A new herbal extract carbon nanodots nanomedicine for anti renal cell carcinoma through PI3K/AKT signaling pathway

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Materials

All cells used in this study were from the American type culture collection (ATCC, Manassas, VA and US), including human renal cell carcinoma cells (A498) and human normal hepatocytes (LO2). Re (HPLC ＞ 95%) and paraformaldehyde (4%) were obtained from the Shanghai Yuanye Biotechnology Co., Ltd. (China). The cell counting kit-8 was obtained from the Wuhan Boster Biological Technology Co., Ltd. (China). The FITC annexin V apoptosis detection kit I (CAT: 556547) was from the BD Biosciences Pharmingen (USA). The DNA content quantitation assay (cell cycle) detection kit, anti-fluorescence attenuation sealing agent, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), crystal violet water solution (0.1%), minimum essential medium (MEM), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), and 2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol (Triton X-100) were obtained from the Beijing Solarbio Science & Technology Co., Ltd. (China). The enhanced mitochondrial membrane potential assay kit (JC-1), fluo-4 calcium assay kit, and diethylpyrocarbonate water (DEPC water) were obtained from the Shanghai Beyotime Biotech Co., Ltd. (China). The roswell park memorial institute medium (RPMI 1640), penicillin-streptomycin solution and certified fetal bovine serum (FBS) were obtained from the VivaCell, Shanghai (China). The phosphate buffered solution (PBS) was obtained from the Hyclone (USA). The primary antibodies (including PI3K, AKT, p-AKT, p21, CDK2 and CCNA2) were purchased from the Proteintech, Wuhan (China). The p-PI3K was purchased from the Cell Signaling Technology (USA). The secondary antibodies CY3-Goat anti-mouse and CY3-Goat anti-rabbit were purchased from the Wuhan BaiQiandu Biotechnology Co., Ltd. (China). The trizol was obtained from the Thermo Scientific (USA). The Transzol Up Plus RNA Kit, PrimeScript^{TM} RT Reagent Kit with gDNA Eraser (Perfect Real Time) and the TB Green Premix Ex Taq II (Tli RNaseH Plus) were obtained from the TaKaRa (Japan). The Four-week-old female BALB/c nude mice were purchased from the Liaoning Changsheng Biotechnology Co., Ltd. The 5-fluorouracil was purchased from the Tianjin Jinyao Pharmaceutical Co., Ltd. The water used in all experiments was ultrapure water.

Devices

The Shimadzu UV-2550 ultraviolet-visible spectrophotometer was used to obtain UV-vis absorption spectra and a VERTEX 70 (resolution: 2 cm^{-1}, coadding times: 32 times) fourier transform infrared spectrometer (FTIR) (Bruker, Germany) was used to obtain the infrared spectra. The Tecnai F20 electron microscope (FEl Company, Netherlands) was used to obtain transmission electron microscopy (TEM) images of the Re-CDs. The infinite M200 PRO (TECAN, Switzerland) was utilized to obtain fluorescence spectra and a FLS 920 steady-state/transient fluorescence spectrometer (Edinburgh, Scotland) was used to obtain the fluorescence lifetime and quantum yield. The ESCALAB 250x photoelectron spectrometer (Thermo Scientifc, USA) was used to perform X-ray photoelectron spectroscopy. The biological images were acquired by an inverted microscope (Nikon, Japan). The fluorescence photos were obtained by a fluorescence inverted microscope (Life Technologies, USA). The experimental data of apoptosis and cell cycle were obtained by a flow cytometry (BD, USA). The information on gene expression was obtained by a qPCR instrument (Bio-rad, USA). Blood routine examination was performed using PE-6800 hematology analyzer (PROKAN, China).

Table S1. After fittings with a third-order decay exponential function (Fig. 1c), three fluorescence lifetimes (τ) were obtained as followed.

