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Evaluation of the influence of selected stilbenes on neuroprotection against stroke with a focus on inflammatory signaling

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THÈSE DE DOCTORAT

Évaluation de l'influence de stilbènes sélectionnés sur la neuroprotection contre l'AVC avec un focus sur la signalisation inflammatoire

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**Présentée en vue de l'obtention du
grade de docteur en Sciences de la Vie et
de la Santé d'Université Côte d'Azur et du
grade de docteur d'Université Masaryk**

Dirigée par : Dr. Catherine Heurteaux /
Prof. Assoc. Jan Hošek

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Université Masaryk

Devant le jury, composé de :

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Maria-Grazia De Simoni, Prof., IRCCS, IT
Pavel Babica, Prof. Assoc., MUNI, CZ
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DISSERTATION THESIS

Evaluation of the influence of selected stilbenes on neuroprotection against stroke with a focus on inflammatory signaling

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the degree of Doctor in Life and Health
Sciences from University Côte d'Azur and
the degree of Doctor from Masaryk University**

Supervised by: Dr. Catherine Heurteaux /
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Resumé

Un grand pourcentage de troubles aigus et chroniques aux niveaux systémique et central s'accompagne d'une réponse inflammatoire excessive ou non résolutive du système immunitaire contribuant au développement de la maladie. L'accident vasculaire cérébral (AVC) ischémique est l'une des maladies les plus dévastatrices au monde où la neuroinflammation caractérisée par l'activation microgliale et la production de médiateurs pro-inflammatoires joue un rôle majeur dans la physiopathologie de l'AVC aggravant son issue. Cette thèse s'intéresse au potentiel anti-inflammatoire des stilbènes naturels et de leur capacité à intervenir dans l'activation médiée par le récepteur toll-like 4 (TLR4) des voies du facteur nucléaire- κ B (NF- κ B) et de la protéine activatrice-1 (AP-1). Plusieurs des 37 stilbènes testés ont montré la capacité : 1./ de réduire remarquablement l'expression des cytokines pro-inflammatoires, comme celui du facteur de nécrose tumorale α (TNF- α) et de l'interleukine-1 β (IL-1 β), 2./ de diminuer l'activité NF- κ B/AP-1, l'activation des protéines kinases activées par des mitogènes (MAPK) ainsi que la dégradation de l'inhibiteur κ B-alpha (I κ B α) dans les macrophages THP-1 et les monocytes THP-1-XblueTM-MD2-CD14. La première partie de cette thèse démontre un puissant effet anti-inflammatoire *in vitro* des stilbènes trouvés dans les plantes alimentaires et médicinales, résultat éventuellement applicable à la prévention des maladies inflammatoires systémiques. Simultanément, ce criblage pharmacologique a conduit à l'identification de composés au potentiel anti-inflammatoire le plus prometteur, parmi lesquels macasiamenene F (MF), stilbène prénylé très peu étudié de *Macaranga siamensis*. Le potentiel anti-inflammatoire de MF a ensuite été étudié au niveau du système nerveux central (SNC) sur le modèle de cellules BV-2 et de microglie primaire de souris stimulées par le lipopolysaccharide (LPS). MF a montré la capacité de réduire l'inflammation à n'importe quel stade du traitement. MF utilisé en prétraitement a radicalement réduit l'expression des gènes et des protéines du TNF- α et de l'IL-1 β . De plus, MF a montré les effets cytoprotecteurs contre la mort de la microglie induite par le LPS. Lorsqu'il est appliqué en co- et post-traitement, MF a pu réduire l'inflammation, ce qui indique son utilité potentielle dans les troubles cérébraux aigus, tels que l'ischémie cérébrale. Par conséquent, la capacité de MF à contrecarrer d'autres mécanismes délétères de la cascade ischémique, tels que le stress oxydatif et l'excitotoxicité induite par le glutamate, a été ensuite étudiée. Bien que l'action antioxydante de MF ait été démontrée mineure, MF a exercé la capacité de protéger les neurones corticaux en cultures primaires contre les dommages excitotoxiques induits par le N-méthyl-D-aspartate (NMDA). Pour tester si MF pourrait antagoniser les conditions ischémiques délétères, les neurones corticaux traités par MF ont été exposés à une privation d'oxygène-glucose (OGD) de 1 h/2 h (modèle de l'OGD). Enfin, MF a été formulé dans

des liposomes dans le but d'améliorer sa solubilité dans l'eau et de tester *in vivo* sur le modèle murin d'occlusion de l'artère cérébrale moyenne (MCAo).

Mots-clefs : stilbènes, inflammation, macasiamenene F, ischémie, neuroprotection

Abstract

An enormous percentage of acute and chronic disorders at the systemic and central levels is underlined by an excessive or non-resolving inflammatory response of the immune system contributing to disease development. Ischemic stroke is undoubtedly one of the worst devastating disorders worldwide where neuroinflammation characterized by microglial activation and production of proinflammatory mediators plays a crucial role in stroke pathobiology worsening its outcome. This thesis deals with the anti-inflammatory potential of natural stilbenes and their ability to intervene in toll-like receptor 4 (TLR4)-mediated activation of nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) pathways. Several of 37 tested stilbenes displayed the ability to remarkably reduce the expression of proinflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), dampen up-stream the NF- κ B/AP-1 activity, as well as the activation of mitogen-activated protein kinases (MAPKs) or inhibitor κ B-alpha (I κ B α) degradation in THP-1 macrophages and THP-1-XblueTM-MD2-CD14 monocytes. The first part of this thesis demonstrates the potent *in vitro* anti-inflammatory effects of stilbenes found in food and medicinal plants, eventually applicable to the prevention of systemic inflammatory disorders. Simultaneously, this pharmacological screening led to the identification of compounds with the most promising anti-inflammatory potential, among them, macasiamenene F (MF), very low investigated prenylated stilbene from *Macaranga siamensis*. MF anti-inflammatory potential was next studied at the central nervous system (CNS) level, on the model of lipopolysaccharide (LPS)-stimulated BV-2 cells and brain-sorted mouse microglia. MF showed the ability to reduce inflammation at whatever stage of treatment. MF used in pre-treatment radically down-regulated the gene and protein expression of TNF- α and IL-1 β . Additionally, MF showed the cytoprotective effects against LPS-induced loss of microglia. When applied as co- and post-treatment, MF was able to inhibit the ongoing inflammation, which indicates its potential usefulness also in acute brain disorders, such as cerebral ischemia. Therefore, the ability of MF to counteract other deleterious mechanisms of the ischemic cascade, such as oxidative stress and glutamate-induced excitotoxicity, was further investigated. Although the antioxidant action of MF was determined as poor, MF exerted the capability to protect primary cortical neurons against *N*-methyl-D-aspartate (NMDA)-induced excitotoxic damage. To evaluate the ability of MF to antagonize the ischemic conditions, MF-treated cortical neurons were exposed to 1 h/2 h Oxygen Glucose Deprivation (OGD)-challenge (OGD model). Then, MF was formulated into liposomes for the purpose of improving its water solubility and further testing *in vivo* on the model of middle cerebral artery occlusion (MCAo).

Keywords: stilbenes, inflammation, macasiamenene F, ischemia, neuroprotection

Abstrakt

Vysoké procento akutních i chronických chorob je doprovázeno zvýšenou nebo přetrvávající zánětlivou reakcí imunitního systému přispívající k rozvoji onemocnění, a to jak na systémové úrovni, tak na úrovni centrálního nervového systému (CNS). Ischemická cévní mozková příhoda je celosvětově jednou z nejčastějších příčin úmrtí a trvalého postižení. Zánět CNS charakterizovaný aktivací mikroglíí a produkcí prozánětlivých mediátorů hraje klíčovou roli v patologii cévní mozkové příhody a vede k zhoršení jejích následků. Tato práce se zabývá protizánětlivým potenciálem přírodních stilbenů a jejich schopností zasáhnout do aktivace drah jaderného faktoru κB (NF- κB) a aktivátorového proteinu 1 (AP-1) zprostředkované toll-like receptorem 4 (TLR4). Více z 37 testovaných stilbenů prokázalo schopnost: 1./ výrazně snížit expresi prozánětlivých cytokinů tumor nekrotizujícího faktoru α (TNF- α) a interleukinu 1 β (IL-1 β), 2./ tlumit signální dráhu NF- κB /AP-1, 3./ snížit aktivaci mitogenem aktivovaných proteinových kináz (MAPKs) a degradaci inhibitoru κB alfa (I $\kappa\text{B}\alpha$) v makrofázích THP-1 a monocytech THP-1-XblueTM-MD2-CD14. První část této práce demonstruje výrazné *in vitro* protizánětlivé účinky stilbenů vyskytujících se v potravinách a léčivých rostlinách, s potenciálním využitím v prevenci systémových zánětlivých chorob. Tento farmakologický screening vedl k identifikaci sloučenin s nejslibnějším protizánětlivým potenciálem, mezi nimi málo známý prenylovaný stilben macasiamenene F (MF) z *Macaranga siamensis*. Antiflogistický potenciál MF byl dále studován na úrovni CNS, a to na modelu lipopolysacharidem (LPS) stimulovaných buněk BV-2 a primárních myších mikroglíí. Látka MF prokázala schopnost snížit zánět v jakékoli jeho fázi. MF aplikovaná před LPS výrazně snížila genovou a proteinovou expresi TNF- α a IL-1 β . Táto látka zároveň prokázala cytoprotektivní účinky vůči LPS-indukované smrti mikroglíí. Kromě toho, MF dokázala tlumit probíhající zánět, a to jak při aplikaci souběžné s LPS, tak v aplikaci po LPS. Tudíž by mohla být potenciálně využita také u akutních poruch CNS doprovázených zánětem, jako je ischemická mrtvice. Z tohoto důvodu byla dále zkoumána schopnost MF zpomalit efekt jiných destruktivních mechanismů ischemické kaskády, jako je oxidační stres a glutamátem indukovaná excitotoxicita. Ačkoli je antioxidační účinek MF slabý, látka MF projevila schopnost chránit primární kortikální neurony před excitotoxickým poškozením indukovaným *N*-methyl-D-aspartátem (NMDA). Schopnost MF tlumit následky ischemie byla následně testována na modelu primárních kortikálních neuronů vystavených 1 h/2 h deprivaci kyslíku a glukózy (model OGD). V rámci práce byly také připraveny a testovány různé liposomální formulace této látky, a to za účelem zvýšení její solubility v hydrofilním prostředí pro další testování na myším *in vivo* modelu okluze střední mozkové tepny (model MCAo).

Klíčová slova: stilbeny, zánět, macasiamenene F, ischemie, neuroprotektce

Proclamation

I hereby declare that the submitted dissertation thesis is my own work elaborated under the supervision of my thesis directors Assoc. Prof. RNDr. Jan Hošek, Ph.D., Dr. Catherine Hurteaux, DR CRNS, and thesis consultant Dr. Nicolas Blondeau, DR CNRS, using the literary sources correctly cited and listed in the reference list. I hereby confirm that the electronic and paper versions of the thesis are identical.

In Brno,

.....

author's signature

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Research background

At the arrival to the Czech laboratory at Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno (since 1st July 2020 part of Masaryk University) in 2016, my team was focusing on the anti-inflammatory, antioxidant, and antidiabetic properties of food and plant polyphenolic compounds. The goal of my PhD project supervised by Assoc. Prof. Jan Hošek was to carry out a comparative in vitro pharmacological screening investigating the effects of a panel of 37 stilbenoids on inflammatory TLR4 signaling with a focus on I κ B α /NF- κ B and MAPK/AP-1 pathways. My work was a part of a bigger project (GAČR 16-07193S), whose aim was to evaluate the potential health benefits of stilbenes in food and medicinal plants and the impact of their metabolization in the human body on their biological activity. My work contributed to the identification of stilbenoids with the most significant anti-inflammatory potential against lipopolysaccharide (LPS)-induced systemic inflammation of THP-1 human macrophages and THP-1-XblueTM-MD2-CD14 monocytes, and led to the selection of the most promising molecules for further in vitro and in vivo studies.

Apart from the anti-inflammatory effects, some stilbenoids have been reported to display neuroprotective properties in the CNS pathologies linked with a strong inflammatory component, such as stroke or Alzheimer's disease (Akinwumi et al., 2018). Therefore, we started collaborating with the team of Drs. Catherine Heurteaux and Jean Mazella at the Institute of Molecular and Cellular Pharmacology (IPMC CNRS), in Valbonne, France. The research of this team is oriented on the development of new therapeutic strategies against two major brain pathologies - depression and ischemic stroke. In the case of stroke, this team focuses on the neurovascular protection by alternative plant-based strategies NeuroAid (MLC601/MLC901), as well as by TREK-1 channel modulators (spadin and its derivatives). Another focus is the neuroprotection related to the nutrition, by the treatment with polyunsaturated omega-3 fatty acids, such as alpha-linolenic acid (ALA), in relation to neuroinflammation.

In 2017, thanks to the awarded Grant of French Government by the French Embassy in Prague, Czech Republic, and the hospitality of Dr. Heurteaux and Dr. Mazella, I received the opportunity to further develop my PhD project in their team within the frame of the joint thesis ("cotutelle") program. The aim of my work in the French laboratory was to evaluate the ability of selected plant stilbenoid macasiamenene F (MF) to antagonize the inflammatory and ischemic conditions at the CNS level against LPS-activated microglia and OGD-challenged primary cortical neurons, respectively. Since 2017, my studies had a character of alternated half-year stays in the French and in the Czech laboratory.

