Chemical and Biological Investigation of KEAP1–NRF2 Pathway and Role of Sesamin in Oxidative Stress and Ageing

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Submitted by:

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CANDIDATE'S DECLARATION

I, Dhatri Upadhyay (/24/MSCCHE/57), student of M.Sc. Chemistry hereby certify that the work which is being presented in the dissertation entitled Chemical and Biological Investigation of KEAP1–NRF2 Pathway and Role of Sesamin in Oxidative Stress and Aging in partial fulfilment of the requirements for the award of the Degree of Master of Science, submitted to the Department of Applied Chemistry, Delhi Technological University, is an authentic record of my work carried out under the supervision of Prof. Archna Rani.

I have not submitted the matter presented in this dissertation for the award of any other degree of this or any other Institute.

Place: Delhi
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Date: 31st May 2026

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CERTIFICATE

I hereby certify that the dissertation titled Chemical and Biological Investigation of KEAP1–NRF2 Pathway and Role of Sesamin in Oxidative Stress and Ageing, which is submitted by Dhatri Upadhyay (DTU/24/MSCCHE/57) to the Department of Applied Chemistry, Delhi Technological University, Delhi, in partial fulfilment of the requirements for the award of Master of Science. This dissertation embodies the results of original work and studies carried out by the student; the contents of this dissertation do not form the basis for the award of any other degree to the candidate or anybody else from this or any other University/Institution.

Place: Delhi
Date: 31st May 2026

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Dhatri Upadhyay

ABSTRACT

The Kelch-like ECH-associated protein 1 and nuclear factor erythroid 2-related factor 2 (KEAP1–NRF2) signalling axis is the most critical and conserved cellular defence system against redox perturbations. Under basal conditions, NRF2 is constitutively ubiquitinated and targeted for proteasomal degradation through its interaction with KEAP1, a Cullin 3-based E3 ubiquitin ligase adaptor. Upon exposure to oxidative or electrophilic stimuli, cysteine residues within KEAP1 are covalently modified, disrupting its interaction with NRF2. Consequently, NRF2 escapes proteasomal degradation, translocate to the nucleus, and heterodimerizes with small musculoaponeurotic fibrosarcoma (sMAF) proteins to drive transcription of antioxidant response element (ARE)-dependent cytoprotective genes.

Sesamin, a lipophilic lignan abundantly present in sesame seeds (Sesamum indicum), has emerged as a biologically significant natural compound with well-documented antioxidant, anti-inflammatory, and neuroprotective properties. This activation then stimulates an increase in the amount of NRF2 transported into the nucleus, a decrease in the amount of KEAP1 in cells, and an increase in the number of ARE-regulated cytoprotective genes expressed by the cell.

The biological significance of the NRF2 pathway extends to multiple organ systems. With age, there is a decline in NRF2 activity resulting in the accelerated pace of cellular senescence, development of the senescence-associated secretory phenotype (SASP), neurodegeneration, sarcopenia, impaired lacrimal and salivary gland functioning, and hearing loss. Data from both in vitro and in vivo experimental settings establish that treating rats or providing a diet that includes components proven to restore NRF2 activity can lessen some of the effects associated with advanced age. Because of sesamin's demonstrated ability to modulate NRF2, it is an attractive candidate for developing a natural therapeutic approach to counteract the effects of aging due to oxidative stress and neurodegeneration. This dissertation incorporates redox chemistry, molecular biology, and natural product pharmacology to present a complete view of the KEAP1–NRF2–sesamin axis and its role in maintaining healthy ageing.

LIST OF ABBREVIATIONS

AD: Alzheimer's Disease
AHL: Age-related Hearing Loss
AKT: Protein Kinase B
ALS: Amyotrophic Lateral Sclerosis
ARE: Antioxidant Response Element
DO-Im: 2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolide
CNC : Cap'n'Collar protein family
DMF: Dimethyl Fumarate
EpRE: Electrophile-Responsive Element
ERK: Extracellular Signal-Regulated Kinase
GCL: Glutamate-Cysteine Ligase
GCLC : Glutamate-Cysteine Ligase Catalytic Subunit
GCLM: Glutamate-Cysteine Ligase Modifier Subunit
GR : Glutathione Reductase
GSH : Reduced Glutathione
GSSG : Oxidized Glutathione
HO-1 : Heme Oxygenase-1
IL-1β : Interleukin-1 Beta
IL-6 : Interleukin-6
KEAP1 : Kelch-like ECH-Associated Protein 1
LCST : Lower Critical Solution Temperature
MAPK: Mitogen-Activated Protein Kinase
MDA : Malondialdehyde
NF-κB : Nuclear Factor Kappa B
NLRP3: NLR Family Pyrin Domain Containing 3
NQO1 : NAD(P)H Quinone Dehydrogenase 1
NRF2 : Nuclear Factor Erythroid 2-Related Factor 2
PD : Parkinson's Disease
PI3K : Phosphoinositide 3-Kinase
PKC : Protein Kinase C
ROS : Reactive Oxygen Species
SASP : Senescence-Associated Secretory Phenotype
sMAF : Small Musculoaponeurotic Fibrosarcoma protein
SOD : Superoxide Dismutase
SSM : Sesamin
TNF-α : Tumour Necrosis Factor-Alpha
TXNRD1 : Thioredoxin Reductase 1
UC : Ulcerative Colitis
UPS : Ubiquitin-Proteasome System

CONTENTS

CHAPTER 1: INTRODUCTION

Every organism alive today lives in a highly oxidising environment, including the air that enables aerobic organisms to survive and one of the greatest molecular threats to their existence: oxidative stress. Since the development of oxygenic photosynthesis approximately 2.4 billion years ago, life has faced the challenge of managing some of the byproducts from oxygen metabolism. Byproducts produced by reactions involving reactive oxygen exist as a consequence of mitochondrial respiration, inflammatory responses, and biotransformation of xenobiotics, desaturates — all represent significant threats to the structure and function of cellular macromolecules, and collectively the altered proteins, peroxidised lipids, and damaged nucleic acids all lead to a cellular state of oxidative dysregulation that increases cellular ageing and disease formation if not controlled. (Matsumaru and Motohashi, 2021).

The KEAP1–NRF2 signalling pathway is a primary regulator of the antioxidant system within cells, acting as both a sensor for oxidation and a way to produce antioxidants when the cell becomes oxidised. Under unstressed conditions, the protein NRF2 is kept at low steady-state levels by continuously being broken down (through constitutive ubiquitination) following its ubiquitination by the KEAP1-Cullin 3 E3 ubiquitin ligase. As a result, NRF2 levels are appropriately maintained at the low levels necessary for normal cellular functioning, while avoiding any additional metabolic costs incurred by maintaining excess amounts of NRF2 in the cell. In contrast, when ROS and/or other oxidizing agents impair the cellular redox system, specific cysteine residues within KEAP1 are then chemically altered and will not be able function properly or ubiquitinate NRF2, resulting in newly synthesized NRF2 accumulates and travels to the nucleus to activate transcription of many cytoprotective genes by binding to antioxidant-response elements located within their corresponding promoter sequences (Yamamoto et al 2018).

Given this background, there has been significant interest from both scientific and pharmaceutical communities to discover and characterize natural compounds that activate (i.e., stimulate) the KEAP1–NRF2 pathway. One of the most chemically complex and biologically active natural NRF2 activators characterized to date is sesamin. This lignan has a tetrahydrofurofuran-type structure and is found in large amounts within sesame seeds (Sesamum indicum L.). The molecular arrangement of sesamin consists of two methylenedioxy phenyl groups and a symmetric tetrahydrofuran core; thus, sesamin is an

unusual compound, as it exhibits superb metabolic stability and lipid solubility, which should help distribute it to various tissues, including the brain. In addition to its well-established antioxidant and anti-inflammatory effects, sesamin has now been demonstrated to stimulate NRF2 signalling through the AKT and ERK kinase pathways and to stimulate NRF2-Cnc-dependent transcription in neuronal cells even in the absence of any significant oxidative stress, suggesting that sesamin may act as a proactive and not just reactive neuroprotective agent (Bai et al., 2019; Le and Inoue, 2021).

1.1 Oxidative Stress and Reactive Oxygen Species

Reactive Oxygen Species (ROS) consist of a wide range of chemically responsive molecules formed from aerobic metabolism. Reactive Oxygen Species (ROS) that will be discussed in this chapter include superoxide anion $O_2^{\bullet -}$, hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), singlet oxygen ($^1O_2$), and hypochlorous acid (HOCl). They are an uncharacteristic group of molecules since they differ in reactivity, distance of diffusion, and target molecules. The inter-conversion of these species through both enzymatic and non-enzymatic reactions establishes a fluid oxidative network within the cell.

Under normal conditions, the predominant source of ROS is the mitochondria. The imperfections associated with electron transfer to proton translocation within the mitochondria's electron transport chain will leak electrons after passing through Complexes I and III (leaking) to directly react with molecular oxygen to form superoxide. The percentage of leakages from the total electron transport chain to produce superoxide appears to be small (0.2%-2%); however, the cumulative production of ROS from these leakages becomes significant as the average cell uses approximately 2×10^{12} molecules of oxygen each day. Other potential cellular sources include: phagocytes (especially NADPH oxidases - NOX enzymes), xanthine oxidase during purine catabolism, cytochrome P450 enzymes metabolizing xenobiotics and lipoxygenases metabolizing arachidonic acid.

The effects of excessive levels of reactive oxygen species (ROS) are far-reaching and can be serious in how they impact the protein oxidation process, leading to carbonyl group formation, sulfenic acids, disulfide bonds and nitro tyrosine adducts that will negatively impact enzyme function, receptor function and protein structure. Lipid peroxidation caused by the reaction of hydroxyl radicals with polyunsaturated fatty acids can lead to the production of reactive intermediates such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which create covalent adducts with proteins and DNA.

Oxidative DNA damage appears in a variety of ways including 8-oxoguanine lesions, abasic sites, single-strand breaks and double-strand breaks, all of which require repair for genomic stability to remain intact. When the repair mechanisms become overwhelmed or less efficient with age, oxidative DNA damage increases, contributing to mutagenic changes, cellular senescence and the potential for developing cancer.

However, it is critical to recognize that ROS are not just agents of disease. When at controlled physiological concentrations, such as hydrogen peroxide and superoxide, they play an important role as second messengers in the signalling pathways that mediate cellular proliferation, differentiation, immune responses, and autophagy. Both the signalling nature of ROS and their damaging effects illustrate how essential it is to keep redox balance within cells. Therefore, redox homeostasis, which is defined as the dynamic balance of oxidative stress generation and the antioxidant ability to neutralise it, is critical to understanding both normal cellular physiology and pathophysiology.

1.2 Antioxidant Defence Systems

In reaction to the ongoing problem of oxidative stress, cells have developed an extensive and complex antioxidant defence system that works on several levels, from the immediate enzymatic suppression of ROS through to the transcriptional induction of genes that will allow them to survive. This antioxidant defence system may be classified as either enzymatic or non-enzymatic, with both playing a unique but complementary role in determining how effective the antioxidant properties of a cell are.

Enzymatic antioxidant systems are made up of three main classes of enzymes. Superoxide dismutase (SODs) catalyse the disproportionation of superoxide to hydrogen peroxide, with three mammalian isoforms: cytosolic Cu/Zn-SOD (SOD1), mitochondrial Mn-SOD (SOD2), and extracellular EC-SOD (SOD3) providing compartment-specific protection. Catalase acts predominantly in the peroxisome and efficiently decomposes H_2O_2 into water and oxygen thus protecting cells against the unwanted build-up of H_2O_2. The glutathione peroxidase (GPx) enzymatic family, in particular GPx1 and GPx4, reduces both H_2O_2 and lipid peroxides to water and lipid alcohol, respectively by consuming reduced glutathione [GSH], which will subsequently recycle to GSH through the action of glutathione reductase using NADPH as an electron source, thereby linking the GSH cycle with the metabolic status of the cell.

Antioxidants can be endogenous or dietary in nature and work by donating electrons to quench (neutralize) reactive oxygen species (ROS). Glutathione (GSH), which is a tripeptide comprised of three amino acids (γ-glutamylcysteinylglycine), exists at millimolar concentrations within virtually all mammalian cells and is considered the most plentiful intracellular antioxidant. The thiol group of GSH represents the primary reducing equivalent for many antioxidant reactions, and the ratio of GSH/GSSG serves as an excellent marker of the oxidative/reductive balance of a cell. Another important redox buffering mechanism is the thioredoxin system, which uses NADPH through the action of thioredoxin reductase to help keep proteins reduced and to regenerate the peroxiredoxin enzymes, which are the main enzymes used to remove H_2O_2 in the nucleus and the cytosol.

