T.C.

CANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

ELUCIDATION OF THE THERAPEUTIC ACTION MECHANISMS OF STRIGOLACTONES AGAINST NEUROINFLAMMATORY DISEASES: POTENTIAL INHIBITORY EFFECTS ON NLRP3 INFLAMMASOME AND MICROGLIA ACTIVATION

MASTER OF SCIENCE THESIS

GİZEM ANTİKA

Thesis Supervisor

PROF. DR. TUĞBA TÜMER

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Gizem ANTİKA

06/01/2022

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Çanakkale, January 2022

ÖZET

NÖROİNFLAMATUAR HASTALIKLARA KARŞI STRİGOLAKTONLARIN TERAPÖTİK ETKİ MEKANİZMALARININ AYDINLATILMASI: NLRP3 İNFLAMAZOM VE MİKROGLİA AKTİVASYONU ÜZERİNE POTANSİYEL İNHİBİTÖR ETKİLERİ

Gizem ANTİKA
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Nöroinflamasyon süreci, zararlı bir uyarana karşı öncelikle Merkezi Sinir Sistemi (MSS)'nin bağışıklık hücreleri olan mikroglia tarafından yönlendirilir. Mikroglial hücrelerin uzun süreli aktivasyonu beynin mikroçevresinde kronik düşük dereceli dejenerasyona neden olabilir. Bu inflamatuar döngü sonunda nöronal bozulmaya ve hücre ölümüne yol açabilir. Kronik inflamasyonun sürecinin hızlanmasına sebep olan önemli mekanizmalardan biri de pro-IL-1β'nin proteolitik olarak aktifleşmesine yol açan ve sonunda proinflamatuar hücre ölümü ile sonuçlanan NLRP3 inflamazom aktivasyonudur. Aktifleşen mikroglial hücrelerin proinflamatuar yanıtının yanı sıra, antiinflamatuar M2 ve antioksidan Mox fenotipleri olarak bilinen alternatif aktifleşmiş mikroglial fenotipleri vardır. Ayrıca, mikroglial hücreler, nöronal rejenerasyonu teşvik etmek için BDNF gibi nörotrofinler üretebilir. Strigolaktonlar (SL), karotenoid türevli lakton moleküllerinden oluşan fitohormonlardır. 2020 yılında yayınlanmış bir çalışmamız, model SL analoğu GR24'ün memeli beyin hücreleri üzerindeki anti-nöroinflamatuar etkisinin ilk kanıtını sunmuştur. Bu tez çalışmasının sonuçlarına göre, SL analoglarından IND ve EGO10'un çok düşük dozlarda NLRP3 aracılı IL-1β salgılanımı üzerinde gösterdikleri güçlü inhibitör etkileri, bu bileşiklerin ileride

MSS ile ilgili hastalıklar için NLRP3 inhibitörü olabileceklerini düşündürmektedir. Dört SL analoğu, özellikle 4Br-debranone, Nrf2 sinyal yolağını mRNA seviyesinde teşvik ederek mikroglial hücrelerin M1 fenotipten Mox fenotipine polarize olmasını sağlamıştır. GR24'ün BDNF ile ilişkili antiinflamatuar etkisi ve mikroglia aracılı nörotoksisiteye karşı gösterdiği nöroprotektif etkisi de bu çalışma ile açıklanmıştır. Bu sonuçlar SL'lerin nöroinflamasyona karşı aday terapötik ajanlar olarak geliştirilebileceğini göstermektedir.

Anahtar Kelimeler: Nöroinflamasyon, Strigolaktonlar, NLRP3 inflamazom, Mikroglial fenotipler, Nöroproteksiyon, Nöronal hayatta kalma

ABSTRACT

ELUCIDATION OF THE THERAPEUTIC ACTION MECHANISMS OF STRIGOLACTONES AGAINST NEUROINFLAMMATORY DISEASES: POTENTIAL INHIBITORY EFFECTS ON NLRP3 INFLAMMASOME AND MICROGLIA ACTIVATION

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Master of Science Thesis in Molecular Biology and Genetics

Advisor: Prof. Dr. Tuğba TÜMER

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The neuroinflammation process is primarily driven by microglia which is the immune cells of CNS against a harmful stimulus. The prolonged inflammatory activation of microglia may result in degeneration in the microenvironment of the brain and neuronal cell death. One of the important mechanisms in the acceleration of chronic inflammation is the activation of NLRP3 inflammasome which leads to release of active IL-1β and eventually results in proinflammatory cell death. Besides the proinflammatory response of activated microglial cells, there are alternative phenotypes of microglia which are antiinflammatory M2 and antioxidant Mox phenotypes. Moreover, microglial cells can produce neurotrophins such as BDNF to promote neuronal regeneration. Strigolactones (SLs) are phytohormones consisting of carotenoid-derived lactone molecules. Our previously published data (Kurt et al. 2020) presented the first evidence of the anti-neuroinflammatory effect of GR24 on mammalian brain cells. In the current thesis study, we evaluated SL analogs with different pharmacophores (GR24, EGO10, 4Br-debranone, IND) against microglial activation. According to the in vitro results, the strong inhibitory effects of IND and EGO10 at very low doses on NLRP3-mediated IL-1β secretion

suggest that they can be developed as an NLRP3 inhibitor for CNS-related diseases. The four SL analogs, especially 4Br-debranone, can switch the microglia from M1 to Mox phenotype by promoting the Nrf2 signaling pathway. The BDNF-related antiinflammatory effect of GR24 in microglial cells and its neuroprotective effect against microglia-mediated neurotoxicity were also elucidated in this study. Overall, these results may suggest that SLs can be developed as candidate therapeutic agents against neuroinflammation.

Keywords: Neuroinflammation, Strigolactones, NLRP3 inflammasome, Microglial phenotypes, Neuroprotection, Neuronal survival

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ABBREVIATIONS

siRNA	Small interfering ribonükleic acid
BCA	Bicinchoninic acid
COX-2	Cyclooxygenase-2
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxyde
FBS	Fetal Bovine Serum
HO-1	Heme Oxygenase-1
IL-1β	Interleukine 1 Beta
iNOS	Inducible Nitric Oxide Synthase
LPS	Lipopolysaccharide
MTT	[3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide]
NO	Nitric Oxide
NQO1	NAD(P)H dehydrogenase [quinone] 1
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
TrkB	Tropomyosin receptor kinase B
SDS	Sodium Dodecyl Sulfate
BDNF	Brain-Derived Neurotrophic Factor
TNF-α	Tumor Necrosis Factor-alpha
NLRP3	Nod-like receptor family, pyrin domain-containing 3
mRNA	Messenger ribonucleic acid
Nrf2	Nuclear factor erythroid 2-related factor 2
ATP	Adenosine 5′-triphosphate
ASC	Apoptosis-associated speck-like protein containing a CARD

Gclc	Glutamate-cysteine ligase catalytic subunit
Srxn-1	Sulfiredoxin
TGF-β	Transforming growth factor beta
ROS	Reactive Oxygen species
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
TLR4	Toll-Like Receptor
Aβ	Amyloid beta
PRRs	Pattern-Recognition Receptors
DAMP	Danger-Associated Molecular Patterns
PAMPs	Pathogen-Associated Molecular Patterns

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CHAPTER 1
INTRODUCTION

Today, millions of people worldwide are suffering from neurodegenerative brain diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), stroke, and depression lead to reduced quality of daily life. Inflammation in the peripheral and the central nervous system (CNS) is thought to be a crucial risk factor in these neurodegenerative disorders. Additionally, immune responses in neurodegeneration are now progressively moving toward the central point of researches about CNS diseases (Pizza et al., 2011). Neuroinflammation is taken into account as chronic activation of the immune system within the CNS in response to a harmful stimulus. This brain immune activation leads to numerous sequential events including infiltration of immune cells to the CNS, activation of resident macrophages of CNS, increased release of pro-inflammatory signaling molecules, which eventually induce neuroinflammatory brain disorders (Streit, 2006).

Microglia are CNS-resident macrophage cells that exhibit specific actions on supporting inflammation, tissue remodeling, synaptic plasticity, and neurogenesis depending on the situation. Under neurodegenerative conditions, microglial cells are activated and simultaneously promote the release of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α. Continuous secretion of these molecules causes chronic neuroinflammation that leads to neuronal damage and loss which are eventually linked with brain disorders (Park et al., 2015). Moreover, other specialized CNS cells such as cerebral endothelial cells that regulate the Blood-Brain Barrier (BBB) also secrete neuroinflammatory responses against external stimuli which leads to disruption of CNS homeostasis and increased BBB permeability (Lee et al., 1993). Besides neurons and astrocytes, microglia cells produce brain-derived neurotrophic factor (BDNF) protein which is a neurotrophic factor in neuronal survivor, differentiation, and synaptic development, as a major source (Ferrini & De Koninck, 2013). In chronic inflammation, low secretion of BDNF causes

neurodegenerative diseases such as AD, PD, and Huntington disease. It was recently identified that BDNF has an anti-inflammatory effect on cyclooxygenase-2 (COX-2), which was mediated through EPO/Shh signaling pathway in microglia (Lai et al., 2018), the studies investigating BDNF-TrkB signaling against microglial activation are limited.

Besides, in the inflammatory processes, activation of nod-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome supports the host defense. Several studies suggest that excessive activation of NLRP3 complex initiates the progression of inflammatory diseases including neurodegenerative ones (Fan et al., 2017). Therefore, the identification of anti-inflammatory lead compounds that target the unregulated activation of NLRP3 inflammasome would be very important for effective anti-neuroinflammatory candidate development.

Certain metabolites such as auxin, gibberellic acid, and abscisic acid are present in the mammalian nervous system as well as plants. Therefore, investigations that link phytohormones with CNS actions are a new aspect in the literature (Nthenge-Ngumbau & Mohanakumar, 2011). Strigolactones (SLs) are a class of carotenoid-derived lactone molecules that have a regulatory role in plants. Our previously published data (Kurt et al., 2020) is the first evidence of the biological effects of SLs on mammalian brain cells. Therefore, elucidation of anti-neuroinflammatory effects and action mechanisms of SL analogs on CNS cells in a more detailed manner is needed to develop new candidate neuroprotective agents.

1.1. Inflammation and Neurodegenerative Diseases

Inflammation is a universal response of the immune system to dangerous factors in the form of physical, chemical, or infective such as trauma, high-fat diet, tobacco, stress, radiation, or pathogens (Bennett et al., 2018). Pathogens (pathogen-associated molecular patterns, PAMPs) and endogenous stress signals (danger-associated molecular patterns, DAMPs) are evolutionarily conserved

structures that were sensed by host cells through germline-encoded pattern-recognition receptors (PRRs). Innate immune system cells such as monocytes, macrophages, or lymphocytes principally express PRRs which stimulate these cells and lead to the processing of inflammatory mechanisms (Portou et al., 2015).

The inflammation process in short term is known as acute inflammation protect the body to get rid of invading pathogens or recover the damaged cells as a defense mechanism of the immune system (Netea et al., 2017). However, failure of defeating infections agents, defects in immune response, or exposure to lifestyle risk factors enforce the homeostasis changes and lead to long-lasting inflammation called chronic inflammation, *see in Figure 1*. According to The World Health Organization (WHO), chronic diseases were reported as the highest epidemiologic risk to human health (Organization, Canada, & Canada, 2005).

The rate of diseases associated with chronic inflammation is expected to rise consistently for the next 30 years in the United States. Therefore, investigations of new therapeutic strategies against chronic diseases with no cure including autoimmune diseases, cancer, or neurodegenerative diseases are of great importance to the world population (Pahwa et al., 2020).

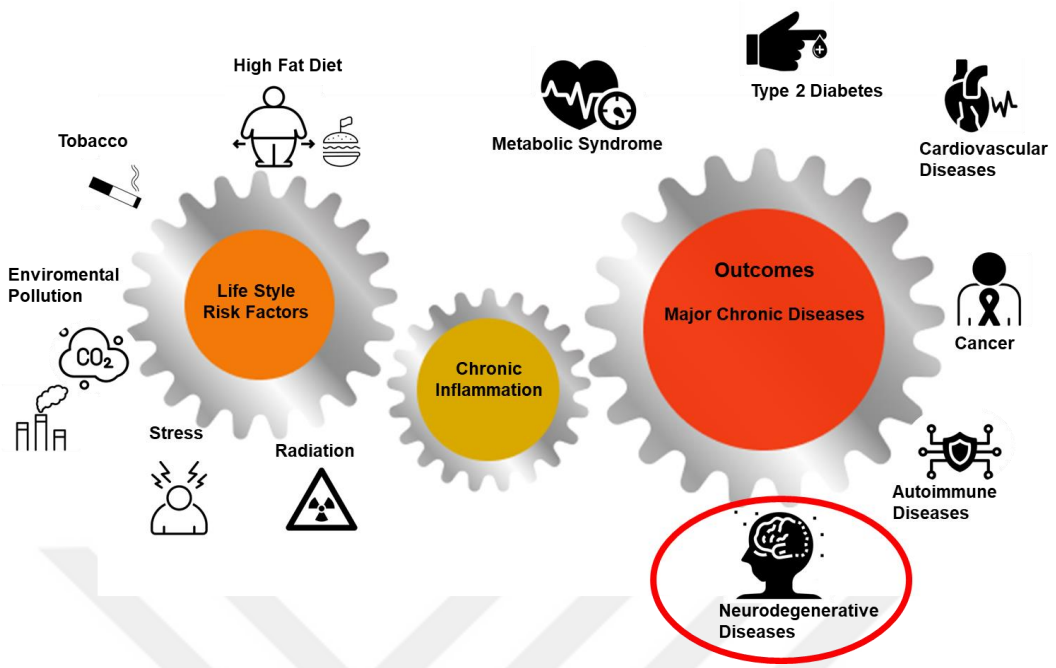


Figure 1. The gearwheel-like progression of chronic inflammatory diseases including neurodegenerative diseases.

The currency of neurodegenerative diseases is globally increasing for dementia in the aging population due to peripheral infections or priming of innate immune responses in the Central Nervous System (CNS) to damage (Cova et al., 2017). There is an inseparable interlink between the immune and nervous systems through the meningeal vessels that connect the brain with the lymphatic system. Therefore, the innate immune response in CNS plays a pivotal role in the progression of neuroinflammation-associated diseases such as Multiple Sclerosis (MS), Alzheimer's Disease (AD), and Parkinson's Disease (PD) (Stephenson et al., 2018).

1.1.1. Neuroinflammation in CNS

Neuroinflammation is the process of an immune response against a stimulation which leads to the generation of proinflammatory molecules by the activation of the cells responsible for the immune response in the brain. Neuroinflammation as a risk factor for CNS triggers immune cells in CNS named

microglia and leads to neuronal cell death. In the chronic form of this process, neuroinflammation is eventually associated with the hallmarks of AD, PD, multiple sclerosis, and even depression pathologies (Jha et al., 2012). Neuroinflammation is a complex system associated with many differentiated cell types such as microglia, astrocytes, brain endothelium, macrophages, and mast cells.

Interactions of CNS Cells

Interconnected interactions of CNS cells that have unique roles in brain health should primarily be explained before understanding the molecular mechanism of neuroinflammation.

CNS is consisted of the brain and spinal cord whose general functions are achieving, processing, and replying to sensory information. Vertebrate CNS stem cells are differentiated into neurons that transmit the information through their axon and dendrites and glia referred to as nerve glue (McKay, 1997). The glia/neuron cell ratio was estimated as 3/1 in the human brain. The huge glial population is subdivided into astrocytes, oligodendrocytes, and microglia with specific functions to cooperate with neurons as guardians. Astrocytes modulate neuronal activity by defensing against oxidative stress, transferring the neurotransmitters, and regulating ion homeostasis (Bélanger & Magistretti, 2009). Oligodendrocytes are myelinating cells of CNS that provide abutment and insulation to axons of neurons (Bradl & Lassmann, 2010). Microglial cells are myeloid cells of CNS which are resident inflammatory cells that provide immunologic defense in the brain. Additively, microglia cells are an important member of the CNS because they are the first and foremost defense system against any stimulus, and they also interact with other glial cells for the progression of the immune response. Therefore, synergic interactions among glia and neurons are crucial for a lot of important features in brain health and disease (Minghetti et al., 2007). Glial activation, neuronal death, and disruption in Blood-Brain Barrier (BBB) are the main issues of neuroinflammation that occur within the interlocked connections of CNS cells, illustrated in Figure 2 (Gorter et al., 2015).

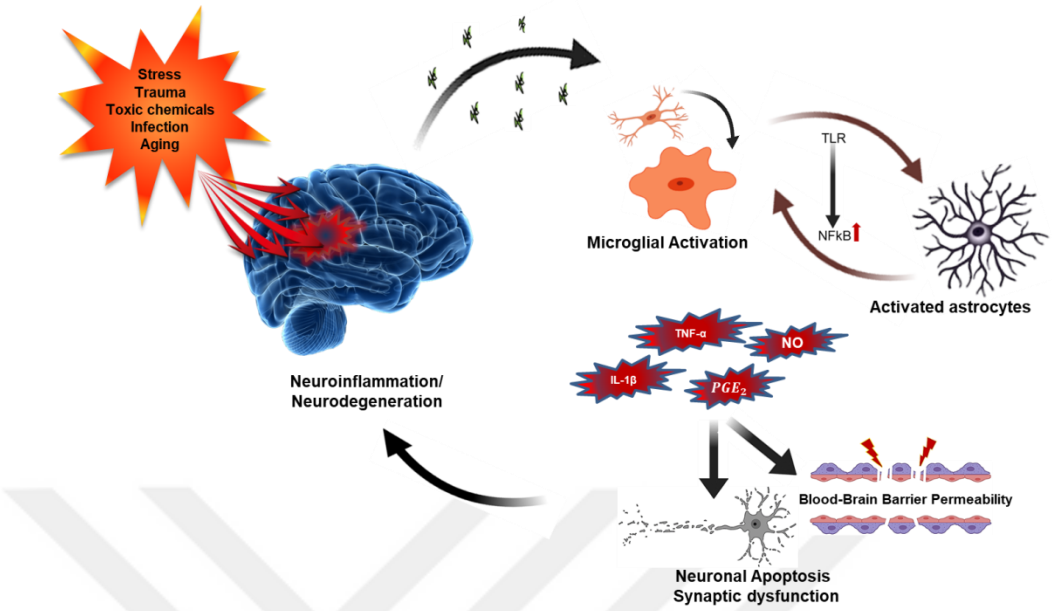


Figure 2. The interdependent pathway of neuroinflammation in CNS.

1.2. Glial Activation

Glial activation is a response of astrocytes and microglia to any neural damage with morphological and physiological changes. As a response, reactive glial cells can produce nitric oxide, cytokines, neuropeptides, or growth factors to protect neurons and eliminate danger factors. However, glial activation in the high level or long term disrupts brain homeostasis, changes the neuronal microenvironment, and plays role in neurodegeneration (Raivich et al., 1999). Moreover, an *in vitro* study reported that astrocytes triggered the proinflammatory response of microglia cells (Solà et al., 2002). The stimulatory relationship of astrocyte-microglia reactivation exacerbated neurological pathologies detected in MS, AD, and stroke. Furthermore, a clinical study by Nordengen et al., demonstrated that glial activation increased the Aβ deposition and shifted the synaptic homeostasis to a harmful stage in AD patients with dementia (Nordengen et al., 2019).

1.2.1. Microglial Activation

In the healthy CNS, microglia constantly investigates the microenvironment in a resting state. Microglia with branched morphology are characterized as 'inactive' forms in the brain. Microglia cells change their morphology in any situation affecting brain homeostasis and become 'active' by inducing the expression of antigens on the cell surface (Ponomarev et al., 2005). The innate immune response of activated microglia may result in favorable or detrimental events depending on the intensity and period of the stimuli. The activated phenotype of microglia is called as proinflammatory M1 phenotype which may give damage to the brain for long period. LPS (lipopolysaccharide) which is a part of the plasma membrane of gram-negative microorganisms used to induce the M1 phenotype in microglial cells as a pathogen-associated molecular pattern (PAMP) *in vitro*/*in vivo* (Gurley et al., 2008).

Toll-Like Receptor 4 (TLR4) is a pattern recognition receptor (PRR) expressed in microglia which recognize the LPS and initiates the inflammatory cellular mechanism. LPS-TLR4 receptor interaction activates the inflammation-associated transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Lester & Li, 2014). Activated microglia cells induce the expression of transcription factors such as NF-κB (Glass et al., 2010). This stimulation lead to an increment in the expression level of the iNOS (inducible nitric oxide synthase) gene causes nitric oxide (NO) release in the environment. Clinically, the increased number of microglial cells expressing iNOS and the high level of nitrite have been observed in PD patients (Hunot et al., 1996). Besides iNOS, COX2 is another local mediator enzyme of inflammation and neurotransmission which is known as the main target of nonsteroidal anti-inflammatory drugs (Luisa Minghetti & Levi, 1998).

