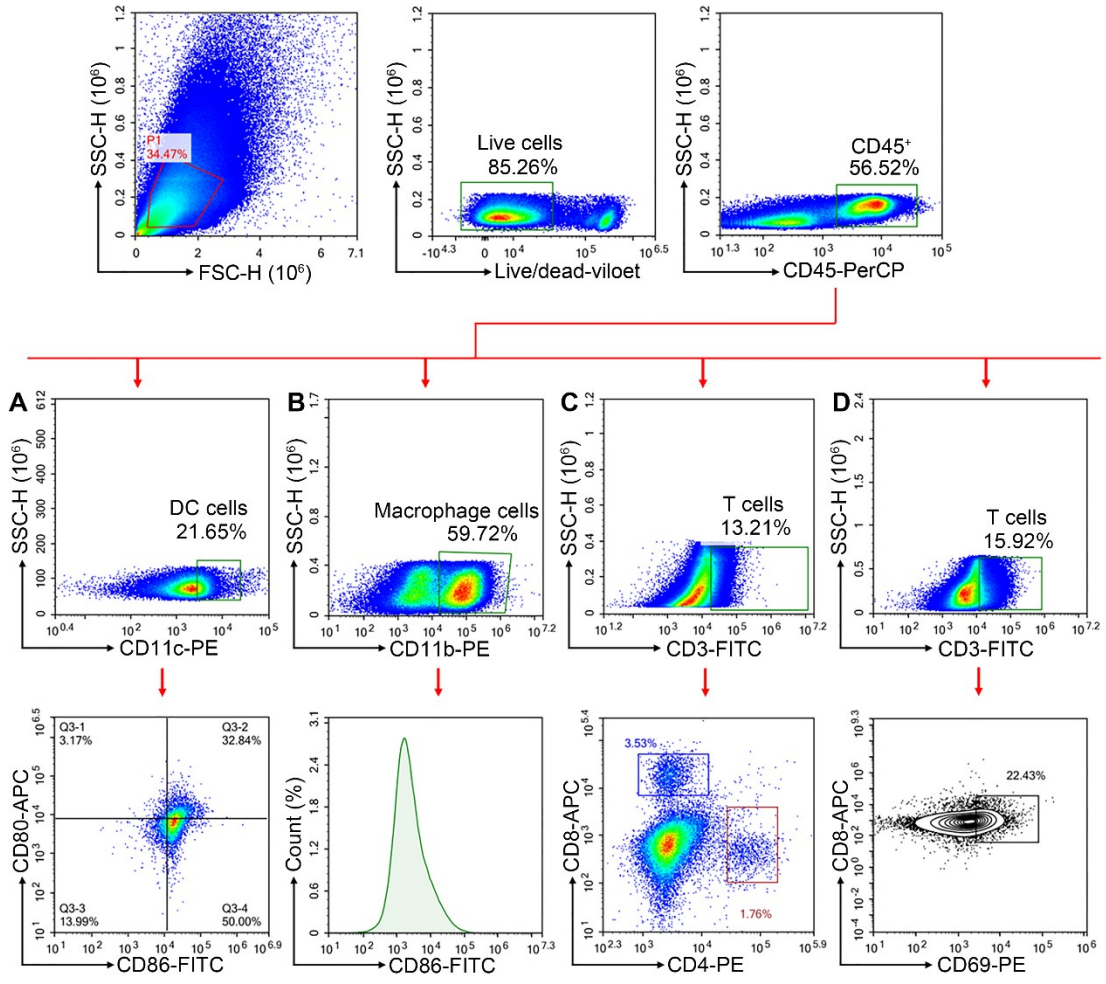


Fig. S14 Gates setting for flow cytometry analysis of DCs (A), macrophage (B), CD4^{+}CD8^{+}T cells (C), and CD69^{+} in CD8^{+} T cells (D).

