A Novel Cationically Enframed High Density Aromatic Peptide, A2, Mitigates Mitochondrial Dysfunction and Promotes Cell Survival Via Reduction of ROS and Maintenance of Mitochondrial Inner Membrane Potential in a Cell Starvation Model

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Introduction

Mitochondrial Dysfunction

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 occurs as a result of: a loss of maintenance of the electrical and chemical transmembrane potential of the inner mitochondrial membrane; changes in the function of the electron transport chain; or a reduction in the transport of critical compounds into mitochondria. [1] Consequently, these changes reduce the efficiency of oxidative phosphorylation as well as the production of adenosine-5′-triphosphate (ATP). Reduction in mitochondrial efficiency and function is a factor of aging [2], and nearly all chronic diseases including: neurodegenerative diseases; cardiac diseases; diabetes; autoimmune diseases; neurobehavioral and psychiatric diseases; and musculoskeletal diseases. [3]

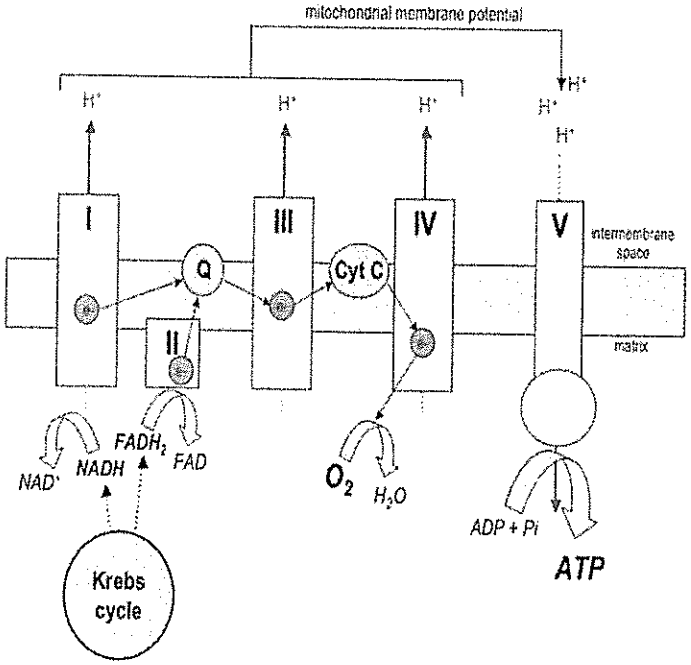


Figure 1: Overview of the mitochondrial electron transport chain. Electrons from NADH are donated to NADH-Q oxidoreductase (Complex I) while electrons from FADH 2 are donated to Succinate-Q reductase (Complex II). Electrons are shuttled through redox centers within both Complexes I and II. and are subsequently accepted by oxidized ubiquinone (Q), a lipophilic electron carrier. Upon accepting two electrons, reduced Q (ubiquinol) transfers its electrons to Q-Cytochrome c oxidoreductase (Complex III). Within Complex III electrons are cycled to the soluble single electron carrier, Cytochrome c (Cyt C). Four molecules of Cyt C successively reduce molecular oxygen (O 2) at Cytochrome c Oxidase (Complex IV) liberating two molecules of water. Complexes I, III, and IV contribute to the pumping of protons (H +) and establishment of a proton electrochemical gradient across the inner mitochondrial membrane (represented as horizontal gray bar). Dissipation of this proton gradient is facilitated by ATP Synthase (Complex V) and is coupled to the production of ATP. Source: ATP (Source: 4)

Mitochondria are composed of an outer mitochondrial membrane and inner mitochondrial membrane (IMM) which is unique in its high concentration of proteins and cardiolipin. [4] The ability of the mitochondria to produce ATP is directly related to the ability of the electron transport chain (ETC) to produce a proton gradient across the IMM. This potential allows protons to translocate through ATP synthase and thus convert adenosine diphosphate (ADP) into ATP. (Figure 1) [5] A negative byproduct of oxidative phosphorylation in the ETC is the production of reactive oxygen species (ROS), which is increased dramatically during ETC dysfunction. These highly reactive free-radicals have the ability to damage cellular lipids, proteins, and DNA. [1] Therefore, it is potentially important to target IMM with small molecules to increase conductance and increase ETC capacity.