The antioxidant network is not static in terms of its capacity, but rather is dynamically adjusted based on prevailing oxidative conditions. The primary mechanism through which this adaptive modulation occurs is through the KEAP1–NRF2 pathway, which senses changes in redox status and coordinates the transcriptional upregulation of both non-enzymatic and enzymatic antioxidant systems. This regulatory pathway provides a sophisticated, rapid, and proportional method for increasing cellular antioxidant capacity in response to an increase in oxidative stress.

1.3 KEAP1–NRF2 Signalling Pathway

The KEAP1–NRF2 system represents the most critical and evolutionarily conserved cellular mechanism for sensing and responding to oxidative and electrophilic stress. It functions as a thiol-based sensor-effector apparatus, in which specific cysteine residues in the KEAP1 protein serve as molecular sensors for oxidants and electrophiles, while NRF2 functions as the transcriptional effector that drives cytoprotective gene expression.

Under homeostatic, unstressed conditions, the cellular abundance of NRF2 is maintained at very low levels through its constitutive ubiquitination and subsequent proteasomal degradation. This is achieved through the interaction of NRF2 with KEAP1, which serves as the substrate adaptor for a Cullin 3-based RING-box protein 1 (CUL3-RBX1) E3 ubiquitin ligase complex. The DLG and ETGE motifs within the Neh2 domain of NRF2 engage the Kelch-repeat domain of KEAP1, positioning NRF2 for efficient ubiquitination on multiple lysine residues within the Neh2 domain. Ubiquitinated NRF2 is

then rapidly degraded by the 26S proteasome, resulting in a protein half-life of approximately 15–20 minutes under basal conditions (McMahon et al., 2003; Itoh et al., 2003).

Upon exposure to ROS, electrophiles, or other oxidative stressors, the thiol groups of sensor cysteine residues in KEAP1 — most notably Cys151, Cys273, and Cys288 — undergo chemical modification, including oxidation to sulfenic acids, formation of disulfide bonds, or covalent alkylation by electrophiles. These modifications induce conformational changes in KEAP1 that disrupt its ability to efficiently ubiquitinate NRF2. The existing NRF2 molecules that are already complexed with KEAP1 remain temporarily sequestered, while newly synthesized NRF2 escapes ubiquitination and accumulates in the cytoplasm. This rapidly accumulating pool of NRF2 then translocate to the nucleus, facilitated by its nuclear localization signals, where it heterodimerizes with small musculoaponeurotic fibrosarcoma (sMAF) proteins — sMafF, sMafG, and sMafK. The KEAP1–NRF2 pathway is very important because it helps cells detect and respond to the damage caused by reactive oxygen species (free radicals) and chemicals that cause damage to cells.

The NRF2-sMaf complex binds to specific gene sequences called antioxidant response elements (AREs; consensus sequence 5'-TGACnnnGC-3') or electrophile-responsive elements (EpREs) located upstream of genes responsible for protecting cells from injury and initiates transcription of those genes (Itoh et al., 1997; Motohashi et al., 2004).

The ability of KEAP1 to sense cysteine residues is unique, and has led to the concept of a 'cysteine code'. Different types of chemicals that cause injury (including electrophiles and oxidants) react with different residues of cysteine, and thus there are different patterns of modification leading to activation of different subsets of NRF2 target genes (i.e. a precisely aligned and stimulus-specific gene regulation). Electrophiles are classified into four groups according to the KEAP1 cysteine code and which types of modification were made to the cysteines affect the functional consequences of activation of NRF2 (Suzuki et al., 2019). This chemical specificity at the sensor protein level allows cells to produce an appropriate, well-timed, and tailored response to the type of oxidative or electrophilic injury.

1.4 Structure and Function of KEAP1

Kelch-like ECH-associated protein 1 is a 69 kDa protein belonging to the BTB-Kelch family of proteins. Its domain architecture is modular and functionally organized, with each domain contributing distinct roles to the overall function of the protein as both a substrate adaptor for E3 ubiquitin ligase activity and a redox sensor. KEAP1 contains five functional domains: an N-terminal region (NTR), a BTB (Broad complex, Tramtrack, and Bric-à-brac) domain, an intervening region (IVR), a double-glycine repeat (DGR) or Kelch domain, and a C-terminal region (CTR).

The BTB domain mediates homodimerization of KEAP1 and is responsible for its interaction with Cullin 3, the scaffold component of the E3 ubiquitin ligase complex. Structural studies have revealed that CUL3 interacts with the BTB domain of KEAP1 through a conserved interface that positions the substrate-binding Kelch domain optimally for NRF2 ubiquitination. The homodimerization of KEAP1 through its BTB domain creates a two-headed molecular structure, often depicted as a 'molecular clamp,' in which each KEAP1 monomer uses its Kelch domain to engage one of the two binding motifs (ETGE and DLG) within the Neh2 domain of a single NRF2 molecule. This bivalent interaction mode is essential for productive ubiquitination of NRF2.

The Kelch domain, consisting of six tandem Kelch repeats, forms a six-bladed β-propeller structure that constitutes the substrate recognition module of KEAP1. The top face of this β-propeller engages the ETGE and DLG motifs of NRF2, with the ETGE interaction being particularly high-affinity and contributing the majority of binding energy. Structural analyses have also revealed that this Kelch domain can accommodate small molecule inhibitors that compete with NRF2 for KEAP1 binding, providing a rational basis for the development of PPI (protein-protein interaction) disruptors as NRF2 activators.

The IVR region includes many important cysteine residues of the sensor proteins found in the KEAP1 protein family, such as cysteine 273 (Cys273) and cysteine 288 (Cys288), which are necessary for inhibiting the transcription factor NRF2 under basal conditions and for detecting certain classes of electrophilic compounds. The BTB region contains cysteine 151 (Cys151), which preferentially reacted with highly reactive electrophilic compounds including some classes of isothiocyanates and heavy metals. The spatial arrangement of the sensor cysteine residues between the different proteins of the KEAP1 family allows them to respond to a wide range of chemical stressors, and the difference in the shape of the protein depending on which cysteine is modified may be one

reason why there is such a precise ability to regulate NRF2 target genes based on the protein being activated by specific electrical signals.

1.5 Structure and Function of NRF2

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a 66-68 kDa basic leucine zipper (bZIP) transcription factor that belongs to the cap'n'collar (CNC) family of proteins. The CNC family is characterized by a unique N-terminal region, the CNC domain, of about 43 amino acids which is required for the family and helps determine DNA binding specificity. In mammals, there are four members of the CNC family: NRF1, NRF2, NRF3 and the p45 subunit of the NF-E2, of which NRF2 is the most extensively studied because it is the most prominent regulator of the antioxidant response.

NRF2 protein contains 7 domains that are functionally defined, Neh1- Neh7 (NRF2-ECH homology domains 1-7). The Neh1 domain includes the CNC-bZIP region, which contains the DNA binding and heterodimerisation sites with sMAF proteins. It includes the NLS which is responsible for transporting NRF2 to the nucleus when activated. The N-terminus Neh2 domain is the major regulatory domain that binds to KEAP1. It has two critical binding sequence regions, an ETGE and a lower affinity DLG sequence, both of which are involved in the bivalent KEAP1 interaction model which is necessary for ubiquitin mediated degradation.

The Neh3, Neh4 and Neh5 domains together form the transactivation domain that brings coactivators and elements of the basal transcription machinery together to activate transcription. The Neh3 domain is involved in the binding of a chromatin remodelling factor called chromo-ATPase/helicase DNA-binding (CHD6) protein. The Neh4 and Neh5 domains bind the CREB binding protein (CBP) and related coactivators. The Neh6 domain harbours a DSGIS motif which is recognized by the β-TrCP E3 ubiquitin ligase when phosphorylated by glycogen synthase kinase 3β (GSK-3β), thus offering an alternative, KEAP1-independent, mechanism for NRF2 degradation. The Neh7 domain binds to the retinoid X receptor alpha (RXRα) in a way that can negatively regulate the transcriptional activity of NRF2.

The expression of NRF2 is not a single switch-on or off mechanism, but is regulated by several layers of control and is a graded response. Several post-translational modifications of NRF2 such as phosphorylation by MAPK, PKC, CKII, and others, can increase the stability of NRF2 and its nuclear localisation. Acetylation of NRF2 by CBP/p300 enhances NRF2 binding to AREs. On the other hand, GSK-3β-mediated phosphorylation and the subsequent binding to SCF/β-TrCP and degradation of KEAP1 is a KEAP1-independent way to inhibit NRF2 activity in the presence of insulin signalling or elevated growth factor levels. These are multiple inputs that are integrated and ensure that NRF2 activity is finely tuned to the metabolic and signalling environment of the cell.

1.6 Oxidative Stress and the Aging Process

For more than 60 years, oxidative stress and aging have been one of the main focus points of biogerontology. Denham Harman first proposed the free radical theory of aging in 1956, which proposed that free radical damage to cellular components was the main cause of aging. Although later decades of work have been used to elaborate and nuance this perspective, the overall conclusion of redox dysregulation as a central mechanism in the aging process has consistently been shown in a variety of model systems and human investigations.

There are several lines of evidence that would suggest that the intracellular redox balance becomes more oxidized as the person gets older. As people get older, their mitochondria become damaged. This can happen by increasing numbers of mutations in mitochondrial DNA, progressive cross-linking of mitochondrial proteins and carbonylation of mitochondrial proteins, and peroxidation of membrane lipids in mitochondria. All these alterations together will result in a less efficient electron transport and higher leakage of electrons, causing increased ROS production. At the same time, the activity of key antioxidant enzymes (Cu/Zn-SOD, Mn-SOD, catalase and glutathione peroxidase) usually decreases in the old tissues, thus impairing their ability to cope with the rising oxidative challenge. Intracellular glutathione levels are markers of the ability to decline markedly with age in various tissues, especially the hippocampus.

The reduction in NRF2 activity is yet another important component of the redox decline of "aged" cells. Transcriptional activity and expression of NRF2 is documented to be decreased in old aged tissues across several species including humans. The decreased NRF2 function could be due to the up-regulation of miRNAs that degrade NRF2 mRNA, down-regulation of NRF2 promoter activity, up-regulation of NRF2 repressors such as KEAP1, and down-regulation of nuclear coactivators. The net result is progressive depletion of the antioxidant reserve that gets more vulnerable to oxidative damage the older the cells get (Zhang et al., 2015; Matsumaru and Motohashi, 2021).

Oxidative stress in aging tissues is a cause and effect of the cellular senescence, a condition of permanent cell cycle arrest caused by accumulation of DNA damage, telomere erosion and activation of oncogenes. Senescent cells also produce a differential secretion of pro-inflammatory cytokines, matrix metalloproteinases and growth factors, referred to as senescence-associated secretory phenotype (SASP), and have reduced activities of NRF2. A tissue microenvironment of chronic inflammation, generated by SASP, causes oxidative stress and senescence of surrounding cells by paracrine interactions, leading to a vicious cycle of tissue dysfunction. Senescent cells have also been found to build up in aged tissues, and their targeted clearance through the use of senolytic drugs confirms their ability to extend health span in mouse models (Baker et al., 2011; Matsumaru and Motohashi, 2021).

1.8 Introduction to Sesamin

Sesamin (chemical name: (3R,3aR,6S,6aR)-hexahydrofuro[3,2-b]furan-3,6-diyl bis(1,3-benzodioxole-5-carboxylate); molecular formula: $C_{20}H_{18}O_6$; molecular weight: 354.35 g/mol) is the most abundant lignan in sesame seeds (Sesamum indicum L., family Pedaliaceae) and sesame oil, typically comprising 0.5–1.0% of the total oil content. The tetrahydrofurofuran-type lignans are tetracyclic compounds with two methylenedioxyphenyl (piperonyl) groups attached to a symmetric bicyclic tetrahydrofuran core unit in a particular stereospecific arrangement. The addition of the methylenedioxy groups makes sesamin different from other sesame lignans and is responsible for its biological activity and metabolic stability.

Sesamin is synthesized in sesame plant from phenylalanine via the phenylpropanoid pathway. The major monolignol precursor, coniferyl alcohol, is oxidized to a diligent protein to yield (+)-pinoresinol, which is reduced by pinoresinol/lariciresinol reductase to (+)-lariciresinol, and then to (+)-secoisolariciresinol. The formation of the methylenedioxy bridges is done by cytochrome P450 enzymes (CYP81Q1 in case of sesame) introducing the furfuran ring system that is characteristic for sesamin. This biosynthetic pathway is found only in the Pedaliaceae family and is responsible for the high level of sesamin in sesame compared to other oilseed crops.