Experimental section

Materials

All reagents were of analytical grade and used without further purification. Ultrapure water purified by Milli-Q purification system (Merck Corporation, China) was used in all experiments. $MnCl_2 \cdot 4H_2O$, 1,10-phenanthroline, sodium valproate, sodium 4-phenylbutyrate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich and used as received without further purification. Annexin V conjugated with fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) were purchased from KeyGen Biotech. Co. Ltd. (Nanjing, China). Genomic DNA mini preparation kit was purchased from Tiangen Biotech (Beijing) Co., Ltd. cGAS (ab252416), STING (ab239074), TBK1 (ab40676), p-TBK1 (ab109272), IRF3 (ab68481), caspase 3 (ab32351), cleaved-caspase 3 (ab32042), PARP1 (ab191217), cleaved-PARP1 (ab32503), Bax (ab32503), Bcl-xl (ab32370), γ-H_2AX (ab81299), HDAC1 (ab109411), HDAC2 (ab32117), GAPDH (ab181602), α-tubulin (ab52866) antibodies and goat anti-rabbit HRP (IgG H&L) (ab97051) secondary antibody were purchased from Abcam. p-STING (19781T) and p-IRF3 (29047T) antibodies were purchased from Cell Signaling Technology, Inc. CD11c-PE (12-0014-82), CD-86-FITC (11-0862-82), CD80-APC (17-0801-82), MHC-II-PE (12-5321-82), and CD206-APC (17-2061-82) antibodies were purchased from BioLegend. CD69-PE (PE-65105), CD8-APC (APC-65069), CD4-PE (PE-65104), CD3-FITC (FITC-65133), and CD11b-PE (PE-65055) antibodies were purchased from Proteintech. Type I IFN, IFN-β, TNF-α and IL-6 ELISA kit were purchased from Elabscience Biotechnology CO., Ltd.

Elemental analysis (C, H and N) was performed using a CHNO-Rapid elemental analyzer. The content of Mn was determined on an inductively coupled plasma mass spectrometer (ICP-MS) using a standard Plasma-Quad II instrument (VG Elemental, Thermo OptekCorp.). Infrared spectra were attained using a 'Bruker ALPHA spectrometer' in the wavenumber range of 4000–400 cm^{-1}. UV-visible absorption spectra were recorded on a PerkinElmer Lambda 35 spectrophotometer. The data of MTT assay were determined using a Tecan Sunrise ELISA Reader at 570 nm. Flow cytometric analysis was performed by using Cytomics FC500 Flow Cytometry (Beckman Coulter Ltd.). X-band continuous wave electron paramagnetic resonance (EPR) measurements were collected on a Brüker E500 EPR spectrometer at a microwave frequency of 9.85 GHz using a liquid-nitrogen cooling system (100 K). Western blotting was carried out on the Bio-Rad mini-PROTEAN tetra system and Bio-Rad Powerpack Universal. Images were captured using a Chemiscope 3400 mini (Clinx science instrument co. Ltd).

Synthesis of MnPC

MnPC was prepared referring to a literature method.[1] Specifically, 1,10-phenanthrolin (1.07 g, 1.5 mmol) in MeOH (10 mL) was added dropwise to a clear pink solution of $MnCl_2$ (0.50 g, 1 mmol) in MeOH (20 mL), which yielded a yellow precipitate. The suspension was centrifuged and yellow supernatant was discarded. The

solid residue was washed with MeOH (10 mL × 3) and diethyl ether (5 mL × 3) before being dried in vacuo. Crude products (1.51g, 75.41%) were obtain. The products were further purified by boiling in MeOH/ultrapure water (1:1 *v/v*) solution containing HCl (1%). The solution was filtered quickly and placed at 4 ℃ to give purified products, which was dried in vacuo. Yield 1.25 g, 82.78%. Elemental analysis found (calcd) for $C_{24}H_{16}Cl_2MnN_4$: C, 58.87 (59.15); H 3.06 (3.17); N, 11.97 (11.82). IR (ν, cm^{-1}): C=N (Vs) 1511.67, 1419.30, 1346.98; C–C (Vs), 1140.85, 1101.69, 1044.60; Mn–Cl (Vs), 782.06, 723.99; Mn–N (Vs), 635.32, 474.39.

