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Review

Inhibitory effects of sulforaphane on NLRP3 inflammasome activation

Cagla Kiser [a,b], Ceren Perihan Gonul [a,b], Melis Olcum [a], Sermin Genc [a,b,c,*,1]

[a] Izmir Biomedicine and Genome Center, Dokuz Eylul University Health Campus, Izmir, Turkey
[b] Izmir Biomedicine and Genome Institute, Dokuz Eylul University Health Campus, Izmir, Turkey
[c] Department of Neuroscience, Health Science Institute, Dokuz Eylul University, Izmir, Turkey

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ABSTRACT

SFN, a dietary phytochemical, is a significant member of isothiocyanates present in cruciferous vegetables at high levels in broccoli. It is a well-known activator of the Nrf2/ARE antioxidant pathway. Long since, the therapeutic effects of SFN have been widely studied in several different diseases. Other than the antioxidant effect, SFN also exhibits an anti-inflammatory effect through suppression of various mechanisms, including inflammasome activation. Considerably, SFN has been demonstrated to inhibit multiple inflammasomes, including NLRP3 inflammasome. NLRP3 inflammasome induces secretion of pro-inflammatory cytokines and promotes inflammatory cell death. The release of pro-inflammatory cytokines enhances the inflammatory response, in turn leading to tissue damage. These self-propelling inflammatory responses would need modulation with exogenous therapeutic agents to suppress them. SFN is a promising candidate molecule for the mitigation of NLRP3 inflammasome activation, which has been related to the pathogenesis of numerous disorders. In this review, we have provided fundamental knowledge about Sulforaphane, elaborated its characteristics, and evidentially focused on its mechanisms of action with regard to its anti-inflammatory, anti-oxidative, and neuroprotective features. Thereafter, we have summarized both *in vitro* and *in vivo* studies regarding SFN effect on NLRP3 inflammasome activation.

1. Sulforaphane

1.1. Definition

Sulforaphane (SFN) is a plant-derived compound belonging to the family of isothiocyanates (ITC) with the chemical structure 4-methyl-sulfinyl butyl isothiocyanate or 1-isothiocyanate-4-methyl-sulfinyl butane ($C_6 H_{11}NOS_2$) (Vanduchova et al., 2019). It is an aliphatic lipophilic organosulfur molecule with a low molecular weight of about 177, 29 (Houghton et al., 2016). Due to its lipophilic characteristic and small molecular size, SFN is passively absorbed by cells (Uddin et al., 2020).

ITCs are chemicals that originate within the plants of the *Brassica* genus of the *Cruciferae* family, involving a wide range of vegetables, namely broccoli, cabbage, brussels sprouts, cauliflower, and mustard greens (Palliyaguru et al., 2018). Early reports on the isolation of SFN in cruciferous plants exist in the 1950s (Kjær et al., 1958). Thereafter, SFN isolated and eluted from broccoli and Phase II inducer and anti-cancer effect were determined in the 1990s (Ullah, 2015). To date, many preclinical studies have been published that provide mechanical evidence to support the effects of SFN on different diseases. (Jiang et al., 2018).

Abbreviations: Aβ, Amyloid-β; ACE2, angiotensin-converting enzyme 2; AP, acute pancreatitis; APP, Amyloid-beta precursor protein; ARE, antioxidant responsive element; ASC, apoptosis-associated speck-like protein containing a CARD; BBB, blood-brain barrier; BDNF, brain-derived neurotrophic factor; BITC, benzyl isothiocyanate; BMDM, bone-marrow-derived macrophage; CARD, caspase recruitment domain; CNC, Cap 'n' Collar; CNS, central nervous system; COVID-19, coronavirus disease 2019; DAMP, damage-associated molecular patterns; DR, diabetic retinopathy; FB, Fernblock®; GSDMD, Gasdermin D; GST, glutathione S-transferase; HDAC, histone deacetylase; HFD, high-fat diet; HO-1, Heme oxygenase-1; IκB, inhibitor of kappa B; ITC, isothiocyanates; Keap1, Kelch-like ECH-associated protein 1; lncRNAs, long non-coding RNAs; LPS, lipopolysaccharide; MCAO, middle cerebral artery; miRNA, microRNA; MSU, monosodium uric acid; NAFLD, non-alcoholic fatty liver disease; Neh2, Nrf2-ECH homologous domain); NF-κB, nuclear factor-kappa B; NLRP3, NLR family pyrin domain containing 3; Nrf2, Nuclear Factor Erythroid 2-related factor 2; PAH, pulmonary arterial hypertension; PAMP, pathogen-associated molecular patterns; PYD, pyrin domain; ROS, reactive oxygen species; rtPA, recombinant tissue plasminogen activator; SFN, sulforaphane; TLR4, Toll-like Receptor 4.

* Corresponding author at: Izmir Biomedicine and Genome Center, Dokuz Eylul University Health Campus, Mithatpasa St. 58/5, 35340 Balcova, Izmir, Turkey.
E-mail addresses: sermin.genc@deu.edu.tr, sermin.genc@ibg.edu.tr (S. Genc).
[1] web: http://ibg.org.tr/research/research-programs/genc-lab/.

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1.2. Pharmacology and pharmacokinetics

ITCs are formed from glucosinolates presented in cruciferous vegetables (Palliyaguru et al., 2018) *via* the activity of the myrosinase enzyme (Tozser and Benko, 2016). Glucosinolates are comprised of nitrogen and sulfur along with a variable side chain (Briones-Herrera et al., 2018). SFN is converted from its inactive precursor glucoraphanin (Dinkova-Kostova et al., 2017) through myrosinase activity (Tozser and Benko, 2016). This converting process occurs following the disruption of plant cell walls *via* chewing, slicing or biting. Because myrosinase is released and activated when tissue is damaged (Vanduchova et al., 2019). In accordance with the fact that there is no corresponding homolog of myrosinase in humans, the required enzymes are available within the microbiome reservoir of human gastrointestinal parts. Nevertheless, providing direct ITCs has a greater bioavailability aspect than glucosinolates (Palliyaguru et al., 2018). This indicates that glucosinolates are required to be metabolized into ITCs so that regarding compounds could be absorbed (Palliyaguru et al., 2018). When biologically active SFN is formed, it passes through *via* passive diffusion, attaches to plasma protein thiols and passes the cell membranes to further process (Patel et al., 2018).

SFN undergoes further metabolism through the mercapturic acid pathway starting with glutathione conjugation. All metabolites within this conversion process have been determined in urine and plasma following the ingestion of cruciferous food (Palliyaguru et al., 2018). In studies of mice, it has been determined that metabolites of SFN exhibit a heterogeneous tissue distribution. Once absorbed, SFN can readily pass the blood-brain barrier (BBB) and also accumulate within the central nervous system (CNS) (Uddin et al., 2020), indicating that it is also a possible bioactive effector in the CNS (Huang et al., 2019).