Sesamin is a lipophilic compound, of low water solubility, generally stocked and diluted in organic solvents (e.g. DMSO) for experimental use at 10-20 mM. It is lipophilic (log P ~ 3.5) and is therefore able to passively diffuse across biological membranes, enabling penetration into the intracellular space and the blood brain barrier, which is beneficial for its possible use in neuroprotection. Following oral administration, sesamin appears in the bloodstream in the small intestine, and is extensively metabolized to catechol derivatives in the liver by cytochrome P450 (CYP) enzymes, such as CYP3A4, CYP2C9, and CYP2C19. These catechol metabolites have biological activity, and could play a role in the in vivo activity of sesamin. Humans that ingest sesame have been estimated to have a bioavailability of ~25–35% and plasma levels of sesamin and metabolites in the micromolar range upon ingestion of a sesame rich meal.

Traditionally, Asian, African, and Middle Eastern medicine systems have relied on sesame seeds for their purported health-promoting properties, such as antihypertensive, anti-arthritic, and hepatoprotective effects. These traditional applications have been substantiated and expanded into the modern scientific realm, revealing the antioxidant, anti-inflammatory, lipid-lowering, antihypertensive, immunomodulatory and anticancer properties of sesamin and its related lignans. In particular, the ability to modulate key signalling pathways that are involved in ageing and neurodegeneration like the KEAP1–NRF2 pathway, the NF-κB inflammatory pathway and several kinase-mediated survival pathways is of current interest in sesamin.

1.9 Sesamin as an NRF2 Activator

The activation of the KEAP1–NRF2 signalling axis by sesamin was first hinted at by a study that showed sesamin and its metabolites could induce the expression of ARE-dependent genes such as HO-1 in neurons (Hamada et al., 2011). Further studies have gradually clarified the molecular mechanisms of this activation, which are complicated and involved, and the interaction between sesamin and the NRF2 regulatory circuit.

Sesamin's NRF2 activity proceeds at least two independent but synergistic mechanisms. Sesamin first activates the PI3K/AKT and MEK/ERK pathways, thereby leading to NRF2 phosphorylation. The phosphorylation of NRF2 by AKT at specific Ser and Thr residues and by ERK1/2 at multiple sites increases the stability of NRF2 by inhibiting the recognition of NRF2 by the β-TrCP degradation machinery and increasing the nuclear accumulation of NRF2. At the same time, sesamin at high doses exhibits the ability to lower the steady state protein level of NRF2, which leads to decreased ubiquitination and degradation of NRF2. These all culminate in an overall sustained and elevated nuclear NRF2 level and ARE responsiveness.

An important aspect that sets sesamin apart from many other currently known NRF2 inducers is sesamin's ability to enhance NRF2/Cnc-dependent transcription in neurons derived from Drosophila adult brain, even without exposure to exogenous oxidative stress (Le and Inoue, 2021). Therefore, this data suggest that sesamin does not necessitate exposure to pre-existing oxidative stress; rather, sesamin is considered a "proactive" (not just reactive) NRF2 activator and has the potential to work as a preventive stabilization methodology for people who may be at risk of developing an oxidative-stress-related pathology. Individuals identified as "at risk" may include elderly individuals and/or early-stage neurodegenerative disease processes; prevention of oxidation damage to all cells would enable many of these individuals to use sesamin to build up a ready supply of antioxidants before substantial oxidation has the potential to cause irreversible damage.

1.10 Objectives of the Study

The following are the aims of this dissertation:

1. To chemically analyse the KEAP1-NRF2 signalling pathway's redox chemistry (KEAP1 cysteine modification), the molecular mechanisms by which NRF2 stabilizes and translocates to the nucleus, how NRF2 promotes ARE-dependent genes.

2. To review and assess the biological importance of the KEAP1-NRF2 pathway in aging, senescent cells, and in age-related diseases in various organ systems.

3. To explore the activity of sesamin in activating NRF2 signaling by examining AKT and ERK pathways and by changing KEAP1 protein levels.

4. To test the ability of sesamin to protect neurons in the context of dependent transcription of neuronal cells, and to identify whether sesamin has different effects based on neuronal subtype.

5. To discuss sesamin's potential therapeutic effects as a natural NRF2 activator for preventing oxidative stress-associated aging and neurodegenerative diseases; to identify current research gaps, as well as future research directions.

CHAPTER 2: LITERATURE REVIEW

The KEAP1/NRF2 pathway is emerging as a key participant in cellular stress response and that targeting this pathway has merit for the development of new therapies for diseases. This chapter presents a thorough examination of significant findings in order to develop an understanding of current knowledge of the KEAP1/NRF2 pathway, its chemical mechanisms of KEAP1 activation, the anti-oxidative and anti-inflammatory properties of sesamin, and the neuroprotective benefits associated with NRF2 activation via sesamin.

2.1 Previous Studies on KEAP1–NRF2

The establishment of the KEAP1–NRF2 pathway as a modulator of cellular defence against oxidative stress was possible in several studies conducted in the late 1990s and early 2000s. Research conducted by Itoh and colleagues (1997) established that NRF2 operates as a transcription factor to promote the expression of phase II detoxifying and antioxidant enzymes. It binds to antioxidant response elements (AREs) in their respective promoters (ARE-dependent gene expression). The two studies conducted by Itoh and colleagues (1999) that isolated KEAP1 as a negative regulator of NRF2 and showed that modification of cysteines within the KEAP1 protein by reactive oxygen species (ROS) alters KEAP1 to release NRF2 of that repression were pivotal studies contributing to our current understanding of KEAP1/NRF2 signalling. Finally, work conducted by both McMahon et al. (2003) and Nguyen et al. (2003) provided additional evidence that KEAP1 acts as a substrate adaptor for the Cullin 3-based E3 ubiquitin ligase to facilitate the ubiquitin-mediated degradation of NRF2.

Through the completion of genome-wide chromatin immunoprecipitation studies, the full extent of NRF2 target genes was defined, and numerous investigations uncovered the molecular mechanisms that have defined NRF2 as the master driver of the cellular transcriptional response to oxidative stress. The paper authored by Tonelli et al. (2018) was particularly important because it involved an exhaustive assessment of NRF2 target genes whereby a comprehensive list of over 250 NRF2 target genes was reported to encompass roles in antioxidant defence, detoxification, glutathione metabolism, NADPH production, proteostasis, autophagy and iron metabolism. Thus, NRF2 is now considered a key regulator of the cellular transcriptional response to oxidative stress (the "master regulator" of antioxidant transcriptional responses).

The KEAP1-NRF2 pathway is essential for maintaining physiological health, particularly in terms of preventing cellular damage and promoting cellular growth. Utilizing genetic mouse models, scientists were able to study how Nrf2 knockout mice were significantly more susceptible to chemical carcinogens, and to agents that would induce oxidative stress, and to agents that induce inflammation. This is direct evidence of the importance of NRF2, as a cytoprotective mechanism, in vivo. When scientists created Keap1 knockout mice (mice that are constantly expressing NRF2), these newborn mice all died shortly after birth due to hyperkeratosis of the oesophageal and forestomach tissue, caused by unregulated levels of NRF2, illustrating that appropriate regulation of NRF2 is equally important during development and for healthy physiology. Scientists have determined that Keap1 knockdown (Keap1-KD) mice (mice that express low levels of KEAP1, and continue to express NRF2, but at moderate, steady levels) would be a more valuable genetic model for studying the positive effects associated with NRF2 activation, without the negative effects experienced by Keap1 knockout mice. Scientists have shown that NRF2 activation can help protect mice against numerous aging-associated diseases and disorders, such as hearing loss (age-related), and salivary gland dysfunction, as well as cognitive decline, and a number of other phenotypes associated with aging (Oishi et al., 2020; Wati et al., 2020; Uruno et al., 2020).

There is great complexity to the connection between cancer and NRF2; it can depend upon the circumstances. When NRF2 is activated in normal cells, it is thought to have cytoprotective effects and inhibit the development of cancerous tumours at the very beginning stages. However, in many types of cancers such as lung cancer, head and neck cancer, and urinary tract cancers, somatic mutations that give function to NRF2 or inhibit the function of KEAP1 are common. When cancer cells have already established themselves, they derive a selective survival benefit from being continuously activated via NRF2 because of an increased ability to handle oxidative stress, a metabolic reprogramming to utilize resources via anabolic pathways (building things up), and the production of proteins that transport drugs (known as efflux transporters) out of the cell or detoxify (render inactive) chemotherapeutic agents (Kitamura & Motohashi, 2018). This "NRF2 Addiction" in cancer represents an enormous clinical obstacle that is receiving more attention from researchers pursuing the development of NRF2 inhibitors to use as adjuvant therapies in the treatment of cancer.

The aging-specific aspects of KEAP1–NRF2 regulation have been systematically investigated by Matsumaru and Motohashi (2021) in a comprehensive review that examined the pathway's contributions to aging in sensory organs, glandular structures, skeletal muscle, and the central nervous system. This work established a conceptual framework in which the progressive decline in NRF2 activity with advancing age — mediated by transcriptional, post-transcriptional, and post-translational mechanisms — creates a state of antioxidant insufficiency that amplifies age-related tissue damage and dysfunction. Importantly, this review also demonstrated that pharmacological restoration of NRF2 activity, whether through electrophilic NRF2 inducers, disruption of the KEAP1-NRF2 protein-protein interaction, or natural dietary compounds, can attenuate multiple age-related phenotypes, establishing the conceptual basis for NRF2-targeted anti-aging interventions.

2.2 Chemistry of KEAP1 Activation

The chemical basis of KEAP1 sensing of oxidants and electrophiles has been a subject of intensive investigation. The key insight that emerged from early biochemical studies is that KEAP1 contains an unusually high number of cysteine residues — 27 in human KEAP1 — with several of these displaying markedly enhanced reactivity relative to the average protein thiol. This enhanced reactivity reflects the microenvironmental influence of neighbouring basic amino acids (Lys and Arg residues) on the pKa of nearby cysteine thiols, lowering it from the typical value of approximately 8.3 in unstructured peptides to values as low as 5–6 in certain structural contexts. At the physiological intracellular pH of 7.2–7.4, these low-pKa cysteines exist predominantly in their thiolate anion form ($–S^-$), which is substantially more nucleophilic than the protonated thiol form (–SH) and therefore much more reactive toward electrophilic and oxidizing reagents.

Depending on the modifying agent's reactivity, there are multiple types of reactions associated with the thiol chemistry of KEAP1 cysteine modifications. The reaction of hydrogen peroxide and organic hydroperoxides with KEAP1 cysteines proceeds via formation of a sulfenic acid (–SOH) as the initial step of sulfonic acid ($–SO_2H$) or sulfonic acid ($–SO_3H$) formation due to oxidative stress at the more extensively modified sites. When trying to increase the extent of oxidation, intramolecular disulfides are generated between different KEAP1 cysteines, including Cys273 and Cys288 within the IVR domain and Cys151 and other Cys residues within the BTB domain. The formation of

intramolecular disulfides results in a conformational change in KEAP1, which inhibits its ability to promote NRF2 ubiquitination. In addition to sulfenic acid, KEAP1 cysteines can undergo reversible glutathionylation by forming mixed disulfides with glutathione, which can temporarily prevent KEAP1 from performing its normal functions and protect sensor cysteines from becoming permanently hyperoxidized.

Key electrophile regulators of KEAP1 are shown to have electrophilic modulation of KEAP1 (e.g., chemical electrophiles may irreversibly modify functional site thiol (-SH) adducts on KEAP1). Among these electrophiles are naturally occurring foods high in antioxidants like sulforaphane and synthetic drugs such as the proton pump inhibitors omeprazole and esomeprazole, as well as environmental toxicants (in particular, those found in gasoline and other combustibles) that cause some cancers. The characterization of the cysteine code - where individual electrophiles characteristically covalently attach to different subsets of KEAP1 cysteine residues to activate distinct sets of NRF2 response element (ARE) target genes - provided a significant advancement in understanding the specificity of the electrophilic counterattack response (Suzuki 2019). The KEAP1 cysteine code has been stratified into four distinct electrophile "groups" based on which specific KEAP1 cysteine residues (See Table 1): Electrophiles A - ultimately react primarily with Cys151; B - primarily react with Cys273 and Cys288; C - react with both Cys151 and Cys273/288, and; D - react with KEAP1 characteristically throughout the entire protein and all four groups share the common characteristic that they modify the redox-sensitive thiol (–SH) constituents of cysteines on KEAP1.

The structural consequences of KEAP1 cysteine modification have been investigated by crystallography, cryo-electron microscopy, and various biophysical techniques. Modification of Cys273 and Cys288 in the IVR domain disrupts the ability of KEAP1 to maintain NRF2 in the ubiquitination-productive conformation by altering the geometry of the two-site NRF2 binding arrangement. Specifically, the bivalent binding model requires that the ETGE motif of NRF2 be bound to one KEAP1 Kelch domain and the DLG motif to the Kelch domain of the other KEAP1 in the homodimer, positioning multiple lysine residues in the Neh2 domain of NRF2 within reach of the ubiquitination machinery. Disruption of this geometry by KEAP1 cysteine modification prevents the proper alignment of NRF2 for ubiquitin transfer, explaining how relatively subtle chemical modifications to KEAP1 can have profound effects on NRF2 stability and transcriptional activity.