Synthesis of MnPVA

MnPVA was prepared according to a reported method.[2] Sodium valproate (1.32 g, 2 mmol) and $MnCl_2$ (0.5 g, 1 mmol) was dissolved in 20 mL of MeOH/ultrapure water (4:1 *v/v*) and refluxed at 80 ℃ for 30 min. 1,10-Phenanthrolin (0.72 g, 1 mmol) was dissolved in MeOH (5 mL) and added dropwise to the reaction mixture. After stirring for 3 h, the solution was centrifuged to remove any insoluble residues. The collected solution was placed at 4 ℃ for 24–72 h to give a yellow precipitate, which was filtered and washed with water (5 mL × 2), dichloromethane (5 mL × 2), diethyl ether (10 mL × 3), and dried in vacuo. Yield 0.35 g, 63.45%. Elemental analysis found (calcd) for $C_{28}H_{40}MnN_2O_5$: C 62.45 (62.33), H 7.58 (7.47), N 5.21 (5.19). IR (ν, cm^{-1}): H_2O (Vs), 3387.94; CH (Vs), 2959.35, 2927.28, 2867.72; C–O (Vas), 1579.96; C–O (Vs), 1415.09; C=N (Vs), 1540.00, 1509.90, 1448.74; C–C (Vs), 1217.63, 1140.51, 1109.43; Mn–O (Vs), 844.05, 755.91, 725.60; Mn–N (Vs), 643.32, 546.93.

Single crystal X-ray diffraction

Single crystals of MnPC and MnPVPA were obtained in their mother liquor after standing at 4°C for several days. X-ray diffraction data were collected using Bruker SMART APEX II CDD area-detector diffractometer. The crystals were measured at graphite-mono-chromated Mo Kα radiation (λ = 0.71073 Å) at 296(2) K using φ-ω scan technique. Data were corrected for absorption effects using the multi-scan method implanted in the software (SADABS).[3] The diffraction data were integrated by using the SAINT. The structures of MnPC and MnPVA were solved through direct methods and all the non-hydrogen atoms were refined anisotropically on F2 by the full-matrix least-squares technique using the SHELXL-2014 crystallographic program package.[4] All the hydrogen atoms were generated geometrically and refined isotropically using the riding model.

Cell culture conditions

HepG2, MDA-MB-231, 4T1, PANC-1, HK-2, PBMC, and THP-1 cells were obtained from ATCC. HepG2, MDA-MB-231, and PANC-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). 4T1, THP-1 cells and PBMCs were cultured in RPMI 1640 1X medium. HK-2 cells were cultured in DMEM/F12 medium. All media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/mL penicillin-streptomycin, and the cells were grown in a humidified 5% CO_2 (*v/v*) incubator at 37℃.

Cell viability assay

The cytotoxicity of the compounds was determined by the MTT assay. Briefly, HepG2 (4×10^3 cells/well), MDA-MB-231 (6×10^3 cells/well), PANC-1 (8×10^3 cells/well), 4T1 (4×10^3 cells/well) and HK-2 (4×10^3 cells/well) were seeded into 96-well plates and grown for 24 h at 37 ℃. The cells were treated with various concentrations of the compounds, respectively, for 72 h. MTT solution (20 μL, 5 mg kg^{-1} in 1 × PBS) was added to each well and the plates were incubated for 4 h. At the end of incubation, the medium was removed and DMSO was added (150 μL per well) to dissolve the purple formazan crystals. The absorbance was measured at 570 nm using a Varioskan flash multimode reader (Tokyo, Japan).

Cellular accumulation

MDA-MB-231 cells (6×10^5 cells/well) were seeded into 6-well plates and incubated for 24 h. The cells were treated with Mn complexes for 24 h. Each sample was washed with PBS twice, trypsinized and collected into microtubes. The cells were lysed in radio immunoprecipitation assay (RIPA) buffer. The concentration of protein was measured using the BCA protein quantitation kit. The sample was digested with concentrated nitric acid (100 μL) at 95 ℃ for 2 h, hydrogen peroxide (30%, 50 μL) at 95 ℃ for 1 h, concentrated hydrochloric acid (100 μL) at 95 ℃ until the total volume was less than 50 μL. The solution was diluted to 1 mL with ultrapure water, and the content of Mn was determined by ICP-MS.

