Nitrogen trapping as a therapeutic strategy in tumors with mitochondrial dysfunction

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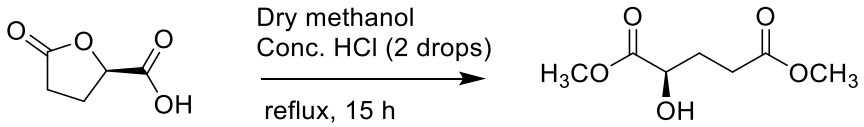
Supplementary Methods

Synthesis of dimethyl L-2HG (dimethyl (2R)-2-hydroxypentanedioate)

To a stirred solution of 1.00 g (7.69 mmol) of (2*R)*-5-oxooxolane-5-carboxylic acid in 10 mL of dry MeOH was added 2 drops of concentrated HCl and refluxed for overnight. The reaction mixture was cooled to 0 °C and quenched with solid $NaHCO_3$ and filtered. The filtrate was concentrated under diminished pressure and the residue was further purified through silica gel column chromatography. Eluted with 2:3 ethyl acetate-hexanes afforded the desired dimethyl (2*R*)-2-hydroxypentanedioate compound as a viscous colorless liquid (1.30 g, 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.88-1.97 (m, 1H), 2.12-2.20 (m, 1H), 2.39-2.54 (m, 2H), 3.94 (brs, 1H), 3.66 (s, 3H), 3.78 (s, 3H) and 4.23 (dd, 1H, *J* = 4.2 and 7.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.4, 29.5, 51.8, 52.8, 69.6, 173.7 and 175.1.



Nutrient dependency studies

Glucose, pyruvate and glutamine dependencies of the isogenic cell pairs of UOK262 and UOK268 were tested. 5,000 cells/well in 96-well plates were cultured for 72 hr in the standard medium (4.5 g/L glucose, 1 mM sodium pyruvate, and 2 mM glutamine) except with one

modification as specified in the figure legends. The relative cell growth was determined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G7570).

Analysis of necrotic regions on tumor H&E sections

Hematoxylin and eosin (H&E) stained tumor sections were photographed and 8-10 images per tumor were taken. Analysis of images was performed with ImageJ software. Regions with at least 5 cells showing cell shrinkage, pyknosis, and/or karyorrhexis phenotype was considered as necrotic region. Using the ImageJ, region of interest (ROI) was drawn around the perimeter of necrotic region. % necrotic area was calculated based on the total necrotic area divided by the total evaluated area. The average number of necrotic regions per frame was also measured and plotted.

Stable isotope labeling using amino acids in cell culture (SILAC) metabolic flux assay

For metabolic tracing experiments, 500,000 143B-wt cells stably transfected with SLC1A3 transporter were seeded in a 6 cm dish in DMEM supplemented with 10% dialyzed fetal bovine serum (FBS). Next day, cells intended for phenformin or dmaKG + phenformin treatment were treated with 100 μM phenformin overnight. On the next day, all of the plates were replaced with fresh DMEM/10% dialyzed FBS with indicated the vehicle, dmaKG, phenformin or dmaKG + phenformin for 6h. In the last 30 minutes of treatment, 500 μM 13C4,15N-aspartate (Cambridge Isotopes Laboratories) was added. At the end of treatment, cells were washed with saline and metabolites were extracted from cells in 80% methanol in water containing 1 μg/ml D8-valine and D8-phenylalanine in proportion to the number of cells. Relative metabolite abundances were calculated by integrating ion peak area and normalized to internal standards. The glutamate-15N/aspartate-13C4,15N ratio was calculated and plotted.

Steady state metabolomic analysis

The metabolite profiling was performed on various platforms as described below.

The metabolomic analysis of the UOK268 cell pair (Figure S1B, S5A) and the UOK262 cell pair (Figure S1A) were performed on a GC-MS/MS platform. 1.5 x 10^5 cells/well were plated in a 6-well plate and treated as mentioned in the figure legends. The cells were washed in saline before being scraped in 300 μL of ice-cold solution (-80 ^{0}C) containing 3 parts of methanol, 1 part of water and norvaline (1 μg in 300 μL). The cell solution was added to Eppendorf tubes containing chloroform (-80 ^{0}C, 300 μL), and this mixture was vortexed for 10 minutes and spun down at the maximum speed for 10 minutes (4 ^{0}C). The top layer comprised of polar metabolites was transferred to a GC-MS vial and the samples were further analyzed as previously described (Davidson et al., 2016).

The metabolomic analysis of the UOK262 cell pair (Figure 3A-B, 3F, S5A) and 143B-wt cells (Figure S8) were performed using a LC-MS system comprised of an Ultimate 3000 (Thermo Fisher Scientific) coupled to a Q ExactiveTM HF-X orbitrap mass spectrometers (Thermo Fisher Scientific). 2.5 x 10^5 cells/well were seeded in a 6-well plate and treated as mentioned in the figure legends. Cells were rapidly isolated by scraping in an extraction solution of 80% methanol containing inosine-15N4, thymine-d4 and glycocholate-d4 internal standards (Cambridge Isotope Laboratories; 250 µL/ well/ 2.5 x 10^5 cells of a 6-well plate). The samples were centrifuged (10 min, 9,000 x g, 4°C) and the supernatants were injected directly onto a 150 x 2.0 mm Luna NH_2 column (Phenomenex). The column was eluted at a flow rate of 500 µL/min with initial conditions of 10% mobile phase A (20 mM ammonium acetate and 20 mM ammonium hydroxide in water) and 90% mobile phase B (10 mM ammonium hydroxide in 75:25 v/v acetonitrile/methanol) followed by a 10 min linear gradient to 99% mobile phase A. MS analyses were carried out using electrospray ionization in the negative ion mode using full scan

analysis over m/z 70-750 at 60,000 resolution with 100 ms max IT and 1e6 AGC. Additional MS settings were ion spray voltage, 3.8 kV; capillary temperature, 350°C; probe heater temperature, 320 °C; sheath gas, 50; auxiliary gas, 15; and S-lens RF level 40. Targeted processing of a subset of known metabolites and isotopologues was conducted using TraceFinder software (Thermo Fisher Scientific). Peak identities were confirmed using reference standards.

