The Synergistic Effects of Protandim® Tri-Synergizer®

Objective: To evaluate the synergistic effects of Protandim® Nrf2 Synergizer®, Protandim® NRF1 Synergizer®, and Protandim® NAD Synergizer®.

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Reference: LifeVantage Corp., Lehi, Utah 84043, LV-43

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Introduction

Aging is a complex process driven by diverse changes in genetic, molecular, biochemical, and cellular events. This multifactorial process is ultimately characterized by a gradual decline in physiological functions, which ultimately compromises the ability of an organism to survive with increasing age.

There are currently many different theories as to why we age. However, no one theory can account for all the changes known to occur. Most likely, all of the different theories have an additive effect and contribute to the declines associated with aging.

To date, there are currently three theories of aging that have been the most extensively studied:

1. The oxidative stress theory of aging
2. The mitochondrial theory of aging
3. The sirtuin theory of aging

1. OXIDATIVE STRESS THEORY OF AGING AND THE NRF2 PATHWAY

The oxidative stress theory of aging states that as the body ages, it starts to accumulate free radicals and other oxidants. Normally, the body can neutralize these free radicals naturally to protect cellular integrity and health. But if left unchecked and free radicals continue to increase, this can have very serious consequences for the cell in the form of oxidative stress.

Oxidative stress can ultimately lead to oxidative damage by attacking and damaging essential biological structures of the cell, compromising cellular function. When enough cells are affected, this cumulative damage can impact tissues and organ systems. This might show up as reduced physical performance, higher inflammatory responses, or decreased metabolic efficiency. In a person, this could be felt as fatigue or lower energy levels, slower recovery after exercise or stress, visible signs of aging, and reduced resilience to everyday physical or environmental stressors.

Antioxidants are the cell's primary defense against free radicals and other oxidants. There are two major classes of antioxidants: 1) dietary antioxidants obtained through food and nutritional supplements and 2) endogenous antioxidants produced by the body.

It has long been debated which class of antioxidants is more important in human health. However, a recent review of the scientific literature led by the United States National Institutes of Health's National Center for Complementary and Integrative Health concluded that "rigorous trials of antioxidant supplements in large numbers of people have not found that high doses of antioxidant supplements prevent disease." Thus, much attention has shifted to the body's endogenous antioxidant and detoxification systems.

Endogenous antioxidants are antioxidants made by the body and are the primary line of defense against oxidative stress. In general, endogenous antioxidants either prevent oxidants from being formed or remove them. Endogenous antioxidants form a complex network of antioxidant metabolites and enzymes, and these networks work together, throughout the cell, to neutralize oxidants and protect important biological structures from oxidative damage.

These intertwined and highly integrated networks most frequently garner their reducing equivalents from cellular energy production that are passed throughout the network. One major advantage of these antioxidant systems is that they can pass energy back and forth, a process also known as a redox cycle. Whereas most dietary antioxidants can only be used once and destroyed, endogenous antioxidants can use the redox cycle to neutralize numerous oxidants because they are constantly being regenerated back to their 'active' or reduced state.

Endogenous antioxidants can also be upregulated in times of increased oxidative stress. The primary pathway responsible for upregulating endogenous antioxidants and other detoxification pathways is the NRF2 cellular signaling pathway. Under normal, or low oxidative stress, the NRF2 transcription factor is normally found anchored to another protein in the cell membrane called KEAP1 (Kelch-like ECH-Associated Protein 1). Under times of oxidative stress, oxidants and other electrophiles release NRF2 from KEAP1. NRF2 then translocates to the nucleus where it binds to its specific promoter sequence (Antioxidant Response Element - ARE) to activate its target antioxidant and other detoxification genes. (Figure 1)

With age, the activity of this NRF2 cellular signaling pathway has been shown to decrease—both in its ability to sense oxidative threats and upregulate its target genes. When this slows down, cellular damage accumulates and leads to signs of aging.