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Abbreviations

AA	arachidonic acid
AAPH	2,2'-azobis(2-methylpropionamide) dihydrochloride
AD	Alzheimer's disease
AGE	advanced end glycation products
AIS	acute ischemic stroke
ALA	alpha-linolenic acid
Apaf-1	apoptotic protease activating factor-1
AP-1	activator protein 1
AREs	antioxidant response elements
Arg1	arginase 1
ATF-2	activating transcription factor 2
ATP	adenosine triphosphate
BAFF	B-cells activating factor
Bax	Bcl-2-associated X protein
BBB	blood-brain barrier
Bcl-2	B-cell lymphoma 2
BCR	B-cell receptor
BDNF	brain-derived neurotrophic factor
CAA	cellular antioxidant activity
CAD	caspase-activated DNase
CAMs	cellular adhesion molecules
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CD	cluster of differentiation; Crohn's disease
CKD	chronic kidney disease
CMP	cytidine monophosphate
CNS	central nervous system
cPLA2	cytosolic phospholipases A2
COX-1/2	cyclooxygenase 1/2

CREB	cAMP-response element binding protein
CTP	cytidine triphosphate
CVDs	cardiovascular diseases
DAMPs	damage-associated molecular patterns
DBP	diastolic blood pressure
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DCs	dendritic cells
DHA	docosahexaenoic acid
DM(2)	diabetes mellitus (type 2)
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
eNOS	endothelial nitric oxide synthase
EPA	eicosapentaenoic acid
EPR	enhanced permeability and retention
ERK1/2	extracellular signal-regulated protein kinase ½
ET	endovascular thrombectomy
FDA	Food and Drug Administration
FIZZ1	resistin-like molecule alpha1
GABA	gamma-aminobutyric acid
γGCS	γ-glutamylcysteine synthetase
GDNF	glial cell-derived neurotrophic factor
GLT-1	glutamate transporter 1
GR	glucocorticoid receptor; glutathione reductase
GRE	glucocorticoid response elements
GSH	glutathione
GSHPx	glutathione peroxidase
GSK-3β	glycogen synthase kinase 3 beta
GSSG	glutathione disulfide
HBV, HCV	hepatitis B virus, hepatitis C virus
HD	Huntington's disease
HMG-CoA	β-hydroxy β-methylglutaryl-coenzyme A

HO-1	heme oxygenase 1
HPLC	high-performance liquid chromatography
HRESIM	high resolution electrospray ionization mass spectrometry
IBDs	inflammatory bowel diseases
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon gamma
IGF-1	insulin-like growth factor 1
IL	interleukin
IL-1RA	IL-1 receptor antagonist
iNOS	inducible nitric oxide synthetase
IκBα	inhibitor κ B-alpha
IKK	inhibitory kappa-B kinase
I/R	ischemia/reperfusion
IRAKs	IL-1 receptor-associated kinases
JAK-STAT	Janus kinase/signal transducer and activator of transcription
JNKs	c-Jun amino-terminal kinases
LDH	lactate dehydrogenase
LOXs	lipoxygenases
LPS	lipopolysaccharide
LTs	leucotrienes
MAPKs	mitogen-activated protein kinases
MCAo	middle cerebral artery occlusion
MCP-1	monocyte chemoattractant protein-1
MedDiet	Mediterranean diet
MF	macasiamenene F
MHC II	major histocompatibility complex II
MIP-1α	macrophage inflammatory protein-1 alpha
MKP-1	mitogen-activated kinase phosphatase-1
MMPs	matrix metalloproteinases
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MyD88	myeloid differentiation 88

NADPH	dihyronicotinamide-adenine dinucleotide phosphate
NAFLD	non-alcoholic fatty liver disease
NCX	sodium-calcium exchanger
NE	norepinephrine
NETs	neutrophil extracellular traps
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NKs	natural killers
NLR	NOD-like receptors
NMDA	<i>N</i> -methyl-D-aspartic acid
NMR	nuclear magnetic resonance
nNOS	neuronal nitric oxide synthase
NOD	nucleotide-binding oligomerization-domain
NOX	NADPH oxidase
Nrf2	nuclear factor erythroid 2–related factor 2
NSAIDs	nonsteroidal anti-inflammatory drugs
OGD	oxygen-glucose deprivation
(ox)LDL	(oxidized) low-density lipoprotein
PAL	phenylalanine ammonia-lyase
PAMPs	pathogen-associated molecular patterns
PARP	poly (ADP-ribose) polymerase
PD	Parkinson’s disease
PEG	polyethylene glycol
PGC-1α	PPAR γ coactivator 1 α
PGs	prostaglandins
PINK1	phosphatase and tensin homolog (PTEN)-induced kinase 1
PMA	phorbol myristate acetate
PPARγ	peroxisome proliferator-activated receptor gamma
PPP	pentose phosphate pathway
PRRs	pattern recognition receptor
PI3-K	phosphatidylinositol 3-kinase
RANKL	receptor activator of NF- κ B ligand

RES	reticuloendothelial system
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSV	resveratrol
(r)tPA	(recombinant) tissue plasminogen activator
SAPKs	stress-activated protein kinases
SBP	systolic blood pressure
SEAP	secreted embryonic alkaline phosphatase
SIRT-1	Sirtuin 1
SLPI	secretory leukoprotease inhibitor
SOD1/2	superoxide dismutase 1/2
SRs	scavenger receptors
STAIR	Stroke Therapy Academic Industry Roundtable
TAK1	transforming growth factor beta-activated kinase 1
TAL	tyrosine ammonia-lyase
TCR	T-cell receptor
TGFβ	transforming growth factor beta
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
TRAF6	tumor necrosis factor receptor (TNFR)-associated factor 6
TRPA1	transient receptor potential ankyrin 1
TRPV1	transient receptor potential vanilloid 1
TXA₂	thromboxane A ₂
UC	ulcerative colitis
VEGF	vascular endothelial factor
WHO	World Health Organization
Ym1	heparin-binding lectin

INTRODUCTION

Drastic changes in human ecology, including diet, physical activity, psycho-emotional stress, population density, and microbial exposure, have dramatically shifted the spectrum of human diseases over the past century. These modern human diseases seem to have two features in common: they involve disruption of homeostasis and they are nearly universally associated with inflammation, as well as with disturbances in its resolution.

The etiologies of inflammation can be of both infectious and non-infectious character. Microbial (bacterial, viral, or protozoal) infection may affect in general any weakened organ and tissue of the human body and trigger an inflammatory reaction with the aim to destroy a harmful agent, resolve the problem and restore homeostasis. However, non-resolved acute inflammation, as well as a long-term mild inflammatory reaction may become persistent, contributing to a variety of chronic diseases, such as atherosclerosis, cardiovascular diseases, diabetes mellitus type 2 (DM2), obesity, or even depression. Moreover, the ongoing systemic inflammatory state may contribute to the development of brain inflammation, called neuroinflammation. Neuroinflammation is a common feature of several chronic neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), or multiple sclerosis. Moreover, neuroinflammation also occurs in acute disorders of the central nervous system (CNS), such as traumatic brain injury and ischemic stroke, and takes part in neuronal tissue damage and death of the most important CNS cells – neurons.

Although the inflammatory response processes depend on the precise nature of the initial stimulus and its location in the body, they all share several common features, including the activation of monocytes and microglia representing the first line of defense and maintaining homeostasis in the organisms. At molecular level, toll-like receptor (TLR)-mediated activation of nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF- κ B) and mitogen-activated protein kinase (MAPK) inflammatory pathways leading to the expression of pro-inflammatory cytokines have been identified for numerous diseases with strong inflammatory component, including stroke.

Stroke represents the main cause of death after ischemic heart disease worldwide, and it is estimated to sustain by 2030. It is also one of the leading causes of disability what represents an enormous socioeconomic impact and burden for the human population.

Based on the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2017, the global number of new stroke events has increased by 75% from 6.8 million of new events in 1990 to 11.9 million in 2017 (Avan et al., 2019). The acute ischemic stroke that represents around 70–80% of cases is caused by decreased blood flow due to the brain artery occlusion and leads to the lack of supply of oxygen and glucose into ischemic area. Rapidly diffusing ischemic cascade triggers a complex of interconnected molecular mechanisms leading to the swift development of pathological signs with a relatively short treatment window. Prompt restoration of blood flow is essential for avoiding serious sequels. Despite quickly developing medical approaches, the statistics are not encouraging, and most people do not escape the detrimental effects of cerebral ischemia, one third of patients die and another third keeps the sequels for the rest of life. Therefore, several promising neuroprotective agents that could antagonize the deleterious events of ischemic stroke, promote recovery, or prevent a recurrent stroke have been identified at the preclinical level. However, all these molecules have failed in clinical trials for several reasons, such as poorly reproducible preclinical experiments or failure of the drugs to penetrate the blood-brain barrier (BBB). Thus, thrombolytic drug recombinant tissue plasminogen activator (rtPA) still remains the only Food and Drug Administration (FDA)-approved therapy.

On the other hand, statistics from the last years indicate that 50–90% of all stroke cases may be attributed to potentially modifiable risk factors, such as hypertension, cardiac causes, DM2, obesity, high cholesterol levels, and could be therefore reduced to certain degree by dietary interventions, smoking cessations, reduced alcohol intake, and elevated physical activity (Barnes, 2013; O'Donnell et al., 2016). Increasing scientific evidence emphasizes the health benefits of the Mediterranean diet (MedDiet) based on a modest intake of saturated fats, but rich on poly-unsaturated omega-3 and omega-6 fatty acids, vitamins, minerals, and phenolics, such as stilbenoids, and propose MedDiet as a pattern of balanced nutrition contributing to cardiovascular health and stroke prevention. Studies on humans underline the importance of plant nutrients and polyphenols in daily food intake (Lakkur and Judd, 2015; Paterson et al., 2018).

Stilbenoids, as natural plant polyphenols, are integral components of food (*e.g.* different edible berries, grapes, peanuts, etc.), beverages (white tea, red wine), and many other medicinal plants from Moraceae, Fabaceae, and Euphorbiaceae families. Stilbenoids

have been shown to be potent antioxidants and anti-inflammatory agents, displaying a plethora of biological effects, including cardioprotective and neuroprotective actions. This suggests that stilbenoids may represent an important component of the human diet preventing cardio- and cerebrovascular diseases, as well as neurodegenerative disorders. Therefore, stilbenoids could be of interest also as a supplementary treatment for numerous diseases where the inflammatory component plays an essential role in disease progression.

Macaranga siamensis S.J.Davies (Euphorbiaceae) is recently (2001) identified Thai plant species containing a variety of novel prenylated stilbenoids firstly described in 2015 and whose biological activity has been poorly investigated to date. Anyway, several *Macaranga* species have a long-standing ethnic history and found a large use among indigenous people of South Asia and islands of Pacific ocean. *Macaranga* spp. has been used also as anti-inflammatory agent for treatment of wounds, cuts, boils, swellings, and sores (Phommart et al., 2005). Therefore, *M. siamensis* with its unexplored content arouses a scientific interest and we tried to describe its potential health benefits in inflammatory diseases at both systemic and CNS level.

The work of my thesis stands at the interface of these three domains: **inflammation, neuropathology** (*e.g.* stroke), and **the role of stilbenoids in both**.

OBJECTIVES

This work has been developed in collaboration between the Czech Department of Molecular Pharmacy at Masaryk University in Brno, that deals with the anti-inflammatory potential of natural polyphenols, including stilbenoids and the French team of Dr. Catherine Heurteaux and Dr. Jean Mazella focusing on neuropeptides, brain diseases, including ischemic stroke and their therapeutics at IPMC CNRS, Valbonne, France. This dissertation thesis has an exploratory character and comprises four main objectives.

The major objective of the work realized in the Czech laboratory was to evaluate the anti-inflammatory potential of a set of 37 non-prenylated and prenylated stilbenoids against LPS-induced inflammation-like response of monocytes and macrophages *in vitro*. This first part focused on LPS-induced stimulation of TLR4 signaling leading to activation of I κ B/NF- κ B and MAPK/AP-1 pathways. These two pathways have been identified to be activated in numerous acute and chronic inflammatory diseases at the both systemic and CNS levels. Their activation leads to the expression of several inflammatory cytokines. The intervention of selected stilbenoids into NF- κ B/AP-1 signaling was assessed together with their up-stream signalization and down-stream effect on transcription of pro-inflammatory cytokines IL-1 β and TNF- α . The aim was to compare the effects of selected stilbenes with the marketed corticoid drug prednisone and the most investigated stilbenoid *trans*-resveratrol, as well as select the most promising agents for further analysis on *in vitro* and *in vivo* models of particular inflammatory diseases.

This preliminary analysis allowed us to identify the most promising agent – recently (2015) discovered and very low investigated prenylated stilbene macasiamenene F (MF) originated from plant *Macaranga siamensis* S.J.Davies (Euphorbiaceae). This stilbene was investigated in the second part of the project developed in the French laboratory IPMC CNRS within the frame of my joined doctoral studies.

The goal of the second project part was to determine if MF could exert the antiphlogistic effects also at CNS level and possibly find usefulness in the treatment of brain disorders accompanied by a strong inflammatory component. The microglial activation mediated through TLR4 signaling is a hallmark of brain inflammation in several acute and chronic CNS disorders, including ischemic stroke and AD, respectively.

Therefore we firstly aimed to evaluate the effect of MF on LPS-challenged microglia using a set of *in vitro* and *ex vivo* of experiments.

The third aim of this work was to evaluate the influence of MF on neuroprotection against ischemic stroke. The ischemic cascade that develops in penumbra after vessel occlusion is characterized not only by neuroinflammation but also by the excitotoxic state and oxidative stress triggered in the first minutes and hours after stroke event. Therefore we aimed to evaluate also the antioxidant effects of MF on AAPH-challenged BV-2 microglia. The potential of MF to protect against *N*-methyl-D-aspartic acid (NMDA)-induced excitotoxic cell death was evaluated on the model of primary cortical neurons. Altogether, to observe the overall protective effects of MF against ischemic stroke, we optimized *in vitro* model of stroke – model of primary cortical neurons exposed to 1 h/2 h of oxygen-glucose deprivation (OGD).

Finally, in order to resolve a poor water solubility of MF for possible *in vivo* administration and find a way to effectively deliver MF to the brain, the fourth objective of this work was focused on the development of safe MF liposomal formulations. After verifying their safety and confirmation of their effectiveness, this project should proceed with *in vivo* experiments of MF on mouse MCAo model.

THEORETICAL PART

1. INFLAMMATION

Inflammation represents an adaptive response of body tissues to the perturbations in homeostasis, such as infection, injury, tissue stress, and exposure to contaminants. Under physiological conditions, this reaction is beneficial, well-coordinated, and self-regulated and flows into tissue repair. However, when the inflammatory response turns uncontrolled, deregulated, and prolonged, inflammation may develop chronic and lead to severe pathologies (Kotas and Medzhitov, 2015). The inflammatory reaction is a complex system of immunological and physiological events that are coordinated by signaling molecules, such as cytokines, chemokines, biogenic amines, and eicosanoids (Bianchi, 2007). The molecular and cellular response to inflammation is mediated by innate immune receptors recognizing pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) with the aim to recruit immune cells and destroy the harmful agents. This manifests by physiological changes that may vary from local vascular responses to the increase in body temperature. We distinguish five cardinal signs of inflammation: heat (calor), pain (dolor), redness (rubor), swelling (tumor), and loss of function (function laesa). Whereas acute inflammation is often the consequence of microbial infection or abrupt tissue injury, chronic inflammation appears to be associated with tissue or organ malfunction and homeostatic disbalance of a physiological mechanism (Fig. 1; Medzhitov, 2008).

