ATR-101 disrupts mitochondrial functions in adrenocortical carcinoma cells and *in vivo*

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Supplemental Materials and Methods

1. Experimental animals and tissue analysis

1.1 H295R cell xenografts

Male CB17 severe combined immunodeficiency (SCID) mice were obtained from Harlan and were fed a standard diet and maintained under a 12h:12h light:dark cycle. At 6 weeks of age, cell suspensions (10^8 cells in 200 µl) were injected subcutaneously into the rear flank of the mice. To measure the effects of ATR-101 on xenograft establishment, the sizes of swelling produced by cell implantation were measured twice during the week after cell injection, and the mice were randomized into groups that had similar distributions of the sizes of the swellings. To measure the effects of ATR-101 on xenograft growth, the mice were randomized into groups that had similar distributions of xenograft sizes with an average of 100 mm^3. Each group was administered vehicle or the indicated amounts of ATR-101 by oral gavage. The sizes of the xenografts were measured three times each week using calipers.

1.2 Measurement of Urinary free cortisol

Urine was collected by housing the mice individually without bedding for 2 hours at different times after beginning administration of vehicle or ATR-101. The levels of urinary free cortisol were measured using DetectX Cortisol Enzyme Immunoassay Kits (Arbor Assays, Cat# K003-H1/H5). Briefly, the stored urinary samples were thawed and diluted in the supplied assay buffer. 50 µl of diluted urine was used to measure the cortisol level using the protocol provided by the manufacturer.

1.3 BrdU incorporation in xenograft cells

The mice were injected intraperitoneally with 0.2 mL bromodeoxyuridine solution (BrdU, 10 mg/ml). After 6 h the xenografts were harvested, embedded in OCT and frozen. BrdU incorporation was detected using a monoclonal antibody directed against BrdU (Roche, Cat# 11170376001) as described (Ji, et al. 2007). Briefly, xenograft sections were immersed in 4% paraformaldehyde for 15 minutes at room temperature and

washed in PBS. The fixed sections were incubated with 1% Triton X-100/ 0.1M PBS for 15 min to permeabilize cell membranes, followed by 10 min incubation in 1N HCl on ice and 20 min in 2N HCl at 37˚C to make BrdU incorporated in DNA accessible to the antibody. The sections were incubated in 0.1M Borate buffer for 5 min at room temperature, followed by 30 min in 0.3% H2O2 at room temperature to inhibit endogenous peroxidases. The sections were blocked with 5% normal serum in PBS for 1 hour at room temperature, followed by overnight incubation with mouse anti-BrdU antibody diluted 1:100 at 4°C. Subsequently, the sections were incubated with biotinylated anti-mouse IgG (Vector, Cat# FMK-2201) diluted 1:250 for 30 min at room temperature. Finally, the sections were stained for peroxidase activity using an ABC kit (Vector, Vectastain ABC Peroxidase Kit, PK-6101) following the manufacturer's instructions.

1.4 Histology and anti-Ki-67 Immunocytochemistry of xenograft sections

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer thick sections were stained with hematoxylin and eosin (H/E). The immunocytochemistry was performed as described (Luconi, et al. 2010). Briefly, xenograft sections were incubated with 1% Triton X-100/ 0.1M PBS for 15 min to permeabilize the membranes. Then, the sections were incubated in 0.1M Borate buffer for 5 min at room temperature, followed by 30 min in 0.3% H2O2 at room temperature to inhibit endogenous peroxidases. The sections were blocked with 5% normal serum in PBS for 1 hour at room temperature, followed by overnight incubation with mouse anti-human Ki-67 primary antibody (DAKO, Cat# M724029-2) diluted 1:100 at 4°C. The sections were incubated with biotinylated anti-mouse IgG (Vector, Cat# FMK-2201) diluted 1:250 for 30 min at room temperature. Finally, the sections were stained for peroxidase activity by using an ABC kit (Vector, Vectastain ABC Peroxidase Kit, PK-6101) following the manufacturer's instructions.