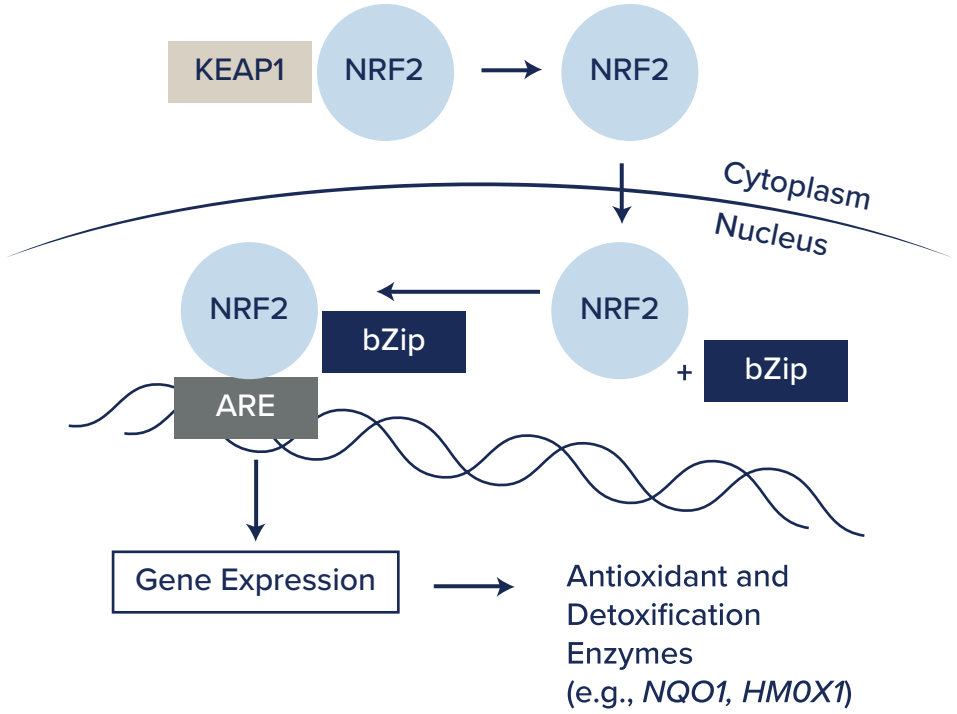

Figure 1. Activation of the NRF2-pathway under oxidative stress parameters.

2. MITOCHONDRIAL THEORY OF AGING AND THE NRF1 PATHWAY

Another leading theory suggests aging begins in the mitochondrion. Mitochondria, the power plants of the cell, are essential for cellular function. It is estimated that about 95% of the energy we need to function every second of every day is produced by mitochondria. The mitochondria produce energy (ATP) by breaking down the food we eat and capturing high-energy electrons in the process.

When mitochondria function properly, the energy released from the electrons powers ATP production. The mitochondria then attach these electrons to molecular oxygen that get detoxified to water. However, this process isn't 100% efficient. Even in young, healthy mitochondria, electrons can escape this process and potentially form free radicals and other oxidants.

The mitochondrial theory of aging states that as we age, our mitochondria become even less efficient. They produce less energy and more free radicals and other oxidants. Reduced energy production compromise's cellular function. And increased free radicals and other oxidants cause more damage to structures of the cell, including mitochondria. This leads to a vicious downward spiral of even less mitochondrial efficiency and an increase in the overall cellular burden of oxidative stress. It creates a negative feedback loop inside your cells, which can lead to persistent or increased fatigue, reduced stamina or endurance, slower recovery after exercise or stress, and feeling like energy levels decline more quickly over time.

A major cellular signaling pathway involved in mitochondrial health is NRF1/NFE2L1 (nuclear factor erythroid 2-related factor 1). The NRF1 cellular signaling pathway directly or indirectly regulates a number of genes involved in mitochondrial health, turnover, and biogenesis. These include another protein named NRF1 (Nuclear respiratory factor-1). NRF1 activates the expression of key genes involved in metabolism, cellular growth, energy production, and mitochondrial DNA transcription and replication.

Together with NRF2, NRF1 also provides the essential function of coordinating gene expression between nuclear and mitochondrial genomes. An additional protein shown to support mitochondrial health is PGC1-α (peroxisome proliferator-activated receptor gamma coactivator-1-alpha). PGC1-α is a transcriptional co-activator that regulates genes involved in energy metabolism and binds to NRF1 to amplify or boost its activity. It is the master regulator of mitochondrial biogenesis and turnover. (Figure 2)