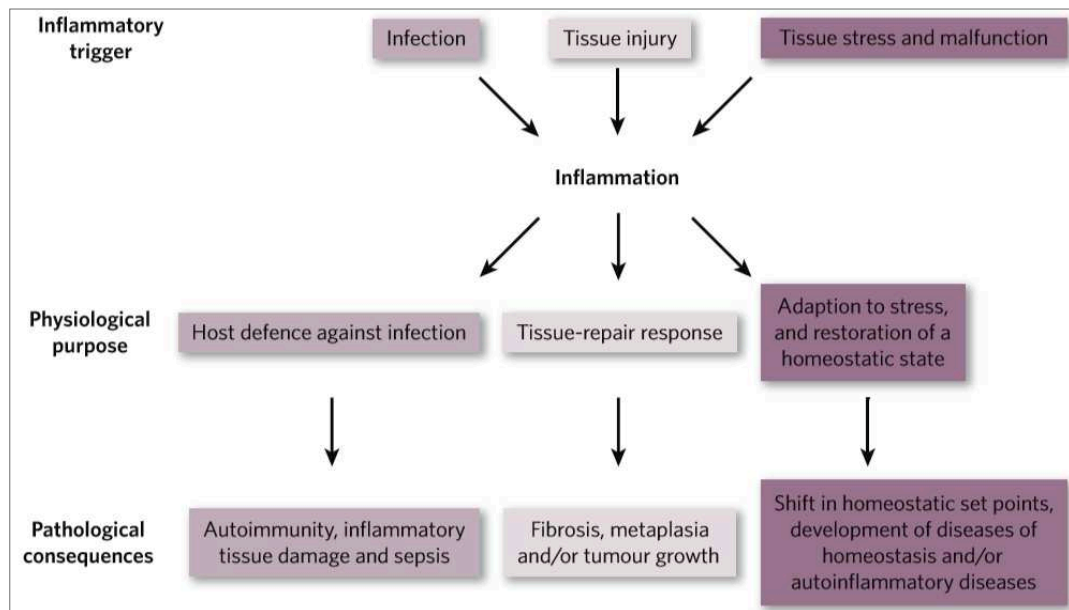


Fig. 1: Causes, physiological and pathological outcomes of inflammation from (Medzhitov, 2008)

1.1 Acute inflammation

The acute inflammatory response initiated by pathogen or tissue injury comprises the coordinated delivery of leukocytes to the site of action. This response is characteristic for microbial infections (particularly bacterial) that are recognized by the innate immune system receptors of tissue-resident macrophages and mast cells, such as Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs). These immune cells further produce a myriad of inflammatory mediators, including chemokines, cytokines, eicosanoids, and vasoactive amines to attract other cells of the immune system to the infected area, starting with neutrophils (Chen et al., 2017). Due to selective molecules on the endothelium of blood vessels, such as selectins and integrins being recognized by neutrophil chemokine receptors, neutrophils are able to selectively pass from vessels to the extravascular tissue. By direct contact with a pathogen or through the action of cytokines secreted by tissue-resident cells, they become activated. Then, they are ready to destroy the invading pathogens by releasing the toxic contents from their granules, including reactive oxygen and nitrogen species (ROS and RNS), elastase, proteinase 3, and cathepsin G. Unfortunately, these highly effective agents do not distinguish between microbial and host targets, so collateral damage of the host tissues is unavoidable (Nathan, 2006). Other important agents capable of phagocytosis and participating in the elimination the harmful elements are macrophages. Together with neutrophils, they play an inevitable role in the early-phase of inflammation and call other agents of adaptive immunity to help them in the resolution of inflammation. The favorable response to acute inflammation consists in the elimination of infectious agents, resolution of the inflammatory state beginning in general in the first few hours after the inflammation onset and initiation of tissue repair that is provided essentially by tissue-resident macrophages (Serhan and Savill, 2005).

Depending on the type of microorganisms and affected organs, the acute inflammation may manifest at both CNS and systemic levels in different forms, such as meningitis or encephalitis (Selim et al., 2007; Tunkel and Scheld, 1993), bronchitis and pneumonia (Park et al., 2016; Sattar and Sharma, 2020), myocarditis and endocarditis (Haddad et al., 2007; Vilcant and Hai, 2020), hepatitis A–E (Manka et al., 2016), cystitis (McLellan and Hunstad, 2016), etc. The most common are bacterial infections caused by gram-positive or gram-negative bacteria. Gram-negative bacteria (e.g. *Klebsiella*

pneumoniae, *Pseudomonas aeruginosa*, *Yersinia pestis*, etc.) are considered to be more pathogenic and more resistant to antibiotics because of the different compositions of the cell envelope. Unlike Gram-positive bacteria having a thick layer of peptidoglycan on their plasma membrane, Gram-negative bacteria possess on the thin layer of peptidoglycan an additional outer membrane with the endotoxin LPS, responsible for their pathogenic potential and massive clinical manifestations in humans (Wang and Quinn, 2010). Therefore, Gram-negative bacteria have often been implicated in the pathogenesis of severe sepsis and septic shock (Alexandraki and Palacio, 2010). Moreover, highly elevated peripheral concentrations of circulating LPS may disrupt the blood-brain barrier (BBB) and promote inflammation at CNS level, so-called neuroinflammation (Banks et al., 2015).

LPS alone consists from several components, including lipid A (called endotoxin), a non-repeating "core" oligosaccharide, and a distal polysaccharide, referred also as O-antigen (Fig. 2). Lipid A has been described as toxic even after bacterial lysis by immune cells. In animal and human cells, lipid A is recognized by the plasma membrane protein TLR4 (Raetz and Whitfield, 2002).

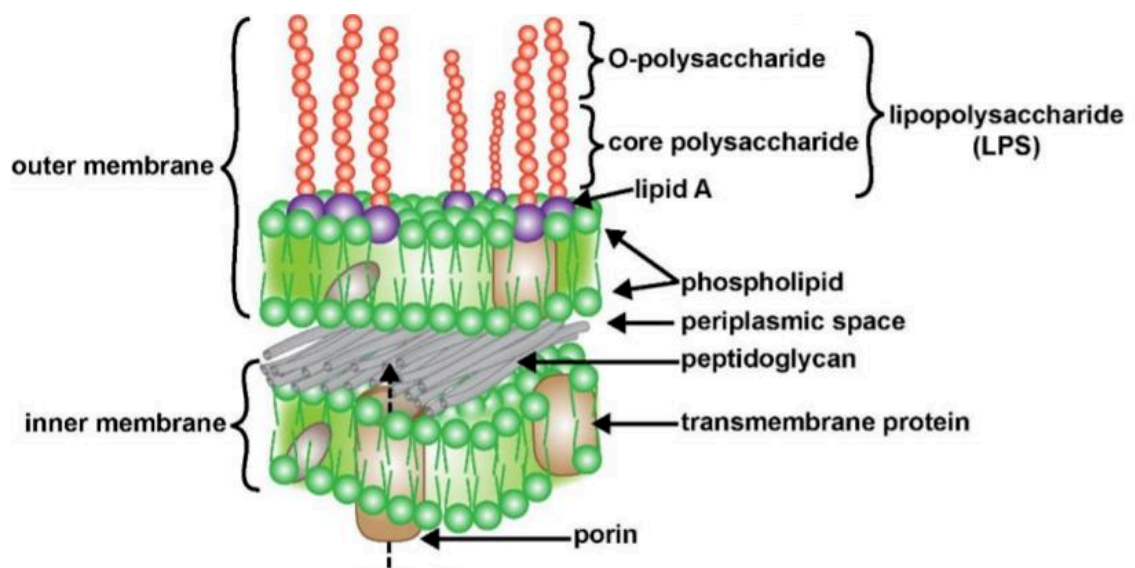


Fig. 2: Typical structure of anaerobic Gram-negative bacterial cell membrane with LPS-coated surface from (Alexandrov et al., 2020).

1.2 Chronic inflammation

The basal state of cells may alter in the response to changes in their environment and switch to stressed, necrotic, or apoptotic program. The tissue-resident macrophages monitor rigorously the environment in an effort to maintain this basal state and respond immediately to any alterations with the aim to eliminate the homeostasis disrupters and restore the initial conditions. Whereas the transition of answers within cells occurs mostly in a switch-like (or all-or-none) manner that prevents the generation of mixed messages, the state of tissue may grade in time. For instance, tissue containing a different number of dead cells may develop a malfunction to different degrees. This tissue malfunction may further induce para-inflammation (low-grade form of inflammation) in order to restore homeostasis and its functionality (Aran et al., 2016; Fulda et al., 2010). Nevertheless, when this malfunction sustains, para-inflammation may turn into inflammation or become chronic in the case of a prolonged exposure (Chen and Xu, 2015; Xu et al., 2009).

In general, chronic inflammation is referred to as slow, long-term inflammation lasting for several months to years that may result from failure of eliminating the agent causing an acute inflammation, recurrent episodes of acute inflammation, an autoimmune disorder, or prolonged exposure to a low level of a particular irritant (Pahwa et al., 2020). Nowadays, several chronic inflammation-associated diseases linked with conditions such as continuous intake of high-calories nutrients and a low level of physical activity, exposure to toxic compounds, allergens, stress, or aging. Diseases linked with such conditions – obesity, DM2, atherosclerosis, asthma, chronic respiratory diseases, heart diseases, stroke, neurodegenerative disorders, or even cancer – are accompanied by chronic low-grade inflammation. The World Health Organization (WHO) ranks chronic inflammatory diseases as the greatest threat of human health. Worldwide, 3 of 5 people die due to above-mentioned chronic inflammatory diseases. Moreover, persisting chronic inflammation can contribute to further progression of the disease and cause for example insulin resistance, or development of metabolic syndrome (Kotas and Medzhitov, 2015; Medzhitov, 2008). This may further trigger the cascade of associated pathologies, such as hypertension, acute cardio- and cerebrovascular diseases. Moreover, ongoing systemic inflammation of the kind observed in metabolic diseases such as DM2 or obesity can lead to the chronic state of neuroinflammation and ultimately result in neuronal cell death (Nicolas et al., 2017; Skaper et al., 2018)

1.3 Monocytes / macrophages

Monocytes are important cells of the innate immune system representing approximately 5–12% of the total leucocyte population depending on the adaptive immune system response. Their major role consists of the host defense against infection and invading pathogens, but also in the activation of adaptive immune system response (Karlmark et al., 2012). Monocytes are derived from a common myeloid progenitor in bone marrow in the presence of CCR2 chemokine receptor that is necessary for their exit to the peripheral blood (Serbina and Pamer, 2006). Once released, they circulate in the blood for several days before they enter specific tissue and differentiate to macrophages. Circulating monocytes give rise not only to a variety of tissue-resident macrophages throughout the body (*e.g.* alveolar macrophages), but also to dendritic cells (DCs, *e.g.* Langerhans cells in the skin), and osteoclasts (Fogg et al., 2006). The CNS also contains various macrophage subsets, including meningeal, perivascular and choroid-plexus macrophages. Although CNS-resident microglia have a different origin, bone marrow-derived monocytes are able to enter the CNS and differentiate into microglia as well (Perry and Teeling, 2013).

Morphologically, monocytes possess an amoeboid shape with unilobar nuclei and non- or low-granulated cytoplasm which classifies them to the subgroup of agranulocytes. Their role consists in phagocytosis of microbes or foreign particles, their digestion, and destruction. This microbe uptake can be direct or indirect through the opsonization of pathogen by specific antibodies or parts of complement. After their digestion, fragments of microbial agents can be trafficked and presented on the cell surface. This second major role of macrophages is called the “antigen-presentation”. They are further important producers of cytokines, such as tumor necrosis factor alpha (TNF- α), interleukin 1 (IL-1), or interleukin 6 (IL-6). Approximately half of monocytes is stored as a reserve in the spleen. During inflammation, the circulating monocytes are recruited to the affected area by different chemokines such as CCL2 (also known as MCP-1, monocytes chemoattractant protein), CCL7 (also known as monocyte-chemotactic protein 3), leukotriene B₄, etc. The activated monocytes accumulate in the inflamed tissue and together with tissue-resident macrophages shift to pro-inflammatory M1 phenotype producing the pro-inflammatory cytokines and iNOS (Karlmark et al., 2012).

Monocytes were originally identified by their large expression of CD14 cell surface marker that is a part of the receptor for lipopolysaccharide. However, a different expression of other antigenic intra- and extracellular markers has shown their heterogeneity. This feature influences their migration patterns and different roles during homeostasis and inflammation (Geissmann et al., 2003). According to differential expression of CD14 and CD16 (also known as Fc γ RIII) surface molecules, two main subsets of monocytes are distinguishable, called as classical and non-classical. Recently, a third subset referred to as an intermediate was identified as well. Classical CD14⁺⁺CD16⁻ monocytes (expressing CD14 but not CD16) represent around 80–95% of circulating monocytes, their role consists mostly in phagocytosis of foreign pathogens. Intermediate CD14⁺⁺CD16⁺ monocytes express both CD14 and CD16, their role is pro-inflammatory and contribute to some pathologies, such as atherosclerosis. Last, non-classical monocytes referred as CD14⁺CD16⁺⁺ cells, express high CD16 and low CD14, their role consist in patrolling of the environment (Pedraza-Sánchez et al., 2013; Yang et al., 2014). In mice, these similar subsets are referred to as Ly6C^{hi}, Ly6C^{mid} and Ly6C^{low} cells (Table 1). The heterogeneity in the expression of antigen markers among human, mouse, and rat species was mentioned in Karlmark et al. (Karlmark et al., 2012).

Monocyte subset	Chemokine receptors	Corresponding ligands	Functions
<i>Classical monocytes</i> Mouse: Ly6C ^{hi} Human: CD14 ⁺⁺ CD16 ⁻	CCR1 CCR2 CXCR2	MCP-2, MIP-1 α (CCL3), CCL5 MCP-1 (CCL2), MCP-3 (CCL7), MCP-5 CXCL1, CXCL2, CXCL3	Phagocytosis
<i>Non-classical monocytes</i> Mouse: Ly6C ^{low} Human: CD14 ⁺ CD16 ⁺⁺	CX3CR1 CCR5 CCR6	CX3CL1 (Fractalkine) CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 CCL20 (MIP-3 α)	Patrolling and tissue repair
<i>Intermediate monocytes</i> Mouse: Ly6C ^{mid} Human: CD14 ⁺⁺ CD16 ⁺	CCR2 ^{high} CX3CR1 ^{low} CCR2 ^{mid} CX3CR1 ^{high} CCR5	MCP-1 (CCL2), MCP-3 (CCL7), MCP-5 MCP-1 (CCL2), MCP-3 (CCL7), MCP-5 CX3CL1 CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5	Pro-inflammatory

Table 1: Monocyte associate chemokines and chemokine receptors (adapted from Karlmark et al., 2012; Yang et al., 2014)

Before undertaking an appropriate action, macrophages have to decipher if cells to be removed are died by necrosis or apoptotic mechanisms through detection of phosphatidylserine at the surface of these cells. According to this, they further decide about the silent removal of dead cells or continuation of the inflammatory response. Apart from the first-line defense against pathogens, macrophages play a crucial role in tissue repair, the final phase of favorable inflammatory response. After the destruction of harmful agents, granulocytes promote the switch in lipid mediators from pro-inflammatory arachidonic acid-derived prostaglandins (PGs) and leukotrienes (LTs) to molecules called lipoxins. This step is pivotal for the transition from inflammation to resolution. Lipoxins are endogenous anti-inflammatory and pro-resolving molecules that cease the recruitment of new neutrophils to sites of inflammation and reperfusion injury (Chandrasekharan and Sharma-Walia, 2015). Simultaneously ongoing biosynthesis of pro-resolution signaling molecules resolvins and protectins contribute to regulation of critical cellular events during the resolution phase. Resolvins and protectins are generated from omega-3 unsaturated fatty eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively. Their activity stimulates the initiation of a programmed death by apoptosis (Serhan and Savill, 2005). Lipoxins further reduce vascular permeability and promote the non-phlogistic infiltration of monocytes. The activated macrophages then ingest and clear apoptotic neutrophils, produce anti-inflammatory and reparative cytokines, such as transforming growth factor-beta 1 (TGF- β 1), and initiate tissue remodeling (Levy et al., 2001). The anti-inflammatory program ends up when macrophages come out from the tissue through the lymphatics (Serhan and Savill, 2005).

