Supporting Information

A $CaCO_3$-based synergistic immunotherapy strategy for treating primary and distal tumors

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Reagents and Materials.

Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), ammonia bicarbonate (NH_4HCO_3), and copper chloride ($CuCl_2$) were purchased from China National Pharmaceutical Group Corporation, China. 1, 2-dioleoylsn-glycero-3-phosphate (sodium salt) (DOPA) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000) DSPE-PEG2000 were purchased from Avanti. 1, 2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Corden Pharma Switzerland LLC. Cholesterol was purchased from Xi'an ruixi Biological Technology Co., Ltd. The INCB24360 analogue, 4-amino-N-(3-chloro-4-fluorophenyl)-N'-hydroxy-1,2,5-oxadiazole-3-carboximidamide (IDO5L) (IDOi) was purchased from Medchemexpress (MCE). Fetal bovine serum (FBS) was purchased from Biological Industries. Trypsin-EDTA was purchased from Gibco, USA. RPMI 1640 medium was purchased from Key Gen Biotech. Co., Ltd. The methylene blue and 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-diphenytetrazoliumromide (MTT) was purchased from Beijing Solarbio Science & Technology Co., Ltd. Reactive Oxygen Species Assay Kit (2,7-dichlorofluorescin diacetate, DCFH-DA) was purchased from Beyotime (Nantong, China). Live & Dead Viability/Cytotoxicity Assay Kit for Animal Cells was purchased from Key Gen Biotech. Co., Ltd. (Nanjing, China). The Mouse TNF-α ELISA Kit were purchased from Boster Biological Technology Co., Ltd, China. Glass Bottom dishes were purchased from Cellvis, Mountain View, CA. 96-well plates were purchased from Hangzhou Xinyou Biotechnology Co., Ltd, China. Plastic centrifuge tubes were purchased from GeneBrick Bioscience LLC. All the aqueous solutions used in experiments were prepared using deionized water (18.2 MΩ cm) obtained from a Milli-Q water purification system. All chemicals were of analytical grade and were used without further purification. Transmission electron microscopy (TEM) was carried out on a HT7700 electron microscope (Hitachi, Japan). Dynamic lighting scattering (DLS) measurements were performed on a S3 Malvern Zeta Sizer Nano (Malvern Instruments). The absorbance was measured in a microplate reader (Synergy 2, Biotek, USA) for the MTT assay. Confocal fluorescence imaging studies were performed using a TCS SP8 confocal laser scanning microscope (Leica, Germany).

Cell lines and animals.

4T1 cells were cultured in RPMI 1640 medium containing 10% FBS and 100 U mL^{-1} of 1% antibiotics (penicillin/streptomycin) and were maintained at 37 °C in a 5% CO_2/95% air humidified incubator (SANYO).

Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU2022113). All animal experiments were conducted and obeyed the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, Biology Institute of Shandong Academy of Science, China.

Synthesis of the $CaCO_3$ nanoparticles.

$CaCO_3$ nanoparticles were synthesized by the gas diffusion reaction. 220 mg $CaCl_2 \cdot H_2O$ was dissolved in 100 mL ethanol in a glass bottle covered by aluminium foil with several pores. Then, 8 g dry ammonia bicarbonate (NH_4HCO_3) was put together with the $CaCl_2$ solution in a vacuum drying chamber for 24 h. $CaCO_3$ nanoparticles were obtained and separated by centrifugation at 12000 rpm.

Synthesis of the $CaCO_3$/Cu nanoparticles.

Similarly, 220 mg $CaCl_2 \cdot H_2O$ and 5 mg $CuCl_2$ were dissolved in 100 mL ethanol in a glass bottle covered by aluminium foil with several pores. Then, 8 g dry ammonia bicarbonate (NH_4HCO_3) was put together with the $CaCl_2$ solution in a vacuum drying chamber for 24 h. $CaCO_3$/Cu nanoparticles were obtained and separated by centrifugation at 12000 rpm.

Synthesis of the $CaCO_3$/Pt and $CaCO_3$/Cu/Pt nanoparticles.

1.25 mg oxaliplatin was dissolved in 1 mL DMF. Then 5 mg $CaCO_3$ or $CaCO_3$/Cu was weighed and added into the above solution and then treated by ultrasound. The mixture was stirred at room temperature for 24 h, and centrifuged at 12000 rpm to obtain $CaCO_3$/Pt or $CaCO_3$/Cu/Pt.

Synthesis of PEGylated nanoparticles.

The above prepared nanoparticles were dissolved in 0.2 mL ethanol and mixed with 0.25 mL DOPA solution (2 mg/mL in chloroform). Then the mixtures were treated by

ultrasonic for 20 min. The obtained turbidized solution (called A) was centrifuged to remove free DOPA, and then dispersed into chloroform. Then, DPPC, cholesterol, and DSPE-PEG in chloroform (4:4:2) were added to solution A and stirred overnight. The chloroform is evaporated to obtain PEGylated $CaCO_3$, $CaCO_3$/Cu, $CaCO_3$/Pt or $CaCO_3$/Cu/Pt nanoparticles. Notably, the materials used in the following experiments were all PEGylated.

MB degradation assay.

MB was employed to determine the generation of •OH. MB was added into the $CaCO_3$ and $CaCO_3$/Cu solutions with H_2O_2. After incubated for 60 min, the solutions were centrifugated and the UV-Vis absorbance spectra of the supernatants were detected.

MTT assay.

To evaluate the cytotoxicity of materials, 4T1 cells were dispersed in 96-well plates at 37 ℃ for 24 h. After that, the cells were incubated with different concentrations (0.2, 0.5, 1.0, 1.5 mg/mL) of materials for 2 h, and then incubated for another 24 h. The MTT solution (150 μL, 0.5 mg/mL in PBS) was further added to each well and incubated for 4 h. After removing the remaining MTT medium, 150 μL of DMSO was added to each well. The absorbance was measured at 490 nm with microplate reader.

Live/dead staining assay.

4T1 cells were cultured in confocal dishes for 24 h. The cells were divided into 5 groups: 1, PBS; 2, $CaCO_3$; 3, $CaCO_3$/Pt; 4, $CaCO_3$/Cu; 5, $CaCO_3$/Cu/Pt. The materials (1.0 mg/mL) dissolved in 1640 were added into the dishes. And the cells were further incubated for 2 h. All the cells were stained with live/dead cell staining assay kit for 30 min and further imaged by the laser scanning confocal microscope.

Detection of intracellular ROS.

4T1 cells were cultured in confocal dishes for 24 h. The cells were first incubated with DCFH-DA for 15 min. Then the cells were divided into 5 groups and treated with: 1, PBS; 2, $CaCO_3$; 3, $CaCO_3$/Pt; 4, $CaCO_3$/Cu; 5, $CaCO_3$/Cu/Pt for 1 h, respectively. Subsequently, the cells were washed with PBS solution and further imaged by the laser scanning confocal microscope.

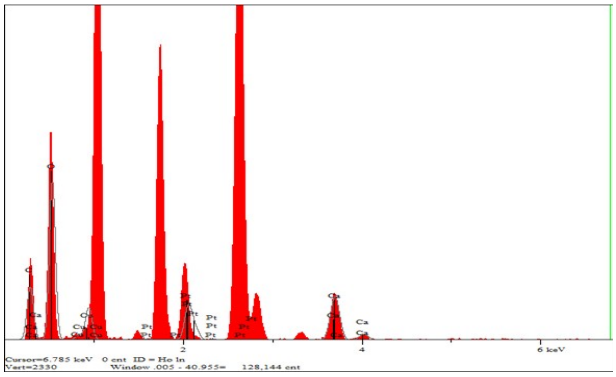
Detection of CRT protein and HMGB1.

The immunofluorescence staining assay was performed to study the CRT of 4T1 with different treatments. Cells were divided into 5 groups and treated with: 1, PBS; 2, $CaCO_3$; 3, $CaCO_3$/Pt; 4, $CaCO_3$/Cu; 5, $CaCO_3$/Cu/Pt for 2 h. After fixed and permeated, the cells were treated with blocking reagent. Cells were stained with anti-CRT-Alex 647 at room temperature for 2 h and Hoechst 33342 for 5 min. Then the cells were studied by the laser scanning confocal microscope for imaging the CRT. Or cells were incubated with primary antibodies (anti-HMGB1) for 1 h and stained with the second antibody (DyLight 550) for 1 h and stained with Hoechst 33342 for 5 min. Then the cells were studied by the laser scanning confocal microscope for imaging the HMGB1.