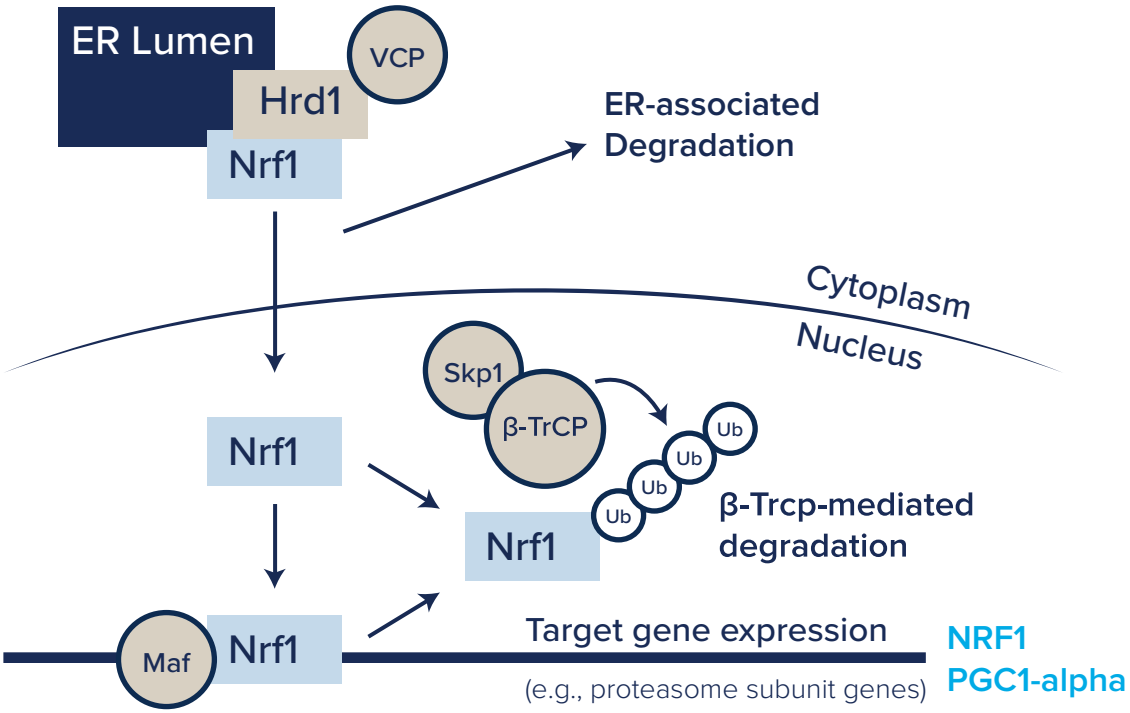

Figure 2. Activation of the NRF1 cellular signaling pathway.

3. SIRTUIN THEORY OF AGING AND THE NAD PATHWAY

Another major theory of aging is the sirtuin theory of aging. This theory came out of studies examining the health benefits of caloric restriction. Caloric restriction is the process whereby caloric intake is restricted by as much as 40-60%. In numerous experimental models, animals put on calorie-restricted diets experienced significant increases in maximum lifespan. It was determined that a family of proteins called “sirtuins” was absolutely required to experience the increase in lifespan brought on by caloric restriction.

As researchers began to understand the molecular biology of these sirtuins, they found that the enzymatic activity for most of them required the molecule NAD+ (nicotinamide adenine dinucleotide). NAD+ is an essential molecule for many biochemical reactions, most notably metabolism of food for energy.

NAD+ coexists in cells in two forms: 1) a molecule called NAD+ and 2) a molecule called NADH (reduced form of NAD+). It is the ratio of these two forms of NAD (the so-called NAD+/NADH ratio) that is important for controlling a number of other cellular signaling pathways.

Under normal conditions, the cell very tightly regulates the NAD+/NADH ratio. However, changes in your metabolism can impact this ratio. As the ratio of NAD+/NADH changes, NAD+ levels increase. This means there is proportionately more NAD+ floating around the cell than NADH. This is biologically important because an increase in NAD+ has been shown to activate several key enzymes, including sirtuins.

Cellular NAD+ is made in two ways through the NAD+ biosynthetic pathway: 1) the de novo and 2) the salvage pathways. In humans, de novo synthesis of NAD+ involves the amino acid, tryptophan. Quinolinic acid is generated directly from tryptophan. The quinolinic acid is then converted to nicotinic acid mononucleotide (NAMN) by the transfer of a phosphoribose moiety. An adenylate moiety is then transferred to form nicotinic acid adenine dinucleotide (NAAD). Finally, the nicotinic acid moiety in NAAD is amidated to nicotinamide (NAM), ultimately forming nicotinamide adenine dinucleotide (NAD).

The salvage pathway in humans involves recycling individual components of NAD+ containing a pyridine base. The three vitamin precursors used in these salvage metabolic pathways are nicotinic acid (NA), nicotinamide (NAM), and nicotinamide riboside (NR). These compounds can be taken up from the diet and/or produced within cells and by digestion of cellular NAD+ and are termed vitamin B3 (niacin) or NR. These precursors can take up extracellular NAD+ from their surroundings, and both NAM and NR can be absorbed from the gut. (Figure 3)