Quantification of DNA-bound Mn

MDA-MB-231 cells (6×10^5 cells/well) were seeded into 6-well plates and incubated for 24 h. The cells were treated with Mn complexes for 24 h. The cells were harvested by trypsinization, washed twice with PBS. Cell pallets were lysed in DNAzol (1 mL genomic DNA isolation reagent, ThermoFisher Scientific) supplemented with RNAse A (100 μg mL^{-1}). The genomic DNA was precipitated from the lysate with absolute ethanol, washed twice with 75% ethanol and resuspended in NaOH (8 mM). The DNA concentration was determined using nanodrop spectrophotometer and the amount of Mn bound to DNA was quantified by ICP-MS.

Cell cycle analysis

MDA-MB-231 cells (6×10^5 cells/well) were seeded into 6-well plates and allowed to resume exponential growth for 24 h. The cells were treated with Mn complexes for 24 h and then harvested by trypsinization, washed twice with PBS, resuspended in 70% ethanol and kept overnight at 4 ℃ for fixation. After washing with PBS, all the cells were resuspended in RNase A solution (100 μL) with propidium iodide (40 μg mL^{-1}). The data were collected by a flow cytometer (BD LARFortessaTM, USA), and cell cycle profiles were modeled using Modfit LT 2.0 software (Variety Software House, Inc., Topsham, ME, USA).

Annexin V/PI apoptosis assay

MDA-MB-231 cells (6×10^5 cells/well) were seeded into 6-well plates and

allowed to resume exponential growth for 24 h. The medium was replaced with the flesh one containing various concentrations of the complexes. After incubation for 48 h, the cells were washed with PBS twice, trypsinized, resuspended in binging buffer (400 μL), and stained with annexin V-FTIC (6 μL) and PI (6 μL) for 45 min at room temperature in the dark. The fluorescence was immediately analyzed by flow cytometry.

Mitochondrial membrane potential (MMP) assay

MMP was evaluated by confocal imaging via JC-1 staining. MDA-MB-231 cells were seeded in a cell culture dish (φ 2 mm, NEST) and allowed to grow overnight at 37 ℃. The cells were treated with $MnCl_2$, MnPC, and MnPVA (6 μM) respectively for 48 h at 37 °C. Then, the cells were stained with JC-1 (Beyotime Biotechnology) following the manufacture's protocol. In brief, 8 mL of ultra-pure water was used to dilute 50 μL of JC-1 (200 ×), and 2 mL of JC-1 dye buffer (5 ×) was added to obtain the JC-1 dyeing working solution. After rinse two times with incomplete culture medium, the cells were irradiated with a laser at 450 nm (30 J/cm^2, 300 s). Confocal imaging was immediately carried out with Zeiss LSM-710. The excitation wavelength was at 488 nm, and the imaging band path for green channel was 535-560 nm, while that for red channel is 580-640 nm.

Interactions with DNA

CT-DNA stock solution was prepared with Tris-HCl buffer solution (5 mM Tris-HCl, 50 mM NaCl, pH 7.4), which was stored at 4 °C overnight and used within 1 week. The concentration of CT-DNA was determined by a nanodrop spectrophotometer at 260 nm, taking 6600 M^{-1} cm^{-1} as the molar absorption coefficient. EB-DNA solution was pre-incubated at 37 °C for 30 min, and then the complex (10, 20, 30, 50, 60, 70, 80, 90, 100 μM) was added to the EB-DNA solution. The mixture was incubated at 37 °C for 24 h and the fluorescence (λ_{ex} = 485 nm, λ_{em} = 510−800 nm) of the EB-DNA system was measured by fluorescence spectroscopy.

Detection of intracellular ROS

Intracellular ROS levels were detected by using ROS fluorescence probe 2′,7′-dichloro-fluorescein diacetate (DCFH-DA) (Invitrogen). MDA-MB-231 cells were plated in 6-well culture plates and allowed to resume exponential growth overnight, respectively. The cells were treated with each Mn complex (12 μM) or PBS for 18 h. The cells were washed with PBS (1 mL × 2) and incubated with DCFH-DA (10 μM) at 37 °C for 30 min in the dark and washed twice with PBS. Confocal imaging was immediately carried out with Zeiss LSM-710.