In the case of sustained or excessive response, they can become maladaptive and it may lead to the development of chronic disorders. An example is obesity-induced insulin resistance accompanied by macrophage accumulation and inflammation in adipose tissue (Shimobayashi et al., 2018). Interestingly, macrophages, as well as brain microglia assisting the adaptation of tissues to stress conditions may be “misused” by tumor cells, which can recruit and use them as a source of growth factors, angiogenic factors, and chemokines. This points out a role of macrophages and microglia also in tumorigenesis and metastasis (Wu and Watabe, 2017).

1.4 Microglia

Although there are also other macrophages in the CNS, microglia represent the greatest part of this cell population. Microglia are the CNS resident mononuclear phagocytes, widely distributed throughout the brain and the spinal cord. Their number represents approximately 10–15% of the total cell population of CNS, depending on the adaptive immune system (Bazan et al., 2012; Carson et al., 2006). Morphological appearance can vary across regions (Harry and Kraft, 2012) and change of shape occurs also after the activation of microglia by inflammatory stimuli. According to the shape, microglia have been classified into three types: amoeboid, ramified, and reactive. Amoeboid microglia have been found in the developing brain, their large spherical cell body and short processes are similar to reactive microglial cells. During the postnatal stages, the amoeboid microglia transform into ramified resting microglia what is a relatively permanent population in the adult CNS. Upon stimulation, the ramified microglia undergo a set of morphological and functional changes in order to mobilize the cellular and molecular defense system of the CNS (Dheen et al., 2007).

Unlike peripheral tissue macrophages, microglia are derived from yolk sac myeloid progenitors in the brain parenchyma during embryonic development (Ginhoux et al., 2010). Microglia actively monitor the brain microenvironment. Their adequate function is pivotal for the homeostasis of the CNS in both health and disease. Resting microglia also participate in maintaining the neurovascular integrity. In addition to their immune functions, emerging data show their role in the control of neuronal proliferation and differentiation, as well as in the formation of synaptic connections. Upon activation by inflammatory stimuli, they mobilize the cellular and molecular CNS defense system and aim to eliminate the harmful elements (Chen et al., 2016; Dheen et al., 2007; Ginhoux et al., 2013). Activation of microglia is a hallmark of brain pathology. Major functions of activated microglia, similarly to monocytes, are phagocytosis and presenting the antigen on their surface (Dheen et al., 2007). Dysregulated microglial activation and microglia-induced inflammation have been observed in most of the brain pathologies suggesting that microglia may exert direct effects on neurons, and thus contribute to disease progression (Perry et al., 2010).

In order to maintain tissue homeostasis, microglia acquire a diversity of phenotypes and shift their functions according to the local environment regulated particularly by neurons and astrocytes *via* interactions with both soluble and membrane-bound mediators, such as chemokine CX3CL1, neurotransmitters, neurotrophins, or CD22. Microglia receive these signals by a variety of receptors (CX3CL1R, CD45R, CSF1R, CD200R, etc.) expressed on their surface (Orihuela et al., 2016; Ransohoff and Cardona, 2010). Neurotropic factors released by neurons contribute to the quiescent state of microglia and dampen inflammation in the CNS (Perry and Teeling, 2013).

We distinguish two main microglia phenotypes – **pro-inflammatory M1 phenotype** which is activated by bacterial LPS, endogenous cytokines like interferon gamma (IFN- γ), TNF- α , or by the presence of DAMPs and PAMPs (Fig. 3) (Kiernan et al., 2016). Microglia recognize noxious stimuli using receptors, such as TLRs, NLRs, and scavenger receptors (SRs) (Ransohoff and Brown, 2012). M1-activated microglia express a variety of specific receptors (CD16, CD32, CD86), present antigens, produce pro-inflammatory cytokines (TNF- α , IL-1 α , IL-1 β , IL-6, IL-12, IL-23), redox molecules (inducible nitric oxide synthase, iNOS; nicotinamide adenine dinucleotide phosphate oxidase, NADPH oxidase), chemokines (CCL2, CCL5, CCL20, CXCL1, CXCL9, CXCL10), major histocompatibility complex II (MHC II), and others (Orihuela et al., 2016; Salvi et al., 2017). It is necessary to mention that microglia share many phenotypic characteristics with peripheral macrophages and produce similar M1 associated factors (Orihuela et al., 2016).

The shift to the **anti-inflammatory M2 microglial phenotype** is associated with the resolution of inflammation and tissue repair. Different cellular markers were identified as ‘M2’ specific. The enzyme arginase 1 (Arg1) is one of the best characterized. Arg1 converts arginine to proline and ornithine which can contribute to wound healing and matrix deposition (Munder, 2009). Interestingly, while M1 microglia use the same substrate arginine for the production of nitric oxide (NO), M2 microglia can effectively outcompete iNOS to downregulate NO production (Morris, 2007). Other M2 markers include a heparin-binding lectin Ym1, resistin-like molecule alpha1 (FIZZ1), and a mannose receptor CD206 (Fig. 3) (Hung et al., 2002; Stahl and Ezekowitz, 1998). This alternative microglial activation classified as ‘M2a’ is induced by IL-4 and IL-13 through IL-4R α and triggers downstream *e.g.* Arg1 upregulation, inhibition of NF- κ B, and

production of scavenger receptors for phagocytosis (Cherry et al., 2014; Martinez et al., 2008). The second state of alternative activation classified as ‘M2c’ is based on macrophages exposed to IL-10, TGF- β , or glucocorticoids (Martinez et al., 2008). A third sub-class of M2 activation was observed following exposure to the immune complexes and stimulation of TLR. This class termed as ‘M2b more resembles to M1 macrophages, because of the lack of any M2 specific markers, such as Arg1, Ym1, or FIZZ1, but presents higher levels of MHCII and CD86. However, M2b microglia express some typical M2 markers, such as IL-10 and IL-12.

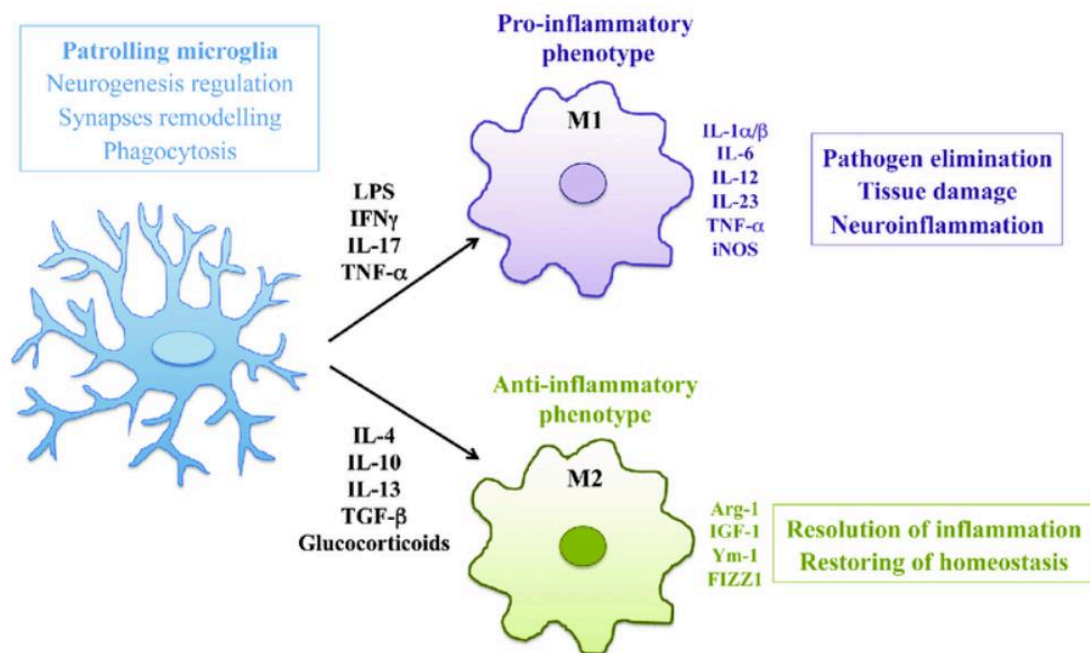


Fig. 3: Microglial activation and polarization during neuroinflammation according to the local environment in CNS from (Salvi et al., 2017).

Additionally, microglia express diverse receptors for neurotransmitters, such as norepinephrine (NE) and γ -aminobutyric acid (GABA) possessing immunomodulatory effects and being able to suppress the production of pro-inflammatory cytokines. For instance, microglia and astrocytes being in intimate communication, express GABA_A and GABA_B receptors. The activation of these receptors dampens LPS or IFN- γ -mediated inflammation by inhibition of NF- κ B and p38 MAPK (Perry and Teeling, 2013).

1.5 Inflammatory mediators

Inflammatory mediators are molecules responsible for a signal transduction during inflammation. According to their different chemical features and biological functions, we distinguish several groups of cytokines, chemokines, vasoactive amines and peptides, components of the complement, proteolytic enzymes, and lipid mediators (Medzhitov, 2008).

First, the most known group of lipid mediators includes eicosanoids and platelet-activating factors. They are derived from cell membrane phospholipids, such as phosphatidylcholine. The cascade begins with intracellular Ca^{2+} ions influx that activates cytosolic phospholipase A_2 (cPLA₂) (Gijón and Leslie, 1999), thus promoting the generation of lysophosphatidic and arachidonic acid (AA) (Fig. 4). AA is then metabolized by cyclooxygenases 1 and 2 (COX-1 and COX-2), which generate eicosanoids prostaglandins (PGs), prostacyclins, and thromboxanes (TXAs). The main prostaglandins PGE_2 and PGI_2 contribute to vasodilation, and PGE_2 alone is also a potent algesic (causing pain) agent. AA can be also processed by lipoxygenases (LOXs), what leads to the generation of leukotrienes (LTs) and lipoxins. Whereas LTs are majorly involved in allergy and asthma, anti-COX-1 and anti-COX-2 agents have been largely implemented in the treatment of pain and several acute and chronic inflammatory diseases that will be detailed later (Bennett and Gilroy, 2016).

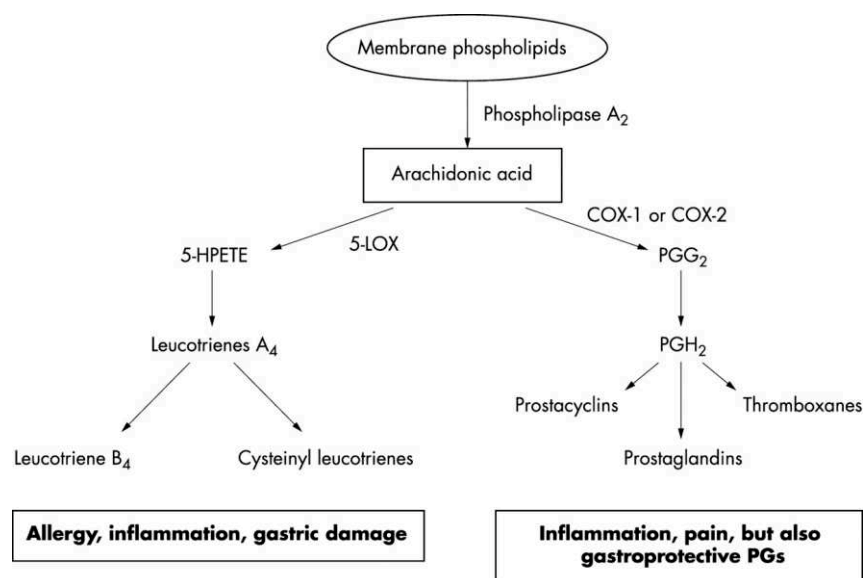


Fig. 4: COX and LOX signaling from (Martel-Pelletier et al., 2003). 5-LOX - lipoxygenase 5; 5-HPETE - 5-hydroperoxyeicosatetraenoic acid; COX-1/2 – cyclooxygenases 1/2; PGG₂ and PGH₂- prostaglandins G₂ and H₂.

Platelet-activating factors, another subgroup of lipid mediators, are produced from lysophosphatidic acid. They are responsible for activation of several processes of inflammatory response, such as platelet activation, modulation of vascular permeability, vasodilation and vasoconstriction (depending on the context), and recruitment of leukocytes (Camussi et al., 1990).

Cytokines, the second group of inflammatory mediators secreted by several types of cells participating in inflammation, primarily by macrophages and mast cells, have also context-dependent role. Whereas proinflammatory cytokines (IL-1 β , IL-6, TNF- α , IFN- γ , etc.) alert the immune system to the presence of potential danger, anti-inflammatory cytokines (IL-1 receptor antagonist, IL-1RA; IL-4; IL-10; TGF- β) favor antibody responses. Their dysregulated production may lead to immunopathology. They have several roles, including the induction of acute “alarming” phase inflammation, activation of endothelium, and leukocytes. The responsiveness to the major pro-inflammatory cytokines IL-1 β and TNF- α , participating to a large extent in both systemic and neuroinflammation is ubiquitous and induces the expression of other inflammatory cytokines, like IL-6 and chemotactic cytokines (chemokines) (Le Thuc et al., 2015).

The third group is represented by chemokines, playing a role of “chemical guides” in cellular communication and recruitment of immune cells, including neutrophils and macrophages towards their producing source (Moser and Willimann, 2004). Over 50 chemokines were divided into 4 families of CXC, CC, CX3C, and C family according to their structures. Chemokines contribute to physiological brain functions maintaining the crosstalk among neurons, glia, and peripheral immune cells, as well as to controlled and adapted inflammatory response (Le Thuc et al., 2015). In the case of prolonged or inadequate response, their contribution in neuroinflammation becomes harmful and promotes tissue injury from two main reasons: 1/ chemokines drive the infiltration of the brain parenchyma and 2/ increased levels of chemokines themselves are neurotoxic (Conductier et al., 2010; Le Thuc et al., 2015). Le Thuc et al., 2015, reviewed the role of the most studied chemokines CX3CL1, CCL2 (MCP-1), and CXCL12 participating in the inflammatory response in acute and chronic CNS diseases, such as stroke and AD, respectively. Several chemokines from CXC and CC families are also involved in distinct stages of wound healing process, such as hemostasis, proliferation, and remodeling (Ridiandries et al., 2018).

The fourth group of vasoactive amines includes histamine and serotonin that are produced by mast cells, basophils, or from platelets degranulation. Both histamine and serotonin increase vascular permeability, dilate arterioles and capillaries, contract nonvascular smooth muscles. In extreme situations, their massive release can be largely pernicious causing the extent vascular dilatation and respiratory collapse, such as the effect of histamine in the anaphylactic shock requiring immediate medical intervention (Abdulkhaleq et al., 2018; Branco et al., 2018).