Considerably, it has been determined to exhibit bioactivity in relatively low concentrations (Patel et al., 2018). Recent studies of clinical assessments have demonstrated that the oral dose of L-SFN ranges from 22 mmol to 592.25 mmol (Mazarakis et al., 2020). The toxicity of dietary compounds is, presumably, worth considering. SFN has been previously demonstrated not to be assessed as cytotoxic until 20 μM. between 20 and 40 μM concentrations, SFN has been cytotoxic in *in vitro* studies. By virtue of the fact that neither *via* through the nourishment of broccoli nor the regarding available supplements could provide such level of SFN levels (Houghton et al., 2013).

1.3. Mechanisms of action

1.3.1. Anti-oxidant effect (Nrf2)

Nrf2, Nuclear Factor Erythroid 2-related factor 2, is a crucial transcription factor in the regulation of oxidative stress response (Silva-Islas and Maldonado, 2018) by which activating various downstream cytoprotective and anti-oxidant enzymes, including Heme oxygenase-1 (HO-1) and glutathione S-transferase (GST) (Uddin et al., 2020). It belongs to Cap 'n' Collar (CNC) transcription factors group (Silva-Islas and Maldonado, 2018).

Nrf2 level is tightly controlled *via* several mechanisms, a key mediator is Kelch-like ECH-associated protein 1 (Keap-1) protein which binds to Nrf2 as well as to actin filaments in the cytoplasm through Neh2 (Nrf2-ECH homologous domain) phosphorylation site (Karan et al., 2020). Under physiological conditions with no cellular stress, Keap-1 promotes Nrf2 degradation by ubiquitin-proteasome pathway to maintain low cellular levels of Nrf2 (Houghton et al., 2016), which contributes to the rapid turnover of Nrf2 with a half-life less than 30 min (Patel et al., 2018).

As an electrophilic or oxidative stress signal is detected by Keap-1 and it dissociates from Nrf2 (Houghton et al., 2016). Direct alteration of cysteine thiol residues of Keap-1 at positions 151, 273, and 288 by Nrf2 inducers results in the release of Nrf2 (Houghton et al., 2016) due to a conformational change in Keap-1 (Silva-Islas and Maldonado, 2018). Once released from the Nrf2-Keap-1 complex, Nrf2 translocates to the nucleus, heterodimerizes with other basic leucine zipper proteins and binds antioxidant responsive element (ARE) sequence within the promoter regions of its corresponding cytoprotective target genes (Houghton et al., 2016), and stimulates their transcriptional activations (Silva-Islas and Maldonado, 2018). Nrf2 regulates expression of more than 1000 genes (Silva-Islas and Maldonado, 2018). Nrf2 target genes are involved in significant pathways, namely antioxidant response, proliferation, cell survival, metabolism, and immune response (Silva-Islas and Maldonado, 2018). Nrf2-dependent genes express a wide range of functionally variable enzymes and also proteins involved in cytoprotection (Dinkova-Kostova et al., 2017). Nevertheless, nuclear translocation of Nrf2 requires a series of phosphorylation and acetylation by kinases and acetylases, respectively (Silva-Islas and Maldonado, 2018).

Following the entrance to the cell, SFN reacts with Keap-1 (Dinkova-Kostova et al., 2017). SFN promotes chemical modification of cysteine residues of Keap-1, preventing the Keap-1-dependent Nrf2 degradation cycle, leading to Nrf2 accumulation (Dinkova-Kostova et al., 2017). The main cysteine residue for SFN to target is C151 residue, among several other cysteine residues of Keap-1 (Dinkova-Kostova et al., 2017). Here, an important concept is that C151 is a critical cysteine and highly reactive one in Keap-1 (Dinkova-Kostova et al., 2017). SFN also induces the expression of Nrf2 mRNA by reducing DNA methylation of Nrf2 promoter in neuroblastoma cells (Zhao et al., 2016). Besides this Nrf2 induction, other possible effects such as the direct anti-oxidant effect should be examined in future studies.

1.3.2. Anti-inflammatory properties

Activation of Toll-like Receptor 4 (TLR4) promotes proinflammatory responses through cytokine production and releases the following activation of nuclear factor-kappa B (NF-κB). SFN suppression on oligomerization of TLR4 contributes to its anti-inflammatory action (Houghton, 2019; Youn et al., 2010). NF-κB is an inducible transcription factor present in many cell types in inactive cytoplasmic form with its inhibitor protein inhibitor of kappa B (IκB). Bacterial lipopolysaccharide (LPS) and cytokines are the primary activators of the NF-κB signaling pathway. Upon activation, NF-κB translocates to the nucleus and upregulates the various pro-inflammatory genes in innate immune cells. In a basic understanding, SFN prevents NF-κB translocation and DNA binding activity (Patel et al., 2018). SFN also stimulates anti-inflammatory response through Nrf2 activation (Mazarakis et al., 2020). HO-1 has been identified as the Nrf2 target gene, but HO-1 also contributes to the anti-inflammatory action of SFN independent from the Nrf2 signaling pathway. (Houghton, 2019).

1.3.3. Autophagy promoting effect

Autophagy is a process of degradation of aggregated and degenerated proteins, damaged organelles (Klomparens and Ding, 2019), as well as Amyloid-β (Aβ) aggregates, clearing the injured part in order to prevent any further tissue degeneration. As a significant neuroprotective aspect, SFN positively regulates autophagy in various cells, including neurons (Klomparens and Ding, 2019). The presence of the ARE binding site in autophagy genes and decreased expression of autophagy genes in Nrf2 knockout cells support the effect of SFN through Nrf2 (Pajares et al., 2016). In addition, absence of Nrf2 enhanced aggregation of Amyloid-beta precursor protein (APP) due to impairments in the autophagy pathway in the mutant mouse model of proteinopathy. However, SFN promotes autophagy in neuronal cells independently of its Nrf2 inducing effect (Jo et al., 2014). These findings suggest that several intracellular signaling pathway mediates autophagy inducing effect of SFN and activation of autophagy by SFN may contribute clearance of aggregated proteins in neurodegenerative diseases.

1.3.4. Mitoprotective effects

A significant aspect of SFN is to protect mitochondrial function within neurons. Mitochondrial homeostasis is vital in neurons as neurons exhibit high metabolic activity in accordance with energy

requirements in neurons (Klomparens and Ding, 2019). Moreover, the Nrf2 pathway upregulates numerous genes involved in mitochondrial biogenesis, protects mitochondria from damages, and also inhibits ATP, decreasing the effects of toxins (Klomparens and Ding, 2019). SFN exerts mitoprotective effect through Nrf2/ARE dependent and mitochondrial fission modulation in different cells (de Oliveira et al., 2018; O'Mealey et al., 2017). Apart from neuronal cells, the mitoprotective effect of SFN is observed in other cells, such as the liver and endothelium (Tubbs et al., 2018; M. Zhang et al., 2020).