The concept of hormesis — the phenomenon by which low-dose exposures to toxicants or stressors elicit beneficial adaptive responses while high-dose exposures are harmful — is particularly relevant to the activation of NRF2 by electrophiles. The electrophilic counterattack response, first described by Talalay and colleagues, represents a classic hormetic mechanism in which low-level exposure to reactive compounds activates the NRF2-dependent antioxidant program, rendering cells resistant to subsequent higher-level oxidative insults. Sesamin, given its structural features including the methylenedioxy groups that can potentially undergo metabolic activation to quinone methide species, may participate in electrophilic activation of KEAP1 cysteines in addition to its kinase-mediated mechanism of NRF2 activation, though direct evidence for covalent KEAP1 modification by sesamin or its metabolites requires further investigation.

2.3 Sesamin and Antioxidant Activity

The antioxidant properties of sesamin have been extensively investigated using a range of in vitro and in vivo experimental systems. Early studies established that sesamin could scavenge free radicals directly, though its radical scavenging potency in simple in vitro assays was generally lower than that of well-established antioxidants such as vitamin E. From a biological standpoint, a more important mechanism of sesamin is its ability to induce endogenous systems of antioxidants, which are able to produce chronic and amplified antioxidant actions, far greater than those produced by direct scavenging.

Bai et al. (2019) provided a particularly detailed examination of the effects of sesamin on oxidative stress, induced by H_2O_2, in Caco-2 colorectal adenocarcinoma cells and in DSS-induced acute colitis mouse models. The authors established that sesamin pretreatment significantly decreased the accumulation of intracellular reactive oxygen species (ROS) in a dose-dependent manner; protection began to reach statistical significance at concentrations equal to 20 μM, with maximum protection occurring at 40-80 μM. Mechanistically, sesamin elevated intracellular GSH levels by approximately 2.5-fold at 40 μM and 3-fold at 80 μM without affecting GSSG levels, indicating enhanced de novo GSH synthesis rather than recycling of oxidized glutathione. The importance of GSH in mediating sesamin's cytoprotective effect was confirmed by the observation that pretreatment with L-buthionine sulfoximine (BSO), a specific inhibitor of GCL and therefore of GSH synthesis, markedly attenuated sesamin's protective effect against H_2O_2-induced cell death.

Luciferase reporter assays using an ARE-driven reporter construct demonstrated that sesamin significantly increased NRF2 transcriptional activity at 8 hours post-treatment, with the effect most pronounced at concentrations of 20–80 μM and diminishing at later time points (16 and 24 hours), consistent with the transient nature of NRF2 activation that is characteristic of a well-regulated stress response (Bai et al., 2019). Quantitative reverse-transcription PCR analysis confirmed corresponding increases in the mRNA levels of NRF2 target genes including HO-1 (approximately 2-fold increase), NQO1 (approximately 1.5-fold), GCLC (approximately 1.7-fold), GCLM (approximately 1.4-fold), and GR (approximately 2.2-fold) at the same time point. Western blot analysis confirmed corresponding increases at the protein level, with nuclear NRF2 protein showing a particularly strong increase (approximately 3-fold at 40–80 μM) that was already apparent at 2 hours post-treatment and further enhanced by 8 hours.

The in vivo relevance of sesamin's antioxidant and NRF2-activating effects was established in the DSS-induced acute colitis mouse model. A dose of 3% DSS in drinking water for C57BL/6 mice resulted in significant colonic damage (colitis) characterized by weight loss, bloody diarrhoea, shortened colon length, typical histological changes (epithelial destruction and crypt abscess) and increases in spleen weight, levels of certain cytokines (IL-6, IL-1β, TNF-α), inhibition of colonic glutathione and superoxide dismutase activity, and increased levels of malondialdehyde. Intragastric administration of sesamin at either 50 or 100 mg/kg/day significantly decreased all of the above parameters measured, with 100 mg/kg/day virtually restoring levels of pro-inflammatory cytokines and antioxidant markers to near control levels. Sesamin at 50 mg/kg produced a greater beneficial effect than 5-aminosalicylic acid (5-ASA) — which is today’s typical treatment for patients with ulcerative colitis — after they had recovered from DSS withdrawal (Bai et al., 2019). Also, Hamada et al. (2011) showed for the first time that a metabolite of sesamin could stimulate HO-1 expression in neuroblastoma (PC12) cells via NRF2/ARE activation; this was significant for both establishing sesamin’s ability to activate the NRF2 pathway in a neuronal cell and confirming that sesamin metabolites (which are produced when sesamin is metabolized by the liver and intestines) contribute to the overall biological effects of sesamin in the diet. Finally, Lahaie-Collins et al. (2008) demonstrated that sesamin could also alter the expression of numerous genes involved in the oxidative stress response in dopaminergic cells exposed to MPP^+, including tyrosine hydroxylase,

superoxide dismutase, catalase, and inducible nitric oxide synthase; thus, indicating that sesamin has a potentially extensive protective effect on dopaminergic neurons.

2.4 Sesamin in Neuroprotection and Aging

Many studies have been conducted on the neuroprotective properties of sesamin and different experimental systems have produced overlapping results that suggest that the KEAP1–NRF2 pathway is the major mechanism through which this compound protects the nervous system. Neuronal cells are particularly vulnerable to oxidative damage due to their high metabolic rate, limited regenerative capacity, high content of polyunsaturated fatty acids in membrane phospholipids, and relatively modest constitutive antioxidant defences compared to astrocytes and other glial cells. The progressive accumulation of oxidative damage in neurons over decades of life is now recognized as a major contributor to age-related cognitive decline and the pathogenesis of neurodegenerative diseases.

The most comprehensive analysis of sesamin's in vivo neuroprotective effects across aging biology was provided by Le and Inoue (2021), who employed Drosophila melanogaster as a model organism to investigate the effects of sesamin feeding on age-related neuronal loss and NRF2/Cnc-dependent transcription in adult brains. Drosophila offers unique experimental advantages for aging research, including a short lifespan, well-established genetic tools, and high degree of conservation of fundamental cellular processes with mammals. The study used a Sod1 mutant background (a fly model of accelerated aging and oxidative stress due to loss of Cu/Zn superoxide dismutase activity) to evaluate sesamin's capacity to suppress aging-related phenotypes including locomotor decline, protein aggregate accumulation in muscles, and loss of dopaminergic neurons in the brain.

Le and Inoue (2021) first established that sesamin feeding at 2 mg/mL (dissolved in 0.5% DMSO in the food medium) significantly stimulated ARE-GFP reporter expression in whole bodies, brains, and gut of adult Drosophila compared to DMSO vehicle controls, with the NRF2/Cnc-dependent transcriptional activation being particularly prominent in neuronal cells. Using neuron-type-specific Gal4 drivers to direct expression of the ARE-GFP reporter selectively in defined neuronal populations, they found that sesamin strongly activated NRF2/Cnc-dependent transcription in glutamatergic neurons (which represent the majority of excitatory neurons in the brain), cholinergic neurons (critical for learning and memory), and dopaminergic/serotonergic neurons (relevant to Parkinson's disease pathology), but showed no significant effect in GABAergic neurons or mushroom body

neurons. This remarkable neuron-type specificity of sesamin's NRF2-activating effects raises intriguing questions about the differential expression or activity of signalling components that mediate sesamin's effects in different neuronal subtypes.

The observation that sesamin stimulated NRF2/Cnc-dependent transcription in Drosophila neurons in the absence of exogenous oxidative stress — an experimental condition designed to reveal the intrinsic NRF2-activating capacity of sesamin independent of any reactive chemistry — is particularly significant. This major finding shows how sesamin differs from the variety of electrophilic NRF2 activators (e.g., sulforaphane, curcumin, etc.) that use their electrophilic properties to react with covalent adducts on cysteine residues at the KEAP1 protein to activate NRF2. Instead of using the electrophilic properties of sesamin to make covalent adducts, it activates NRF2 via receptor and/or kinase-mediated signalling events, which may be initiated in the absence of redox stress. This proposes that sesamin's NRF2-activating actions are mediated through AKT and ERK activation, and do not require covalent modification of the cysteines on KEAP1 (Bai et al., 2019).

Studies have documented that sesamin has a broader neuroprotective effect in aging animal models. For example, Hou et al. (2003) demonstrated that sesame antioxidant components, including sesamin, inhibited LPS-induced NO production by BV2 microglial cell cultures, indicating that the sesame antioxidants can reduce neuroinflammation. Additionally, Lahaie-Collins (2006) shown when PD model cells were exposed to sesamin, that they were able to modulate the activity of a large number of neuroprotective markers, supporting the conclusion that sesamin can also provide neuroprotection through the activation of multiple antioxidant, anti-inflammatory, and survival signalling pathways with NRF2 activation as the common hub for these diverse actions.

2.5 KEAP1–NRF2 in Diseases

The KEAP1–NRF2 signalling pathway plays an important part in the development of many different types of human disease due to its essential function as a cellular defence against stress and maintaining cellular stability. The type of disease documented to result from NRF2 dysfunction includes every type of human organ system, from excess to not enough NRF2. That is, diseases caused by excess oxidative stress feature too little NRF2 for cytoprotection, while disorders caused by excess activation of NRF2 include certain cancers that utilise NRF2 to survive and resist therapy.

When it comes to neurodegeneration, there is strong evidence of the role of Nrf2 (nuclear factor-E2-related factor 2) as an important part in the processes involved. Postmortem tissue samples from patients with Alzheimer's disease (AD) have shown increased levels of markers for oxidative stress, including protein carbonyls, 8-oxoguanine, 4-HNE adducts and isoprostanes, along with decreased levels of antioxidant enzyme activity and reduced levels of glutathione. Independent studies have consistently found that there are changes in both expression and activity of Nrf2 in AD brain tissues, but the exact nature and degree of these changes may vary by brain region and stage of the disease process. During early or mild cognitive impairment (MCI), it is possible to see reversible upregulation of Nrf2 target genes as a mechanism for protecting against increasing oxidative stress. However, during later stages of AD, cumulative damage to the regulatory machinery of Nrf2 may ultimately lead to an irreversible loss of Nrf2 function. Genetic studies using mouse models investigating Nrf2 have been particularly informative: Nrf2 knockout mice have greater pathology related to AD in multiple models of amyloid precursor protein (APP)/tauopathy, whereas Keap1 knockdown mice that have moderate Nrf2 activation show reduced amyloid plaque burden, lessened neuroinflammation and improved cognitive function in the App-NL-G-F knock-in model (Uruno et al., 2020).

The KEAP1-NRF2 system is disturbed in the pathogenesis of Parkinson's disease, as already established in human post-Mortem Collections and various animal models. Dopaminergic neurons in the substantia nigra are commonly reported to be selectively vulnerable to oxidative stress in PD, due to the fact that they have a high intrinsic oxidative burden stemming from the metabolism of dopamine which produces hydrogen peroxide (H_2O_2) and quinones, thereby allowing KO-PD neurons to depend heavily on NRF2 receptor function. Treatment with NRF2 activating agents, such as 6-MSI and dimethyl fumarate, has been shown to protect the dopaminergic neuron population and reduce neuroinflammation in PD in MPTP mice and 6-OHDA PD mouse models (Morroni et al., 2014; Lastres-Becker et al., 2016).

The Nrf2 protein is also found to be important when studying cardiovascular disease. The effects of oxidative stress can lead to many diseases associated with the heart including oxidative damage (cellular injury caused by free radicals), atherosclerosis (plaque build-up in the arteries), high blood pressure, ischemia-reperfusion injury (cell damage due to low blood flow) and heart failure (inability of the heart to pump enough blood). Nrf2 protects the heart by reducing the amount of oxidative injury that occur to the

cells lining blood vessels, reducing the amount of oxidized LDL cholesterol in the blood, inhibiting the activation of NLRP3 inflammatory proteins, and maintaining normal functioning of mitochondria within heart muscle cells.

There is increasing recognition of the role of NRF2 in metabolic disorders such as obesity and diabetes. Activation of NRF2 leads to improved insulin sensitivity, increased glucose uptake, suppression of fat formation, and decreased inflammation in fat cells. However, long-term activation of NRF2 could disturb the body's balance (metabolic homeostasis) by increasing glucose use by rapidly dividing cells and blocking the breakdown of fat. Therefore, the relationship between NRF2 and metabolism is very complex, and the use of NRF2 for treating metabolic disorders must be carefully thought out with respect to when and how long NRF2 is activated as well as which type of tissue is affected by NRF2 activation.

