The imipridone ONC213 targets α-ketoglutarate dehydrogenase to induce mitochondrial stress and suppress oxidative phosphorylation in acute myeloid leukemia

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Materials and Methods

Primary AML Samples

Clinical samples were screened for gene mutations by PCR amplification and automated DNA sequencing, and for fusion genes by real-time RT-PCR, as described previously.[1,2]

Western Blot

Anti-PARP, -MCL-1, -BCL-2, -β-actin, -BAX, and -BCL-xL were purchased from Proteintech (Chicago, IL, USA). Anti-BIM, -cleaved (cf) caspase 3, -eIF2-α, -p-eIF2-α, -ATF4, -α-KGDH, and -CHOP were purchased from Cell Signaling Technologies (Danvers, MA, USA). Anti-p-AKT(S473), -p-AKT(T308), -DR5, and -TRAIL were purchased from Affinity Biosciences, Zhenjiang, Jiangsu, China. Anti-VDAC1 was purchased from Biomake.cn (Shanghai, China). Anti-AKT, -BAK, and -total OXPHOS cocktail were purchased from Abcam (Cambridge, MA, USA).

Pharmacokinetics

Eight-week-old BALB/c female mice (Charles River Laboratories, Wilmington, MA, USA) (n=2 mice/time point) were treated with one oral dose of 50 mg/kg ONC213. Plasma samples were collected at 0.5, 1, 2, 4, 6, 8, and 24 h. Pharmacokinetic samples were analyzed by a validated LC/MS-MS method.[3,4] ONC201 was used as an internal standard.

Seahorse Analysis

Cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse XFe24 flux analyzer (Agilent Technologies, Santa Clara, CA, USA), as previously described.[5] Cells were attached to CellTak-coated plates and then incubated with XF Base medium supplemented with 2 mM glutamine, 1 mM Na-pyruvate, and 10 mM glucose at pH 7.4 to measure mitochondrial function. Basal OCR and ECAR were assessed and then oligomycin (final concentration, 1 μM), FCCP (0.5 μM for MV4-11 and J000106565 and 1 μM for THP-1 cells), and rotenone/antimycin A (0.5 μM each) were added at the indicated times to measure the OCR.

Mitochondrial Enzyme Activity Assays

Citrate synthase activity was measured, as previously described,[6] with modifications. Cells were lysed in lysis buffer (25 mM Tris-HCl (pH 7.5) and 0.5% β-dodecyl maltoside). The assay was initiated by adding acetyl-CoA to the cell lysate, 1 mM Ellman's reagent (5′5′-dithiobis-2-nitrobenzoic acid) and 1 mM oxaloacetate, followed by measuring absorbance at 412 nm. Activity was calculated using an extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ for DTNB. MV4-11 cells were treated with 500 nM ONC213, lysed by sonication in phosphate buffer saline (PBS), and then resuspended in 0.5% β-dodecyl maltoside. Bovine heart mitochondria, used as a positive control for all assays. NADH dehydrogenase (Complex I) and succinate dehydrogenase (Complex II) were assayed by following the reduction of 2,6 dichlorophenoindophenol (DCPIP; Fluka, Buchs, Switzerland) at 600 nM. Reactions were started with 150 mM NADH or 10 mM succinate (pH 7.4), respectively, in 25 mM Tris buffer (pH 7.4) containing 2.5 mg/mL fat-free BSA, 1 mM antimycin A, and 50 mM DCPIP for 1 min. For NADH dehydrogenase, measurement of the samples was zeroed against an identically prepared cuvette containing 10 mM rotenone. The specificity of the succinate dehydrogenase assay reaction was confirmed by testing with 1 mM 2-thenoyltrifluoroactone. Calculations for complexes I and II were done using an extinction coefficient of 21 $mM^{-1}cm^{-1}$ for DCPIP. Cytochrome *c* oxidase activity was measured spectrophotometrically by following the changes in cytochrome *c* absorbance after the sample was added. Bovine cytochrome c was prepared at 1 mM and reduced with 5 μL/mL 1 M DTT, then diluted to 50 μM in 25 mM Tris (pH 7.4) buffer. Baseline absorbance at 550 nm was at least 10-fold higher than that at 565 nm. Cytochrome *c* mixture was added to a cuvette, and the reaction was started by adding sample, with total volume being 1 mL, and mixing by pipetting. The decrease of absorbance at 550 nm was measured for 1 min. Cytochrome *c* oxidase activity was calculated using an extinction coefficient of 19.6 $mM^{-1}cm^{-1}$ for reduction/oxidation of cytochrome *c*. For each treatment condition, equal volumes of freshly

prepared MV4-11 lysate, corresponding to 2 million cells, were added to each well of a 96 well plate. Three replicate data points and 3 controls were prepared for each treatment. Substrate, 1.5 µl of α-ketoglutarate (1 M, pH 7.4 in water) was added to the lysates and, at start time, the volume brought to 150 µl with Reaction Buffer, containing NAD+ (1 mg/ml), thiamine pyrophosphate (200 µM), coenzyme a (100 µM), $MgCl_2$ (1 mM), and fatty acid free BSA (1 mg/ml) made in 25 mM Tris (pH 7.4). Reactions were incubated for 30 min at room temperature. Fifty µl of freshly prepared Developer Buffer, containing iodonitrotetrazolium chloride (3 mM), and phenazine methosulfate (5 µM) was added to all reactions to identically prepare NADH standards, and incubated for an additional 5 min to allow color to develop before reading at 500 nm. Controls without α-ketoglutarate, but otherwise identical, were subtracted as background and activities were calculated as nmol NADH formed per min per mg protein. The α-KGDH activity was normalized by citrate synthase activity and expressed as a ratio of α-KGDH activity to citrate synthase and computed for each treatment condition and normalized to the untreated control in each experiment.