1.5 TUNEL staining of xenograft sections

Apoptotic cells were detected in xenografts using the Cell Death Detection kit (TUNEL staining, Roche). Briefly, fixed xenograft sections were incubated in 0.1% Triton X-100 /

0.1% sodium citrate 2 minutes on ice to permeabilize the cells followed by 30 min in 0.3% H2O2 at room temperature to inhibit endogenous peroxidases. Then, the sections were rinsed twice with TBS [10 mM Tris-HCl, 0.9% NaCl (pH 7.4)]. TUNEL staining was then performed according to the manufacturer's instructions. The nuclei were stained by dipping the slides in Mayer's hematoxylin solution (Thermo Scientific, 7211) for 30 seconds. After drying the slide, the sections were mounted in permount mounting medium (Fisher Scientific, SP15-100). Digital images of the cells were obtained using an Olympus microscope equipped with a color digital CCD camera. Apoptotic cells in the cortex of the xenografts within 2mm from the surface were quantified by counting the percentage of TUNEL-positive cells relative to the total number of nucleated cells in randomly selected fields.

1.6 Visualization of lipofuscin in the adrenal cortex

Six female Dunkin-Hartley guinea pigs (BW 500-600 g) were administered vehicle or 100 mg/kg/day ATR-101 orally for two weeks. The adrenals were harvested, embedded in OCT and frozen. Cryosections were fixed in 1% (wt/vol) formaldehyde/PBS paraformaldehyde for 10 minutes and mounted into 40% glycerol/TBS mounting medium. The fixed sections were imaged within 2 h by fluorescence microscopy (485±20 nm excitation, 560±20 nm emission) to detect autofluorescence. The autofluorescence spectrum of lipofuscin was analyzed using a Leica SP5X Inverted 2-Photon FLIM Confocal microscope.

1.7 Sudan Black B staining of adrenocortical sections

Sections of the adrenal cortex frozen in OCT were mounted onto superfrost slides and were fixed in 1% (wt/vol) formaldehyde/PBS for 1 min at room temperature and then washed three times (approx.1 min) at room temperature, with PBS. Sections were then incubated for 5 min in 50% ethanol and then for another 5 min into 70% ethanol. The sections were stained with 0.7% Sudan Black B in 70% ethanol for 5 min and were then submerged in 50% ethanol and washed in distilled water to remove unbound dye. The stained sections were mounted in 40% Glycerol/TBS mounting medium.

2. Indicators of cell viability

2.1 Measurement of cellular reducing activities

Cellular reducing activities were measured by bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promaga, Cat# G3580) as well as resazurin-based PrestoBlue reagent (Molecular Probes, Cat# A-13261) according to the manufacturer's instructions. Briefly, H295R cells were seeded in 96-well plates 24 hours before the assay. The cells were cultured with the indicated concentrations of ATR-101 for 10 minutes. MTS or PrestoBlue reagent was added to the wells and the absorbance at 490nm was measured in the MTS reduction assay and the fluorescence at λex/λem = 584/612 nm was measured in the PrestoBlue assay at 15 minute intervals for 4 hours using a SpectraMax M5 plate reader (Molecular Devices).

2.2 Measurement of ATP levels

The ATP levels were measured using the CellTiter-Glo® Assay kit (Promega). Cells were cultured in white 96-well plates at 2 x 10^3 cells per well in 100 µl medium. After 24 hours, the reagents indicated were added. At the times indicated, the plates were transferred to room temperature for 30 minutes. 100 µl CellTiter-Glo® Reagent was added to each well. The plates were incubated in the dark for 15 minutes at room temperature. The Luminescence signals were detected at 500 nm using SpectraMax M5 plate reader (Molecular Devices). The luminescence signals from wells containing medium without cells were used to subtract the background.