Western blotting

MDA-MB-231 and THP-1 cells were seeded into 10 cm cell culture dishes and allowed to resume exponential growth for 24 h. The cells were treated with each Mn complex for 24 h and then harvested, washed with ice-cold PBS twice, lysed in RIPA buffer (150 - 200 μL), and kept at 4 ℃ for 30 min. The supernatant was collected after

centrifugation (1.2×10^4 rpm, 4 °C, 15 min) and the total protein content was quantified by Bradford's assay. Equal quantity of protein (60 μg) was reconstituted in loading buffer (5% DDT, 5 × Laemmli Buffer) and heated at 95 ℃ for 10 min. The protein mixtures were separated by 8–12% SDS-PAGE, and electrophoretically transferred onto a PVDF membrane. After blocking in 5% BSA in TBST solution (1 × TBS with 0.1% Tween-20) for 1 h at ambient temperature, the membranes were rinsed twice with TBST before incubation with primary antibodies in appropriate dilutions at 4 ℃ overnight. The membranes were washed three times with TBST, and incubated with peroxidase-conjugated secondary antibodies in washing buffer for 1 h, and washed three times with TBST. The bolts were incubated with Luminata Crescendo Western HRP substrate (Merck Millipore) and imaged.

ELISA assay

The amount of TNF-α, IL-6 and IFN-β secreted by drug-treated MDA-MB-231 cells or IFN-I secreted by drug-treated THP-1 cells into the conditioned medium supernatant at 24 h was measured using the commercially available human dsDNA, TNF-α, IL-6, IFN-β, and IFN-I sandwich ELISA screening kits (Pierce) according to the manufacturer's protocol. Calibration standards provided by the kits were reconstituted and diluted in conditioned medium.

Cytotoxicity of activated PBMCs against tumor cells

MDA-MB-231 cells were seeded in the bottom layer of the Transwell chamber (3.0 μM) at a certain density and DMEM complete medium containing each Mn complex was added to the cells respectively. After incubation overnight, human PBMCs were seeded in the upper layer at the ratio of 1:10 (MDA-MB-231: PBMCs) and cultured for 48 h, with cell void of PBMCs or/and the complex as the reference. At the end of incubation, the upper layer of Transwell chamber was removed and MTT solution was added. After incubation for 4 h, the medium was removed and DMSO (300 μL) was added. The absorbance was measured on a microplate reader at 570 nm.

Inhibition of HDACs

The HDAC activity of the complexes were determined in vitro using Fluor-de-Lys® HDAC fluorometric activity assay kit (Enzo Life Science). MDA-MB-231 cells were seeded in 96-well plates at the density of 8×10^3 cells/well and allowed to resume exponential growth for 24 h. The medium was replaced with the fresh one containing tested complex and incubated for 48 h according to the manufacture's protocol. Fluorescence was measured using a Fluoroskan Ascent FL (Labsystem, Finland) plate reader with excitation at 360 nm and emission at 460 nm.

Maturation of BMDCs in vitro

Bone marrow-derived dendritic cells (BMDCs) were derived from C57BL/6 mice. In brief, bone marrow cells were withdrawn from the femurs and tibias, and red blood cell (RBC) lysate was added to remove the red blood cells. Mouse bone marrow cells were seeded into 10 cm cell culture dishes in complete RPMI-1640 medium containing

10% FBS with GM-CSF (20 ng mL^{-1}) and IL-4 (10 ng mL^{-1}) for DC differentiation. On day 3, the culture medium was replaced with fresh culture medium containing GM-CSF and IL-4. On day 5, the cells were collected, washed with PBS twice, and seeded into 6-well plates. MDA-MB-231 cells were seeded into 6-well plates at a density of 6 × 10^5 cells per cell and incubated for 24 h. The medium was replaced with the fresh one containing the indicated concentration of Mn complex and incubated for 24 h. The supernatant of MDA-MB-231 cells was collected and co-cultured with immature BMDCs for 24 h. The presence of the co-stimulatory molecules CD11c, CD86, CD80, and MHC-II on BMDCs were analyzed using flow cytometry.

