THE MITOCHONDRIAL EFFECTS OF NELFINAVIR IN HUMAN BRAIN
MICRO VASCULAR ENDOTHELIAL CELLS

A THESIS
SUBMITTED ON THE EIGHTH DAY OF MAY 2017
TO THE PROGRAM OF NEUROSCIENCE
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
OF THE SCHOOL OF SCIENCE AND ENGINEERING
OF TULANE UNIVERSITY
FOR THE DEGREE OF
MASTER OF SCIENCE IN NEUROSCIENCE

BY

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ABSTRACT

Objectives. Human Immunodeficiency Virus (HIV) infects immune cells and lowers cell-mediated immunity leading to acquired immune deficiency syndrome or AIDS. The virus causes damage/dysfunction of helper T cells, macrophages, and dendritic cells. One of the long-term complications of untreated AIDS is severe cognitive impairment caused by HIV-associated dementia (HAD). Highly Active Antiretroviral therapy, the HAART regimen, which inhibits virus replication has been shown to reduce the incidence of HAD. HAART includes several mechanistically diverse classes of drugs such as protease inhibitors, nucleoside inhibitors of reverse transcriptase, and non-nucleoside reverse transcriptase inhibitors. The protease inhibitor, nelfinavir, is used in HIV therapy in order to mitigate the effects of the HIV virus by breaking down HIV-1 and HIV-2 proteases, which are essential to the replication of the virus within the host cell. HAART has decreased the incidence of HAD yet milder cognitive dysfunction, considered to be a consequence of anti-HIV drug toxicity, often manifests. Previous studies have shown that protease inhibitors may play a role in causing oxidative stress in endothelial cells. The present study involves understanding the effect of nelfinavir on mitochondrial oxidative stress and its role in the injury to human brain microvascular endothelial cells that form the blood-brain barrier.

Methods and Results. Our studies utilized primary human brain microvascular endothelial cells (hBMECs). We performed measurements of mitochondrial
superoxide levels (ESR Spectroscopy), oxygen consumption rates or OCR (Seahorse XF-Extracellular Flux Analyzer and Mito Stress Test Assay), cell viability/proliferation (CCK8 based Cellular Viability Assay). Sub-therapeutic doses of nelfinavir (1 µmol/L) increased the cell proliferation whereas therapeutic (3-5 µmol/L) and supra-therapeutic (10 µmol/L) doses of nelfinavir reduced the cell viability. In addition, treatment with sub-therapeutic levels of nelfinavir has no effect on the levels of mitochondrial superoxide in hBMECs but therapeutic and supra-therapeutic levels of nelfinavir increased mitochondrial superoxide levels. Measurements of OCR showed that sub-therapeutic doses of nelfinavir enhanced the basal and maximal respiration in hBMECs. In contrast, therapeutic concentration of nelfinavir reduced ATP production and spare respiratory capacity although basal respiration, proton leak, and non-mitochondrial respiration were unchanged. However, supra-therapeutic dose of nelfinavir significantly reduced basal respiration, ATP production, and spare respiratory capacity accompanied by reduced non-mitochondrial respiration and proton leak.

Conclusions. We identified that nelfinavir treatment was associated with a decrease in cellular proliferation at therapeutic and supra-therapeutic levels. Furthermore, we identified an increase in mitochondrial superoxide species in cells treated with nelfinavir in concentrations beyond therapeutic levels which was accompanied by a decrease in basal respiration, ATP production, and mitochondrial spare capacity. These results are indicative of nelfinavir causing cellular cytotoxicity in BMECs that are likely mediated by mitochondrial oxidative stress and impaired mitochondrial respiration.
ACKNOWLEDGMENTS

I would like to extend my sincere gratitude to Dr. Katakam for mentoring me throughout my undergraduate and graduate studies at Tulane. I would also like to thank my colleagues in Dr. Prasad Katakam’s laboratory, Venkata N. Sure (Doctoral Student in Pharmacology), Nicholas R. Peterson (M.S. Pharmacology), and Graham Unis (M.S. Pharmacology) for their help throughout my thesis research work. This research work was supported by the Louisiana Board of Regents Support Fund-Research Competitiveness Subprogram (LEQSF-2014-17-RD-A-11), American Heart Association National Center Scientist Development Grant (14SDG20490359), and National Institute of Health RO1 Grant (National Institute of Neurological Disorders and Stroke and National Institute of General Medical Sciences: R01NS094834) awarded to Dr. Prasad V. Katakam.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>1 Background</td>
<td>1</td>
</tr>
<tr>
<td>1.1 HIV Associated Dementia</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Blood-Brain Barrier and Microvascular Endothelial Cells</td>
<td>3</td>
</tr>
<tr>
<td>1.3 Mitochondrial ETC and Oxidative Stress</td>
<td>6</td>
</tr>
<tr>
<td>2 Introduction</td>
<td>11</td>
</tr>
<tr>
<td>3 Methods and Materials</td>
<td>13</td>
</tr>
<tr>
<td>3.1 BMEC Cell Culture</td>
<td>13</td>
</tr>
<tr>
<td>3.2 Cellular Viability</td>
<td>14</td>
</tr>
<tr>
<td>3.3 ESR Spectroscopy</td>
<td>15</td>
</tr>
<tr>
<td>3.4 Mitochondrial Respiration</td>
<td>16</td>
</tr>
<tr>
<td>4 Results</td>
<td>20</td>
</tr>
<tr>
<td>5 Discussion</td>
<td>26</td>
</tr>
<tr>
<td>6 Limitations</td>
<td>31</td>
</tr>
<tr>
<td>7 References</td>
<td>32</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Figure 1: Transverse Section of BBB</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Figure 2: Electron Transport Chain and ROS Production</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>Figure 3: SOD Mechanism</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>Figure 4: BMEC Digital Phase Contrast Images</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Figure 5: Seahorse Mito Stress Test</td>
<td>18</td>
</tr>
<tr>
<td>6</td>
<td>Figure 6: Cell Viability</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>Figure 7: Mitochondrial Superoxide Production</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>Figure 8: Oxygen Consumption Rate with 10 µM Nelfinavir Treatment</td>
<td>23</td>
</tr>
<tr>
<td>9</td>
<td>Figure 9: Basal Respiration and ATP Production</td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>Figure 10: Spare Capacity and Non Mitochondrial Respiration</td>
<td>24</td>
</tr>
<tr>
<td>11</td>
<td>Figure 11: Maximal Respiration and Proton Leak</td>
<td>28</td>
</tr>
</tbody>
</table>
I. BACKGROUND