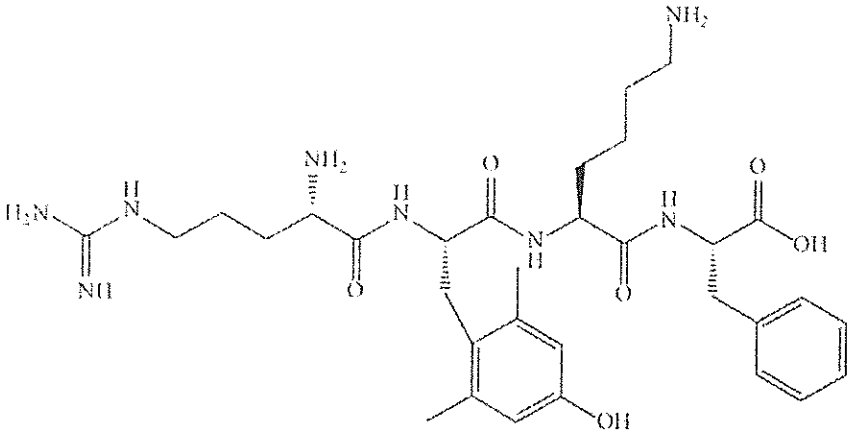
Diphenylalanine

Diphenylalanine (FF) is a self-assembling peptide which is known to improve conductance, thereby, have the potential to be used for IMM targeting. However, FF is very toxic and has antibacterial capabilities, where FF shown to nano-assemble in an E. coli culture inhibited bacterial growth and triggered upregulation of stress-response regulons, inducing substantial disruption of bacterial morphology, and causing membrane permeation and depolarization.[7] In addition, delivery of FF into the mitochondrial matrix of mammalian cells using triphenyl phosphonium conjugated FF (TPP-FF), resulted in self-assembly of TPP-FF in mitochondrial matrix, disruption of IMM and cell death [8], suggesting that FF should be targeted away from the matrix. Specifically, it must be of interest to target FF to IMM.

Szeto-Schiller Peptides

Presently, the Szeto-Schiller (SS) (SS-31 and SS-20) peptides represent a new approach to targeting IMM. The structural motif of these SS peptides centers on alternating aromatic residues and basic amino acids. They can target and reduce mitochondrial ROS, which inhibits mitochondrial permeability transition and cytochrome c release, thus reducing oxidant-induced cell death. [9] Both SS-31 and SS-20 can interact with mitochondrial cardiolipin to maintain mitochondrial structure and integrity. Previous studies have shown that SS-31 treatment

improves ATP production, reduces mitochondrial ROS production, and decreases oxidative damage in different cell culture and animal models. SS-31, being an antioxidant, has been demonstrated to be highly effective in several animal models associated with mitochondrial dysfunction and oxidative stress, such as ischemia-reperfusion injury and heart failure. [10]. However, direct conjugation of FF to SS peptides is likely to disrupt the unique alternating aromatic –cationic structure (Figure 2), which was proposed for SS peptides, interfering with SS functions.



SS-31

Figure 2

Chemical structure of SS-31. The peptide sequence is D-Arg-Dmt-Lys-Phe-NH_2. Alternating aromatic rings differ from FF peptides. Dmt represents the non-standard, antioxidant amino acid dimethyltyrosine. (Source: 9)

Cardiolipin

Cardiolipin (CL) is a type of diphosphatidylglycerol lipid which constitutes twenty percent of the inner mitochondrial membrane. Recent studies have brought attention to cardiolipin as a unique target for promoting mitochondrial efficiency. Cardiolipin is important for cristae curvature and is necessary for optimal activity of the respiratory complexes and the assembly of supercomplexes. [4,11] CL is highly acidic and has a head group (glycerol) that is esterified to two phosphatidylglyceride backbone fragments rather than one, resulting in a very specific ultrastructure and role in mitochondrial function. The diphosphatidylglycerol structure combined with four acyl chains gives cardiolipin its dimeric nature, which is unique among phospholipids resulting in a highly specific conical structure. [12] CL has been shown to interact with a number of inner mitochondrial membrane (IMM) proteins, enzymes and metabolite

carriers, including complexes I through IV in the ETC. (Figure 3)

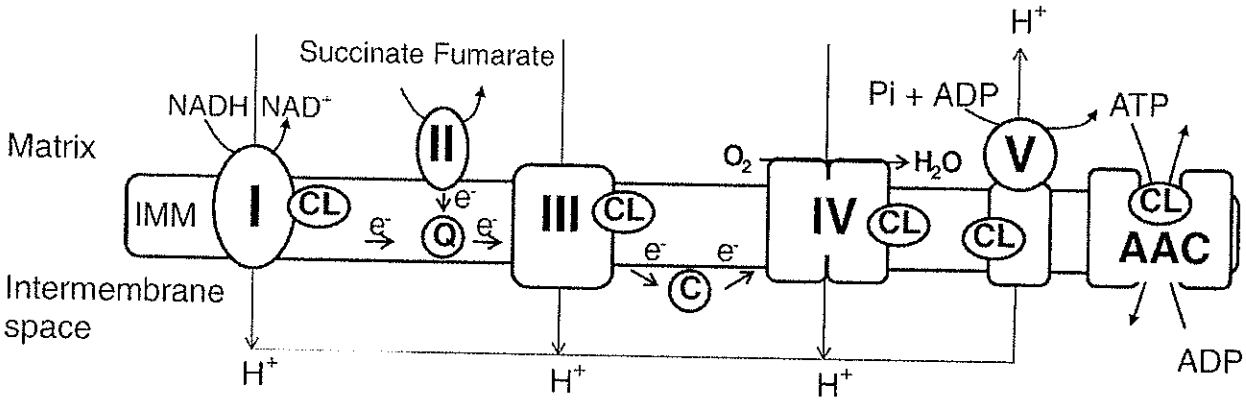


Figure 3: Interaction of cardiolipin with oxidative phosphorylation complexes. The dimerically cross-linked phospholipid structure of CL provides increased stability of complexes I, II, III, and IV. (Source: 12)