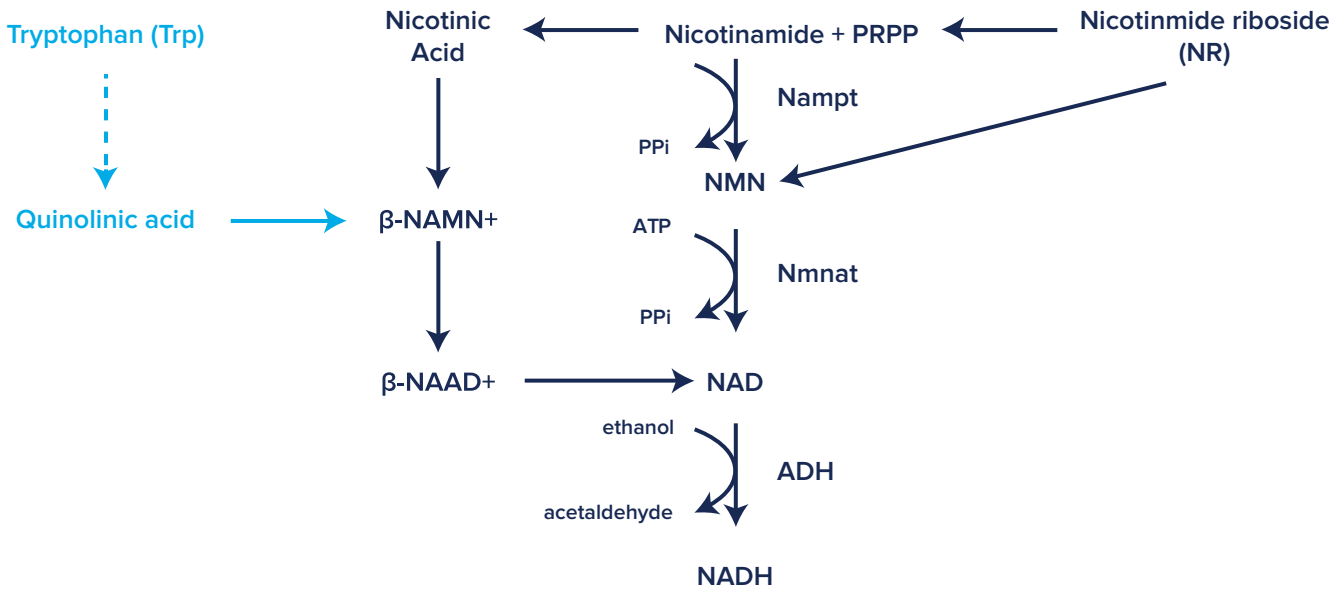

Figure 3. The de novo and salvage pathways of NAD+ and NADH production.

Despite the presence of the de novo pathway, salvage reactions are essential in humans and are, thus, more important. This high requirement for NAD+ results from the constant consumption of the NAD+ in cellular reactions, such as post-translational modifications, including those modulated by sirtuins.

NAD+ production and availability naturally decline with age due to reduced efficiency in the salvage pathway and increased consumption of cellular stress responses. This decline can impact cellular energy production, mitochondrial function, and the activity of NAD+-dependent enzymes like the sirtuins. As a result, key cellular maintenance processes, especially autophagy, may become less efficient over time. Autophagy is responsible for clearing damaged proteins and cellular components from the cell; therefore, reduced NAD+ levels can contribute to the gradual accumulation of cellular damage associated with aging. Supporting adequate NAD+ levels may help maintain metabolic function and promote healthy cellular renewal processes, including autophagy.

THE LIFEVANTAGE® PROTANDIM® FAMILY OF PRODUCTS

The LifeVantage Protandim family of products has been specifically formulated to maximize support of the body's natural cellular functions. By using nutrients in the exact right ratios and amounts, the formulas target and activate specific cellular signaling and biochemical pathways related to oxidative stress, optimal mitochondrial function, and sirtuin production.*

Protandim Nrf2 Synergizer was specifically designed using five specific herbal extracts that have been clinically shown to activate the NRF2 pathway. Ultimately, when the NRF2 protein is activated, it will go into the nucleus of the cell, activate protective genes, and bring cells to a heightened state of readiness to react quickly to insults that enter the cell. In other words, it reawakens the NRF2 pathway and brings it back to its full potential.*

Protandim NRF1 Synergizer was specifically formulated to target mitochondrial health. When activated, the NRF1 transcription factor has been shown to remove damaged mitochondria while also promoting biogenesis, which is the process of making new, healthy mitochondria within the cell. Protandim NRF1 Synergizer was specifically formulated to 1) target and activate the NRF1 protein and increase mitochondrial biogenesis and 2) deliver nutrients that support maintenance proteins and help fuel ATP production to support overall mitochondrial function.*

Protandim NAD Synergizer was designed to target cellular signaling pathways and upregulates genes involved in the salvage pathway for NAD+. By activating these genes, NAD+ levels increase and, ultimately, stimulate sirtuin activity, which plays a key role in supporting cellular health processes, including mitochondrial function and autophagy.*

To study the interaction and synergies between the three Protandim products, known as Protandim Tri-Synergizer, research was performed to determine the combination's impact on gene expression for targets known to be affected by Protandim Nrf2 Synergizer (*NQO1, HMOX1*), Protandim NRF1 Synergizer (*NRF1, PGC1-α*), and Protandim NAD Synergizer (*NAMPT, NMNAT1*). Global sirtuin activity, autophagy activity, and the ratio of NAD+/NADH were also investigated in HepG2 liver cells.*

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METHOD

1. Dose Response/Viablity Studies

The Cell-Titer-Glo Luminescent Cell viability assay (Promega) is a method to determine the number of viable cells in culture based on quantitation of the ATP present, as an indicator of metabolically active cells. Cells were seeded at a density of 400 cells/well (C2C12) or 700 cells/well (HepG2) in 384-well white-walled Cultur-Plate-384 cell culture plates (Perkin-Elmer, Waltham MA, Catalog #6007680) and exposed to test agent 18 hours later. Cells were allowed to grow in the presence of test agent for 24 hours for dose-course studies, at which point Cell-Titer-Glo (Promega Corp., Madison WI, Catalog #G7571) reagent was added at a volume equal to the cell culture medium in the plate (50µL + 50µL), according to manufacturer's instructions. Luminescence was read on an Envision 2104 multilabel reader (Perkin-Elmer, Waltham WI). A 700 nm filter was used to reduce background signals greater than 700nM (bioluminescence of luciferase is 562nm).

