Electronic Supplementary Information

Novel Copper-Based and pH-sensitive Nanomedicine for Enhanced Chemodynamic Therapy

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1. Materials

Sodium carbonate anhydrous (Na_2CO_3), ethanol, sodium diethyldithiocarbamatre (DDTC), and copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) were purchased from Sinopharm Chemical Reagent Co. (China). Human serum abumin (HSA,20%) was obtained from CLS behring. Vorinostat was obtained from Selleckchem (Munich, Germany). Methylene blue (MB), neocuproine and fluorescein isothiocyanate (FITC) were provided by Aladdin Chemical Co. Ltd. Molecular probe 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), Lyso-Tracker (Red) and cell lysis buffer for Western and IP were obtained from Beyotime Biotechnology (China). Amplite ™ fluorimetric HDAC activity assay was provided by AAT Bioquest, Inc (America). 2′,7′- dichlorofluorescin diacetate (DCFH-DA), Calcein-AM/propidium iodide (CA/PI) staining agents and CCK-8 were purchased from Jiangsu KeyGEN BioTECH Corp., Ltd. Roswell Park Memorial Institute (RPMI) 1640 Medium, Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin solution, trysin-EDTA solution, and fetal bovine serum (FBS) were purchased from ThermoFisher Scientific Inc. All the animals used in this study were obtained from Yangzhou university Medical Center (Yangzhou, China). All operations on animals are in accordance with the management practices of Institution Animal Care and Use Committee (IACUC) of Nanjing University.

2. Synthesis and characterization of CuNPs and V-CuNPs

Synthesis of CuNPs and V-CuNPs

Briefly, 1mL HSA and 1mL Na_2CO_3 solution (1 M) were dissolved in 20 mL deionized water and stirred for 20 min at 37℃, Then 10 mL $CuSO_4$ solution (50 mM) and 10 mL vorinostat solution (dissolved in methanol, 3.0 mg/mL) were added alternatively for ten cycles (1mL in one cycle) to the above mixture to form V-CuNPs. After stirring for 30 min at 37℃, the solution was cooled to room temperature. Then, free vorinostat and ions were removed by an ultrafiltration device (Millipore 8400, ultrafiltration membrane MW:30 KD) and the final sample was stored at 4°C for further applications. CuNPs was prepared in the similar way. 10 mL $CuSO_4$ solution (50 mM) was added dropwise to HSA/Na_2CO_3 mixture solution, and cooled to room temperature and removed free ions by the ultrafiltration. FITC-encapsulated CuNPs (FITC@CuNPs) was prepared via similar methods.

Characterization of CuNPs and V-CuNPs

Particle size distributions and zeta potentials of CuNPs and V-CuNPs were detected by DLS (NanoBrook 90 plus Zeta, Brookhaven, USA), transmission electron microscopy (TEM). Lattice information of V-CuNPs was also characterized by TEM (JEM-200CX). Vorinostat loaded V-CuNPs was confirmed with Fourier transform infrared spectroscopy (FT-IR, NEXUS870, Wisconsin), Powder X-ray diffraction (XRD, CAD4/PC, Enraf Noius). The concentration of vorinostat of nanoparticles detected by High Performance Liquid Chromatography (HPLC, Shimadzu LC-20AT).

Encapsulation Efficiency of V-CuNPs

To detect the concentration of vorinostat, briefly, 50 μL sample was dissolved in 950 μL hydrolysis solution (HCl (10 mM): MeOH = 1:1), then the concentration of vorinostat was determined by HPLC. The drug encapsulation efficiency was calculated according to the following formula: Encapsulation Efficiency = (amount of vorinostat in nanoparticles / total amount of vorinostat input during sample preparation) x 100%. The concentration of copper ions was detected by forming $Cu_2(DDTC)_2$ complex with DDTC and determined by UV-Vis absorption at 452 nm, the concentration of cooper ions was calculated according to a calibration curve of known concentrations.

Degradation Experiments of CuNPs and V-CuNPs

Briefly, prepared hydrolysis solutions ($V_{methanol}$: V_{H2O} = 1: 2) of pH = 7.4 and 5.0, mixed CuNPs or V-CuNPs samples with 2 mL hydrolysis solution of different pH. The mixed solutions stand for 2 hour and took picture.

pH sensitive Release Profile

1 mL of V-CuNPs (0.5 mg/mL of vorinostat) were dialyzed (Mw cutoff = 14 kDa) in 200 mL PBS solutions (pH 7.4, 6.5, or 5.0) containing 1% (v/v) Tween 80 at 37 ℃. At predetermined time intervals, 1 mL of the dialysate was removed and 1 mL of fresh PBS solution was added. The concentration of vorinostat was measured by HPLC (Phase A: 0.1% phosphate (H_3PO_4) in water; Phase B: acetonitrile; ZORBAX SB-Aq C18 HPLC column (Agilent) was used with a gradient of B 25 % at 1.0 mL min^{-1} flow rate).

Detection of Cu^+ in CuNPs and V-CuNPs

Firstly, ethanol solution of the neocuproine reagent (10 mM), $CuSO_4$ solution (10 mM), and KH_2PO_4 buffer solution (pH 6.2) were prepared. V-CuNPs and CuNPs were diluted to the concentration of 10 mM with deionized water. Then 900 μL neocuproine solution, 1 mL buffer and 1 mL ethanol were mixed, 100 μL $CuSO_4$, CuNPs or V-CuNPs was added in mixed solutions and reacted for 20 min. Finally, the

absorption from 360 nm to 600 nm was detected by UV−vis spectroscopy.