The metabolomic analysis of the 143B cell pair (Figure 3C-D) was performed on a LC-MS/MS platform with the following protocol. 5 x 10^5 cells were seeded per 60 mm dish and treated as mentioned in the figure legends. The metabolites were extracted into 80% LC-MS grade methanol containing 500 nM internal standards (Catalog # MSK-A2-1.2; Cambridge Isotope Laboratories; 500 µL/ 5 x 10^5 cells) and analyzed at the Whitehead Institute Metabolite Profiling Core Facility (Massachusetts Institute of Technology).

The aspartate levels in xenograft tumors and mouse organs (Figure 6C,E) were measured using hydrophilic interaction liquid chromatography on a LC-MS/MS platform. Metabolites from finely chopped and ground tumor or tissues (20 mg) of UOK262, 143B-CytB and 143B-wt in vivo efficacy studies were extracted with 80% methanol comprising d8-Phenylalanine and d8-Valine. The volume of solvent used for extraction is normalized to the protein concentration of the lysates. Later, samples were analyzed on LC-MS/MS for all amino acids. The peaks of targeted molecules were monitored in the positive ion mode on TSQ Quantiva triple quadrupole MS.

Measurement of 2HG enantiomers

Chirality of 2-hydroxyglutarate (2-HG) was determined using a GC-MS/MS system (Intlekofer et al., 2015). 5 x 10^5 cells were plated per 60 mm dish and treated with or without dmaKG (6.6 mM) for 2.5 hr. Metabolites were extracted with ice-cold 80:20 methanol:water.

After overnight incubation at −80° C, cell extract was harvested, sonicated, and centrifuged at 21,000 g for 20 min at 4° C to precipitate protein. Extracts were then dried in an evaporator (Genevac EZ-2 Elite). Dried samples were derivatized with 100 µl of freshly prepared 50 mg/ml (+)-diacetyl-L-tartaric anhydride (DATAN, Sigma) in dichloromethane-acetic acid (v/v=4:1) at 75° C for 30 min. After cooling to room temperature, derivatized samples were dried under nitrogen at room temperature and resuspended in 200 µl of UltraPure water (18.2 MΩ, PureLab) prior to LC-MS/MS analysis. Analysis was performed on a Thermo Vantage triple-quadrupole mass spectrometer operating in SRM and negative ionization modes. LC separation was using an Acquity UPLC HSS T3 analytical column (2.1×100 mm, 1.8 µm, Waters) with an Agilent 1260 infinity binary pump. Mobile phase A was 125 mg/l ammonium formate in water adjusted to pH 3.5 with formic acid, mobile phase B was methanol, and flow rate was 0.3 ml/min. Initial conditions were 3% B for 5 min, then increased to 80% B at 5.5 min and held for a further 2.5 min. 10 min of re-equilibration time was used to ensure retention time stability. The column temperature was held at 40° C. Samples were kept at 4° C and the injection volume was 5 µl. MS source parameters were spray voltage: 2500 V; capillary temperature: 300 °C; vaporizer temperature: 250° C; sheath gas pressure: 50 psi; aux gas pressure: 40 psi. The compound specific S-lens value was 37 V for 2HG. Individual reactions monitored and collision energies (CE) for 2HG were *m/z* 363.0 → 147.1 (CE: 12 V)*, 129.1 (CE: 27 V), with * indicating the primary transition used for quantification. The identities of D- and L-2HG enantiomers were determined by comparing to the retention times of the derivatized pure standards and additionally confirmed by spike-ins of derivatized pure standards into experimental samples. Chromatograms were acquired and processed with XCalibur and TraceFinder software (ThermoFisher).

NAD/NADH assay

The levels of NAD+ and NADH were measured by the NAD/NADH-Glo Assay (Promega, G9071). Briefly, the UOK262 cell pair were treated with dmaKG for the indicated times. Cells were scraped in PBS and harvested by centrifugation for 1 minute at 300 x g. Pelleted samples were lysed in 100 µL of ice cold lysis buffer (1% Dodecyltrimethylammonium bromide (DTAB) in 0.2 N NaOH diluted 1:1 with PBS) and stored at -80 °C. To measure NADH, 20 µL of the cell lysate were heated at 75 °C for 30 min to degrade NAD+. To measure NAD+, samples were diluted 1:1 with 0.4 N HCl and incubated at 60 °C for 15 min to degrade NADH. Following incubations, 20 µL of 0.25 M Tris/ 0.2 N HCl and 20 µL of 0.5 M Tris were added to the NADH and NAD+ samples, respectively. Manufacturer's instructions were then followed to measure NAD+/NADH.

Glucose uptake study

UOK262-ev and FH cells were plated in a 12-well plate ($2x10^5$ cells/well). Next day, cells were treated with dmaKG (6.6 mM) for 3, 6, and 9 hr. Subsequently, the media was replaced with glucose-free DMEM after a single wash with PBS. Cells were incubated for 1 hr followed by the addition of (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose) (2-NBDG) (150 µM) for 30 mins. At the end, cells were collected by trypsinization, washed in PBS, and stained with propidium iodide before analysis using the BD Fortessa X-20 cell analyzer.

References

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