	Fitting equation	**R^2**	**$\tau 1$**	**$\tau 2$**	$\tau 3$	$\bar{\tau}$
Re-CDs	y=0.63138*exp(-x/3.88562)+6.79186*exp(-x/1.19665)+9.49993*exp(-x/1.19679)+0.00163	0.99812	3.88562	1.19665	1.19679	1.49726

Table S2. Particle size statistics table of the Re-CDs.

	Area	Mean	Length		Area	Mean	Length
1	0.178	75.137	2.238	**26**	0.192	78.678	2.549
2	0.132	73.355	1.639	**27**	0.196	109.164	2.591
3	0.178	57.708	2.238	**28**	0.136	124.138	1.785
4	0.143	84.57	1.832	**29**	0.136	115.63	1.785
5	0.178	60.933	2.273	**30**	0.158	115.479	2.041
6	0.27	83.374	3.469	**31**	0.18	121.6	2.395
7	0.161	65.974	2.033	**32**	0.158	126.514	2.089
8	0.201	99.33	2.591	**33**	0.158	95.966	2.066
9	0.23	86.205	2.963	**34**	0.163	104.925	2.129
10	0.161	63.218	2.058	**35**	0.18	52.29	2.38
11	0.178	77.154	2.273	**36**	0.158	84.197	2.087
12	0.138	78.757	1.775	**37**	0.223	62.22	2.952
13	0.247	65.133	3.259	**38**	0.114	70.198	1.476
14	0.225	100.962	2.933	**39**	0.202	114.568	2.657
15	0.203	67.79	2.683	**40**	0.174	87.864	2.306
16	0.269	58.444	3.568	**41**	0.218	103.746	2.907
17	0.132	91.05	1.728	**42**	0.142	69.257	1.867
18	0.214	107.562	2.795	**43**	0.114	85.476	1.476
19	0.203	92.183	2.65	**44**	0.18	96.121	2.362
20	0.181	62.606	2.37	**45**	0.23	55.408	3.051
21	0.219	66.616	2.918	**46**	0.203	93.486	2.667
22	0.23	77.708	3.051	**47**	0.241	69.826	3.191
23	0.258	90.982	3.378	**48**	0.209	119.814	2.732
24	0.181	116.155	2.389	**49**	0.225	80.082	2.963
25	0.285	85.022	3.748	**50**	0.148	86.056	1.897

Table S3. Comparison of administration dose and inhibition rate of carbon nanodots from different extract sources and the Re-CDs.

Precursor	Method	Cancer application	Reference
Ginsenoside Re	Hydrothermal	When the administration dose was 90 μg/mL, the survival rate of the A498 cells was 28.6 ± 1.3%.	This research
Riboflavin	Hydrothermal	When the administration dose was 100 μg/mL, the survival rate of the 4T1 cells was 31%.	Yue J, et al.[1]
Curcumin	Hydrothermal	After 48 h treatment, the IC50 of the HepG2 cells was 267.73 μg/mL.	Ilhan H.[2]
Ellagic acid	Hydrothermal	When the administration dose was 400 μg/mL, the survival rate of the 4T1 cells was less than 20%.	Ye X, et al.[3]

References

[1] Yue J, Li L, Jiang C, Mei Q, Dong WF, Yan R. Riboflavin-based carbon dots with high singlet oxygen generation for photodynamic therapy. *J Mater Chem B.* **2021,** 9(38):7972-7978.

[2] Ilhan H. Nanoarchitectonics of the Effects of Curcumin Carbon Dot-Decorated Chitosan Nanoparticles on Proliferation and Apoptosis-Related Gene Expressions in HepG2 Hepatocellular Carcinoma Cells. *ACS Omega*. **2023,** 8(37):33554-33563.

[3] Ye X, Qu Z, Wu Y, Zhao S, Mou J, Yang S, Wu H. Nitrogen-doped carbon dots derived from ellagic acid and L-tyrosine for photothermal anticancer and anti-inflammation. *Biomater Adv.* **2024,** 163:213951.