Activation of proinflammatory transcription factors also increases the production of other proinflammatory molecules containing reactive oxygen species (ROS), reactive nitrogen species, interleukins (IL), and tumor necrosis factor-alpha (TNF-α) in microglial cells. There are several pieces of evidence indicating that these proinflammatory molecules released from microglia to the brain

microenvironment cause neurotoxicity and degeneration in neurons (Gao et al., 2002).

1.2.2. Microglial Phenotype Polarization

Recent studies determined that microglia retain tissue homeostasis and defend the CNS with different phenotypes under different conditions. Although activated microglia is destructive for neurological diseases, the primary function of microglia with the M1 phenotype is the clearance of foreign molecules for the protection of CNS. Moreover, macrophage/microglia can also be polarized to M2 (antiinflammatory), or Mox (antioxidant) phenotypes depending on the variety of stimuli (Kadl et al., 2010).

IL-4 is known as one of the main inducers for M2 polarization which leads to the reduction in proinflammatory cytokine signaling. Microglia M2 phenotype produce anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β to providing suppression of neuroinflammation, maintainance of homeostasis, and protection of nerve tissues. The M2 phenotype polarization is characterized by the expression of specific markers such as Arg1, CD206, Fizz1, and Ym1 genes (Z. Chen & Trapp, 2016). It was reported that the microglia population in the M1 phenotype was much more dominated than the M2 phenotype in the situation of spinal cord injury. Change in the homeostasis of microglial polarization led to the decreased phagocytosis of damaged myelin and apoptotic cell which is associated with neurotoxicity (Kroner et al., 2014). On the other side, the prevalence of M2 phenotype microglia may worsen the progression of some neovascular diseases such as glioblastoma multiform because of their pro-angiogenic and protumorigenic features (L. He & Marneros, 2014).

In a recent study of Kadl et al., the novel macrophage phenotype was identified as Mox phenotype that developed in the oxidative microenvironment. Macrophages with Mox phenotype are represented by an obvious upregulation of Nrf2 as a key regulator in return for oxidized phospholipids. Moreover, the novel

phenotype was characterized by the expression of Nrf2-mediated genes including HO-1, NQO1, sulfiredoxin (Srxn-1), glutamate-cysteine ligase catalytic subunit (Gclc), and Thioredoxin reductase 1 (Kadl et al., 2010). In literature, a few studies investigated the action mechanism of antiinflammatory molecules for the polarization of the activated microglia to Mox phenotype. The Mox phenotype polarization has great importance in the amelioration of oxidative stress which cooperates with inflammation. An *in vivo* study showed that $\alpha 7$ nicotinic acetylcholine receptor expressed in periphery suppressed inflammation and stimulate the Mox phenotype polarization in microglia for neuroprotection of the brain against perinatal hypoxia-ischemia in neonatal mice (Hua et al., 2014). These different microglial activation states are dynamically and continuously shifting based on the feature of the stimulant and inflammatory responses. Furthermore, the discovery of therapeutics that control the balance between different microglial phenotype populations could advance the repairing of nervous system diseases (Hu et al., 2015).

Cytoprotective Transcription Factor Nrf2

The transcription factor nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) gene so-called Nrf2 is a common cytoprotective pathway that regulates the expression of antioxidant and detoxification genes (Q. M. Chen & Maltagliati, 2018). Nrf2 transcription factor is activated in oxidative stress conditions and enhanced its expression with positive feedback. The Nrf2 activation promotes the expression of an array of cytoprotective genes containing the antioxidant response element (ARE) promoter region. NAD(P)H quinone oxidoreductase (NQO1) and glutamate-cysteine ligase catalytic subunit (Gclc) are Nrf2-mediated detoxification genes that play multiple roles in the redox environment. NQO1 detoxifies reactive molecules by reducing the ubiquinone and vitamin E derivatives to antioxidant forms and scavenging superoxides. Moreover, Nrf2 tightly regulates the synthesis of glutathione by modulating the Gclc gene which is necessary for the detoxification of the xenobiotics (Q. M. Chen & Maltagliati, 2018).

Heme oxygenase-1 (HO-1) and sulfiredoxin-1 (Srxn1) are Nrf2-mediated antioxidant genes. HO-1 mitigates ROS generation by degrading free heme molecules that

accumulated after tissue damage and also promote mitochondrial biogenesis. At the organism level, HO-1 is responsible for cardioprotection and tissue repair as well as regulation of the inflammatory process (Otterbein et al., 2016). Nrf2 also activates the transcription of the Srnx1 gene which mitigates oxidative stress after oxygen/glucose deprivation to keep the balance of the cellular oxidation/reduction in astrocytes (Zhou et al., 2015).

1.3. NLRP3 Inflammasome Activation: A Key Inflammatory Mechanism

Another crucial mechanism in the acceleration of chronic inflammation in CNS is the activation of NLRP3 inflammasome which is consist of nod-like receptor protein NLRP3, adaptor protein ASC, and procaspase-1. Inflammasome complex is stimulated by an array of substances that arise throughout infections, tissue damage, or metabolic instability. The NLRP3 inflammasome mechanism during these pathological conditions disrupt the homeostasis of cells and the microenvironment and cause cellular death. The process damages tissues and accelerates the progression of several chronic diseases including gout, atherosclerosis, metabolic syndrome, and neurodegenerative diseases (Gustin, 2016). NLRP3 inflammasome activation is an essential regulator of IL-1β which is involved in neuroinflammation-caused diseases such as AD. The misfolded amyloid proteins trigger the inflammasome activation in different parts of the body; Aβ in AD, pancreatic islet amyloid polypeptides in type-2 diabetes, and superoxide dismutase 1 in amyotrophic lateral sclerosis (Masters & O'Neill, 2011).

1.3.1. Molecular Mechanism of NLRP3 inflammasome activation in Microglia

The multiprotein complex is primed by microbial or endogenous molecules such as LPS, TNF-α, or IL-1β as a first signal that induces NLRP3 and pro-IL-1β expression through activation of NF-κB signaling pathway. Then the NLRP3 inflammasome is activated to form the complex by a second signal which implicates PAMPS, and crystals. The signal 2 factors such as adenosine 5′-

triphosphate (ATP), pore-forming toxins, or viral RNA trigger the recruitment of ASC and caspase-1 proteins, see in *Figure 3*. (He et al., 2016). In addition, recent *in vitro* studies proved that the inflammasome mechanism is also activated in microglial cells after exposure to neuropathological proteins such as prion protein or Aβ (Halle et al., 2008; Shi et al., 2012).

NLRP3 inflammasome complex is assembled to activate caspase-1 to the cleaved form in the cell. The active caspase-1 proteolytically cleaves pro-IL-1β into the active IL-1β which is simultaneously secreted from cells (Figure 3). The caspase-1 dependent IL-1β release performs an indispensable role in the generation of pyroptosis which is an inflammatory programmed-cell death (Latz et al., 2013). The released mature IL-1β binds to the Interleukin-1 receptor of surrounding cells and triggers the stress response. The strong triggering role of IL-1β accelerates the propagation of chronic inflammation. Furthermore, caspase-1 activation is involved in pore formation, membrane rupture, and pyroptosis.

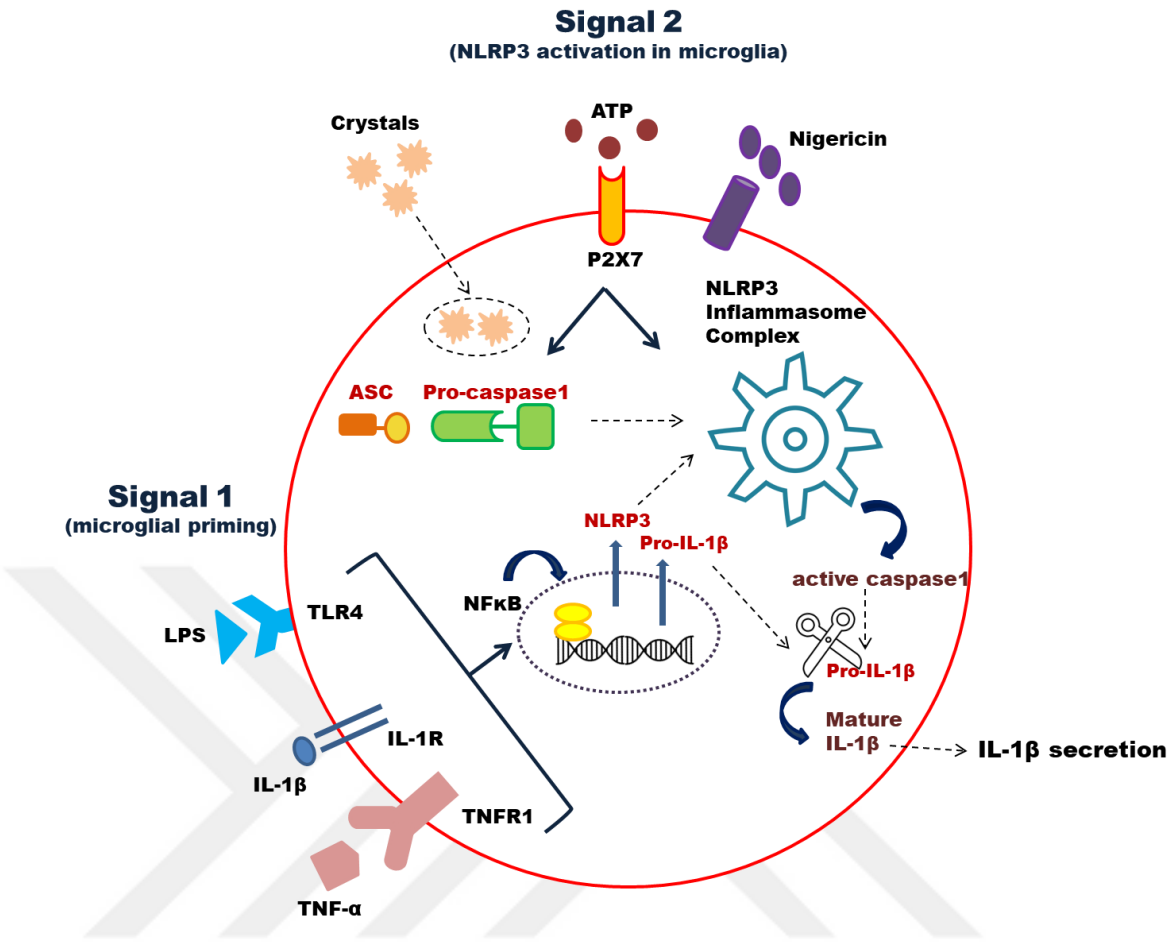


Figure 3. The molecular mechanism of NLRP3 activation by common triggers in microglia cells.

During NLRP3 inflammasome activation, the fluxing balance of K^+ and Ca^{2+} ions shifted when the signal 2 factors trigger the microglial cells. For instance, ATP is recognized by the ionotropic P2X7 receptor which induced the opening of K^+ efflux channels and results in an increased level of IL-1β and IL-18 release (Pétrilli et al., 2007). Nigericin, a pore-forming toxin, induces the NLRP3 inflammasome by permeabilizing the cellular membrane to K^+ efflux. NLRP3 inflammasome complex is assembled after the cytosolic K^+ level is decreased by triggers that target plasma membrane permeability such as ATP and nigericin Another special process in NLRP3 inflammasome activation is mitochondrial damage due to increased ROS production by stimulation with ATP or nigericin in microglial cells (Perregaux & Gabel, 1994).

1.4. Neuronal Health in Inflammation

The important regulators of neuronal survival, development, function, and plasticity are known as neurotrophins or nerve growth factors (NGF). Brain-derived neurotrophic factor (BDNF) is one of the vital NGF that functions in neuronal survival and synaptic strength and plasticity related to learning and memory. BDNF expression is regulated with TrkB receptor tyrosine kinase with positive feedback. TrkB-BDNF signaling initiates the beneficial biological cascade in neurons (X. Song et al., 2016). BDNF is synthesized by neurons, astrocytes, and microglial cells.

Microglial BDNF was first demonstrated in microglia cultures and different parts of CNS during various neurological disorders (Ferrini & De Koninck, 2013). The reduced BDNF expression level has been detected in some animal neurodegenerative disease models including Hungtington disease, PD, and AD (Jiao et al., 2016). However, a large amount of BDNF was suggested to be expressed and secreted from different cell types found in cerebral arterioles such as microglia when nerve cells are damaged by ischemic stroke (Béjot et al., 2011). Besides the inflammatory responses of activated microglia, they also augmented the secretion of specific messengers, including BDNF for neuroprotection (T. Miwa et al., 1997).

However, the prolonged inflammatory activation of microglia may result in chronic low-grade degeneration in the brain microenvironment, neuronal disruption, and cell death. Therefore, researches have been focused on the inhibition of microglia-mediated neurotoxicity by a natural product. The new therapeutic agents against neuroinflammation should be targeted specifically on microglia-mediated neurotoxicity by promoting the production of NGFs such as BDNF (Kaur & Prakash, 2017).

1.5. Sitrogolactones

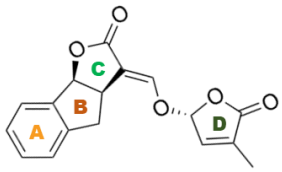
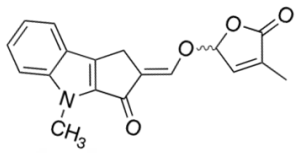
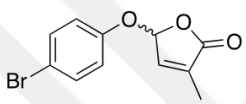
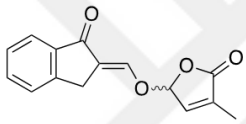
Phytohormones are chemicals produced in very low concentrations by plants for the adaptation to environmental stimuli and also required for plant development. Strigolactones (SLs) are a class of carotenoid-derived lactone molecules as a novel phytohormone type that have a regulatory role in plants. It

was first discovered in 1966 as molecules that enable the germination of Striga plants, a root parasite. Later, the release of SLs by plant roots was reported to stimulate hyphal branching in arbuscular mycorrhizal fungi and increase the effectiveness of symbiosis (C. Cook et al., 1966). Since 2008, SLs have been proven to play an important role in the control of shoot and root branching, fruit ripening, secondary growth, aging, and stress response in plants (Umehara et al., 2010).

The recently elucidated biological properties have led to the exploration of various natural and synthetic SL (analogs) with different bioactiphores. The chemical structure of SLs originates from a core of the tricyclic ABC lactone ring system attached to a butanolide D-ring by an enol-ether bridge (Table 1). The chemical structure of GR24 is classified as a canonical strigol-type SL while EGO10, 4Br-debranone and IND have different bioactivities with unique combinations of C-, D-lactam modifications (Bürger & Chory, 2020). IND is a novel synthetic SL analog derived from indanone with B, C, and D ring systems. There is still no study that examined the pharmacological effect of IND on a mammalian cell system including cancer models (Mwakaboko, Zwanenburg, & physiology, 2011). Moreover, the anti-inflammatory activity of SL analogs including EGO10, IND, and 4Br-debranone has not been investigated on neuroinflammation models yet.

Table 1

The chemical composition of SL analogs used in this study

Chemical Structure	Compound	IUPAC Name
	GR24	(±) (3a*R*,8b*S*,*E*)-3-((((*R*)-4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)methylene)-3,3a,4,8b-tetrahydro-2*H*-indeno[1,2-*b*]furan-2-one
	EGO10	(±)(*E*)-4-methyl-2-(((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)methylene)-1,4-dihydrocyclopenta[*b*]indol-3(2*H*)-one
	4Br-debranone	(±) 5-(4-bromophenoxy)-3-methylfuran-2(5*H*)-one
	IND	(±) (*E*)-3-methyl-5-((1-oxo-1,3-dihydro-2*H*-inden-2-ylidene)methoxy)furan-2(5*H*)-one

1.6. The Aim of The Study

In this thesis, the four SL analogs with different bioactiphores GR24, EGO10, 4Br-debranone, and indanone-derived SL (IND) were studied on LPS-induced microglia and neuronal cells for the elucidation of their anti-neuroinflammatory activities and mechanism of action. The multi-target effects of the four SL analogs on neuroinflammation were elucidated for the first time by focusing on NLRP3 inflammasome activation, microglial phenotype polarization, BDNF, and Nrf2 related pathways, and neuronal survival in vitro. SIM-A9 mouse microglial cell line was selected for the LPS-induced neuroinflammation model. In addition, the NLRP3 inflammasome activation model was conducted on SIM-A9 microglial cells for the first time by ATP/nigericin induction after LPS treatment.

In the scope of thesis studies;

a- Nontoxic/safe doses of SL analogs; EGO10, 4Br-debranone, and IND were determined by cytotoxicity tests on SIM-A9 microglial cells,

b- The mechanism of SL analogs on nitrosative stress and pro-inflammatory factors were elucidated on LPS-induced SIM-A9 cells at gene and protein levels using Real Time-PCR, ELISA, and Western Blot techniques,

c- The potential inhibitory effects of four SL analogs on LPS+ATP or LPS+nigericin-induced NLRP3 inflammasome activation were elucidated by analyzing the level of mature 1L-1β secretion using ELISA assay in SIM-A9 microglial cells.

d- The modulatory effects of SL analogs on microglial phenotype polarization were analyzed at gene expression level by RT-qPCR techniques using specific markers of M1, M2, and Mox phenotype on LPS-induced SIM-A9 cells,

e- The molecular mechanism of GR24 on Nrf2 dependent anti-neuroinflammatory effect was investigated by using siRNA gene silencing technique on SIM-A9 cells,

f- The relation between the anti-inflammatory effect of GR24 and the BDNF pathway was investigated by using a TrkB receptor inhibitor drug on LPS-induced SIM-A9 cells. The change in the anti-inflammatory effect of GR24 on SIM-A9 cells with or without TrkB inhibitor was analyzed by RT-qPCR technique as a new insight for the neuroprotective effects of SLs for both neurons and microglia,

g- The indirect effects of the four SL analogs on microglia-mediated neurotoxicity were determined by using the conditioned medium model on SH-SY5Y neuronal cells.

CHAPTER 2
PREVIOUS STUDIES

Phytohormones are special molecules for the mammalian brain because of their capability to cross the BBB. Therefore, they have the potential to accumulate in the brain and affect brain functions. Indeed, some studies have shown that detectable amounts of gibberellic acid, and indole-3 acetic acid cumulated in the brains of mice that were fed with a diet rich in these phytohormones (Nagashayana et al., 2000). A recent study showed that abscisic acid, a phytohormone, ameliorated learning and memory problems by modulating PPAR-β/δ receptors and the PKA signaling pathway in rats with the AD model. However, there was no evidence related to the biological activities of SLs, recently identified phytohormones, on CNS cells in case of neuroinflammation until our previous study (Kurt et al., 2020). .The initial studies with SLs on mammalian cells have primarily focused on anticancer activities of SL analogs. Pollock et al., (2012) showed that GR24, the most widely used SL analog, inhibited the growth and survival of breast cancer cells and mammospheres by inhibiting PI3K/AKT activation and regulating stress-related pathways (C. B. Pollock et al., 2012). Moreover, an in vivo study demonstrated the anticancer effect of SLs on the breast cancer xenograft model, by affecting the microtubule bundling (Mayzlish-Gati et al., 2015). Following in vitro study by the same research group have shown that SL analogs significantly suppressed the proliferation of prostate, colon, lung, breast, melanoma, osteosarcoma, and leukemia cell lines (Pollock et al., 2014). Additively, those SL analogs induced the cell cycle arrest and activation of apoptosis through the stress-related MAPK. Another study showed that SLs induced DNA damage and inhibited DNA repair in cancer cells which results in increased genome inconstancy and cell death (Michael P. Croglio et al., 2016).

Shortly after cancer-related studies, the favorable effects of GR24 on glucose metabolism were demonstrated in rat L6 skeletal muscle cells (Modi et al., 2017). However, studies about the effects of SLs against inflammation were not found in literature until 2018. The inhibitory effect of GR24 was identified on the release of certain pro-inflammatory factors and migration of neutrophils in

fluorescence-marked zebrafish (Zheng et al., 2018). The marked potency of GR24 in the suppression of LPS-induced inflammation and neuroinflammation was demonstrated in macrophages and microglial cells respectively by our research group (Kurt et al., 2020; Tumer et al., 2018).

