Electronic Supplementary Information

Quecretin@UiO-66 NPs Combined with Chloroquine to Optimize Tumor Therapy by Dual Autophagy-Ubiquitination System Blockade

Yinzhu Chen, †Feiyi Yan, †Yue Yang, Lipeng Zhang, xuepeng Teng, *Shuaiyu Wang, * and Tianlong Liu*

1Laboratory of Veterinary Pathology and Nanopathology, College of Veterinary Medicine, China Agricultural University, No.2 West Road Yuanmingyuan, Beijing 100193, P.R. China; 2China National center for food safety risk assessment, No.37, Guangqu Road, Chaoyang District, Beijing 100022, P.R. China.
† These authors contributed equally.

Correspondence: Tianlong Liu
Laboratory of Veterinary Pathology and Nanopathology, College of Veterinary Medicine, China Agricultural University, No.2 West Road Yuanmingyuan, Beijing 100193, P.R. China
Tel +86 010 62732843
Fax +86 010 62732843
Email: liutianlong@cau.edu.cn
Figure S1  The DLS analysis of UiO-66 and Que@UiO-66 NPs

Figure S2  Zeta of UiO-66 and Que@UiO-66 NPs
**Figure S3**  Hemolysis rate of UiO-66 in gradient concentrations

**Figure S4**  Histological picture of organs (H&Estain, 10×)
**Figure S5**  Picture of western blot (p62)

**Figure S6**  Picture of western blot (LC3BI and LC3BII)

**Figure S7**  Picture of western blot (loading control)
Materials and methods

Materials
UiO-66 was bought from Shiyanjia Lab (www.shiyanjia.com), Quercetin (>98% purity), chloroquine (>99% purity) were purchased from MedChemExpress (Shanghai, China).

Preparation of Que@UiO-66 NPs
100mg of UiO-66 and 100 mg of Que were poured into 10 mL ethanol, ultrasonic blended and shaken for 10h, 4°C in shaker and then centrifuged at 10000 rpm for 20 min. The mixture was washed three times with ethanol and freeze dried.

Characterization of Que@UiO-66 NPs
The crystallinity and surface morphology of the UiO-66 and the prepared Que@UiO-66 NPs were detected using X-ray diffraction (XRD, igaku Smart Lab SE), scanning electron microscopy (SEM, HITACHI S-3400N), and transmission electron microscopy (TEM, HITACHI H-7500). UiO-66 and Que@UiO-66 NPs were characterized by Fourier Transform Infrared Spectroscopy (FTIR). The drug loading efficiency of Que@UiO-66 NPs was analyzed via ultraviolet spectrophotometry (HITACHI, U-3900), because Que has characteristic absorption peak at 370nm. The particle size distribution and zeta potential of UiO-66, Que@UiO-66 were detected by dynamic light scattering (DLS, Malvern Zetasizer3000HS).

In Vitro Antitumor Activities
Murine breast cancer cells (4T1) were purchased from Shanghai Gaining Biological Technology Co. Ltd (Shanghai, China) and used in this study. Cell culture medium was Roswell Park Memorial Institute (RPMI)-1640, 10% fetal bovine serum (FBS) and 0.5%(v/v) penicillin (10,000 unit), and 0.5% (v/v) streptomycin (10,000 unit), all were bought from Cytiva. The cell was maintained at 37 °C in a 5% CO2 incubator.

Cell viability was assessed using an ATP assay kit was purchased from Beyotime, (CellTiter-Lumi™ Steady Luminescent Cell Viability Assay Kit) to reflect the number of living cells. The quantification of whole cell ATP was performed according to the manufacturer’s protocol. Cells were seeded in 96-well plates (5 × 104 cells per well) and incubated for 18 h before treatment.
Next the culture medium was withdrawn and fresh medium containing Que@UiO-66 NPs and the combination of Que@UiO-66 NPs and CQ with a series of concentrations were added to each well, the concentration of CQ kept 30µM. After 24hrs incubation, the luminescence of each well was tested by microwe plate reader (Tecan, Spark).

Apoptosis of 4T1 cells were conducted using the AnnexinV-FITC/PI apoptosis kit (Procell, China). 4T1 cells were seeded in 6-well plates at a density of 2×105 cells/well for 24 hrs. The medium was then exchanged for fresh medium containing UiO-66, Que@UiO-66 NPs, Que@UiO-66 NPs combined with CQ, UiO-66 combined with CQ (UiO-66, Que@UiO-66 NPs dose: 500µg/mL, CQ concentration: 30µM), cells treated without treatment were settled as control group. After 24 hrs. incubation, the cells were stained for flow cytometric analysis. Flow cytometric assay was performed by FACSVerse cytometer (BD Biosciences) and the data were analyzed by FlowJo software (Treestar).