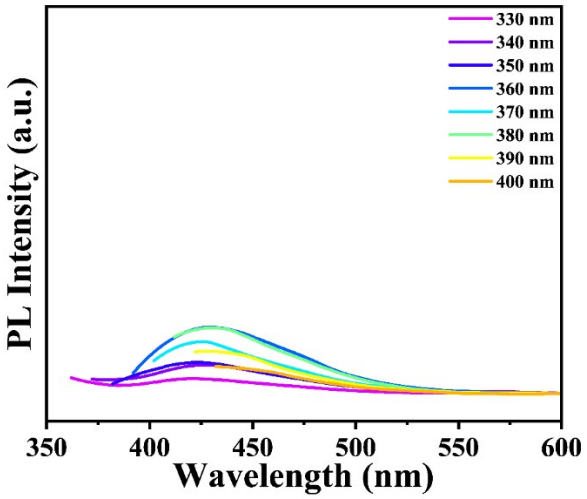

Figure S1. The fluorescence spectra of the Re dissolved in methanol (0.45 mg/mL).

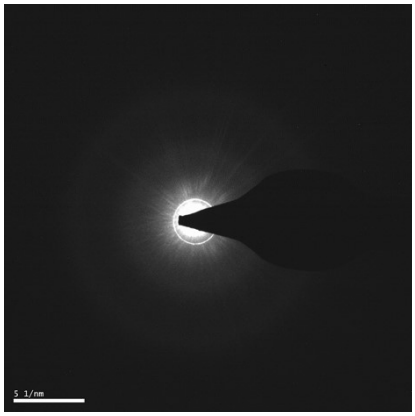

Figure S2. SAED image of the Re-CDs. The scale bar is 5 1/nm.

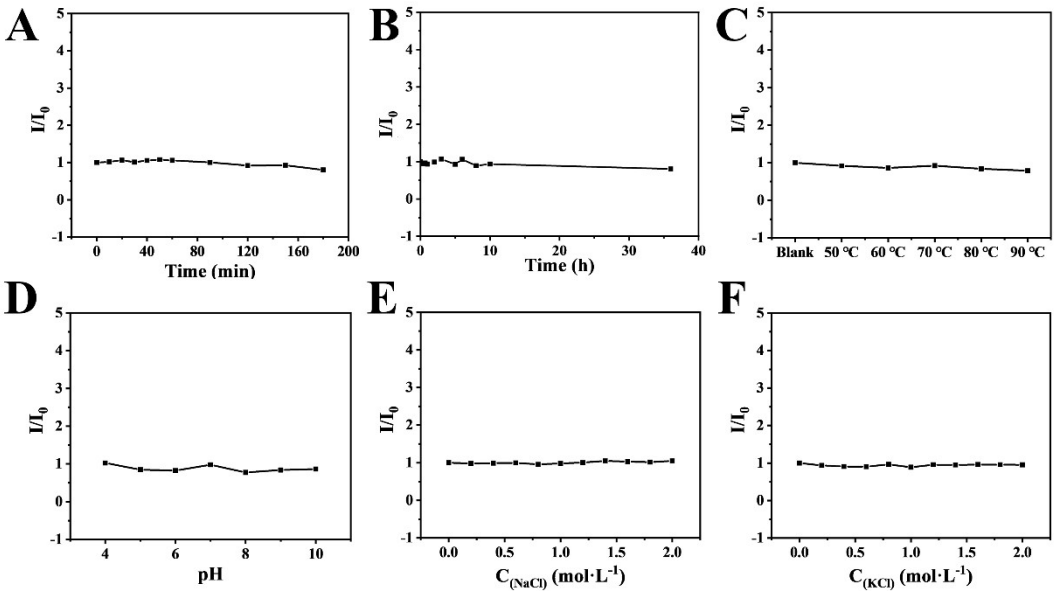

Figure S3. (A) Calculated chart of the fluorescence intensity of the Re-CDs solution (0.5 mg/mL) after irradiation under 365 nm UV lamp at different times. (B) Calculated chart of the fluorescence intensity of the Re-CDs solution (0.5 mg/mL) under room temperatures at different times. (C) Calculated chart of the fluorescence intensity of the Re-CDs solution (0.5 mg/mL) at different temperatures. (D) Calculated chart of the fluorescence intensity of the Re-CDs solution (0.5 mg/mL) at different pH values. (E) Calculated chart of the fluorescence intensity of the Re-CDs solution (0.25 mg/mL) at different NaCl concentrations. (F) Calculated chart of the fluorescence intensity of the Re-CDs solution (0.25 mg/mL) at different KCl concentrations.