In our previous study in 2018, it has been shown for the first time that GR24 had anti-inflammatory, antioxidant, and cytoprotective effects on LPS-induced macrophage cell lines (Tumer et al., 2018). Consequently, our previously published data in 2020 demonstrated that GR24 had a strong anti-neuroinflammatory effect on LPS-induced microglial activation, nitrosative stress, and pro-inflammatory cytokine release by regulation of NF-κB, Nrf2, and PPARγ signaling pathways. In addition, we also elucidated that GR24 has a BBB-protective effect in LPS-induced brain endothelial cells by promoting the gene expression level of a tight junction protein (occludin) and decreasing the permeability level of LPS-damaged BBB, *in vitro*. (Kurt et al., 2020). Therefore, studies relating the SLs on neuroinflammation and their action mechanisms are still limited in the literature. However, it is suggested that SLs would be promising candidate agents against neuroinflammation-related diseases such as AD (Dell'Oste, Spyrakis, & Prandi, 2021). Recent in vitro studies in the scope of neuroinflammation investigated the effects of natural compounds on microglial phenotype polarization and Nrf2-dependent action mechanism in microglial cells. In the study of Eren et al., (2018), sulforaphane which is a phytochemical suppressed the LPS-induced microglial activation and oxidative stress through the Nrf2 signaling pathway in the N9 microglial cell line. Additively, the M1 phenotype hallmarks including the high level of IL-1β and TNF-α gene expression and secretion as well as NO and ROS production were significantly suppressed by sulforaphane treatment in microglial cells. Moreover, sulforaphane significantly enhanced the Mox phenotype polarization in LPS-induced microglial cells by upregulating Nrf2-mediated genes including NQO1, HO-1, Gclc, and Srxn1. (Eren et al., 2018). On the other hand, there are many in vitro and in vivo studies that focused on the suppression of NLRP3 inflammasome activation in macrophages/microglia cells by the treatment of phytochemicals (Fan et al., 2017; J. Lee et al., 2016; Wei et al., 2018). A recent in vitro study by Tufekci et al (2021)

indicated that sulforaphane inhibited the NLRP3 inflammasome activation in LPS and ATP-induced N9 microglial cells (Tufekci et al., 2021). The prominent NLPR3-induced mature IL-1β secretion and caspase-1 activity were significantly decreased by 5 μM of sulforaphane. Moreover, NF-kB nuclear translocation, NLRP3-caused pyroptotic cell death and mitochondrial ROS production were all suppressed by sulforaphane in microglial cells (Tufekci et al., 2021). In a recent study, Lai et al. have firstly elucidated the anti-neuroinflammatory effect of minocycline, an FDA-approved drug,on microglial activation through the BDNF-EPO-Shh signaling pathway in microglial cells (Lai et al., 2018). The LPS-induced COX-2 mRNA and protein expressions were mitigated while BDNF expression was upregulated by minocycline treatment in BV-2 microglial cells. Blocking the BNDF signaling with the treatment of a TrkB inhibitor revealed that the inhibitory effect of this drug on COX-2 was regulated with BNDF signalling patway in BV-2 cells. Furthermore, Lai et al., (2018) suggested that the increased level of BDNF expression by therapeutic agents in microglial cells may inhibit neuroinflammation while protecting neuronal survival (Lai et al., 2018). Even though the helpful effects of BDNF within the CNS have been identified, there are limited studies that investigate the BDNF-mediated anti-neuroinflammatory effects of a potential therapeutic compound like phytochemicals. An in vivo study suggested that neuroinflammation-related learning and memory impairment could be protected via ROS-BDNF-TrkB pathway with the treatment of silibinin, a flavanoid in LPS-treated rats (X. Song et al., 2016).

The concomitant interactions among glia and neurons are crucial for brain health and disease progressions (L. Minghetti et al., 2007). In the study of Eren et al., (2018), the detrimental effects of activated microglia on neuronal survival and neurite number were assessed in vitro by treating SH-SY5Y neuronal cells with LPS-induced microglial conditioned media and coculture model (Eren et al., 2018). This study showed that the neurotoxicity of LPS-induced N9 microglial secretomes on SH-SY5Y neuronal cells was decreased by 5 μM of sulforaphane treatments in both coculture and conditioned medium models (Eren et al., 2018).

As mentioned above, recent studies investigated the therapeutic effects of molecules against neuroinflammation in microglial cells by targeting different key

mechanisms including microglial phenotype polarization, NLRP3 inflammasome activation, Nrf2 signaling, or neuroprotective BDNF-TrkB pathways.

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals, Kits, and Required Products

All materials used in this study are classified according to their usage areas and listed in Table 2.

Table 2

List of chemicals, kits, and required products

	Code	Items
Cell Culture Chemicals	D6429	Dulbecco's Modified Eagle's Medium-high glucose
	PBP01	Dulbecco's PBS
	DMP17	Dulbecco's Modified Eagle's Medium without phenol red
	11320074	Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12
	10270106	Fetal Bovine Serum (FBS)
	15140122	Penicillin-Streptomycin
	35050061	GlutaMAXTM Supplement
	26050088	Horse Serum Heat Inactivated
	L6529	Lipopolysaccharides from Escherichia coli O55:B5
	A6419	Adenosine 5′-triphosphate disodium salt hydrate
	4312	Nigericin sodium salt – TOCRİS
	HY-12678-5	Entrectinib
	10496	Sulforaphane
	M5655	MTT(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue)
	T8154	trypan blue solution
	SC-358801	Dimethyl Sulfoxide (DMSO), cell culture reagent
	25200056	Trypsin-EDTA (0.25%), phenol red
	31985047	Opti-MEM™ I Reduced Serum Medium 500ml

	"Table 1 continue"	
Kits	Q10210	QubitTM RNA BR Assay Kit
	23227	Pierce™ BCA Protein Assay Kit
	78835	NE-PER Nuclear and Cytoplasmic Extraction Kit
	48300	Total RNA Purification Plus Kit
	4368814	High-Capacity cDNA Reverse Transcription Kit
	BMS6002	IL-1 beta Mouse ELISA Kit
	BMS607-3	TNF alpha Mouse ELISA Kit
qPCR and siRNA Gene Silencing Studies	4444557	TaqMan™ Fast Advanced Master Mix
	4331182	TaqMan™ Gene Expression Assay, Mm00440502_m1-iNOS
	4331182	TaqMan™ Gene Expression Assay, Mm02619580_g1- Actbβ
	4331182	TaqMan™ Gene Expression Assay, Mm03294838_g1- COX-2
	4331182	TaqMan™ Gene Expression Assay, Mm00434228_m1- IL-1β
	4331182	TaqMan™ Gene Expression Assay, Mm00443258_m1 TNF-α
	4331182	TaqMan™ Gene Expression Assay, Mm00477784_m1- Nfe2l2
	4331182	TaqMan™ Gene Expression Assay, Mm01253561_m1-Nqo1
	4331182	TaqMan™ Gene Expression Assay, Mm00516005_m1-Hmox1
	4453320	TaqMan® Gene Expression Assays,Mm00802655_m1-Gclc
	4453320	TaqMan® Gene Expression Assays, Mm00475988_m1-Arg1
	4453320	TaqMan® Gene Expression Assays, Mm01329362_m1Mrc-1
	4448892	TaqMan® Gene Expression Assays, Mm00769566_m1Srnx1
	11668027	Lipofectamine ™ 2000 Transfeksiyon Reaktifi
	4390843	Silencer ™ Negatif Kontrol siRNA
	4390771	Pre-designed Silencer® Select siRNA, 5 nmol
Western Blot	34580	SuperSignalTM West Pico PLUS Chemiluminescent
	sc-47778	ß-Actin (C4) Antibody, Santa Cruz
	sc-7271	NOS2 (C-11) Antibody, Santa Cruz
	sc-365949	Nrf2 (A-10) Antibody, Santa Cruz
	sc-376861	Cox-2 Antibody (H-3), Santa Cruz
	NB7160	Secondary Antibody Goat Anti Rabbit, NOVUS

3.1.2. Essential Equipment

This thesis study was carried out with the equipment found in the Molecular Biology and Genetics Research Laboratory at Çanakkale Onsekiz Mart University. The equipment used in this study is listed in Table 3.

Table 3

The list of the essential equipment

Brand	Model	Equipment
Tecan	Infinite® M200 PRO	Microplate Reader
Inivitrogen	Qubit 4	Fluorometer
LI-COR Biosciences	C-DiGit ®	Blot Scanner
Thermo Fisher Scientific	Applied Biosystems™ 7500	Real-Time PCR Systems
Esco	CCL-170T-8	Cell Culture CO2 Incubator
Esco	Class II BSC	Biological Safety Cabinets
Major-Science (MS)	MW-23	Waver Shaker
BioSan	TS-100	Thermo-Shaker
Hanna Instruments	HI-2211	pH meter
Hettich Zentrifugen	MIKRO 200R	Refrigerated Micro Centrifuge
Hettich Zentrifugen	Universal 320	Standart Centrifuge
BioRad	-	Vertical Electrophoresis System
BioRad	T100™	Thermal Cycler
Thermo Fisher Scientific		Wet Electroblotting System

3.1.3. Supply of SL Analogs Used in the Study

SL analogs used in the study; GR24, 4Br-debranone, IND, and EGO10 were purchased from Strigolab company in Italy (https://strigolab.eu/).

3.2. Methods

3.2.1. Cell Growth Studies

According to the appropriate growth conditions specified in the ATCC, SIM-A9 microglia cells were growth in DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) medium including 10% fetal bovine serum (FBS), 5% horse serum, and 1% penicillin-streptomycin in an incubator comprising 5% CO_2 and 75% humidity at 37 °C. SH-SY5Y human neuroblastoma cell line were growth in DMEM medium with 10% FBS, and 1% penicillin-streptomycin.

These cells were harvested with Trypsin-EDTA and plated for the following studies when they have reached 80% density. Inflammatory in vitro model in SIM-A9 cells was established by the treatment with Lipopolysaccharide (LPS) for 12 hours (gene and protein expression analysis) or 24 hours (cell viability and NO assays) as optimized for SIM-A9 cells in our previously published data (Kurt et al., 2020). Additionally, SIM-A9 cells were treated with only DMSO as an untreated control and DMSO+LPS as a LPS-induced control group in each experiment.

3.2.2. NLRP3 Inflammasome Activation Model in SIM-A9 cells

To activate the molecular mechanism of the NLRP3 inflammasome, there are two key steps known as priming mostly by LPS induction (signal 1) and the second one (signal 2) is the activation step by ATP or nigericin (NIG). For priming step, the optimum time to induced the IL-1β secretion by 1 µg/ml of LPS stimulation was determined as 24 hours. SIM-A9 cells were treated firstly with LPS in the presence or absence of 5 and 10 µM of IND and EGO10, 20 and 50 µM of 4Br-debranone, 10 and 20 µM of GR24, 5 µM of sulforaphane as a positive control or DMSO as a vehicle control (J. Lee et al., 2016; Tufekci et al., 2021) for 24 hours. After LPS priming, the treatment process followed with 1 mM of ATP or 10 µM of nigericin for 40 minutes to switch on the NLRP3 inflammasome mechanism. The cells and supernatants were collected to evaluate the effects of SL analogs against NLRP3 inflammasome activation in microglia cells by ELISA, Griess and Western Blot analysis.

3.2.3. Suppression of BDNF-TrkB pathway in SIM-A9 cells

First of all, BDNF gene expression levels were analyzed in SIM-A9 cells which were treated with LPS and SL analogs by RT-qPCR technique (as described in section 3.2.7). GR24 at 50 µM was selected for further analysis as a BDNF-promoter SL analog and then, its anti-inflammatory effect on COX-2 gene was analyzed by RT-qPCR technique. The optimum concentration of entrectinib, a TrkB receptor inhibitor was assessed by MTT cell viability assay at the dose range of 1 nM to 10 µM. Then, RT-qPCR analysis for COX-2 gene for 50 nM-10 µM doses of entrectinib within LPS treatment were performed in SIM-A9 cells. Finally, the inflammation model with suppressed-BDNF signalling pathway was conducted on SIM-A9 cells with pre-treatment of 1 µM of entrectinib followed by LPS treatment in the presence or absence of GR24. After 12 hours of GR24 treatment, gene expression analysis of these samples was carried out for BDNF and COX-2 genes to estimate the potential BDNF-mediated anti-inflammatory effect of GR24.

3.2.4. MTT Cell Viability Assay

The MTT [3- (4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide] method is a spectrophotometric test that can sensitively measure cell proliferation. Tetrazolium dye reacts with succinate dehydrogenase (SDH), a mitochondrial enzyme in living cells, and is reduced to formazan, which can be measured spectrophotometrically. As a result, the blue-purple colored, water-insoluble formazan substance is formed. The MTT reduction capability of cells is considered as a measure of cell viability; therefore, the dye density correlates with the number of viable cells.

The MTT assay was established to decide the safe doses of SL analogs on SIM-A9 microglia cells. Firstly, cells were seeded into 24-well plates at a concentration of $4x10^5$ cells/well and incubated for 24 hours. After that, SIM-A9 cells were treated with EGO10, IND, and 4Br-debranone at concentrations varying between 1-100 µM and 1 µg/mL LPS whose concentration had been optimized for the cell line in our previous study (Kurt et al., 2020). The treated cells were incubated for 24 hours under appropriate conditions, and then the medium of each

sample was aspirated gently. The 20 µL MTT at 0,1 mg/mL concentration was simulatenously added to each well and incubated for two hours at 37 ° C, 5% CO_2, and 75% humidity conditions. After that, the medium was aspirated and the formazon formed by living cells were dissolved with 200 µL of DMSO for 10 minutes. Cell survival rates were quantified at 570 nm and 620 nm (background) using an ELISA reader.

3.2.5. NO Release Measurement and IC_{50} Determination

NO release levels were determined by analyzing the media collected from the inflammation model of SIM-A9 microglia cells according to the Griess method (Griess, 1879). As an analytical chemistry method, Griess test determine the nitrite ion (NO^{2-}) in solution. The chemical composition of Griess reagent is 0,2% N-(1-naphthyl) ethylenediamine dihydrochloride, 5% phosphoric acid, and 2% sulfanilamide. A red-pink azo dye is formed via the reaction of the NO^{2-} ion found in the medium with the Griess reagent. Different doses of SL analogs were administered to SIM-A9 cells together with 1 µg/mL of LPS and incubated for 24 hours. After 24 hours, 100 µL medium for each sample and sodium nitrite ($NaNO_2$) standards which were prepared in the range of 0-100 µM concentration was mixed with Griess reagent at equal amounts and incubated in a 96-well plate for 10 minutes at room temperature and in a dark environment. The absorbance of color that formed after incubation was quantified at 520 nm using a spectrophotometer with a microplate reader. The calculated NO concentrations were normalized by cell viability percentages. IC_{50} and ANOVA analyzes were performed with GraphPad Prism 8 program.

3.2.6. TNF-α ve IL-1β ELISA Tests

ELISA is one of the commonly used immunological tests to detect protein, antigen, and antibody levels in biological samples. The principle of the Sandwich ELISA method is that the target sample captured by primary antibody and then the secondary antibody bind to the antibody-antijen complex. Due to secondary antibody is fixed with horseradish peroxidase (HRP) which is an enzyme bind to

the substrate solution, can be measured colorimetrically. First of all, SIM-A9 cells were seeded at a density of $4x10^5$ cells/well and incubated for 24 hours. Subsequently, media was exchanged with DMEM media without serum, and selected doses of SL analogs (5 and 10 µM for both EGO10 and IND, and 20 and 50 µM for 4Br-debranone) with LPS were applied to the SIM-A9 cells. After 24 hours of incubation, the collected media were analyzed using Invitrogen mouse IL-1β and TNF-α ELISA kits pursuant to the protocols provided by the manufacturer. The concentrations of IL-1β and TNF-α secreted into the medium were normalized to the total cellular protein concentration of each sample measured by the BCA assay. The IL-1β ELISA assay was also carried out for the samples of LPS+ATP/nigericin-induced SIM-A9 cells which were treated with the aforementioned doses of SL analogs.

3.2.7. Gene Expression Analysis

SIM-A9 cells were treated with certain doses of SL analogs and after incubation time, the total RNA of each sample was segregated from the cell pellet by the NORGEN Total RNA Purification Plus kit according to the protocols provided by the manufacturer. The cDNA synthesis was actualized using the ABI-High-Capacity cDNA reverse transcriptase kit, according to the manufacturer's instructions. Relative gene expression levels of samples were analyzed by Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) that were performed by using cDNAs and specific TaqMan primers for SIM-A9 cells.

Total RNA Isolation

SIM-A9 cells were treated with LPS (1 µg/mL) and certain doses of SL analogs for 12 hours (time optimizations were made in our previously published study (Kurt et al., 2020). Following incubation, the old media was removed and the cells were collected using trypsin-EDTA and then centrifuged at 5000 RPM for 5 minutes. RNA isolation was performed according to the procedure of NORGEN Total RNA Purification Plus kit. 350 µL of Buffer RL containing 1% β-mercaptoethanol was added to the cell pellet and mixed gently for 5 minutes. The

cell lysate was transferred to tubes with the special gDNA removal column provided by the kit and centrifuged at 14,000 g for 1 minute. Then the gDNA removal columns were discarded and %100 ethanol was mixed with the flowthrough in the %60 ($V_{sample}/V_{ethanol}$) ratio by vortexing. This mixture was transferred to tubes with an RNA purification column and then, centrifuged at 5,000 g for 1 minute. After the flowthrough, each sample was removed, than 400 µL of washing solution A was added to the columns and centrifuged at 14,000 g for 1 minute. After repeating this step 3 times, RNA columns were transferred into elution tubes, 25 µL of elution solution A was transferred through the filter of columns and centrifuged at 200 g for 2 minutes and then at 14,000 g for 1 minute.

Fluorometric Quantification of RNA

Measurement of RNA concentration were carried out using the Qubit RNA BR Assay Kit by the Invitrogen Qubit Fluorometer according to the protocols presented by the manufacturer.

cDNA Synthesis

The synthesis of cDNA from the obtained RNA samples was executed by utilizing the ABI-High-Capacity cDNA reverse transcriptase kit including 18 nucleotides long oligo d (T) primer in line with the manufacturer's instructions. cDNAs of each sample were synthesized at a concentration of 1500 ng/10 µL and then kept at -20 °C for following qPCR method.

RT-qPCR Technique

RT-qPCR is a quantitative real-time method that uses fluorescent-labeled probes to monitor multiplications of target DNA sequence occurring in the polymerase chain reaction. The method can also be used to detect expression levels of RNA transcripts which were reverse transcripted to complementary DNA (cDNA). The amount of PCR products are directly associated with the signal created by the fluorescent dye-labeled probes (Kubista et al., 2006). qPCR reactions

were accomplished using TaqMan™ Fast Advanced Master Mix in line with the manufacturer's recommended instructions. The cDNAs obtained from SIM-A9 cells were diluted to 10 ng in the well and then qPCR reactions were performed using TaqMan probes specific for iNOS, IL-1β, COX-2, TNF-α, NQO1, HO-1, Gclc, Srxn1, Arg1, CD206, and BDNF and Nrf2 genes. B-actin was utilized as the housekeeping gene. The Ct mean values were normalized against the Ct mean of β-actin (threshold value) using the comparative delta-delta Ct (ΔΔCt) method (Livak & Schmittgen, 2001). Then, the ΔΔCt value of the LPS-induced control group was used as the calibrator.

3.2.8. Analysis of Protein Expression Levels

SDS-PAGE and Western Blot techniques are generally preferred to identify target proteins in the protein mixture isolated from cell lysate. Separation of proteins occurs according to their size and then they need to be transferred from gel to the membrane. Firstly, each protein in cell lysate were dissociated based on their molecular weight using sodium dodecyl sulfate (SDS)-Polyacrylamide gel electrophoresis (PAGE) method. Specific target protein expression levels were presented immunologically by the Western Blot technique by use of specific antibodies.

Cell Lysate Preparation for SDS-PAGE

SIM-A9 cells were plated into 6-well plates at a concentration of $7.5x10^5$ cells/well and then cultivated for 24 hours. After 24 hours, the media of each well was renewed with DMEM media involving 1% penicillin without serum, and SIM-A9 cells were treated with LPS and SL analogs; 4Br-debranone (20 and 50 µM), EGO10, and IND (5 and 10 µM). After 12 hours of incubation, cells were rinsed with PBS, and then cell lysates were collected by using RIPA solution. The whole-cell lysate protein samples were used to analyze iNOS, COX-2, and β-actin expression levels. The effective doses of SL analogs were applied to SIM-A9 cells with LPS for 4 hours afterward 40 minutes ATP induction. The nuclear and cytoplasmic protein fractions of the treatment groups were seperated by the NE-

PER® kit. Protein concentrations of the protein samples were measured via the BCA protein determination kit (Wiechelman et al., 1988).

