Discovery a novel human lactate dehydrogenase A (LDHA) inhibitor as an anti-proliferation agent against MIA PaCa-2 pancreatic cancer cells

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Materials and cell culture

Compound 3 (3-((3-carbamoyl-7-(2,4-dimethoxypyrimidin-5-yl)quinolin-4-yl)amino)benzoic acid) was synthetized following the procedures reported by Chai et al. 1. Identified compounds 5-13, with the purity > 95 %, used in the experiment were purchased from local agent supplier. Stock solutions were prepared by dissolving the compounds as 20 mM in DMSO and stored at -80 °C. Human pancreatic cancer cells MIA PaCa-2 was obtained from American Type Culture Collection (ATCC). The cells were cultured in DMEM containing 10 % fetal calf serum at 37 °C in 5% C02, 95% humidified air. Purified LDH-A (from human liver, item no. 355-50) was obtained from Lee Biosolutions (St Louis, Missouri, USA).

Anti-proliferation assay

The anti-proliferation activities were determined by using MTT assay. A suspension of MIA PaCA-2 cancer cells (5000/well in 90 μL medium) were seeded in 96-well plates and cultured for 24 h. The compound solutions (200 μM) were prepared by diluting the stocking solution in PBS buffer. Then compounds (10 μL/well) were added to the 96-well plates (90 μL medium), which were incubated for another 72 h. Then, 10 μL of MTT solution was added to each well. After 3 h of incubation, solvent was removed and 100 μL DMSO was added to each well. The absorbance of each wells were read at wavelength of 570 nm.

After the most potent compound 12 was identified, the exact IC50 value for compound 12 was determined. The procedures are similar as above described, eight different concentrations of compound 12 ranging from 400–3.125 μM (10 μL/well) were added into the 96-well plate (90 μL medium). The OD values were read in the Microplate Reader, and the results were expressed as IC50 values, which were the mean values derived from three independent experiments.

Lactate Measurement
MIA PaCa-2 cancer cells were treated with different concentrations of compound 12 for 10 h in serum-free medium, the lactate production was measured by lactate assay kit (Biovision, CA, USA). Meanwhile, total proteins of cell lysates were collected and quantified for normalization of lactate release.

**Seahorse XF24 experiment**

The key parameters of glycolytic function was assessed using a Seahorse XF24 glycolysis stress kit. This technology is widely used to measure oxygen consumption rate (OCR) expressed in pmol/min and extracellular acidification rate (ECAR) expressed in mpH/min.

In this experiment, MIA PaCa-2 (40000/well) were added into the wells, after the cells were attached, an additional 100 μL growth medium was added and the cells were incubated for 24 h at 37 °C in 5% CO2 atmosphere. Then the cells were treated with various concentration of compound 12 for 10 h, and then cells were subjected to the XF24 extracellular flux analyzer for the measurement of OCR and ECAR.

**Apoptosis detection by flow cytometry**

MIA PaCa-2 cancer cells were seeded at a density of 5 x 10⁵ cells/mL on a six well plate and were allowed to grow overnight. Then treated with 5, 10 and 15 μM compound 12 for 10 h at 37 °C. Cells were trypsinized and washed with cold PBS for three times, then collected the cells, which were resuspended in 1 x Annexin binding buffer to ~5×10⁵ cells/mL for 100 μL. To 100 μL of cell suspension, 10 μL Annexin V and 5 μL PI working solution were added, and incubated for 15 min at dark. After the incubation, 400 μL PBS was added to each sample, and was gently mixed and analyzed immediately on the flow cytometer.

**LDHA Enzymatic Assay**

The experiment was performed in 96-well plates, 10 μL volume with the following final enzyme and buffer: 50 mM Hepes (pH 7.2), 0.01% (v/v) TritonX-100, 0.01% (0.1 mg/mL) Bovine Gamma Globulin, 2 mM DTT, 1 nM LDHA, 50 μM NADH, and 50 μM pyruvate. Compounds in 100% DMSO with 1:2 serial dilutions (final DMSO = 1%). For the enzyme reaction, serially diluted compounds were added to a mixture of enzyme and NADH. The assay plates were then incubated at room temperature for 10 minutes and a baseline read was conducted on the Microplate Reader with excitation at 340 nm and emission at 480 nm to identify any compounds which interfere with NADH fluorescence. Following the baseline read, pyruvate was added to the assay plates and the plates were read with excitation 340 nm and emission 480 nm for 10 minutes every 2.5 seconds. A suitable linear timeframe was selected (150-400 s) to calculate the slope of each concentration tested. The curve bottom was set to the
background rate (initial 5 second recording prior to addition of pyruvate) and curve top was set to no inhibitor (DMSO only) control wells rate.

**Molecular docking**

Molecular docking was conducted by a Surflex Dock program in the Sybyl-X 2.0 package. The crystal structure of LDHA was obtained from the Protein Data Bank (PDB ID: 4QT0). The crystal structure of LDHA was prepared with all H added and charge added by AMBER7 FF99 method. The structures of small molecular database was download from Zinc database (http://zinc.docking.org/). Firstly, the commercial available database was filtered, the remaining compounds were subjected to the polar H adding and being energy optimized with a tripos force field and charged optimized with Gasteiger-Huckel method. The protomol was generated in ligand mode with the threshold kept at 0.50 and the bloat 0. Ring flexibility was considered and other parameters during the docking program were determined through a number of attempts.

![Binding modes of the compound 3 in the active site of LDHA. Compound 3 formed hydrogen bonds with the residues Asp 52, Arg 99, Arg 112 and Gly 97.](Image)

**Fig. S1** Binding modes of the compound 3 in the active site of LDHA. Compound 3 formed hydrogen bonds with the residues Asp 52, Arg 99, Arg 112 and Gly 97.

Reference: