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Supporting Information

Cu(I)-based Fenton-like agent inducing mitochondrial damage for photo-assisted enhanced chemodynamic therapy

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Experimental Section

Materials

All of the chemicals and reagents employed in this work were used without further purification. $Cu(NO_3)_2 \cdot 3H_2O$, triphenylphosphine (TPP) oxalic dihydrazide, 4-dimethylaminocinnamaldehyde and methylene blue (MB) were purchased from Bide Pharmaceutical Technology Co. Ltd. (Shanghai, China). Annexin V-FITC/propidium iodide, Calcein-AM/propidium iodide (CA/PI) staining agents, 2′,7′-dichlorodihydrofluoresceinn diacetate (DCFH-DA) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) assay kit were purchased from Beyotime Biological Technology Co., Ltd. (Shanghai, China). MitoTracker Red CMXRos was obtained from Yeasen Biological Technology Co., Ltd. (Shanghai, China). SOSG (singlet oxygen sensor green) was purchased from Thermo Fischer Scientific (MA, USA). Hela cells were obtained directly from the cell bank of Shanghai Institute of Life Sciences (China) and cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium which containing 10 % FBS (Gibco) plus 1 % penicillin.

Instruments

Single crystal X-ray diffraction measurements were carried out using an XtaLAB Synergy-DS (Cu) X-ray Source (λ = 1.54184 Å). Absorbance and fluorescence spectra were recorded on UV−vis spectrophotometer (UV-2600i, Shmadzu) and fluorometer (Cary Eclipse, Agilent Tech). Elemental analyses for C, H and N were recorded on a Perkin-Elmer 2400II elemental analyzer. Powder X-ray diffraction was measured on a Rigaku D/max diffractometer equipped (Cu-Kα, λ = 1.54056 Å). XPS spectra were measured by Electron spectrometer ESCALAB 250Xi. EPR spectroscopy was conducted on A300 equipment (Bruker, GER). The inductively coupled plasma mass spectrometry (ICP-MS) data were obtained on a FLexar-NexION300X ICP-MS instrument (PerkinElmer Inc., USA).

Synthesis of ligand DC-OD and DC-OD-Cu $[Cu_2(DC\text{-}OD)(PPh_3)_4]\cdot 2NO_3\cdot 2CH_3OH$

Ligand **DC-OD** was first obtained by refluxing 4-dimethylaminocinnamaldehyde and oxalyl dihydrazide in ethanol at a molar ratio of 2:1. For **DC-OD-Cu**, a mixture of **DC-OD** (43.2 mg, 0.1 mmol) and PPh_3 (131 mg, 0.5 mmol) in 5 mL methanol was added to an aqueous solution of 5 mL $Cu(NO_3)_2 \cdot 3H_2O$ (38.0 mg, 0.2 mmol) with stirring at room temperature for 30 min, then, the solution was cooled and filtered. Several days later, red block crystals of **DC-OD-Cu** suitable for X-ray structure analysis were crystallized from the solution after slow evaporation at room temperature, and collected by filtration, washed with ethanol and dried in air. Yield: 55 %. Elemental analysis data: calculated (%) for $C_{98}H_{96}Cu_2N_8O_{10}P_4$ (1796.81): C, 65.45; H, 5.34; N, 6.23. Found (%): C, 65.12; H, 5.62; N, 6.29. HRMS (ESI): m/z calculated for $[Cu_2(PPh_3)_2(DC\text{-}OD)]^+$: 1083.27; found 1083.26. IR (KBr ν/cm^{-1}): 3437 w, 3053 w, 1594 vs, 1480 s, 1436 vs, 1321 m, 1228 w, 1162 vs, 1095 s, 998 w, 809 m, 744 s, 695 vs, 519 vs, 424 w.

Crystallographic analysis

Single-crystal data of **DC-OD-Cu** were collected on a Rigaku SuperNova diffractometer equipped with Cu Kα radiation (1.54178 Å) at 100 K. The structure was solved by direct methods and refined with the full matrix least squares methods on F^2 with the SHELXT-2015[1] and SHELXL-2018[2] programs. All non-hydrogen atoms were refined with anisotropic displacement coefficients, and all hydrogen atoms were generated geometrically. The detailed crystallographic data and structure refinement parameters are summarized in Table S1. Selected bond lengths and angles for **DC-OD-Cu** are provided in Table S2. Crystallographic data for the structural analyses have been deposited at the Cambridge Crystallographic Data Centre (CCDC reference number: 2191945).

Detection of extracellular ROS

Hydroxyl radicals were detected firstly through the UV-vis absorption spectra of methylene blue (MB) at 665 nm. Briefly, 22.5 μL **DC-OD-Cu** (2.0 mM, DMF), 90.0 μL H_2O_2 (2 mM) and 60.0 μL MB (2 mM) was added to PBS (0.2 M, pH = 7.4) maintain a total volume of 3.0 mL, which was incubated in dark at 37 °C water bath. Additionally, the radical scavenger 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was used to detect the production of •OH radicals *in vitro* with EPR spectroscopy. A mixed solution containing

DC-OD-Cu solution (50 μM), H_2O_2 (1 mM), and DMPO (25 mM) was formed quickly. Immediately, the solution was transferred to a quartz capillary tube and EPR signals were recorded at room temperature. A mixed solution of the same concentration of H_2O_2 and DMPO was measured as a control to test the EPR signal under the same conditions. On the other hand, the production of $^1O_2$ was detected using SOSG probe upon white LED light (400-700 nm, 20 mW/cm^2) irradiation. Typically, 25 μM **DC-OD-Cu** and 25 μM SOSG were mixed in PBS (0.2 M, pH = 7.4), and then the fluorescence intensity at 535 nm was recorded with light duration. Meanwhile, the EPR spectroscopy measurements were carried out on a Bruker Model A300 spectrometer at 298 K after mixing **DC-OD-Cu** (25 μM) and TEMP (50 μM) under white LED light.

Cytotoxicity of DC-OD-Cu by MTT Assay

HeLa cells were seeded in 96-well plates (10^4 cells per well) in 5 % CO_2 at 37 °C atmosphere for 24 h in dark, then, cells were per-incubated with or without H_2O_2 (100 mM) for 2 h. After discarding the H_2O_2-containing medium, fresh cell culture medium containing different concentrations of **DC-OD-Cu** (0, 1.0, 2.0, 4.0 and 6.0 μM, PBS, 7.4) was added to each well for 6 h incubation. Subsequently, the cells were irradiated with/without white LED light (20 min, 400-700 nm, 20 mW/cm^2) and further cultured for another 42 h. Lastly, 20 μL of standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added into each well and further incubated for 4 h. Afterwards, discard the medium and 100 μL DMSO was added into every well to dissolve formed formazan crystals. Absorbance at 570 nm of each well was measured on an enzyme-labeling instrument and the data was recorded using SPSS software. Experiments were performed 3 times as a parallel test.

Cellular ROS Generation by DC-OD-Cu

DCFH-DA was used for monitoring intracellular ROS generation. Briefly, the HeLa cells were planted into confocal dishes at a density of 1×10^5 cells/dish and incubated overnight at 37 °C in a humidified atmosphere containing 5 % CO_2 in dark. Afterwards, the cells were pre-cultured for another 1 h in the presence or absence of fresh 1640 medium containing 100 μM H_2O_2. Then, the cells were incubated with **DC-OD-Cu** (2.0 μM) for 4 h, or irradiated with white LED light (20 min, 400-700 nm, 20 mW/cm^2), and then incubated for 4 h. Finally, the level of intracellular ROS was detected by Two-photon Confocal Scanning Laser Microscope photon (Leica TCS SP8 DIVE, GER) after 20 min incubation with DCFH-DA.