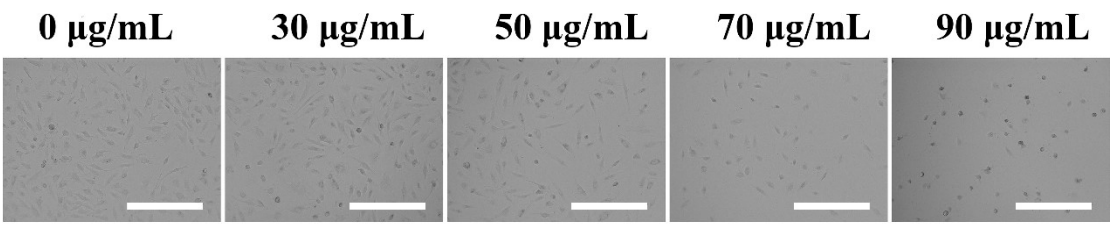

Figure S4. Microscopic morphological images of the A498 cells after 48 h co-incubation with different concentrations of the Re-CDs (0, 30, 50, 70 and 90 μg/mL) at 37°C. The scale bar is 400 μm.

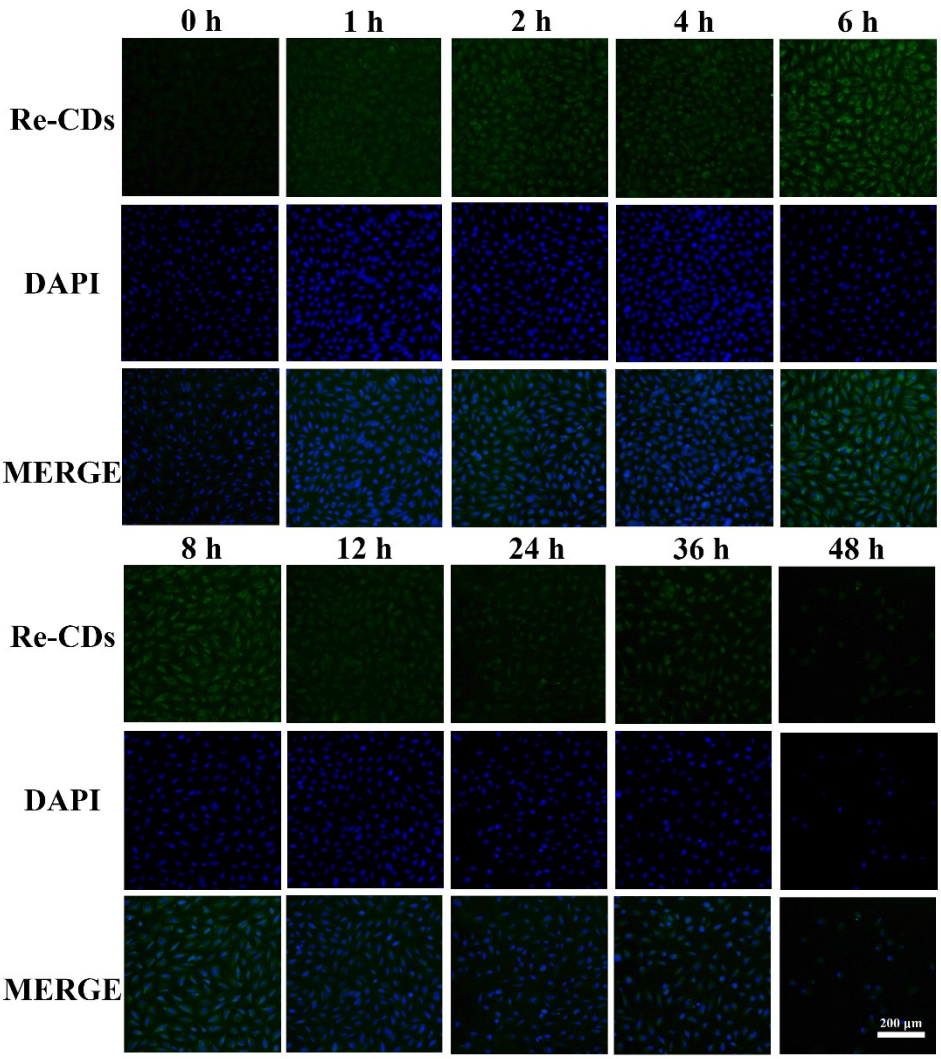

Figure S5. Fluorescence images of the A498 cells after incubation with the Re-CDs (100 μg/mL) at different times (0, 1, 2, 4, 6, 8, 12, 24, 36 and 48 h) at 37°C (DAPI, Ex = 360 nm; Re-CDs, Ex = 470 nm). The scale bar is 200 μm.