2.6 Research Gaps

In spite of the large body of knowledge on the KEAP1-NRF2 pathway and the antioxidant effects of sesamin, a lack of research remains that limits our ability to fully understand how sesamin can be used therapeutically and how NRF2-targeting approaches could be applied to age-related diseases and neurodegenerative disorders.

To our knowledge, while evidence for the kinase-mediated activation of NRF2 by sesamin through AKT and ERK has been shown in a Caco-2 cell line (Bai et al., 2019), the upstream receptor or membrane-level interactions that mediate this kinase signal as a result of sesamin are not yet clearly defined. Sesamin is a lipophilic compound and may bind to membrane receptors by changing membrane lipid composition or by entering cells and interacting with intracellular signal proteins. However, the precise molecular target(s) responsible for initiating AKT and ERK phosphorylation remain unidentified. Once established, the primary molecular target of sesamin will provide an improved understanding of sesamin's mechanism of action, which may also allow for the design of more potent and specific analogues.

The specificity of sesamin's NRF2-activating properties across different types of neurons within Drosophila adult brains is the second notable observation, as sesamin was significantly able to activate NRF2 activity in glutamatergic, cholinergic, and dopaminergic neurons but not in GABAergic neurons; however, the molecular mechanism for this neuron-type specificity remains unclear. There may be several contributing factors that will

need to be investigated further in future studies, including differences in expression of signalling molecules, metabolism of sesamin in different types of neurons, and basal levels of NRF2/Cnc that are present prior to sesamin treatment, all of which will have an effect on how easily activation of NRF2 occurs after sesamin administration.

While in vitro and Drosophila in vivo studies provide strong evidence to support that sesamin can activate NRF2, there is currently limited experimental data examining the ability of orally administered sesamin to activate NRF2 using pharmacodynamic studies conducted using naturally aged mammals. Therefore, future studies are needed to determine whether being able to orally administer sesamin at physiologically relevant doses will activate NRF2 signalling in the brains of naturally aged mammals, and whether these NRF2-dependent changes will produce sufficient functional improvements in this specific population.

Fourthly, there is presently no systematic assessment of potential interactions between the NRF2-activating effects of sesamin and the chronic inflammation associated with aged tissues as a result of SASP. Since NRF2 activation is responsible for suppressing the transcription of NF-κB-driven inflammation-related genes, sesamin might be considered to have a potential effect for reducing SASP in senescent cells. However, no studies have been conducted to compare the effectiveness of sesamin against the antioxidant benefits of sesamin, nor has a proper dose-response determination been made so that sesamin can be utilized maximally as an anti-aging intervention while minimizing the possibility of negative side effects or complications. These areas of research should be pursued so that a case can be made for sesamin's use in the treatment of geriatric conditions.

CHAPTER 3: CHEMICAL MECHANISM OF KEAP1–NRF2

The KEAP1-NRF2 pathway represents an integral method of communication through chemicals – a dialogue between reactive oxygen (ROS) or electrophile species and the cystine residues (which are sensitive to oxidation) of the KEAP1 protein. To truly understand how cells sense oxidative stress and subsequently activate an appropriate transcriptional response, it is necessary to have a thorough understanding of this molecular conversation at the level of each individual chemical reaction, bond formation, and conformation alteration. This chapter provides a detailed analysis of the chemistry underpinning each major step in the KEAP1–NRF2 signalling cascade, from the initial generation and chemistry of reactive oxygen species through the thiol modifications of KEAP1, the stabilization and nuclear translocation of NRF2, the biochemistry of glutathione metabolism, and the kinase-mediated regulatory circuits involving AKT and ERK that modulate NRF2 activity. The molecular mechanism by which sesamin engages and activates this pathway is discussed in the final section.

3.1 Redox Chemistry and ROS Generation

The chemistry of superoxide generation begins at the electron transport chain, where thermodynamically unfavourable single-electron reductions of dioxygen occur at multiple sites. At Complex I (NADH: ubiquinone oxidoreductase), electrons can leak from the flavin mononucleotide (FMN) cofactor or from iron-sulphur clusters, particularly under conditions of high mitochondrial membrane potential and $NADH/NAD^+$ ratio that slow the forward electron flow. Ubiquinol-cytochrome c reductase, or Complex III, produces an ubisemiquinone intermediate during the Q cycle, some of which can leak their second electron to generate superoxide. Superoxide leakage follows a stoichiometric relationship with the electron carriers' reduction state and also depends on the concentration of oxygen around these electron carriers. Superoxide production at Complexes I and III is generated primarily within the mitochondrial matrix and partially in the intermembrane space, which restricts the effects of these superoxide to the compartment of the mitochondria for the most part.

The enzymatic dismutation of superoxide to hydrogen peroxide, catalysed by superoxide dismutase, follows the reaction: $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$. The rate constant for this reaction is approximately $2 \times 10^9\ M^{-1}s^{-1}$ for the enzymatic reaction, vastly

exceeding the rate of spontaneous dismutation. The product H_2O_2 is considerably less reactive than superoxide and diffuses readily across biological membranes, allowing it to function as a second messenger. However, in the presence of transition metal ions — particularly Fe^{2+} and Cu^{+} — H_2O_2 undergoes Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\bullet}OH + OH^{-}$) to generate the hydroxyl radical (•OH), the most potent and non-selective oxidant encountered in biological systems with a rate constant for reaction with biological molecules exceeding 10^{10} $M^{-1}s^{-1}$.

The hydroxyl radical reacts with virtually every class of biological molecule at near diffusion-limited rates. Its reaction with polyunsaturated fatty acids initiates a self-propagating chain reaction of lipid peroxidation through the sequence of hydrogen abstraction, oxygen addition to the carbon-centred radical, and chain transfer reactions that generate multiple reactive aldehydic byproducts. Its reaction with deoxyribose in DNA generates strand breaks, while its reaction with guanine produces 8-oxoguanine, the most abundant and best-studied form of oxidative DNA base damage. Its reaction with amino acid residues in proteins produces protein carbonyls, methionine sulfoxide, and tyrosine nitration products that serve as biomarkers of oxidative stress and contribute to loss of protein function.

3.2NRF2 Stabilization and Nuclear Translocation

The stabilization of NRF2 as a result of the KEAP1 being inactivated and then transported into the nucleus does not occur diffusely but rather through several active-regulatory mechanisms that provide direction for the transport of NRF2 to the appropriate intracellular location and align NRF2 with its correct association with chromatin. The molecular understanding of this process provides insight into the sophistication of the NRF2 regulatory process as well as potential avenues for therapeutic intervention.

Under normal conditions, newly synthesized NRF2 is rapidly captured by KEAP1 in the cytoplasm. The interaction is initiated by high-affinity binding of the ETGE motif (sequence: DEETGE) within the Neh2 domain of NRF2 to the bottom face of the Kelch β-propeller of one KEAP1 molecule in the homodimer. This initial interaction has a Kd of approximately 5 nM, making it one of the tightest protein-protein interactions known for a regulatory complex. Subsequent engagement of the lower-affinity DLG motif (Kd approximately 1 μM) with the Kelch domain of the other KEAP1 molecule in the homodimer positions multiple lysine residues in the Neh2 domain — particularly Lys44,

Lys50, Lys52, Lys53, Lys56, Lys62, Lys68 — within proximity of the ubiquitin transfer machinery for polyubiquitination. This 'two-site binding, one-target ubiquitination' model explains why a single KEAP1 homodimer can efficiently ubiquitinate a single NRF2 molecule: the bivalent interaction provides the geometric precision required for productive ubiquitin transfer.

When KEAP1 cysteine modifications reduce the efficiency of this ubiquitination process, newly synthesized NRF2 accumulates in the cytoplasm and acquires additional post-translational modifications that promote its nuclear entry. Phosphorylation of NRF2 at specific serine residues by PI3K/AKT downstream kinases (including p70 S6 kinase) and by ERK1/2 demonstrates the capacity to promote NRF2 nuclear accumulation through multiple mechanisms including masking of a KEAP1-dependent cytoplasmic retention sequence and enhancing interaction with importins. The importin α/β-mediated nuclear import pathway recognizes the NLS within the Neh1 domain of NRF2, and the NLS accessibility and activity are enhanced by the phosphorylation events associated with active NRF2 signalling.

Once in the nucleus, NRF2 must locate and bind ARE-containing promoters with within the scope of chromatin, which presents both physical barriers to transcription factor binding and regulatory opportunities. NRF2 heterodimerization with sMAF proteins (sMafF, sMafG, sMafK) is an absolute requirement for ARE binding, as neither NRF2 nor sMAF alone can efficiently engage the ARE sequence. The bZIP domains of both NRF2 and sMAF contribute to the leucine zipper-mediated heterodimer formation, while the adjacent basic regions cooperate to contact the major groove of the ARE DNA sequence. The CNC domain of NRF2 provides additional contacts that confer specificity for the ARE/EpRE sequence compared to the related Maf response element (MARE) bound by sMAF homodimers. The recruitment of coactivators including CBP/p300 to the NRF2-sMaf complex further amplifies transcriptional activation by stabilizing the preinitiation complex and promoting chromatin remodelling at ARE-containing promoters.

The nuclear export and subsequent cytoplasmic degradation of NRF2 following resolution of the oxidative stress signal is mediated by the leucine-rich nuclear export sequence (NES) within the Neh5 domain, which, when activated by Crm1/exportin-1-dependent export mechanisms, returns NRF2 to the cytoplasm where it can again be captured and ubiquitinated by the newly regenerated (reduced) KEAP1. This nuclear-cytoplasmic shuttling mechanism ensures that NRF2 activity is precisely controlled in both

time and magnitude, with activation being transient in proportion to the duration and severity of the oxidative insult.

3.3 Glutathione Metabolism

Glutathione is the most common antioxidant found within cells, and it serves both as an intracellular antioxidant (the most plentiful) and as a substrate for a great number of detoxification reactions. Its tripeptide structure, featuring the unusual γ-glutamyl isopeptide linkage that confers resistance to most intracellular proteases, allows it to maintain high steady-state concentrations of 1–10 mM in most mammalian cells. The central role of glutathione in the NRF2 defence program — NRF2 simultaneously upregulates the biosynthetic enzymes, recycling enzymes, and cystine transport systems required for maintaining high glutathione levels — exemplifies the integrated nature of the NRF2 antioxidant response.

The biosynthesis of glutathione proceeds in two ATP-requiring enzymatic steps. In the first and rate-limiting step, glutamate-cysteine ligase (GCL, also known as gamma-glutamylcysteine synthetase) catalyses the condensation of L-glutamate and L-cysteine to form gamma-glutamylcysteine. The GCL holoenzyme consists of the catalytic subunit (GCLC, 73 kDa) and the modifier subunit (GCLM, 31 kDa). GCLC alone possesses catalytic activity, while GCLM, which has no catalytic activity of its own, enhances GCLC activity by increasing the affinity for substrate glutamate, decreasing the inhibition constant for the feedback inhibitor GSH, and stabilizing the holoenzyme complex against thermal inactivation. Both GCLC and GCLM genes contain functional AREs in their promoter regions and are strongly induced by NRF2 activation. In the second step, glutathione synthetase (GS) catalyses the condensation of gamma-glutamylcysteine with glycine to produce GSH, using ATP as an energy source. GS is also a NRF2 target gene, though with lower induction compared to the GCL subunits.

3.Molecular Mechanism of Sesamin

Based on the Mechanistic Data discussed, the Molecular Mechanism of Action for Sesamin Activation of the KEAP1-NRF2 Pathway is By Kinase-Dependent Mechanism and is Initiated at or near the Plasma Membrane and Progresses Through Intracellular Signalling Pathways to Result in NRF2-Dependent Gene Expression. This Mechanistic Pathway is Unlike That of Classical Electrophilic NRF2 Inducers Like Sulforaphane, with Implications for Both Its Safety and Broader Range of Biological Effects.

It seems that the event that causes sesamin to activate NRF2 is through phosphorylating AKT and ERK1/2. There is currently not enough information about the signal that connects sesamin to kinase activation, but researchers have many ideas based on the chemistry of sesamin and other plant-derived lignans. One possibility is that sesamin touches lipid rafts on the outside of cells or attaches itself to associated membrane proteins to change their shape so they can communicate with phosphoinositol-3-kinase (PI3K) or Ras. Another possibility is that sesamin has the potential to passively cross into the plasma membrane because it has a fat-soluble nature and then subsequently affect how far aminergic growth factor receptors move laterally in a side-to-side manner or become clustered together so they can interact better with the ligand that activates them from inside the cell (i.e., use their own ligands much more quickly). Additionally, sesamin's metabolites may directly activate enzymes or receptors by acting as reducing agents or mimicking a ligand. The primary metabolites produced from sesamin via CYP450-mediated demethylation (e.g., catechol) are both reducing agents.