1.3.5. Neurogenesis promoting effects

A decline in adult neurogenesis has been linked with numerous neurodegenerative and psychiatric diseases. SFN demonstrates a significant aspect of promoting the generation of neurons *via* increasing neuronal expression of brain-derived neurotrophic factor (BDNF) (Kim et al., 2017). Further, SFN increases Wnt signaling within neural stem cells, leading to upregulation of proliferation of stem cells and their differentiation to neurons (Han et al., 2017). (Klomparens and Ding, 2019). SFN also promotes neurogenesis in mice with Alzheimer's disease-like lesions induced by combined administration of aluminum and D-galactose (R. Zhang et al., 2014). The neurogenesis-inducing effect of SFN in humans should be investigated in future studies.

1.3.6. Epigenetic mechanisms

SFN has been determined to affect post-translational modifications (Houghton et al., 2013) and modulate epigenetic mechanisms influencing activation or silencing of genes in cancer (Su et al., 2018). Inhibition of histone deacetylase (HDAC) is mainly related to the chemoprotective effect of SFN. It demonstrates chemo-preventive aspects through HDAC inhibition in several cancer types (Royston et al., 2018). HDAC inhibitory effect of SFN is not limited to cancer cells; it has been observed in dendritic cells, cortical neurons and neural crest cells (Kim et al., 2017; Qu et al., 2015; Yuan et al., 2018). On the other hand, SFN has been also stated as a potential regulatory agent for DNA methylation in both development and progression of cancer (Su et al., 2018). Also, SFN has been reduced global kidney DNA methylation in hypertensive rats (Senanayake et al., 2012).

1.3.7. microRNA and long- noncoding RNA

A variety of microRNAs (miRNA) is known to be targeted by SFN in cancer states of cells, including miR21 and miR200c, where SFN reduces the viability of cancerous cells and promotes their apoptosis (Dacosta and Bao, 2017). Increased pro-inflammatory miR-146a levels by Aβ1-42 treatment were significantly attenuated by SFN in the human THP-1 cell line (Tozser and Benko, 2016). Further, LPS-induced increase in pro-inflammatory miR-155 has been decreased *via* SFN treatment in murine microglia (Eren et al., 2018). These findings confirmed that SFN exhibited an anti-inflammatory effect by modifying miRNA expression. SFN also alters long non-coding RNAs (lncRNAs) in cancers and other chronic diseases (Mishra et al., 2019). SFN has been suppressed the increased expression of lncRNA LINC01116 in human prostate cancer cell line (Beaver et al., 2017).

1.3.8. Chemoprevention, cancer-associated inflammation

SFN exhibits significant chemopreventive effective characteristics *via* epigenetic modifications and following Nrf2 activation (Jiang et al., 2018). Enhanced Nrf2 signaling and DNA damage repair by SFN interfere with cancer stem cells as well as carcinogen detoxification (Klomparens and Ding, 2019). SFN demonstrates an inhibitory effect on tumor development initiation and increases the sensitivity of cancer cells to chemotherapeutics interfering with various signaling pathways, including induction of cell cycle arrest and apoptosis and anti-inflammatory action (Jiang et al., 2018).

1.3.9. Anti-microbial effects

SFN displays anti-microbial effects on o pathogenic microbe within the gut. SFN has been demonstrated a direct anti-microbial effect on *Helicobacter pylori* bacterium *via* activating Nrf2 (Houghton, 2019). Clinical trials with broccoli sprout also decreased three markers of *H. pylori* infection within eight weeks (Yanaka et al., 2009). Besides its anti-microbial activity, antiviral effect of SFN has been observed against respiratory syncytial virus and HIV (Cho et al., 2009; Furuya et al., 2016).

1.4. Clinical trials

Preclinical studies have revealed SFN is a powerful potential therapeutic molecule in many diseases. Subsequently, several clinical studies started in healthy people and patients with different diseases, including cancer, inflammatory disease, Alzheimer's disease, autism and schizophrenia (Patel et al., 2018). Broccoli, broccoli sprout homogenate, broccoli seed extract, and broccoli sprout extract have been used in these clinical trials. Among the 76 clinical trials of SFN, 47 are completed and 13 are recruiting patients according to data recruited from the ClinicalTrials website of the U.S. National Library of Medicine (Medicine, 2021). Administration of broccoli sprouts in hot water was safe and well-tolerated, but interindividual differences in bioavailability were found in healthy persons (Kensler et al., 2005). Referring to previously conducted studies, SFN treatment has been efficient in diabetes, cancer, pulmonary disease, skin disorder and schizophrenia (Palliyaguru et al., 2018; Patel et al., 2018). Besides, the consumption of broccoli sprouts during 10 weeks decreases inflammatory markers IL-6 and C-reactive protein suggesting that it can be efficient in diseases with inflammatory pathogenesis (Lopez-Chillon et al., 2019).

2. NLRP3 inflammasome

NLR family pyrin domain-containing 3 (NLRP3) inflammasome is a part of the innate immune system that fights pathogens and maintains homeostasis (Danielski et al., 2020). The innate immune system is the first actor that responds to intruders (Hillion et al., 2020). These intruders can be microorganisms, or microorganism derived molecules such as bacteria, fungi, viruses as pathogen-associated molecular patterns (PAMPs) to cause inflammation; or dead or dying cells' particles as damage-associated molecular patterns (DAMPs) that cause sterile inflammatory responses (Pittman and Kubes, 2013). Both DAMPs and PAMPs are sensed by the cell *via* receptors, and this recognition activates intracellular signaling pathways. For NLRP3 inflammasome, JNK, MAPK and NF-κB pathways are in action. These pathways trigger or inhibit the formation of the NLRP3 inflammasome (Liu et al., 2020).

NLRP3 inflammasome comprises three elements: NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC) and procaspase-1. NLRP3 is the nucleation element that is in the center of the inflammasome. NLRP3 consists of 3 domains: C-terminal leucine-rich repeats (LRRs), NACHT domain, N-terminal pyrin domain (PYD). ASC nucleates around NLRP3 with its N-terminal PYD and C-terminal caspase recruitment domain (CARD), and it recruits procaspase-1 that consists of a CARD and a caspase domain (Yang et al., 2019).