HIV Associated Dementia

The viral tropism of Human Immunodeficiency Virus (HIV) is seen in CD4+ T cells, macrophages, as well as microglial cells. One of the components of the HIV-associated dementia (HAD) is the HIV’s effect on microglia promoting neurotoxicity and causing cognitive decline. The histological pathology of HAD is characterized by monocytes and macrophages entering into the cellular nervous system (CNS), abnormalities of dendritic processing, glial toxicity, and degradation of myelin sheaths(1-3). The primary symptoms of HAD are motor dysfunction, speech problems, and behavioral change. HAD develops late in HIV-1 infection after severe deterioration of the immune system.

HAD progresses in 4 stages. Prior to stage 1, there is affliction associated with difficulty attending to simple tasks and loss of rudimentary conversational ability. These lapses will progress to a greater degree at stage 1 and multi staged tasks become very difficult. These early stages do not show motor dysfunction or severe behavioral change however speech problems slowly become evident. During stage 2 there is lack of fine motor control as well as noticeable impairment in mental and cognitive performance. At stages 3 and 4 abnormalities in motor function are evident as patients often lose balance. Simultaneously abnormalities in speech control and cognitive assessment are noticeable and often severe. Neuroimaging techniques, such as computerized tomography (CT) and magnetic resonance imaging (MRI) show widened cortical sulci as well as
enlarged ventricles (2). Additionally there is often deterioration of the white matter systems while less commonly the basal ganglia and thalamus (2). While these cannot conclusively determine one is infected with HAD they are evident of cerebral encephalopathy.

Highly Active Antiretroviral therapy, the HAART regimen, which inhibits virus replication has been shown to reduce the incidence of HAD. However, patients with AIDS receiving HAART still suffer from milder forms of cognitive disorders, which have been proposed to be due to drug-toxicity of members of HAART. Although the severity of HAD has decreased with the use of HAART, persistence of milder forms may indicate that one or multiple treatments may contribute to neurocognitive decline. Since the inclusion of protease inhibitors (PIs) in HAD treatment, rates of cognitive dysfunction have significantly improved. However, the use of PIs has also been associated with several metabolic disorders characterized by oxidative stress in endothelial cells (4-7). PIs function by selectively blocking viral replication by binding to viral proteases and inhibiting proper proteolytic cleavage, thus not allowing the production of a mature virion. They have been shown to induce lipodystrophy, insulin resistance syndrome and hyperlipidemia which are all related to neurocognitive decline (8). Antiretroviral drugs have also been shown to increase the production of reactive oxygen species (ROS) and cause cell damage (9). However, the underlying mechanisms for the deleterious actions of nelfinavir have not been characterized. Moreover, the characterization of nucleoside reverse transcriptase inhibitors and non-nucleotide reverse transcriptase on cognitive dysfunction has not been fully understood. It is understood that HIV enters the brain early on in infection primarily by disruption of the blood-brain barrier (BBB) and then passes
through these infected cells. Infected microglia further propagate the infection by causing cellular dysfunction and apoptosis. Thus, HAD is associated with the immune activation of macrophages and microglia which secrete toxins into cerebral tissue thus inducing metabolic encephalopathy.

**Blood-Brain Barrier (BBB) and Brain Microvascular Endothelial Cells**

Blood delivers oxygen and nutrients to all tissues of the body. The BBB is composed of microvasculature that supplies these nutrients to the brain. These brain microvessels are specialized to restrict permeability of various ions and molecules that easily diffuse across endothelial cells in other vascular beds. This BBB separates the circulating blood from the brain extracellular fluid (BECF) and is critical in protecting the brain from toxic substances. Precise control of this movement is essential to CNS homeostasis and disruption of the BBB leads to pathological states.

The microvasculature is surrounded by two different basement membranes (BM), the inner vascular BM and the outer parenchymal BM. These BMs are important for providing an anchor for many signaling processes while also providing an additional barrier for substrates to cross before reaching neural tissue. The protective nature of the barrier is due to the physical and metabolic properties of brain microvascular endothelial cells (BMECs), which have unique properties (10,11) when compared to peripheral endothelial cells (ECs). BMECs contain protein complexes that restrict movement of solutes between the blood and brain. Pericytes are contractile cells that surround the abluminal walls of the microvasculature and are embedded in the vascular BM. They contain contractile proteins which contract in order to control the diameter of the capillary (12). Astrocytes surround the BBB, and are a major glial cell that extends
polarized cellular processes called ‘foot processes’. These processes almost completely surround the vascular tube. They provide a cellular link between neuronal circuitry and blood vessels which allow them to relay signals to regulate blood flow in response to neuronal activity (13). This includes regulating the contraction and dilation of pericytes and smooth muscle cells that surround the vessels.