Separation of Proteins by SDS-PAGE

Protein lysates obtained from SIM-A9 microglia cells were separated by vertical electrophoresis technique using a discontinuous buffer system as stated in (Laemmli, 1970). The separating and stacking solutions were prepared according to Table 4.

Table 4

Gel components in SDS-PAGE method

Stock Solutions	**Separating Gel (8,5%)**	**Separating Gel (12%)**	**Stacking Gel (4%)**
Gel Solution (mL)	8,5	12	1,3
Distilled Water (mL)	13,55	10,04	6,1
Seperating Gel Buffer (mL) Tris-HCl, pH:8,8	7,5	7,5	-
Stacking Gel Buffer (mL) Tris-HCl, pH:6,8	-	-	2,5
10% SDS (mL)	0,3	0,3	100
10% APS (mL)	0,15	0,15	0,05
TEMED (mL)	0,015	0,015	0,01

Cell lysates were diluted 1,33 fold with sample dilution buffer and boiled at 100 ℃ for 2 minutes. Then, the protein samples were taken immediately onto ice

and were immediately loaded into the wells at the same concentrations with the help of the Hamilton syringe.

Western Blot Analysis

Target protein levels were determined immunologically by the Western Blot technique using specific antibodies for each protein (Kyhse-Andersen & methods, 1984). Analyzes of iNOS and COX-2 proteins were performed in lysates obtained from LPS induced-SIM-A9 cells while Nrf2 cytoplasmic protein expression analyzed in NLRP3-induced cells. As Housekeeping gene, β-actin levels were measured to normalize the levels of cytoplasmic fraction/whole cell lysate. For Western blot analysis, the gel was removed from the apparatus after electrophoresis, kept in transfer buffer for 10 minutes, and a sandwich model was created using a cassette as shown in Figure 4.

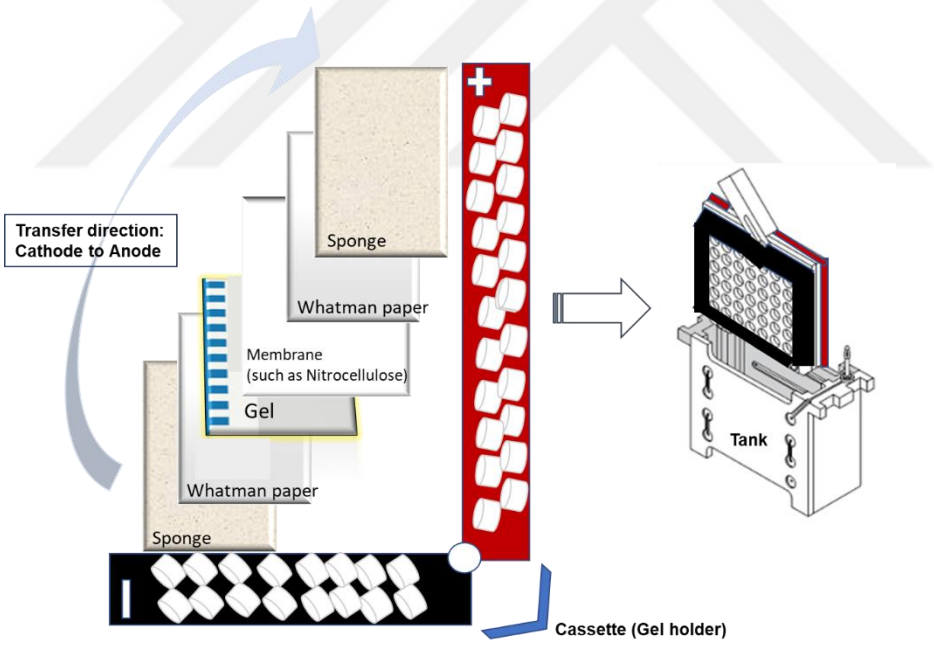


Figure 4. Preparation of an Electroblotting Sandwich to transfer the proteins from a gel to a nitrocellulose membrane.

The sandwich in the sealed cassette was placed into the electrophoretic transfer system and filled with cooled transfer buffer. After the system was connected to the power supply, the protein transfer continued for 2 hours at 90 volts. At the end of 2 hours, the protein-containing surface of the membrane was incubated with blocking solution which is 5% skim milk powder in 20 mL Tris Buffer Saline Tween for 1 hour on a constant speed shaker to cover the protein-free areas on the membrane. The membrane was then incubated overnight with the primary antibody. The membrane was cleaned 3 times for 5 minutes with TBST and incubated for 1 hour with secondary antibody (anti-rabbit/mouse IgG-HRP) coupled with the marker enzyme Horseradish Peroxidase. The membrane was then incubated with Western Blotting Luminol Reagent for 5 minutes in dark. The protein bands in the membrane were imaged on the C-DiGit® Blot Scanner and densitometrically construed with Image Studio Digits software.

3.2.9. siRNA Transfection to silence Nrf2 gene expression

SIM-A9 microglia cells were seeded in 6-well plates at a concentration of $7.5x10^5$ cell/well and incubated for 15 hours to reach a density of 50-60%. After 15 hours, the media of the cells were replenished with Opti-MEM ™ I Reduced Serum media. On the other hand, control siRNA and Nrf2 siRNAs with a concentration of 5 nmol were diluted to a concentration of 20 pmol/µL using Opti-MEM media. For each well, the oligomer-Lipofectamine® 2000 complex was prepared in the ratios of 5 µL of 20 pmol/µL siRNA, 5 µL of Lipofectamine® 2000 transfection reagent, and 240 µL of Opti-MEM ™ I Reduced Serum and then applied to the cells gently. After 12 hours of transfection, cell media were replenished with DMEM media which include 10% FBS and 1% penicillin. The most effective dose of GR24, 20 µM, was applied to control-siRNA and Nrf2-siRNA groups together with LPS and incubated for 4 hours. At the end of incubation, cells were harvested with trypsin-EDTA and RNA isolation was accomplished as detailed in Section 3.2.7.1. cDNA synthesis from these RNA samples was performed at a concentration of 1500 ng/10µL as described in section 3.2.7.3. Subsequently, qPCR analyses (as described in section 3.2.7.4) were performed using TaqMan probes specific for

Nrf2, HO-1, and IL-1β genes to evaluate the effects of GR24 on gene expression levels in Nrf2 gene silenced-SIM-A9 cells.

3.2.10. Conditioned Media Assay

The protective effects of SLs on both glial and neuronal cell cultures were investigated according to the studies of Lai et al., 2018 and Eren et al., 2018. SIM-A9 microglia cells were seeded on a 6-well plate at the concentration of $1x10^6$ cell/well and after 24 hours incubation, the cells were treated with LPS and the optimum doses of SL analogs (10 µM of IND and EGO10, 20 µM of GR24, and 50 µM 4Br-debranone) for 24 hours. SH-SY5Y neuronal cells were also seeded on a 24-well plate at the concentration of $4x10^5$ cell/well at the same time. After 24 hours of incubation, the media of SIM-A9 cells were removed and filtered using a 0,22 µm filter, and these cleared conditioned media were transferred onto SH-SY5Y cells and incubated for 24 hours. The cell viability of SH-SY5Y cells was examined with MTT cytotoxicity test.

3.2.11. Statistical Analysis

All data were reported as mean ± SEM and each experiment was reiterated at least three times. For statistical analysis, the GraphPad Prism 8 software was utilized to ascertain the significant difference by one-way analysis of variance (ANOVA) analysis which was subsequently continued with Tukey's post hoc test for comparison of three or more groups (*$p< 0.02$, **$p< 0.005$, ***$p < 0.001$).

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Results

The thesis work includes in vitro investigation of SL analogs with four different bioactiphores on microglia and neuronal cells. In this part, we presented the in vitro effects of SL analogs against neuroinflammation by focusing on microglial activation, NLRP3 inflammasome mechanism, BDNF signaling, and microglial phenotype polarization in SIM-A9 microglia cells and neuronal survival in SHSY5Y cells.

4.1.1. Viability Tests and Dose Determination Studies

The non-toxic doses of SL analogs on SIM-A9 microglia cells were determined by the MTT cytotoxicity test. The appropriate LPS concentration to stimulate the inflammatory mechanism in SIM-A9 cells was determined as 1 µg/mL as represented in our previous study (Kurt et al., 2020). MTT assay was performed simultaneously after SIM-A9 microglia cells were treated with varied doses of SL analogs and 1 µg/mL LPS for 24 hours. Indanone-derived SL (IND) and EGO10 at 20 µM concentration decreased the cell viability by 50% and 52% compared to control, respectively. 4Br-debranone at the concentrations of 20 µM and 50 µM did not have a toxic effect on the cell viability of SIM-A9 cells (Figure 5). Therefore, 5 and 10 µM concentrations were selected as safe doses for EGO10 and IND, while 10, 20, or 50 µM concentrations have been often used for 4Br-debranone. Additively, the further unknown activities of GR24 in the molecular mechanism of neuroinflammation were studied at the dose range of 5-50 µM according to our previous study (Kurt et al., 2020).

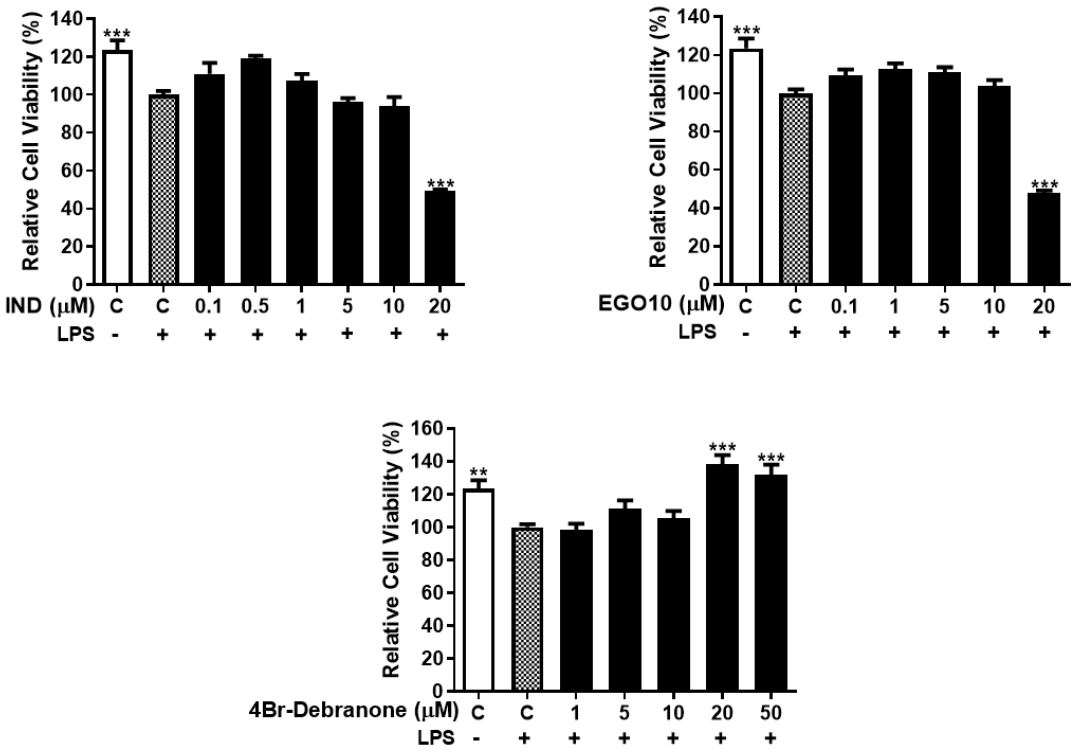


Figure 5. Effects of SL analogs on cell viability of SIM-A9 microglia cells. C: Control, IND, EGO10, and 4Br-debranone: SL analogs. $**p < 0.005$, $***p < 0.001$.

4.1.2. Effects of SL Analogs on NO Release in LPS-Induced SIM-A9 Microglia Cells

The half-maximal inhibitory concentrations (IC_{50}) of SL analogs were analyzed to reveal their suppressive effects on LPS-induced NO release in SIM-A9 microglia cells. SIM-A9 cells were treated with different doses of SL analogs and LPS for 24 hours. Hereafter, the cell media of each sample were tested by using Griess reagent to detect the concentration of NO secreted from cells. The IC_{50} value of 4Br-debranone for NO inhibition in microglial cells was detected as 12,73 μM, while the IC_{50} values of EGO10 and IND were found at much lower concentrations as 1,72 μM and 1,02 μM, respectively (Figure 6).

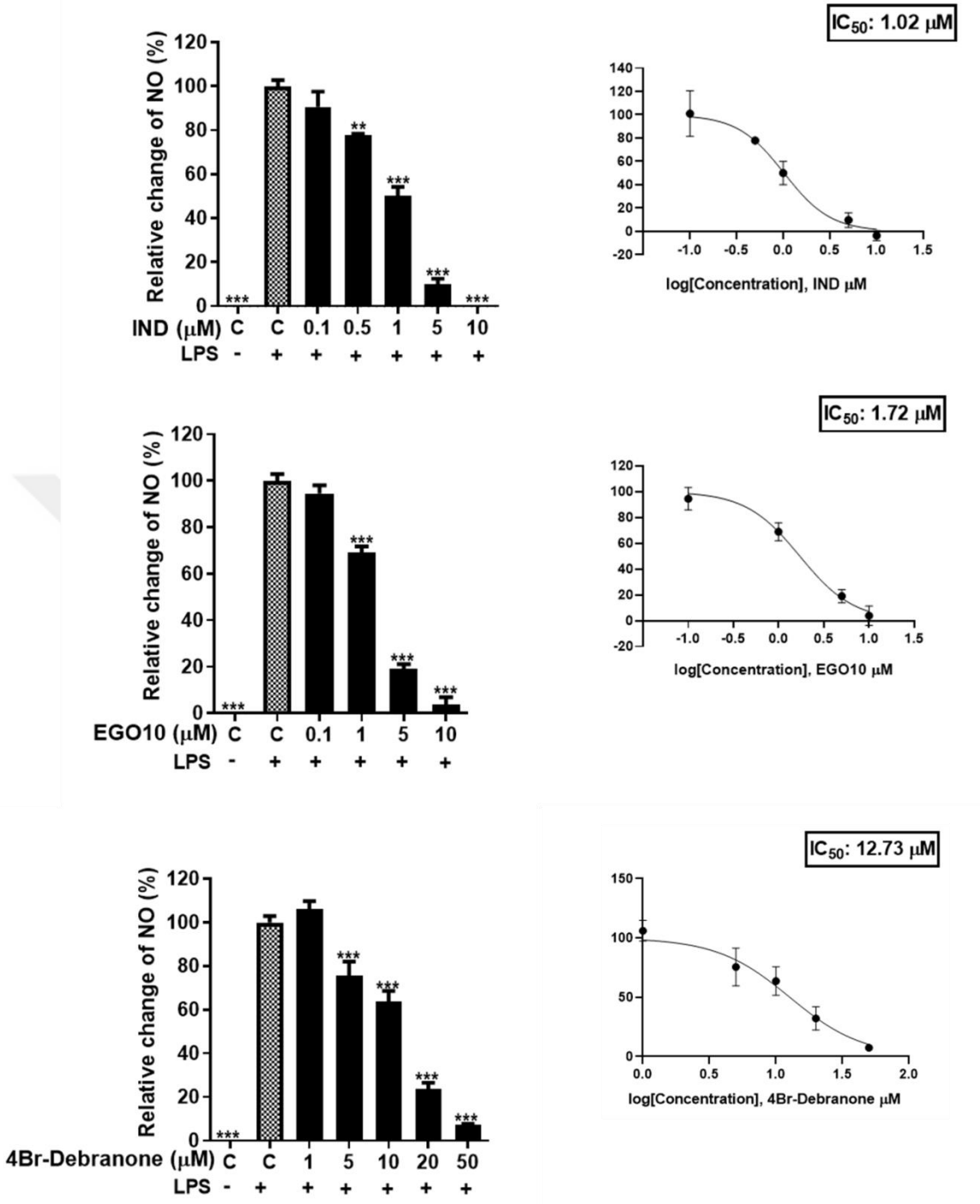


Figure 6. Effects of SL analogs on the level of NO release in SIM-A9 microglia cells. C: Control, IND, EGO10, and 4Br-debranone: SL analogs. ***$p < 0.001$.

4.1.3. Effects of SL Analogs on IL-1β and TNF-α Proinflammatory Cytokine Release in LPS-Induced Microglia Cells

The suppressive effects of SL analogs on the secretion level of IL-1β and TNF-α proinflammatory cytokines in SIM-A9 cells were identified by utilizing ELISA assay. Primarily, SIM-A9 cells were treated with these SL analogs at safe doses and LPS (1 μg/mL) for 24 hours. As a result, 5 and 10 μM doses of EGO10 decreased the level of LPS-induced IL-1β release by 46% and 66%, while IND decreased its level by 55% and 64% at the same concentrations, respectively. Moreover, the level of IL-1β release was diminished as 74% by the treatment with 50 μM of 4Br-debranone in SIM-A9 cells compared to control (only LPS treated group) (Figure 7).

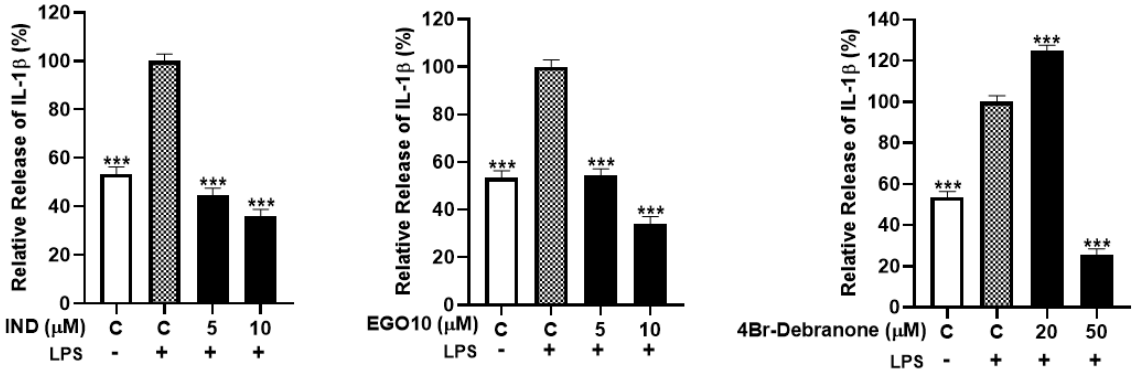


Figure 7. Effects of IND, EGO10, and 4Br-debranone on the level of LPS-induced IL-1β release in SIM-A9 microglia cells. C: Control. ***$p < 0.001$.

After 24 hours of incubation after SL treatments in SIM-A9 cells, 5 and 10 μM doses of EGO10 significantly suppressed LPS-induced TNF-α release by 75% and 80%, respectively, compared to control (Figure 8). As represented in Figure 8, the level of LPS-aroused TNF-α release in SIM-A9 cells was decreased by 50% by the treatment of IND at 5 μM dose while its 10 μM concentration inhibited the TNF-α release as 91% compared to control. Moreover, SIM-A9 cells treated with 4Br-debranone at concentrations of 20 and 50 μM dose-dependently alleviated the

level of LPS-induced TNF-α release by 30% and 83% compared to the control group (Figure 8).

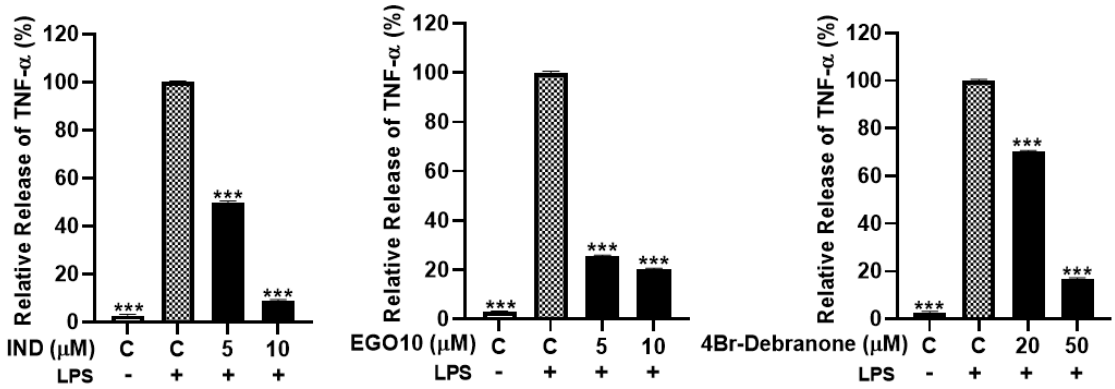


Figure 8. Effects of IND, EGO10, and 4Br-debranone on the level of LPS-induced TNF-α release in SIM-A9 microglia cells. C: Control. ***$p < 0.001$.