NLRP3 inflammasome activation requires two steps (Fig. 1). The first step is the priming step which requires pattern recognition receptors TLR4, NOD2, TNFR and IL-1R. This pattern recognition induces NLRP3 activation and ASC phosphorylation *via* NF-κB activation (Bauernfeind et al., 2009). The second step is the activation step, which includes inflammasome complex formation with NLRP3, ASC and procaspase-1. In the activation step, intracellular ATP, potassium efflux and mitochondrial reactive oxygen species (ROS) could be an inducer of inflammasome complex formation (Wu et al., 2020). This leads to activation of caspase-1, and consequently, caspase-1 contributes to maturation and secretion of IL-1β and IL-18 (Schroder and Tschopp, 2010; Sepehri et al., 2017; Shao et al., 2015). Gasdermin-D (GSDMD) is another protein that is cleaved by caspase-1 that triggers pyroptosis, a type of cell death that generates pores on the cell membrane. N domains

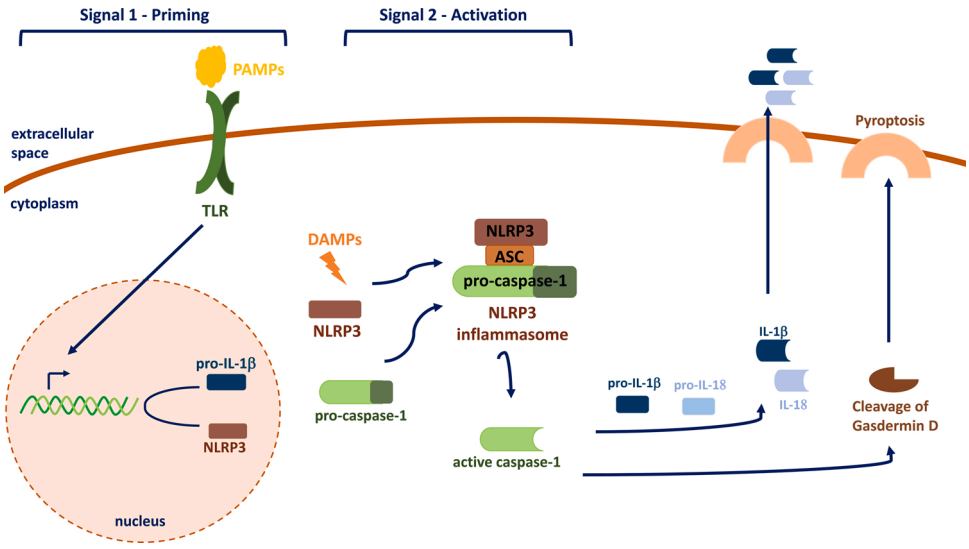

Fig. 1. NLRP3 inflammasome activation. NLRP3 inflammasome complex is activated through PAMPs and DAMPs. Inflammasome complex consists of NLRP3, ASC and Caspase-1 proteins, and once it is activated, Caspase-1 of the complex cleaves precursor of IL-1β and IL-18 in the cell, providing mature IL-1β and IL-18, which are then secreted from the cell. Additionally, activated Caspase-1 protein might also lead to pyroptotic cell death through Gasdermin D.

of GSDMD are shared/conserved upon gasdermin protein family and they are essential for pore formation. Cleaved N domains move to plasma membrane, oligomerize and bind membrane lipids, phosphoinositides and cardiolipin, which as a result disrupt membrane structure. This disruption induces formation of pores that have 10−14 mm diameter and triggers pyroptotic cell death. Gasdermin-N domains act like B-barrel PFPs, or can introduce a new way of pore formation, shown by structural studies. Generated pores allow release of pro-inflammatory cytokines IL-1β and IL-18 (Ding et al., 2016).

Pyroptosis is a type of programmed cell death which is mediated by inflammatory caspases. In the context of NLRP3 inflammasomes, inflammasome-activated caspase-1 starts a cellular process that results in pore formation, which makes the cell susceptible to killing by phagocytosis. Pyroptosis share similar characteristics with apoptosis as they both are caspase-dependent and DNA damage and nuclear condensation can be observed (Jorgensen and Miao, 2015).

There are two other activation mechanisms for NLRP3 complex formation. The noncanonical pathway is activated by cytoplasmic LPS gram-negative bacteria and needs caspase-11 in mice (Groslambert and Py, 2018) and caspase-4 and caspase-5 in humans (Groslambert and Py, 2018; Souza et al., 2015). The third activation mechanism, the alternative pathway, is initiated by the TLR4 pathway and finalized with NLRP3 activation *via* caspase-8. While pyroptosis is observed in a non-canonical way similar to the canonical pathway, pyroptosis is not seen in alternative activation of the inflammasome.

The primary positive regulators of NLRP3 inflammasome activation are potassium (K+) efflux, calcium (Ca2+) influx, lysosome destabilization and rupture, mitochondrial ROS and mitochondrial DNA damage (Kelley et al., 2019). Negative regulators are substances with similar structures to PYD or CARD domains that block nucleation of the core NLRP3 inflammasome, autophagy, cytokines, nitric oxide and miRNAs (Caballano-Infantes et al., 2017; Giorgi et al., 2018). In a disease state, the balance between activation and inhibition of NLRP3 inflammasome is unbalanced (Leemans et al., 2011).

Post-translational modifications regulate NLRP3 inflammasome activation. Phosphorylation at Ser5 inhibits the activation, while phosphorylation at Ser 198 induces activation. Ubiquitinylation is another important regulator since NLRP3 is ubiquitinylated in resting-state macrophages. Its deubiquitinylation leads to priming and activation (Juliana et al., 2012). Sumoylation also has a similar effect; desumoylation by SENP6 and SENP7 activates NLRP3 (Barry et al., 2018).

3. *In vivo* effects of SFN on NLRP3 inflammasome

3.1. Stroke

Stroke is a severe common neurological disorder with high mortality and long-term disability worldwide. Approximately 80 % of strokes are ischemic nature and occur following interruption of the cerebral blood flow from thromboembolic occlusion of cerebral arteries (Phipps and Cronin, 2020). Recombinant tissue plasminogen activator (rtPA) is a unique approved drug for the treatment of ischemic stroke; however, rtPA can be applied to a small portion of patients (Xiong et al., 2019). For this reason, it is necessary to develop new drugs that can be used widely in stroke patients. Intra-arterial occlusion of the middle cerebral artery (MCAO) using nylon filament is the most accepted animal model of ischemic stroke in rodents for drug studies (Hermann et al., 2019).

The mechanisms underlying neuronal death in cerebral ischemia are complex and not fully understood but involve excitotoxicity, oxidative stress, apoptosis and inflammation (Khoshnam et al., 2017). Inflammation plays an essential role in the pathogenesis of ischemic stroke, and the presence of inflammation is the key factor for clinical prognosis (Mo et al., 2020). After ischemia, the production of cytokines, including IL-1β and IL-18 from injured neuronal and glial cells, are activated and initiates cellular responses leading to neuronal cell death and BBB disruption (Mo et al., 2020; Nakamura and Shichita, 2019). Recent studies supported the role of NLRP3 inflammasome in the pathogenesis of stroke. Increased expression of inflammasome-related proteins up to seven days has been reported in the experimental stroke model. NLRP3 deficiency in knockout animals or suppression of NLRP3 with NLRP3 inhibitor MCC950 altered clinical findings and reduced infarct volumes (Ismael et al., 2018; Yang et al., 2014). In a human post-mortem study, elevated levels of NLRP3, IL-1β, caspase-1 and ASC were found in the ipsilateral side of the brain in stroke patients. Targeting the NLRP3 inflammasome by phytochemicals could be a promising strategy for the treatment of ischemic stroke (Hung et al., 2020).