The downstream signalling pathway will follow the same steps through the established signalling pathways (PI3K/AKT and MEK/ERK) to produce phosphorylated Akt (at Thr308) and phosphorylated ERK1/2 (at Thr202/Tyr204) regardless of how they start off. Each pathway will target GSK3β and inhibit its function through Akt phosphorylation, which prevents GSK3β from phosphorylating NRF2 and leading to its β-TrCP dependent degradation. Active ERK1/2 will phosphorylate and stabilize NRF2 at multiple sites and promote the import of NRF2 into the nucleus. At the same time, possibly as a result of increased NRF2 activity, the levels of KEAP1 will decrease; thus, reducing the KEAP1 dependent ubiquitinylation of NRF2 and increasing the NRF2 response. All these combined effects produce a proportional increase in nuclear NRF2 protein levels (approximately 3x at 40-80 μM sesamin) after 8 hours and a subsequent increase in ARE dependent gene expression.

The finding that sesamin can activate NRF2/Cnc-dependent transcription in Drosophila neurons without the requirement of oxidative stress (Le and Inoue, 2021) is in line with the aforementioned kinase-mediated mechanisms, as growth factors have been shown to activate kinase cascades independent of redox chemistry. In contrast, if sesamin were an electrophilic KEAP1 modifier, one would expect its NRF2 activations to rely on the formation of reactive metabolites and would not occur without metabolic activation. Therefore, the ability of sesamin to activate NRF2 in both the absence of oxidative

conditions as shown in the Drosophila models provides compelling evidence for the superiority of the kinase-mediated mechanism. This is further supported by the fact that sesamin activates NRF2 in particular types of neurons in Drosophila brains, likely due to the differential expression of proteins that participate in the relevant signal transduction pathways rather than as a result of non-specific chemical reactivity, which would be predicted to affect all cell types equally.

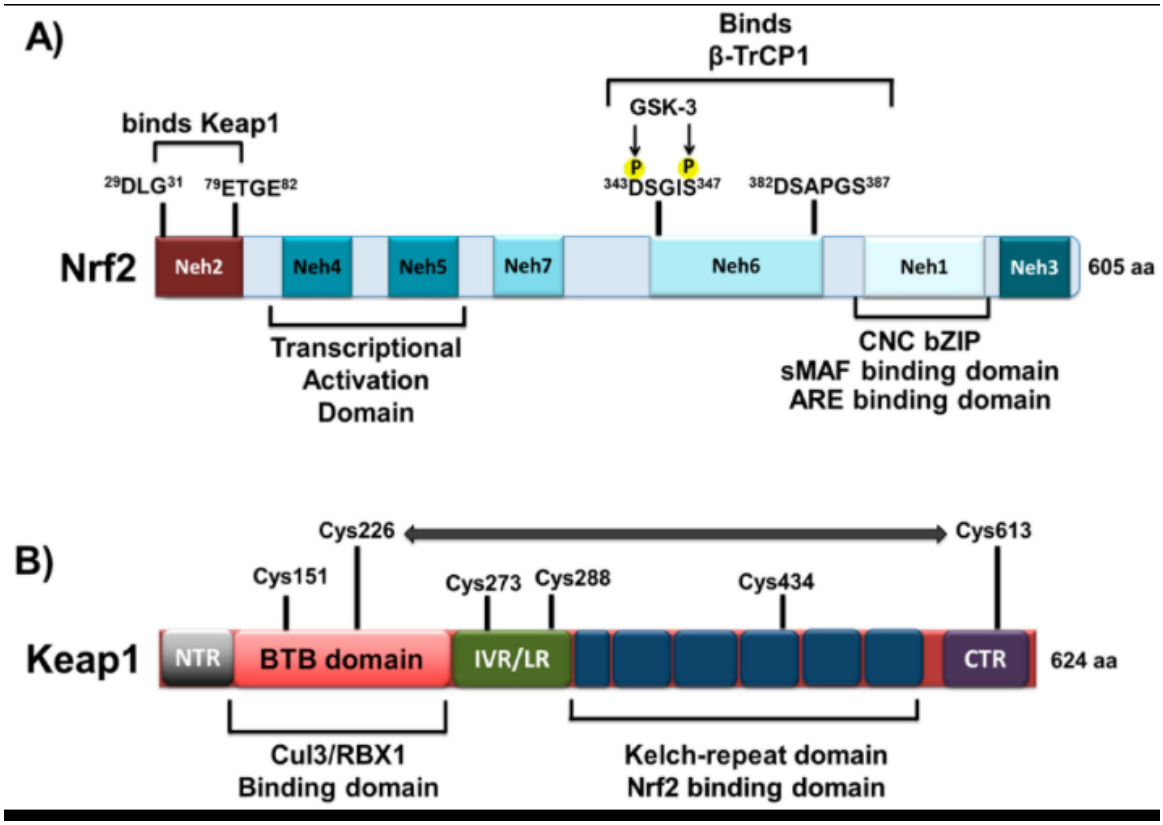

FIG – STRUCTURE OF Nrf2 AND Keap 1 PROTEINS

CHAPTER 4: BIOLOGICAL AND THERAPEUTIC IMPORTANCE

The biological importance of the KEAP1–NRF2 signalling pathway exceeds its well-known function as a transcriptional regulator for antioxidant genes. The NRF2 transcription factor has broad pleiotropic effects on cellular physiology, and by regulating many of its own target genes, the NRF2 pathway also regulates the cellular processes of detoxification, anti-inflammatory signalling, metabolism and autophagy, all of which have critical implications for health and disease across multiple organ systems. Increasing evidence that activation of the NRF2 signalling pathway by natural compounds like sesamin can protect from inflammation, ageing, neurodegeneration and many other types of disease has resulted in a great deal of interest in this signalling pathway for potential therapeutic applications. This chapter reviews systematically all the biological and therapeutic angles of this pathway.

4.1 Role in Inflammation

The biological processes of oxidative stress and inflammation are intimately connected, where each process will strengthen the effects of the other and cause additional stress, in turn. For example, the presence of reactive oxygen species (ROS) as a result of oxidative stress leads to the activation of multiple NF-κB-dependent genes that encode for inflammatory proteins. In response to pro-inflammatory cytokines, both neutrophils and macrophages produce ROS as part of their oxidative burst, adding further to the oxidative load on tissue. The KEAP1–NRF2 pathway serves as a critical molecular switch that can interrupt this cycle by simultaneously reducing oxidative stress and suppressing inflammatory gene transcription through multiple mechanisms.

The anti-inflammatory effects of sesamin have been demonstrated in multiple experimental contexts. In the DSS-induced colitis model, sesamin at 50 and 100 mg/kg significantly reduced colonic levels of IL-6, IL-1β, and TNF-α, with the 100 mg/kg dose restoring all three cytokines essentially to control levels (Bai et al., 2019). This anti-inflammatory effect was accompanied by restoration of colonic NRF2 signalling and antioxidant enzyme activity, indicating that the anti-inflammatory activity is at least partly mediated through NRF2-dependent mechanisms. Earlier studies demonstrated that sesamin could inhibit LPS-induced inflammation in various cell types and animal models, including suppression

of nitric oxide production by microglial cells (Hou et al., 2003) and inhibition of LPS-induced acute lung injury through inhibition of the TLR4 signalling pathway. Sesamin also protects human endothelial cells against HMGB1-induced vascular inflammatory responses, suggesting potential cardiovascular anti-inflammatory activity (Lee et al., 2013).

The connection between NRF2 and the activation of the NLRP3 inflammasome is of increasing interest given the importance of liver cells in aging-related inflammation. The NLRP3 inflammasome is a complex made up of many proteins, and its role is to detect signals that indicate cellular damage (pathological), thus resulting in the stimulation of caspase-1 and the maturation of pro-inflammatory cytokines IL-1β and IL-18. It is imperative that more investigators recognize the NLRP3 inflammasome as a significant contributor to ongoing, sterile, age-related inflammatory processes within tissues. One-way NRF2 inhibits the activation of the NLRP3 inflammasome is to promote pathways that reduce the level of ROS produced within the mitochondria since ROS are responsible for causing NLRP3 activation; NRF2 activation also promotes TXNIP downregulation and reduces potassium efflux from cells, both of which contribute to inhibiting NLRP3 activation. Collectively, the inhibition of NLRP3 seems to play a role in the actions of sesamin in age-related inflammatory disease.

4.2 Role in Ageing and Longevity

Across several model organisms such as yeast, nematodes, fruit flies and rodents, the connection between KEAP1-NRF2 pathway and aging has been researched extensively. All data support that appropriate NRF2 activity is conserved as a key determinant of lifespan and health span in all organisms. These studies have also revealed the important nuance that the relationship between NRF2 activity and longevity is not monotonic but follows an inverted U-shaped curve, with mild-to-moderate NRF2 activation being beneficial for longevity and health while excessive or persistent NRF2 activation can paradoxically accelerate aging and impair fitness.

In Caenorhabditis elegans, the NRF2 ortholog SKN-1 is a well-established positive regulator of lifespan and stress resistance. Constitutive nuclear accumulation of SKN-1, achieved either genetically or through pharmacological inhibition of the KEAP1 ortholog WDR-23, extends lifespan and increases resistance to multiple stressors. Interestingly, the mechanisms by which SKN-1 extends lifespan are partially distinct from those mediating its effects on stress resistance, as demonstrated by the observation that DAF-16/FoxO

overexpression can rescue the shortened lifespan but not the oxidative stress hypersensitivity of skn-1 mutants (Tullet et al., 2017). In Drosophila, keap1 loss-of-function mutations extend lifespan and increase oxidative stress tolerance in males, and mild NRF2/Cnc activation extends lifespan by modulating ROS levels and attenuating aging-related phenotypes, while strong activation paradoxically accelerates aging through metabolic deregulation (Sykiotis and Bohmann, 2008; Tsakiri et al., 2019).

In mammals, comparative studies have revealed a positive correlation between NRF2 activity and species lifespan. Lewis et al. (2015) demonstrated that naked mole-rats (Heterocephalus glaber), which live approximately 10 times longer than predicted by body size, have constitutively elevated NRF2 activity compared to shorter-lived rodents, and that there is a significant positive correlation between NRF2 activity and lifespan across nine rodent species. Constitutive activation of NRF2 has been observed in approximately 95% of bird species, which are characteristically long-lived relative to their body size, and may represent an adaptive mechanism counterbalancing the high ROS levels associated with flight metabolism (Castiglione et al., 2020).

The age-related decline in NRF2 activity in mammalian tissues is well documented and has been attributed to multiple mechanisms. Numerous microRNAs that exert their effects by targeting mRNA through binding to the 3′ UTR of the NRF2 gene product are found to be positively regulated in the aged tissues and have been linked to decreased levels of NRF2 protein, with a specific emphasis on miR-146a, miR-28, and miR-200a (Linna-Kuosmanen et al., 2018; Smith et al., 2015). In aged cells, there is a relative resistance of the Kelch domain of KEAP1 to oxidative inactivation, as compared with young cells, potentially resulting from age-dependent changes to the redox proteome and thus accessibility of KEAP1 sensor cysteine residues. In addition to the observed increased expression of KEAP1 in some aged tissues, these observations further favour the degradation of NRF2.

Findings suggest numerous therapeutic benefits due to this research project. Re-establishing NRF2 activity in older biological systems through medication has potential for reversing multiple signs of aging. Twelve weeks of providing aged (old) laboratory mice with a natural product called sulphoraphane increased NRF2, mitochondria, heart, exercise ability, glucose tolerance and skeletal muscle satellite cell activation—a major and systemic improvement in many different aspects of the physical aging process, demonstrating that NRF2 has the ability to improve multiple facets of healthy aggressive

aging. The life expectancy of male mice given a natural compound called Protandim to increase NRF2 was longer, possibly the strongest direct evidence of the ability of NRF2 to change an aging pattern in an animal (Strong et al., 2016). These investigations offer an evidence base for further evaluation of sesamin as a dietary component to activate NRF2 to promote healthy aging.

4.3 Neuroprotective Effects

The CNS is one of the most metabolically active and vulnerable to oxidative stress of all organs and tissues in your body — while it only represents 2% of your entire body mass, it utilizes 20% of the body's oxygen. Because neurons have a very high level of mitochondrial respiration, little capability of regenerating themselves, a large amount of oxidizable polyunsaturated fatty acids, and relatively low levels of basal antioxidant enzyme activities compared to astrocytes, they are particularly susceptible to oxidative damage. Age-related, progressive accumulation of oxidative damage coupled with a decrease in NRF2-mediated antioxidant defence results in conditions conducive to neurodegenerative pathogenesis in the ageing brain.