4.1.4. Effects of SL Analogs on The Gene Expression Levels of Proinflammatory Factors in LPS-Induced Microglia Cells

The M1 (proinflammatory) phenotype was induced by LPS induction in SIM-A9 cells. SIM-A9 microglia cells were treated with the selected concentrations of SL analogs with 1 μg/mL LPS for 12 hours. The relative mRNA expression levels of pro-inflammatory factors (markers of M1 phenotype) including IL-1β, TNF-α, İNOS, and COX-2 were detected by way of the RT-qPCR technique.

According to the statistical analysis, 5 and 10 μM of EGO10 decreased the LPS-excited iNOS mRNA expression level by 67% and 61%, while the same doses of IND caused a dose-dependent reduction by 70% and 82%, respectively (Figure 9). iNOS mRNA expression levels in LPS-induced SIM-A9 cells were also significantly suppressed as 53%, 44%, and 57% by the treatment with 4Br-debranone at 10, 20, and 50 μM doses compared to control, respectively (Figure 9).

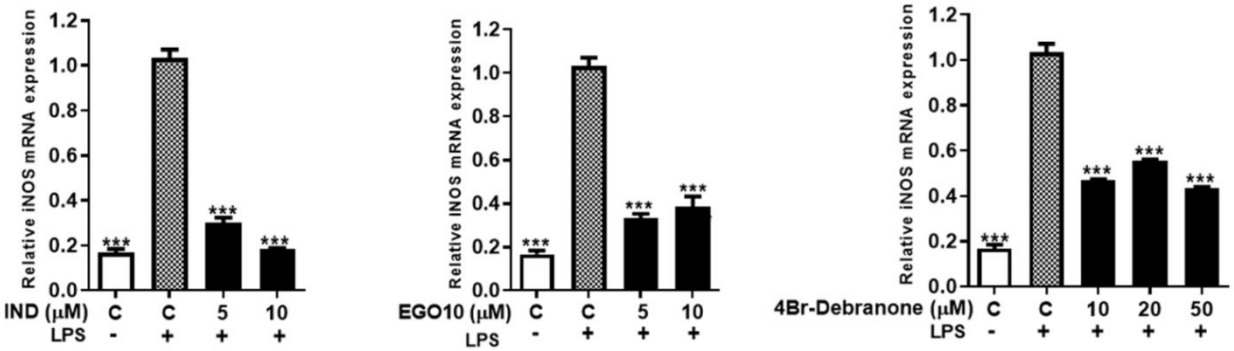


Figure 9. Effects of IND, EGO10, and 4Br-debranone on proinflammatory iNOS mRNA expression levels in LPS-induced SIM-A9 cells. C: Control. ***$p < 0.001$.

In Figure 10, EGO10 at 5 and 10 μM concentrations significantly restrained the IL-1β mRNA expression level by 78% and 87% while IND inhibited its level by 85% and 87% at the same doses, respectively in LPS-induced SIM-A9 cells. LPS-induced IL-1β mRNA expression level was significantly and dose-dependently suppressed by 10, 20, and 50 μM doses of 4Br-debranone by 67%, 79%, and 95% as compared to control, respectively (Figure 10).

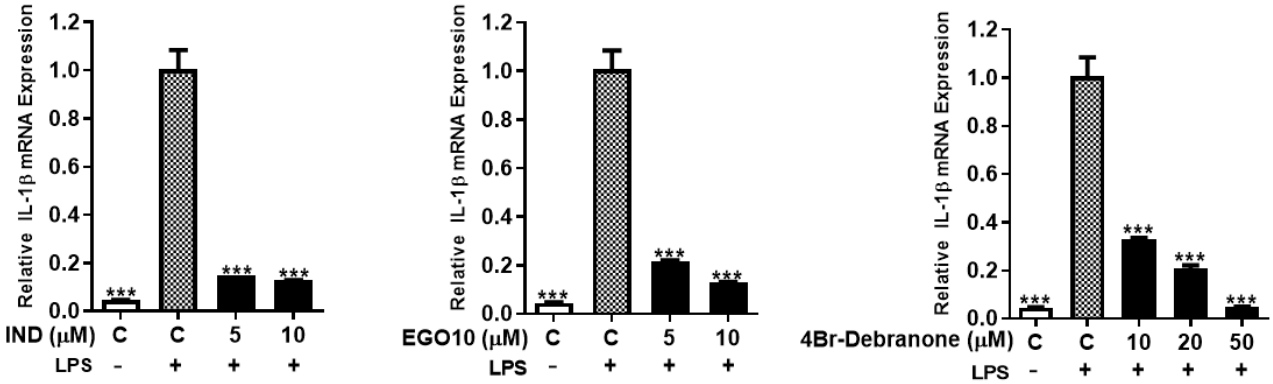


Figure 10. Effects of IND, EGO10, and 4Br-debranone on proinflammatory IL-1β mRNA expression levels in LPS-induced SIM-A9 cells. C: Control. ***$p < 0.001$.

Furthermore, 20 and 50 μM doses of 4Br-debranone significantly suppressed the LPS-induced TNF-α mRNA expression level by 27% and 29% as compared to the control, consecutively (Figure 11). EGO10 at both doses (5 and 10 μM) did not suppress the TNF-α mRNA expression level in LPS-induced SIM-A9 cells. However, IND at only 5 μM concentration significantly decreased the LPS-induced TNF-α mRNA expression level by 15% relative to control (Figure 11).

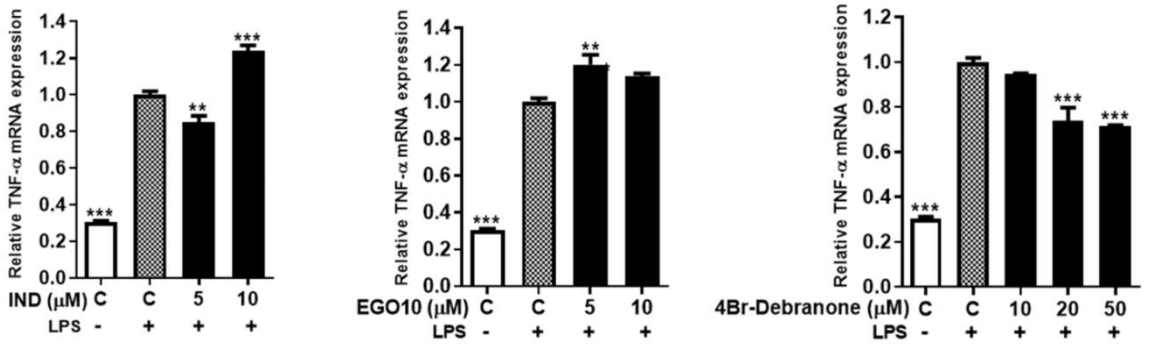


Figure 11. Effects of IND, EGO10, and 4Br-debranone on proinflammatory TNF-α mRNA expression levels in LPS-induced SIM-A9 cells. C: Control. $^{**}p < 0{,}005$, $^{***}p < 0{,}001$.

As represented in Figure 12, LPS-induced COX-2 mRNA expression level was suppressed as 62%, 54%, and 46% by the treatment with 10, 20, and 50 μM of 4Br-debranone, respectively in SIM-A9 cells. Treatments with EGO10 and IND at 5 μM significantly decreased the COX-2 mRNA expression level by 70% and 68%, respectively, while both of these SLs at 10 μM showed 68% downregulation compared to control (Figure 12).

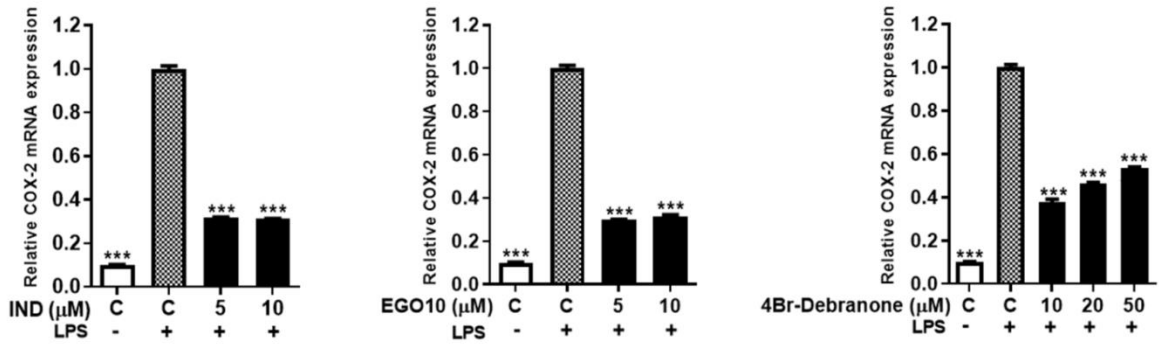


Figure 12. Effects of IND, EGO10, and 4Br-debranone on LPS-induced COX-2 mRNA expression levels in SIM-A9 cells. C: Control. ***$p < 0{,}001$.

4.1.5. Effects of SLs on The Protein Expression Levels of Pro-inflammatory Factors

The anti-inflammatory effects of IND, EGO10, and 4Br-debranone on the protein expression levels of iNOS and COX-2 enzymes were detected by Western Blot analyzes. As demonstrated in Figure 13, only 4Br-debranone at 50 μM concentration significantly suppressed the COX-2 protein level by 17% as compared to control.

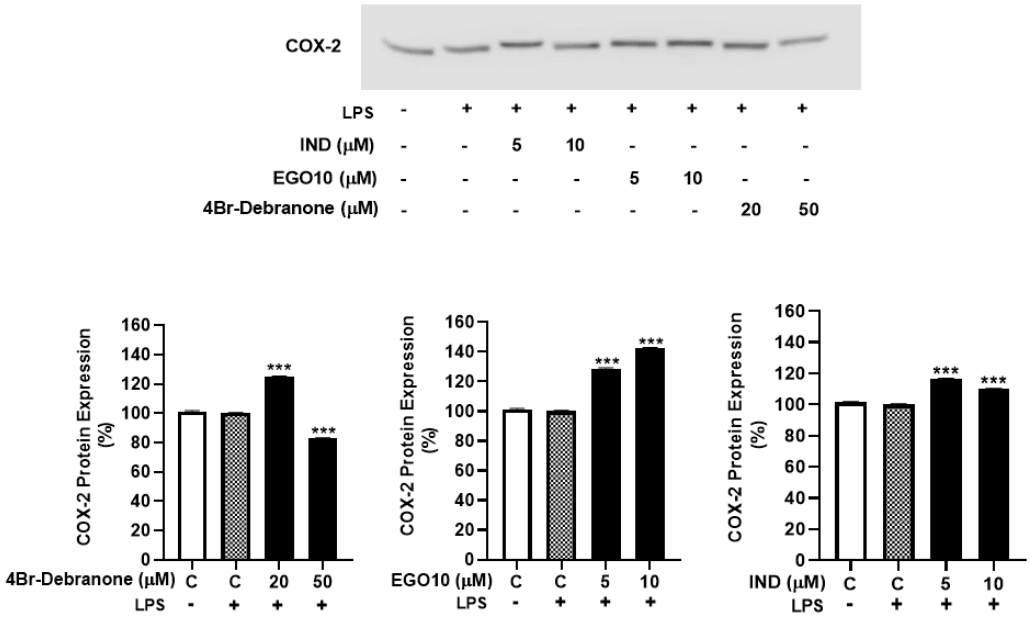


Figure 13. Effects of 4Br-debranone, EGO10, and IND on the COX-2 protein expression level of LPS-induced SIM-A9 cells. C: Control. ***$p < 0{,}001$.

According to Figure 12, 50 μM of 4Br-debranone significantly suppressed the LPS-induced iNOS protein expression level by 23%, while 10 μM of IND decreased its level by 9% as compared to control. On the other hand, EGO10 did not change the level of iNOS protein expression at both concentrations (Figure 14).

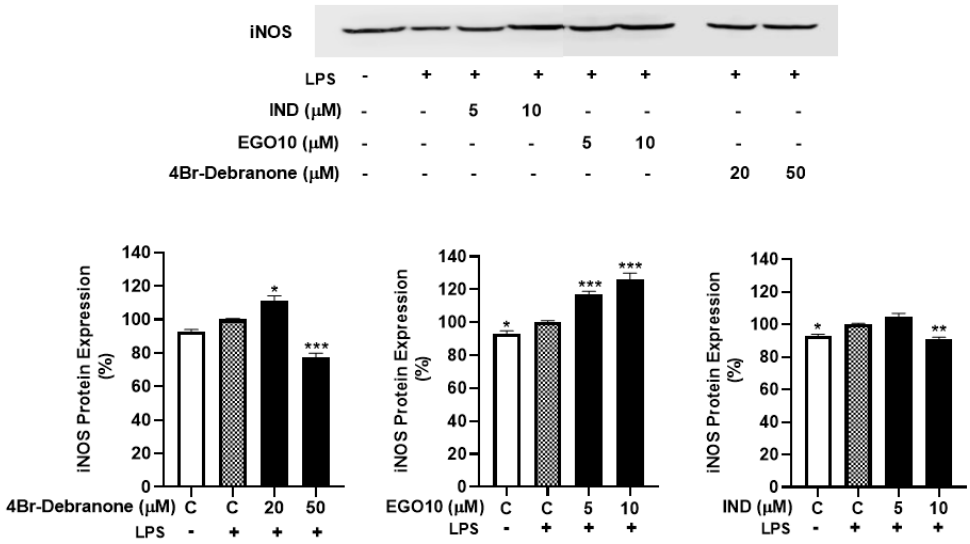


Figure 14. Effects of 4Br-debranone, EGO10, and IND on LPS-induced iNOS protein expression levels in SIM-A9 cells. C: Control. *$p < 0,02$, **$p < 0,005$, ***p $< 0,001$.

4.1.6. The molecular mechanism of GR24 on Nrf2-dependent anti-neuroinflammatory effect in SIM-A9 microglia cells

Nrf2 silencing studies were conducted to evaluate the anti-neuroinflammatory effect of GR24 is whether through the Nrf2 pathway in LPS-primed SIM-A9 microglia cells.

For this purpose, 20 μM of GR24 (selected as the most effective dose), was applied to Control siRNA and Nrf2 siRNA-transfected groups with LPS and incubated for 4 hours. The Nrf2 mRNA expression of Nrf2 gene-silenced groups was significantly blocked as; 9,2-fold in LPS-treated control, 5,6-fold in GR24-treated group, and almost completely in the nontreated control as compared to their control siRNA-transfected groups (Figure 15 (A)).

In Control siRNA transfected group, 20 μM of GR24 increased the HO-1 mRNA expression level by 4,1-fold as compared to the control. However, the same treatment of GR24 increased its level by only 1,5 fold in Nrf2-silenced and LPS-induced SIM-A9 cells (Figure 15 (B)). GR24 treatments on Nrf2 siRNA and

control siRNA-transfected SIM-A9 cells repressed the LPS-induced IL-1β gene expression by 47% and 43% as compared to their control groups, respectively (Figure 15 (C)).

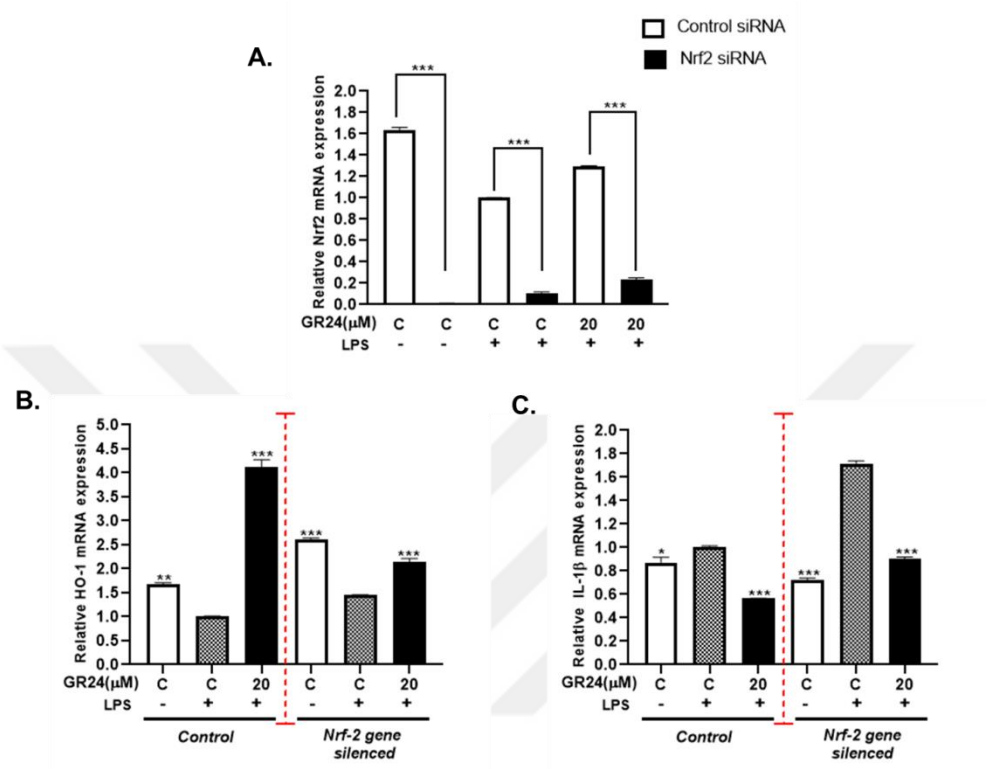


Figure 15. The relative mRNA expression levels of (*A*) Nrf2, (*B*) HO-1, and (*C*) IL-1β in Nrf2 silenced SIM-A9 cells after 4 hours of GR24 treatment. C: Control. *p < 0,02, **p < 0,005, ***p < 0,001.

4.1.7. Interaction between the Anti-Inflammatory Effect of GR24 and the BDNF Signalling Pathway in Microglia Cells

To elucidate the supportive effect of GR24 on the expression level of neurotrophic factor BDNF gene, SIM-A9 cells were treated with LPS and GR24 at 20 and 50 µM doses for 12 hours. As a result of RT-qPCR analysis, treatment with 50 µM of GR24 gave rise to a significant upregulation on the BDNF mRNA expression level by 4,6-fold as compared to control (Figure 16 (A)). Additively, only LPS treatment also lead to a 1,9-fold improvement in BDNF gene expression

level relative to nontreated control. The relation between the anti-inflammatory effect of GR24 and BDNF signaling was further examined on the gene expression of the COX-2 gene. After that, the suppressive effect of GR24 at 50 µM was shown on the proinflammatory COX-2 gene by 78% decrease in SIM-A9 cells (Figure 16 (B)).

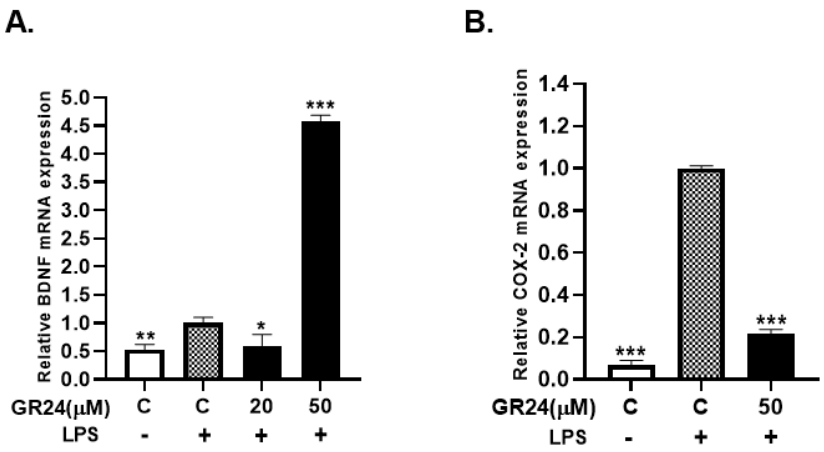


Figure 16. The effects of GR24 on (*A*) BDNF and (*B*) COX-2 gene expression in LPS-induced SIM-A9 cells. C: Control. ***$p < 0{,}001$.