2. Gene Expression in HepG2 Cells

HepG2 cells were seeded at 200k cells per well. After 18 hours of incubation, cells were treated and, at appropriate time points, cells were lysed with lysis buffer. RNA isolations were conducted per manufacturer's recommendations using the PureLink Mini RNA kit (Thermo, Cat. No. 12183025). RNA was eluted with RNase free water and quantified by spectrophotometer. Reverse transcription reactions were performed using 1µg of RNA using the Vilo Superscript system (Thermo, Cat. No. 11756500). A six-fold dilution was made of each cDNA in PCR-grade water, and 2µL of this solution was carried forward into qRT-PCR. Reactions were carried out in 20µL total volume, made up as follows: 2µL cDNA, 6µL PCR-grade water, 1µL gene-of-interest primer (FAM label), 1µL GAPDH primer (VIC label), 10µL Taqman Fast Advanced Master Mix (Thermo Fisher Scientific, Catalog #4444963). Reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR Instrument (Thermo Fisher Scientific) under the following conditions: 50°C – 2 minutes, 95°C – 20 seconds, 40 cycles of (95°C – 3 seconds, 60°C – 30 seconds). Threshold cycle (CT) was determined by the instrument software. Differences in threshold cycle between the gene of interest and GAPDH (ΔCT) were determined for each sample and used to determine fold induction of each gene of interest, compared to untreated controls.

PCR primers were purchased from Thermo Fisher Scientific as follows:

PPARGC1A (PGC-1α): Hs0106724 human (FAM labeled)
NRF-1: Hs00602161 human (FAM labeled)
NQO1: Hs01045993 human (FAM labeled)
HO1: Hs011102250 human (FAM labeled)
NAMPT: Hs00237184 human (FAM labeled)
NMNAT1: Hs00276702 human (FAM labeled)
GAPDH control: 4326315E human (VIC labeled)

3. Genes of interest

a. NRF2-pathway genes:

i. *NQO1* (NAD(P)H dehydrogenase quinone oxidoreductase-1)—This gene encodes a cytoplasmic 2-electron reductase. This FAD-binding protein forms homodimers and reduces quinones to hydroquinones. This helps detoxify harmful quinones and reduce oxidative stress.

ii. *HMOX1* (heme oxygenase-1)—This gene encodes heme oxygenase, an essential enzyme in the breakdown of heme, a component of hemoglobin and other heme-containing proteins. Free heme can be toxic and pro-oxidative if it accumulates in cells. It is considered a cytoprotective and stress-response enzyme.

b. NRF1-pathway genes:

i. *NRF1* (nuclear respiratory factor-1)—This gene encodes a protein that homodimerizes and functions as a transcription factor which activates the expression of some key metabolic genes regulating cellular growth and nuclear genes required for respiration, heme biosynthesis, and mitochondrial DNA transcription and replication.

ii. *PGC1-α* (peroxisome proliferator-activated gamma coactivator-1-alpha)—The protein encoded by this gene is a transcriptional coactivator that regulates the genes involved in energy metabolism. This protein interacts with PPAR-α, which permits the interaction of this protein with multiple transcription factors. This protein can interact with and regulate the activities of cAMP response element binding protein (CREB) and nuclear respiratory factors (NRFs). This is important because it acts as a master regulator of cellular energy production and coordinates multiple pathways and transcription factors to support mitochondrial function, metabolic efficiency, and overall cellular health.*

c. NAD-pathway genes:

i. *NAMPT* (nicotinamide phosphoribosyltransferase)—This gene encodes a protein that catalyzes the condensation of nicotinamide with 5-phosphoribosyl-1-pyrophosphate to yield nicotinamide mononucleotide (NMN), one step in the biosynthesis of nicotinamide adenine dinucleotide (NAD).

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ii. *NMNAT1* (nicotinamide nucleotide adenylyltransferase-1)—This gene encodes another enzyme which catalyzes a key rate-limiting step (NMN to NAD+) in the biosynthesis of NAD.