Cell uptake assay

HeLa cells (approximately 5×10^5 cells per mL) were seeded in 10 cm culture dishes and incubated overnight. Then the cells were treated with **DC-OD-Cu** (1.0 μM) for 2 h, 4 h, 6 h, 8 h and 10 h respectively. Then, the culture medium containing the **DC-OD-Cu** was removed, washed three times with PBS to completely remove residual **DC-OD-Cu**, and the cells were then digested with trypsin and counted. For whole cell copper measurement, the HeLa cells were digested and collected directly. For mitochondria copper measurements, the mitochondria of cells were extracted and collected by centrifugation using the Cell Mitochondria Isolation Kit (Beyotime BioTECH Shanghai, China). All samples were digested in HNO_3 (65 %, 300 μL)/H_2O_2 (30 %, 100 μL) at room temperature and quantified by ICP-MS analysis.

Cell apoptosis and live/dead assay

HeLa cells were seeded with 5×10^4 cells per well in a 6-well plate and incubated 24 h in dark, followed by per-incubation with or without H_2O_2 (100 mM) for 2 h. Then, discard the H_2O_2-containing medium, HeLa cells were treated in a medium containing **DC-OD-Cu** (5.0 μM) for 6 h in dark. Subsequently, cells were washed twice with PBS. After replacing with fresh medium further for irradiation by a white LED light (0 or 20 min, 400-700 nm, 20 mW/cm^2), the cells were cultured for another 30 min. Additionally, the cells were washed twice with PBS, collected by trypsin solution and washed twice with ice-cold PBS. Finally, the gathered cells were stained with Annexin V-FITC/PI staining solution for 20 min under dark environment and then analysed by flow cytometry (FACS Aria II flow cytometry, BD Biosciences, San Jose, USA). The data were processed and analysed by FlowJo 7.6 software. For live/dead staining assay, follow the above steps exactly, just

change the dye to Calcein-AM (2 μg/mL) and PI (5 μg/mL) to each well for 20 min incubation. After multiple rinses with PBS, the dead and living cells were detected on Cell Imaging Multi-Mode Reader System (BioTek, citation 5, USA).

Mitochondrial membrane potential assay

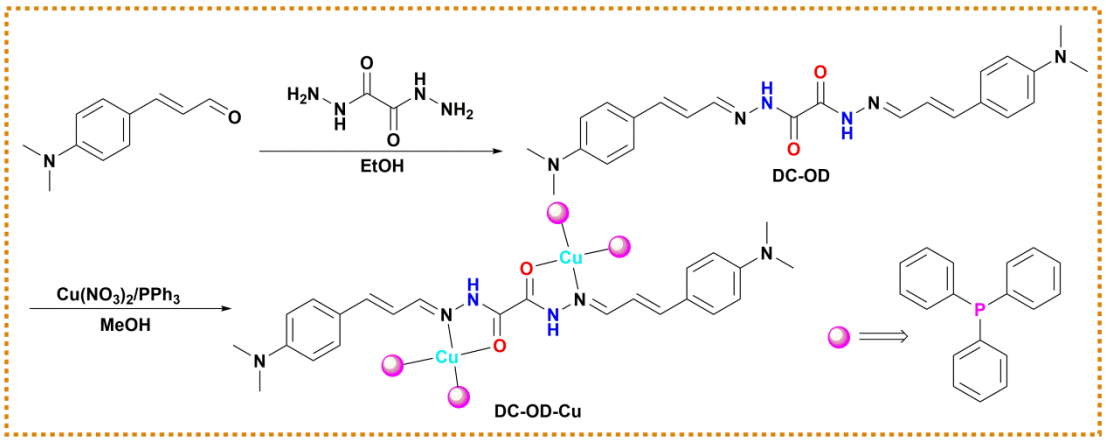
HeLa cells were seeded with 5×10^4 cells per well in a 6-well plate and incubated 24 h in dark, followed by per-incubation with or without H_2O_2 (100 mM) for 2 h. Then, discard the H_2O_2-containing medium, HeLa cells were treated in a medium containing **DC-OD-Cu** (5.0 μM) for 6 h in dark. Then the cells were washed twice with PBS. After replacing with fresh medium further for irradiated with/without a white LED light (20 min, 400-700 nm, 20 mW/cm^2), the cells were cultured for another 30 min. Afterward, the cells were washed twice with PBS, followed by addition of JC-1 (5 μg/mL) staining dye. After 20 minutes incubation, the culture medium of all samples was removed and the cells were washed twice by PBS, and re-suspended in PBS for immediate flow cytometry analysis.