In the following, SIM-A9 cells were pre-treated with an FDA-approved TrkB receptor inhibitor (entrectinib) to suppress the BDNF-TrkB signaling pathway. Primarily, SIM-A9 cells were pretreated with entrectinib at the dose range of 1 nM – 10 µM for 1 hour before the 24 hours of LPS stimulation. Entrectinib did not decrease the cell viability of LPS-induced SIM-A9 cells at any concentration represented in Figure 17 (A). Then, 3 different concentrations of entrectinib were selected to elucidate its effect on COX-2 gene expression. According to Figure 17 (B), 50 nM of encrectinib increased the COX-2 gene expression by 1,5-fold while it leads to a 1,3-fold decrease at 10 µM concentration as compared to control. However, 1 µM of entrectinib did not significantly change the COX-2 gene expression level in LPS-triggered SIM-A9 cells. Thus, we selected 1 µM

concentration of entrectinib due to properly compare the antiinflammatory effect of GR24 on the COX-2 gene with or without blocking the BDNF pathway.

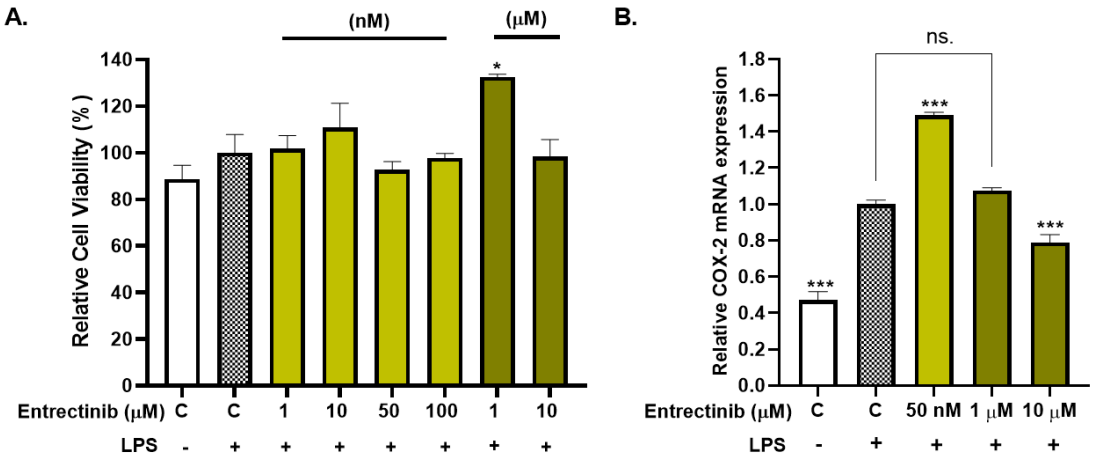


Figure 17. The effect of entrectinib (TrkB receptor inhibitor) on (*A*) cell viability and (*B*) COX-2 gene expression at different concentrations in LPS-induced SIM-A9 cells. C: Control. *$p < 0{,}02$, ***$p < 0{,}001$.

As represented in Figure 18, 50 µM of GR24 mitigated the LPS-induced COX-2 mRNA expression level by 4,7-fold in comparison to only the LPS-treated control. However, the same treatment of GR24 decreased the mRNA expression level of the COX-2 gene by 1,5-fold in SIM-A9 cells treated with LPS after pre-treatment of entrectinib as compared to the TrkB-BDNF pathway-blocked control group. On the other hand, 1 hour of pre-treatment with entrectinib did not impressed the expression level of the COX-2 gene in SIM-A9 cells contrasted with control (Figure 18).

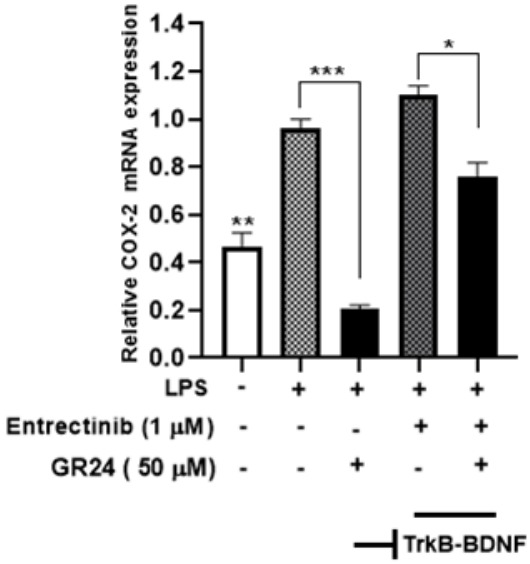


Figure 18. Change in the anti-inflammatory effect of GR24 on COX-2 gene expression after pre-treatment of entrectinib in LPS-induced SIM-A9 cells. C: Control, Entrectinib: TrkB receptor inhibitor to block BDNF signaling. *$p < 0,02$, **$p < 0,005$, ***$p < 0,001$.

4.1.8. Neuroprotective effect of SLs with Microglial Conditioned Media

The effect of the SL analogs on cell viability was elucidated in SH-SY5Y neuroblastoma cells by MTT assay. As represented in Figure 19 (A), GR24 and 4Br-debranone significantly supported the cell viability of SH-SY5Y cells by 2,5 and 4,6-fold compared with control (only DMSO), respectively. After that, the effect of LPS-induced microglial activation on neuronal cell viability with or without SL treatment was assessed by using a conditioned medium assay. SIM-A9 cells were previously treated with LPS and SL analogs for 24 hours. The filtered supernatant of SIM-A9 cells (conditioned medium) was transferred onto SH-SY5Y cells simultaneously and incubated for 24 hours.

Only the LPS-treated microglial medium increased the cell viability of SHSY5Y cells by 2,6-fold as compared to control which includes only DMSO treated-microglial conditioned medium (Figure 19 (B)). 10 µM IND and LPS-treated microglial medium augmented the cell viability of SHSY5Y cells by a 1,1-fold as well as the 5 µM of sulforaphane (positive control), compared to control

including LPS-treated microglial medium (Figure 19 (B)). EGO10 at 10 µM and 4Br-debranone at 50 µM-treated microglial secretomes showed a mild proliferative effect on SHSY5Y cells by 1,2 fold contrasted with the control group (C_{+LPS}). Treatment with 20 µM of GR24 led to a significant microglia-mediated protecting effect on SH-SY5Y cells by 1,5-fold compared to control.

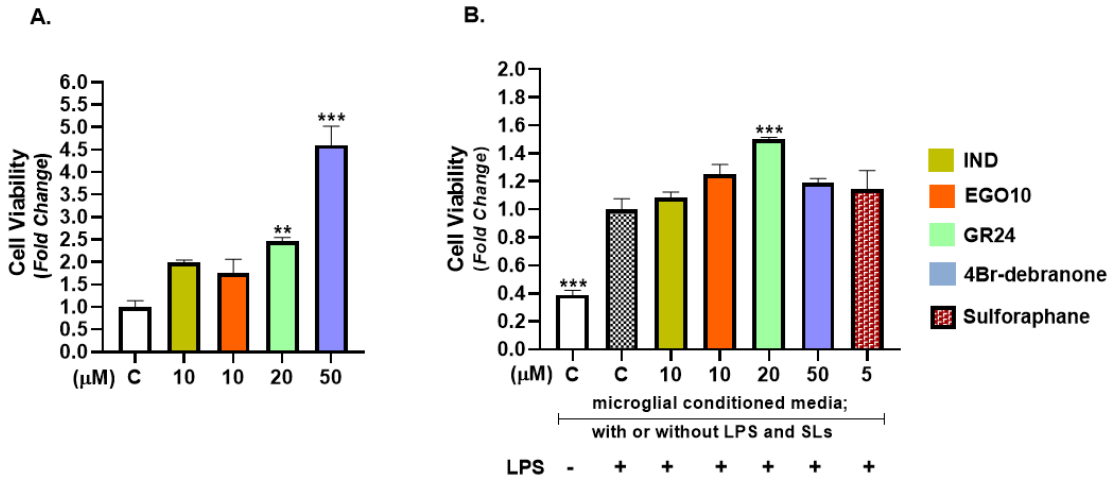


Figure 19. The effects of SLs on the cell viability of SH-SY5Y cells (*A*) and the undirect effects of SLs on neuronal cell viability through reactive-microglial cell secretome (*B*). C: Control. $^{**}p < 0{,}005$, $^{***}p < 0{,}001$.

4.1.9. The modulatory effect of SL analogs on M2 microglial phenotype polarization in LPS-Induced Microglia Cells

The modulatory effects of SL analogs on the gene expression levels of anti-inflammatory M2 phenotype and antioxidant Mox phenotype markers were elucidated in LPS-induced SIM-A9 microglia cells that represented the M1 phenotype. SIM-A9 cells were treated with SL analogs and LPS for 12 hours.

As represented in Figure 21, the gene expression levels of M2 phenotype markers including Arg1 and CD206 decreased after LPS stimulation as 40% and 47% in SIM-A9 cells compared to nontreated control, respectively. However, IND increased the gene expression level of Arg1 by 1,8 fold at 5 µM and the level of CD206 gene by 1,7 fold at 10

μM concentration in LPS-induced SIM-A9 cells (Figure 20). EGO10 at 5 and 10 μM doses significantly augmented the mRNA expression level of CD206 by 1,3 and 1,4 fold, respectively whereas it did not show any change on Arg1 gene at the same doses.

4Br-debranone at 50 μM concentration solely increased the gene expression level of CD206 by 1,3 fold in LPS-induced SIM-A9 cells (Figure 20). GR24 did not induce the gene expression level of Arg1 but its 20 μM contributed to a significant increment in CD206 gene expression as 1,3-fold compared to the control (Figure 20).

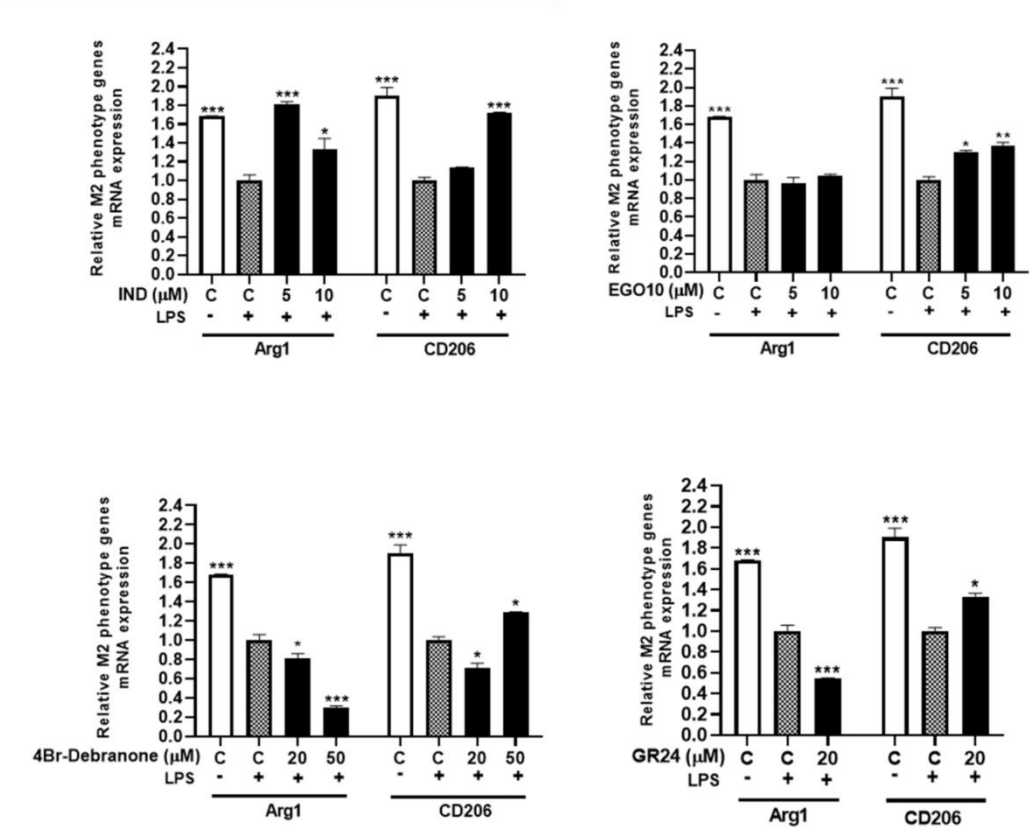


Figure 20. Effects of IND, EGO10, 4Br-debranone, and GR24 on mRNA expression levels of M2 phenotype markers in LPS-induced SIM-A9 cells. C: Control. *$p < 0,02$, **$p < 0,005$, ***$p < 0,001$.

4.1.10. The modulatory effect of SL analogs on Cytoprotective Genes to Induce Mox Microglial Phenotype Polarization

The effects of SL analogs on Mox phenotype markers including Nrf2 and its downstream enzymes HO-1, NQO1, Srxn1, and Gclc were determined at mRNA expression level in LPS-primed SIM-A9 microglia cells with RT-qPCR analysis. After 12 hours of treatment with 4Br-debranone at concentrations of 10, 20, and 50 μM, Nrf2 mRNA expression level was significantly and dose-dependently increased by 1,3, 1,5, and 2,2-fold in LPS-induced SIM-A9 cells, respectively (Figure 21). EGO10 at 5 and 10 μM doses significantly increased Nrf2 mRNA expression level by 1,2 and 1,4-fold as compared to the LPS-treated control, respectively. IND at 5 μM did not generate a significant difference in the level of Nrf2 mRNA expression. However, its 10 μM concentration significantly increased the level by 1,3-fold compared with control.

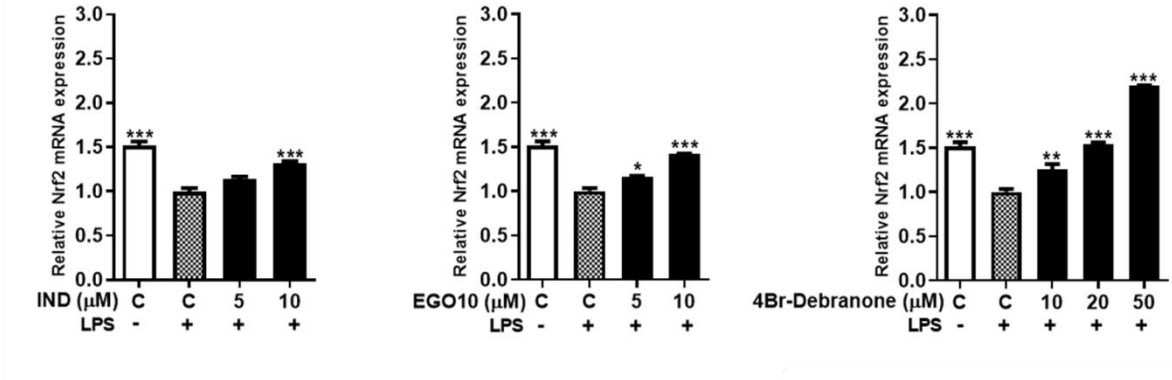


Figure 21. The promoting effects of IND, EGO10, and 4Br-debranone on Nrf2 mRNA expression levels in LPS-induced SIM-A9 cells. C: Control. *$p < 0,02$,**$p < 0,005$, ***$p < 0,001$.

EGO10 and IND improved the gene expression level of NQO1 dose-dependently by 3,5 and 2,8-fold at 5 μM while 5,9 and 6,5-fold at 10 μM dose in LPS-induced SIM-A9 cells, respectively (Figure 22 (A)). Moreover, IND at 5 and

10 μM concentrations significantly enhanced the Gclc mRNA expression level by 1,3 and 1,5-fold as compared to control, respectively (Figure 22 (B)). However, EGO10 significantly enhanced the Gclc expression by 1,2-fold at only 10 μM concentration in LPS-induced SIM-A9 cells.

4Br-debranone at 10, 20, and 50 μM doses significantly elevated the mRNA expression level of NQO1 by 2,0, 2,9, and 15,4-fold in LPS-induced SIM-A9 cells, respectively (Figure 22 (*A*)). The SL analog at 20 and 50 μM doses also promoted the Gclc mRNA expression by 1,4 and 2,0-fold compared with control, respectively (Figure 22 (*B*)).

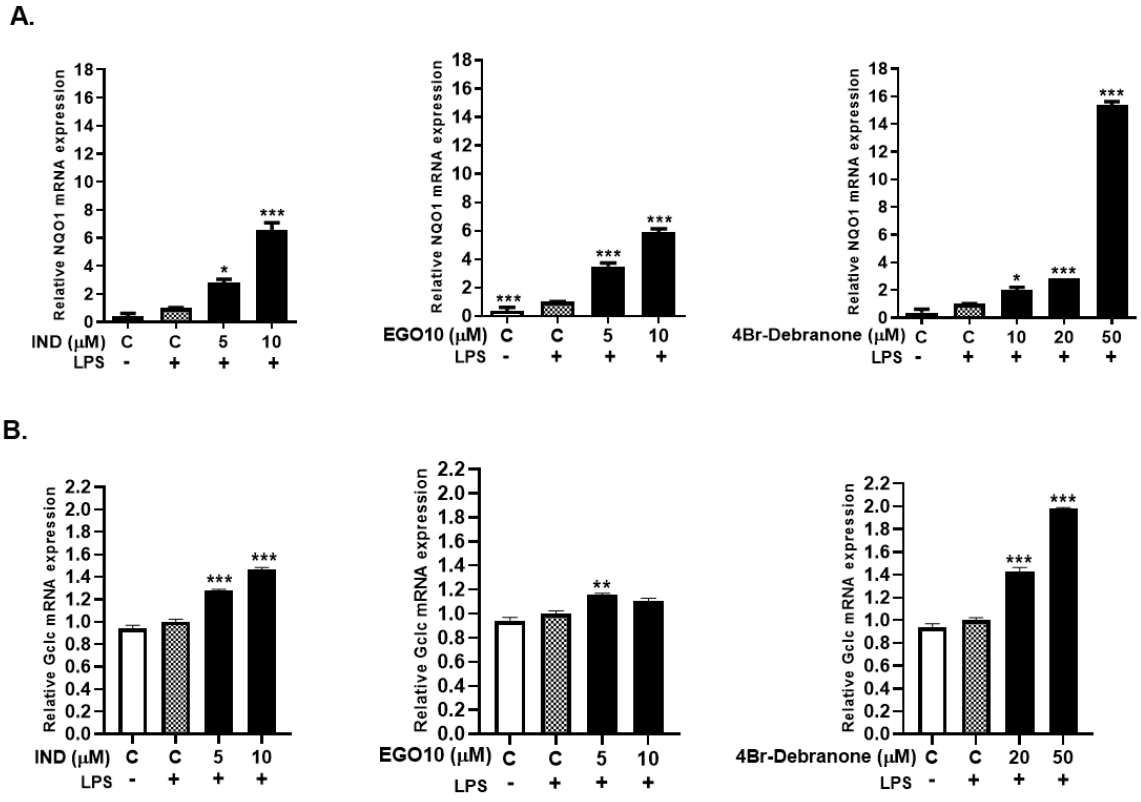


Figure 22. Effects of IND, EGO10, and 4Br-debranone on Nrf2-dependent detoxification enzymes; NQO1 and Gclc mRNA expression levels in SIM-A9 cells. C: Control. *$p < 0,02$,**$p < 0,005$, ***$p < 0,001$.

The expression levels of HO-1 which is an Nrf2-dependent antioxidant gene were upregulated by indanone at both doses as 1,3-fold while 10 μM of EGO10 increased its level as 1,1-fold as compared to only LPS-induced SIM-A9 cells. The gene expression level of Srxn1 was significantly enhanced by the treatments of IND as 2,3-fold at both 5 and 10 μM doses as compared to control. EGO10 at 5 and 10 μM also significantly upregulated the Srnx1 expression as 1,4 and 1,8-fold, respectively (Figure 23 (B)). Furthermore, 4Br-debranone at 10, 20, and 50 μM doses significantly and dose-dependently increased the HO-1 mRNA expression level by 2,0, 3,8, and 6,0-fold respectively, while upregulating the Srxn1 gene expression by 2,4 and 3,8-fold (for 20 and 50 μM) as compared to control (Figure 23).

