Electronic Supplementary Material (ESI) for RSC Advances.

Supplementary Information

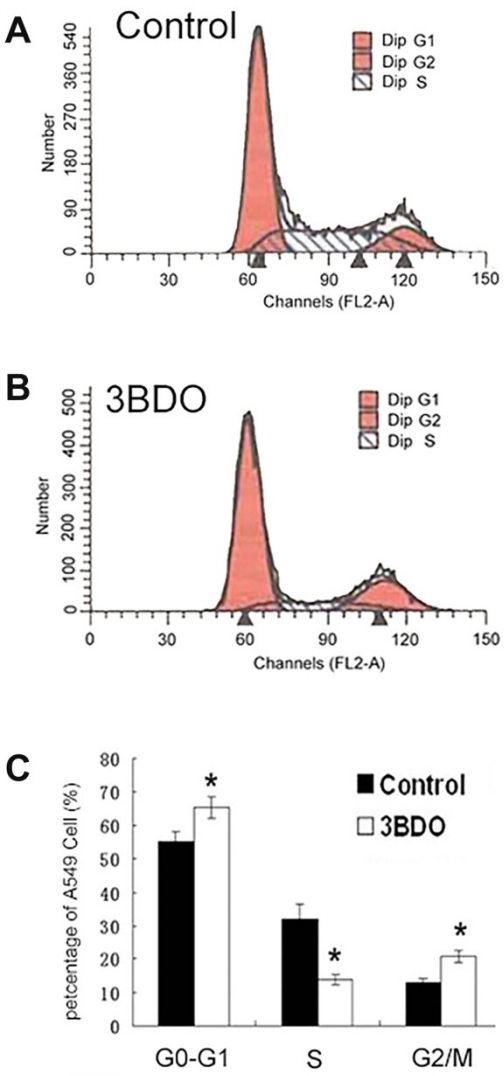

Fig.S1. Effect of 3BDO on cell cycle distribution of A549 cells at 48h. A-B: A549 cells were treated with or without 180 μM 3BDO. C: quantification of cell cycle distribution. (*$p<0.05$ vs control in the same cycle phase, n=3)

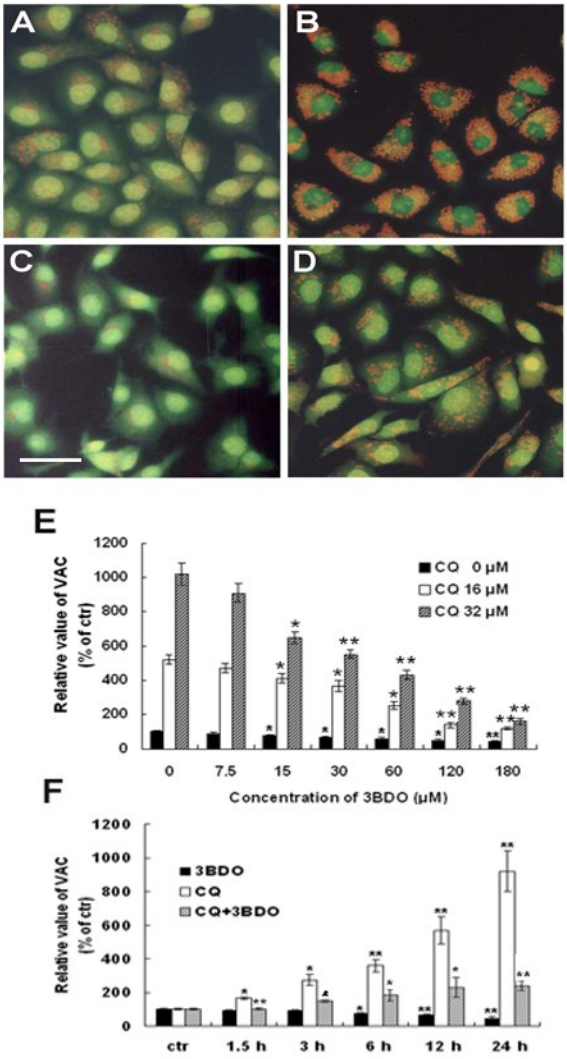

Fig.S2. The observation of acidic vacuoles acumination in A549 cells using AO staining (A-D) and neutral red uptake method (E-F). Red dots within A549 cell line indicated acumination of acidic vacuoles. A: DMSO; B: CQ (32 μM); C: 3BDO (180 μM); D: CQ (32 μM) +3BDO (180 μM).The relative absorbance (OD=540nm) of neutral red uptake were shown (E-F). (E) After treatment of different concentrations of 3BDO (0, 7.5, 15, 30, 60, 120 and 180 μM) for 24 h. (F) The different treatment time at 1.5, 3, 6, 12 and 24 h at 3BDO 180 μM. (Scale bars: 20μM;* p<0.05 and ** p<0.01 *vs* control, n=3)