The BMECs are connected by tight junctions (TJ) and adherens junctions (AJ) (2). The TJs are composed of smaller subunit proteins such as claudins and occludins. Claudins are important for limiting paracellular ion movement with high selectivity. Occludins are important in tight junction stability and maintenance of the barrier properties of the tight junctions (2).

These TJs are present throughout the endothelial barrier preventing the paracellular diffusion. Under normal conditions, most solutes cannot cross this barrier unless there are selective transporters present. AJs are important for cell-cell crosstalk, utilizing the actin cytoskeleton of adjacent cells to initiate and maintain direct contact. They are composed of Junctional Adhesion Molecules (JAMs), Ve-cadherins and catenin proteins. JAMs are important for localizing cell to cell contact, establishing cell polarity, and play a role in initiating tight junction formation (2). The Ve-cadherins control the cohesion of intercellular junctions. Catenin proteins linking the Ve-cadherins to the actin cytoskeleton are important for Ve-cadherin cell adhesion properties.

In addition to the junctional complexes that form the BBB, the cytoskeleton of brain endothelial cells play an important role in maintaining inter-endothelial junctional stability (2). The cytoskeleton is composed of actin filaments, microtubules and intermediate filaments. The actin filaments interact regularly with cadherin and occluding
proteins and promote membrane adhesion. The microtubule system is essential for the rapid assembly of actin filaments. It is composed of polymers of α-tubulin and β-tubulin; and assembled through the formation of rigid hollow rods from the nucleus to the periphery. Intermediate filaments play a role in the reorganization of the actin and microtubule complexes (2).

**Figure 1: Schematic of Transverse Section of Blood-Brain Barrier.** BMECs shown with components of TJs and AJs that restrict solute flow across endothelium. TJs composed of actin, occluding and claudins. AJs composed of JAMs, catenin, and cadherins. Diffusion across the barrier occurs by substrate specific receptors such as GLUT-1 for glucose, P-GP for the efflux of waste, and AQP-4 for modulation of water movement. Physiological regulation of the permeability occurs in both astrocytes and endothelium through various factors such as VEGF, bFGF, GDNF, and TGFβ. (Figure courtesy of Popescu et al, J Neurol Soc 2009)

The junctional complex and cytoskeleton of brain endothelial cells contribute to BBB functionality under normal physiological conditions. However, in a disease state the integrity of this barrier is disrupted. At the cellular level, this is largely due to the
formation of inter-endothelial gaps due to disruption of the TJ and AJ adhesive properties as well as changes in the organization of the actin cytoskeleton. Disruption within the brain endothelial cells causes dysfunction in cellular metabolism and signaling. BMECs have very low rates of transcellular and paracellular diffusion due to these protein complexes.

This restrictive nature allows for movement across the endothelium to be regulated by cellular transport proteins. The two categories of transporters are efflux transporters and nutrient transporters. Efflux transporters are polarized to the surface of the lumen and transport lipophilic molecules. Nutrient transporters facilitate the movement of specific nutrients from the BBB to the CNS, and also remove specific waste products. Most of these transport processes require energy expenditure and therefore BMECs are thought to contain more mitochondria than other ECs (14).

While nelfinavir has been shown to increase oxidative stress in vasculature, the implications have not been fully understood in BMECs. Because of the protection offered by the BBB, little nelfinavir is initially able to cross into brain tissue. However, damage to the BBB caused by the cytotoxicity of nelfinavir will expose the neuronal tissue to nelfinavir thus impacting the cognitive function. In addition, BBB opening under disease conditions will damage the brain parenchyma through recruitment of leukocytes and edema from vasculature (2,15).

**Mitochondrial Electron Transport Chain and Oxidative Stress**

Mitochondria power the many active processes in BMECs that are essential to maintain the BBB function. The Mitochondrial electron transport chain (ETC), the critical component of functioning mitochondria, consists of four protein complexes and
spans across the inner mitochondrial membrane. It accepts electrons from reducing equivalents NADH and FADH₂. NADH donates electrons at Complex I and FADH₂ donates electrons at Complex II. These electrons pass through the complexes of the ETC and ultimately reduce O₂ to H₂O. Complexes I, III, and IV allow for protons to be pumped from the mitochondrial matrix into the inner mitochondrial membrane, and this generates a proton motive force that is fundamental to ATP production.

The major source of ROS from the ETC comes from complexes I and III (16,17). Complex I is the initial entry point for electrons from NADH. In states of high NADH/NAD⁺ ratios these electrons will be passed from Complex I to O₂ to form superoxide (18). Additionally, when succinate levels are high while NADH/NAD⁺ is low, electrons coming from FADH₂ will backflow to complex I and produce superoxide (19,20). Complex III also contributes to mitochondrial ROS through inhibition of the Q cycle, where electrons are passed between ubiquinol and cytochrome C (21,22). This can generate superoxide in both the matrix and inner mitochondrial membrane based on whether uncoupling occurs at the matrix or inter-membrane space (23,24).

Oxidative stress is a condition under which cells are compromised due to either an excess generation of reactive oxygen species (ROS) or an inability to degrade the ROS. The normal redox states of cells is disrupted due to the excessive reactive free radicals damaging the cellular components (3). Exposure to the nelfinavir drug has been shown to cause oxidative stress in many cell types including endothelial cells (1). At the level of mitochondria, oxidative stress has been shown to occur when electrons in the electron transport chain prematurely reduce oxygen to ROS such as superoxide that promotes cytotoxicity if not properly scavenged by the endogenous ROS scavengers. The first line
of defense against oxidative damage are superoxide dismutases (SOD), the enzymes that catalyze the “dismutation” of superoxide to hydrogen peroxide (H$_2$O$_2$) (3). Disruption of this antioxidant capacity has been implicated in many neurodegenerative diseases (25). The H$_2$O$_2$ formed by the SODs is further degraded by peroxidase and catalase to water and oxygen.