Fenton reaction and therapetic effects of CuNPs and V-CuNPs

MB (25 μM) were mixed with H_2O_2 (10 mM) and V-CuNPs (0.5 mM) for different times (0, 5, 15, 20, 30 and 60 min), and the remaining MB was measured at 650 nm by UV−vis spectroscopy. The cytotoxicity of V-CuNPs nanomedicine was detected by CCK-8. CT26 cells were seeded into 96-well plates (5 x 10^3 cells per well) and incubated for 12 hours. Then, the CT26 tumor cells were incubated with different concentration of CuNPs, free vorinostat and V-CuNPs, respectively ([vorinostat]=2.5, 5.0, 10, 20 and 30 μg mL^{-1}, [copper ions] ≈ 2.5, 5.0, 10, 5.0 and 10.0 μg mL^{-1}) and V-CuNPs (equivalent copper ions and vorinostat) for 24 hours. After that, the cell medium was replaced by 100 μL fresh culture medium and incubated for another 24 hours. The medium was removed, added 110 μL CCK-8 solution (10 μL CCK-8 to 100 μL medium), and incubated at 37 ℃ for 30 min. The absorbance at 450 nm was detected by microplate reader (Molecular Devices SpectraMax M3). Cells without treatment were used as control. Cell viability was calculated by flowing formula:

$$\text{Cell viability (\%)} = (OD_{sample} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\ \%$$

*OD_{sample}, the absorbance of sample treated cells at 450nm; OD_{blank}, the absorbance of blank at 450nm; $OD_{control}$: the absorbance of control at 450nm.

CT26 cells were seeded into 6-well plates with a density of 2 x 10^3 cells per well for 24 hours, and incubated with CuNPs, free vorinostat (5 μg/mL) and V-CuNPs for 24 hours. After that, the supernatant was discarded, and the cells were washed twice with PBS, replaced with fresh medium, then incubated for 10 days. During this process, the medium was changed frequently. Finally, the cell clones were stained with crystal violet dye for counting.

CT26 cells were seeded in 12-well plates (1 x 10^5 cells per well) for 24 hours. After that, the tumor cells were incubated with vorinostat, CuNPs or V-CuNPs with the same concentration of vorinostat (20 μg/mL) and copper ions (20 μg/mL) for 24 hours. Then, the treated cells were washed with PBS, and stained with AM/PI fluorescent dyes to detect live and dead tumor cells.

Cellular Uptake

CT26 cells were seeded in confocal dish with a density of 6 x 10^4 cells and incubated for 12 h. Then the medium was discarded, and the cells were incubated with FITC@CuNPs ([FITC] = 1 μM) for 0.5, 2 or 12 h. After the cells were washed with PBS, DAPI and Lyso-tacker were used to label nucleus and lysosome. Finally, the cells were observed with Olympus FV3000 LSCM.

To detect concentration-dependent cellular uptake, CT26 cells were seeded in 6-well plates (2 x 10^5 cells per well). After attachment, the cells were treated with different concentrations of FITC-loaded nanocarrier ([FITC] = 0, 0.5, 1, 2, 4 1 μM) for 5h. The treated cells were washed with PBS twice and analyzed by flow cytometry (FL1 channel, BD FACs Calibur).

ROS Detection *in Vitro*

DCFH-DA probe was used to detect intracellular ROS generation. Briefly, CT26 cells was incubated with different concentrations of V-CuNPs ([Copper ions] ≈ 5, 10, 20, 30 and 40 μg/mL, [vorinostat]= 5, 10, 20, 30 and 40 μg/mL)，V-CuNPs ([vorinosta10, 20, 30 and 40 μg/mL) or CuNPs ([Copper ions] =10, 20, 30 and 40 μg/mL) for 10 hours. Then, the treated cells washed with PBS twice and incubated with DCFH-DA fluorescent probe for 30 min. Finally, the cells were washed with PBS and the intracellular ROS generation was detected with fluorescence microscopy.

HDAC Inhibition Assay

The HADC activity was detected by Amplite ™ fluorimetric HDAC activity assay and it was determined by monitoring the green fluorescence enhancement with excitation at 490 nm and emission at 525 nm. The HDAC activity of CT26 cells after incubation with CuNPs, free vorinostat or V-CuNPs was determined by the following method. Firstly, CT26 cells were seeded in 6-well plates (5 x 10^5 cells per well) for 12 hours, and incubated with PBS, CuNPs, free vorinostat (15 μg/mL) or V-CuNPs for 15h. Then, the treated cells were washed twice with PBS, treated with trypsin and then counted. Taking 5 x 10^5 cells of each well and added 100 μL cell lysis buffer to obtain cells extract. Finally, the cells extract was centrifuged at 10000 g for 5 min and the HDAC activity of supernatant was detected by HDAC activity assay.

Pharmacokinetic Profiles of Free Vorinostat and V-CuNPs

The mice were intravenously injected with V-CuNPs and free vorinostat at a dose of 2 mg/kg body weight, respectively (n = 4). Then, 0.5 mL of blood samples were collected from the retro-orbital plexus of eyes at different time points (0.5, 1, 2, 4, 8, 12 and 24 h) after injection. The samples were added with hydrolysate solution (DMSO:10 mM HCl = 1:1, 0.5 mL) and ultrasounded for 5 min, then added with 1 mL methanol for vibration. The mixed solutions were centrifuged at 10000 rpm for 10 min, and the supernatant was collected and detected the concentrations of vorinostat with HPLC.

***In vivo* therapeutic effects of V-CuNPs**

The mice were subcutaneously implanted with 1 x 10^5 CT26 cells per mouse at the left hind leg. When

the tumor grew approximately to 40 mm^3, CT26 tumor-bearing mice were randomly divided into seven groups (n = 7). The tumor bearing mice were treated with: Control group (intravenous injection with PBS), free vorinostat (1 mg/kg of vorinostat, intravenous and intratumoral injection), CuNPs (1 mg/kg of copper, intravenous and intratumoral injection) and V-CuNPs (1 mg/kg of vorinostat and 1 mg/kg of copper, intravenous and intratumoral injection) every 3 days. The tumor size and body weight were recorded every two days. The tumor size was calculated by the following equation: tumor volume V = $AB^2/2$ mm^3 (A, the longest dimension; B, the shortest dimension). At the end of the therapy, blood samples were collected and all the mice were sacrificed. The blood samples were centrifuged at 12000 rpm for 10 min to obtain serum for biochemistry test. The tumors and main organs (heart, live, spleen lung and kidney) were dissected for histopathological analysis with hematoxylin and eosin (H&E) staining. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining and Ki67 immunochemistry assays were performed to detect apoptotic and proliferative tumor cells, respectively.