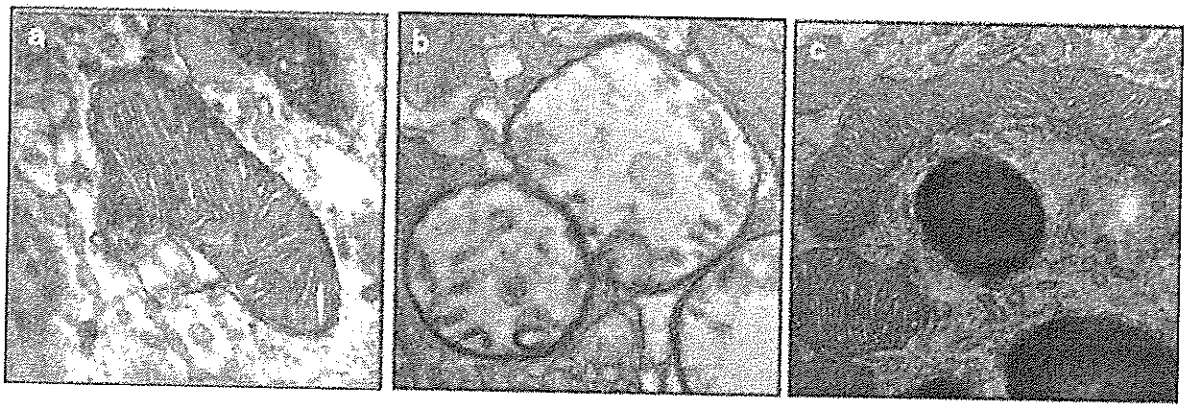


Figure 4: SS-31 protects the mitochondrial structure in retinal pigment epithelium (RPE) cells in diabetic mice. Mice were fed either a normal diet (ND) or a diabetic diet (DD). The DD mice also received streptozotocin (STZ). These mice then received saline or SS-31 applied as eye drops daily, starting at 12 weeks. Representative electron microscopic images of RPE mitochondria at 32 weeks in (a) ND, (b) DD+STZ plus saline, and (c) DD+STZ plus SS-31 mice. (Source: 4)

Previous literature on the impact of type 2 diabetes on mitochondria in mice suggests that SS-31 has the ability to restore mitochondrial morphology. Figure 4a shows mitochondria in mice fed a normal diet. Figure 4b shows mice given a diabetic diet and streptozotocin which reduces insulin secretion. There is a clear deformation of the mitochondria in this image, where the mitochondria are swollen, and nearly all cristae architecture is lost. However, figure 4c shows mitochondria in mice who received the diabetic diet and streptozotocin, but additionally they were exposed to SS-31. The image shows restored mitochondria morphology and a clear

increase in cristae from the previous image (5b). This suggests that SS-31 has the ability to reverse the effects that diabetes has on the mitochondria. Figure 5 demonstrates a possible interaction between SS-31 and CL, where SS-31 sits on top of CL. This may allow for the increased cristae formation in the mitochondria shown in figure 4.

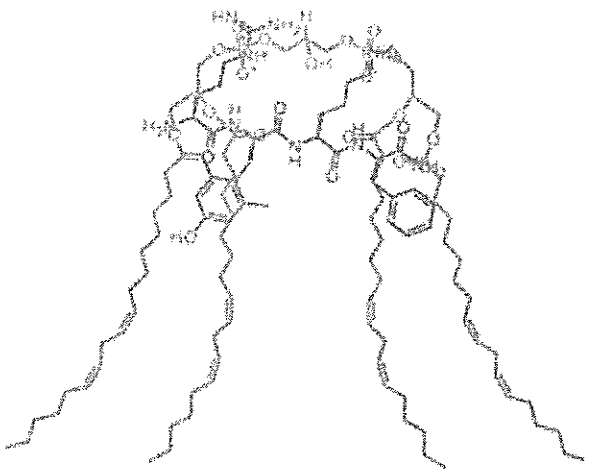


Figure 5. Previously proposed model of interaction between SS-31 (blue) and CL (red) demonstrating favorable electrostatic interactions between Arg/Lys and the phosphoglyceride moieties of CL and hydrophobic interactions between aromatic side chains and phospholipid acyl chains. SS-31 is selectively targeted to CL rather than the mitochondrial matrix and does not alter CL composition. (Source: 11)

Serum Starvation

Serum is added to culture media in order to provide a mixture of nutritional growth factors. It also acts as a buffer to cell culture conditions which could cause disruption to cell growth or toxicity. Such conditions include pH change, proteolytic activity, or the presence of heavy metals or endotoxin. [13] Thus, when serum is removed from cell culture, cells will inevitably die. Serum starvation is a common model used throughout various research experiments as it provides an easy way to observe changes in cells during cell death, without any complication through the use of chemical toxins. Serum starvation was applicable in our experiment as it provided a basis for comparison across many procedures. Previous studies have shown that serum starvation results in mitochondrial dysfunction, decreased membrane potential, as well as formation of ROS and oxidative stress. Thus, it provides a simple model to study mitochondrial dysfunction in cell cultures.

