**Supplementary information**

# Dichloroacetic acid and rapamycin synergistically inhibit tumor progression

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## Materials and methods

### 1. Cell culture

MDA-MB-231, 4T1, CT26, B16F10, and Hepa1-6 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). MDA-MB-231, B16F10, and Hepa1-6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), and 4T1 and CT26 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640. Full cell-culture mediums were supplemented with 10% (0.1 g/mL) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

### 2. Reagents and antibody

The following reagents were used: rapamycin (Selleckchem, #S1039, USA), everolimus (#T1784, Topscience, China), Dichloroacetic acid (DCA) (#sc-214877, Santa Cruz, USA), Trichloroacetic (TCA) (#T834457, Merklin, China), 4. Sulforhodamine B (SRB) (#259262, J&K Scientific, China), BCA assay kit (#P0012, Beyotime, China), protease inhibitors (#14001, Bimake, USA), phosphatase inhibitors (#15001, Bimake). The following antibodies were used: Rabbit polyclonal anti-phospho-PDH-s232 (#AP1063, Sigma, USA), anti-phospho-PDH-s293(#AP1062, Sigma, USA), anti-phospho-PDH-s300(#AP1064, Sigma, USA), anti-phospho-S6 (#4858, Cell Signaling Technology (CST), USA), anti-PARP (#9532, CST, USA); Mouse monoclonal anti-PDHA1 (#sc-377092, Santa Cruz, USA), anti-ribosomal protein S6 (#sc-74459, Santa Cruz, USA), anti-Vinculin (#sc-73619, Santa Cruz, USA). HRP-linked anti-rabbit IgG (#111-035-003, Jackson Immunoresearch, USA) and HRP-linked anti-mouse IgG (#115-035-003, Jackson Immunoresearch, USA).

### 3. Western blot

Cells were collected and extracted using lysis buffer (150 mmol/L NaCl, 1% (volume fraction) Triton X-100, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L ethylene glycol bis(2-aminoethyl)tetraacetic acid (EGTA), 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerolphosphate, 20 mmol/L Tris-HCl, pH 7.5, with protease inhibitor cocktail). After adding lysis buffer, cell lysates were subjected to appropriate sonification on ice 10–15 times (2 s at a time, avoid proteins denaturation). And then, cell lysates were centrifuged at 10000*g* for 10 min at 4 ℃. Protein concentration was tested using BCA assay kit, and the supernatant were denatured with 5× SDS loading buffer. Samples were analyzed using the SDS page. Immunoblot was performed by immersing the gel in transfer buffer for 1 h at 100 V and transferring the proteins onto a 0.45 μm PVDF membrane. The membrane was blocked in 5% non-fat milk and incubated with relative antibodies.

### 4. Sulforhodamine B (SRB)-based cell-viability assay

Sulforhodamine B (SRB) assays were performed as described by Orellana and Kasinski (2016) with minor modifications. Briefly, 2000 cells in 100 μL medium were seeded in 96-well plates per well. After cells attachment, 2× concentrations of rapamycin and DCA in 100 μL medium were added. After 48 h treatment, cells were fixed with 10% TCA in culture medium at 4 ℃ for 1 h. Fixed cells were washed with deionized water three times and stained with 0.04% SRB in 1% acetic acid for 30 min. Stained 96-well plates were washed with 1% acetic acid three times as soon as possible and air-dried at room temperature. Stained dye was dissolved in 50 μL 10 mmol/L Tris (pH10.5) and detected using a plate reader (Cytation5) at 510 nm. Assays were performed using at least three biological replicates.

### 5. Cell-cloning formation

Two hundred cells were seeded in each well of 12-well plates. After leaving cells to attach for 24 h, 2 mmol/L dichloroacetic acid (DCA) and 10 nmol/L rapamycin was added to the culture medium. The culture medium containing the indicated reagents was refreshed every three days. When cells clones were clearly visible, we used SRB to stain and quantify the clones, as described above. Cell plates containing clones were imaged and counted.

### 6. Animal study

Male BALB/c mice (6–8 weeks old) were obtained from the Dalian medical university and maintained under specific pathogen-free (SPF) conditions. $1\times10^5$ 4T1 cells were injected subcutaneously into hind leg groin. Rats were divided into four groups: control (no treatment), DCA alone, rapamycin alone and DCA cooperate with rapamycin. Treatments were performed when volumes of tumor nudes were looked clearly. DCA (0.75 g/L) was added to the drinking water as previously described (McMurtry, Bonnet et al., 2004). Rapamycin (2 mg/kg) was dissolved in DMSO/PEG300/deionized water (20/40/40, v/v/v, 0.5 mg/mL) and administered intraperitoneally (IP) once daily. Tumors were measured using a caliper every two days, and tumor volume was calculated using standard formula $0.5\times L\times W^2$, where $L$ is the longest diameter

and *W* is the shortest diameter. The tumors were removed, photographed and weighed 18 d after injection. All institutional and national guidelines for the care and use of laboratory animals were followed.

## 7. Statistical analysis

Data were processed using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA) and are presented as mean±standard deviation (SD). A two-tailed unpaired Student's *t*-test (normal distribution) was used to assess the significance of differences between the two groups. $P<0.05$ was considered statistically significant.

## References

Mcmurtry MS, Bonnet S, Wu X, et al., 2004. Dichloroacetate prevents and reverses pulmonary hypertension by inducing pulmonary artery smooth muscle cell apoptosis. *Circ Res*, 95(8):830-840. https://doi.org/10.1161/01.RES.0000145360.16770.9f

Orellana EA, Kasinski AL, 2016. Sulforhodamine B (SRB) assay in cell culture to investigate cell proliferation. *Bio Protoc*, 6(21):e1984. https://doi.org/10.21769/BioProtoc.1984