Fifth, vasoactive peptides can be either maintained in their active form and warehoused in the secretory vesicles (*e.g.* substance P) or produced from inactive precursors *via* proteolysis what is the case of kinins, fibrin degradation products, or fibrinopeptides A and B. Substance P is the product of sensory neurons and inflammatory cells, such as macrophages, eosinophils, or dendritic cells. While substance P actively participates in inflammatory diseases of the respiratory, gastrointestinal, and musculoskeletal systems (O'Connor et al., 2004), other vasoactive peptides, such as fibrin degradation products are generated in the proteolytic reaction starting with the coagulation factor XII (Hageman factor), thrombin or plasmin in the coagulation cascade, cause vasodilation and increase vascular permeability. Moreover, Hageman factor activates the kallikrein-kinin cascade that leads to the production of bradykinin causing pain, one of the main physiological sign of inflammation (Jennewein et al., 2011; Medzhitov, 2008).

Sixth important mediators are the complement components C3a, C4a, and C5a, referred also as anaphylatoxins. All of them, but mostly C5a induce granulocyte and monocyte recruitment, trigger mast-cell degranulation leading to the release of histamine and thereby affect the vasculature (Ricklin and Lambris, 2013).

The last group consists of proteolytic enzymes, such as elastin or matrix metalloproteinases (MMPs), degrades the extracellular matrix and membrane proteins. They are involved in a series of reactions, including host defense, tissue remodeling, and leukocyte migration. In conclusion, we point out that inflammation is a highly complex process involving the cooperation of many immune cells and cellular mediators. Therefore, it would be interesting to understand completely the logic and hierarchy of individual inflammatory mediators (Nissinen and Kähäri, 2014).

1.6 TLR4 activation of NF- κ B/AP-1 signaling

TLR4 is a 95 kDa transmembrane protein, member of TLR family, belonging to a bigger family of pattern recognition receptors (PRRs). Cells of innate immune system use PRRs as sensors detecting molecules of “uninvited guests” - pathogens. They are typically expressed by monocytes, macrophages, dendritic cells, or neutrophils enabling them to identify two main classes of possibly dangerous molecules: DAMPs and PAMPs. The expression of TLR4 protein referred also as cluster of differentiation 284 (CD284), is encoded by the TLR4 gene having a locus on human chromosome 9. TLR4 is an indispensable receptor for LPS, but also several viral proteins, low-density lipoproteins (LDLs), and heat-shock proteins belong to its ligands. Stimulation of TLR4 leads to the activation of NF- κ B/AP-1 signaling which is essential for multiple immunological transcriptional programs. The inflammatory responses to microbes are predominantly managed by the innate immune cells through the NF- κ B canonical pathway (Medzhitov and Horng, 2009), while the activation of adaptive immune cells is triggered *via* non-canonical (alternative) signaling (Kaileh and Sen, 2012). Both pathways seem to be involved in the development of secondary lymphoid organs and tissues, such as lymph nodes and Peyer’s patches (Alcamo et al., 2002; Cildir et al., 2016).

The canonical NF- κ B pathway responds to diverse stimuli, including ligands of mentioned PRRs and TLR4, but also to ligands of TNF receptor (TNFR) family members, T-cell receptor (TCR) and B-cell receptor (BCR). We focused essentially on the activation of TLR4 by LPS that leads to the formation of the complex between LPS binding pocket and secreted glycoprotein MD-2 *via* an extracellular domain of TLR4. The presence of TLR4 co-receptor CD14 is important for the recognition of LPS. The interaction among CD14, TLR4, and MD2 further induces myeloid differentiation 88 (MyD88)-dependent signaling from the cell surface, which activates the IL-1 receptor-associated kinases (IRAKs) 1, 2 and 4. Then, the signal is transmitted through TNF receptor-associated factor 6 (TRAF6), TGF- β -activated kinase 1 (TAK1) and further divide between two separate pathways: I κ B/NF- κ B and MAPK/AP-1 signaling (Fig. 5).

The main role of I κ B in the I κ B/NF- κ B complex is to inhibit the activation of NF- κ B and prevent its translocation to the nucleus. I κ B is upstream regulated by the inhibitory κ B kinase (IKK) complex which consists of three subunits. IKK- γ has mostly regulatory

function, while IKK- α and IKK- β subunits are catalytically active. Upon stimulation, the IKK β phosphorylates I κ B α at Ser32 and Ser36 residues. This phosphorylation triggers the polyubiquitination and proteasome-mediated degradation of I κ B proteins, resulting in the translocation of activated NF- κ B from the cytoplasm to the nucleus, its DNA binding, and induction of NF- κ B-dependent gene transcription, including transcription of pro-inflammatory cytokines TNF- α , IL-1, and IL-6 (Gamble et al., 2012). Although the degradation of I κ B is the main step in NF- κ B activation, multiple mechanisms are critical in the regulation of NF- κ B transcriptional activity, such as ubiquitination, sumoylation, or phosphorylation of upstream and downstream mediators, including NF- κ B proteins themselves (Pasparakis et al., 2006). Worthy to mention that NF- κ B regulates also genes involved in the regulation of ROS in cell and ROS have various inhibitory or stimulatory roles in NF- κ B signaling (Morgan and Liu, 2011). The canonical pathway activates NF- κ B heterodimers, predominantly the p50/p65 (RelA) and p50/c-Rel dimers. RelA is a critical effector liable for transcription of proinflammatory proteins and survival genes (Lawrence, 2009; Liu et al., 2017).

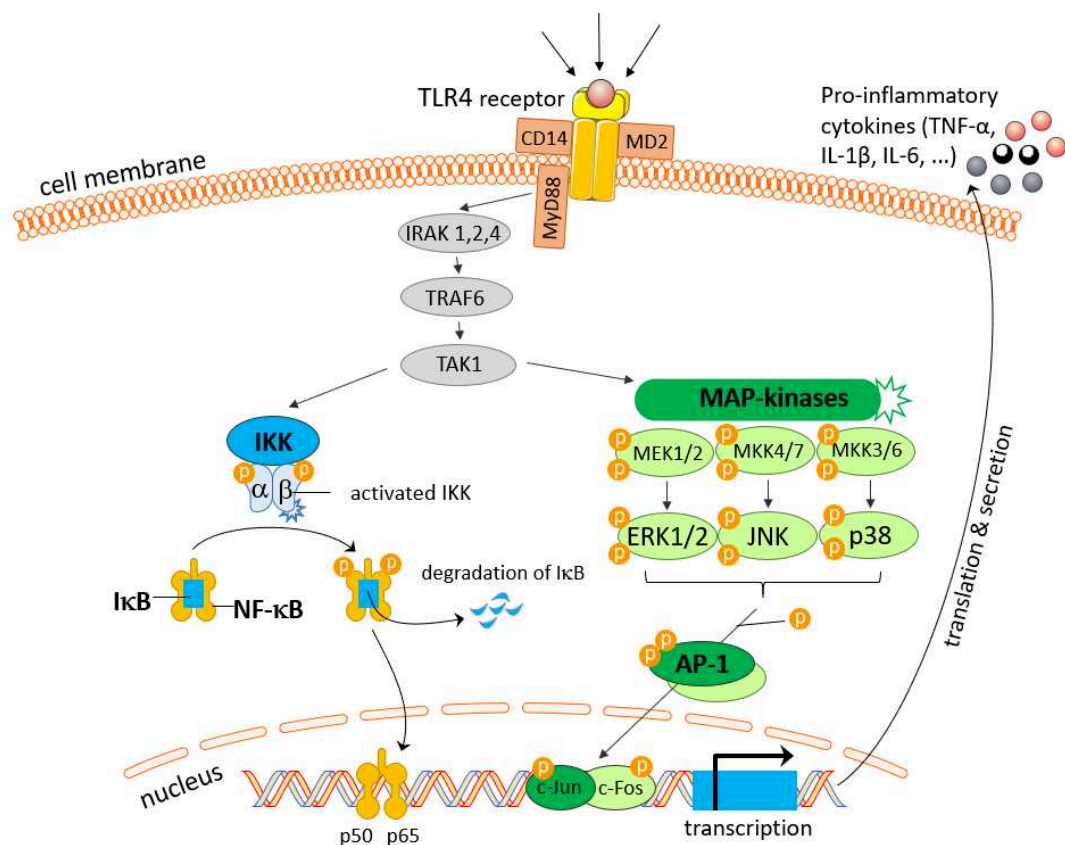


Fig. 5: LPS-induced TLR4 activation of NF- κ B/AP-1 signaling pathways. LPS stimulation of TLR4 receptors leads to the activation of IKK/NF- κ B and MAPKs/AP-1 pathways, and further transcription of pro-inflammatory cytokines, including TNF- α , IL-1 β and IL-6.

The “non-canonical” NF- κ B signaling is triggered upon stimulation of the tumor necrosis factor superfamily receptors (TNFRs), such as CD40, receptor activator of NF- κ B ligand (RANKL), B-cells activating factor (BAFF), but does not respond to TNF- α ligand. The stimulation of the non-canonical pathway leads to the activation of p52/RelB dimers of NF- κ B. This pathway is generally slower and longer-lasting, liable for the regulation of lymph-organogenesis and B-cells activation (Lawrence, 2009; Sun, 2011).

To understand the NF- κ B function and its activation in various pathologies, different cell models such as THP1-XBlueTM-MD2-CD14 cells and transgenic mice were prepared. The gene knockout of Rel/NF- κ B signal transduction pathway in mice includes several options, such as knockout of the genes encoding the transcription factors (Rel/NF- κ B), inhibitors (I κ B), and kinases (IKK) (Pasparakis et al., 2006). From genetic experiments in mice, it is clear that NF- κ B activation has complex roles in the inflammatory response and does not have to be necessarily proinflammatory. Lawrence et al., 2001, suggested in their study that NF- κ B could have a dual role and be involved in both the acute inflammation onset and its resolution *via* regulation of anti-inflammatory genes, such as IL-10 and induction of leukocyte apoptosis (Lawrence et al., 2001; Lawrence and Fong, 2010).

When focusing on another branch of TLR4 signaling (Fig. 5), the activation of TAK1 triggers also the phosphorylation of MAPKs and activation of different kinases: the extracellular signal-regulated protein kinase 1/2 (ERK1/2), the c-Jun amino-terminal kinases (JNKs, also called stress-activated protein kinases, SAPKs), and the p38 MAP kinases (p38). All of them differentially regulate many cellular functions including inflammation (Peroval et al., 2013). ERK1/2 are effectors of the Ras and c-Raf proto-oncoproteins activated in response to mitogenic stimuli. Contrarily, the JNKs/SAPKs and the p38 MAPKs are activated preferentially by environmental stress and inflammatory cytokines of the TNF family (Kyriakis, 2018). MAPKs lead to the activation and nuclear translocation of AP-1 subunits such as c-fos, c-Jun, and activating transcription factor 2 (ATF-2). The binding of AP-1 to DNA responsive elements results in macrophages in the initiation of transcription of the proinflammatory cytokines (e.g. IL-1, IL-6, IL-12, IL-23, and TNF- α) (Roy et al., 2016).

Noteworthy, several studies discuss that the SAPK/JNK and p38 MAPK pathways may be involved in the activation of NF- κ B in the cytoplasm, its translocation into nucleus, as well as in modulation of its transactivating potential in the nucleus (Schulze-Osthoff et al., 1997). The potential crosstalk between ERK and I κ B α /NF- κ B pathways has been also suggested (Seo et al., 2013).

The TLR4-mediated activation of I κ B α /NF- κ B and MAPKs/AP-1 was described for numerous inflammation-based pathologies (Liu et al., 2017). Moreover, increasing occurrence of studies suggesting the crosstalk between these two pathways underlines their important contribution in the inflammatory response and regulation of transcription of inflammatory molecules (Ramalingam et al., 2020). Therefore one of the aims of this work was to look more in detail on the ability of stilbenoids to intervene in the mechanisms of this signaling.

1.7 Pathologies linked with inflammation

Inflammation accompanies several acute and chronic pathologies at both the peripheral and CNS levels. Although the acute inflammation is mostly caused by infectious agents, it is also an important component of non-infectious pathologies, such as stroke, traumatic brain injury, and hemorrhage (Goerge et al., 2008; Skaper et al., 2018). Inflammatory process occurs also on the background of several chronic and autoimmune disorders, including allergic asthma, chronic obstructive pulmonary disease (Moldoveanu et al., 2008), atherosclerosis (Libby et al., 2002), rheumatoid arthritis (Smolen et al., 2018), or inflammatory bowel diseases (Vochyánová et al., 2015). The engagement of the canonical NF- κ B and MAPK/AP-1 signaling at molecular level is not exception for these diseases (Lawrence, 2009). Moreover, the crosstalk of immune cells between systemic and central level is important fact that should not be overlooked (Kempuraj et al., 2017). In our project, we focused on the intervention of stilbenoids into inflammation in general, and then more specifically in neuroinflammation.

1.7.1 Systemic inflammatory diseases

Inflammation may theoretically affect any weakened tissue and organ, bronchi and lungs belong to the most susceptible to exposure to bacterial and viral pathogens, environmental pollutants, as well as cigarette smoking. Redundant acute inflammation may

lead to lung injury and impair gas exchange. Unresolved lung injury accompanied by chronic inflammation is typical for chronic obstructive pulmonary disease (COPD), asthma, or cystic fibrosis (Brusselle and Bracke, 2014; Crooks et al., 2000; Leitch et al., 2008; Riise et al., 1995). Excessive long-term smoking may overload the airways with macrophage, neutrophil, and T lymphocyte infiltration and products of their activation – free oxygen radicals, proteases, and cytokines, such as TNF- α , IL-6 and IL-8 (Kawayama et al., 2016).

At gastrointestinal level, both upper and lower part of tract may become irritated and inflamed under some pathological conditions and result in the form of pharyngitis, esophagitis, gastritis, etc. Pancreatitis is one of the most common gastrointestinal causes of hospitalization in the US that may be a result of pancreatic duct obstruction, trypsinogen gene mutation, or alcoholism (Manohar et al., 2017). The incidence of acute pancreatitis ranges from 4–45 per 100,000 patients per year (Chen et al., 2017). Pancreatitis is characterized by destruction of acinar cells, activation of NF- κ B, MAPK, and Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathways and inflammatory cells, including macrophages, neutrophils, and granulocytes secreting the inflammatory cytokines (Zheng et al., 2013). The cytokines further activate pancreatic stellate cells which can promote chronic pancreatitis, and contribute to the development of pancreatic cancer, one of the most lethal of malignancies and the fourth most common cause of death from cancer in the US (Yadav and Lowenfels, 2013). The uncontrolled septic or aseptic inflammation may flow also into a massive loss of hepatocytes, metabolic changes, and at the end the indefinite damage to the liver. Inflammation of both origins increases a risk of chronic liver diseases, such as non-alcoholic fatty liver disease (NAFLD) or viral hepatitis (Brenner et al., 2013). Infectious hepatitis is caused primarily by hepatitis B or C virus (HBV or HCV), both releasing PAMPs and leading to serious complications including cirrhosis, liver injury, or eventually hepatocellular carcinoma (Riaz et al., 2011). The liver affected by aseptic alcoholic or nonalcoholic steatohepatitis, or injured by long-term use of drugs is the source of massive release of DAMPs, which are together with PAMPs responsible for TLR4-mediated set-off of the inflammasome (Szabo et al., 2007). The colon and small intestine may be also hit by chronic inflammatory bowel diseases (IBDs) being often conditioned by environmental (*e.g.* diet, altered microbiota function) and genetic factors resulting to dysfunctional interactions between gut bacteria

and mucosal immune system (McGuckin et al., 2009). IBDs mainly comprise the ulcerative colitis (UC) and Crohn disease (CD). A key aspect in the immune system's response to microbes is the recognition of microbial agents via PRRs, such as TLRs and NLRs, triggering the NF- κ B and MAPK activation, secretion of IL-1 β , IL-18, and NLR-mediated caspase-1 activation (Fukata and Arditi, 2013).