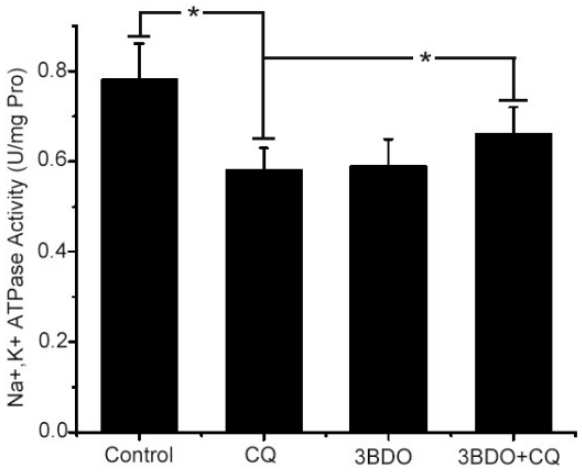

Fig.S3. The effect on Na^+, K^+-ATPase after incubation with CQ, 3BDO and CQ combination with 3BDO for 24 hours. (* p<0.05 and ** p<0.01*vs* control, n=3)

2. Materials and methods

2.1. Reagents, chemicals and preparation of drugs.

The culture medium RPMI 1640 was obtained from Gibco BRL Co. (Grand Island, USA), bovine calf serum from Beijing dingguo changsheng biotech CO.LTD (Beijing, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) was purchased from Amresco Company, Acridine orange (AO) from Fluka Incorporation. Chloroquine diphosphate (Sigma, USA) was dissolved in distilled water as a stock solution at a concentration of 0.1 M. 3BDO was dissolved in dimethyl sulfoxide (DMSO) as a stock solution at a concentration of 0.2 M. The final concentration of DMSO all used in the experiment was below 0.1% in culture medium (v/v) and it did not affect cell viability.

2.2. Cell cultures.

Human lung cancer cell line A549 was cultured in RPMI 1640 medium, supplemented with 10% (v/v) Bovine Calf Serum and 80 U/ml gentamicin at 37ºC in a 5% CO^2 humidified environment. The cells were seeded at a density of 6250/cm^2 into plates or appropriate dishes to attach for 24 h for the following assays.

2.3. Observation of cell morphological changes.

A549 cells were seeded into 24-well plates for 24 h and then treated with DMSO 0.09% (v/v, as control), or CQ 32 μM, or 3BDO 180 μM, or both CQ 32 μM and 3BDO 180 μM at 24 h. Cell morphological changes were observed and photographed with Phase Contrast Microscope (Nikon, Japan).

2.4. MTT assay for cell viability.

Cells were seeded in 96-well plates at the density of 6250/cm^2. After 24 h, cells were treated with DMSO 0.09% (v/v, as control), CQ 32 μM or various concentrations of 3BDO, for 6, 12, or 24 h. The cell viability was determined by MTT assay following the procedure as described previously[18]. The light absorptions were measured at 570 nm using SpectraMAX 190 microplate spectrophotometer (GMI co., USA).

2.5. LDH assay for drug toxicity.

The detection was performed by using Lactate Dehydrogenase (LDH) kit (ZhongSheng Co., BeiJing, China) according to the manufacturer's instructions. Briefly, cell culture medium of A549 cells was collected after treatment with DMSO 0.09% (v/v, as control), or CQ 32 μM, or 3BDO 180 μM, or both CQ 32 μM and 3BDO 180 μM for 24 hours. Released LDH in the medium from cells catalyzes the oxidation of L-lactate to pyruvate with concomitant reduction of NAD+ to NADH. The LDH activity can be calculated from the rate of NADH increase in absorbance at 340 nm. Light absorption was analyzed at 340 nm using Cintra 5 UV-visible spectrometer (GBC co., Australia). LDH activity was calculated by the formula:

LDH (U/L) = (ΔA sample/min - ΔA blank/min) × F

F = 1000 ×V total / (Vsample × extinction coefficient)

The extinction coefficient of mmol NADH at 340 nm is 6.3

2.6. Acridine orange (AO) staining for lysosomal vacuolation

Acidic vacuoles were detected with AO staining and were observed under a fluorescence microscope. Briefly, cells were cultured in fresh medium in 24 well plate with DMSO 0.09% (v/v, as control), CQ 32 μM, 3BDO 180 μM, or both CQ 32 μM and 3BDO 180 μM for 24 h. Then the cells were stained with AO 0.1 mg/ml at room temperature for 1 minute. Subsequently the plate was washed once with PBS. At last the cells were observed and photographed under an Olympus BH-2 fluorescence microscope.

