Novel Mango Ginger Bioactive (2,4,6-trihydroxy-3,5-diprenyldihydrochalcone) Inhibits Mitochondrial Metabolism in Combination with Avocatin B

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Supplementary Information

Part 1: Methods

Avocatin B Extraction

Hass avocado seeds were air dried, crushed and placed in glass bottles with ethyl acetate in a 2:1 solvent: seed ratio. The bottles were sealed and rotated on a 120 Vac Benchtop Roller (Wheaton; Millville, NJ) for 24 hours. Extracts were then gravity filtered and the solvent was evaporated using a Rotavapor® R-100 rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) twice. The crude extract was purified using flash chromatography using a silica (Fisher Scientific; Mississauga, ON) column and ethyl acetate as the mobile phase. Column fractions were analyzed for purity using thin layer chromatography (eluent: ethyl acetate, R_f = 0.30) and visualized using p-anisaldehyde stain (Fisher). All purified samples were characterized by ^{1}H NMR. Samples were dissolved to 2 mg/mL in deuterated chloroform ($CDCl_3$) and transferred to an NMR tube. The samples were then analyzed using an Avance 400 MHz

spectrometer (Bruker; Billerica, Massachusetts) and recorded as parts per million (ppm). Purified Avo B was dissolved in DMSO for experimental use.

Combination Index Calculations

CI values were calculated using the Chou-Talalay Combination Index Method, using the following formula based on the based on the median-effect equation

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

where $(Dx)_1$ is a concentration of the first drug individually that inhibits a system by x%, and $(Dx)_2$ is a concentration of the second drug individually that inhibits a system by the same x%. The numerator, $(D)_1$ and $(D)_2$, are the drug concentrations in the combination that also inhibit x%.

Respirometry

Cell Culture

Cell viability was determined using trypan blue 0.4% cell stain (Gibco) and counting using a hemocytometer. Cells with ≥95% viability were used for cell culture. OCI-AML2 and OCI-AML3 cells were cultured in 100 mm cell culture dishes (Corning) at a density

of 0.5×10^6 cells/mL in 10mL of IMDM media; 10 million cells were required for each treatment. The desired treatment (1μM M1, 2μM Avo B, 1μM M1 + 2μM Avo B, or DMSO) was added and the plates were incubated in 5% CO_2 at 37°C for 1 hour.

Permeabilization

After incubation, the cells required for 1 treatment were collected and centrifuged at 1200 rpm for 5 minutes. The media was then removed, and the pellet was resuspended in 1mL of PBS. The pellet was then transferred to a 1.5mL microcentrifuge tube and centrifuged at 1200 rpm for 5 minutes in a microcentrifuge. Following the second centrifugation, the PBS wash was carefully removed, and the pellet was suspended in 500 μL permeabilization buffer (80mM KCl and 250mM sucrose in PBS) containing 0.01% digitonin (Sigma-Aldrich). The mixture was then agitated gently for 3 minutes, centrifuged at 1200 rpm for 5 minutes, and recentrifuged for one more cycle after the permeabilization buffer was removed. The pellet was then suspended in 150μL of MiR05 respiration buffer and injected into the Oroboros Oxygraph-2k (Oroboros Instruments; Innsbruck, Austria) chambers. The Oroboros Oxygraph-2k chambers

contained 2mL of MiR05 mitochondrial respiration medium (0.5mM EGTA, 3mM $MgCl_2$, 60mM lactobionic acid, 20mM taurine, 10mM KH_2PO_4, 20mM HEPES, 110mM D-sucrose, 1 mg/mL bovine serum albumin (BSA) in ddH_2O) [93]. Basal respiration was measured after injection, once steady-state respiratory flux was obtained.

Electron Transport Chain Activity

Mitochondria Rich Fraction Preparation

Using AML2 cells, 15x10^6 cells were collected and centrifuged for 5 minutes at 1200 rpm. The cells were washed with 1mL PBS for 5 minutes at 1200 rpm; the supernatant was discarded, and the pellet was retained. The pellet was then flash frozen in liquid nitrogen, thawed on ice, and resuspended in 10mM of ice-cold hypotonic Tris HCl buffer. The cells were homogenized with 3 pulses on Fisherbrand Model 120 Sonic Dismembrator (Fisher Scientific), with each pulse consisting of 3 seconds on, 3 seconds off at 45% amplitude. The cell homogenate was mixed thoroughly with 200µL of a 1.5M sucrose solution and centrifuged at 600g for 10 minutes at 2ºC using a microcentrigue. The supernatant was then collected in a 1.5mL microcentrifuge tube

and centrifuged at 14000g for 10minutes at 2ºC. The supernatant was discarded, and the pellet was resuspended in 150μL of 10mM ice-cold hypotonic Tris HCl buffer and divided into aliquots for protein estimation.

BCA Assay

The total protein content of the mitochondria fraction was quantified using the BCA protein assay. BSA standards at 0, 20, 40, 60, 80, and 100 μg/mL were created and 10μL of each standard was plated in triplicate in a 96-well plate. The mitochondrial rich fraction was diluted 10x using ddH_2O, and 10μL of this dilution was added to the 96-well plate in triplicate. Bicinchoninic acid (BCA) working reagent was then prepared containing 50 parts BCA (Sigma-Aldrich) to one-part 4% copper II sulphate (Sigma-Aldrich). The BCA working reagent was added to each BSA standard and sample well in the 96-well plate. The optical density was measured at 527nm using the Synergy HT spectrophotometer. Protein content of the sample was estimated using the standard curve.

Supplementary Figure 1

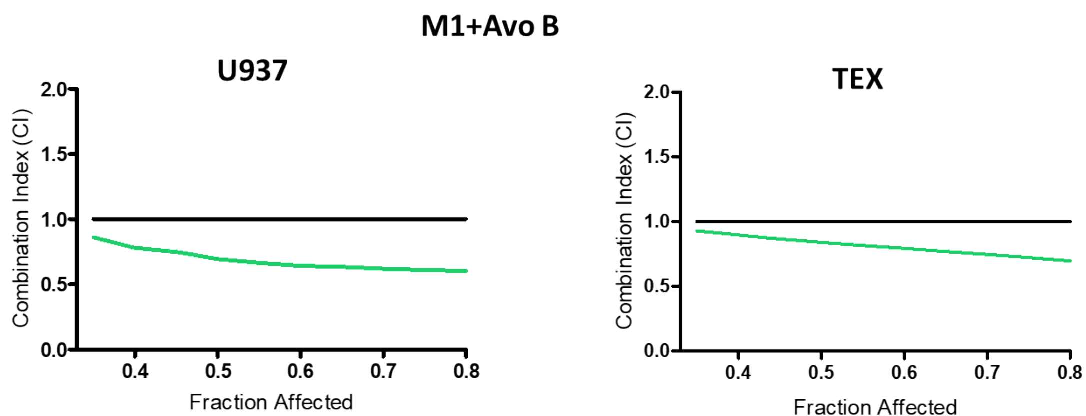

Figure S1: M1 interactions with Avo B in U937 and TEX cells. Equal molar concentrations of M1 and Avo B were incubated with U937 (left) or TEX (right) leukemia cells and cell viability was measured after 72 h by flow cytometry using 7AAD. Combination index (CI) values, which assesses drug-interaction effects, were calculated using the CompuSyn software. CI values of <1, >1 or equal to 1 denote statistical synergy, antagonism, or additivity, respectively. All experiments are n=3, data is mean ± SD. Representative figures shown.