The therapeutic effect of SFN in stroke has been studied in an animal model of disease. Systemically administered SFN decreases cerebral infarct volume, and pretreatment of SFN protects cerebral microvasculature through activating Nrf2/HO-1 signaling pathway in focal ischemia model (Alfieri et al., 2013; Srivastava et al., 2013; Zhao et al., 2006). SFN treatment also exerts protective benefit in the cerebral thrombosis model (Gillespie et al., 2018). In a recent study, the inhibitory effect of SFN on NLRP3 inflammasome has been reported in the

MCAO model of stroke (Yu et al., 2017). SFN was administered intraperitoneally after 60 min of occlusion as a single dose. SFN treatment reduces infarct volume and improves clinical findings and inhibits the increased expression of NLRP3, cleaved caspase-1, IL-1β and IL-18 levels in the brain of mice. Multiple possible mechanisms such as Nrf2 and autophagy activation underlying the NLRP3 inhibitory effect of SFN in ischemic stroke should be evaluated in further studies (De Muylder, 1987; Hung et al., 2020; Sivandzade et al., 2019). In addition, target cells of SFN in the inhibition of NLRP3 inflammasome could be determined (Lenart et al., 2016; Voet et al., 2019).

3.2. Retinal vascular disorders

Retinal vascular disorders, such as diabetic retinopathy and retinal vein occlusion, are common causes of vision loss (Park, 2016). Inflammation and oxidative stress are the main factors that can worsen the pathology of these diseases. Injuries caused by lack of nutrients in diabetic retinopathy (DR) or retinal ischemia/reperfusion (IR) results in activation of NLRP3 inflammasome as injury sensors (Gong et al., 2019; Li et al., 2019). The study by Devi et al. is one of the first studies that show the causative role of NLRP3 inflammasome in increased stress, inflammation, and cell death (Devi et al., 2012). The levels of IL-1β and IL-18 secreted by NLRP3 inflammasome are elevated by the ROS-TXNIP pathway in retinal cells (W. Chen et al., 2017). Microglial activation is also a result of injury-associated inflammation; in DR cases, microglial activation and cell death can be seen even before the vascular endothelial cell abnormalities (Park, 2016).

DR is a complication that can be seen in type 1 and 2 diabetes mellitus, which affects the eyes. It results from increased blood sugar levels and the leading cause of vision loss in many countries (Esmaili and Boyer, 2018). Inflammation, oxidative stress and hypoxia were reported to be the factors that can worsen DR. Oxidative stress can be a consequence of elevated glucose levels in the circulation, which causes over-produced inflammatory mediators to damage the retinal cells. There are also studies that show that ROS accumulation is one of the major factors that affect DR development, followed by increased levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. It was also shown that oxidative stress could induce the activation of the NLRP3 inflammasome, which affects DR pathology (Li et al., 2019).

Known anti-inflammatory and anti-oxidant effects of SNF lead scientists to consider it as a therapeutic reagent for diabetic nephropathy and diabetic cardiomyopathy in the experimental setup. In the article by Li et al., the mechanism of how SFN affects DR pathology was studied. They used male Sprague-Dawley mice and induce DR by using 65 mg/kg streptozotocin (STZ). Five groups of mice (control, SFN1, STZ, SFN1 + STZ, and SFN0.5 + STZ) were experimented for 12 weeks. Two doses of SFN (0.5 or 1 mg/kg) and STZ were injected each day intraperitoneally for the experimentation period. After treatment, retinal tissue samples were tested for cytokine (TNF-α, IL-1β, and IL-6) and NLRP3 levels. Reduced cytokine levels and downregulation in the expression of NLRP3 and other inflammasome components upon SFN treatment show its protective effects in DR treatment. In addition, activation of the Nrf2 signaling pathway, which is activated against oxidative stress, with the induction of DR and reduced damage by inhibition of NLRP3 inflammasome, allows further understanding of the mechanism of DR pathology (Li et al., 2019).

In another study, SFN administration inhibited the change in the retinal thickness, which happened due to IR injury and decreased retinal ganglion cell death. SFN treatment also reduced the pro-inflammatory cytokine levels, downregulation in the NLRP3 inflammasome components and decrease in microglial activation. These results were similar with the NLRP3 knockdown group, suggesting that neuroprotection by SFN is through NLRP3 inflammasome inactivation in glaucoma (Gong et al., 2019).

3.3. MSU-induced inflammation

Monosodium uric acid (MSU) induced inflammation is known to cause diseases such as peritonitis and gout (Busso and So, 2010; Yang et al., 2018). Gout, the most common form of arthritis, has been prevalent for more than a decade. MSU deposition in the joints triggers an immune response and result in chronic arthritis (Yang et al., 2018). This immune response starts with the activation of macrophages by MSU crystals and follows with the secretion of cytokines and recruitment of neutrophils. The most critical cytokine in MSU-induced inflammation is IL-1β, which induces activation of IL-1 and MyD88-dependent NF-κB signaling pathways (Shang et al., 2019). It was also shown that IL-1β secretion in acute gout is a result of NLRP3 inflammasome activation, suggesting the role of MSU-crystals in inflammasome activation (Busso and So, 2010). One of the recent studies by Yang et al. demonstrates the effect of SFN on the MSU-induced acute gout model in mice. SFN treatment one hour before induction resulted in a decrease in IL-1β levels and inhibition of NLRP3 inflammasome activation, with attenuated symptoms observed in mice. These outcomes further show the role of NLRP3 inflammasome in the gout inflammatory pathway and how SFN can alleviate the inflammation (Yang et al., 2018). In another study where MSU-induced peritonitis model used, SFN treatment was applied for 3 days. Results were similar; IL-1β secretion was reduced and NLRP3 inflammasome was inhibited in SFN injected peritonitis model. SFN also attenuated the mitochondrial ROS levels induced by rotenone which also inhibited the secretion of IL-1β, allowing further understanding on how SFN blocks the NLRP3 inflammasome activation (Lee et al., 2016b).

3.4. High-fat diet (HFD)

Non-alcoholic fatty liver disease (NAFLD) is caused by excess accumulation of fat in the liver and can result in steatosis or liver failure. In addition to excessive fat, inflammation and cell death that follows the liver injury are also seen in the patients who have NAFLD (Rossato et al., 2020). NLRP3 inflammasome complex is known to be activated by the free fatty acids. After activation by high-fat diet (HFD), proinflammatory cytokines like IL-1β and TNF-α are recruited, and the liver is further damaged by triglyceride accumulation and triggered cell death (Csak et al., 2011). An increase in the level of NLRP3 inflammasome components in case of liver damage (Ganz et al., 2011) and IL-1β knock-out mice being protected from liver fibrosis in a severe case of NAFLD further validate their role in disease progression (Kamari et al., 2011). Since there is a limited number of treatments for this disease, inhibition of inflammasome is one of the important targets. The study by Yang et al. is focused on using SFN for that aim. They showed that orally administered SFN for 9 weeks decreased hepatic steatosis scores of HFD fed mice. Also, mRNA levels of inflammasome components such as ASC and caspase-1 and caspase-1 enzyme activity were also lowered by SFN treatment. In addition, it was demonstrated that SFN helped reducing NLRP3 inflammasome-caused damage by inducing the autophagy through activation of AMPK-Ulk1 pathway. AMPK pathway activation recovered mitochondrial dysfunction and regulated HFD induced NLRP3 inflammasome activation. Although the gender differences in SNF response should be studied, the given results provide an insight for mechanisms on how SFN inactivates NLRP3 inflammasome (Yang et al., 2016).