Statistical Analysis

All data were shown as mean ± SD or mean ± SEM. Statistical significance between two groups was analyzed by two-tailed Student's t test.

Table.1 Four batches of V-CuNPs preparation including approximate 1:1 (m/m) of vorinostat and copper ions.

V-CuNPs	Batch 1	Batch 1	Batch 1	Batch 1
Vorinostat (mg/mL)	1.26	1.35	1.29	1.48
Copper ions (mg/mL)	1.29	1.37	1.30	1.46

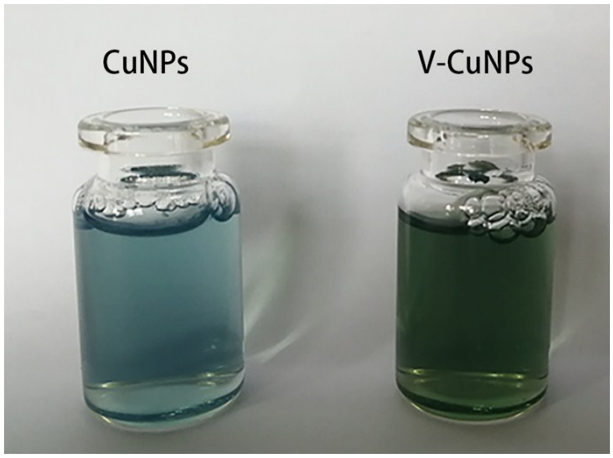

Fig. S1 Images of CuNPs and V-CuNPs.

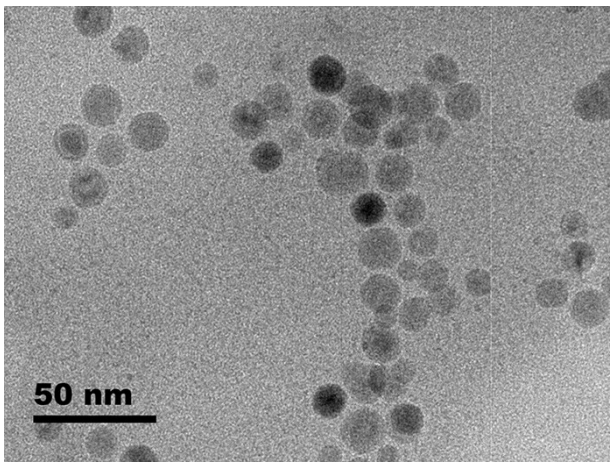

Fig. S2 Transmission electron microscope (TEM) image of CuNPs.

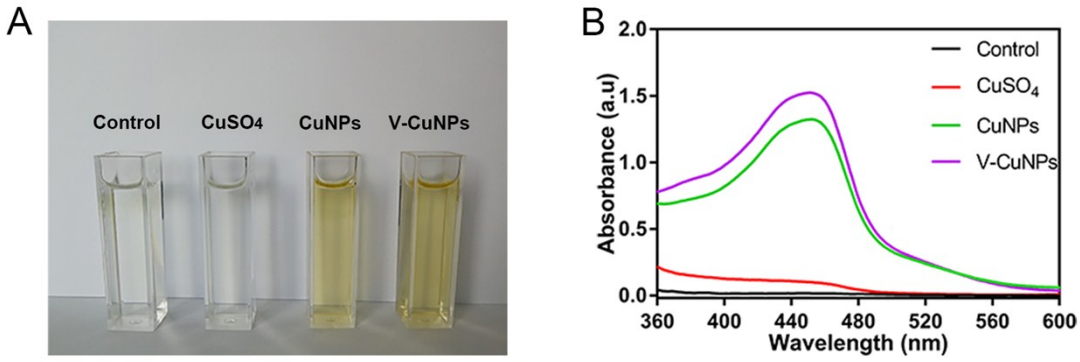

Fig. S3 (A) Images of Cu^{+}-neocuproine complex. (B) UV absorption of various solutions.

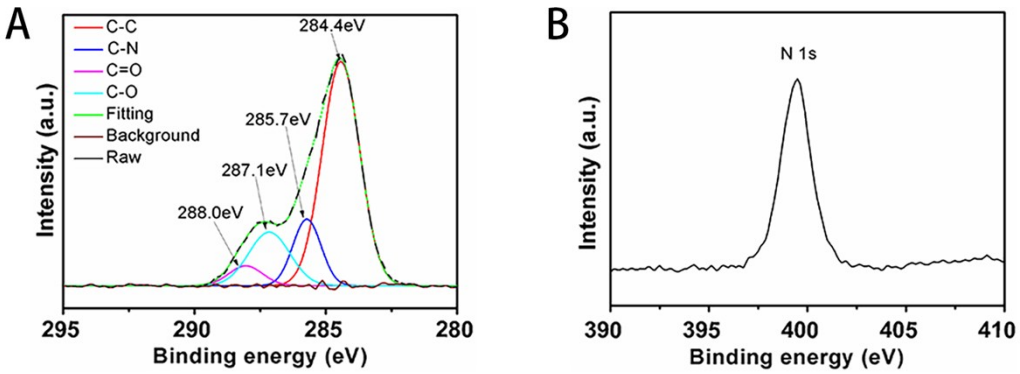

Fig. S4 (A-B) C 1s and N 1s XPS spectrum of V-CuNPs.