**Figure 2: Electron transport chain and ROS production.** Electrons from NADH are donated to complex I and electrons from FADH$_2$ are donated to Complex II to ultimately reduce O$_2$ to H$_2$O. Local environmental conditions can compromise this process allowing for premature reduction of O$_2$ to O$_2^-$ This can occur at Complex I and Complex III. The
O$_2^-$ that does not escape the mitochondria is reduced to H$_2$O$_2$ by MnSOD in the matrix and CuZnSOD in the inter-membrane space (Figure courtesy of Gutterman et al, Antioxidants and Redox Signaling 2011)

![Superoxide Dismutase Diagram](image)

Figure 3. Conversion of O$_2^-$ to H$_2$O$_2$ and subsequent enzyme mediated catalysis to H$_2$O and O$_2$. (Figure courtesy of Kenneth Today, Textbook of Bacteriology 2011)

Measurement of ROS was difficult because of the short half-life of superoxide and also lack of specificity of detection systems. One approach to detect ROS was to add fluorescent dyes sensitive to oxidative radical species and determine the rate of oxidation. However, these dyes would have to compete with SOD, therefore only a small percentage could be detected. Additionally, the half-life of the radical species is very short. Furthermore, the fluorescent dyes were also not specific to superoxide. Our experiments involve tagging the anionic ROS with stabilizing “probes” that can be quantified using electron spin resonance (ESR) spectroscopy, also known as electron paramagnetic (EPR) spectroscopy (26). These probes are specific to superoxide and are sensitive to measure small concentrations of ROS produced in the cells.
Free radicals are harmful to cells because they react with amino acids of proteins, damage DNA, and oxidize polyunsaturated fatty acids in lipids causing cellular dysfunction (27,28). Therapeutic plasma nelfinavir concentrations are usually 5 µmol/L and can go up to 7-8 µmol/L. The generation of ROS in the mitochondria has been considered one of primary mechanisms leading to cytotoxicity and leading to the arrest of the cell cycle at the G1 phase (29). Interestingly, sub-therapeutic doses of nelfinavir had a protective effect against mitochondrial induced apoptosis in oxygen-glucose deprivation studies (30). The mechanism of these protective effects has been attributed to mcl-1, an anti-apoptic mitochondrial membrane protein as well as enhanced ERK1/2 phosphorylation (29).
II. INTRODUCTION

In this study, we examined the effects of Nelfinavir on the mitochondrial function to understand how cellular bioenergetics are altered in BMECs. Mitochondrial dysfunction is an important instigator of cytotoxicity. By analyzing mitochondrial oxygen consumption rates we can determine the cellular metabolism and the potential role it plays in cytoprotective / cytotoxic signaling in BMECs. Previous studies by Dr. Mondal have found that nelfinavir induces oxidative stress in human brain microvascular endothelial cells (1). Previous studies also showed that the recruitment of inflammatory cytokines to these damaged regions caused an increase in cell adhesion molecules (CAMs), which tightly bind the circulating monocytes as well as the endothelium. Secretion of a transcription factor by nelfinavir promotes adhesion of the integrins expressed on the monocytes to adhere to the endothelial cells and promote transendothelial migration (1). Grigorian et al. showed that exposure to nelfinavir significantly increased ROS within cerebral endothelial cells (5). These studies have shown decreased Notch4 expression, which is a vital protein used in cellular signaling. They have also reported that Nelfinavir impairs the Notch signaling pathway, which promotes neuronal function and development. Studies have shown that that antiretroviral therapy has been associated with significant side effects such as heart disease, diabetes, and cardiovascular complications due to an increase in mitochondrial oxidative stress (31) through production of superoxide by NADPH oxidases (32). However, the exact mechanisms underlying the nelfinavir-induced mitochondrial dysfunction in BBB
endothelial cells have not been examined to date. Cerebrovascular dysfunction has been implicated in the decline in the cognitive function and dementias. Thus, understanding the mechanisms of nelfinavir-induced endothelial injury is essential to prevent cognitive disorders in patients with HIV infection. We hypothesize that nelfinavir induced impairments of mitochondrial respiration and mitochondrial oxidative stress mediate the cellular injury in brain microvascular endothelial cells.
III. METHODS AND MATERIALS

Primary Human Brain Microvascular Endothelial Cell Culture

Primary human brain microvascular endothelial cells (ACBRI 376, passage 3) were purchased from Cell Systems Corporation (CSC). The hBMECs were transferred into flask containing 10 ml complete CSC media. Cells were passaged at a ratio of 1:3 when 90-95% confluent. Early passaged cells were stored periodically in nitrogen tanks (Taylor Wharton, Minnentonna, MN, USA) using freezing media. All cell culture methods are performed in a SterilGARD III Advance fume hood (The Baker Company, Sanford, ME, USA) at room temperature (21°C).

These cells tested negative for HIV Serologic Test, HIV PCR Test, and Test of frozen cells for Mycoplasma spp. They tested greater than 95% positive by immunofluorescence for the Cytoplasmic VWF/Factor VIII Test and Cytoplasmic uptake of Di-I-AC-LDL Test. These cells demonstrate specific markers of differentiation such as interdigitated cell contact, desmosomes and ZO-1 protein epitopes. These cells are used extensively in studying AIDS-related BBB Dynamics (Cell Systems™).