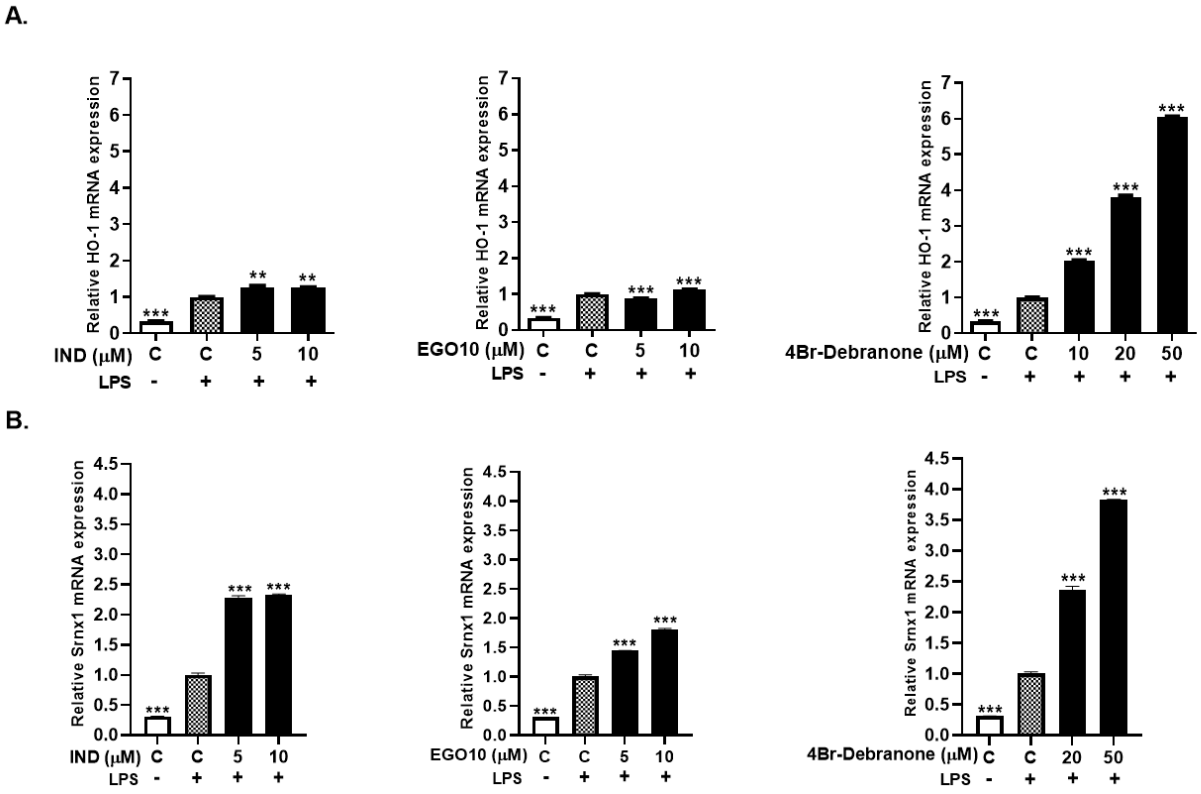


Figure 23. Effects of IND, EGO10, and 4Br-debranone on Nrf2-dependent antioxidant enzymes; (*A*) HO-1 and (*B*) Srxn1 at mRNA expression levels in SIM-A9 cells. C: Control. $^{**}p < 0{,}005$, $^{***}p < 0{,}001$.

GR24 at the concentration of 20 µM significantly increased the gene expression of Gclc by 1,5-fold, but not the Srxn1 gene when compared with control in LPS-induced SIM-A9 cells (Figure 24).

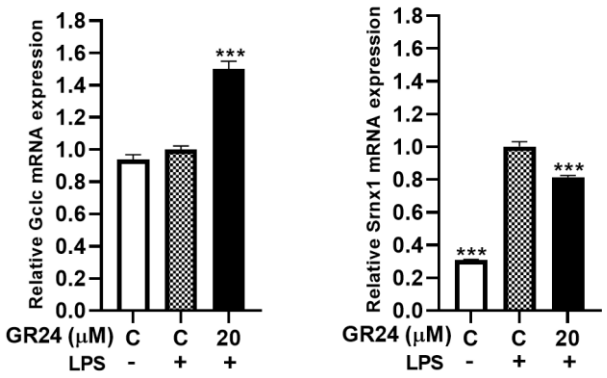


Figure 24. Effect of GR24 on Mox phenotype markers including Gclc and Srxn1 at mRNA expression level in LPS-induced SIM-A9 cells. C: Control. ***$p < 0{,}001$.

4.1.11. The Potential Inhibitory Effect of SLs NLRP3 Activation in Microglia Cells

SIM-A9 microglia cell submitted to a priming step of 24 hours with LPS and followed by 40 minutes 1 mM of ATP induction which is a general protocol for the activation step of NLRP3 inflammasome (Figure 25 (A)). The supernatants of each sample were gathered to measure the IL-1β and NO levels by ELISA and Griess assays, respectively.

Inhibitory effects of SLs on NLRP3 Inflammasome Activation in Microglia

The ATP treatment (signal 2) following LPS presented a remarkable aggravation in the level of IL-1β secretion by 63% upregulation compared to only LPS-treated SIM-A9 cells (Figure 25 (B)). As illustrated in Figure 25 (B), GR24 and EGO10 at a 10 µM almost completely inhibited the NLRP3-dependent IL-1β release in LPS+ATP-induced SIM-A9 cells. The IL-1β secretion level was

significantly and dose-dependently suppressed by 5 and 10 µM of IND as 27% and 47%; 20 and 50 µM of 4Br-debranone as 74% and 84%, sequentially compared with the LPS/ATP-induced control. Moreover, 5 µM of sulforaphane (positive control) suppressed the NLRP3-induced IL-1β level by 87% while 5 µM of EGO10 lead to an 80% decrease in ATP+LPS-induced control.

As seen in Figure 25 (C), 10 µM of nigericin (signal 2) stimulation after LPS (signal 1) increased the IL-1β level by 23% as compared to only the LPS-treated control. GR24 at 10 µM concentration did not show any change in IL-1β release in LPS+NIG-induced SIM-A9 cells. 4Br-debranone at 20 and 50 µM doses significantly and dose-dependently suppressed IL-1β release by 16% and 54% compared to control in LPS+NIG-induced SIM-A9 cells. Treatments with the 5 µM of IND and EGO10 completely inhibited LPS+NIG-induced IL-1β release as well as sulforaphane (5 µM) treatment.

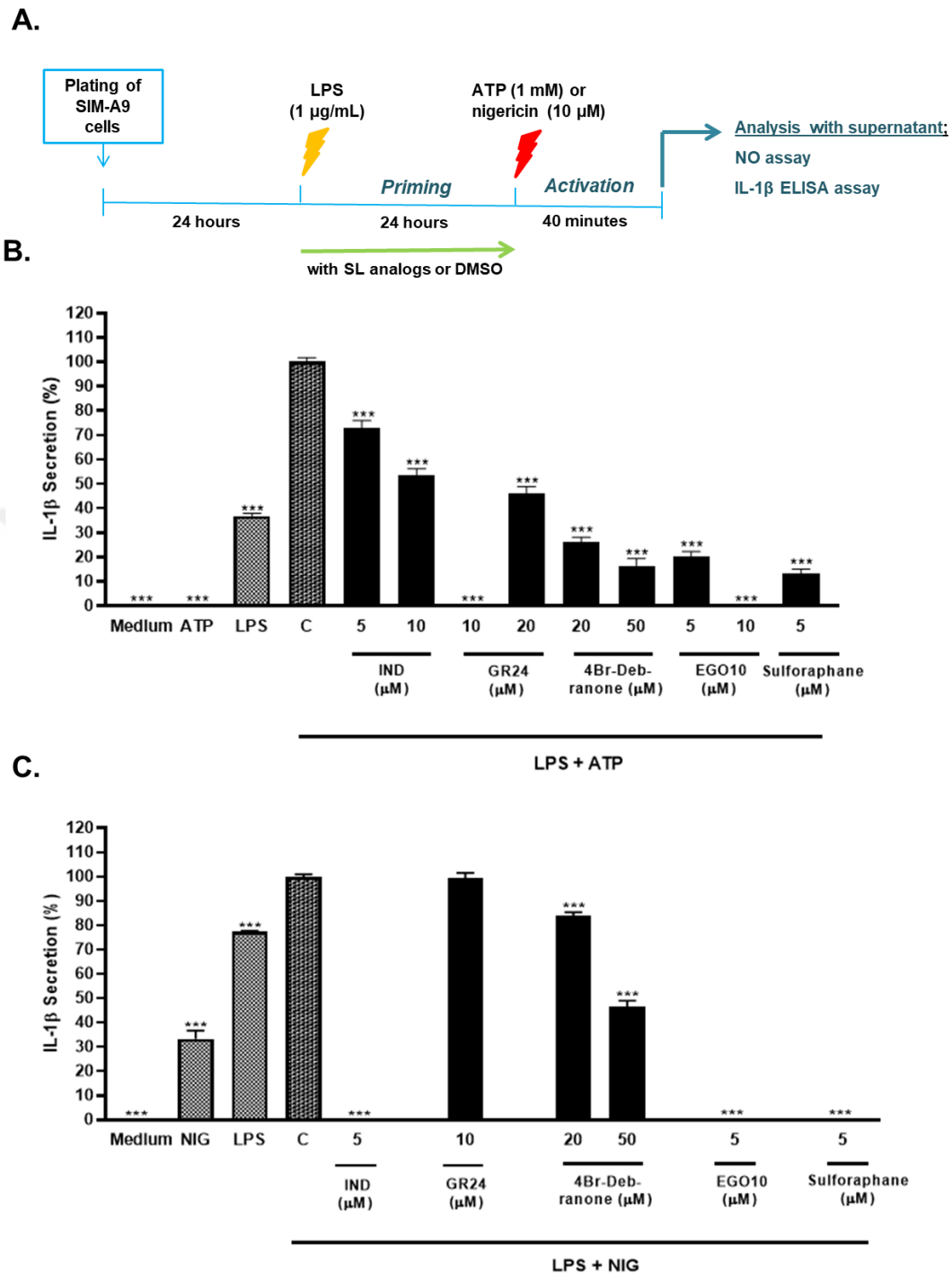


Figure 25. The action of SLs on the NLRP3 inflammasome model. (A) In vitro protocol for NLRP3 inflammasome activation in SIM-A9 cells and inhibitory effects of SLs on NLRP3 inflammasome-induced IL-1β release after stimulation with LPS and (B) 1 mM ATP or (C) 10 µM of nigericin (NIG). Medium: including only DMSO, C: LPS+ATP-treated control. ***$p < 0{,}001$.

SLs Inhibited the NO Secretion in NLRP3 Inflammasome Model of Microglia

ATP stimulation after LPS treatment (control) significantly increased the NO level by 30% relative to only LPS-treated cells in SIM-A9 cells (Figure 26 (A)). IND at 5 and 10 µM doses suppressed the NO secretion level by 82 and 90%, GR24 at 10 and 20 µM by 58 and 85%, 4Br-debranone at 20 and 50 µM by 59 and 93% as compared control, respectively. The LPS+ATP-induced NO levels were also significantly suppressed with the treatment of 5 µM of EGO10 by 76% while its 10 µM caused a complete inhibition in SIM-A9 cells. Sulforaphane at 5 µM decreased the level of LPS+ATP-induced NO release by 49% in SIM-A9 cells (Figure 26 (A)).

In Figure 26 (B), 10 µM of nigericin stimulation after LPS treatment did not change the level of NO secretion as compared to only LPS-induced control. IND and EGO10 decreased the NIG+LPS-induced NO secretion level by 52% and 20% at 5 µM concentration as well as 68% and 44% at 10 µM respectively. GR24 at 10 and 20 µM repressed the NO level by 37 and 65%; 4Br-debranone at 20 and 50 µM by 57 and 77%, as compared to control, respectively. Besides that, 5 µM sulforaphane showed a 33% reduction in NIG+LPS-induced SIM-A9 cells.

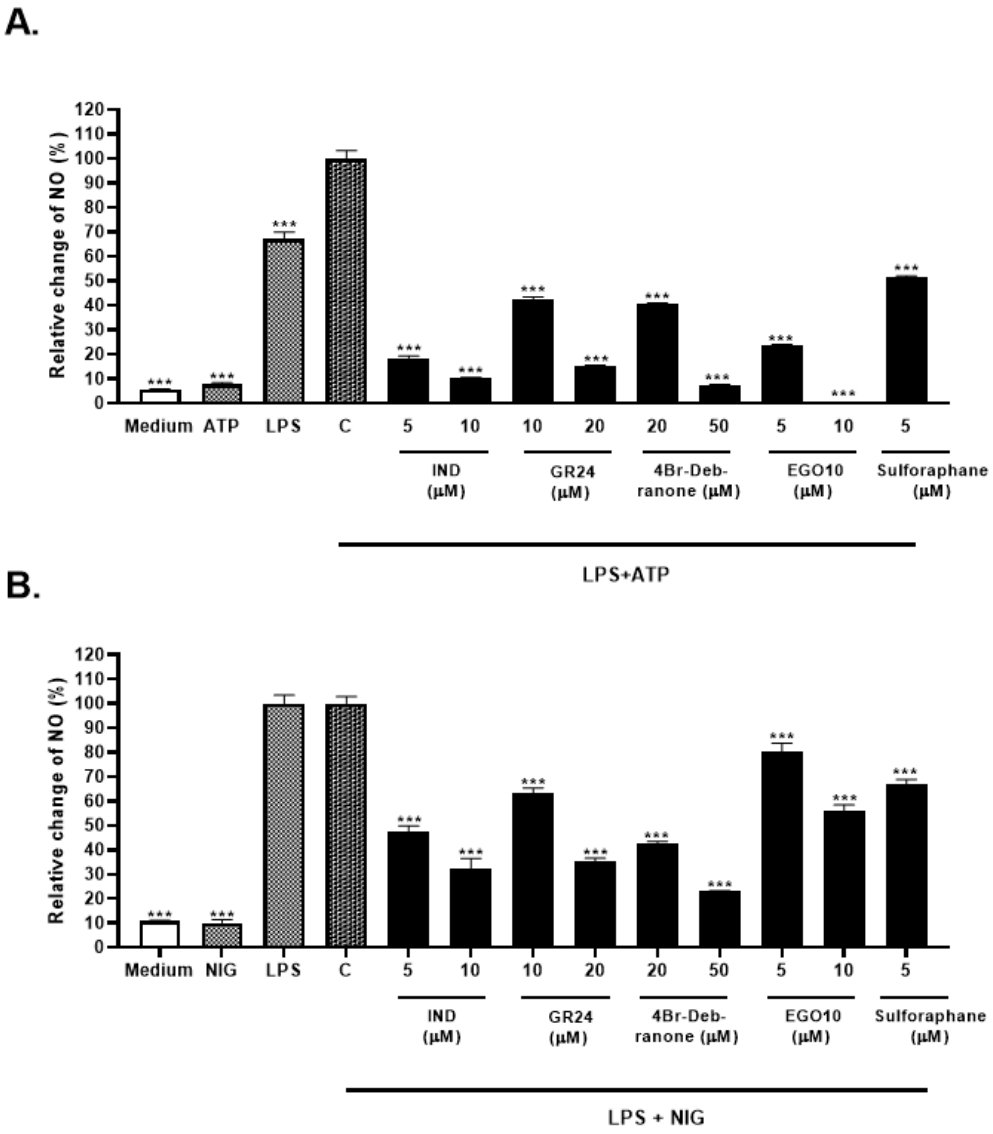


Figure 26. The effects of SL analogs on NLRP3 inflammasome-induced NO release after stimulation with LPS and (A) 1 mM ATP or (B) 10 μM of nigericin (NIG). Medium: including only DMSO, and C: LPS+ATP-treated control. ***$p < 0{,}001$.

4.1.12. Neuroprotective effects of SLs on Nrf2 Protein Expression against Microglial NLRP3 Inflammasome Activation

SIM-A9 cells were treated with one of the selected doses of each SL analogs and LPS for 4 hours and then pursued with ATP induction for 40 minutes. The cytoplasmic protein samples were analyzed by the Western blot method for Nrf2 and β-actin house-keeping protein (Figure 27). The relative Nrf2/β-actin

protein expression ratio of SIM-A9 cells (only DMSO, untreated control) was significantly decreased by 0,9-fold after induction of LPS and ATP (control). Similar to the positive control, 5 µM sulforaphane (2,3-fold increase), EGO10, and GR24 at 10 µM activated the Nrf2 protein expression by 1,9, and 2,0-fold, respectively in NLRP3-induced microglial cells. IND enhanced its expression by 1,2-fold contrasted with control. Furthermore, treatment with 50 µM of 4Br-debranone lead to the most effective enhancement (2,8-fold) in Nrf2 protein expression level in LPS+ATP-activated SIM-A9 cells as compared to the control.

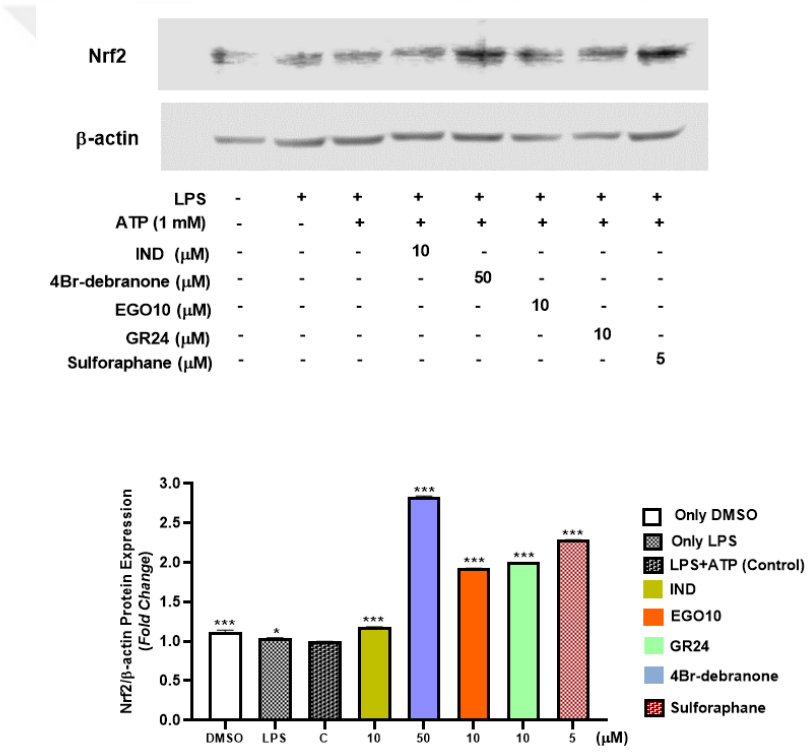


Figure 27. Post-translational Nrf2 Promoting Effect of SLs on NLRP3 inflammasome-activated SIM-A9 cells. Medium: including only DMSO, and C: LPS+ATP-treated control *$p < 0,02$, ***$p < 0,001$.

4.2. Discussion

Carotenoids are pigments that are widely found in plants and their positive effects on human health have been known for years. Apocarotenoids have smaller

structures and different biological properties which are formed as a result of the breakdown of carotenoids. SLs are recently discovered apocarotenoid-derived plant hormones. Essentially, SLs were first discovered in 1966 as molecules that promote germination of the parasitic Striga plant. However, their inclusion into the phytohormone class and the structural and functional elucidation of more than 20 natural SL derivatives based upon the 2010s (Waters et al., 2017). For this reason, the number of studies assessing the molecular effects of SLs in mammalians is limited in the literature. Although the studies in the literature showing the effects of SLs on mammalian cells are extremely limited, they are generally gathered around their anticancer properties (also see *Chapter 2*). The anti-inflammatory and Nrf2-related cytoprotective effects of a model synthetic SL analog, GR24, were elucidated for the first time on macrophage cells which are one of the most important defense elements of the systemic circulation by our research group (Tumer et al., 2018). In the aforementioned study, the therapeutic effects of GR24 through AKT activation were also demonstrated on an inflammatory insulin resistance model in muscle and liver cells. According to in silico analyzes in the same study, GR24 was defined as a drug-compatible small molecule and it has the potential to cross BBB. After that, we published the first data demonstrating that the suppressive effect of GR24 on proinflammatory mediators and also its cytoprotective effect through the Nrf2 signaling pathway on LPS induced SIM-A9 mouse microglial and bEnd.3 BBB endothelial cell lines (Kurt et al., 2020).

Research demonstrating the interaction of plant hormones with the mammalian brain is quite interesting, for instance, the mammalian brain can synthesize ABA under stress conditions (Qi et al., 2015). Therefore, our previous reports about the effects of GR24 on neuroinflammation in vitro model encouraged us to further examined the effects of SLs with different bioactiphores on microglial cells which is the main character mediating the inflammatory mechanism in CNS. ADME analyses of three different SL analogs; EGO10, IND, 4Br-debranone showed that their drug similarity properties are higher than the reference molecules and GR24 according to the Lipinski and Veber rules of 5. Moreover, the molecular weights of each SL analogs are less than 500 g/mol which is suggested as a critical

point for BBB permeability in drug designing. The four SL analogs were recommended for polar surface area and lipophilicity for cellular permeability criteria according to the in silico analysis. Therefore, the four SL analogs with different pharmacophores (Table 1) were selected to investigate their potential neuroprotective effects on CNS-related cell lines. As a first step, the effects of SLs on NO/iNOS signaling, a nitrosative stress pathway, were investigated in SIM-A9 microglial cells.