NRF2 serves a neuroprotective function that has been studied in detail using animal models of Alzheimer's disease and Parkinson's disease. In the APP/PS1 double-transgenic mouse model of Alzheimer's disease, Nrf2 was found to be a critical factor in regulating amyloid plaque accumulation, tau hyperphosphorylation, neuroinflammation and cognitive deficits. In contrast, the activation of NRF2 through Keap1 knockdown significantly reduced the number of plaques, decreased the expression of pro-inflammatory genes, increased glutathione synthesis and improved memory function in the hippocampus (Uruno et al., 2020). These findings establish NRF2 as both a modifier of AD pathology and a potential therapeutic target. In mouse models of PD, Nrf2 deficiency exacerbates MPTP-induced dopaminergic neuron loss and neuroinflammation, while NRF2-activating compounds, including dimethyl fumarate and 6-MSITC, protect dopaminergic neurons and improve behavioural outcomes (Lastres-Becker et al., 2016; Morroni et al., 2014).

The neuroprotective effects of sesamin have been most comprehensively documented in the Drosophila ageing model by Le and Inoue (2021). This study demonstrated that sesamin feeding at 2 mg/mL suppressed multiple ageing-related phenotypes in adult flies, including age-dependent accumulation of damaged proteins in muscles and neuronal loss in the brain, particularly affecting dopaminergic neurons. The

suppression of these ageing phenotypes was accompanied by stimulation of NRF2/Cnc-dependent transcription in multiple types of neurons, including glutamatergic and cholinergic neurons, which are the primary excitatory neurotransmitter systems in the brain and are selectively vulnerable in AD, and dopaminergic/serotonergic neurons, which are selectively lost in PD.

The finding that sesamin activates NRF2/Cnc-dependent transcription in neuronal cells even in the absence of exogenous oxidative stress is particularly significant from a neuroprotective perspective. Most known NRF2-activating electrophiles, while effective at inducing antioxidant defences, require their electrophilic or pro-oxidant chemistry to activate KEAP1, which means that they are activating the antioxidant response by creating a low-grade chemical stress. In contrast, sesamin's kinase-mediated mechanism of NRF2 activation — which operates through normal signal transduction pathways without requiring KEAP1 cysteine modification — is fundamentally a physiological stimulus rather than a chemical stressor. This distinction is clinically important because it suggests that sesamin could be used at lower doses and for longer periods without the toxicological concerns associated with electrophilic NRF2 inducers.

The apparent specificity of sesamin's NRF2-activating effects according to neuron type, namely activating glutamatergic, cholinergic and dopaminergic/serotonergic but not GABAergic neurons in the Drosophila brain, warrants additional evaluation relative to the relative vulnerability of the different neural substrates involved with neurodegenerative disease. The types of neurons that are lost in the greatest proportions in Alzheimer's disease include cholinergic neurons originating in the basal forebrain, and glutamatergic neurons located within the entorhinal cortex and hippocampus; in contrast, individuals with Parkinson's disease exhibit pronounced losses of dopaminergic neurons situated in the substantia nigra pars compacta. As an NRF2 activator that specifically activates NRF2 in the most highly vulnerable neuron types, sesamin may confer neuroprotective effects specifically to the neuronal types that are at greatest risk for age-associated neurodegeneration. Further study is needed on the underlying mechanism that accounts for this specificity (e.g., differential expression of upstream signalling pathways, differing rates of sesamin metabolism in the various neuron types, etc.).

Sesamin could function as a neuroprotectant via other mechanisms that might exist in addition to NRF2 action. Sesamin has been shown to inhibit cytochrome P450 enzymes that are responsible for the catabolism of vitamin E, possibly making this lipid-soluble

antioxidant more bioavailable for free radical scavenging in neurally localised tissues. Additionally, sesamin inhibits NF-κB-dependent expression of pro-inflammatory genes; decreases the production of nitric oxide from activated microglia; and influences mitochondrial function in neuronal cells. Thus, while sesamin's neuroprotective properties are likely multi-faceted, ultimately, they all seem to depend on NRF2 activation for an overall effect.

4.4 Role in Cancer and Other Diseases

The intricate relationship that exists between NRf2 and the development of cancer is bidirectional and complex in nature. The NRf2 pathway can serve two functions as an existing tumour suppressor in healthy cells and a pro-survival mechanism, which is taken advantage of by already established malignant cells. In normal tissues and in the early stages of carcinogenesis, NRF2 acts as a tumour suppressor by preventing oxidative DNA damage that drives mutagenesis, by enhancing the detoxification of carcinogenic electrophiles through induction of glutathione S-transferases and NQO1, and by reducing chronic inflammation that promotes tumour initiation and progression. Multiple preclinical investigators have established that dietary NRF2 activators, including sulforaphane, curcumin, and sesamin, can inhibit chemical carcinogenesis in animal models.

In contrast, in established tumours — particularly those harbouring somatic loss-of-function mutations in KEAP1 or gain-of-function mutations in NRF2 — constitutive NRF2 activation serves as a selective survival advantage. Persistent NRF2 activation in cancer cells enhances antioxidant capacity, drives metabolic reprogramming toward anabolic biosynthesis, promotes autophagy, and induces expression of drug efflux transporters and detoxification enzymes that mediate multidrug resistance. This 'NRF2 addiction' phenotype is observed in subsets of non-small cell lung cancer, squamous cell carcinoma of the head and neck, and urothelial carcinoma, among other tumour types, and is associated with poor prognosis and resistance to platinum-based chemotherapy and other first-line treatments (Kitamura and Motohashi, 2018).

In cardiovascular disease, NRF2 activation provides protection against multiple pathological processes. The induction of HO-1 by NRF2 in vascular endothelial cells reduces the expression of cell adhesion molecules (ICAM-1, VCAM-1) that recruit monocytes to sites of vascular inflammation, thereby attenuating the early stages of atherosclerosis. NRF2-induced antioxidant defences protect against LDL oxidation, a key

step in atherogenic plaque formation. In the heart, NRF2 activation during ischemia-reperfusion injury reduces infarct size by limiting oxidative damage during the burst of ROS generation that occurs upon blood flow restoration. Sesamin's reported cardioprotective effects, including blood pressure reduction and lipid-lowering activity, may reflect its capacity to activate NRF2 in vascular and cardiac tissues.

In type 2 diabetes and metabolic syndrome, NRF2 activation improves insulin sensitivity, reduce β-cell oxidative stress, and suppress adipose tissue inflammation. The NRF2 target gene NQO1 plays a particularly important role in metabolic regulation by maintaining the redox state of ubiquitous enzyme cofactors and by supporting mitochondrial electron transport efficiency. Sesamin has been reported to improve lipid metabolism by inhibiting hepatic Δ5 and Δ6 fatty acid desaturases, enzymes that generate highly unsaturated fatty acids that are substrates for lipid peroxidation, suggesting a dual mechanism by which sesamin reduces oxidative substrate availability while simultaneously enhancing the capacity to neutralize the ROS that do form.

4.5 Therapeutic Potential of Sesamin

The convergence of evidence from multiple experimental systems pointing to sesamin as a safe, orally bioavailable, brain-penetrant NRF2 activator with a kinase-mediated mechanism of action positions it as a highly attractive candidate for therapeutic development within the scope of oxidative stress-related aging and neurodegenerative diseases. There are multiple distinctive characteristics of sesamin that set it apart from other known NRf2 agonists and increase its overall potential for therapeutic use.

One unique quality of sesamin is how it activates NRf2 through a mechanism that is not considered electrophilic but rather kinase-mediated. As a result, this leads to a safer agent overall; in comparison, there are many existing types of NRf2 inducers that are classified as being electrophilic agents, such as sulforaphane and bardoxolone methyl, that form semi-permanent covalent bonds (adducts) with cysteine residues in a variety of proteins, including KEAP-1 due to their electrophilic nature. Hence, when these agents are administered there is potential for significant toxicity, i.e., one reported side effect related to bardoxolone methyl is gastrointestinal toxicity, that can limit the ability to use them at therapeutically effective doses. Sesamin's ability to utilize the PI3K, AKT, MEK, and ERK pathways for activation will likely provide for a larger therapeutic window of sesamin with fewer off-target side effects than other NRf2 agonists are capable of giving.

Fourth, the combined NR2-activating, anti-inflammatory, lipid-lowering, and antioxidant properties of sesamin suggest that sesamin could have synergistic or otherwise complementary effects on the complex, multi-factorial mechanisms involved in aging and age-related diseases where one mechanistic approach alone is unlikely to provide the necessary benefit. Future clinical studies exploring sesamin's efficacy in conditions of high oxidative stress and low NRF2 levels—such as mild cognitive impairment, sarcopenia, and metabolic syndrome in older adults—would provide valuable information from which to determine whether sesamin would be beneficial as a dietary supplement for promoting healthy aging.

CHAPTER 5: DISCUSSION

The information gathered and reviewed during this investigation provides a strong rationale for the importance of the KEAP1-NRF2 pathway to both cell chemistry and organism (or the) aging process. In this context, sesamin is recognized as an important natural activator that impacts the KEAP1-NRF2 pathway and has future therapeutic applications. The next section provides a framework to connect the previous chemical and biological insights into a single conceptual model, places the relevance of the identified key items in relation to the current state of scientific knowledge and defines potential future directions for research.

5.1 Summary of Findings

The key conclusions from this research can be summarized as follows. The KEAP1 - NRF2 pathway operates as an advanced thiol-based redox sensor - effector system with specific cysteine residues on KEAP1 acting as the molecular sensors of oxidative stress and electrophilic stress and NRF2 acting as the transcriptional effector that coordinates a broad-based cytoprotective gene program. In this sensor system, the chemical specificity of the sensor system as described by the 'cysteine code' concept provides cells with the tools to respond to chemically induced stressors with transcriptional responses specific to that stressor. Collectively, NRF2 target genes enhance the cell's ability to produce and recycle glutathione, detoxify ROS, degrade heme and maintain protein quality. This multilayered defence provides cells with protection from oxidative damage.

Over time, the activity of Nrf2 gradually diminishes across different types of age-related tissues and species. This reduction in Nrf2 activity creates a state of antioxidant deficiency, which further exacerbates oxidative damage associated with aging (the aging phenotype) and promotes cellular senescence, inflammation driven by senescence-associated secretory phenotype (SASP) factors, and tissue dysfunction (e.g., age-related hearing loss, dry eye disease, hyposalivation, cognitive decline, Alzheimer's Disease, and Parkinson's Disease). Pharmacological or genetic restoration of Nrf2 activity can significantly alleviate these age-related conditions in aged organisms via the use of either electrophilic inducers, direct inhibition of protein-protein interaction (PPI) disruptors, or natural dietary compounds, thereby demonstrating that Nrf2 activation represents a potential strategy for combating the effects of aging on the human body.

Sesamin, through a kinase-mediated mechanism that involves phosphorylation of AKT and ERK1/2 to activate NRF2, increases nivoosity and induction of its translocation to the nucleus, down-regulation of KEAP1 protein, and strong induction of ARE-dependent genes known to provide cytoprotective effects (HO-1, NQO1, GCLC, GCLM and GR) ultimately results in increases of intracellular GSH levels needed for sesamin to exert cytoprotective effects to oxidative stress. Additionally, sesamin has demonstrated in vivo anti-inflammatory and antioxidant properties via DSS-induced colitis by showing greater reduction on disease activity and improvement of colonic antioxidant status in comparison to 5-ASA. Furthermore, in a Drosophila aging model, sesamin consumption prevented age-dependent neuronal death and accumulation of protein aggregates and stimulated NRF2/Cnc-dependent transcription specifically in glutamatergic, cholinergic, and dopaminergic/serotonergic neuronal populations (the most vulnerable to neurodegeneration associated with aging) regardless of the presence or absence of oxidative stress.

5.2 Chemical Interpretation

From a chemical perspective, the KEAP1–NRF2 system is a remarkable example of the cell's capacity to use thiol chemistry for precise redox sensing and signal transduction. The low-pKa cysteine residues in KEAP1 function as molecular switches whose oxidation state is exquisitely sensitive to the prevailing intracellular redox environment, allowing KEAP1 to function as a real-time monitor of cellular oxidative status. The diversity of chemical modifications that KEAP1 cysteines can undergo — sulfenic acid formation, disulfide bond formation, glutathionylation, and electrophilic alkylation — and the distinct functional consequences of each modification type provide the molecular basis for the stimulus-specific activation of NRF2 target gene subsets that characterises the sophisticated cellular stress response.