Inflammation may strike also the urinary system, presided by the bacterial cystitis, one of the most common pathologies, which can firstly manifest as a moderate discomfort, but swiftly expand to serious problem and became non-responsive to several antibiotics (Nickel, 2007). Kidney inflammation can contribute to the progressive renal injury and eventuate in glomerulonephritis, acute or chronic kidney disease (CKD), or renal failure. CKD is a very common complication of elderly patients affecting approximately 10–12% of the population (Mallappallil et al., 2014). Such a kidney dysfunction is most commonly induced by infection, ischemia/reperfusion, or immune complexes formation/deposition (Ernandez and Mayadas, 2016). Renal tubular epithelial cells are likely important promoters of kidney inflammation, characteristic by leukocyte infiltration and activated NF- κ B and MAPKs signaling (Cassidy et al., 2012; Lee et al., 2019; Sanz et al., 2010).

Last but not least, cardiovascular diseases (CVDs) are the most urgent and chief cause of death and disability worldwide (Sofi et al., 2016). According to WHO, 17.9 million people die every year from CVDs what counts 31% of deaths worldwide. CVDs represent a group of severe pathologies of the heart and blood vessels, including coronary heart disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, and pulmonary embolism. Myocardial infarction, which commonly results from its underlying pathology, coronary atherosclerosis, is the most common cause of cardiac injury and involves the acute loss of many myocardial cells (Jennings et al., 1990). Endogenous ligands, including necrotic cardiac cells, derived from tissue affected by injury are recognized as danger signals by cell surface TLR receptors and trigger post-infarction inflammatory responses by activating NF- κ B signaling (Beg, 2002). CVDs are the cardinal cause of death and disability particularly in patients with DM2 what comprise around two-thirds of diabetic patients (Booth et al., 2006). Among these, approximately 40% die from ischemic heart disease, 15% from other reasons, such as congestive heart failure, and about 10% from stroke (Low Wang et al., 2016). Pointing out that there are approximately 380 million of adults currently living with DM2, CVDs represent a severe

problem in developed countries and a great burden for the healthcare system. Considering that DM also activates the NF- κ B and MAPK inflammatory signaling, this increases already elevated level of inflammation triggered by CVD (Esser et al., 2014).

1.7.2 CNS diseases linked with neuroinflammation

Neuroinflammation is a pathological feature which accompanies several brain diseases and the microglial activation is the main hallmark of this process (Hornedo-Ortega et al., 2018). Both infectious agents and brain injury, such as tissue damage observed following ischemic, traumatic, or excitotoxic brain injury have been identified to set off the inflammatory cascade involving microglial and monocyte component (Allan and Rothwell, 2001; Vezzani and Granata, 2005). Scientific evidence points out the inflammatory reactions in the brain pathologies, such as stroke, which can enhance neuronal excitability, impair cell survival, and increase the permeability of the BBB (Fig. 6).

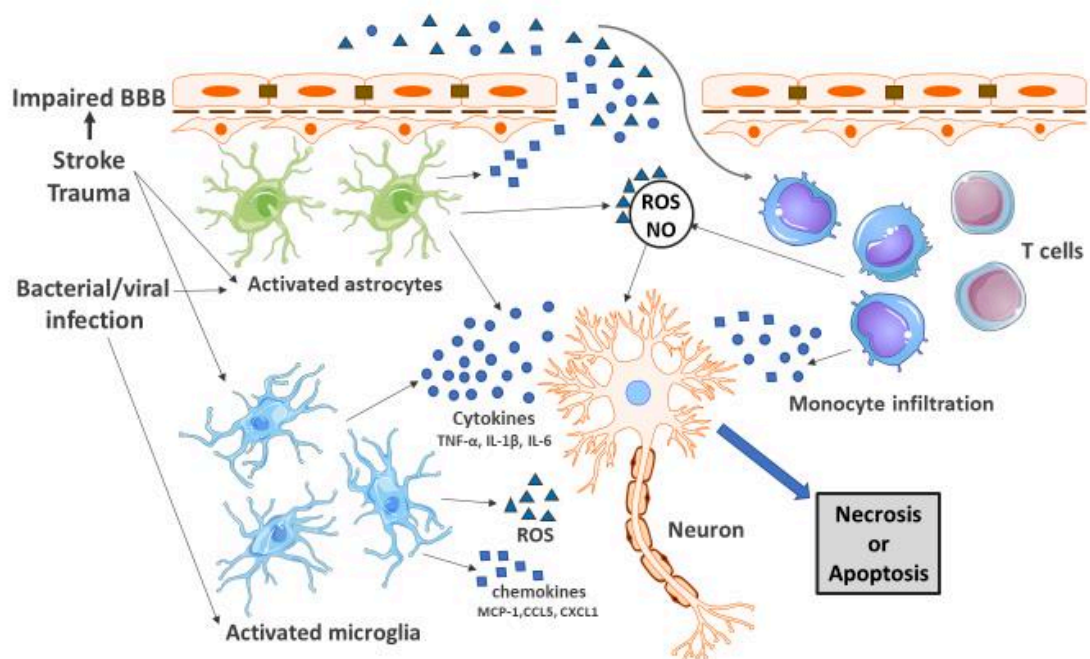


Fig. 6: Effects of neuroinflammation on the activation of immune cells contributing to neuronal damage.

Herrmann et al., 2005, demonstrated the IKK activation in a mouse model of stroke, highlighting the NF- κ B mediated neuronal death following the brain infarct. Thus, the inhibition of IKK activity could reduce the infarct size. Using a model of transgenic mice, it has been shown that the neuronal-specific ablation of IKK2 protects mice from

ischemia-induced brain damage (Herrmann et al., 2005). The inflammatory process related to cerebral stroke will be detailed later. Worthy to note that ischemia (with consecutive inflammation) may affect also other previously mentioned organs, such as heart, lung, and kidney (Hausenloy and Yellon, 2013; Malek and Nematbakhsh, 2015; Weyker et al., 2013).

Chronic inflammation at the CNS level underlines also several autoimmune and neurodegenerative disorders like AD, PD, and Huntington's (HD) characterized by the presence of cellular aggregates and the misfolded proteins β -amyloid, α -synuclein, and huntingtin, respectively (Sastre et al., 2014). Furthermore, inflammation may be a common factor contributing, or predisposing the occurrence of seizures in various forms of epilepsy. Experimentally induced seizures in rodents trigger the prominent inflammatory response in brain areas recruited in the onset and propagation of the epileptic activity (Vezzani and Granata, 2005).

Although the different mentioned systemic and CNS pathologies are treated differently according to the affected mechanism in the human body, all these diseases have a common feature in the form of ongoing inflammation on their background. Therefore, a supplementary safe natural treatment could alleviate the damage caused by inflammation and prevent the aggravation of the tissue/organ injury.

1.8 Anti-inflammatory treatments

Non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids, such as prednisone are the most commonly prescribed and largely commercially produced drugs. Apart from aspirin derived from natural molecule salicylic acid, others are synthetic molecules with numerous side effects (Fig. 7), not completely safe and appropriate for long-term use. Therefore, an alternative drug without or with minimal adverse effects and safe in long-term, would be welcomed in this area. Such a molecule could eventually originate in natural sources, such as medicinal plants containing a plethora of polyphenolic compounds, including stilbenoids known for their anti-inflammatory potential. The therapeutic, but also adverse effects of NSAIDs are related to the inhibition of COX-1 and COX-2 pathways, responsible for biosynthesis of prostaglandins, as previously detailed in the chapter 1.5 (Inflammatory mediators). The most known derivate of natural molecule

salicylic acid originated in white willow (*Salix alba* L., Salicaceae), commercially produced as aspirin, works by non-selective and irreversible inhibition of COX-1 and 2. Moreover, aspirin is an efficient anti-platelet agent used in the prevention of recurrent myocardial infarction and stroke through inhibition of thromboxane A₂ (TXA₂) production (Warner et al., 2011).

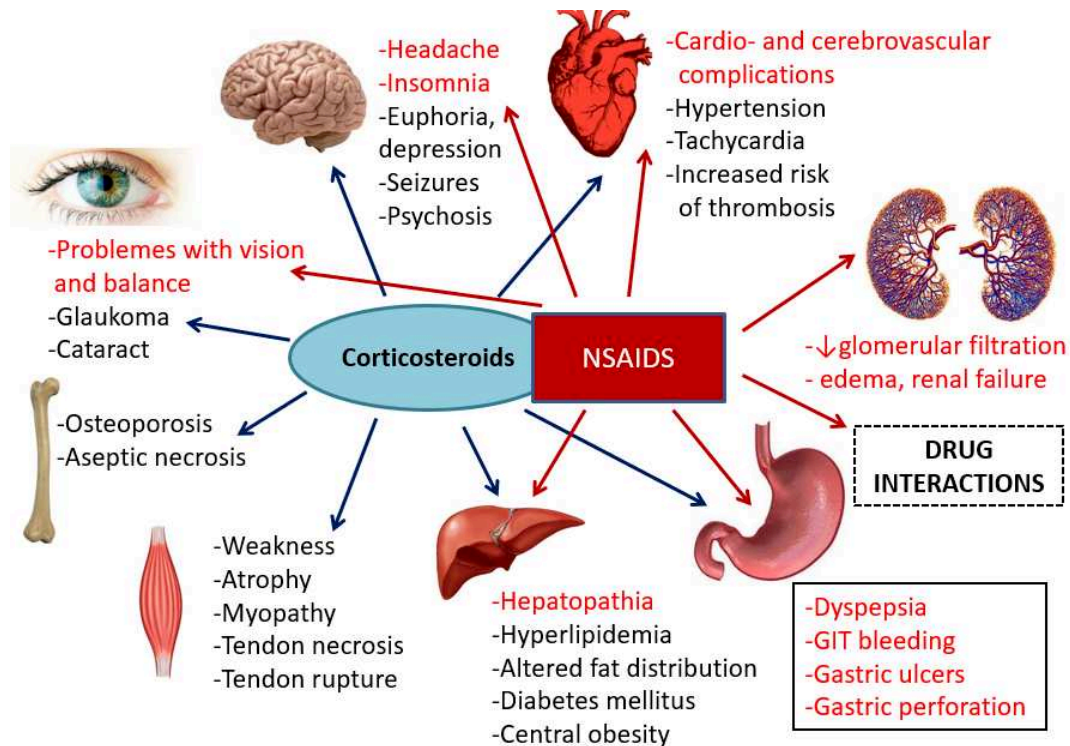


Fig. 7: Side effects of the anti-inflammatory drugs corticosteroids and NSAIDs.

Because COX-1 is constitutively present in the stomach where it protects the gastrointestinal mucosa, severe complications associated with the non-selective COX-1/2 NSAIDs, such as gastritis, stomach bleeding, and eventually peptic ulcers may occur (Matsui et al., 2011). Ibuprofen, ketoprofen, indomethacin, diclofenac, and naproxen are typical representants from this group. Furthermore, NSAIDs can induce adverse renal effects possibly leading to acute and chronic kidney injury. Indeed, PGs regulate also the vasodilation at glomerular level, thus blockage of their production results in reduced renal blood flow (Dixit et al., 2010; Lafrance and Miller, 2009).

The selective inhibition of “inducible” COX-2 is more favorable for the prevention of gastrointestinal side effects. Therefore, COX-2 preferential inhibitors, such as meloxicam, nimesulide, or etodolac, and selective COX-2 inhibitors, such as celecoxib and

rofecoxib were developed. Anyway, the inhibition of constitutive COX-2 in the kidney may transiently increase blood volume and exacerbate existing hypertension by elevation of systolic blood pressure (Harris, 2013). Additionally, the inhibition of endothelial COX-2-derived vascular prostacyclin linked with platelet activation and vasoconstriction contributes to even more serious cardiovascular risks of these therapeutic agents (Funk and FitzGerald, 2007). Last but not least, numerous drug interactions with NSAIDs represent a risk, especially for elderly patients with multiple medications (Wongrakpanich et al., 2018).

NSAIDs are used to treat acute conditions such as pain and migraine, because of their ability to block PGE₂ synthesis and their action on peripheral sensory neurons. Another purpose of NSAIDs is fever reduction, although nor fever neither pain do not have to be exclusively launched by inflammation. For instance, ibuprofen action in its standard single dose of 400 mg/tablet for an adult has primarily analgesic action. To reach the anti-inflammatory effect, the higher dose of 1600–2400mg (400–800mg 4 or 3 times per day) is needed. At the same time, it is the maximal daily dose for an adult patient, which may have serious above-mentioned adverse effects when using in the long-term (Bushra and Aslam, 2010). Therefore, new approaches for development of a safe anti-inflammatory treatment, probably acting by another molecular mechanism, are highly demanded. In spite of this, NSAIDs are widely used also for the treatment of chronic disorders such as rheumatoid arthritis, osteoarthritis, and other inflammatory arthropathies, including psoriatic arthritis, ankylosing spondylitis, etc. Nowadays, also other drugs for the treatment of these and other (*e.g.* Crohn's disease, ulcerative colitis) chronic diseases are available, such as IL-1 receptor antagonists or TNF inhibitors. Although these treatments called „biological“ have been shown to be highly efficient, they represent an enormous financial burden for the health care system and are not accessible to all patients (Binion, 2010; Dinarello et al., 2012; Monaco et al., 2015).

Corticosteroids represent another largely administered group of effective anti-inflammatory drugs used in the treatment of serious acute and chronic pathologies characterized by an excessive and inappropriate response of the immune system. Nevertheless, as potent immunosuppressors, they affect several mechanisms across the whole body causing a bulk of serious adverse effects. Their mechanism of action consists in easy transport across cell membranes (due to their lipophilic character), binding to the

cytoplasmic glucocorticoid receptor (GR), translocation to the nucleus and binding to DNA specific sequences in the promoter region of glucocorticoid response elements (GRE; Fig. 8). This leads to activation of genes encoding anti-inflammatory proteins, such as secretory leukoprotease inhibitor (SLPI), mitogen-activated kinase phosphatase-1 (MKP-1), and I κ B α . Nuclear GR also interacts with co-activator molecules, such as cyclic adenosine monophosphate (cAMP) response element binding protein (CREB)-binding protein (CBP), which is activated by proinflammatory transcription factors, including NF- κ B and AP-1, thus switching off the inflammatory genes that are under control of these transcriptional factors (Barnes, 2006).