Animal Experiments

Animal experiments were consigned to Nanjing OG Science and Technology Service Co., Ltd. BALB/c nude mice (about 6 weeks aged) were purchased from Changzhou Cavens Laboratory Animal Co., Ltd. (Changzhou, China, approval No. SCXK 2021-0013). All animal experiment procedures were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and all *in vivo* experiments were approved by Nanjing Lambda Pharmaceutical Co., Ltd. (Nanjing, China, Approval No. SYXK 2017-0040). HeLa cells (5×10^6) were subcutaneous injected into the nude mice. When the tumor volume reached about 100 mm^3, the tumor-bearing mice were divided into four groups and, respectively, treated with (1) intratumoral injection of saline (0.9 %) at days 1, 3, 5, 7, 9, 11, and 13; (2) 450 nm white LED irradiation for 15 min every 12 h after intratumoral injection of saline (0.9 %) at days 1, 3, 5, 7, 9, 11, and 13; (3) intratumoral injection of **DC-OD-Cu** (30 mg/kg) at days 1, 3, 5, 7, 9, 11, and 13; and (4) 450 nm LED irradiation for 15 min every 12 h after intratumoral injection of **DC-OD-Cu** (30 mg/kg) at days 1, 3, 5, 7, 9, 11, and 13 (20 mW/cm^2). The tumor volume and body weight of each mouse were carefully measured every other day. The tumor volume was calculated using the formula $V = LW^2/2$, where L and W stand for the maximum and minimum diameter of the tumors, respectively. On day 15, all mice were sacrificed.

Statistical Analyses

All data are present as means ± standard deviation (SD). Statistical analysis was evaluated using Student's t test and P value <0.05 was considered with statistically significant difference. A p-value less than 0.05 was regarded statistically significant ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).



Scheme S1. Synthetic routes of **DC-OD** and **DC-OD-Cu**.

Table S1. Crystal data and structure refinement of **DC-OD-Cu**.

Complex	**DC-OD-Cu**
Empirical formula	$C_{98}H_{96}Cu_2N_8O_{10}P_4$
Formula weight	1769.79
Crystal system	Monoclinic
Space group	P21/*n*
a (Å)	14.2262 (1)
b (Å)	21.2688 (1)
c (Å)	15.1530 (1)
α (°)	90.00
β (°)	103.775 (1)
γ (°)	90.00
Volume (Å^3)	4453.04 (5)
Z	2
D_{calc} (g cm^{-3})	1.340
F (000)	1876
Reflections collected	44875
Independent reflections	9374
R_{int}	0.030
Goodness-of-fit on F^2	1.04
R_1, wR_2 [$I >= 2\sigma(I)$]	0.0519, 0.1443
R_1, wR_2[all data]	0.0541, 0.1462

Table S2. Selected bond lengths (Å) and angles (°) for **DC-OD-Cu**.