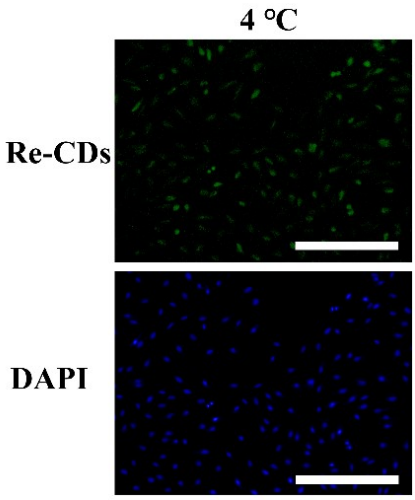

Figure S6. Fluorescence images of the A498 cells after incubation with the Re-CDs (100 μg/mL) at 4°C for 6 h (DAPI, Ex = 360 nm; Re-CDs, Ex = 470 nm). The scale bar is 400 μm.

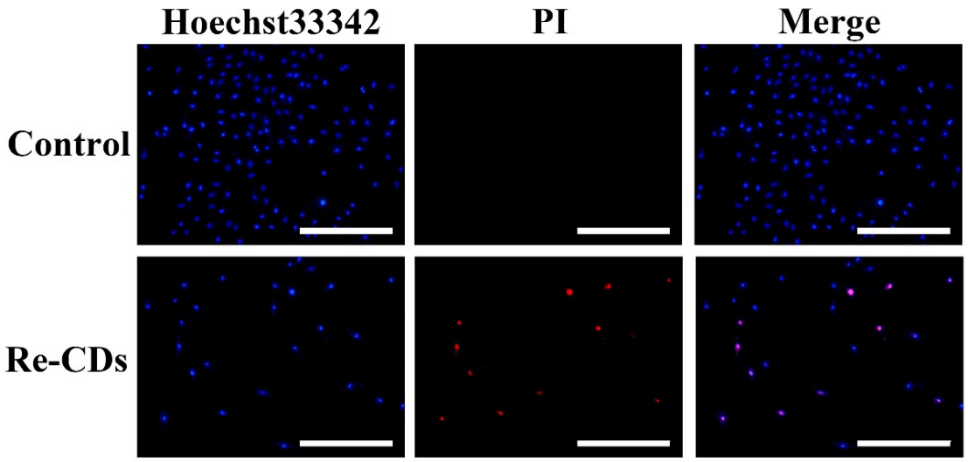

Figure S7. Fluorescence images of hoechst33342/PI staining of the A498 cells after incubation with the Re-CDs (100 μg/mL) at 37°C for 48 h (Hoechst33342, Ex = 360 nm; PI, Ex = 530 nm). The scale bar is 400 μm.

