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 ($\text{CDCl}_3$) 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 median-effect equation

\[
CI = \frac{(D_1)}{(D_x)_1} + \frac{(D_2)}{(D_x)_2}
\]

where \((D_1)\) is a concentration of the first drug individually that inhibits a system by \(x\)% and \((D_2)\) 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 \(\geq 95\)% 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 0.5x10^6 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% CO2 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 MgCl₂, 60mM lactobionic acid, 20mM taurine, 10mM KH₂PO₄, 20mM HEPES, 110mM D-sucrose, 1 mg/mL bovine serum albumin (BSA) in ddH₂O) [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, 15x10⁶ 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 10 minutes at 2°C. 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$_2$O, 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.
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.