Female BALB/c mice (5–6 weeks) were purchased from Vital River Laboratories (Beijing, China). Mice were subcutaneously injected with 4T1 cells (3×107 cells) at the flank. After the tumor volume reached ~5 mm 3, the mice were randomly divided to 4 groups, with six mice in each group. The tumor-bearing mice in the 4 groups were treated with PBS, Que@UiO-66 NPs, CQ and Que@UiO-66 NPs +CQ, respectively every 2 days via intravenous injection. Tumor volume were measured every 2 days and the data were calculated by tumor volume formula (V=L×W2/2). On day 16, mice were euthanized. The tumor tissues of each mouse were collected, weighed and fixed in formalin. Heart, lung, kidneys, spleen and liver were also been fixed. Hematoxylin and eosin (H&E) staining of these tissues were conducted according to standard protocol. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Experimental Animal Ethics Committee in Beijing.

Bio-TEM for the Study of Molecular Mechanism

4T1 cells were seeded in 5-cm culture dishes (Corning, USA) at an initial density of 1×10 5 cells/dish. After 18 hrs of incubation, cells were treated with fresh medium containing Que@UiO-66 NPs(200µg/ml), CQ (30 µM), or their combination [Que@UiO-66 NPs(200µg/ml) and CQ (30 µM),] for 12 hrs. Cells treated with complete medium was set as control. Then, cells were fixed in 2% Paraformaldehyde-2.5% Glutaraldehyde solution, followed by insertion in 2% agarose, post-fixation in osmium tetroxide, and dehydration with an acetone series. Subsequently, the obtained samples were embedded in resin and polymerized at 60 °C for 24 h. The sections were made into ultrathin ones, counterstained with 3% uranyl acetate and 0.3% lead citrate, and then observed under scan electron microscope(HITACHI, H-7500).

Western Blotting for the Study of Molecular Mechanism

4T1 cells were seeded in 6 well culture plate (Corning, USA) at an initial density of 1×10 6 cells/dish. After 18 hrs of incubation, cells were treated with fresh medium containing Que@UiO-66 NPs(500µg/ml), CQ (30 µM), or their combination [Que@UiO-66 NPs(500µg/ml) and CQ (30 µM),] for 24hrs. Proteins were extracted and quantified by the BCA protein assay kit (Jiangsu Cowin Biotec Co.,Ltd). Adding 5× loading buffer to proteins and boiling for 10min to denatured. Then,15 µg proteins was electrophoretically separated using 10% and 15% Bis-Tris SDS-PAGE gel and transferred to polyvinylidene fluoride membrane (PVDF; Millipore). PVDF membranes with proteins were blocked with 5% skim milk powder in TBST for 1 hr. Then, membranes were then incubated at 4 °C overnight with primary antibodies (1:5000 dilution, LC3B, p62, β-actin) followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membranes were washed twice in TBST and captured by ChemiDoc XRS (Sagecreation,China). β-actin was used as a loading control.
ImageJ software was used to semiquantitatively measure the mean gray value and normalize it to that of β-actin.

**Proteasome Activity Assay**

2 × 10^6 4T1 cells were seeded on 6-well plates for 12 hours and then treated with Que@UiO-66 NPs (200μg/ mL) and CQ(30μM), after 24 hours later, discarded medium and collected cells, washed cells with PBS once, then resuspended cells in 0.5% NP-40, centrifuge 10 – 15 minutes at 4°C at 13,000 rpm using a cold microcentrifuge to remove any insoluble material, collected supernatant and transfer to a clean tube. Using the prepared sample to proceed the assay procedure and detection by following the instructions (Proteasome activity assay kit,Abcam).

**Hemolysis Test**

A set of centrifuge tubes were prepared, and 1mL of 2% red blood cell suspension was added respectively. The normal saline group was used as the negative control without hemolysis, and 1% TritonX-100 group was used as the positive control with complete hemolysis. The final concentration of UiO-66 in experimental groups was gradient concentration of 25μg/mL, 50μg/mL, 100μg/mL, 200μg/mL and 500μg/mL. Three groups were set to repeat the experiment. After adding drugs, the centrifuge tube was placed in a 37°C incubator for incubation for 1h, and then the mixture was centrifuged at low speed for 10min. The supernatant was transferred to a 96-well plate. Finally, the absorbance of each supernatant at 545nm was measured with a microplate reader. The relative hemolysis rate was calculated according to the formula.

**Animal Treatment**

Forty female, pathogen free ICR mice aged 7 weeks were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd.. This animal experiment was performed strictly with the guidance approved by of China Agricultural University Animal Centre Laboratory, China. The detailed procedures were described as follows. One group of 8 rats were randomly divided into 5 groups, and UiO-66 was dispersed in 5% glucose at doses of 200mg/kg, 100mg/kg, 50mg/kg, 20mg/kg and 0mg/kg (blank control group), respectively. The death was recorded after 15 days of routine feeding. On the 15th day, blood was collected and the heart, liver, spleen, lung, kidney and brain were fixed. The major organs acquired were utilized to do the histological examination with the direction of standard techniques.