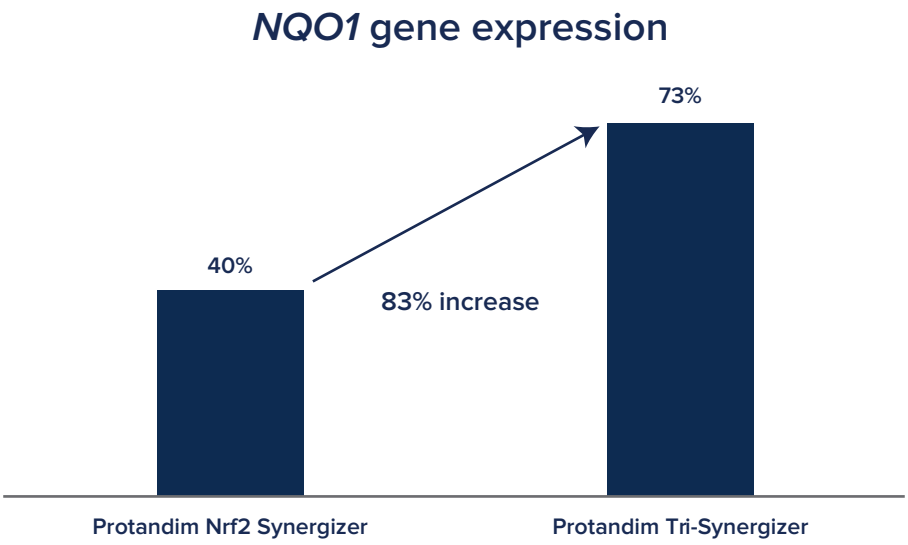
RESULTS AND DISCUSSION

1. Gene expression in HepG2 cells

a. NRF2-pathway influences:

Figure 4 (A and B) illustrates the effect of Protandim Nrf2 Synergizer and Protandim Tri-Synergizer on *NQO1* and *HMOX1* gene expression. Over a 24-hour period, Protandim Nrf2 Synergizer increased *NQO1* gene expression by 40%, whereas Protandim Tri-Synergizer showed a 73% increase. This corresponded to a synergistic 83% increase of *NQO1* expression as compared to Protandim Nrf2 Synergizer alone. A similar pattern was observed for *HMOX1* gene expression. Protandim Nrf2 Synergizer increased *HMOX1* expression by 51%, while Protandim Tri-Synergizer resulted in an 848% increase. This represented a synergistic effect of 1,563% as compared to Protandim Nrf2 Synergizer alone.*

A.



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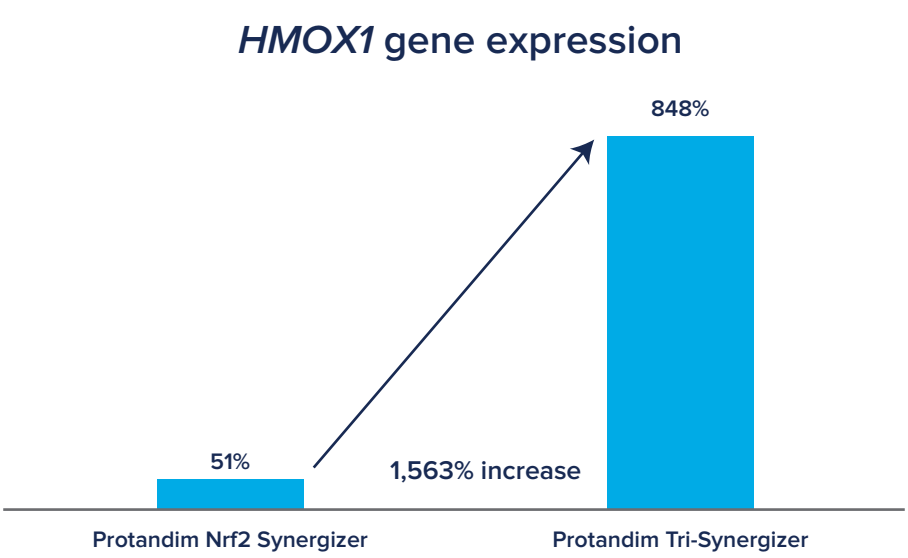

Figure 4A and 4B. Protandim Nrf2 Synergizer vs Protandim Tri-Synergizer and its effects on the antioxidatant genes *NQO1* and *HMOX1* after incubating 10 mcg/ml into HepG2 cells for 24 hours.

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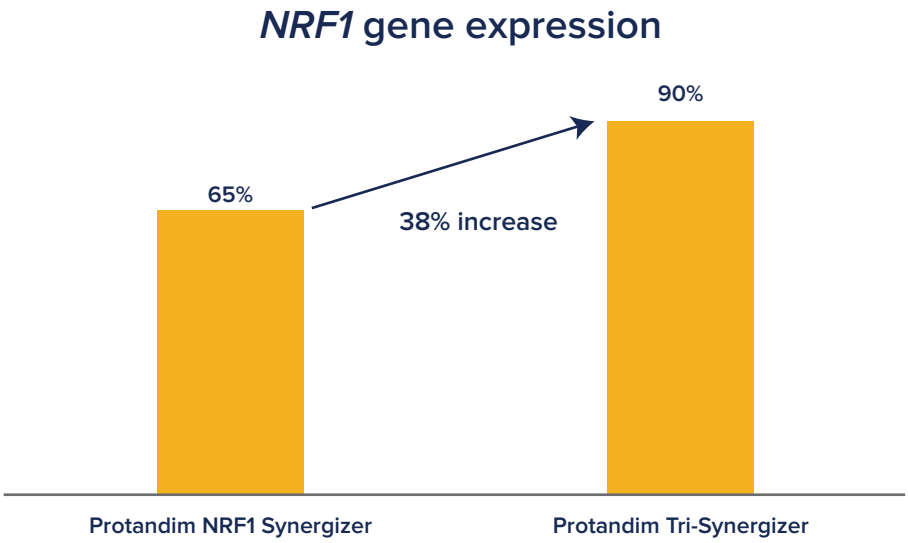
b. NRF1-pathway influences:

Figure 5 (A and B) show the effects of Protandim NRF1 Synergizer and Protandim Tri-Synergizer on *NRF1* and *PGC1-α* gene expression. Over a 24-hour period, Protandim NRF1 Synergizer increased *NRF1* expression by 65%, while Protandim Tri-Synergizer showed an increase of 90%. This represents a synergistic increase of *NRF1* expression of 38% compared to Protandim NRF1 Synergizer alone. A similar effect was observed for *PGC1-α* gene expression. Protandim NRF1 Synergizer increased *PGC1-α* expression by 71% and Protandim Tri-Synergizer by 120%. This corresponds to a synergistic effect of 69% compared to Protandim NRF1 Synergizer alone.*

A.



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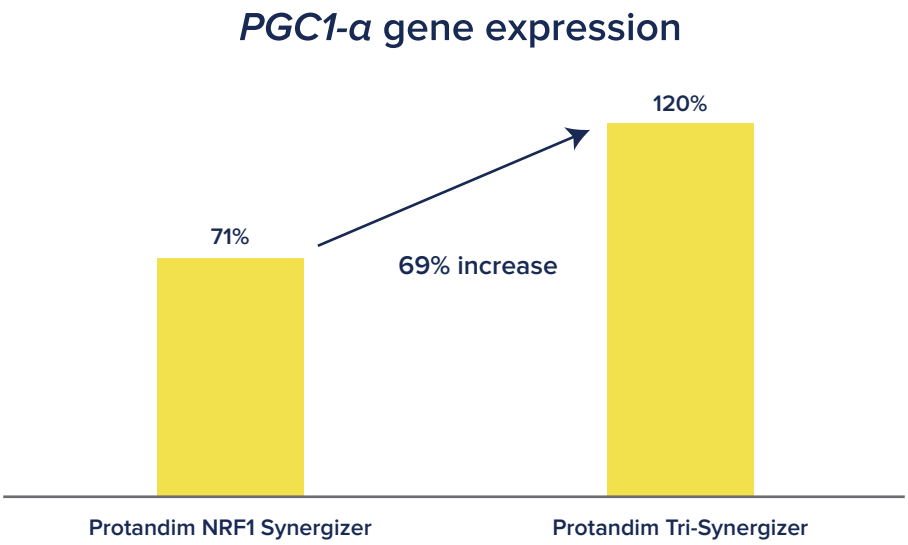

Figure 5A and 5B. Protandim NRF1 Synergizer vs Protandim Tri-Synergizer and its effects on transcription factor *NRF1* gene and master regluator of cellular energy production *PGC1-α* gene expression after incubating 10 mcg/ml into HepG2 cells for 24 hours.

c. NAD-pathway influences:

Figure 6 (A and B) shows the effect of Protandim NAD Synergizer and Protandim Tri-Synergizer on *NAMPT* and *NMNAT1* gene expression. Over a 24-hour period, Protandim NAD Synergizer showed an increase in *NAMPT* gene expression of 12%, while Protandim Tri-Synergizer showed an increase of 128%. This represents a synergistic increase of *NAMPT* expression of 967% compared to Protandim NAD Synergizer alone. A similar effect was seen for *NMNAT1* gene expression. Protandim NAD Synergizer increased *NMNAT1* expression by 12%, whereas Protandim Tri-Synergizer increased it by 227%. This corresponds to a synergistic increase of 1,792% compared to Protandim NAD Synergizer alone.*

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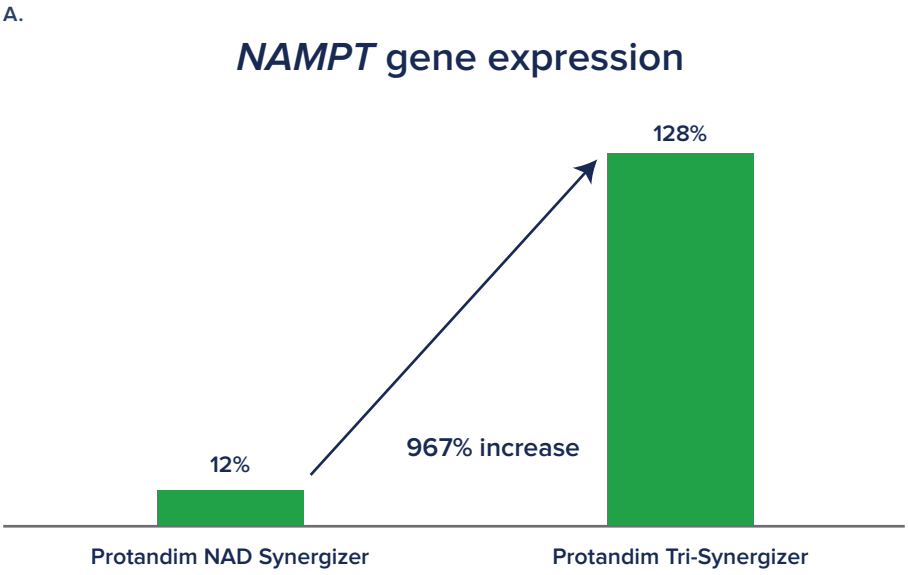