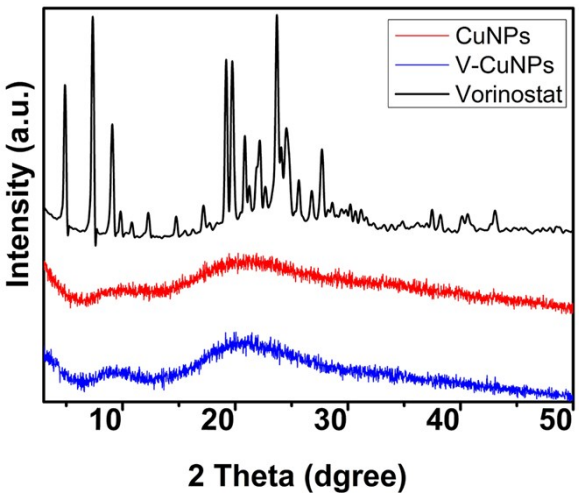

Fig. S5 XRD pattern of pure vorinostat, CuNPs and V-CuNPs.

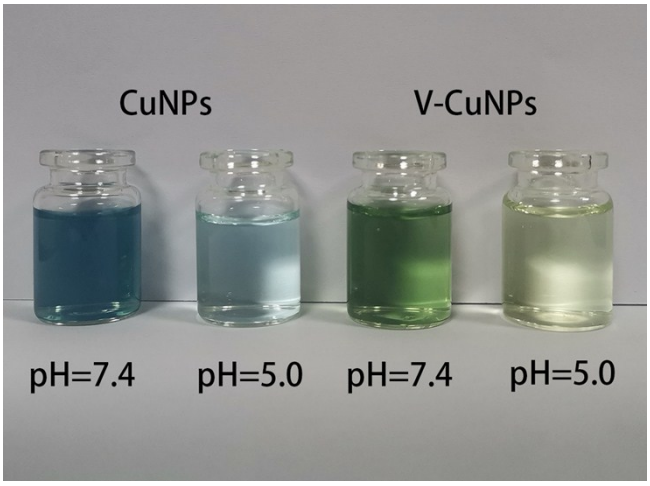

Fig. S6 Degeneration experiment of CuNPs and V-CuNPs at pH = 7.4 or 5.0.

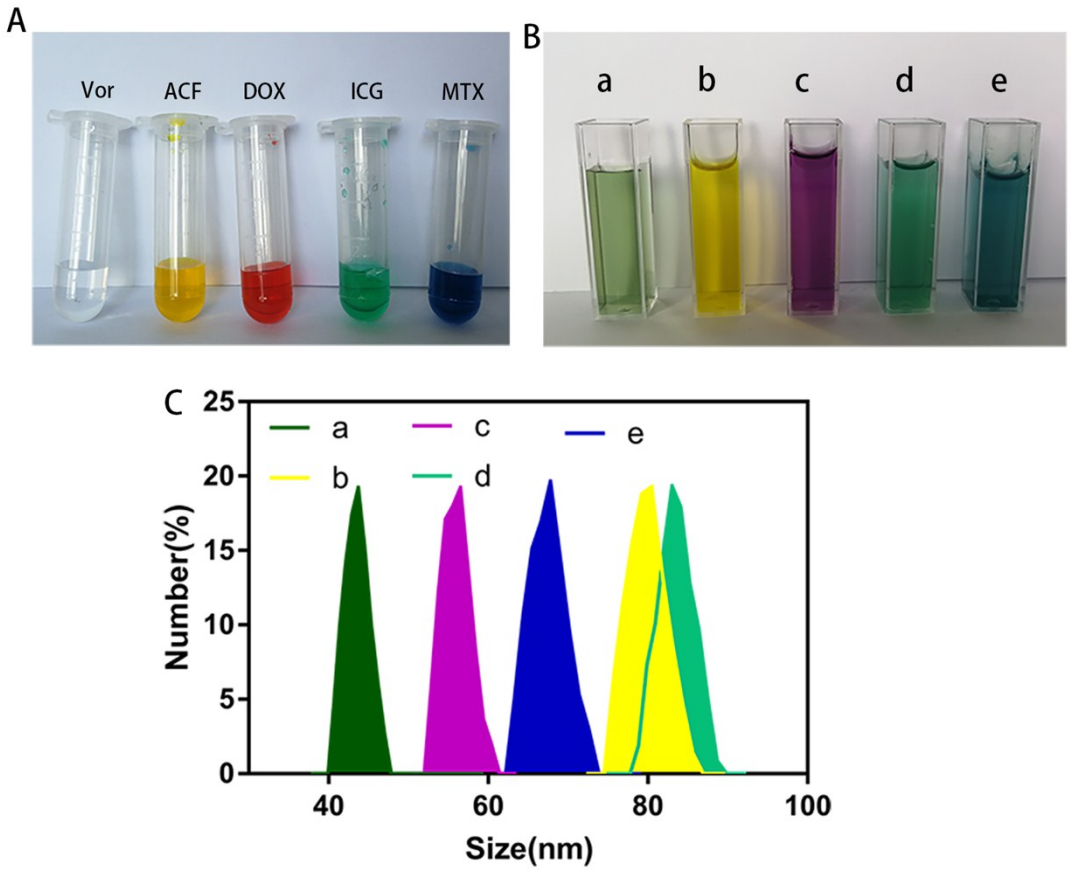

Fig. S7 (A) Images of vorinostat (Vor), acriflavinium chloride (ACF), doxorubicin (DOX), indocyanine green (ICG) and mitoxantrone (MTX) solutions. (B) The appearance of nanoparticles loaded with corresponding drugs and photosensitizers. (C) Dynamic light scattering (DLS) data of various nanoparticles (a, V-CuNPs, b, ACF@CuNPs, c, DOX@CuNPs, d, ICG@CuNPs, e, MTX@CuNPs).