Passaging cells from flask to dishes and plates (P9 to P10).

Passage Reagent Group (PRG) system was purchased from Cell Systems (4Z0-800) and was aliquoted and kept frozen at -20°C. The PRG reagents were thawed in a 37°C water bath. PRG-1 and PRG-2 were kept at 37°C until use. PRG-3 was kept on ice.

Cells were passaged when they reached 90-95% confluence. Medium was decanted from the flask into a waste beaker. Cells were washed with PRG-1. PRG-2 was
immediately added to the flask. After 30 seconds, the PRG-2 was removed from the flask and transferred to 50ml tube on ice. After 90 seconds, PRG-3 was added to take out cells from the flask. Cell suspension was centrifuged at 500 g for 5 minutes at 4°C. After centrifugation, supernatant was discarded and cell pellet was resuspended in desired volume of CSC complete media and plated on to dishes, 96-well plates or seahorse micro culture plates coated with attachment factor. Cells were counted using a Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA, USA) before plating onto 96-well plate or seahorse micro culture plate.

**Figure 4.** (Left) 40X Digital Phase Contrast Image of BMECs at Passage 5 (Right) 6300X Digital Immunofluorescence after staining with ZO-1 monoclonal antibody at Passage 5 (Information taken from website of Cell Systems™)

**Cell Viability.**

Cell Viability was measured using Cell Counting Kit-8 (CCK-8) purchased from Dojindo Molecular Technologies, INC. CCK-8 assay is a sensitive colorimetric assay for the determination of the number of viable cells in cell proliferation and cytotoxicity.
assays. CCK-8 has WST-8 \([2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]\) which produces cell media soluble orange colored formazan product in presence of dehydrogenases in viable cells. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells. The detection sensitivity of CCK-8 is higher than the other tetrazolium salts such as MTT, XTT, MTS or WST-1.

hBMECs were plated on 96-well plates \((10000 \text{ cells/well})\) coated with attachment factor. Cells were pre-incubated for 24 hours in a humidified incubator (e.g., at 37°C, 5% CO\(_2\)) before 24 hour nelfinavir drug treatment \((1.0, 3, 5 \text{ and } 10 \mu\text{mol/L})\). Later, 10 μl of CCK-8 solution was added to each well without introducing the bubbles, since they interfere with the O.D. reading. Cells were incubated for 3 hours in the CO\(_2\) incubator at 37°C with CCK-8 solution. Absorbance was measured at 450 nm and 600 nm using a FLUOStar Optima Plate reader at 37°C. Background absorbance at 600 nm was subtracted from absorbance at 450 nm. The relative cell viability (%) was expressed as a percentage relative to the untreated control cells. The number of experiments for each treatment group was counted based on the average absorbance values of 24 wells containing hBMECs.

**ESR Spectroscopy**

Mitochondrial superoxide production in hBMVEC\(s\) was measured utilizing ESR spectroscopy using the spin probe mito-TEMPO-H \((1\text{-hydroxy-4-[2-triphenylphosphonio)-acetamido]-2,2,6,6-tetramethylpiperidine, 1-Hydroxy-2,2,6,6-tetramethyl-4-[2-(triphenylphosphonio) acetamido] piperidinium dichloride, 50 \mu\text{mol/L}}.\) Spin probe mTH \((\text{mito-TEMPOH, 50 } \mu\text{mol/L})\) and DTPA\((\text{diethylenetriaminepentaacetic}}\)
acid, 0.1 mmol/L) were dissolved in modified Krebs-Hepes (KH) buffer containing (in mmol/l) 99.01 NaCl, 4.69 KCl, 1.87 CaCl2, 1.20 MgSO4, 25 NaHCO3, 1.03 K2HPO4, 20 Na-HEPES, and 11.1 D-glucose, under nitrogen gas bubbling at pH 7.35.

Cells were plated on attachment factor coated 60 mm dishes. After cells reached 90-95% confluency, they were treated with different concentrations of nelfinavir (1, 3, 5 and 10 µmol/L) for 24 hours. Briefly, cells were washed with calcium and magnesium free PBS for 2 times after drug treatment. Later, cells were incubated with KH buffer containing spin probe and DTPA for 2 hour at 37°C. The needle end of 1-ml syringe barrel was cut and the plunger was retracted from the sliced end. Afterwards, cells were scrapped in mito-TEMPOH containing KH and transferred into 1 ml syringes before freezing them in liquid nitrogen. To take measurements, the syringe was gently warmed between the palms of the hands and the plunger pushed the frozen contents into a finger Dewar (Noxygen Science Transfer & Diagnostics, Elzach, Germany) containing liquid nitrogen. Samples were measured using Bruker Bio Spin spectrometer. Afterwards, the finger Dewar was placed into the ESR spectrometer. Mitochondrial ROS was determined using the following ESR settings: center field, 1.99 g; microwave power, 20 mW; modulation amplitude, 2 G; sweep time, 10 s; number of scans, 10; field sweep, 60 G. The amplitude measurements of the ESR spectra were normalized to protein concentrations. The number of experiments for each treatment group was counted based on the number of 10 cm cell culture plates containing hBMECs.

Mitochondrial Respiration.