Structure of A2 (patent pending with Research Foundation of City University of New York)

Short high density aromatic peptides (HDAP) are considered to be excellent semiconductors for long-distance electron transfer. The structure of A2 was designed to bring FF into the mitochondria in a different way than previous studies. Thus, the structure of A2 is biotin-D-Arg-Phe-Phe-D-Arg-NH2. D-Arg was used instead of the L-Arg isomer as D-Arg is not labile to protease cleavage, and it was important to ensure that A2 would remain stable among mammalian proteins.

Purpose of Project

The goal of this project is to target FF peptides to mitochondria without producing cellular toxicity. A high density aromatic peptide, A2, was recently designed which contains FF enframed by cationic amino acids (D-Arg). Objectives of this project included: demonstrating 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.

Methodology

I. Cell Culture

Madin-Darby Bovine Kidney (MDBK) epithelial cells were 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 were then switched to serum free media and observed for 5-7 days. Cells were incubated in 5% CO_2 at 37 °C.

II. A2 uptake study

10 μM A2 was incubated with MDBK cells for 1 hour, followed by removal of media and fixation with 4% paraformaldehyde (PFA). Cells were then 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.

III. A2 mitochondria uptake

10 μM A2 were 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 was removed and cells were fixed with 4 % PFA. Cells were incubated with Streptavidin, Alexa Fluor™ 488 (Molecular Probes) conjugate, and MitoTracker and viewed using a Nikon Eclipse Fluorescence microscope and a water immersion lenses.

IV. Mitochondrial Potential and ROS Formation

MDBK cells were grown in serum free media for 5 days in the presence or absence of A2. They were 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) was used to detect cellular oxidative stress due to its ability to indicate ROS formation.

V. Starvation model

Cells were 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 was removed and cells were fixed in 4% PFA. Cell detection was achieved by staining cells with Methylene Blue Loeffler for 30 minutes. Images were obtained using a Tiffen Zoom Camera, interfaced to PC Image, and images were analyzed using ImageJ NIH software. Experiments were conducted 5 times in triplicate for each experimental condition.

Results

Figure 6 shows the uptake of A2 into MDBK cells. All cells were conjugated with MitoTracker and StreptAvidin. The upper panels demonstrate that mitochondria are present in the cytoplasm of cultured cells. The lower panels show that StreptAvidin was only detected in cells treated with A2. StreptAvidin effectively labeled A2 since A2 contains biotin in its chemical structure.

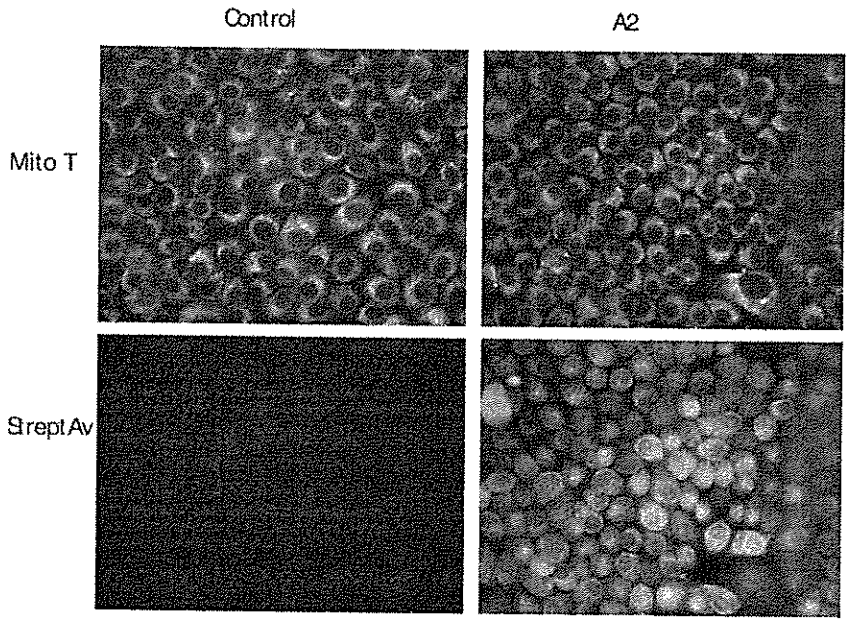


Figure 6:MitoTracker (upper panels) was used to detect cells in a monolayer on a 6 well plate. MitoTracker stains mitochondria in live cells and its accumulation is dependent upon membrane potential. StreptAvidin (bottom panels) was used to detect A2 in the same cells. Streptavidin is a 52.8 kDa protein purified from the bacterium Streptomyces avidinii. Streptavidin homo-tetramers have an extraordinarily high affinity for biotin. With a dissociation constant on the order of ≈10−14 mol/L, the binding of biotin to streptavidin is one of the strongest non-covalent interactions known in nature.

A2 was co-localized with mitochondria to determine whether A2 targets mitochondria. StreptAvidin labeled A2 due to biotin in the structure of A2. Bright red spot aggregates are indicative of mitochondria aggregation/mitophagy and is not labeled by Streptavidin. (Figure7) Mitochondria aggregation occurs when cells undergo oxidative stress. Therefore, A2 was only found in healthy mitochondria. This data shows that biotinylated A2 readily crosses MDBK cell membranes and further A2 targets IMM.