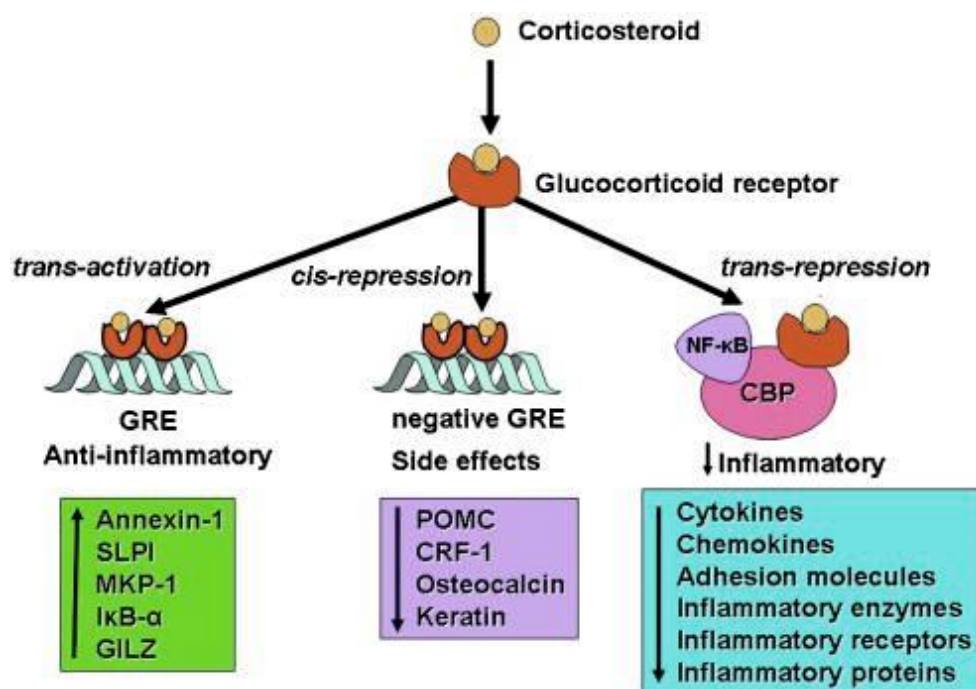


Fig. 8: Corticosteroid regulation of gene expression. CBP - CREB binding protein; SLPI - secretory leukoprotease inhibitor; MKP-1 - mitogen-activated kinase phosphatase-1; I κ B- α - inhibitor of NF- κ B; GILZ - glucocorticoid-induced leucine zipper protein; POMC - proopiomelanocortin; CRF-1 - corticotrophin-releasing factor (Barnes, 2006).

Pleiotropic action of corticosteroid affects the expression of multiple genes responsible for both physiological and pathological features (Aljebab et al., 2017). Osteoporosis, muscle weakness, growth retardation in children, skin fragility, hypertension, metabolic effects, including diabetes, weight gain, and central obesity, but also neuropsychiatric effects, such as anxiety, hypomanic reactions, depression, sleep disturbances are common adverse effects of corticosteroids (Fig. 7). Moreover, strong

immunosuppression enhances the vulnerability of organism to different infections (Yasir et al., 2020).

Therefore, the selective inhibition of pro-inflammatory markers being under NF- κ B and AP-1 transcription control, without undesirable effects on other physiological functions could represent a promising strategy for further scientific explorations. The TLR4-mediated activation of I κ B α /NF- κ B and MAPKs/AP-1 pathways was also our initial point of interest. We focused on the ability of natural polyphenolic molecules from the group of stilbenoids to intervene into these two inflammatory pathways, as well as the most described COX-1/2 and 5-LOX pathways examined within the project.

2. STILBENOIDS

From a chemical point of view, stilbenes are organic compounds sharing a diphenylethylene core that can be differently substituted (Fig. 9). They may exist as two stereoisomers, *cis*- and *trans*-, whereby *trans*- form is more stable and common in nature. Stilbenoids are hydroxylated derivatives of stilbenes that were found in many plants as their secondary metabolites. In plants, they play a role of “phytoalexins” or “plant defenders” whose synthesis *de novo* is regulated by plant tissue in response to a microbial attack with the aim to protect the plant against a harmful pathogen or another environmental stress (Nalli et al., 2016).

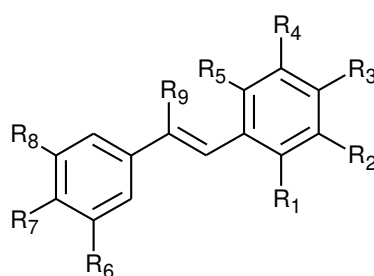


Fig. 9: *trans*-stilbene skeleton with sites of possible substitution

2.1 Origin and role in plants

Stilbenoids are minor but qualitatively important components of food (peanuts, rhubarb, different edible berries), beverages (wine, white tea) and medicinal plants, such as various *Morus*, *Artocarpus*, *Trabopogon*, and *Macaranga* species (Granica et al., 2015; Hošek et al., 2019). The most known and studied stilbenoid to date is *trans*-resveratrol (*trans*-3,5,4'-trihydroxystilbene, RSV). RSV was found in a variety of about 70 plant species and fruits, particularly in grapes (*Vitis vinifera*; around 150-780 µg/100 g), red wines (80-2700 µg/150 mL), peanuts (*Arachis hypogaea*; raw up to 180 µg/100 g, roasted 1.8-7.9 µg/100 g, peanut butter 15-75 µg/100 g) (Neves et al., 2012; Sanders et al., 2000), berries (*Vaccinium* spp.), cocoa (*Theobroma cacao*; cocoa powder 127-227 µg/100 g, dark chocolate 25-43 µg/100 g) and other plants such as Japanese knotweed (*Polygonum cuspidatum*), typical for Japanese diet (Neves et al., 2012). Several other stilbenes, such as piceatannol, pterostilbene, piceid, α -viniferin, and vitisin A, have been also found to be constitutively present in *Vitis* spp., and their synthesis in plants may be induced by toxins and fungal infection (Akinwumi et al., 2018; Pawlus et al., 2012). Interestingly, RSV has been directly linked with the resistance of grapes against plant pathogens such as *Botrytis*

cinerea or *Plasmopara viticola*, and its increased production in the plant has been observed also after exposition to exogenous stimuli like UV light (Hasan and Bae, 2017). This has been supported by other studies where the biosynthesis of RSV, *trans*-arachidin-1, *trans*-3'-isopentadienyl-3,5,4'-trihydroxystilbene, and arahypin-7 has been elicited in peanut hairy root culture by methyl jasmonate and cyclodextrin (Pilaisangsuree et al., 2018).

Pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene), a dimethyl ether analog of RSV, another more intensively investigated stilbene, has been found in species such as *Pterocarpus marsupium* and different types of blueberries (*Vaccinium* spp.) (Lin et al., 2020). Hydroxylated RSV derivative, piceatannol (*trans*-3',4',3,5-tetrahydroxystilbene) was found apart from grapes, in rhubarb (*Rheum* spp.), passion fruit, and white tea (Dvorakova and Landa, 2017), pinosylvin (3,5-dihydroxystilbene) in pine (*Pinus* spp.) and *Cajanus cajan* (Hammerbacher et al., 2011), rhapontigenin, desoxyrhapontigenin, and *trans*-gnetol were isolated from *Gnetum* spp. (Dvorakova and Landa, 2017). Moreover, other stilbenes containing plants are cultivated in Asia as folk remedies for commercial purposes, including *Rheum undulatum*, *Rhodomyrtus tomentosa*, *Melaleuca leucadendron*, and *Euphorbia lagascae* (Reinisalo et al., 2015).

Stilbenoids are formed in plants through the phenylpropanoid pathway (Fig. 10). The biosynthesis starts by conversion of phenylalanine to cinnamic acid, or tyrosine to *p*-coumaric acid, continues by formation of cinnamoyl-CoA, *p*-coumaroyl-CoA or caffeoyl-CoA that are further converted to stilbenes after addition of three malonyl-CoA units (from acetate pathway) and catalytic activity of stilbene synthase (Akinwumi et al., 2018). The stilbene core can undergo further modifications such as methylation, prenylation, glucosylation or isomerization to *cis*-stilbene (Tab. 2). The prenyl unit (3-methylbut-2-en-1-yl) is a 5-carbon substituent synthesized *via* the isoprenoid mevalonate (Fig. 11) or deoxyxylulose (non-mevalonate) pathway (Fig. 12). The prenylation then occurs due to stilbenoid-specific enzyme prenyltransferase and may increase the lipophilicity of stilbene, and thus improve its passage through the cell membrane (Hošek et al., 2019). Moreover, the prenylation is recognized as cell intrinsic and ubiquitous process of all eukaryotic cells, where prenylation plays role in post-translational modification of some proteins, as necessary step for their proper functioning (Palsuledesai and Distefano, 2015).

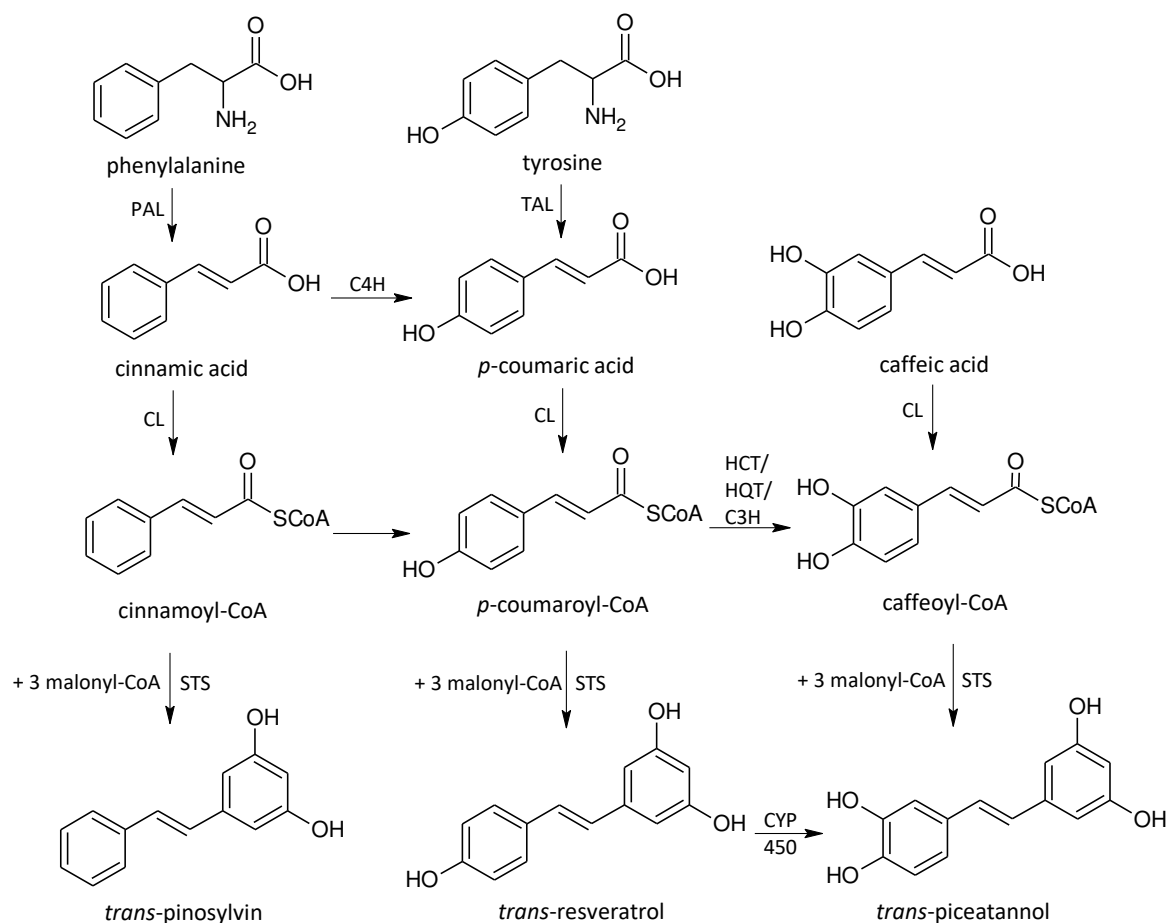


Fig. 10: Biosynthesis of stilbenoids in plants via phenylpropanoid (shikimate) pathway. PAL-phenylalanine ammonia-lyase, TAL-tyrosine ammonia-lyase, C4H-cinnamate-4-hydroxylase, C3H-coumaroylshikimate-3'-monooxygenase, HCT-hydroxycinnamoyl-CoA shikimate hydroxycinnamoyl transferase, HQT hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase, CL 4-coumarate: coenzyme A ligase, STS stilbene synthase. 3 malonyl-CoA units comes from the acetate pathway. Adapted from (Hammerbacher et al., 2011)

Reaction	Enzyme	Stilbene product
Methylation	methyltransferase	<i>trans</i> -pterostilbene, <i>trans</i> -isorhapontigenin <i>trans</i> -pinosylvin monomethyl ether, etc.
Prenylation	prenyltransferase	arachidin-1, mappain, macasiamenenes, schweinfurthins, chiricanine A, etc.
Glucosylation	glucosyltransferase	<i>trans</i> -piceid, <i>trans</i> -astringin, <i>trans</i> -reveratrolside, mulberroside A, etc.
Isomerization	isomerase	<i>cis</i> -stilbenes: <i>cis</i> -resveratrol, combretastatin A-4, macasiamenene C-E, H-J, etc.
Oligomerization	peroxidases	<i>trans</i> - ϵ -viniferin, vitisin A, pallidol hopeaphenol, quadrangularin A, etc.

Table 2: Possible modifications of stilbene core in plants, adapted from (Chong et al., 2009).

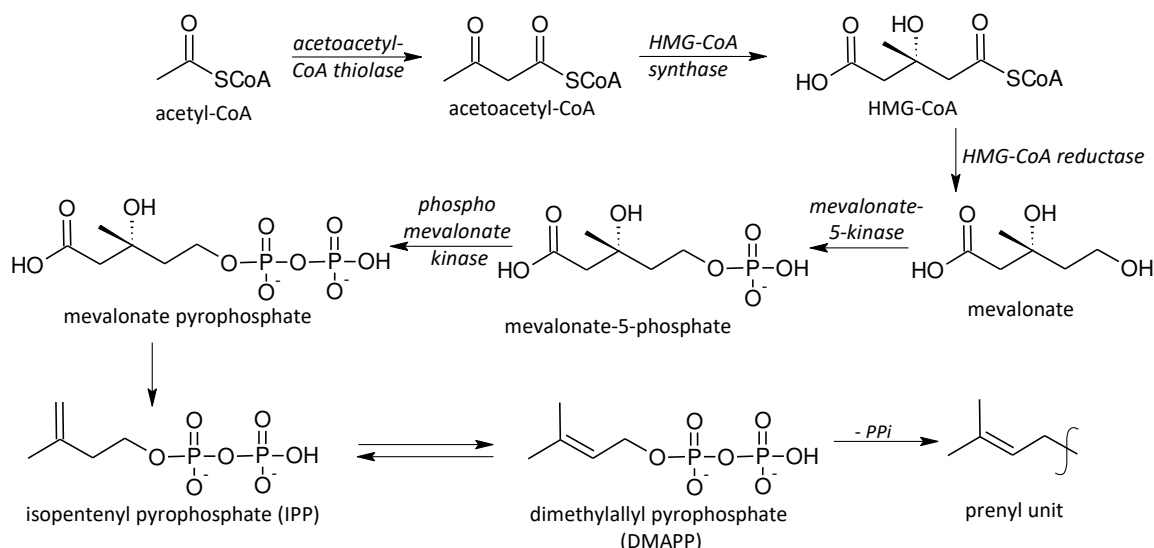


Fig. 11: Biosynthesis of prenyl unit via mevalonate pathway from (Eisenreich et al., 2001). HMG-CoA synthase/reductase- β -Hydroxy β -methylglutaryl-coenzyme A synthase/reductase.