The Seahorse Bioscience XFe24 extracellular flux analyzer was used to measure the oxygen consumption rate (OCR) as an indicator of mitochondrial respiration.
Seahorse XF24 cell culture microplates were coated with attachment factor. On the first day, hBMECs were seeded (80,000 cells per well) in cell culture microplates and placed in the CO₂ incubator (5% CO₂, 37°C). Second day, cells were treated with different concentration of Nelfinavir (1.0, 3, 5 and 10 µmol/L) or vehicle (DMSO) for 24 hours. On the day of assay, cells were washed with 500 µl of XF assay media (glucose 25 mmol/L, glutamate 4 mmol/L, pyruvate 10mmol/L, pH 7.4) before keeping the cells in non-CO₂ incubator for 20 minutes with 525 µl of assay media. Oligomycin, FCCP and Rotenone/antimycin stock solutions were prepared according to manufacturer instructions using XF assay media. Drug solutions were loaded in the sensor cartridge of seahorse utility plate that was hydrated for 24 hours with XF calibrant in non-CO₂ incubator. After calibration, microculture plate with cells were inserted in the seahorse XF24 flux analyzer to measure oxygen consumption rate (OCR) at different time intervals. The assay protocols consisted of three cycles of baseline measurements followed by three cycles for each treatment. Basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and nonmitochondrial respiration were measured by sequential injections of respiratory modulators (Fig. 5). These respiratory modulators target components of the electron transport chain (ETC) in the mitochondria to reveal key parameters of metabolic function. Oligomycin (1 µmol/L) inhibits ATP synthase (complex V), and the decrease in OCR following injection of oligomycin correlates to the mitochondrial respiration associated with cellular ATP production. Carbonyl cyanide-4(trifluoromethoxy) phenylhydrazone (FCCP) (1.5 µmol/L) is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential. As a result, electron flow through the ETC is uninhibited, and oxygen is maximally consumed.
by complex IV. The FCCP-stimulated OCR can then be used to calculate spare respiratory capacity, defined as the difference between maximal respiration and basal respiration. The third injection was a mix of rotenone (0.5 µmol/L), a complex I inhibitor, and antimycin A (0.5 µmol/L), a complex III inhibitor. This combination shuts down mitochondrial respiration and enables the calculation of nonmitochondrial respiration driven by processes outside the mitochondria. OCR data was normalized to protein concentration as determined by the Pierce BCA Protein Assay Kit.

Figure 5. Oxygen consumption rate for vascular endothelial cells during Seahorse Extracellular Flux Assay. The treatments are added sequentially (Note: all parameters given are based on the subtraction of non-mitochondrial respiration rates).

1. Oligomycin binds to the Fo protein of ATP synthase and inhibits oxidative
phosphorylation. The difference between OCR before oligomycin treatment and after oligomycin treatment accounts for mitochondrial oxygen usage that is linked to ATP production.

2. FCCP is an uncoupling agent, which acts as an ionophore and disrupts the hydrogen ion protein gradient. This further electron flow through the electron transport chain and the mitochondria attempt to adapt this ATP lack by maximally increasing electron flow. After addition of FCCP we can measure the maximal respiration rate. Moreover, the difference between the maximal respiration rate and basal respiration rate gives us spare capacity which as indication of the cells bioenergetic endurance. Cells with a higher spare capacity can sustain cytotoxic environments better than those with a lower spare capacity.

3. Antimycin A inhibits electron flow from complex b to complex c1 and Rotenone inhibits electron flow from complex 1 to ubiquinone (CoQ). The combination of these drugs effectively completely inhibits electron flow and thus ATP linked mitochondrial respiration.

**Data analysis and statistics.**

Results were expressed as means SE; n indicates the number of independent measurements. Data were analyzed using unpaired t-test and one-way ANOVA with Tukey’s post hoc test. P 0.05 was considered statistically significant.
IV. RESULTS

Effect of nelfinavir on cell viability

We determined cellular viability following exposure to 1 μmol/L (n=8; p<0.05) nelfinavir versus 5 μmol/L (n=8; p<0.05) therapeutic and 10 μmol/L (n=4; p<0.05) supra-therapeutic dosages. Cells were exposed to treatment for 24 hours. Nelfinavir treatment on the hBMECs shows a dose dependent decrease in viability when exposed to therapeutic and supra-therapeutic dosage. However, there is an increase in viability in the sub-therapeutic dosage. This is referred to as a biphasic response because there are two distinct responses based on the concentration of the nelfinavir used (Figure 6).

Figure 6: Nelfinavir shows a biphasic dose dependent response in hBMECs. Nelfinavir drug treatment increased cell viability at lower concentration whereas at higher concentration decreased cell viability.
**Effect of nelfinavir on mitochondrial superoxide**

While the viability assay shows us the effect of nelfinavir on overall cellular proliferation and survival, it does not point to the specific metabolic processes affected by nelfinavir. Because nelfinavir is a protease inhibitor, it may downregulate protein function necessary for metabolic activity. Mitochondrial oxidative phosphorylation activity and free radical production are both indicators of the metabolic activity. In order to determine the effects of nelfinavir on mitochondrial oxidative stress hBMECs were treated with various concentrations of nelfinavir for 24 hours in the present of mitoTEMPO-H, a mitochondria specific spin probe for ROS. Afterwards mitochondrial superoxide measurements were made using ESR Spectroscopy.

**Figure 7. Nelfinavir shows an increase in mitochondrial superoxide production.**

Nelfinavir treatment dose dependently increased mitochondrial superoxide compared to vehicle DMSO.
We found that nelfinavir dose-dependently increases mitochondrial superoxide levels at 5 µmol/L (n=14; p<0.05) and at 10 µmol/L (n=14; p<0.05) nelfinavir compared to vehicle treatment (n=13). The dose dependent increases at 5 and 10 µmol/L show that nelfinavir has direct effect on mitochondrial ETC leading to excessive superoxide generation (Figure 7). We did not see a change in superoxide levels at 1 µmol/L (n=13; p<0.05) or 3 µmol/L (n=14; p<0.05).