Human CLPP WLA Assay

Assay buffer: 50 mM Tris-HCl (pH 7.8) (T5941; Sigma), 10 mM magnesium chloride (AM9530G; Ambion), 5% ultrapure glycerol (15514-011; Invitrogen), 100 mM potassium chloride (5920-1; Caledon), 0.02% Triton X-100, and 1 mM DTT (10197777001; Sigma). A 12 µM stock solution of human CLPP protein (2×) was prepared in assay buffer and then dispensed (25 µL per well) into a black solid-bottom 384 micro-well plate (82051-310; VWR Scientific, Radnor, PA, USA).[7] Compounds were added (0.001-50 µM concentration range) using a D300 digital dispenser (HP, Palo Alto, CA, USA), and the plate was then incubated for 15 min at room temperature. A 90 mM stock of fluorescent tripeptide substrate Ac-WLA-AMC (Boston Chemicals, Whitby, Ontario, Canada) was prepared in 100% DMSO. This was further diluted to a 100 µM stock (2×) of the tripeptide substrate in assay buffer, and then the DMSO concentration of the solution was adjusted to 4% (2×). The reaction was initiated by adding peptide substrate (25 µL per well), followed by incubating at 37°C without shaking for 30 min in the dark. Readings were taken using the BioTek microplate reader (Ext/Ems: 380/460 nm). Data were plotted in GraphPad Prism using log(agonist) versus response-variable slope (four parameters). A control run without compound was used to define 100% activity. Percent activity was normalized by setting the lowest compound concentration equivalent to the control activity at 100%.

Human CLPP Casein-FITC Assay

Assay buffer: 50 mM Tris-HCl (pH 7.8) (T5941; Sigma), 10 mM magnesium chloride (AM9530G; Ambion), 5% ultrapure glycerol (15514-011; Invitrogen), 100 mM potassium chloride (5920-1; Caledon), 0.02% Triton X-100, and 1 mM DTT (10197777001; Sigma). A 2 µM stock solution of human ClpP protein (2×) was prepared in assay buffer and then dispensed (25 µL per well) into a black solid-bottom 384 micro-well plate (82051-310; VWR).[7] Compounds were added (0.003-50 µM concentration range) using an HP D300 digital dispenser. The plate was then incubated for 15 min at room temperature. A 10 mM stock of fluorescent substrate Casein-FITC (C0528, Sigma) was prepared in 100% DMSO. This was further diluted to a 9.6-µM stock (2×) of the substrate in assay buffer, and then the DMSO concentration of the solution was adjusted to 4% (2×). The reaction was initiated by adding peptide substrate (25 µL per well), followed by incubating at 37°C without shaking overnight (21 h) in the dark. Readings were taken using the BioTek microplate reader (Ext/Ems: 380/460 nm). The data were plotted in GraphPad Prism using log(agonist) vs. response—variable slope (four parameters). A control run without compound was used to define 100% activity. Percent activity was normalized by setting the lowest compound concentration equivalent to the control activity at 100%.

Mitochondrial Membrane Potential

MV4-11 cells were treated with vehicle or 250 nM ONC213 for 8 h. As a positive control, MV4-11 cells were treated with 10 µM FCCP for 10 minutes prior to TMRE staining. Cells were stained with 100 nM TMRE for 30 minutes, collected, and washed with PBS. Flow cytometry was used to measure TMRE fluorescence.

Geometric mean was calculated using FlowJo version 10.8.1. Relative TMRE fluorescence was determined with respect to the vehicle treated group.

Total Cellular ROS

MV4-11 cells were treated with vehicle or 250 nM ONC213 for 8 h, or with 10 uM Hydrogen Peroxide for 15 minutes (positive control). Cells were stained with 5 uM Invitrogen CellROX Deep Red for 20 minutes, collected, and washed with PBS. CellROX fluorescence was measured by flow cytometry. Geometric mean was calculated using FlowJo version 10.8.1. Relative CellROX fluorescence was determined with respect to the vehicle treated group.

Electron Microscopy Assessment of Mitochondrial Morphology

MV4-11 and THP-1 cells were treated with ONC213 for 8 h. Cells were pelleted, washed in PBS, and then fixed in 0.1 M cacodylate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde. Samples were post-fixed in reduced osmium tetroxide and contrasted with aqueous uranyl acetate. Dehydration was achieved by a series of ascending concentrations of ethanol to 100%, followed by 100% propylene oxide. Samples were infiltrated with EmBed-812 and polymerized at 80°C. Embedded samples were sectioned at approximately 80 nm on a LeicaUC-7 ultramicrotome (Wetzlar, Germany) and examined in a TF20 transmission electron microscope (Thermo Fisher Scientific, Hillsboro, OR, USA) at 80 kV. Digital micrographs were captured with an Advanced Microscopy Techniques (Woburn, MA, USA) imaging system. Unless otherwise indicated, all reagents were from Electron Microscopy Sciences (Hatfield, PA, USA).

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