NO is produced by the iNOS enzyme and increased under inflammatory conditions which is the predominant source of free radical generation in the brain. Microglia and astrocyte cells release NO at micromolar concentration into the brain microenvironment by the stimulation of iNOS/NO signaling. As a result, this mechanism have an important part in the development and progression of AD by creating a degenerative endless loop (infinite loop) between microglia and neuron cells in the initial stages of AD (Asiimwe et al., 2016). Therefore, suppression of iNOS-mediated NO is thought to be an important therapeutic intervention. In the thesis study, all SL analogs significantly suppressed the LPS-induced NO level at very low doses in SIM-A9 microglia cells. IND and EGO10 (1,02 and 1,72 µM) give the lowest IC_{50} values among all analogs. In our previous study, the IC_{50} value of the 1400W compound, which is a selective iNOS inhibitor, for NO secretion in SIM-A9 cells under the same conditions was 2,23 µM. EGO10 and IND showed a stronger inhibitory effect than 1400W when we compared these two data in LPS-induced SIM-A9 cells. Therefore, it can be determined that whether these compounds are selective or not by further studies and they can be developed as selective iNOS inhibitors. On the other hand, 4Br-debranone gave very low IC_{50} values of 12.7 µM, respectively, in terms of NO suppression capacity. These values are lower than some other selective iNOS inhibitors, such as aminoguanidine (20,1 µM) and L-NIL (27,1 µM), as well as some natural anti-inflammatory compounds such as resveratrol (68 µM) and curcumin (18,5 µM) (Matsuda et al., 2000; Abas et al., 2006).

Increased iNOS mRNA and protein levels have been appointed in brain lesions of patients with many neurodegenerative diseases such as AD and PD. In the thesis study, all SL analogs suppressed the iNOS mRNA level by more than

50% at very low micromolar doses. Although all the three SL analog treatments effectively suppressed the NO secretion and iNOS mRNA expression, the iNOS protein expression was not changed in LPS-induced SIM-A9 cells. These results suggest the probability that these compounds may act as catalytic inhibitors by binding to the active site of the iNOS enzyme. This hypothesis should be supported by pure enzyme investigations.

IL-1β is a first cytokine identified with the role in chronic neuroinflammation that increases its expression in a positive feedback manner and also stimulates the expression of other pro-inflammatory factors in particular TNF-α and COX-2 in microglia or astrocytes. Moreover, several in vivo animal models reported that IL1β and TNF-α elevation induced neuronal damage, leukocyte recruitment, and damage in BBB permeability which may be accompanied by AD pathogenesis (Shaftel et al., 2008). In this study, the protein expression levels of two important proinflammatory factors; TNF-α and IL-1β, which are released out of the cell when they are induced under inflammatory conditions, were ascertained by ELISA assay. All the three SL analogs significantly constrained the release of both IL-1β and TNF-α cytokines in LPS-induced SIM-A9 cells at 24 hours. In particular, EGO10 and IND at 10 μM and 4Br-debranone at 50 μM doses effectively suppressed the level of LPS-induced TNF-α release by 80%, 91%, and 83%, respectively. Although the forceful inhibitory effects of EGO10 and IND in TNF-α release, these analogs could not suppress the TNF-α mRNA expression level as much as demonstrated in TNF-α release. However, 20 and 50 μM of 4Br-debranone significantly restrained the LPS-induced TNF-α mRNA expression level.

IL-1β is an forceful proinflammatory cytokine and attracted attention as the primary increasing mediator in the early period of neuroinflammation. In addition, it increases the permeability of the BBB and allows for the migration of systemic immune system cells to the damaged area (Mendiola & Cardona, 2018). EGO10 and IND at the concentrations of 5 and 10 μM exhibited a significant and similar suppressive effect on IL-1β release compared to the LPS-treated control. Moreover, a strong suppressive effect on IL-1β release was also observed after the treatment of 50 μM of 4Br-debranone. In qRT-PCR analysis, EGO10 and IND significantly

and strongly suppressed the IL-1β mRNA expression level even at their lowest dose (5 μM) while 4Br-debranone leads to a dose-dependent suppression at 12 hours.

Unlike TNF-α, IL-1β is found in the cytoplasm in a biologically inactive form, and activation of caspase-1 by the NLRP3 inflammasome complex are involved in its activation (Gustin, 2016). NLRP3 inflammasome activation which is consists of the node-like receptor protein NLRP3, adapter protein ASC, and pro-caspase-1. The formation of the NLRP3 inflammasome complex is another crucial mechanism involved in the progression of chronic inflammation in the CNS (Halle et al., 2008). In the inflammatory response, the expression of NLRP3 and pro-IL-1β is firstly induced by activation of NF-κB, this step is defined as the priming in macrophages or microglial cells. Then, the NLRP3 multiprotein complex assembles and is activated with a second signal such as ATP, pore-forming toxins (nigericin), or viral RNA (Y. He, H. Hara, & G. Núñez, 2016). Extracellular ATP at millimolar concentrations serves as an NLRP3 agonist and activates this protein complex by facilitating K^+ flux, and cytosolic [Ca^{2+}] level changes. Moreover, nigericin is known as potassium ionophore which triggers K^+ efflux which is a common step in NLRP3 activation and then leads to IL-1β maturation in LPS-induced macrophages (Y. He et al., 2016). In this study, we performed for the first time an *in vitro* NLRP3 inflammasome activation model on SIM-A9 microglia cells. Microglia cells were activated by the stimulation of ATP or nigericin as a secondary stimulus after the priming step with LPS treatment to reveal the potent suppressive effects of SLs on NLRP3 inflammasome activation. In this study, sulforaphane was used as a positive control which is known as an effective NLRP3 inhibitor in microglial cells, *in vitro* (Tufekci et al., 2021). The secretion level of mature IL-1β was quantified from the supernatant samples of SIM-A9 cells by ELISA assay. The IL-1β secretion level was strongly triggered by ATP treatment (signal 2) following LPS as compared to LPS-treated control indicating the NLRP3 inflammasome model is successfully switched on in SIM-A9 cells. GR24 and EGO10 at a physiologically relevant-low dose (10 μM) suppressed NLRP3-dependent IL-1β secretion almost completely in LPS-ATP primed/activated SIM-A9 cells. The efficiency of SL analogs on LPS+ATP-induced NLRP3 inflammasome activation is supported by another potent activator named nigericin (NIG). Accordingly, treatment with NIG

significantly activated the NLRP3 inflammasome mechanism and triggered the IL-1β release in microglial cells. Although GR24 at 10 µM concentration displayed a strong suppressive effect in LPS+ATP stimulation, the compound did not show any change in IL-1β release when the cells were activated by NIG. However, other SL analogs exhibited a significant and consistent suppression profile on NLRP3-induced IL-1β release in NIG+LPS-induced SIM-A9 cells similar to the LPS+ATP model. Treatments with the lowest dose (5 µM) of IND and EGO10 completely inhibited LPS+NIG-induced IL-1β release, similar to the positive control, sulforaphane (5 µM).

The cellular regulatory mechanism induces the production of NO to prevent the secretion of mature IL-1β and IL-18 due to NLRP3 inflammasome activation in long-term LPS-stimulation. Therefore, NO has a regulatory role in NLRP3 inflammasome activation which directly inhibits the active caspase-1 in macrophages (Inflammasome, 2012). Junior et al., (2013) demonstrated that macrophages activate the NLRP3 inflammasome mechanism to suppress the L. Amazonensis (a parasite) infection as well as production of NO. It was suggested that NLPR3-induced IL-1β production restricts the parasite production through a NO-dependent mechanism (Lima-Junior et al., 2013). However, there is still no study investigating the link between NO secretion and NLRP3 inflammasome activation in microglial cells. In the thesis study, we explained that ATP stimulation after LPS treatment (control) significantly increased the NO level as compared to only LPS stimulation in SIM-A9 microglia cells. The result revealed that the microglial immune response against NLRP3 inflammasome activation exaggerated the NO production in microglial cells. Almost all SL analogs showed a strong inhibitory effect on NO release in a dose-dependent manner in LPS+ATP-induced SIM-A9 cells. However, the positive control, sulforaphane at 5 µM showed a weaker inhibitory effect on LPS+ATP-induced NO release than the four SL analogs. SL analogs showed a consistent NO inhibitory profile on NİG+LPS induced SIM-A9 cells, as well as the LPS+ATP-treated group. As a hallmark of neurodegenerative diseases, microglia-mediated neurotoxicity emerges from proinflammatory responses as a result of microglial activation. The microglial response as the first defense against injury or infection is called as M1 phenotype,

however, microglia have also neuroprotective phenotypes (Tang & Le, 2016). The M1 proinflammatory phenotype was induced by LPS stimulation in SIM-A9 cells. All of these SL analogs effectively suppressed the LPS-induced proinflammatory factors characterized in M1 phenotype microglia. Especially, low doses of IND and EGO10 strongly and significantly inhibited M1 phenotype markers containing IL-1β, TNF-α, iNOS, and COX-2 genes at the mRNA expression manner. Although neuronal COX-2 play role in the vasculature and synaptic signaling, COX-2 expression in glial cells is contributed with neuroinflammation (Lopez et al., 2020). COX-2 expression is promoted through NF-κB activation in microglial cells and synergistically prostaglandins, which are the products of COX-2 upregulates NF-κB (López & Ballaz, 2020). In this study, although the three SL analogs effectively suppressed COX-2 at the mRNA level, only 50 μM of 4Br-debranone was capable of suppressing the LPS-induced COX-2 protein production in microglial cells. M2 phenotype of microglia is an alternative activation of microglia with neuroprotective and immunosuppressive responses which is induced by IL-4 or TGF-β. The antiinflammatory M2 phenotype of microglia promotes the expression of Arg1, CD206, and Fizz1 genes. The important point in the polarization of microglia is the balance of M1/M2 phenotype heterogeneity because the M2 phenotype may also be associated with angiogenesis and tumorigenesis due to its proliferative markers. We analyzed the effects of SLs on the M2-phenotype markers in LPS-activated microglia cells. The M2 phenotype was significantly induced by IND which enhanced the gene expression level of Arg1 at 5 μM and CD206 at 10 μM concentration in M1 phenotype (LPS)-induced SIM-A9 cells. Although 4Br-debranone significantly decreased the mRNA expression level of proinflammatory factors representing the M1 phenotype, it could mildly increase the gene expression level of CD206 at 50 μM dose in LPS-induced SIM-A9 cells. Among the three SL analogs, only IND could return the expression levels of the M2 phenotype markers to the normal range in LPS-induced SIM-A9 cells as in the untreated control. Therefore, these results referred that IND may have the moderate ability to switch the M1 phenotype microglia cells to the M2 phenotype. Similar to 4Br-debranone, GR24 did not induce the M2 phenotype at gene expression level for Arg1 but its 20 μM dose led to the mild increment in the gene expression level of CD206 in LPS-induced SIM-A9 cells.

The NFE2L2 gene is a gene that encodes the transcription factor Nrf2. Nrf2 is responsible for controlling the expression levels of the most important antioxidant and detoxification (phase II) genes. As a novel macrophage phenotype, the Mox phenotype that polarized by oxidized phospholipids. Mox phenotype was represented by an obvious upregulation of Nrf2-intervened expression of redox-regulatory genes including HO-1, Srxn-1, Gclc, and Thioredoxin reductase 1 (Kadl et al., 2010). In this study, we identified first time the Mox phenotype switching effect of SLs on inflammation modeled microglial cells. Besides the moderate promoter effect of SLs on the Nrf2 transcription factor, the SL analogs also improved the expression of Nrf2-dependent detoxification genes including NQO1 and Gclc in LPS-induced SIM-A9 cells. The expression levels of HO-1 in LPS-induced SIM-A9 cells were mildly upregulated by indanone at both doses. However, the gene expression level of Srxn1 was strongly ameliorated by the treatments of IND and EGO10. These results showed that the two SL analogs can switch the M1-phenotype of microglia to Mox phenotype by modulating the mRNA expression of these characteristic genes. Furthermore, between these SL analogs, 4Br-debranone showed the strongest enhancing effect on both the Nrf2-dependent antioxidant and detoxification genes in LPS-induced microglial cells. These results suggest that 4Br-debranone might have a potent Mox-switching effect in M1-phenotypic microglia cells to protect CNS from chronic inflammation.

Our previously published data showed the cytoprotective effect of GR24 on the Nrf2 signaling pathway by enhancing the NQO1 and HO-1 gene expression on LPS-triggered SIM-A9 cells (Kurt et al., 2020). In this study, we further showed that the effect of GR24 on Nrf2-dependent two other Mox phenotype markers in addition to NQO1 and HO-1 at mRNA level in SIM-A9 cells. Although the mRNA expression levels of NQO1 and HO-1 were strongly increased by the treatment of 20 μM of GR24 as 13 and 2,4-fold as regards the control in the study of Kurt et al., (2020), GR24 significantly induced the gene expression of Gclc by 1,5-fold but not Srxn1 in LPS-induced SIM-A9 cells**.**

In our previously published data, we have also elucidated that GR24 significantly activated the nuclear translocation of Nrf2 while inhibiting the nuclear activation of NF-κB at 4 hours in LPS-induced SIM-A9 cells (Kurt et al., 2020).

Studies in the literature have shown that there is a cross-talk relationship between Nrf2 activation and NF-κB inhibition (M.-g. Song et al., 2015). Therefore, we investigated the link between the antiinflammatory effect of GR24 and the Nrf2 pathway in SIM-A9 microglial cells by using the siRNA silencing technique. The HO-1 gene-enhancing capacity of GR24 was almost 64% decreased after the Nrf2 gene was silenced in SIM-A9 cells. Interestingly, the LPS-induced IL-1β gene expression was even reinforced in Nrf2 silenced SIM-A9 cells by 1,7-fold relative to the control ($Nrf2^{-/-}$) siRNA group. The result explained the regulatory role of Nrf2 on the inflammatory mechanism. Besides that, GR24 suppressed the LPS-induced IL-1β gene expression at the same level both on Nrf2 siRNA and control siRNA-transfected microglia cells compared to their only LPS-induced control groups. These results suggest that GR24 suppressed the inflammatory factors not through Nrf2 signaling even if it promoted the Nrf2-mediated antioxidant response. We also investigated the effects of SL analogs in Nrf2 protein expression on NLRP3-inflammasome activated microglial cells at 4 hours. All SL analogs significantly increased the ratio of cytoplasmic Nrf2/βactin protein expression while 50 µM of 4Br-debranone lead to the most effective enhancement in Nrf2 protein expression level in LPS+ATP-activated SIM-A9 cells. The Nrf2 mRNA expression in LPS-primed microglia cells and the protein expression in NLRP3-induced cells were consistently promoted with treatments of the four SL analogs. Even though, the cytoplasmic and nuclear proteins of ATP+LPS stimulated microglial cells were isolated to analyze the nuclear translocation of Nrf2 by the Western Blot technique, a sufficient amount of nuclear protein lysate could not be obtained by NEPER kit. Therefore, we could only prove that the production of cytoplasmic Nrf2 protein was significantly enhanced by all SL analogs as well as the positive control (sulforaphane) in NLRP3-activated microglial cells. In further studies, the Nrf2 nuclear translocation and the relation of SLs with the Nrf2 signaling pathway should be elucidated in a more detailed manner on NLRP3 inflammasome-activated microglial cells.

It is known that BDNF is a neurotrophic factor synthesized by neurons, astrocytes, and microglial cells. As a potent resource of BDNF, microglial cells contribute to neuronal network excitability, thus the microglia-neuron interactions

have great importance for neurodegenerative diseases (Jiao et al., 2016). Moreover, Lai et al., (2018) recently elucidated the BDNF-EPO-Shh signaling pathway in the arrangement of inflammatory responses of microglial cells by inhibiting the BDNF receptor also called TrkB (Lai et al., 2018).

Because SLs lead to an effective antiinflammatory effect in microglia, we further elucidated the BDNF-related antiinflammatory effect of a selected SL analog on LPS-induced microglia cells. In this study, the BDNF-TrkB signaling was suppressed by the pre-treatment of entrectinib for the first time in the SIM-A9 microglial cell. Since GR24 is the SL analog whose effect on the mechanism of neuroinflammation has been mostly elucidated, we used this compound to elucidate its relationship with the BDNF pathway. GR24 at 50 µM remarkably improved the BDNF mRNA expression level while considerably suppressing the COX expression in LPS–induced SIM-A9 cells. The suppressive capacity of GR24 on COX-2 mRNA expression (4,7-fold) was remarkably reduced when the BDNF pathway was inhibited (1,5-fold) by entrectinib. These results suggest that the anti-neuroinflammatory effect of GR24 on LPS-induced microglia cells may be related to the TrkB-BDNF pathway. The most important pathological event in the progression of neurodegeneration is neurotoxicity due to microenvironmental factors including ROS production, proinflammatory cytokines and, etc. due to uncontrolled microglial activation. In this thesis study, the protective effects of SLs on neuronal SH-SY5Y cells were elucidated with MTT cell viability assay following the direct and microglia-mediated treatments (microglial conditioned medium) of SLs. Among the four SL analogs, GR24 (at 20 µM) and 4Br-debranone (at 50 µM) significantly enhanced the neuronal cell viability in SH-SY5Y cells. The conditioned medium assay was accomplished to understand whether the potential ameliorative effect of SLs on activated microglial cells also constructively affects neuronal cell viability. SH-SY5Y neuronal cells were treated with microglial secretomes which were simultaneously transferred after SIM-A9 cells were treated with LPS and SLs for 24 hours. Interestingly, LPS treated microglial secretome induced the cell viability of SH-SY5Y neuronal cells contrasted with the nontreated control at 24 hours. The reason for increased cell viability by LPS treatment may be related to LPS-induced BDNF expression in microglial cells (see

Figure 16 (A) and Figure 19 (B)). Activated microglia is known as a major source of proinflammatory response however they also trigger neurotrophin production to promote neuronal regeneration (T. Miwa et al., 1997). In this study, we showed that SLs both suppressed the proinflammatory phenotype of microglia while enhancing the BDNF expression and neuronal cell viability. EGO10 (10 µM) and 4Br-debranone (50 µM)-treated microglial secretomes similarly showed a mild proliferative effect on SHSY5Y cells in comparison to the treatment with only LPS-induced microglial secretome. Eventually, 20 µM of GR24 showed a significant protective effect on SH-SY5Y neuronal cells through microglial cells. Overall, these results suggest that SLs may have a multi-target neuroprotective effect on both microglia and neuron cells but their molecular mechanism should be further investigated in neuronal cells by co-culture studies.

CHAPTER 5
CONCLUSION

Neuroinflammation has been presented as one of the early pathological cases of AD and also different neurodegenerative diseases. New generation disease-modifying therapeutic approaches targeting microglial activation and neuroinflammation may enlighten new routes in the treatment/prevention of these diseases. Strigolactones (SLs) are structurally distinct apocarotenoids and have been currently classified in phytohormones. Although the anticancer effect of SLs was primarily elucidated on mammalian cells, our research group showed their impact on brain cells under inflammatory conditions for the first time in 2020.

In this study, we identified the unknown effects of four SL analogs with different pharmacophores on microglial and neuronal cells under different inflammatory conditions. In LPS stimulated SIM-A9 microglial cells, IND and EGO10 showed potent antiinflammatory effects such as low IC_{50} values for NO release and strong supression in the TNF-α, and IL-1β cytokine release in LPS treated microglial cells. As another inflammatory condition, NLRP3-inflammasome activation was performed in the microglial cell. All SL analogs, GR24, EGO10, IND, and 4Br-debranone remarkably suppressed the ATP-stimulated IL-1β release in microglial cells. Additively, EGO10 and IND almost completely inhibited the NLRP3-induced IL-1β release in microglial cells activated with nigericin following LPS treatment. Furthermore, the four SL analogs showed a dual inhibitory effect on both IL-1β and NO secretion in the NLRP3 inflammasome model. The BDNF-related antiinflammatory effect of GR24 on LPS-induced SIM-A9 cells was elucidated by analyzing the change in the COX-2 gene expression level. As a promising neuroprotective compound, GR24 improved the neuronal viability in the conditioned media assay. Although all SL analogs lead to a transition from M1 to Mox, 4Br-debranone exhibited the most prominent effect for Mox pehnotype polarization in microglial cells. Overall, these results revealed that the four SL analogs have specific and multi-target activities against neuroinflammation, thus they might be developed as candidate neuroprotective agents with further in vitro and in vivo studies.

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