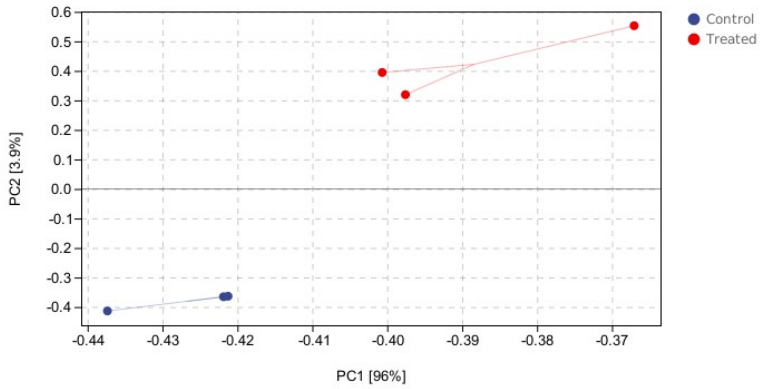

Figure S8. PCA analysis plot of the untreated A498 cells (Control group) versus the Re-CDs (100 μg/mL, 48 h) treated A498 cells (Treated group).

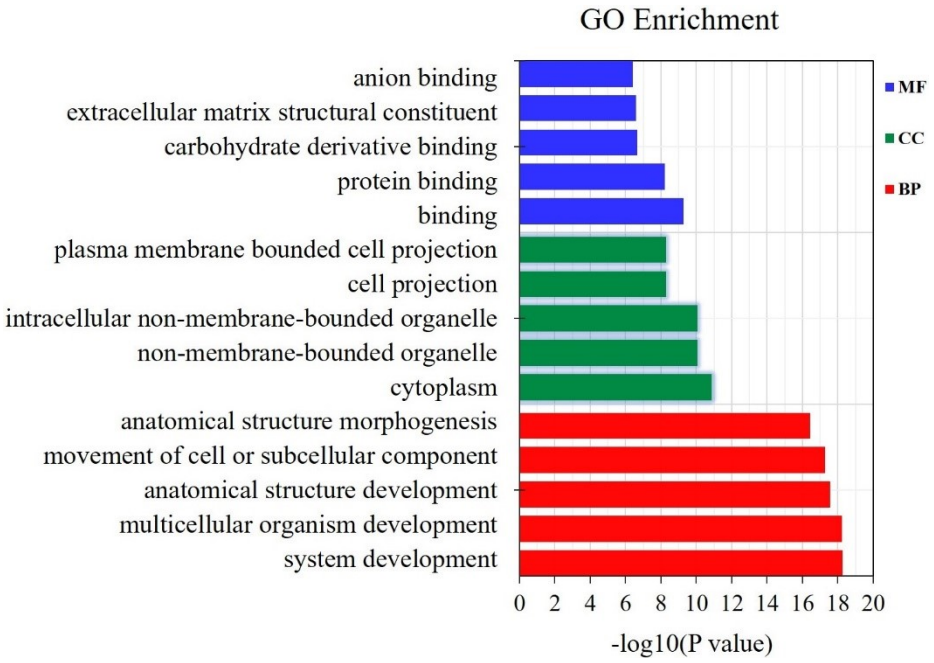

Figure S9. Three biological processes involved in the GO enrichment analysis. The top five items of each biological process are listed in order of the P value.