DC-OD-Cu			
Cu1—P1	2.2540 (6)	Cu1—P2	2.2323 (6)
Cu1—O1	2.1823 (16)	Cu1—N2	2.061 (2)
P2—Cu1—P1	119.14 (2)	N2—Cu1—P1	110.38 (6)
O1—Cu1—P1	109.86 (5)	N2—Cu1—P2	121.26 (6)
O1—Cu1—P2	110.39 (5)	N2—Cu1—O1	77.90 (7)

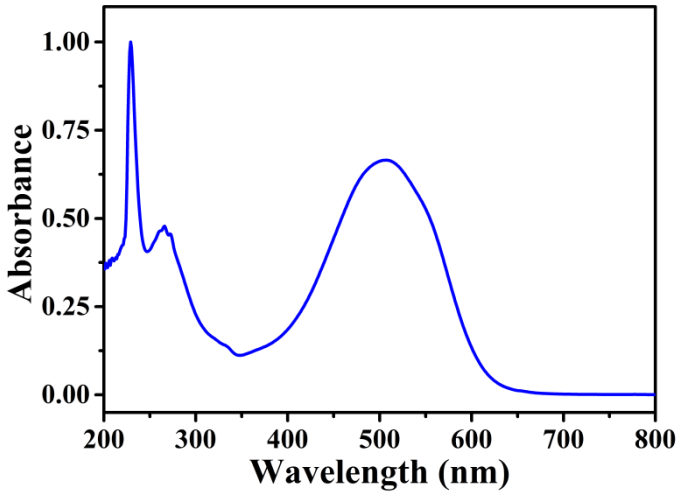


Figure S1. UV-vis absorption spectra of **DC-OD-Cu** (5×10^{-5} M) in dichloromethane at room temperature.

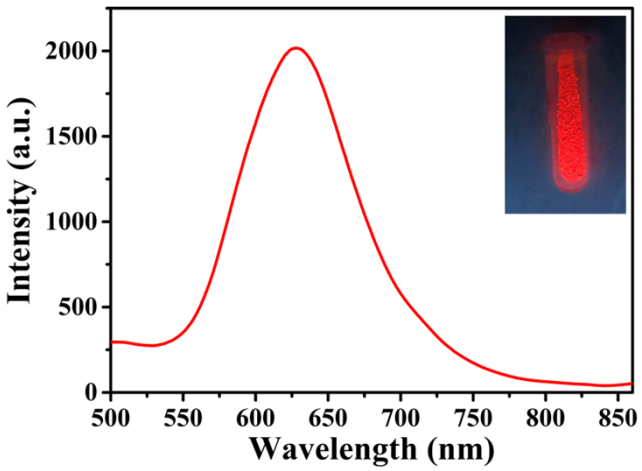


Figure S2. Emission spectra (λ_{ex} = 450 nm) of **DC-OD-Cu** in the solid state at room temperature.

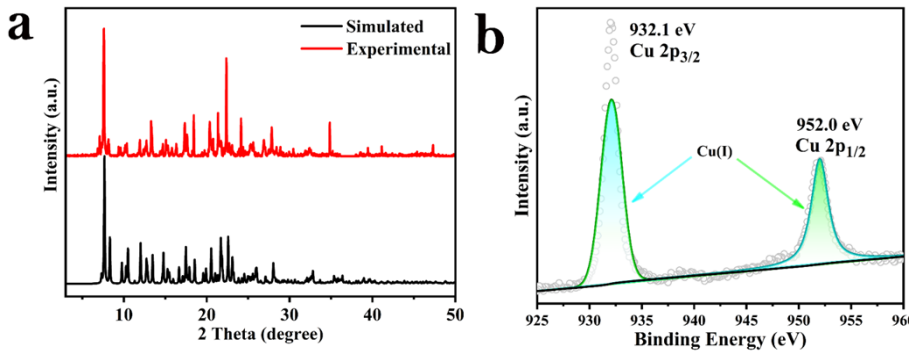


Figure S3. XRD and XPS spectra of **DC-OD-Cu**.