In Vivo Therapeutic Effect.

First, 5×10^5 4T1 cells in 50 μL of serum-free RPMI 1640 medium were subcutaneously injected into the right flank of the Balb/C mice as primary tumors. After 3 days, 5×10^5 4T1 cells were subcutaneously injected into the left flank of the Balb/C mice as distal tumors. Tumor volume (V) of mice were calculated by measuring the length (L) and width (W) and was calculated as $L\times W^2/2$.

The tumor-bearing mice were divided into 6 groups: 1, PBS; 2, $CaCO_3$; 3, $CaCO_3$/Pt; 4, $CaCO_3$/Cu; 5, $CaCO_3$/Cu/Pt; 6, $CaCO_3$/Cu/Pt+IDOi. The different materials (1 mg) were injected into the primary tumors of mice for 5 times every other day. The 200 μg of IDOi was injected intravenously on day 2, 4, 6, 8, 10. Body weights and volumes of primary tumors and distal tumors were recorded every two days. After 14 days, the primary tumors and main organs were collected for H&E staining.



Elt.	Line	Intensity (c/s)	Conc	Units	Error 2-sig	MDL 3-sig	
C	Ka	67.00	15.097	wt.%	.590	.421	
O	Ka	188.73	62.870	wt.%	1.370	.689	
Ca	Ka	77.49	13.272	wt.%	.501	.403	
Cu	Ka	1.28	1.421	wt.%	.965	1.373	
Pt	La	.51	7.340	wt.%	10.074	14.760	
			100.000	wt.%			Total

kV 15.0

Takeoff Angle 35.0°

Elapsed Livetime 50.0

Fig. S1 The energy dispersive X-ray spectroscopy (EDS) of $CaCO_3$/Cu/Pt.

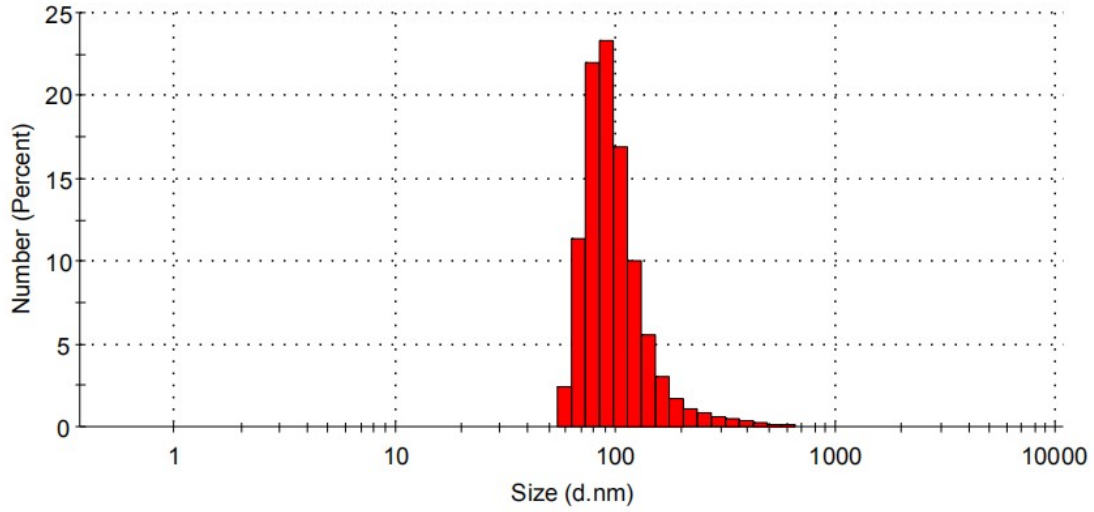


Fig. S2 The hydrodynamic size of $CaCO_3$/Cu/Pt by the dynamic light scattering (DLS).