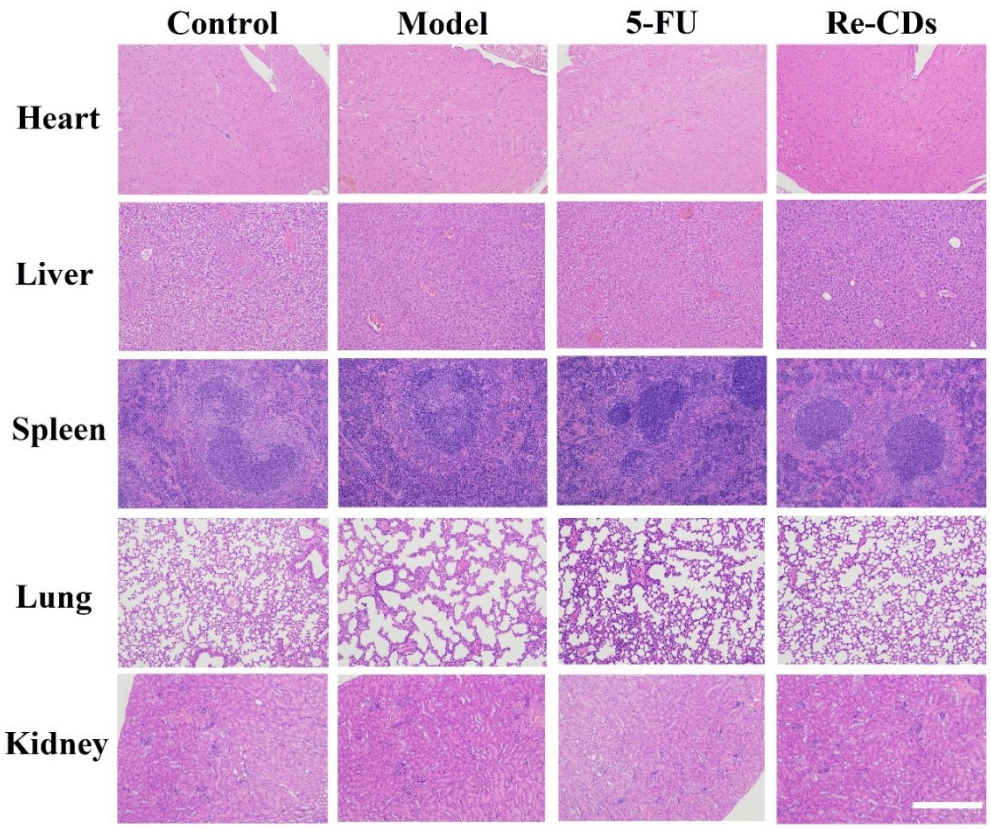

Figure S10. H&E staining images of major organs (heart, liver, spleen, lung and kidney) of mice in each group (Control group, Model group, 5-FU group and Re-CDs group). The scale bar is 400 μm.

On the whole, in the heart, liver, spleen, lungs and kidneys of mice, the cellar nuclei were blue-purple, with regular morphology and uniform chromatin distribution, and the cytoplasm was pale red or pink in color and had uniform texture. Few obvious abnormalities were found between the Re-CDs group and the Control group, indicating that the Re-CDs had no significant toxic effect on these major organs of the mice. In addition, the lung of the mice in the Model group were obviously damaged.