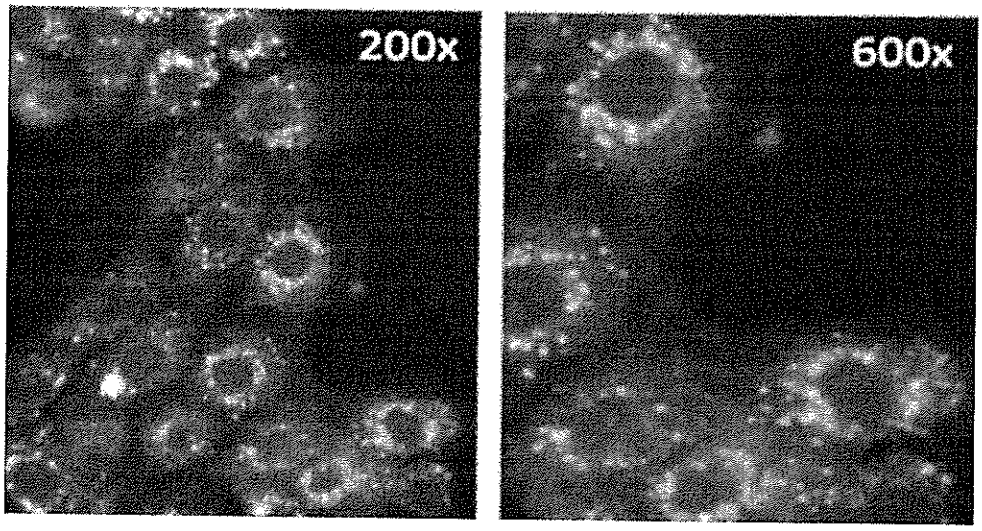


Figure 7: MitoTracker (Red) co-localizes with Streptavidin, Alexa Fluor™ 488 (green) as indicated by the appearance of yellow inside of the cell. The overlap of MitoTracker and StreptAvidin yields the yellow color exhibited above.

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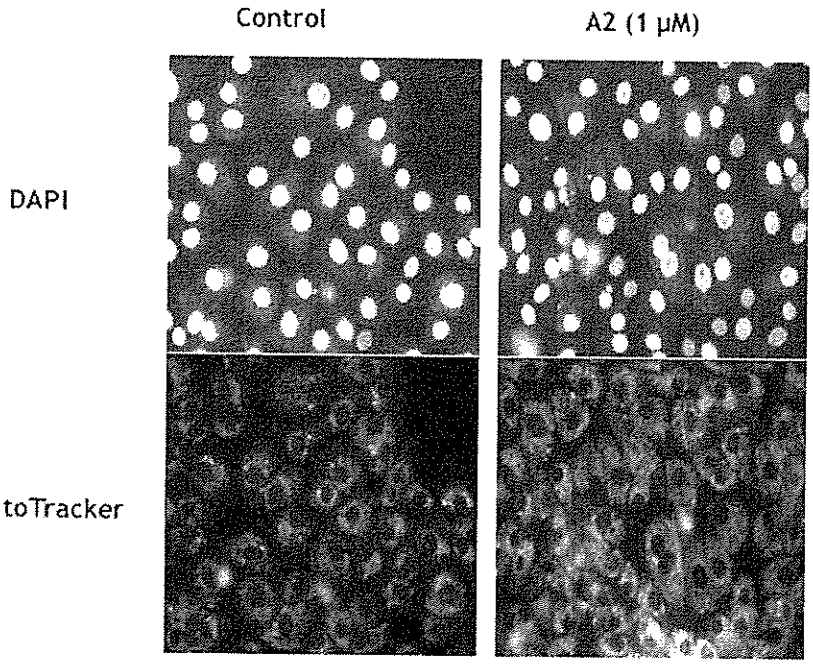


Figure 8: MDBK cells were grown in serum-free media for 5 days in the absence or presence of A2, and then stained with MitoTracker, to detect mitochondrial membrane potential. DAPI, 4',6-diamidino-2-phenylindole, is a fluorescent stain that binds strongly to A-T rich regions in DNA. DAPI was used here to show that cells are present on slides. DAPI labels nuclear regions.