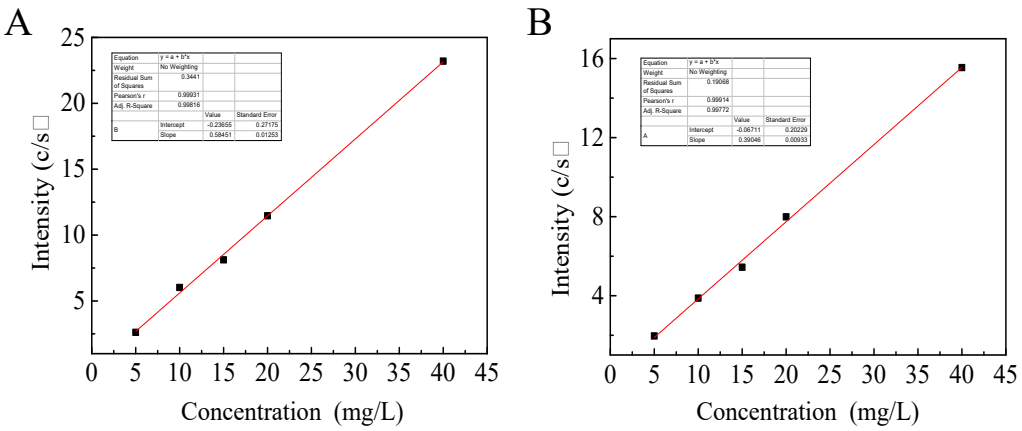


Fig. S3 The standard curves of oxaliplatin (A) and Cu^{2+} (B) by the ICP-AES. The values in the present study are expressed as means ± SD.

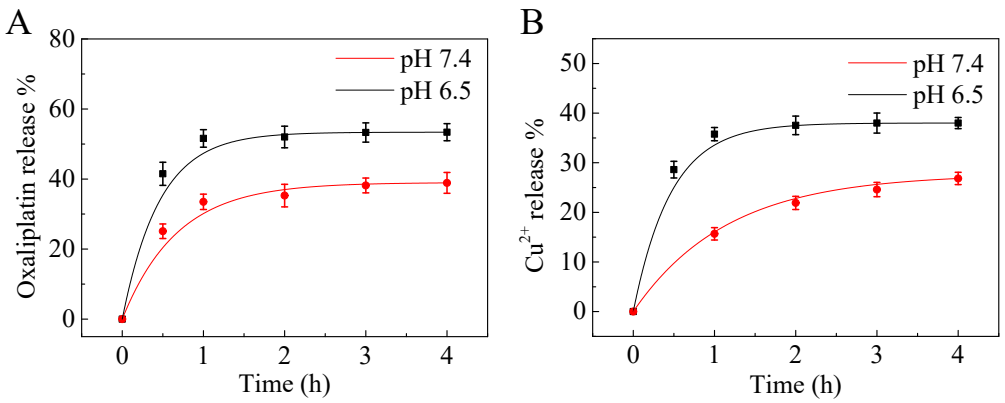


Fig. S4 The release of oxaliplatin (A) and Cu^{2+} (B) the ICP-AES in the condition of pH 7.4 and pH 6.5. The values in the present study are expressed as means ± SD.

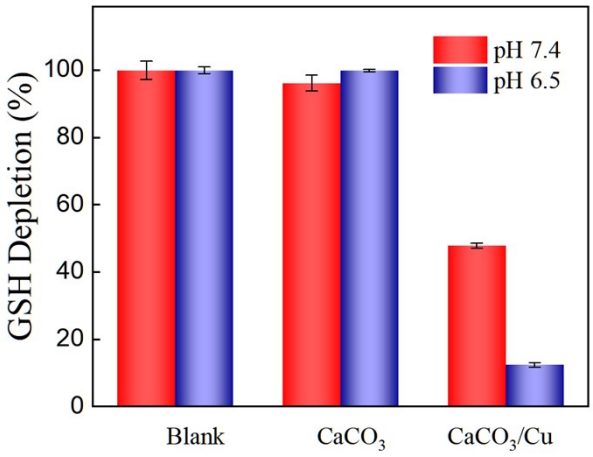


Fig. S5 The GSH consumption of $CaCO_3$ and $CaCO_3$/Cu in different pH. The same concentration of GSH was added to each group. The values in the present study are expressed as means ± SD.

