**High Content Imaging of Mitochondria**

Cells were plated on clear bottomed CellCarrier 96-well plates at 5000 cells per well in complete MEM lacking phenol red and allowed to adhere overnight. The following day cells were treated with B508 or control, over five doses ranging from 100 nM-1 uM for the indicated time. Cells were stained with 500 nM Mitotracker orange CMTM Ros (Invitrogen, M22426) diluted in PBS added directly to the B508 containing medium, followed by Hoescht 33342 at a final concentration of 2 drops/ml (ThermoFisher, Cat# R37605) and Alexa-647 conjugated wheat germ agglutinin. Cells were fixed by addition of 15 uL 37% formaldehyde added directly to labeling medium for 30 minutes at room temp. Medium was removed and cells were washed twice with PBS. For experiments where CMTMRos signal is normalized to mitochondrial content, antibodies for MT-CO2 were added, and plates were washed 3x with PBS. Anti-mouse alexa 488 conjugated secondary antibodies were added for 1 hour and plates were washed 3x with PBS. Plates were imaged on a CX5 high content imager using Thermo Scientific HCS Studio 4.0 Cell Analysis Software at 10x magnification using the 386/440, 485/521, and 650/694 LED light source. Hoechst signal was used for autofocus and 9 images were collected per well. We used cellprofiler to assess total cell count per well based on Hoechst staining and mask the CMTMRos signal to the plasma membrane based on wheat germ agglutinin signal.

**Time-resolved Assessment of Mitochondrial Membrane Potential (ΔΨm) in Living Cells**

PC3 or H460 cells were plated onto 12- or 27-mm Nunc Glass Bottom Dishes at 20% confluence the night prior to imaging. The following day, medium was replaced with MEM lacking phenol red supplemented with pyruvate and glutamine and plates were transferred to a ZEISS LSM 880 laser scanning confocal microscope equipped with an incubator equilibrated to 37°C and 5% CO2 for 20 minutes prior to imaging. Tetramethyl Rhodamine Methyl Ester (TMRE) was added directly to the medium at a final concentration of 10 nM and allowed to equilibrate for 5 minutes prior to image acquisition, with the dye remaining in medium throughout the experiment. Plates were imaged every minute over four z-planes using an LSM-880 equipped with a C-Apochromat 63x/1.2 W autocorr M27 Oil objective with image size of ~150 x 150 μm. TMRE was excited using a 561nm laser line and Hoescht was excited using a 405 nm laser line, with laser power settings between 1-3%, which did not induce phototoxicity over the 1 hour timeframe. Spectral filtering of emission bands was limited to 460/60 for Hoechst, 550-650 nm for TMRE. After collecting images for an initial 10-minute period, cells were treated with B508 at 100 nM for the indicated time and imaged for an additional 50 minutes. To validate that TMRE was used at nonquenching concentrations, we treated control plates of both H460 and PC3 cells with oligomycin at 1 µM or FCCP at 10 μM and observed the expected increase (>1.3-fold) and decrease in TMRE intensity across both cell lines3.

**Analysis of Cell Cycle and BrdU Staining**

Cells were plated on clear bottomed CellCarrier 96-well plates at ~5000 cells per well depending upon proliferation rate in complete RPMI lacking phenol red and allowed to adhere overnight.  The following day cells were treated with compoud over a dose-response range for 24 hours. After 24 hours, medium was replaced with medium containing 0.03 mg/ml BrdU and the equivalent concentration of compound and labeled for 1 hour at 37°C and 5% CO2. For analysis of replication after cell cycle arrest from B508, medium was replaced with medium containing 0.03 mg/ml BrdU without B508 or analogues, and allowed to recover for 1-12 hours. After 1 hour, the plate was washed 2x with 150 uL PBS per well and fixed and permeabilized for 10 minutes with pre-chilled methanol. Methanol was removed and the plate washed twice with PBS.  After washing, 80 uL 1.5 N HCl was added to each well and BrdU was made accessible in 30 minutes. Next, the plate was washed with PBS and 50 µL of Alexa Fluor 488 conjugated Anti-BrdU (R&D Systems, Cat IC7225G) diluted 1:400 in 5% BSA in PBS was added per well, the plate was sealed with reflective plate covers and incubated overnight at 4°C on a rocking plate. The following day, antibody was removed and the plate washed 2x with cold PBS for five minutes each. Cells were stained with Hoescht 33342 diluted 2 drops/ml (ThermoFisher, Cat# R37605) for one hour and washed twice with PBS. Plates were imaged on a CX5 high content imager using Thermo Scientific HCS Studio 4.0 Cell Analysis Software at 20x magnification using the 386/440 and 485/521 LED light source. Hoechst signal was used for autofocus and 9 images were collected per well.