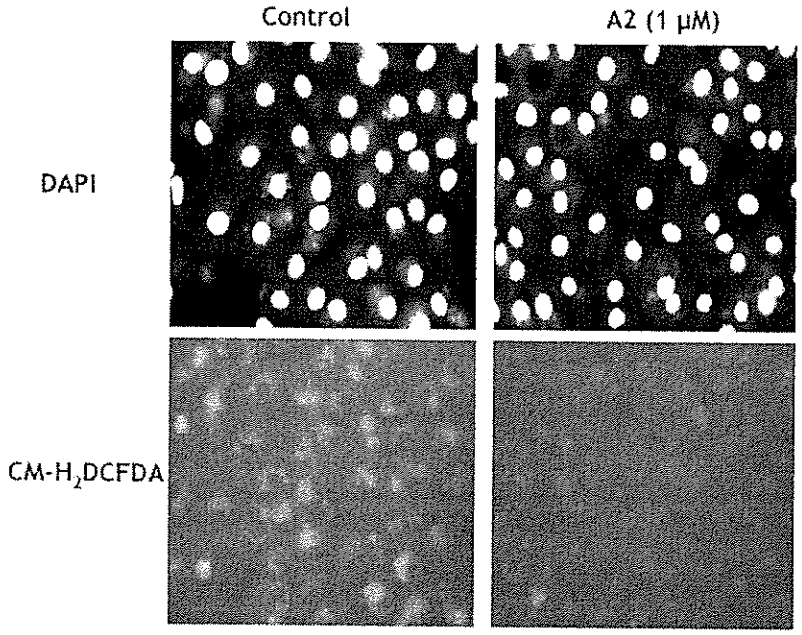
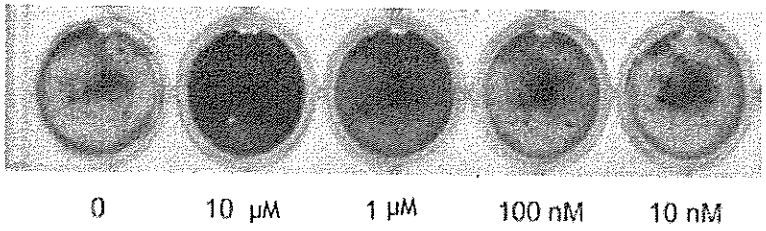


Figure 9: MDBK cells were grown in serum-free media for 5 days in the absence or presence of A2 , and then stained with CM-H_2DCFFDA to detect cellular oxidative stress. DAPI, 4',6-diamidino-2-phenylindole, is a fluorescent stain that binds strongly to A-T rich regions in DNA. DAPI was used here to show that cells are present on slides. DAPI labels nuclear regions.

A2 improved mitochondrial membrane potential compared to untreated controls, as evidenced by the brighter MitoTracker staining (Figure 8). CM-H_2DCFFDA staining was brighter in control preparations compared to those treated with A2 (Figure 9), indicative of antioxidative activity of A2.

a.



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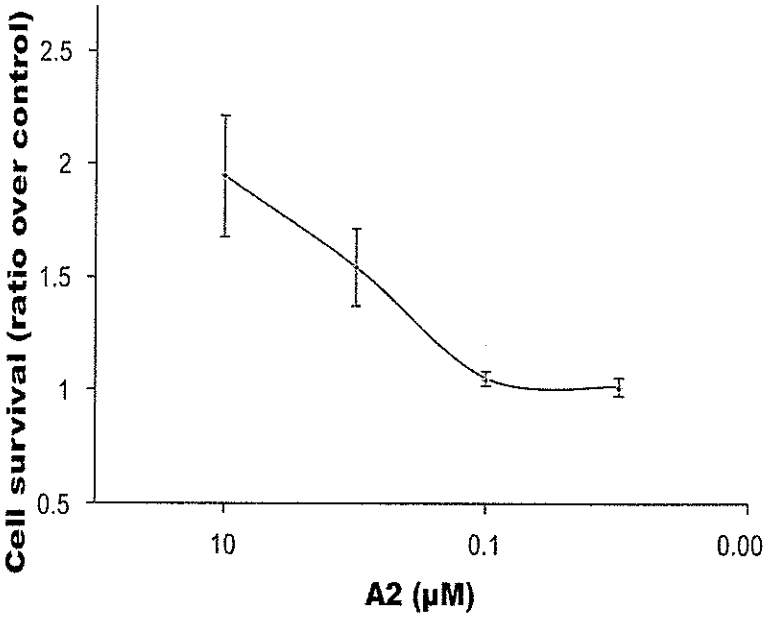

Figure 10: Cells were grown for 5 – 7 days resulting in monolayer disruption, cell detachment, and death, which was easily detected by weak methylene blue. In the presence of A2, cells remained attached and cell monolayer appeared to be intact, as demonstrated by strong methylene blue staining. (a) Samples were analyzed and counted as described in the methodology. Error bars indicate standard error for each specified A2 concentration (n=5). (b)

A2 promotes cell survival as shown in Figure 10a through stringer staining. Figure 10b shows that as the concentration of A2 increases, cell survival increases as well. The data analysis in this figure, indicated a dose-dependent response of A2, with EC_{50} of about 100 nM.

Discussion

Our experimental results support emerging evidence that our peptidyl-like compound, A2, has the ability to increase mitochondrial function, and in doing so decrease the effects of mitochondrial dysfunction. It is worth mentioning that A2 reduces oxidative stress on its own. This is in contrast to the antioxidant component in SS-31 due to the dimethyltyrosine in its chemical motif. [8] Since A2 has no ROS scavenging properties, there is an alternate mechanism by which it reduces ROS production in the IMM.