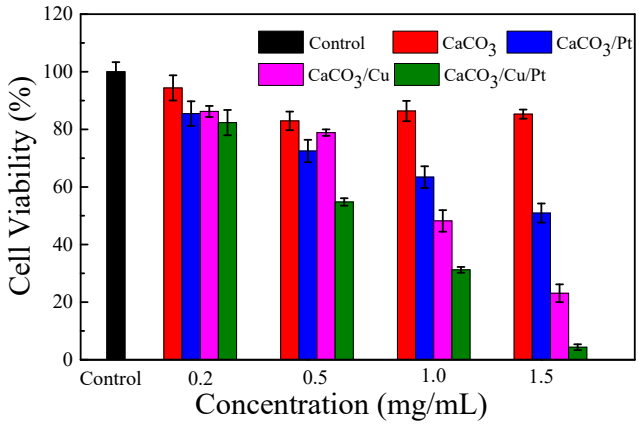


Fig. S6 The cell viability after various treatments. The values in the present study are expressed as means ± SD.

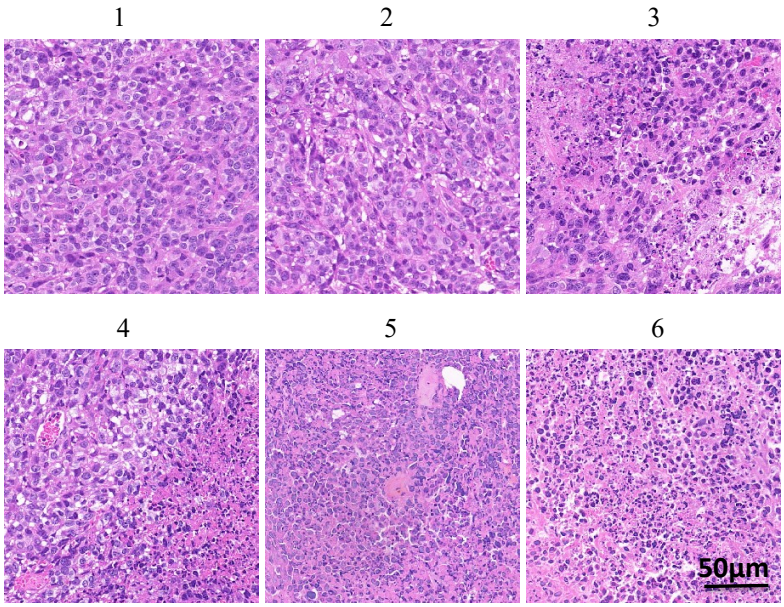


Fig. S7 H&E staining images of the primary tumor slides. 1, PBS; 2, $CaCO_3$; 3, $CaCO_3$/Pt; 4, $CaCO_3$/Cu; 5, $CaCO_3$/Cu/Pt; 6, $CaCO_3$/Cu/Pt+IDOi.

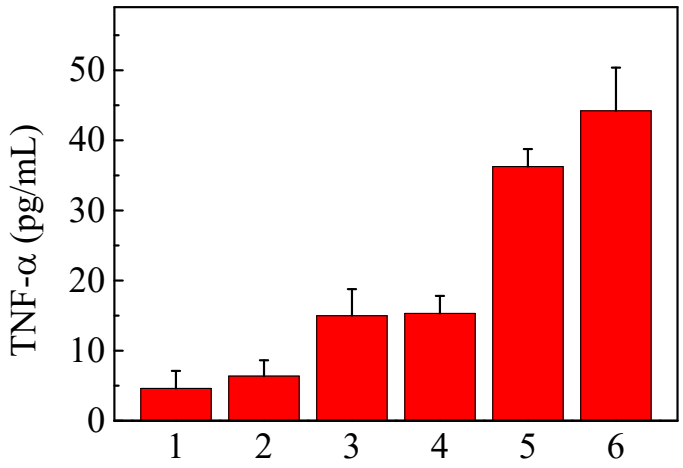


Fig. S8 The levels of TNF-α after various treatments. 1, PBS; 2, $CaCO_3$; 3, $CaCO_3$/Pt; 4, $CaCO_3$/Cu; 5, $CaCO_3$/Cu/Pt; 6, $CaCO_3$/Cu/Pt+IDOi. The values in the present study are expressed as means ± SD.