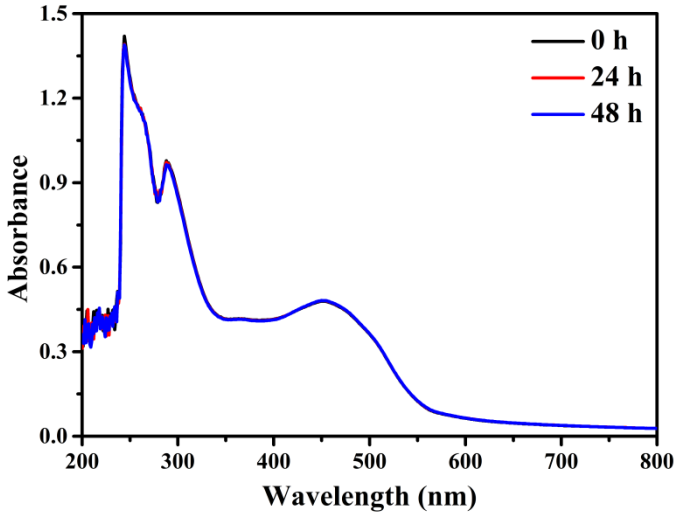


Figure S4. The absorption spectra of **DC-OD-Cu** in RPMI 1640 culture medium over 48 h.

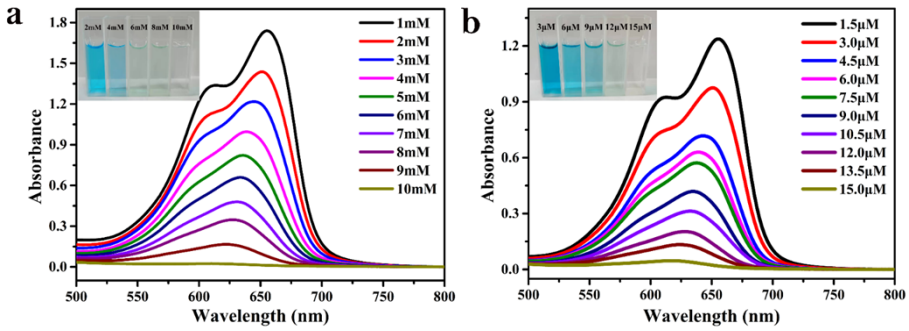


Figure S5. UV-vis absorption spectra of MB degradation after treated at different concentration of H_2O_2 (a, MB: 20 μM, **DC-OD-Cu**: 20 μM) and **DC-OD-Cu** (b, MB: 20 μM, H_2O_2: 10 mM) in Tris-HCl buffer (0.05 M, pH=7.4).

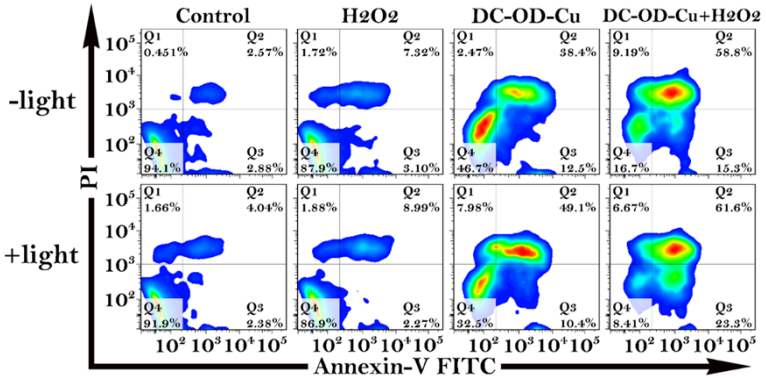


Figure S6. Flow cytometry analysis of HeLa cell apoptosis after different treatment for 48 h.