2.7. Neutral red uptake assay for VAC.

The VAC was determined by neutral red uptake assay as described previously[5] and was appropriately modified. 0.5% neutral red stock solution was prepared in 0.9% saline and filtered. Staining solutions were prepared before each experiment by diluting the stock solution (1:10) in 1% PBS (phosphate-buffered saline). A549 cells had been seeded in 12 well plates at the density of 6250/cm^2 24 h before CQ or 3BDO were added. After incubation with the chemicals for indicated time, the A549 cells were washed twice with PBS and were incubated for 4 minutes with 4 ml staining solution. The cells were washed twice with PBS, and the neutral red sample was extracted from cells by adding 1 ml acidified alcohol (50% of alcohol, 1% of acetic acid, 49% water) per well. The optical density (OD) at 540 nm of samples was determined using Cintra 5 UV-visible Spectrometer.

All assays were performed in triplicate. The OD value of each sample was subtracted from the OD of the well without cells to yield a net OD. The neutral red uptake readings for each sample would be normalized for total protein.

Then cell monolayers were washed once with 1% PBS and lysed with 20 μl of lysis buffer per well. The total protein concentration was determined with the Coomassie Plus protein assay reagent by using BSA as a standard. The value of the VAC for each dish was normalized by dividing the neutral red uptake data by the total protein concentration.

2.8. Na^+, K^+-ATPase activity assay.

The A549 cells were cultured in the medium with DMSO 0.09% (v/v, as control), CQ 32 μM, 3BDO 180 μM, or the combination of CQ and 3BDO for 24 hours. The cells were trypsinized after washed twice with PBS. Then, the cells were homogenized with ultrasonic (400 W, 12 min) in 3 ml buffer (20 mM Tris–HCl, pH 7.0, 10 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 0.34 mM sucrose) on ice. The enzyme samples were used for Na^+, K^+-ATPase activity assay as described in the instruction of the detection kit (Nanjing Jiancheng Biotechnology Institute, China). ATPase may hydrolyze ATP and provide both ADP and inorganic phosphate (Pi), so the enzyme activity was determined by measuring the amount of inorganic phosphate (Pi) liberated from ATP during the incubation of reaction mixture. The optical density was measured at 636 nm (wavelength), using a Cintra 5 UV-visible Spectrometer. The enzyme activity was expressed as Unit per mg of protein (U/mg prot).

2.9. Semi-quantitative reverse transcription-PCR and agarose gel electrophoresis

Total RNA was isolated from cells with the use of Trizol reagent (Invitrogen, USA), and 2 μg of sample was reversely transcribed with M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions. The specific primers for *TGFB2-OT1* are sense strand 5'-GCAGTTTCACCTAAAGAGCAGC-3'，and anti-sense strand 5'-TTCCTTCCCACCTCCACCC-3'. The specific primers for GAPDH are sense strand 5'-ACCACAGTCCATGCCATCAC-3' and anti-sense strand 5'-TCCACCACCCTGTTGCTGTA-3'. The cDNA for the test genes was amplified by PCR using the Takara Taq system (Takara, Japan). The PCR conditions were 94°C for 3 min; 35 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min; and 72°C for 7 min. The PCR products were analyzed on a 2% agarose gel. GAPDH RNA levels were analyzed in parallel to ensure that equal amounts of cDNA samples were loaded for each reaction.

2.10. Flow cytometry analysis of cell cycle

Cells were harvested and then fixed with 70% ethanol, stained with 50 μg/mL propidium iodide (PI) containing 10 μg/mL RNase A at 4 °C for 1 h. The stained cells were analyzed using a FACS Calibur flow cytometer

(BD Bioscience, USA). Cell cycle distribution was analyzed by ModiFit software (BD Bioscience, USA).

2.11. Statistical analysis.

Data were presented as means ± SE from 3 independent experiments and analyzed by Student's t-test. Differences at $p<0.05$ were considered statistically significant.