3.5. Acute pancreatitis

Acute pancreatitis (AP) is an inflammatory pancreatic disease that leads to morbidity and mortality in patients. Inflammation starts in the acinar cells and then spreads to the infected tissue, following by the systematic failure (Iyer et al., 2020). In severe AP (SAP), IL-1β secretion by NLRP3 inflammasomes has been suggested to contribute to the severity of the disease; NLRP3−/− mice have reduced inflammation, and inflammatory cascades were blocked due to reduced IL-1β levels (Fu

et al., 2018). Dang et al. used SFN as a potential therapeutic agent for AP; they treated mice with SFN for 3 days before induction of the disease and found that pancreatic damage was decreased in the treatment group. In addition to the decrease in NLRP3 inflammasome signaling protein levels in acinar cells, they also showed the association of the Nrf2 pathway with inhibition of oxidative stress by SFN and inhibition of the NF-κB pathway, which has a major role in pancreatic inflammation. Overall, they suggest that SFN can alleviate pancreatic damage with its antioxidant and anti-inflammatory effects (Dong et al., 2016).

3.6. Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a disease that is characterized by endothelial cell proliferation, vascular inflammation, remodeling and resulted in right ventricular (RV) failure and eventually death. Aside from mentioned characteristics, pulmonary vascular inflammation and recruited inflammatory cells to the area also contribute to disease pathology (Deng et al., 2019). It was shown that NLRP3 inflammasome and secreted pro-inflammatory cytokines play an important role in inflammation processes and have been targeted for therapy of PAH (Yin et al., 2017). Kang et al. aimed to use SFN as a regulatory molecule for the Nrf2 pathway to inhibit NLRP3 inflammasome activation in hypoxia-induced PAH mice. Upon 4 weeks of SFN treatment, RV dysfunction was partially rescued, and fibrosis was prevented. NLRP3 inflammasome activation was blocked, and IL-1β secretion was reduced with an increase in Nrf2 and its downstream molecule NQO1 at the molecular level. As a result, they stated that inflammation in PAH could be reduced with SFN by activating the Nrf2 pathway, which blocks NLRP3 activation (Kang et al., 2020).

4. *In vitro* studies

The effects of SFN on NLRP3 inflammasome were shown in *in vitro* studies as well. SFN treatment is done after the stimulation of immune cells like microglia, and changes in molecular level are examined. Greaney et al. used bone-marrow-derived macrophages (BMDMs) differentiated from RAW264.7 cells to study the effects of SNF on Nrf2 independent inactivation of NLRP3 inflammasomes. In their results, they showed that ROS production does not reverse the effects of SFN and Nrf2-/- BMDMs still have reduced levels of NLRP3 inflammasomes after SFN pre-treatment, suggesting SFN inhibition of NLRP3 inflammasomes does not depend on the Nrf2 pathway and not affected by ROS production (Greaney et al., 2016). Another study used THP-1 human monocytic cell line to focus on anti-inflammatory effects of SFN upon Aβ stimulation. Upon SFN treatment, it was demonstrated that IL-1β secretion and STAT1 pathway, which was activated by Aβ peptides, were blocked, and the Nrf2 pathway was activated. Nrf2 activator mimicking the effects of SFN provides more evidence for the mechanisms of its anti-inflammatory role in Alzheimer's disease, which makes it a potential therapeutic agent (An et al., 2016).

Recently, our group demonstrated the involvement of the Nrf2 pathway in the anti-inflammatory effects of SFN. Pre-treatment of microglial cells with SFN had protective effects against NLRP3 inflammasome activation with a decrease in IL-1β and NLRP3 expression levels and caspase-1 activity. SFN exhibited this effect modulating different signaling pathways, including NF-κB, Nrf2, HMGB1 (Tufekci et al., 2021). Our other exciting finding is that alteration in miRNA expression by SFN mediates its inhibitory effect on NLRP3 inflammasome activation. SFN decreased miR-155 expression and increased miR-223 expression, which were altered by NLRP3 induction. Functional study with miR-155 mimic and miR-223 antagomir confirmed alteration in miRNAs expression mediates the anti-NLRP3 effect of SFN.

5. Potential use of SFN for COVID-19

The coronavirus disease 2019 (COVID-19) that resulted in a pandemia is characterized by a serious inflammatory state. This state involves hyper-activation of transcription factor NF-κB through cytokines, namely IL-6 (Gasparello et al., 2021). S protein of SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2) receptor of host cells enabling viral entrance during infection. Inhibiting Nrf2 is demonstrated to upregulate ACE2 receptor; on the other hand activating this particular transcription factor decreases levels of ACE2, correlatively decreasing the availability of the receptor for the virus proteins. In line with this, activation of Nrf2 is potent to inhibit NF-κB driven inflammatory response resulted from SARS-CoV-2 infection (Cuadrado et al., 2020). On the other hand, COVID-19 infection during pregnancy is a significant issue as it causes maternal immune activation which may result in development of psychiatric disorders in offsprings. Considering anti-inflammatory and anti-oxidant characteristics of SFN within cruciferous vegetables, it is stated that the dietary use of SFN during pregnancy possibly decreases development of neuropsychiatric disease development in case of COVID-19-infected pregnancy (Hashimoto, 2021). In another study regarding COVID-19 and SFN, a proof-of-principle study of Gasparello *et.al.*, it has been demonstrated that *in vitro* infection of bronchial epithelial IB3-1 cells with SARS-CoV-2 spike protein increases expression of namely IL-6 and IL-8 interleukins (Gasparello et al., 2021), IFNα and IFNγ interferons (Ribeiro et al., 2021) along with several other cytokines and chemokines associated with the severe inflammatory state, referred as cytokine storm (Gasparello et al., 2021). The term cytokine storm can be described as the dramatic heightening of inflammatory response acoompained by un-controlled cytokine and interferon overproduction (Liskova et al., 2021). The aforementioned upregulation in inflammatory response contributes to brain and lung inflammation and multiple organ failure; namely heart, liver and kidney, collaboratively leading to death in COVID-19 cases (Nile et al., 2020). Due to the fact that pro-inflammatory cytokines resulting from cytokine storm and the virus itself are able to cross the BBB and invade the CNS, inevitable neuroinflammation in accompany with disruption in functional units result in neurodegeneration (Ribeiro et al., 2021).