The mechanistic analysis of sesamin's action reveals an interesting departure from the canonical electrophilic activation mechanism. While the molecular structure of sesamin, featuring the electron-rich methylenedioxy groups, would in principle be susceptible to metabolic activation to quinone methide intermediates capable of electrophilic KEAP1 modification, the available experimental evidence points strongly to a predominantly kinase-mediated mechanism. The independence of sesamin's NRF2-activating effects from oxidative conditions, the requirement for AKT and ERK

phosphorylation, and the neuron-type specificity of sesamin's effects in Drosophila — all of these observations are more consistent with receptor- or kinase-mediated signal transduction than with non-specific electrophilic chemistry. While this finding does not rule out the likelihood that sesamin metabolites may activate NRF2 through electrophilic mechanisms in certain cell types, it does suggest that the kinase-mediated route is the predominant mechanism under the conditions of this study.

According to the chemical analyses, the central biological link between NRF2-activation and cytoprotection is glutathione metabolism. The coordinated up-regulation of GCLC, GCLM, GR, and the cystine transporter xCT by NRF2 leads to a robust increase in the capacity of the cell to synthesize glutathione and recycle it. Because of the increase in cellular GSH, the cell will be able to quench ROS directly using the glutathione peroxidase reaction and also provide a substrate for glutathione S-transferases to detoxify xenobiotics that are electrophiles. The work of Bai et al. (2019) shows that BSO pretreatment specifically abolishes the cytoprotective effects of sesamin. This result provides evidence that elevation of glutathione is not just a side effect of NRF2-activation but instead is absolutely necessary for the antioxidant protection offered by sesamin.

5.3 Importance of Natural NRF2 Activators

Natural dietary substances that can activate the KEAP1-NRF2 pathway have been identified and developed and provide a unique bridge between traditional herbal medicine, nutritional science and molecular pharmacology. Supporting this bridge is the fact that, in general, natural NRF2 activators have many advantages over synthetic pharmaceutical products to increase the activity of NRF2, which will be further explained below.

The first advantage that natural NRF2 activators have is the fact that these compounds have been exposed to and evolved over millions of years as part of plant defence strategies against herbivores and pathogens, resulting in very low toxicity for mammals. The historical exposure of humans to sesame through the consumption of sesame seed (which contains a natural NRF2 activator called sesamin) provides an extensive safety record in terms of epidemiological studies which synthetic compounds do not provide. This is important for an application intended to assist the amelioration of the signs and symptoms

of ageing due to the prolonged, chronic nature of these applications resulting in the potential for toxicity to be additive over time.

There are some natural NRF2 activators that have the ability to activate multiple molecular targets simultaneously. This means they have the potential to provide more comprehensive protection against the many different causes of aging than a single-target intervention would provide. One example is the compound sesamin, since it has NRF2-related activities as well as activities that are independent of NRF2, giving it a polypharmacological profile. In terms of neurodegeneration and aging, the pathological processes that occur include oxidative stress, inflammation, mitochondrial dysfunction, protein aggregation, and neuroinflammation. Because a polypharmacological agent would address multiple pathological mechanisms simultaneously, it may provide superior efficacy compared to highly selective single-target agents.

Furthermore, because sesamin is naturally found in sesame seeds and sesame oil, it provides a potentially inexpensive and accessible method for activating NRF2 on a population-wide basis through dietary recommendations or food fortification. Even though pharmacologically effective doses of sesamin in disease states are likely higher than the dietary exposure level, sesamin has a relatively low inherent toxicity profile; therefore, much higher than dietary supplements of sesamin can likely be administered safely. Establishing standardized forms of sesamin with known bioavailability and pharmacokinetic properties would facilitate controlled clinical trials to evaluate the efficacy of sesamin in age-related conditions.

5.4 Future Scope

These results highlight numerous significant avenues for future research that could greatly enhance our knowledge of the therapeutic effects of sesamin as well as the broader use of NRF2 targeting therapeutic strategies in aging and neurodegeneration.

One of the major gaps in chemical research is to identify the direct molecular target of sesamin (i.e., the receptor, enzyme, or signalling protein that is the first molecular interaction of sesamin that activates the AKT and ERK phosphorylation cascade. Various approaches such as photoaffinity labelling of sesamin analogues, thermal proteome

profiling, and drug affinity responsive target stability (DARTS) assays will provide valuable insights into potential protein targets of sesamin. Identification of protein targets will facilitate the identification of the initiate mechanism leading to sesamin-induced NRF2 activation and, furthermore, will enable structure-activity relationship studies to facilitate the development of improved sesamin analogues with greater potency and selectivity.

To establish vital translational evidence to enhance the clinical development of sesamin, it is necessary to investigate the effects of sesamin on neuroprotection in mammalian, in vivo, models of aging (particularly aged mice or possibly using non-human primate models) through the use of the measurement of NRF2 target gene expression levels, levels of markers of oxidative injury, and cognitive behaviour outcomes. These studies should use physiologically relevant doses and regimens of orally administered sesamin to determine whether sesamin (at a dose that can be realistically achieved in the bloodstream of an aged human) activate NRF2 signalling in the aged brain to reduce neuroinflammation, reduce aggregate protein accumulation, and enhance cognitive performance in aged mammals. To achieve these objectives, an optimal dose and formulation of sesamin for neuroprotection in aged mammals must also be determined.

The specificity of sesamin's effect on NRF2 activation in the different types of neurons, as observed in Drosophila (with NRF2 activation observed only in glutamatergic, cholinergic and dopaminergic and not GABAergic), warrants investigation into possible mechanisms conferring this specificity. RNA sequencing analysis of sesamin-responsive versus sesamin-unresponsive neuron populations combined with proteomic analysis of the kinases activated by sesamin could yield putative molecular determinants of sesamin's neuron-type-specific NRF2 activation. Understanding the rationale for sesamin's selective NRF2 effects would be essential for predicting how accurately the results from Drosophila can be applied to mammalian systems, and for determining how the same rationale can be used to develop effective neuroprotective therapies targeting specific neuron types in mammals.

To determine whether sesamin works as a treatment, it would be optimal to conduct clinical studies on older adults with mild cognitive impairment, early Parkinson's disease, or metabolic syndrome; this would then serve as a basis for establishing the pharmacokinetic and pharmacodynamic relationship to utilize rational doses of the product. In such trials, there should also be appropriate biomarkers of NRF2 pathway activation, for example, urinary levels of mRNA of NRF2-related genes in exfoliated urine cells or plasma

concentrations of NRF2 target proteins such as NQO1, in order to confirm that the product produces the desired pharmacodynamic effect.

CHAPTER 6: CONCLUSION

6.1 Overall Conclusion

This dissertation examines both the chemical and biological mechanisms by which the KEAP1-NRF2 signalling pathway is modulated by sesamin as it relates to oxidative stress and ageing. The investigation incorporates a multi-disciplinary approach using mechanistic chemistry, cellular and systems biology, and pharmacology of naturally occurring compounds to create a complete understanding of how cells sense and respond to oxidative stress, how this response deteriorates with advanced age, and how a natural food substance can recover and enhance the functioning of this important cellular defence.

Chemically, KEAP1-NRF2 is structurally composed of a sensitive thiol-based sensing device in which reactive thiols (cysteines) serve as direct sensors for the intracellular oxidative environment (specifically Cys151, Cys273, and Cys288). The "cysteine code" concept demonstrates the chemical complexity of the KEAP1-NRF2 system; different oxidising agents or electrophiles differ in their preference for specific KEAP1 cysteines, and thereby activate distinct subsets of NRF2 target genes. The end result of the NRF2 transcriptional response is an extensive gene expression program that protects the cell from toxic insults through regulating glutathione metabolism, enzymatic neutralisation of reactive oxygen species (ROS), detoxification of toxic substances, and inhibition of inflammatory processes. Collectively, these cytoprotective responses restore redox homeostasis within the cell.

At the biological level, a decline in NRF2 activity with age is a bidirectional, shared vulnerability that increases the sensitivity to oxidative damage, chronic inflammation, cellular senescence, and tissue dysfunction in aged organisms. This deficiency of NRF2 creates a potential source of multiple geriatric pathologies across many organ systems; pharmacological correction of NRF2 deficiency (synthetic NRF2 activators, natural products and genetic methods) has produced improvements in multiple indicators of aging in experimental models. Thus, the KEAP1–NRF2 pathway is a genuine target for therapeutic anti-ageing interventions.

Sesamin is an extraordinary natural NRF2 (nuclear factor erythroid 2-related factor 2) activator based on its unique kinase-mediated mechanism of action (via PI3K/AKT and MEK/ERK signal transduction) that does not involve the typical/standard KEAP1 cysteine

alkylation mechanisms associated with many other known NRF2 activators (i.e., electrophilic inducers). It is a nature-based physiologic NRF2 activator with the capacity to induce antioxidant gene expression long-term without causing oxidative stress. This is evidenced by sesamin's established ability to cross the blood-brain barrier and specifically activate NRF2/Cnc-dependent transcription in neurons that age and degenerate without an accompanying oxidative stress component, thus having a preventative neuroprotection mechanism associated with the development of Alzheimer's and Parkinson's disease. Additionally, sesamin has demonstrated in vivo anti-inflammatory and antioxidant activity, and its long history of safe human dietary use (i.e., 1,000s of years) makes it a compelling/appealing dietary supplement for clinical research/development targeting healthy ageing, and neurological protection.

This paper provides a solid scientific base for understanding how sesamin acts on a molecular level and provides insight into the many ways it can be used biologically. Additionally, the paper presents several important research questions, including identifying sesamin's primary molecular target, whether the results of the neuroprotective Drosophila studies can be extrapolated to mammalian models, and whether supplementing older adults will prove to be an effective treatment for age-related diseases. The answers to these questions will ultimately determine if sesamin can fulfil its promise as a natural therapeutic approach to treating age-related diseases.

6.2 Future Perspectives

The scientific trajectory of KEAP1-NRF2 research and the pharmacology of sesamin suggest numerous exciting future avenues for expanding our understanding and translating the information gained from the mechanistic studies herein into health benefits.

Development of sesamin analogues with enhanced potency, water solubility, and bioavailability represents a significant opportunity for medicinal chemistry. Recognising the primary molecular target of sesamin — when found — will greatly facilitate the use of structure-activity relationship studies to produce optimised derivatives with stronger NRF2 activating activity at lower doses while maintaining the favourable safety profile of the parent compound. The methylenedioxy groups of the core structure of sesamin may be systematically altered to investigate the biological activity of ring-substituted compounds and the stereochemistry of the tetrahydrofuran core may be altered to investigate the stereospecificity of receptor or enzyme interactions.

The newly discovered discipline relating to senolytics and senomorphics (compounds that can specifically kill off or reduce the SASP produced by senescent cells) may prove to be an intuitive therapeutic companion to NRF2 Activators such as sesamin. While senolytics remove the source of ongoing chronic senescence (e.g., inflammation), NRF2 Activators could help to improve the antioxidant defences of non-senescent cells while simultaneously suppressing residual inflammation from the senescent cells. Thus, the potential for senolytics and NRF2 Activators to interact together may render additive or synergistic effects that could manifest in the slowing or reversing of the aging process. Clinical studies of combinations of sesamin with established senolytic agents' dasatinib and quercetin in an aged population with significant senescence will provide important data regarding the long-term safety and efficacy of combination therapies for healthy longevity.

Biomarkers associated with NRF2 activity development would be very beneficial to the emerging field of translational sesamin research as they would facilitate the implementation of sesamin into clinical trials and population studies. Current approaches in using peripheral blood mononuclear cells (PBMCs) or urinary exfoliated cells to determine NRF2 target gene expression appear promising but require further validation. Future applications of plasma proteomics for NRF2-regulated proteins, as well as methods that employ positron emission tomography (PET) imaging together with NRF2-specific radiotracers, may ultimately allow for non-invasive assessment of NRF2 pathway activity in the brains of older adults, thereby enabling researchers to directly measure the pharmacodynamic effects of sesamin in the target organ of greatest clinical importance.

Overall, there is a growing realization of how important the gut microbiome is in modulating both the bioavailability and the biological activity of polyphenols and lignans from dietary sources. This points to the fact that there is a need for a thorough investigation into how sesamin interacts with the gut microbiota; also, we must consider that certain bacterial species in the gut may bio transform sesamin into more active forms, known as enterolignans, and that each individual's microbiome composition may dramatically influence both the systemic pharmacokinetics of sesamin and its biological effects. Thus, using microbiome characterization in clinical studies on sesamin may help us better understand the interindividual variability in response to sesamin, and it may also suggest that microbiome modulation is a potential strategy to enhance the therapeutic properties of sesamin.

To summarise, the KEAP1–NRF2 pathway is an area with much potential in terms of being a research and therapeutic platform; and sesamin, being the natural activator of the pathway, provides an exciting avenue for research and therapy that bridges redox chemistry, ageing biology, and natural product pharmacology. This dissertation furthers our understanding of NRF2 as a master regulator of healthy ageing, and shows sesamin to be a naturally safe, excitingly different from other activators, and an effective way to activate this pathway; these results will lead to further research, ultimately resulting in extending our health span.

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