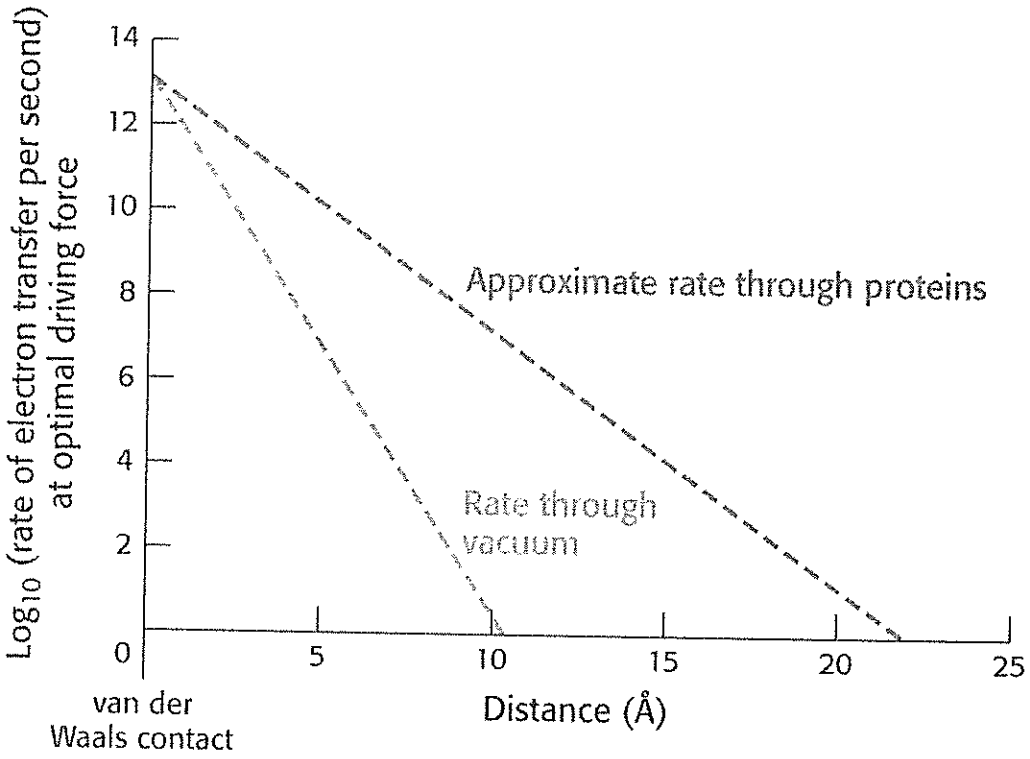

Figure 11: The rate of electron transfer decreases as the electron donor and the electron acceptor move apart. In a vacuum, the rate decreases by a factor of 10 for every increase of 0.8 Å. In proteins, the rate decreases more gradually, by a factor of 10 for every increase of 1.7 Å. This rate is only approximate because variations in the structure of the intervening protein medium can affect the rate.

It is possible that A2 has the ability to increase electron transport rate, and this is what causes the increased conductivity in the ETC. Electrons have the ability to move through space, but as the distance between the electron donor and electron acceptor increases, the rate of electron transfer rapidly decreases (Figure 11). By traveling through proteins, electrons have the potential to travel a farther distance in less time, thus increasing the rate of transfer. I am proposing that A2 allows for an alternate pathway for electrons which provides for a faster rate. Alternatively, A2 may increase the distance which electrons can travel with given energy.

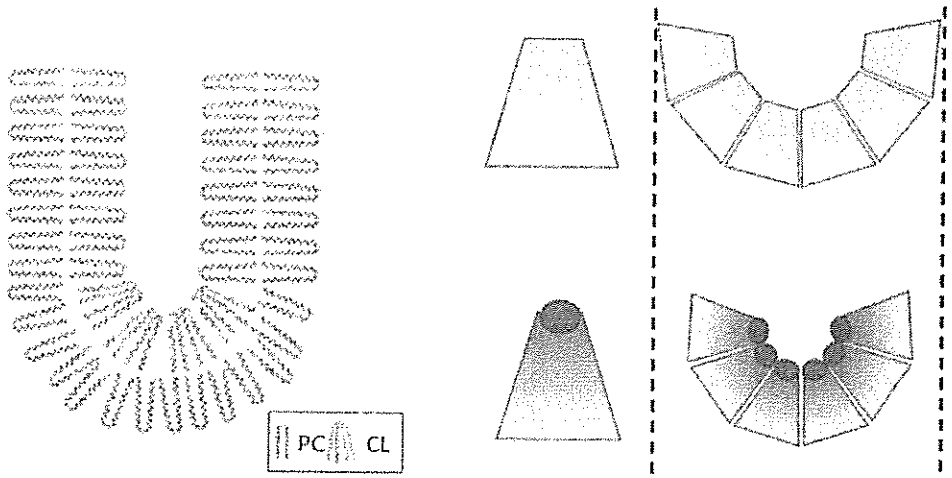

Figure 12: Cardiolipin aids in the formation of mitochondrial cristae. Red circles represent A2, as it allows for a tighter curvature in the IMM. (Source 11)

A2 may be acting in a similar manner to previous studies of SS-31 where the peptide simply maintains the architecture of the mitochondrial cristae. The extensive folding of the IMM increases the total surface area in which oxidative phosphorylation can occur, which optimally drives ATP synthesis. [14] Thus, A2 could be increasing mitochondrial potential through an increase in surface area of the cristae which would provide more surface area for chemical interactions to occur across the IMM.