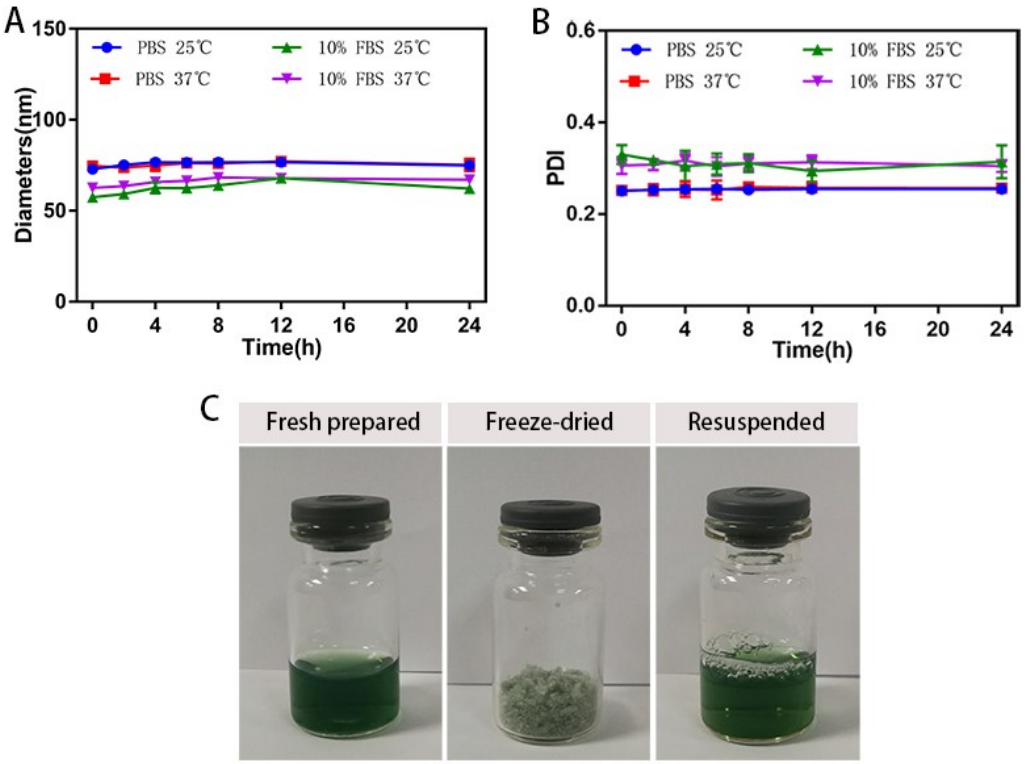

Fig. S8 (A and B) DLS data in number size distribution and polymer dispersity index (PDI) of V-CuNPs incubated with PBS or 10% FBS at 37 or 25 ℃. (C) Images of fresh prepared, freeze-dried and resuspended V-CuNPs. Data were indicated as mean ± SD, n = 3.

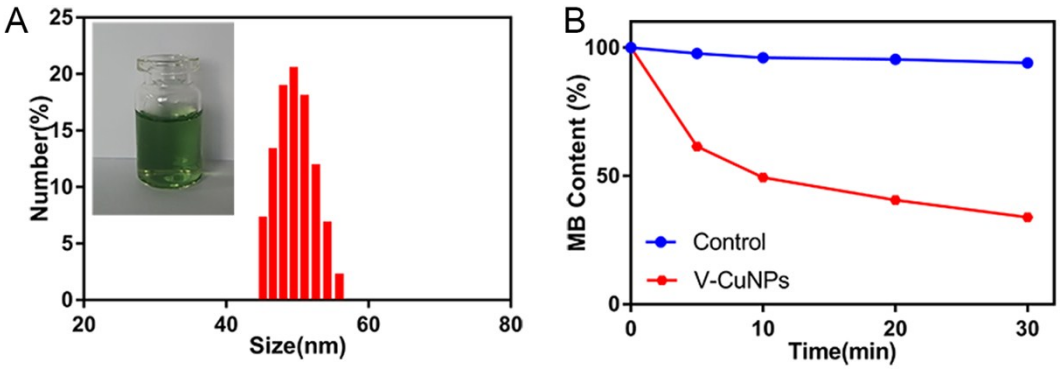

Fig. S9 (A) DLS data of V-CuNPs after stored at 4 ℃ for 3 months. (B) MB degradation curve of V-CuNPs after stored at 4 ℃ for 3 months. Data were indicated as mean ± SD, n = 3.

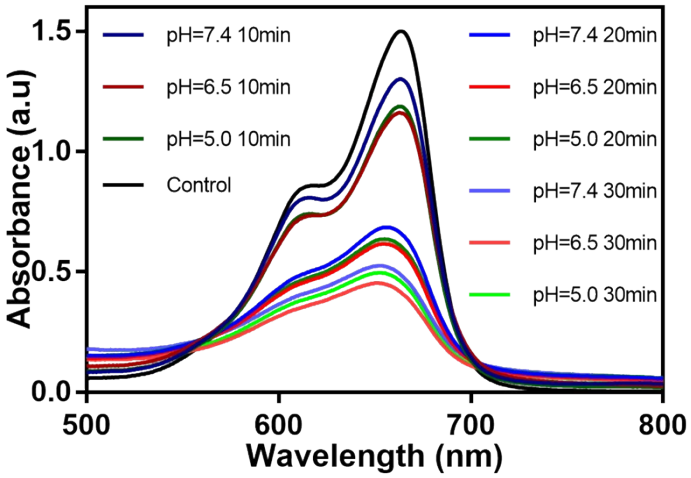

Fig. S10 Degradation rates of MB in the different pH (7.4, 6.5 and 5.0) values.