**High-content Fluorescence Imaging Coupled Metabolic Flux Assay**

The oxygen consumption and extracellular acidification rate across the 10-cell line panel was measured on a Seahorse XFe96 instrument. To account for differences in growth rate, cell density at confluence and error during plating, a protocol that uses cell count to normalize OCR and ECAR was optimized. Titrations of each cell line were first assessed for (1) an ECAR value of at least 10 mpH/min, which is the low end of a potential linear detection range, (2) retained morphology of the plated cells by visual inspection/microscopy, and (3) a cell density that lies within the linear range of a graph of cell seeding density versus ECAR values (**Supplementary Figure 2**). In addition, the concentration of B508 needed to induce 50% inhibition or induction of OCR and ECAR respectively, was assayed to verify saturating concentrations to use across cell lines (**Supplementary Figure 2**). For this assessment, cells were plated on XFe96 plates at a density ranging from 2,000 to 20,000 cells per well. Two days after plating, all cell lines were equilibrated in serum-free DMEM (Sigma-Aldrich D5030) containing 25 mM glucose, 10 mM sodium pyruvate, 2 mM glutamine and 5 mM HEPES pH 7.4 for 1 h before a mitochondrial stress test was performed consisting of 3 min cycles of mixing and 2 min cycles of measurements. Basal respiration rates were measured, followed by sequential injections of oligomycin (2 μM), FCCP (1 μM) and rotenone (2 μM) plus antimycin A (RAA, 2 μM). To measure the acute response to B508 active and inactive analogues, compounds were injected using one of the ports after measurements of basal respiratory rates were complete across an eight point concentration range (**Supplementary Figure 2**). Using the cell dilutions assessed in this fashion, oxygen consumption and extracellular acidification in response to B508 was assessed across the cell line panel as described above. After respirometry, cells were fixed by addition of 30 uL 37% formaldehyde for 30 minutes, washed twice with PBS, stained with Hoechst 33342 diluted 2 drops/ml (ThermoFisher, Cat# R37605) for one hour and washed twice with PBS and imaged in 100 uL PBS after . Plates were imaged on a CX5 high content imager using Thermo Scientific HCS Studio 4.0 Cell Analysis Software at 2x magnification using the 386/440 and 485/521 LED light source. Hoescht signal was used for autofocus and 1 image was collected per well. We used cellprofiler to assess total cell count per well based on Hoechst staining to mask individual cells. Oxygen consumption and extracellular acidification was normalized to the total number of cells identified in this field of view. For calculation of mitochondrial OCR (mitoOCR) and glycolytic ECAR (glycol ECAR), we used the following equations with a BF factor of 2.6 mM/pH and Kvol of 1.60 where Rot/AA represents values after treatment with rotenone and antimycin a.

eq 1: PER (pmol H+/min) = ECAR (mpH/min) × BF (mmol/L/pH) ×Vol measurement chamber (µL) × Kvol

eq 1: PER (total) = glycoPER + mitoPER

eq 2: mitoPER = PERbasal – PERRot/AA

eq 3: mitoOCR = OCRbasal – OCRRot/AA

eq 4: CCF [Equation]

eq 5: GlycoPER (pmol/min) = PER (pmol/min) – mitoPER (pmol/min) ≈≈ ECAR2-DG