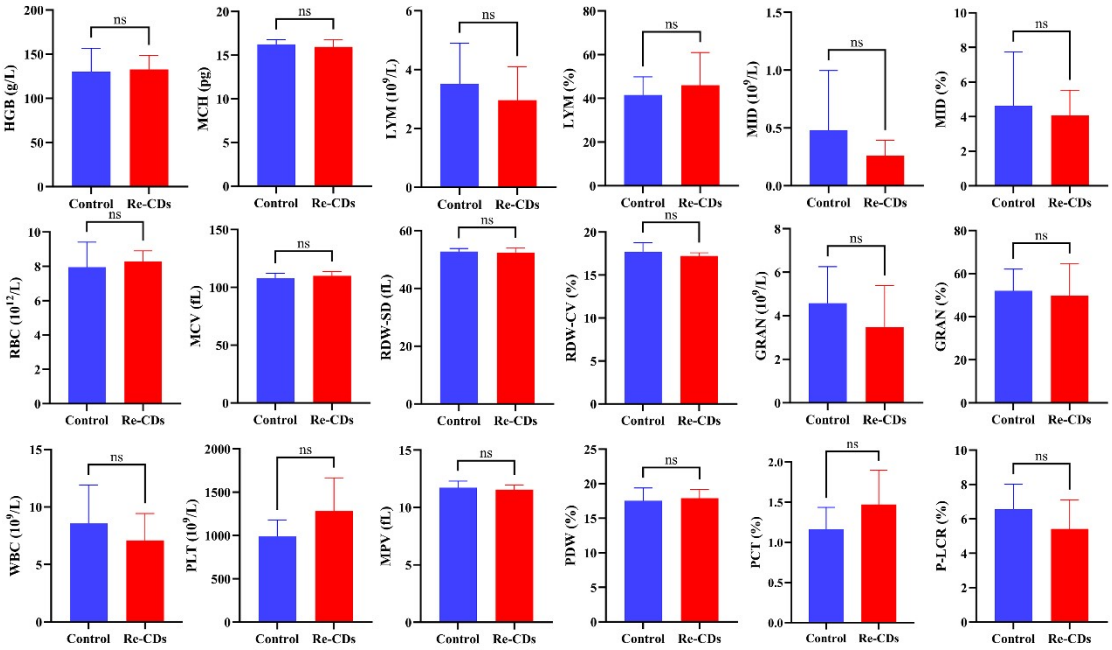

Figure S11. Statistical histogram of the blood index changes of the mice in the Control group and the Re-CDs group via routine blood test.

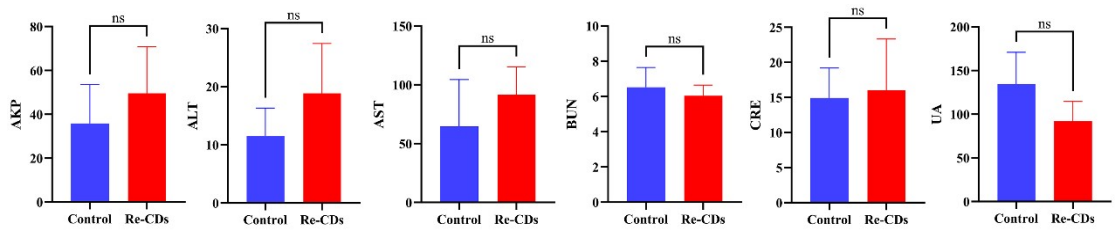

Figure S12. Statistical histogram of the serum biochemical index changes of the mice in the Control group and the Re-CDs group.

Table S4. The primer sequences for the realtime-PCR.

Primer	Species	Quantitative RT-PCR primer
GAPDH-Forward	Human	CTGGGCTACACTGAGCACC
GAPDH- Reverse	Human	AAGTGGTCGTTGAGGGCAATG
COL6A3- Forward	Human	CTGTTCCTCTTTGACGGCTCA
COL6A3- Reverse	Human	CCTTGACATCATCGCTGTACTGA
ITGA1- Forward	Human	CTGGACATAGTCATAGTGCTGGA
ITGA1- Reverse	Human	ACCTGTGTCTGTTTAGGACCA
ITGB6- Forward	Human	GAGGACTACCCGGTGGATTTG
ITGB6- Reverse	Human	TCCTTTATTGTGTTGAGGTCGTC
PIK3CA- Forward	Human	CCACGACCATCATCAGGTGAA
PIK3CA- Reverse	Human	CCTCACGGAGGCATTCTAAAGT
AKT3- Forward	Human	AATGGACAGAAGCTATCCAGGC
AKT3- Reverse	Human	TGATGGGTTGTAGAGGCATCC
CDKN1A- Forward	Human	CGATGGAACTTCGACTTTGTCA
CDKN1A- Reverse	Human	GCACAAGGGTACAAGACAGTG
CDK2- Forward	Human	CCAGGAGTTACTTCTATGCCTGA
CDK2- Reverse	Human	TTCATCCAGGGGAGGTACAAC
CCNA2- Forward	Human	GGATGGTAGTTTTGAGTCACCAC
CCNA2- Reverse	Human	CACGAGGATAGCTCTCATACTGT