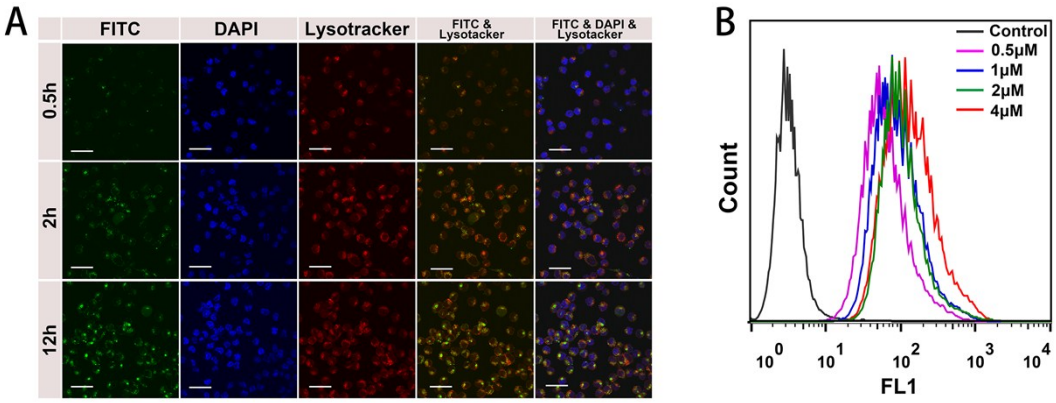

Fig. S11 (A) Laser scanning confocal microscope (LSCM) images of CT26 cells after incubation with FITC@CuNPs, and stained with Lyso-tracker and DAPI, Sale bar, 40 μm. (B) Flow cytometry of CT26 cells treated with different concentrations of FITC@CuNPs).

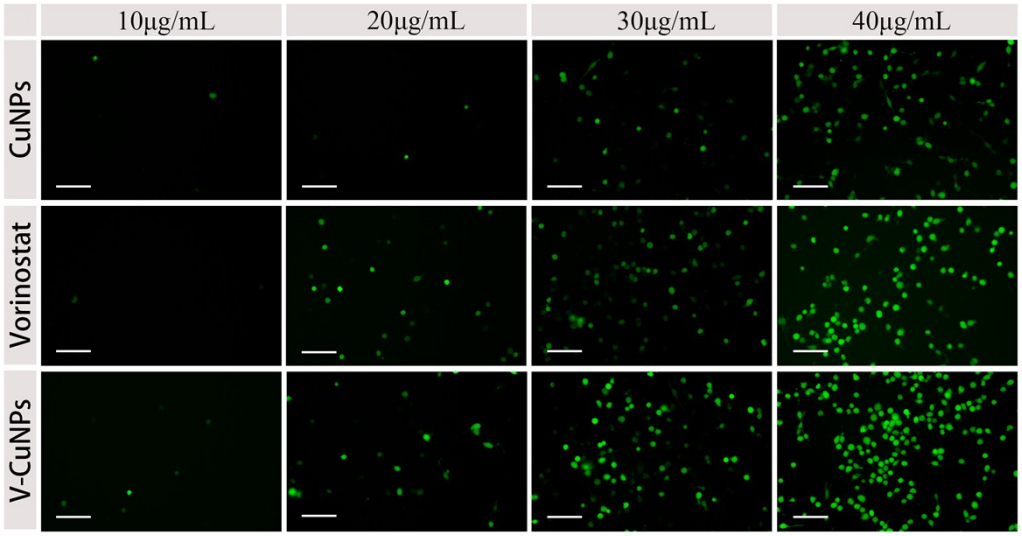

Fig. S12 Fluorescence images of CT26 cells stained with DCFH-DA (green) after treatment with different concentrations of CuNPs ([Copper ions]=10, 20, 30 and 40 μg/mL), free vorinostat ([vorinostat] = 10, 20, 30 and 40 μg/mL) or V-CuNPs ([Copper ions] ≈ 10, 20, 30 and 40 μg/mL, [vorinostat] = 10, 20, 30 and 40 μg/mL) for 12 h. Sale bar, 100 μm.

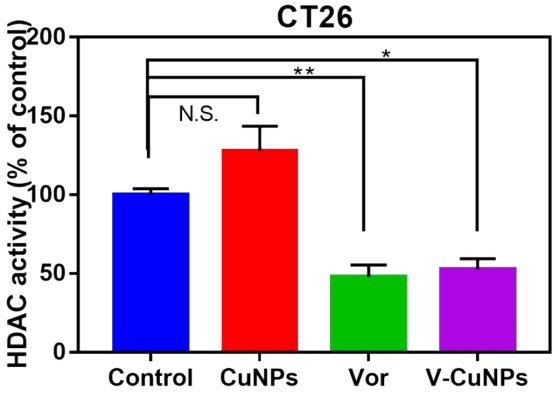

Fig. S13 The HDAC activity of CT26 cells after treated with CuNPs, vorinostat or V-CuNPs, respectively. Data were indicated as mean ± SD, n = 3. *, $p < 0.05$, **, $p < 0.01$, N.S. represented nonsignificance.

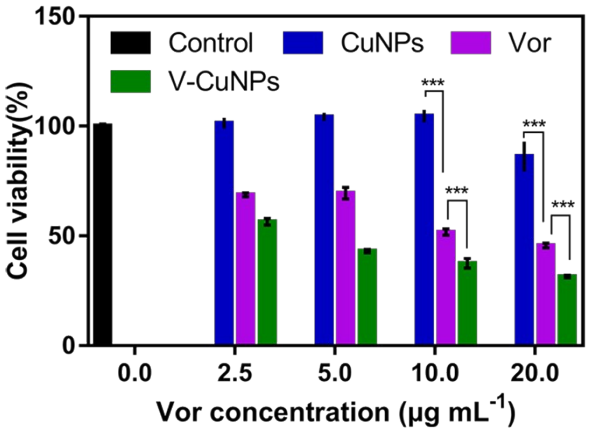

Fig. S14 *In vitro* cytotoxicity of CuNPs, free vorinostat and V-CuNPs with different concentrations against CT26 tumor cells. Data were indicated as mean ± SD, n = 3. ***, $p < 0.001$.