Even though the exact mechanism SFN acting on COVID-19 effects has not been revealed yet, it is suggested that known mechanisms of action of SFN as Nrf2 activation or NF-κB inhibition might be the explanation; still, SFN treatment demonstrated to reverse this upregulation of aforementioned cytokines during infection. They demonstrated that IL-6 and IL-8 mRNA levels elevated *via* S-protein, however, SFN treatment decreased their expression. Further, this study demonstrates that SFN prevents mRNA accumulations of IL-6 and IL-8 cytokines, and also interfere with release of cytokines in a dose-dependent manner. Overall, it is stated that use of SFN can be utilized in terms of dietary intervention to attenuate severe inflammation resulted from COVID-19 infection and it is significant to provide its clinical use containing combined therapy.

6. Use of SFN in combined treatments and SFN-like molecules

SFN is also used in combination with other compounds like plant extract. The study by Serini et al. used Fernblock® (FB), a patented extract obtained from *Polypodium leucotomos* which showed to have anti-inflammatory properties, in combination with SFN, to investigate their anti-aging and anti-inflammatory effects using melanoma cells and keratinocytes. Their results showed that both SFN and FB alone and combined have protective effects against inflammasome activation and oxidative stress; NLRP3, ASC, and cleaved caspase-1 expression were decreased with SFN and/or FB treatment. They also stated that inhibition of NLRP3 inflammasome activation was significantly higher when combined treatment was applied. Overall, their study demonstrates that SFN, with or without FB, is a potent dietary supplement that can be used as an inhibitor for inflammatory microenvironments that can be seen in melanoma (Serini et al., 2020).

Benzyl isothiocyanate (BITC) is a phytochemical like SFN that can

also be found in cruciferous vegetables. Likewise, it has anti-inflammatory and anti-oxidative activities. Although there are not many studies present for the anti-inflammatory effects of BITC, its similarity to SFN makes it a potential target for therapeutic approaches. Few studies which focus on BITC try to understand its anti-inflammatory role in LPS-stimulated cells. To examine its neuroprotective effects, Lee et al. used the BV2 microglial cell line for *in vitro* studies. With 1 h of BITC treatment, it was shown that NLRP3 inflammasome activation and IL-1β secretion were inhibited in addition to the inactivation of the NF-κB pathway, which was previously activated by LPS. ROS levels were also seen to be decreased, suggesting the involvement of ROS in the anti-inflammatory effects of BITC (Lee et al., 2016a). Another study which used steatohepatitis model mice also showed decreased IL-1β and caspase-1 levels in Kupffer cells of BITC treated group, consistent with the results previously found. Further studies with different disease models can be helpful to understand which pathways or downstream molecules are involved in the inhibition of NLRP3 inflammasome by BITC (Chen et al., 2020).

7. Conclusion and future perspective

SFN is one of the phytochemicals that exhibit anti-inflammatory, antioxidant and cytoprotective effects. Considering *in vitro* and *in vivo* studies summarized in Tables 1 and 2, the anti-NLRP3 effect of SFN makes this phytochemical a potential candidate in the treatment of NLRP3-related diseases. Several other previously conducted studies have confirmed the efficacy of SFN with varying consumption time intervals and doses. It is highly promising and motivating that SFN supplementation, even in the form of broccoli itself, has demonstrated beneficial consequences without any significant side effects.

Table 1
In vivo studies of SFN involving NLRP3 inflammasome.

Organism	Injury Model	SFN/BITC treatment (dose/duration/ route)	Inflammasome components identified	Method(s) of detection	Findings about SFN	Mechanism of inflammasome suppression	References
Mouse (BALB/c)	Cerulean hyperstimulation induced AP	5mg/kg SFN for 3 days before AP induction (ip)	NLRP3, IL-1β, Caspase-1	WB, Q-PCR, ELISA	Decreases NLRP3, casp-1-p20, and IL-1β expression	NF-κB inhibition	(Dong et al., 2016) 27, 847,555
Mouse (C57BL/6)	MSU-induced (5 or 10 mg/mouse) peritonitis model	0.5 or 25 mg/kg SFN for 6 h (ip)	NLRP3, NLRC4, AIM2, IL-1β, Caspase-1	WB, Q-PCR, ELISA	Inhibits caspase-1 and IL-1β secretion, decreases NLRP3 and pro IL-1β gene expression	ROS downregulation	(Lee et al., 2016a, 2016b) 27, 423,466
Mouse (C57BL/6)	HFD	Daily administration of 30 mg/kg SFN for 9 weeks (oral)	IL-1β, Caspase-1, ASC	Immunoblot, ELISA	Decreases ASC, caspase-1, IL-1β gene expression and pro IL-1β protein expression, reduces casase-1 activity	mROS downregulation, AMPK-Ulk1 pathway-activated autophagy	(Yang et al., 2016) 27, 075,683
Mouse (C57BL/6 J, Balb/cJ and Nrf2$^{-/-}$ mice on the C57BL/6 background)	MSU crystal-induced acute gout (ip)	25 mg/kg SFN injection (ip) 4 min before MSU-crystal injecton	IL-1β, Caspase-1, MEK	WB, ELISA	Inhibits caspase-1 and IL-1β cleavage	N/A	(Greaney et al., 2016) 26,269,198
Mouse (C57BL/6)	MSU-induced (2 mg/mL, sc) acute gout model	1, 5, 10, 30 mg/kg SFN administration (oral) 1 h before MSU injection	IL-1β, Caspase-1, ASC TNF-α	Immunoblot, ELISA	Decreases caspase-1 activity and IL-1β levels, suppresses pro-IL-1β and pro-caspase-1 degradation	N/A	(Yang et al., 2018) 29, 340,626
Mouse (Sprague-Dawley)	MCAO model	5 and 10 mg/kg SFN injection (ip) 1 h before MCAO	NLRP3, IL-1β, Caspase-1, IL-18	WB, Q-PCR, ELISA	Decreases cleaved caspase-1, IL-1β and mature IL-18 levels and inhibits NLRP3 gene expression	N/A	(Yu et al., 2017) 28, 189,971
Mouse (Sprague-Dawley)	DR induced with STZ (65 mg/kg)	0.5, and 1 mg/kg/d of SFN for 12 weeks (ip)	NLRP3, IL-1β, Caspase-1, ASC	WB, Q-PCR, ELISA, EMSA	Decreases TNF-α, IL-1β and IL-6 levels, and NLRP3, IL-1β, caspase-1 and ASC protein expression	N/A	(Li et al., 2019) 30, 606,939
Mouse (Sprague-Dawley)	I/R-induced acute glaucoma model, NLRP3 knockdown	Daily 5, 10 and 20 mg/kg SFN administration (oral), for 1 week before acute glaucoma surgery	NLRP3, IL-1β, Caspase-1, ASC, TNF-α	WB, Q-PCR, ELISA, EMSA	Decreases IL-1β, TNF-α, NLRP3, caspase-1 and ASC expression	N/A	(Gong et al., 2019) 31, 266,422
Mouse (C57BL/6)	Su5416 (sc) + hypoxia induced PAH model	0.5 mg/kg SFN injection (sc) for 5 days/4 weeks	NLRP3, IL-1β, TNF-α	WB, Q-PCR	Decreases IL-1β, NLRP3 and TNF-α expression	N/A	(Kang et al., 2020) 32, 108,526
Mouse (C57BL/6)	Steatohepatitis model created with HFCCD	HFCDD supplemented with 1 g/kg BITC (oral) for 9 weeks	NLRP3, IL-1β, Caspase-1	WB, Q-PCR, immunoprecipitation	Decreases IL-1β and caspase-1 protein expression	N/A	(Chen et al., 2020) 32, 126,212

Table 2
In vitro studies of SFN involving NLRP3 inflammasome.