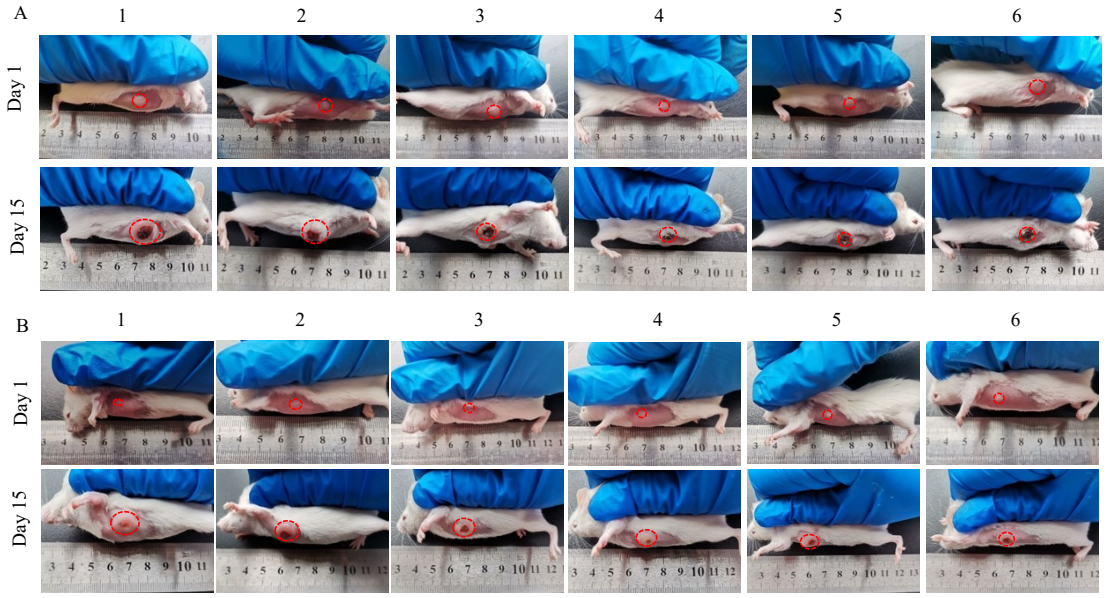


Fig. S9 Photographs of mice with primary (A) and distal (B) tumors on day 1 and day 15. 1, PBS; 2, $CaCO_3$; 3, $CaCO_3$/Pt; 4, $CaCO_3$/Cu; 5, $CaCO_3$/Cu/Pt; 6, $CaCO_3$/Cu/Pt+IDOi.