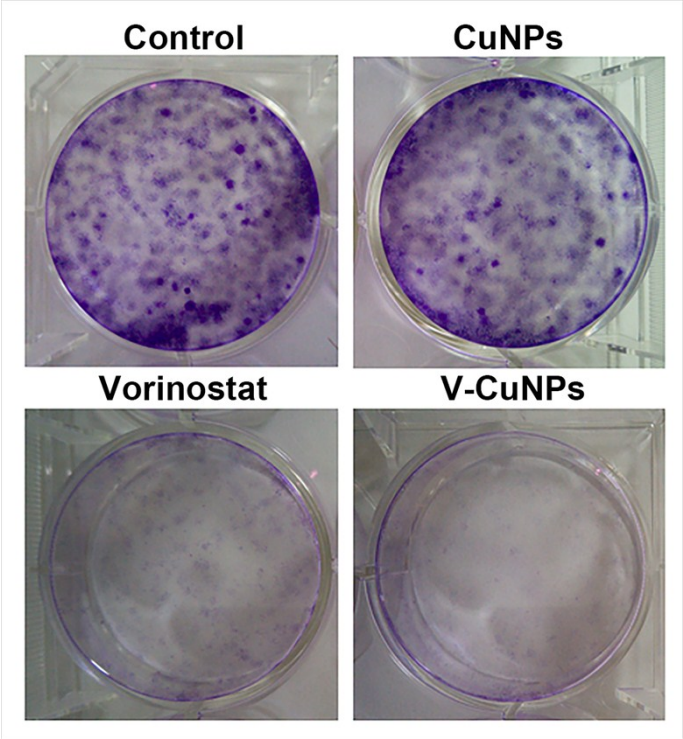

Fig. S15 Colony formation assay of CT26 tumor cells treated with CuNPs, free vorinostat and V-CuNPs.

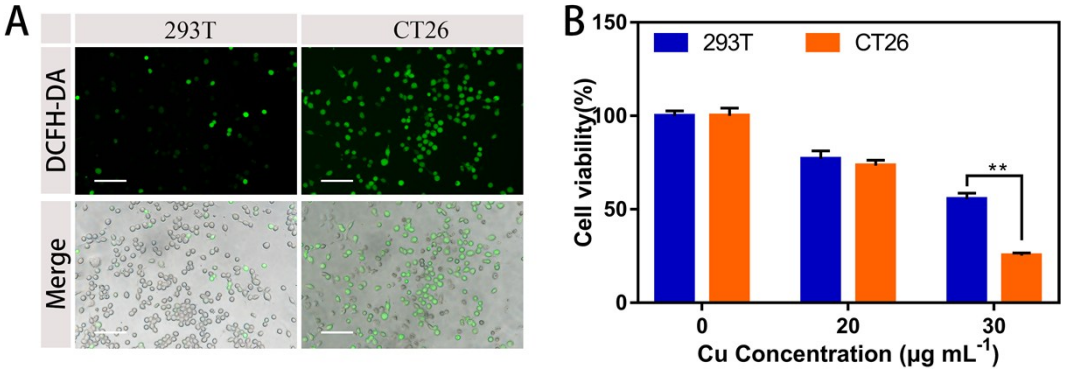

Fig. S16 Fluorescence images of 293T and CT26 cells stained with DCFH-DA (green) after treatment with CuNPs ([copper], 50 μg/mL) for 10 h, respectively. Sale bar, 100 μm. (B) *In vitro* cytotoxicity of CuNPs with different concentrations against 293T and CT26 cells. Data were indicated as mean ± SD, n = 3. **, $p < 0.01$.

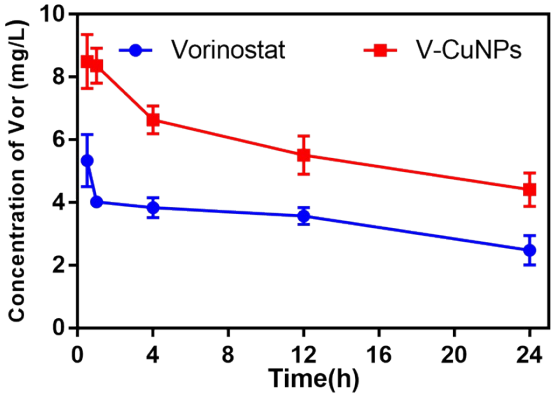

Fig. S17 Pharmacokinetic profiles of free vorinostat and V-CuNPs after intravenous injection (2 mg/kg of vorinostat). Data were indicated as mean ± SEM, n = 4.

Table. 2 Pharmacokinetic parameters of free vorinostat and V-CuNPs. Data were shown as ± SD (n = 4). The pharmacokinetic parameters of vorinostat and V-CuNPs were analyzed by DAS 2.0 software.

Parameters	AUC(0-t)	AUC (0-∞)	AUMC(0-t)	MRT(0-t)	$T_{1/2}$	Cmax
Unit	mg/L*h	mg/L*h	mg/L*h^2	h	h	mg/L
Vorinostat	82.620 ± 6.230	192.899 ± 87.922	878.57 ± 139.563	10.588 ± 0.948	23.342 ± 17.088	5.708 ± 0.931
V-CuNPs	139.175 ±21.208	385.978 ± 188.772	1459.052 ± 309.775	10.416 ± 0.802	36.659 ± 19.525	8.991 ± 1.258

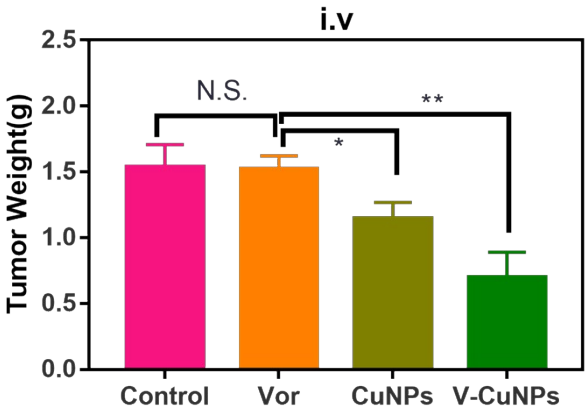

Fig. S18 Tumor weight after 16 days of intravenous administration. Data were indicated as mean ± SEM, n = 7, *, $p < 0.05$, **, $p < 0.01$, N.S. represented nonsignificance.