In vivo toxicity tests

The acute toxicity of the Mn complexes in vivo was firstly tested by using female BALB/c mice. All mice were purchased and housed in polycarbonate cages at 22 ± 2 ℃ and a 12 h dark/12 h light cycle, and were fed with commercial chow and water ad libitum. Mn complexes with different concentrations (5, 10, 20, 40 $mg \cdot kg^{-1}$) were dissolved in PBS. The mice were injected with identical PBS (200 μL) every two days, 6 mice in each group. The mice were injected intravenously with $MnCl_2$, MnPC, and MnPVA in 200 μL of PBS, respectively. The behavior and body weight of the mice were monitored over 14 days. Finally, the LD_{50} values of the complexes were calculated. All the experimental procedures on mice were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Nanjing University and experiments were approved by the Institutional Animal Care and Use Committee of Nanjing University.

In vivo antitumor activity

Female BALB/c mice (aged 6 to 8 weeks, 18–20 g, n = 24) were purchased from Jiangsu Huachuang Xinnuo Pharmaceutical Technology Co., Ltd. All experiments were authorized by the Animal Care and Use Committee of Nanjing University.

The 4T1 cell suspension in PBS (6 × 10^6 cells per mouse) was injected subcutaneously into the hind leg of the mouse. When the tumor grew to a size of 80–150 mm^3 10 days after cell implantation, 48 mice were randomly divided into 4 groups, which were injected via the tail vein with 200 μL of PBS, $MnCl_2$ (1.3 mg $Mn \cdot kg^{-1}$), MnPC (1.3 mg $Mn \cdot kg^{-1}$), and MnPVA (1.3 mg $Mn \cdot kg^{-1}$), respectively, every 2 days. The growth of subcutaneous tumor was monitored, and the body weight of mice was observed every two days as well. The tumor volumes were measured according to the formula: $V = W^2 \times L \times 1/2$, where W is the width and L is the length of the tumor. At the end of the experiments, all mice were sacrificed, and the tumor, major organs were harvested. Immunofluorescence and immunohistochemical analysis were carried out after H&E staining, and the blood were collected for ELISA assay.

In vivo antitumor immunity

Tumor, spleen and lymph nodes were harvested from mice in different groups and homogenized into single cell suspension. Red blood cells were lysed with ACK lysing buffer at room temperature for 5 min twice. The cells were stained with corresponding

surface markers, including that of macrophages polarization ($CD86^+CD206^+$ in $CD11b^+$), mature DC cells ($CD80^+CD86^+$ in $CD11c^+$), T cell ($CD4^+CD8^+$ in $CD3^+$), and T cell activation ($CD69^+CD8^+$ in $CD3^+$) respectively. Finally, samples were tested by flow cytometry analyses. Serum samples were isolated from mice after above treatments and stored at –80 ℃. And the secretion of TNF-α, IL-6, and IFN-I (IFN-β) of serum was detected by ELISA kit according to the manufacturer's protocols.

Tumor tissues were collected on day 20 for γ-H2AX and HDAC1/2 immunohistochemistry. Briefly, tumor tissue slides were blocked in 3% bovine serum albumin solution for 1 h at room temperature, followed by routine deparaffinization and antigen retrieval. Primary antibodies were incubated at 4 ℃ overnight. The tumor tissue slides were washed three times with TBST and stained for 1 h. The tissues were counterstained by Hematoxylin, and the nuclear were stained by DAPI, respectively. The images of the tissues were taken by confocal microscopy.

Statistical analysis

Statistical analysis was processed by Graph-Pad Prism 9 (GraphPad software, Inc., La Jolla, CA, USA). For multiple comparisons, a one-way analysis of variance (ANOVA) with Tukey's post hoc test was used. P-values are presented as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. No statistically significant (n.s.) differences were considered when $p > 0.05$.

References

1 S. Abbas, F. Rashid, E. Ulker, S. Zaib, K. Ayub, S. Ullah, M. A. Nadeem, S. Yousuf, R. Ludwig and S. Ali, J. Iqbal, *J. Biomol. Struct. Dyn.,* 2021, **39**, 1068-1081.

2 H. Abu Ali, M. D. Darawsheh and E. Rappocciolo, *Polyhedron,* 2013, **61**, 235-241.

3 G. M. Sheldrick, *Acta. Crystallogr A.,* 2008, **64**, 112-122.

4 M. Sheldrick, *University of Göttingen,* 1990, **46**.