Research Plan

a) Rationale

Mitochondria are the key organelles required for energy production within the cell. Therefore, when mitochondria exhibit a loss of function, the entire cell becomes endangered. Mitochondrial dysfunction is the underlying cause of nearly all age-related diseases and occurs due to a loss of electrical and chemical transmembrane potential of the inner mitochondrial membrane (IMM), changes in the function of the electron transport chain (ETC), or a reduction of transport of critical compounds into the mitochondria. The ability of the ETC to produce a proton gradient across the IMM is what allows for the production of denosine-5′-triphosphate (ATP). However, a negative byproduct of oxidative phosphorylation in the ETC is the production of reactive oxygen species (ROS), which have the ability to damage cellular lipids, proteins, and DNA. Therefore, the ability to target the IMM with small molecules in order to increase conductance and ETC capacity will has the potential to treat nearly all chronic diseases including: neurodegenerative diseases; cardiac diseases; diabetes; autoimmune diseases; neurobehavioral and psychiatric diseases; and musculoskeletal diseases.

b) Research Objectives

Demonstrate if A2 is cell-permeable; targets mitochondria; and more importantly, if A2 optimizes electron conductance in mitochondria membrane, improves mitochondrial membrane potential, prevents oxidative stress, and prevents cell death during starvation.

c) Procedure, Risk and Safety, Data Analysis

I. Procedure and Data Analysis

Cell Culture

Madin-Darby Bovine Kidney (MDBK) epithelial cells will be grown in Dublecco's Modified Eagle's Medium (DMEM), a high glucose media containing 4.5 g/L of glucose and 2 µM glutamine, and 10% heat inactivated horse serum. Cells will then be switched to serum free media and observed for 5-7 days. Cells will be incubated in 5% CO_2 at 37 °C.

A2 uptake study

10 µM A2 will be incubated with MDBK cells for 1 hour, followed by removal of media and fixation with 4% paraformaldehyde (PFA). Cells will be permeabilized with 0.2% digitonin, incubated with Streptavidin, Alexa Fluor™ 488 (Molecular Probes) conjugate, and viewed using a Nikon Eclipse Fluorescence microscope and a water immersion lenses.

A2 mitochondria uptake

10 μM A2 will be incubated with MDBK cells for 1 hour, followed by removal of the media, 1 hour incubation at -80 °C, and thawing in 4% PFA. Media will be removed and cells will be fixed with 4 % PFA. Cells will be incubated with Streptavidin, Alexa Fluor™ 488 (Molecular Probes) conjugate, and MitoTracker and viewed using a Nikon Eclipse Fluorescence microscope and a water immersion lenses.

Mitochondrial Potential and ROS Formation

MDBK cells will be grown in serum free media for 5 days in the presence or absence of A2. They will be then stained with MitoTracker to detect mitochondrial membrane potential since MitoTracker accumulation is dependent upon membrane potential. $CM-H_2DCFDA$ (chloromethyl 2',7'-Dichlorodihydrofluorescein diacetate) will be used to detect cellular oxidative stress due to its ability to indicate ROS formation.

Starvation model

Cells will be incubated in serum free media for 5 – 7 days in the presence of different concentrations of A2. At the end of the incubation period, media will be removed and cells were fixed in 4% PFA. Cell detection will be achieved by staining cells with Methylene Blue Loeffler for 30 minutes. Images will be obtained using a Tiffen Zoom Camera, interfaced to PC Image, and images will be analyzed using ImageJ NIH software. Experiments will be conducted 5 times in triplicate for each experimental condition.

II. Risk and Safety

1) **Human Subject: N/A**
2) **Vertebrate Animals: N/A**
3) **Potentially hazardous biological agents (PHBA):** Established Madin-Darby Bovine Kidney Epithelial Cells (MDBK line) will be used. ATTC cat #: CCL-22. Personal protective equipment (nitrile gloves,, lab coat, protective eye wear, protective shoes) will be worn when handling PHBAs. PHBA waste will be placed into designed biohazard containers for proper autoclave and disposal by university environmental health and safety department.
4) **Hazardous Chemical / activities / devices:**

1. Dublecco's Modified Eagle's Medium (DMEM)

These are common media for tissue culture cells. It may cause irritation to the eyes, skin, mucous membranes and respiratory tract if inhaled. If eye irritation occurs, one must wash their eye thoroughly with water and seek medical assistance. In the case of skin irritation, the affected area must be washed thoroughly with a large amount of soap and water. If

swallowed, one must wash their mouth with water.

2. 4% paraformaldehyde (PFA)

Flammable solid, may form combustible dust concentrations in air. Harmful if swallowed, causes skin irritation, may cause an allergic skin reaction, causes serious eye damage, harmful if inhaled, may cause respiratory irritation, suspected of causing cancer

3. Fetal Bovine Serum (FBS)

The FBS does not have any health hazards or physical hazards and it is not flammable. However, it may cause eye irritation, skin irritation, and may be harmful by inhalation or ingesting. To minimize risk one must ensure adequate ventilation, wear goggles, gloves, lightweight protective clothing, and prevent from going down the drain by following local regulations.

4. Phosphate-buffered saline (PBS)

It may cause irritation to the eyes and skin through contact. If ingested, PBS may cause irritation to the digestive tract. If inhaled, it may cause irritation to the respiratory tract. If one's eye comes in contact with PBS, one can use water to flush out the chemical. In the case of skin irritation, the chemical can be washed off with water. In the case of ingestion, one may rinse the mouth with water.

5. Streptavidin, Alexa Fluor™ 488

Not a hazardous substance or mixture. Avoid contact and inhalation. Recommended handling with caution.

6. MitoTracker

May cause eye irritation with susceptible persons. May cause skin irritation in susceptible persons. May be harmful by inhalation. May be harmful if swallowed.

7. $CM-H_2DCFDA$

The product contains no substances which at their given concentration, are considered to be hazardous to health. If skin contact rinse with plenty of water. If eye contact rinse cautiously with water for several minutes. Ingestion is not expected to present a significant hazard under anticipated conditions of normal use. Inhalation is not expected to be a hazard under anticipated conditions of normal use of this material.

8. Methylene Blue Loeffler

Flammable liquid and vapor, vapor may cause flash fire, causes eye irritation. May be harmful if swallowed. May cause respiratory tract and skin irritation. Contains material which may cause damage to the following organs: blood, reproductive system, liver, respiratory tract, skin, eyes, central nervous system.

9. A2

The product contains no substances which at their given concentration, are considered to be hazardous to health. If skin contact rinse with plenty of water. If eye contact rinse cautiously with water for several minutes. Ingestion is not expected to present a significant hazard under anticipated conditions of normal use. Inhalation is not expected to be a hazard under anticipated conditions of normal use of this material.

d) Bibliography

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— NO ADDENDUM EXIST —