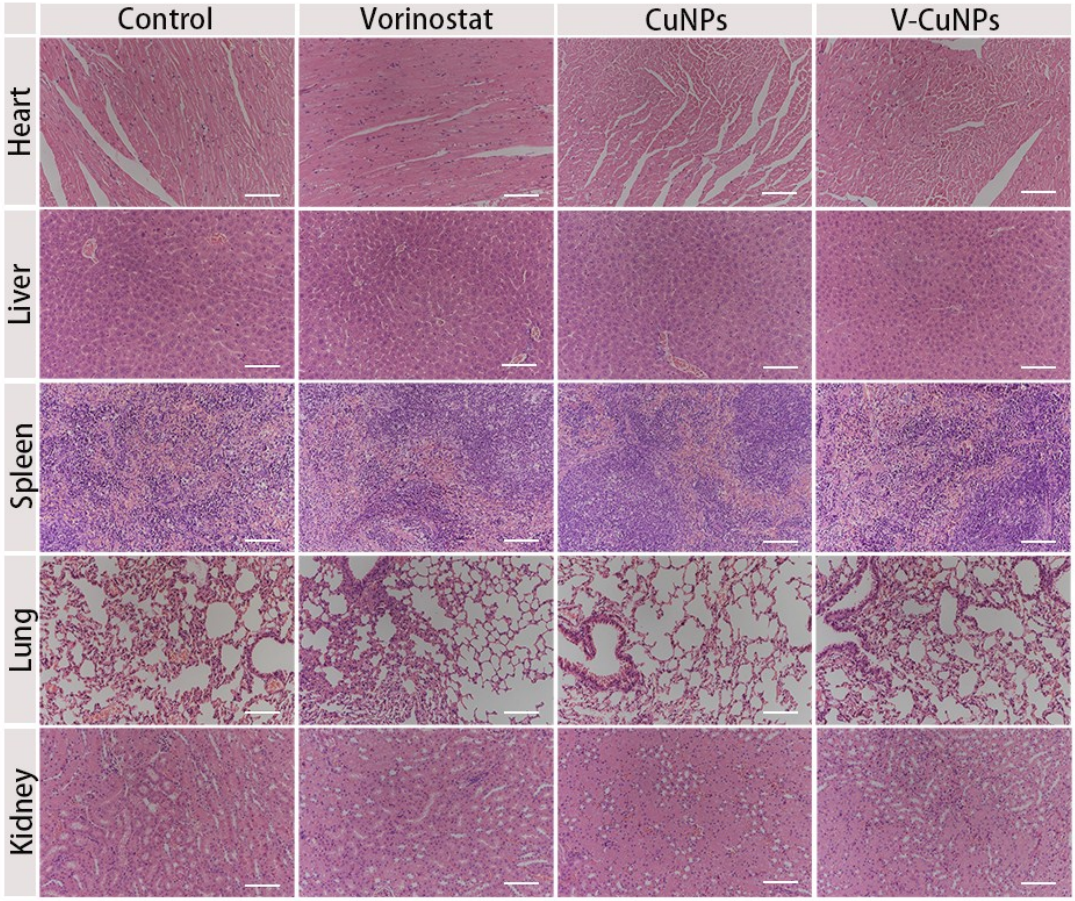

Fig. S19 H&E staining of the major organs after intratumoral administration for 16 days, scale bars 100 μm.

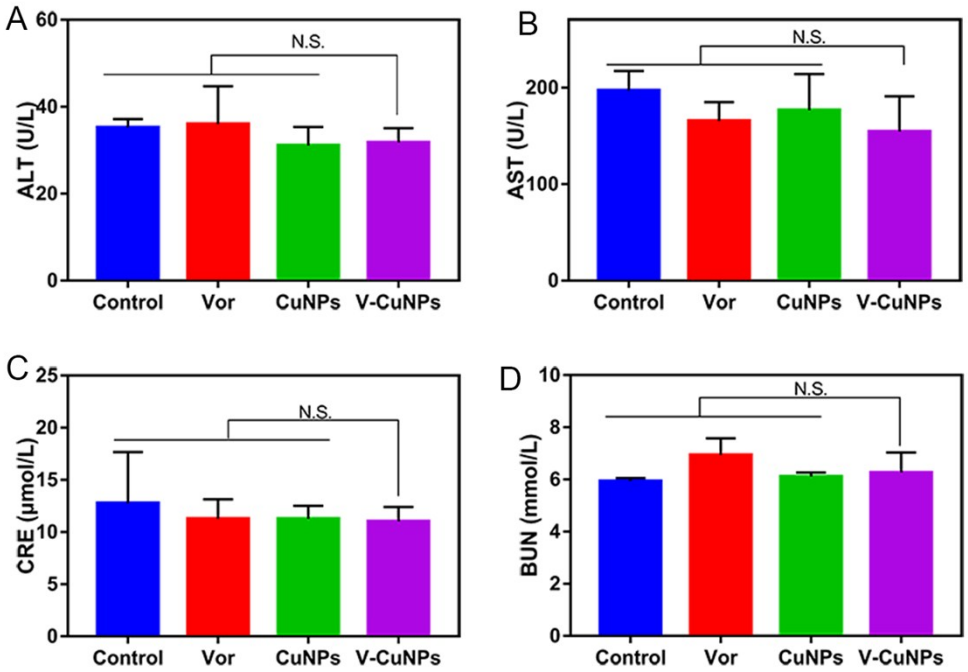

Fig. S20 (A-D) Serum biochemical data of liver and renal functions. Data were indicated as mean ± SD, n = 7, N.S. represented nonsignificance.