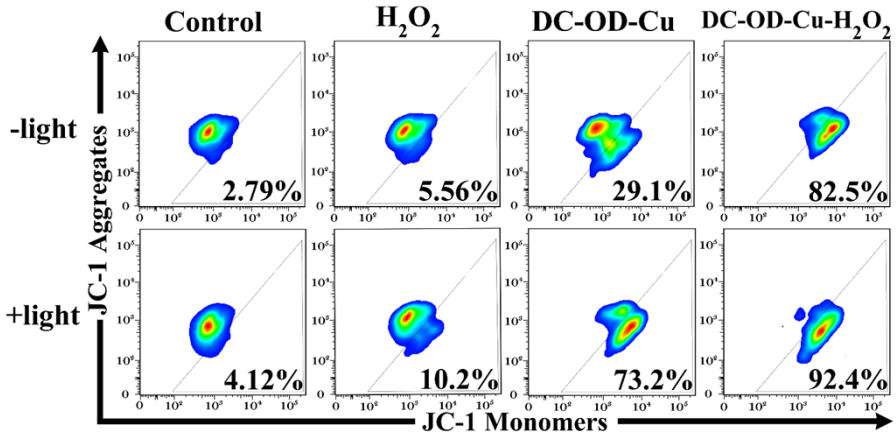


Figure S7. Measurement of mitochondrial membrane potential by flow cytometry in HeLa cells after different treatments.

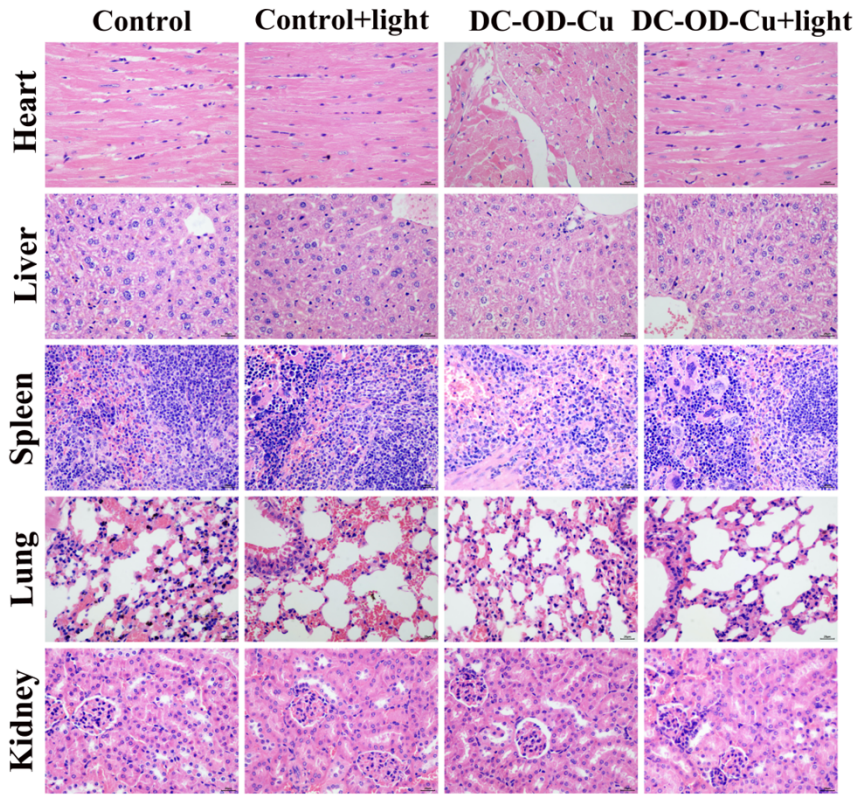


Figure S8. Histopathological analysis results (H&E staining images) of the major organs, heart, liver, spleen, lung and kidneys, of mice that were exposed to different treatments. Scale bar: 20 μm.

References

1 G.M. Sheldrick, *Acta Crystallogr. Sect. A: Found. Adv.,* 2015, **A71**, 3–8.

2 G.M. Sheldrick, *Acta Crystallogr. Sect. C: Struct. Chem.,* 2015, **C71**, 3–8.