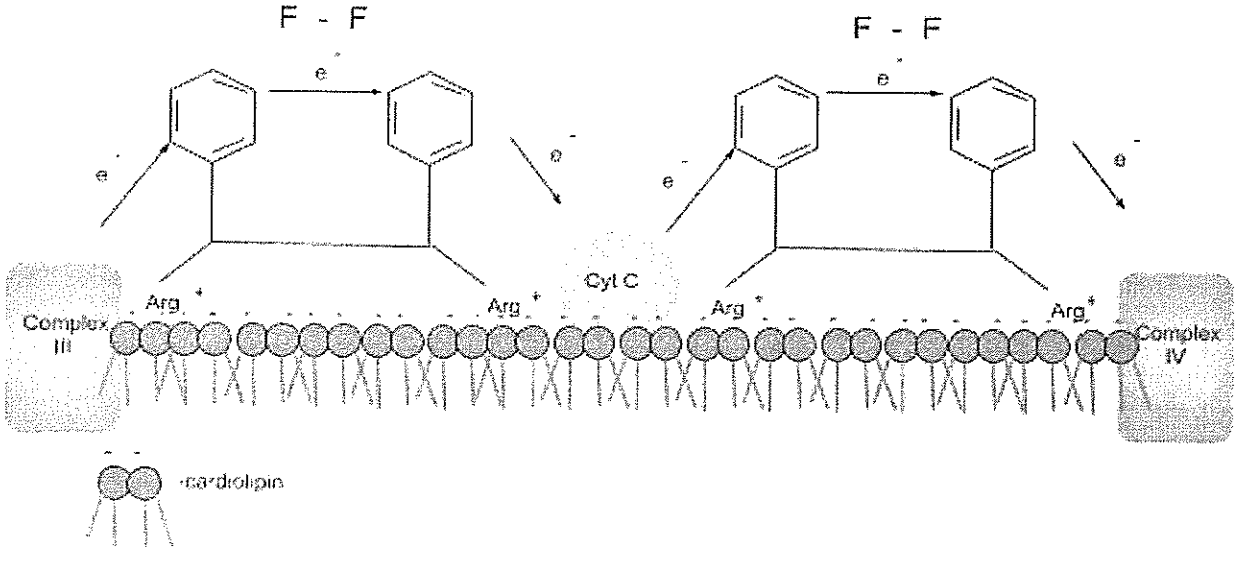

Figure 13: Diagram of how A2 may be working to provide alternative pathway for electron flow across IMM. This model shows the flow of electrons between complex I and complex II of the ETC, but this interaction could occur between any two complexes within the ETC. (Source: student generated)

An alternative model that could demonstrate how A2 aids in mitochondrial dysfunction is through a pathway that the FF aromatic rings may provide. In contrast to the interaction between SS-31 and CL as shown in Figure 4, this model suggests that Arg rests above the CL, and that FF serves as an electrical conduit. While SS-31 only has the ability to target CL, A2 can organize CL. This may allow for increased function of the ETC by allowing for more curvature in the cristae of the IMM. Figure 13 shows the difference in the proposed interaction of A2 with CL when compared to the interaction between SS-31 and CL in figure 4 .

Conclusion and Future Work

The data suggests that A2 promotes cell survival in a serum starvation model. The mechanisms by which this occurs are still unclear, but work is being done to further investigate this process. A2 is able to enter the cell and target the mitochondria. Data also suggests that A2 increases mitochondrial potential across the IMM, possibly even causing hyperpolarization. Additionally, data suggests that A2 reduces the oxidative stress which occurs in the mitochondria of cells during starvation. Future tests could be conducted to observe the effect that A2 has on mitochondrial potential and ROS levels in a model which induces cell death through chemical means. Such a test could be completed through the use of FCCP which is a potent uncoupler of mitochondrial oxidative phosphorylation. FCCP disrupts ATP synthesis by transporting protons across the IMM, which depolarizes mitochondrial membrane potential, dissociating ATP synthase from the ETC. [15] Since our data suggests that A2 can increase membrane potential, it is reasonable to suggest that A2 could reverse the effects of FCCP on the mitochondria.

Future research could be done to observe the effect that A2 has on β-actin within the cells. β-actin is the building block of the cytoplasmic cytoskeleton and plays a crucial role in cell motility, division, and gene expression. [16] However, previous studies have shown that during serum starvation, β-actin levels increase within cells in order to prepare for apoptosis, and

β-actin deficient cells have been reported to be resistant to certain cell death induction. [17] Thus, it would be significant to study if A2 has any effect on β-actin levels within the cells during serum starvation. Furthermore, actin moves to perinuclear space during serum starvation where it constricts the nucleus, causing the nuclear membrane to rupture. [18] Actin cytoskeleton also interacts with the mitochondria which creates a stress pathway of a loss of transmembrane potential and release of pro-apoptotic factors into the cytoplasm. [19] It is worth investigating if the location of actin in cells treated with A2 is different than those in serum starvation conditions since our data suggests that A2 reduces cell death in the starvation model.

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