2.3 Measurement of cell membrane permeability

The permeability of the cell membrane was measured by incubating the cells with 50 nM SYTOX green (Invitrogen, Cat# S7020) for 15-30 min. The fluorescence of SYTOX binding to nucleic acids was visualized by florescence microscopy or measured using a SpectraMax M5 microplate reader (λex/λem = 485/530 nm).

2.4 Propidium iodide and annexin V staining

Cells were stained using the Annexin V Apoptosis Detection Kit I (Becton, Dickinson and Company) in accordance with the manufacturer's protocol. H295R cells were seeded in six-well plates 24h in advance for the experiments. The cells were cultured with vehicle or 30 μM ATR-101 for 20 hours. The cells were washed twice with PBS and incubated with 1X FITC Annexin V solution for 1 hour at 37°C in darkness. After washing with PBS, the cells cells were incubated with 1X PI solution and 5 μg/ml hoechst 33342 for 15 minutes at RT in darkness. The cells were imaged using an inverted florescence microscope and the number of cells labeled by each reagent was counted.

2.5 Analysis of cytochrome c release

Cytosolic proteins were analyzed by western blot using anti-cytochrome c antibody. To investigate the significance of cytochrome c release, H295R cells were pretreated with 100 μM minocycline for 1 h, followed by the addition of vehicle or 30 μM ATR-101 for 20 hours. The cells were collected and washed twice by PBS. The cells were resuspended in 1 mL mitochondria isolation buffer (220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EGTA, 0.2% Fat Acid free BSA, 10 mM HEPES-KOH pH7.5) and homogenized by 15-20 strokes in a Dounce tissue grinder with a tight pestle. The homogenate was centrifuged at 900 g for 10 min at 4 C to pellet nuclei and unbroken cells. The resulting supernatant was transferred to a conical tube and centrifuged (12,000g; 20 min) to pellet the mitochondria. The supernatant (cytoplasm) was collected and the protein concentration was measured. The same amounts (15 μg) of total protein were used for Western blot analysis using an anti-cytochrome c monoclonal antibody (Clone: 7H8.2C12, BD Biosciences). anti-GAPDH antibody was used to compare the amounts of protein loaded.

2.6 Caspase-3/7 activity assay

The effect of ATR-101 on caspase 3 and 7 activities was evaluated using the Caspase-GloW 3/7 kit (Promega North America, Madison, WI) according to the instructions provided by the manufacturer. Briefly, H295R cells (2×103 cells/well in 100μl) were

seeded in a 96-well black walled clear bottom plate (Costar). The cells were incubated for 24 hours and then cultured with 25 μM ATR-101 alone or in combination with 200 μM minocycline for 20 h. The Caspase-GloW 3/7 reagent (100μl/well) was added. The plates were incubated in the dark for 60 minutes at room temperature with gentle agitation. The luminescence was measured using a SpectraMax M5e microplate reader.

3. Analysis of mitochondrial functions and reactive oxygen in cells

3.1 *Mitochondrial membrane potential in cells*

The mitochondrial membrane potential was determined by measuring the fluorescence of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbo-cyanine iodide (JC-1) as well as tetramethylrhodamine, methyl ester (TMRM). The cells were cultured with vehicle or ATR-101 as indicated, followed by incubation with 250 nM JC-1 or 100 nM TMRM for 30 minutes (Perry, et al. 2011). JC-1 and TMRM fluorescence were visualized using an inverted florescence microscope. JC1 fluorescence was measured at λex/λem = 485/530 nm and λex/λem = 500nm/590nm using a SpectraMax M5 plate reader (Molecular Devices). TMRM fluorescence was measured at λex/λem = 544nm/590nm using a SpectraMax M5 plate reader (Molecular Devices). Differences in the mitochondrial membrane potential are reflected in changes in the ratio of JC-1 fluorescence emissions (590 nm / 530 nm) and in the intensity of TMRM fluorescence emissions (590 nm).