Effect of nelfinavir on mitochondrial respiration

Nelfinavir treatment showed a biphasic dose dependent response on mitochondrial respiration. A typical representation of vehicle vs drug treatment (10 µmol/L) on mitochondrial respiration showed in figure 8. It gives information about the fundamental parameters of mitochondrial function like basal respiration, ATP turnover, proton leak, and maximal respiration, or spare respiratory capacity.

Basal respiration was significantly increased in cells treated with 1 µmol/L (n= 57 wells; p<0.05) sub therapeutic concentration whereas 10 µmol/L (n= 30 wells; p<0.05) concentration significantly decreased basal respiration showing biphasic response as shown in fig. 9A. ATP production was significantly decreased in cells treated with 3 (n= 15 wells; p<0.05), 5 (n= 42 wells; p<0.05) and 10 µmol/L compared to vehicular treatment (n= 81 wells; p<0.05) indicating that hBMVEC s have lost their ability to produce ATP to meet energetic needs of cells following higher concentration of drug treatment as shown in fig. 9B.
We found that cells treated with 5 and 10 µmol/L drug concentrations resulted a significant decrease in spare respiratory capacity compared to vehicle shows that cells lost their capability to respond to an energetic demand at higher drug concentrations fig10 A. It also shows cells were not respiring to their theoretical maximum.

Figure 8. Oxygen consumption rates in hBMVECs treated with vehicle-DMSO and nelfinavir (10 µM) treated hBMECs. Cells treated with 10 µM nelfinavir showed reduced oxygen consumption rate compared to vehicle DMSO.
Figure 9: A. Effect of nelfinavir on basal respiration and ATP production. Basal Respiration Rates in hBMVECs showed biphasic response with increased basal respiration at 1 µmol/L and reduced basal respiration at 10 µmol/L (A) Nelfinavir drug treatment dose dependently reduced ATP production in hMBVECs (B).

Figure 10. Effect of nelfinavir treatment on spare capacity and non-mitochondrial respiration. hBMECs treated with higher concentrations of nelfinavir showed reduced spare respiratory capacity (A) and non-mitochondrial respiration (B).
Nelfinavir treatment didn’t show any effect on non-mitochondrial respiration at 1, 3 and 5 µmol/L concentration, however, cells treated with 10 µmol/L concentration showed increased non-mitochondria respiration as shown in fig 10 B.

Nelfinavir treatment showed a biphasic dose dependent response on mitochondrial maximal respiration. Maximal respiration was increased 1 µmol/L drug treatment whereas cells treated with 3, 5 and 10 µmol/L concentration showed decreased maximal respiration as shown in fig 11 A. Proton leak wasn’t effected with nelfinavir drug treatment except at 3 µmol/L concentration where it reduced proton leak in cells as shown in fig 11 B.

**Figure 11.** A. Nelfinavir drug treatment’s effect on maximal respiration and proton leak. hBMECs treated with nelfinavir drugs showed reduced maximal respiration (A) and proton leak (B)
V. DISCUSSION

There are three major findings in this study. First, nelfinavir treatment was associated with a decrease in cellular viability/proliferation at therapeutic and supratherapeutic levels. Second, we identified an increase in the mitochondrial superoxide levels following the treatment with therapeutic levels of nelfinavir. Finally, we observed impaired mitochondrial function characterized by a decrease in basal respiration, ATP production, and spare capacity of mitochondria in response to therapeutic dose of nelfinavir treatment. The findings from our experiments provide evidence that nelfinavir causes cellular cytotoxicity in BMECs mediated by mitochondrial dysfunction and oxidative stress.

The objective of our study was to determine the effect of PIs on the BMECs in order to understand their potential adverse impact on BBB function. BMEC studies are important as they form the BBB and the bioenergetics, metabolism, and signaling processes of these cells play an important pathophysiological role in maintaining CNS homeostasis. The in-vitro findings in our reports have important consequences in the implication of the deleterious effects of PI based treatment associated with HAD. Furthermore, our findings identified the specific biochemical mechanisms associated with these cognitive defects.

Cell viability assay in nelfinavir treated BMECs showed a protective effect at subtherapeutic doses of nelfinavir treatment followed by a decrease in cellular proliferation.
at therapeutic doses at supra therapeutic doses of nelfinavir treatment. This implicates nelfinavir as a drug with potential cytotoxic effects. The protective effects seen at sub therapeutic levels could be the result of the cellular responses to low levels of cytotoxicity as a defense mechanism. This may be similar to the phenomenon often referred to as ‘pharmacological preconditioning’ where a sub-lethal dose of a cytotoxic substance prepares the cells to withstand a lethal injury. The decrease in cellular proliferation following nelfinavir treatment at higher doses confirmed that nelfinavir cytotoxic to BMECs and has the ability to compromise BBB leading to cognitive dysfunction. Findings from our studies are consistent with previous viability studies by Brandmann et al. (J Neurochem 2012) that made measurements of lactate dehydrogenase activity and found nelfinavir induced reduction of cellular viability. In addition, the mitochondrial superoxide measurements were indicative of cellular damage associated with nelfinavir treatment as well. Ranging from sub therapeutic to supra-therapeutic nelfinavir treatment there was an increase in mitochondrial ROS levels. ROS are associated with further cellular damage as free radicals cause abnormal redox reactions that interfere with basal cellular metabolism. Our studies were primarily focused on the mitochondria because of their central role in the cellular responses to injury. Previous research has shown that BMEC oxidative stress is involved in Asperger’s, ADHD, Alzheimer’s, atherosclerosis and depression (17,18). The main oxidants involved in oxidative stress are superoxide anion, hydrogen peroxide, hydroxyl radical, organic hydroperoxide and peroxynitrate. These species are formed in autoxidation reactions through different biochemical mechanisms and at increased levels will trigger cellular apoptosis. We believe that the cellular damage and lack of proliferation at therapeutic and
supra-therapeutic nelfinavir treatment is due to increased mitochondrial ROS levels. Furthermore, because mitochondria produce ROS, they themselves are often the targets of the damaging effects of ROS. Studies have implicated superoxide as having deleterious effects on mtDNA and mitochondrial ion channels, initiating cellular apoptosis(28).