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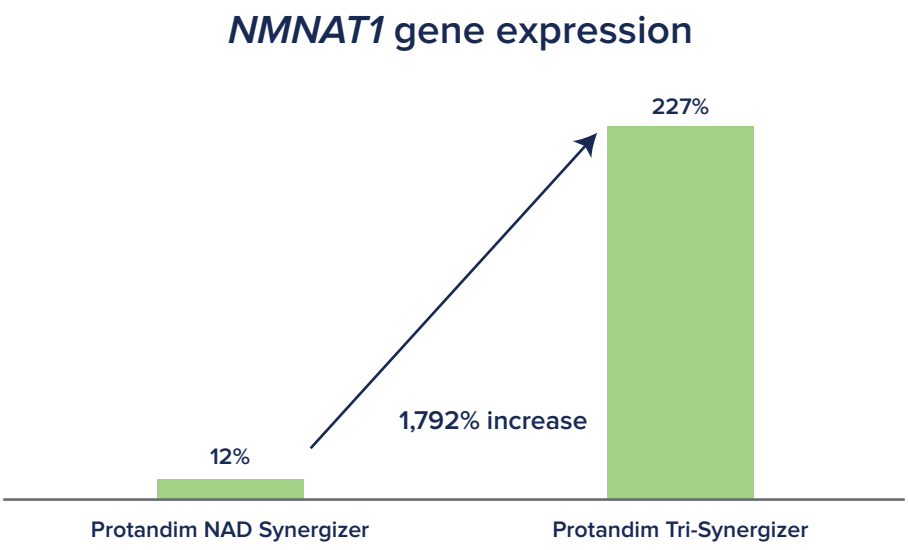

Figure 6A and 6B. Protandim NAD Synergizer vs Protandim Tri-Synergizer and its effects on enzyme *NAMPT* gene and key rate-limiting enzyme *NMAT1* gene after incubating 10 mcg/ml into HepG2 cells for 24 hours.

Conclusion

The interactions and potential synergies between Protandim Nrf2 Synergizer, Protandim NRF1 Synergizer, and Protandim NAD Synergizer were investigated both individually and in combination, referred to as Protandim Tri-Synergizer. Gene expression of targets known to be affected for each pathway—*NQO1* and *HMOX1* for *NRF2*, *NRF1* and *PGC1-α* for *NRF1*, and *NAMPT* and *NMNAT1* for NAD—were analyzed in HepG2 liver cells.

Genes regulated through activation of the antioxidant response element (ARE) by the NRF2 pathway, specifically *NQO1* and *HMOX1*, demonstrated significant synergistic increases of 83% and 1,563% respectively, when treated with Protandim Tri-Synergizer compared to Protandim Nrf2 Synergizer alone. These results are unexpected, as neither Protandim NRF1 Synergizer nor Protandim NAD Synergizer were specifically designed to target the ARE promotor region. While certain components, such as wasabi found in Protandim NAD Synergizer and alpha lipoic acid found in Protandim NRF1 Synergizer, may indirectly influence this pathway through modulation of mitochondrial redox balance, this secondary effect does not fully account for the magnitude of the observed increases.*

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A similar pattern was observed within the NRF1 pathway. Expressions of *NRF1* and *PGC1-α* increased by 38% and 69%, respectively, with Protandim Tri-Synergizer compared to Protandim NRF1 Synergizer alone. NRF1 is a transcription factor that regulates mitochondrial proteins involved in the respiratory chain, while PGC1-α acts as the master coactivator that enhances NRF1 activity, supporting mitochondrial biogenesis. The addition of both Protandim Nrf2 Synergizer and Protandim NAD Synergizer alone does not fully explain the synergistic increase observed in these gene expression levels.*

Consistent findings were also seen in the NAD pathway. Protandim NAD Synergizer targets genes encoding key enzymes in the NAD salvage pathway, including *NAMPT* and *NMNAT1.* When combined as Protandim Tri-Synergizer, expression of *NAMPT* and *NMNAT1* increased by 967% and 1,792%, respectively, compared to Protandim NAD Synergizer alone, further supporting a synergistic effect across pathways.*

Overall, these results demonstrate a strong mechanistic rationale behind combining Protandim Nrf2 Synergizer, Protandim NRF1 Synergizer, and Protandim NAD Synergizer. These pathways are biologically interconnected and collectively influence cellular defenses, mitochondrial function, and energy metabolism. Targeting them simultaneously may provide a more comprehensive, whole-body, systems-level approach, supporting antioxidant defenses, enhancing mitochondrial capacity, and promoting cellular energy production and repair, rather than focusing on a single pathway in isolation. This may help support cellular energy, recovery, resilience against age-related cellular decline, and protection from oxidative stress.*

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