3.2 *Levels of reactive oxygen species*

The levels of reactive oxygen species were measured using the fluorescent probes 2',7'-dichlorofluorescin diacetate (DCFH), MitoSOX and DHE. The cells were cultured under the conditions indicated, followed by incubation with 2.5 μM DCFH, 2.5 μM MitoSOX™ Red or 2.5 μM DHE for 60 minutes. The fluorescence in the cells was visualized by florescence microscopy or measured using a SpectraMax M5 microplate reader (λex/λem = 485 nm/530 nm for DCFH λex/λem = 400 nm/590 nm for MitoSOX™ Red and DHE). 2',7'-dichlorofluorescin diacetate enters cellS and the acetate group is cleaved by cellular esterases, trapping the non-fluorescent 2',7'-dichlorofluorescin in the

cell. Subsequent reaction with reactive oxygen species, particularly hydrogen peroxide and hydroxyl radical, yields the fluorescent dichlorofluorescein. Dichlorofluorescein fluorescence was visualized by florescence microscopy or measured using a SpectraMax M5 microplate reader (λex/λem = 485/530 nm). DHE enters the cell and can be oxidized by reactive oxygen species including superoxide and hydroxyl radical to yield fluorescent ethidium. Ethidium binding to DNA amplifies its fluorescence. MitoSOX™ Red is a derivative of dihydroethidium that preferentially localizes to mitochondria. Oxidation of MitoSOX™ Red by superoxide produces 2-hydroxyethidium, which is excited at 400 nm, in contrast to other ethidium oxidation products.

3.3 Mitochondrial morphology

H295R Cells were cultured on a glass coverslip for 24h. The cells were preloaded with 250 nM Mitotracker Red for 2 hours at 37C. After washing twice with PBS, the cells were cultured with the indicated concentrations of ATR-101 for 6 hours. The cells were rinsed briefly with warm PBS, fixed with 4% paraformaldehyde and stained using anti-cytochrome c antibody.

4. Preparation and analysis of rat liver mitochondrial fractions

4.1 Preparation of rat liver mitochondrial fractions

Mitochondrial fractions were prepared from rat liver as previously described (Frezza, et al. 2007). Briefly, liver fragments were gently homogenised in 220 mM mannitol, 70 mM sucrose, 10 mM HEPES (pH 7.5), 1 mM EDTA and 0.2% (w/v) fatty-acid-free BSA by 15-20 strokes in a Dounce tissue grinder with a tight pestle. The homogenate was centrifuged at 900 g for 10 min at 4 C to pellet nuclei and unbroken cells. The resulting supernatant was transferred to a conical tube and centrifuged at 6200 g for 10 min to pellet the mitochondria. The mitochondrial pellet was washed with and then resuspended in storage buffer (250 mM sucrose, 10 mM HEPES pH 7.5, 1 mM EDTA and 0.2% (w/v) fatty-acid-free BSA, 1mM ATP, 0.08 mM ADP, 5mM Succinate, 2mM phosphate buffer pH 7.4). The freshly isolated mitochondria were used for the analysis of mitochondrial membrane potential and ATP synthesis. Mitochondria that had been

frozen at -80˚C were used to measure the activities of individual complexes. The protein concentrations of the mitochondrial fractions were determined using the Bio-Rad protein assay kit following the the instructions provided by the manufacturer.

4.2 ATP synthesis in mitochondrial fractions

ATP synthesis was measured as described (Wibom, et al. 2002) by placing 50 µg of freshly isolated mitochondria in 100 µl of assay buffer (0.25 M D-Sucrose, 50 mM HEPES-KOH pH7.5, 2mM MgCl2, 1 mM EGTA, 10mM potassium phosphate buffer pH 7.4, 2 mg/ml fatty acid free BSA). The mitochondria were pre-incubated with vehicle or with the indicated concentrations of ATR-101, 5 µM rotenone (Complex I inhibitor) or 10 mM Malonate (Complex II inhibitor) for 30 minutes. ATP synthesis was initiated by adding the indicated substrates (30 mM glutamate / 30 mM malate for complex I pathway; 60 mM succinate for complex II pathway) and 300 µM ADP. After incubation at 25°C for 30 minutes, 100 µl CellTiter-Glo® Reagent was added. The samples were incubated in dark for 15 minutes at room temperature. The luminescence was measured at 500 nm using a SpectraMax M5 microplate reader.