The implementation of HAART has had a significant impact in improving the prognosis of patients diagnosed with HIV-1. This treatment has been implicated vascular dysfunction including hyperinsulemia, hyperlipidemia, and increased blood pressure as potential side effects (1,31). These side effects have been shown to enhance the risk for cardiovascular complications over time, and severely damage the endothelial cells. While there is a correlation between PI activity and vascular dysfunction, the specific deleterious actions have not been properly investigated. Increasing levels of oxidative stress will compromise mitochondrial respiration. The parameters studied in the Mito Stress test indicate a biphasic dose dependent response in overall mitochondrial respiration. At 1 µM nelfinavir, BMECs showed an increase in viability, basal respiration rates, ATP production rate, spare capacity, and FCCP response. Collectively these may be referred to as protective effects. The function of the protective effects is to increase cellular respiration in order to meet any additional demands for energy such as conditions like cell proliferation. Exposure to therapeutic as well as supra-therapeutic levels of nelfinavir reduced the basal respiration indicating oxidative stress. While the superoxide measurements by ESR spectroscopy indicate the production of ROS, the OCR measurements reflect on how nelfinavir affects the oxygen consumption and its specific usage in the cells. Therapeutic concentrations of nelfinavir (3 and 5 µmol/L) had no
effect on basal respiration and non-mitochondrial respiration, but reduced ATP production, maximal respiration, and spare respiratory capacity. This reduction of ATP production with unchanged basal respiration may indicate that nelfinavir promotes reduced efficiency of mitochondria which may possibly due to uncoupling of oxidative phosphorylation. Uncoupling of oxidative phosphorylation could be due to either activation of uncoupling proteins in the inner mitochondria or due to alteration of permeability of inner mitochondrial membrane leading to leak of protons into mitochondrial matrix and loss of proton motive force. However, measurements of proton leak indicated that nelfinavir has no effect on the proton leak but paradoxically reduced the proton leak at 3 µmol/L concentration. Thus, the mechanisms underlying the loss of efficiency of mitochondrial respiration in nelfinavir treated cells is unclear and needs further examination. The loss of efficiency in oxidative phosphorylation could account for reduced maximal respiration and spare respiratory capacity seen in nelfinavir treated BMECs at therapeutic concentrations. Alternatively, direct damage to mitochondrial biogenesis leading to reduced mitochondrial volume caused by excessive ROS generation may also cause reduced spare respiratory capacity in nelfinavir treated cells. Finally, supra-therapeutic concentrations of nelfinavir (10 µmol/L) showed the most dramatic damaging effects on mitochondrial respiration in BMECs involving greatest decrease in all metrics of mitochondrial respiration. At high concentrations, nelfinavir appears to cause damage to mitochondrial by multiple mechanisms including loss of efficiency of mitochondrial respiration, decreased ATP production, and reduced mitochondrial spare capacity. Paradoxically, proton leak and non-mitochondrial respiration appear to be reduced by nelfinavir the underlying mechanisms however remain unclear.
Thus, our experiments showed for the first time that nelfinavir has the ability to reduce the cellular survival and proliferation of BMECs via disruption of mitochondrial respiration and promotion of mitochondrial ROS generation. The effects of nelfinavir on BMECs could possibly underlie the breach of BBB that has direct implication for impairment in cognitive function in HIV infected patients receiving nelfinavir treatment. The future direction of these studies will focus on specific signaling pathways and proteins involved in PI induced changes in mitochondrial function. Furthermore, cytotoxicity studies need to be replicated in primary cultures of cortical neurons to identify similarities and differences in the mechanisms mediating the effects of nelfinavir and its potential to induced neuronal injury. Our studies were conducted in vitro however utilizing animal model in vivo with long term exposure to PIs could further demonstrate the role of PIs in BBB impairments and neurodegeneration.
VI. Limitations

Our data in this study was collected in endothelial cell lines *in vitro* after treatment with various drugs. The drug treatments ranged from 24 to 48 hours before cells were collected for experimentation. Because these studies were conducted *in vitro*, it is difficult to state whether the toxicity of nelfinavir would have the same effects *in vivo*, as they could be moderated by other factors throughout the body. Thus, the use of animal models in studying the effect of PIs on HAD will be useful in future studies.

Our mitochondrial respiration studies provided clear functional deficits in mitochondrial respiration in cells treated with nelfinavir. However, even with the state of the art Seahorse Analyzer technology, the sensitivity of detection of OCR in cells treated with high concentration of nelfinavir posed a problem with paradoxical reduction in non-mitochondrial respiration and proton leak. However, complementary approaches such as mitochondrial ROS measurements and viability assays help identify the technical challenges of OCR measurements.
REFERENCES


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