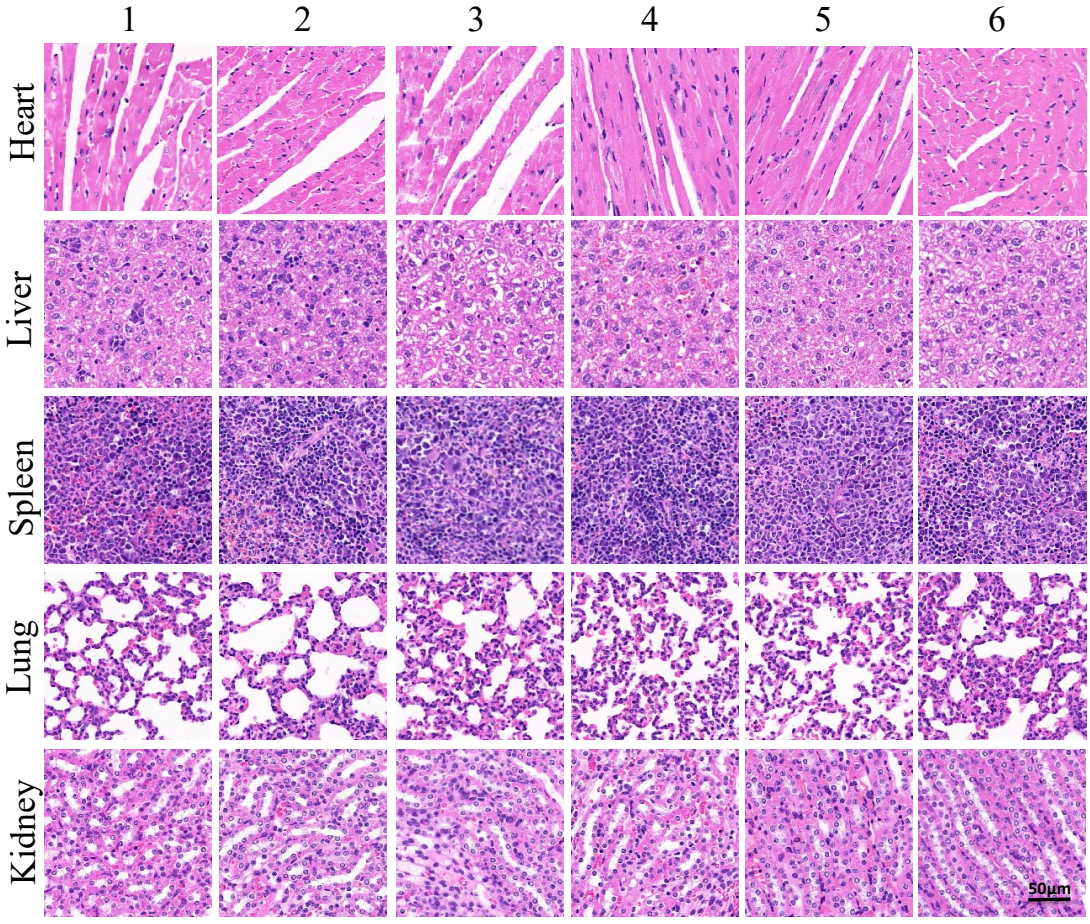


Fig. S10 H&E staining images of five major organs. 1, PBS; 2, $CaCO_3$; 3, $CaCO_3$/Pt; 4, $CaCO_3$/Cu; 5, $CaCO_3$/Cu/Pt; 6, $CaCO_3$/Cu/Pt+IDOi.

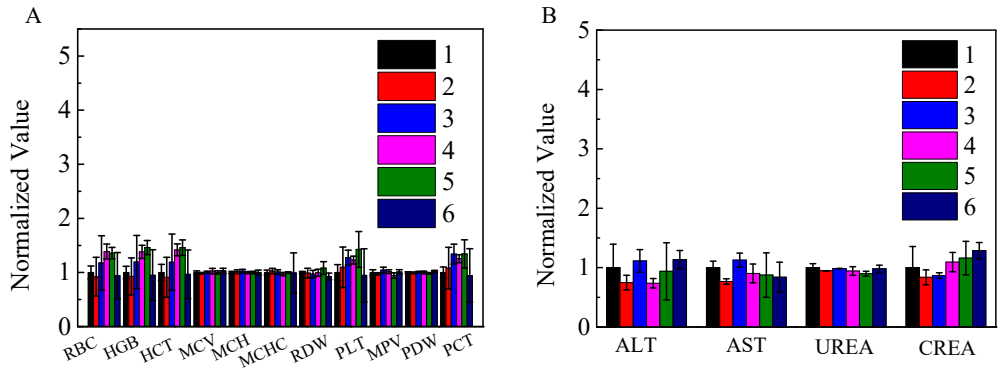


Fig. S11 The blood routine (A) and blood biochemical (B) indexes of different groups. 1, PBS; 2, $CaCO_3$; 3, $CaCO_3$/Pt; 4, $CaCO_3$/Cu; 5, $CaCO_3$/Cu/Pt; 6, $CaCO_3$/Cu/Pt+IDOi. The values in the present study are expressed as means ± SD.