4.3 Complex III and Complex IV activity assays

Complex III and Complex IV activities were measured as described (Spinazzi, et al. 2012) by subjecting 1 µg rat liver mitochondria to 3 freeze-thaw cycles in 25mM potassium phosphate buffer pH 7.5, 5 mmol/L $MgCl_2$, 0.1 mM potassium-EDTA, 0.025% Tween-20. The fragmented mitochondria were pre-incubated with vehicle, 50 µM ATR-101, 10 µg/ml antimycin A (AA), or 3 mM KCN for 30 minutes. To measure cytochrome c reduction by Complex III, 1 mM final concentration potassium cyanide (KCN) and 100 µM oxidized cytochrome c were added to the reaction. The reaction was started by the addition of 100 µM reduced decylubiquinol. To measure cytochrome c oxidation by Complex IV, 0.1 ug of the pretreated mitochondria were combined with reduced cytochrome c at 25 µM final concentration. The changes in the level of reduced cytochrome c were determined by measuring the absorbance at 550 nm.

4.4 F1F0-ATPase activity determination

F1F0-ATPase activity was measured as described (Yilmaz, et al. 2008) by subjecting 1 µg rat liver mitochondria to 3 freeze-thaw cycles in mitochondria isolation buffer (220 mM mannitol, 70 mM sucrose, 10 mM HEPES pH 7.5, 1 mM EDTA and 0.2% (w/v) fatty-acid-free BSA). The mitochondria were pre-incubated with vehicle, 50 µM ATR-101, 4 mM sodium azide (NaN3) or 5 µg/ml oligomycin for 30 minutes. ATP was added to the reactions and F1F0-ATPase activity was determined by measuring phosphate release using the Colorimetric ATPase Assay Kit (Novus Biologicals) following the protocol provided by the manufacturer.

5. Statistical analysis

Two-tailed unpaired Student's *t* tests were used to determine the statistical significance of differences in measured values. The Mann–Whitney non-parametric U-test was used to determine the statistical significance of differences between observations in groups of animals where the variability might not be normally distributed. The Chi-square test was used to determine the statistical significance of differences in the tumor incidence. The SPSS 15.0 program was used for data analysis.

Abbreviations

ACAT: acyl-CoA:cholesterol acyltransferase
ATP: adenosine 5´-triphosphate
ATR-101: N-[2,6-bis(1 -methylethyl)-phenyll-N'-[[1 -[4-(dimethylamino)phenyl]cyclopentyl]methyl] urea
Brdu: 5-bromo-2'-deoxyuridine
CCCP: carbonyl cyanide m-chlorophenylhydrazone
DCFH: 2', 7'-Dichlorodihydrofluorescin diacetate
DCCD: N,N'-dicyclohexylcarbodiimide
DHE: dihydroethidium
DIC: differential interference contrast
DMSO: dimethyl sulfoxide

Everolimus: dihydroxy-12-[(2R)-1-[(1S,3R,4R)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]propan-2-yl]-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-azatricyclo[30.3.1.0 hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentone

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

JC-1: 5,59,6,69-tetrachloro-1,19,3,39-tetraethylbenzimidazolocarbocyanine iodide

MitoSOX: hydroxyethidine

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

OCT: optimum cutting temperature

PI: propidium iodide

Rapamycin: (3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-Hexadecahydro-9,27-dihydroxy-3-[(1R)-2-[(1S,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclohentriacontine-1,5,11,28,29(4H,6H,31H)-pentone

ROS: reactive oxygen species

SCID: severe combined immunodeficiency

TMRM: tetramethylrhodamine methyl ester

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

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