Organism	Treatment	SFN/BITC treatment (dose/duration/ route)	Inflammasome components identified	Method(s) of detection	Results	Mechanism of inflammasome suppression	References
RAW264.7, L929 differentiated into BMDMs	1 mg/mL LPS treatment for 2 h after SFN	50 mM SFN pretreatment for 30 min	IL-1β, Caspase-1, MEK	WB, ELISA	Inhibits caspase-1 and IL-1β cleavage	N/A	(Greaney et al., 2016) 26,269,198
Mouse (BV2 cell line)	Stimulation with 1 μg/mL LPS for 48h	1, 5 and 10 μM BITC treatment for 1 h	NLRP3, IL-1β, Caspase-1	WB, Q-PCR, ELISA, EMSA	Decreases IL-1β and NLRP3 gene expression and caspase-1 protein expression	ROS downregulation, NF-κB inhibition	(Lee et al., 2016a, 2016b) 27, 430,883
Human (THP-1 cell line)	Stimulation with 10 μM Aβ(1−42) peptide for 24 h	1, 2 and 5 μM SFN pretreatment for 30 min	IL-1β	WB, Q-PCR, ELISA	Decreases IL-1β gene expression and NLRP3 protein expression	Downregulation of STAT-1 phosphorylation, Nrf2-mediated HO-1 signaling cascade activation	(An et al., 2016) 26, 827,637
Rat (Müller cell line)	25 mM glucose for 48 h	2.5 μM SFN pretreatment for 48 h	NLRP3, IL-1β, Caspase-1, ASC	WB, Q-PCR, ELISA, EMSA	Decreases TNF-α, IL-1β and IL-6 levels, and NLRP3, IL-1β, caspase-1 and ASC protein expression	N/A	(Li et al., 2019) 30, 606,939
Human (WM115, WM266−4, HaCaT and NCTC2544 cell lines)	Pretreatment of NCTC 2544 cells with 20 ng/mL TNF-α for 16 h for ELISA analysis.	5 and 10 μM SFN and 1 and 2 mg/mL FB alone and/or in combination for 24, 48 and 72 h	NLRP3, IL-1β, Caspase-1, ASC	WB, ELISA	Decreases NLRP3 and ASC, cleaved caspase-1 and IL-1β protein expression	N/A	(Serini et al., 2020) 32, 486,135
Mouse (N9 cell line)	1 μg/mL LPS for 4 h and 5 mM ATP treatment for 1 h after SFN	5 μM SFN pretreatment for 2 h	NLRP3, IL-1β, Caspase-1	WB, Q-PCR, ELISA	Decreases secreted and intracellular IL-1β expression, NLRP3 protein expression and pyroptotic cell death	NF-κB inhibition, increased expression of miRNA-223 and decreased expression of miRNA-155 through Nrf2 activation	(Tufekci et al., 2021) 33,711,331

Clinical applications of SFN have importance, especially in chronic inflammatory diseases. However, the source of L-SFN, dose, and consumption duration of L-SFN establishes difficulties in clinical applications. Although promising results have been obtained from cultured neurons and animal models with SFN administration, predictable and nonpredictable potential adverse effects of SFN administration are also essential and need evaluation. Along with the route of administration and time interval, the source and concentration of SFN should be elaborated. The combination of SFN with an additional compound may increase its effectiveness; it should be tested in future preclinical and clinical trials. Loading of SFN into nano drug delivery carriers such as exosomes may provide advantages such as easily crossing the blood-brain barrier. The use of such drug delivery systems should be tried in future studies. SFN suppresses NLRP3 inflammasome by regulating different intracellular mechanisms (Fig. 2). Additionally, novel mechanisms such as miRNAs, lncRNAs, and tRNAs targeting hundreds of genes involved in mechanisms of action of SFN should be included in future considerations.

Declaration of competing interests

The authors declare that they have no known competing financial

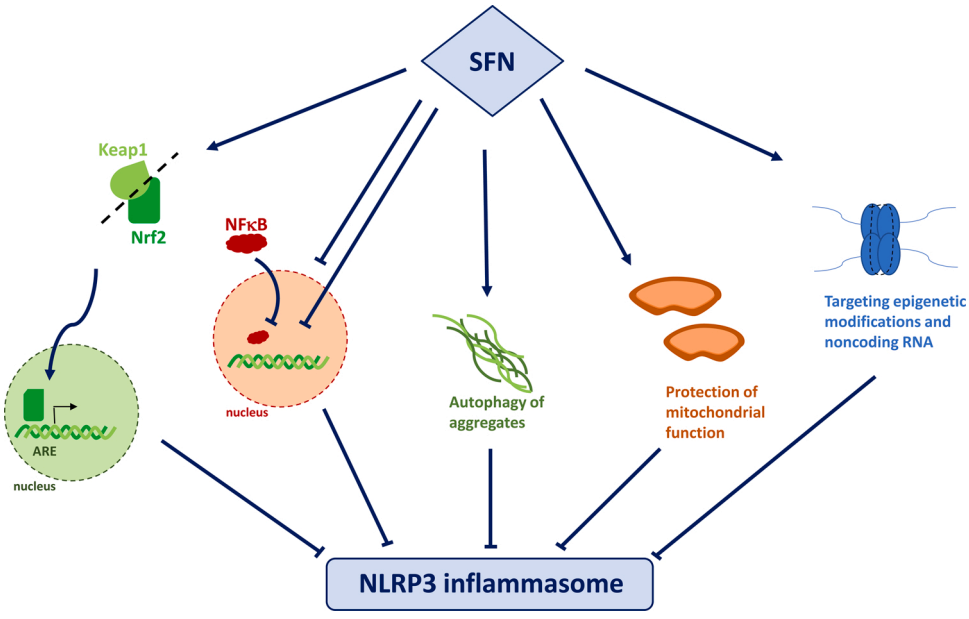

Fig. 2. Mechanisms of SFN acting on NLRP3 inflammasome.

interests or personal relationships that could have appeared to influence the work reported in this paper.

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