

**Inhibition of aerobic glycolysis represses
Akt/mTOR/HIF-1 α axis and restores
tamoxifen sensitivity in antiestrogen-
resistant breast cancer cells**

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Supplementary materials and methods

Sulforhodamine B assay

A quantitative sulforhodamine B (SRB) colorimetric assay [1] was used to determine the inhibition of cellular growth by various compounds. Cells were plated at 1×10^3 cells/well in 96-well plates and grown for 24 h. The cells were treated with 4-OHT at 0, 2.5, 5, or 10 μ M for 24 h. At the end of the incubation, the cells were fixed with 10% trichloroacetic acid (1 h at 4°C), stained for 20 min at RT with 100 μ l of a 0.4% w/v SRB solution (Sigma) in 1% acetic acid, and then quickly rinsed with 1% acetic acid. After air-drying, protein-bound dye was dissolved in 100 ml of 10 mM unbuffered Tris base at pH 10.5. The pink SRB was quantified by measuring the optical density at 540 nm. For each condition, the average cell density and the standard deviation were calculated, from the data points corresponding to the 6 wells.

Cell cycle analysis

The cell cycle analysis was performed by flow cytometry. Cells (1×10^6) were harvested, washed twice in PBS, and fixed in 70% ethanol at 4°C overnight. The fixed cells were stained with propidium iodide (PI)/RNase staining buffer (Becton Dickinson, 550825). Stained samples were analyzed by a FACS Canto II and the percentage of cells in each cell cycle phase was determined using FlowJo software. All samples were measured in triplicates with three different cell lines showed similar results.

Supplementary materials and methods

Measurement of reactive oxygen species

Reactive oxygen species (ROS) production was determined using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE), which can be used to preferentially detect hydrogen peroxide and superoxide, respectively [2]. Cells were incubated in a 96-well plate at 2×10^5 cells/well with the addition of DCFH-DA (50 $\mu\text{g/ml}$) and DHE (10 $\mu\text{g/ml}$), respectively. Intracellular ROS levels were measured by monitoring fluorescence generated from the product via oxidation of either DCFH-DA or DHE at an excitation wavelength of 485 nm and emission wavelength of 535 nm for the product from DCFH-DA and an excitation wavelength of 488 nm and emission wavelength of 605 nm for the product from DHE during a 1-h reaction period [3].

Cellular glutathione concentration measurement

To measure the concentrations of intracellular reduced glutathione (GSH) and oxidized glutathione (GSSG), cells were detached by gentle trypsinization and one millions of cells were collected from each type of cell. The pellet was suspended with cold 5%(w/v) Metaphosphoric acid (Sigma Aldrich, 23927-5) and the suspension was centrifuged. The supernatant was transferred and lysed by sonication. Either total or oxidized glutathione was detected using Glutathione detection kit (Enzo, ADI-900-160) according to the manufacturer's instruction.

Oxygen consumption measurements

XF24 analysis was performed as previously described [4]. Briefly, the XF24 cell culture plates (Part No. 100777-004, Seahorse Bioscience, USA) were shaped to each well of the 24-well plate. Then, the sensor cartridge was placed on top of the plate and incubated at 37°C without CO₂ overnight. The oxygen consumption rate (OCR) was measure by the XF24 analyzer and XF24 software. After measuring the OCR, the XF24 assay results were normalized to cell number. The cell number for each well was counted using a hemocytometer. Live cells were also counted and the XF assay results were normalized to cell number using XF assay software.

Supporting references

1. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275.
2. Rothe G, Valet G (1990) Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescein. *J Leukoc Biol* 47: 440-448.
3. Jung MK, Song HK, Kim KE, Hur DY, Kim T, et al. (2006) IL-18 enhances the migration ability of murine melanoma cells through the generation of ROI and the MAPK pathway. *Immunol Lett* 107: 125-130.
4. Qian W, Van Houten B (2010) Alterations in bioenergetics due to changes in mitochondrial DNA copy number. *Methods* 51: 452-457.