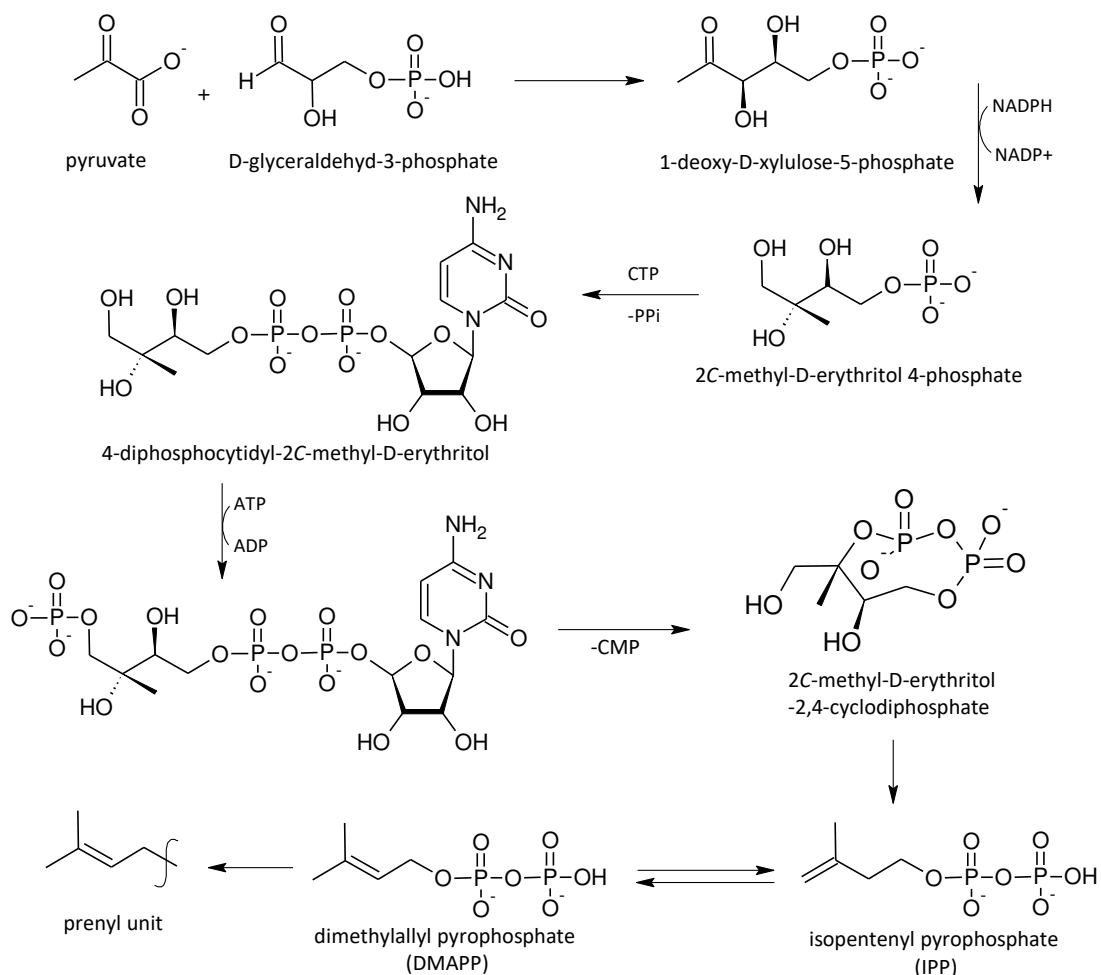


Fig. 12: Biosynthesis of prenyl unit via non-mevalonate pathway adapted from (Eisenreich et al., 2001). CTP-cytidine triphosphate, CMP-cytidine monophosphate, ATP-adenosine triphosphate, ADP-adenosine diphosphate, NADPH/NADP+-(dihydro)nicotinamide adenine dinucleotide phosphate.

2.2 Health benefits

Many polyphenols and stilbenes-rich plants consumed as food, or drunk in the form of tea or red wine take part of diet called “Mediterranean”, which is based on the consumption of vegetables, whole grains, nuts, fish, seafood, olive oil, and moderate intake of red wine. Several studies propose the MedDiet as a promising pattern for the prevention of age-related, cardiovascular, and neurodegenerative disorders (Hornedo-Ortega et al., 2018). It was observed that MedDiet consumption is associated with a reduced risk of dementia, AD, PD, and cognitive decline in general (Alcalay et al., 2012; Anastasiou et al., 2017; Féart et al., 2013). Largely studied role of stilbenoids in the prevention and treatment of cardiovascular diseases arouse interest in the investigation of their possible involvement in the treatment of cerebrovascular diseases, such as stroke.

2.2.1 *Anti-inflammatory effects*

Stilbenoids isolated from plants have shown a variety of health benefits that are based on their strong anti-inflammatory and anti-oxidant effects. Reported possible anti-inflammatory mechanisms of the most known stilbene RSV involve the downregulation of synthesis and release of pro-inflammatory mediators, particularly eicosanoids, inducible nitric oxide synthase (iNOS), MMPs, and inhibition of immune cells activation through its inhibitory effects on NF- κ B and AP-1 pathway and further production of proinflammatory cytokines (de la Lastra and Villegas, 2005). All these effects reviewed by Dvorakova and Landa, 2017, were described similarly on numerous *in vitro* and *in vivo* models. Alongside RSV, two other most investigated stilbenes – piceatannol and pterostilbene – have shown very similar *in vitro* and *in vivo* effects and capability to counteract the NF- κ B/AP-1 pathways, together with COX-1/2 and 5-LOX activity (Dvorakova and Landa, 2017; Leláková et al., 2019). RSV inhibited *in vivo* I κ B α phosphorylation (Kundu et al., 2006a), NF- κ B nuclear translocation and activity (Cichocki et al., 2008), decreases ERK phosphorylation (Kundu et al., 2006a) and AP-1 binding and activity (Kundu et al., 2006a, 2006b). RSV also reduces the expression of proinflammatory cytokines TNF- α , IL-1 β , IL-6, IL-8, and IL-18 (Dvorakova and Landa, 2017), and targets the activity of proinflammatory enzymes COX-1/2 (Baur and Sinclair, 2006; Kundu et al., 2006a).

Very similar effects were described for the trimer of RSV, α -viniferin, on cell model of LPS-activated RAW264.7 mouse macrophages and a mouse model of paw edema

(Chung et al., 2003). The root extract of *V. vinifera* containing seven mono- and oligomeric stilbenoids, such as RSV, piceatannol, *trans-ε*-viniferin, and miyabenol C, exhibits free radical scavenging and cellular antioxidant properties, acting on Nrf2, heme oxygenase-1 (HO-1), γ -glutamylcysteine synthetase (γ GCS) systems, as well as the anti-inflammatory effects downregulating the expression of proinflammatory cytokines, such as IL-1 β (Esatbeyoglu et al., 2016). Several stilbenes exhibited the ability to regulate *in vitro* the activity of 5-LOX and COX-1/2 (Kutil et al., 2015). Piceatannol has been shown as a strong inhibitor of 5-LOX, comparable to commercially used drug zileuton, while pinostilbene displays great *in vitro* inhibitory effects of COX-2. The interactions of these compounds with enzymes active sites have been supported also by *in silico* analysis. Other stilbenes, such as pinosylvin monomethylether, isorhapontigenin, and some prenylated *Macaranga* stilbenes have also display anti-COX-1 and anti-COX-2 activity but are more selective to COX-1 (Hošek et al., 2019; Leláková et al., 2019). Although COX-1 inhibition is linked with gastrointestinal side effects, typical for non-selective NSAIDs, COX-1-dependent synthesis of prostaglandins is implicated in pathological processes such as atherosclerosis, endothelial dysfunction, or neuroinflammation. Therefore selective COX-1 inhibition could find its usefulness (Vitale et al., 2014). Moreover, diarylisoxazole scaffold has been proposed for synthesis of new selective COX-1 inhibitors without effects on gastrointestinal functions (Cingolani et al., 2017).

Stilbenoids arouse interest mostly in the domain of cardiovascular health in relation to platelet aggregation and atherosclerosis, which may have a strong connection with their ability to interfere with arachidonic acid pathway and mechanisms of collagen-induced platelet aggregation (Ko et al., 1999). The ability of stilbenes to intervene in AMP-activated protein kinase/endothelial nitric-oxide synthase (AMPK/eNOS) signaling can have a connection with high blood pressure regulation (Theodotou et al., 2017). Strong antioxidant and anti-inflammatory effects of stilbenoids may stand behind reported protective action against cardiac ischemia/reperfusion (I/R) (Hung et al., 2004; Wu et al., 2017). Worthy to note that all mentioned pathologies are closely related to cerebrovascular diseases and may influence their development. The neuroprotective effects against cerebral I/R injury and neurodegenerative disorders were recorded as well and will be described more in details. Additionally, some stilbenoids display also cancer-preventive potential

(Sirerol et al., 2016) and beneficial effects in diabetes and obesity (Akinwumi et al., 2018; Dvorakova and Landa, 2017).

2.2.2 *Cardioprotective effects*

The cardioprotective effects of stilbenoids involve primarily their potential to improve the high blood pressure, prevent atherosclerosis due to anti-platelet and LDL cholesterol-lowering effects (Akinwumi et al., 2018). More exemplarily, pterostilbene has shown the protective effects against myocardial I/R injury in diabetic rats *via* attenuation of oxidative/nitrosative stress, a decrease of the inflammatory response (Yu et al., 2017), through the AMPK activation (Kosuru et al., 2018), and upregulation of eNOS (F. Liu et al., 2017). Activation of eNOS is also one of the potential mechanisms for blood pressure regulation (McCormack and McFadden, 2013).

The meta-analysis of six randomized clinical trials comprising 247 subjects showed that oral administration of commercially produced RSV in capsules at a higher dose of 150 mg/day decreases significantly systolic (SBP), but not diastolic blood pressure (DBP). Lower doses have no significant effect on SBP, neither DBP (Liu et al., 2015). Alongside, a significant decrease in LDL-cholesterol (Bhatt et al., 2012) and an increase of HDL cholesterol is observed at biochemical level (Movahed et al., 2013). In another randomized double-blinded clinical trial, pterostilbene orally administered in the form of a capsule at the dose of 125 mg twice per day decrease both SBP and DBP while a lower dose of 50 mg twice daily does not have any effect (Riche et al., 2014). RSV, probably due to its potent anti-COX-1 activity inhibits the platelet aggregation induced by collagen, thrombin, adenosine diphosphate (ADP) and epinephrine, a major risk factor of atherothrombosis that can lead to myocardial infarction or stroke. Human cohort study on 50 high-risk cardiac patients confirmed the inhibitory effect of RSV against platelets aggregation in patients sensitive but also resistant to acetylsalicylic acid treatment (Stef et al., 2006).

High plasma levels of triglycerides and low-density lipoproteins (LDL), often nicknamed as "bad" cholesterol, represent another highly important risk factor of CVS diseases. RSV displays the ability to target oxidized LDL (oxLDL) promoting in the systemic circulation the accumulation of inflammatory cells such as macrophages. The elevated concentration of oxLDL in the circulation may lead to the formation of plaques on walls of vessels and cause atherosclerosis. At preclinical level, RSV directly affects

lipolysis *in vitro* in human fat cells (Gomez-Zorita et al., 2013), attenuates *ex vivo* the (oxLDL)-induced apoptosis in vascular endothelial cells (Chang et al., 2011), and inhibits the lipid accumulation *in vivo* (Zang et al., 2006). These results have been confirmed also in clinical trials, where RSV decreased LDL and oxLDL in humans taking daily 350 mg of RSV-enriched grape extract during 6 months (Tomé-Carneiro et al., 2012).

Several studies observed similar effects of other two RSV derivatives – pterostilbene and piceatannol. In a mouse model of high fat-induced atherosclerosis, pterostilbene attenuates plaques formation *via* NF- κ B signaling and suppressed expression of pro-inflammatory cytokines such as TGF- β , TNF α , IL-1 β , and IL-6 (Zhang and Zhang, 2016). Another *in vivo* study demonstrated that treatment by piceatannol at variable doses (15–45 mg/kg) results in reduced LDL-cholesterol levels and lipid peroxidation (Llarena et al., 2016). Although the *in vivo* results of the last two mentioned compounds have to be confirmed at the clinical level, it seems that stilbenoids such as RSV exert their cardioprotective effects by improving anti-inflammatory response and intervening into atherothrombotic signaling (Tomé-Carneiro et al., 2013).

2.2.3 Neuroprotective effects

The protective effects of stilbenoids at the CNS level were mostly investigated on cell and animal models of neurodegenerative diseases, such as PD and AD, but some studies reported also their intervention in acute pathologies, such as cerebral ischemia. Both neurodegenerative disorders and acute brain diseases are linked with oxidative/nitrative stress and mitochondrial dysfunction leading to loss of function and death of neurons. ROS/RNS toxicity is a major pathogenic factor in neurodegenerative disorders, but also in a plethora of non-degenerative brain diseases, such as stroke, epilepsy, hepatic encephalopathy (Ruszkiewicz and Albrecht, 2015). Therefore, stilbenoids thanks to their potent anti-oxidant character may be beneficial in different brain pathologies.

2.2.3.1 Neurodegenerative disorders

Similarly to cardiovascular diseases, RSV effects have been studied the most also at the CNS level. In cell models, RSV promotes clearance of amyloid-beta peptides, the main feature of AD pathology (Marambaud et al., 2005). In neuron-glia co-cultures, RSV

protects dopamine neurons against LPS-induced neurotoxicity through inhibition of microglia-mediated neuroinflammation (Zhang et al., 2010). Pterostilbene displays *in vitro* neuroprotective effects against high glucose- (Yang et al., 2017) and glutamate-induced injury (Wang et al., 2016), prevents cell death and generation of ROS in neurons (Yang et al., 2017), and improves cholinergic transmission *via* inhibition of cholinesterases (Naik et al., 2017).

In animal models, RSV improves cognition and reduced oxidative stress in rats with vascular dementia (Ma et al., 2013). Oxyresveratrol (10 μ M) effectively prevents A β (25-35)-induced neuronal cell damage in rat by reducing glutamate release, ROS production and interfering with the increase of cytosolic calcium concentration (c[Ca²⁺]) (Ban et al., 2006). In *in vivo* model of parkinsonism induced by neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), RSV improves motor coordination and protects against neuronal damage through free radical scavenging (Lu et al., 2008).

In humans, RSV administered orally at a dose of 75 mg twice daily for 14 weeks improves cognition and cerebrovascular function in postmenopausal women (Evans et al., 2017). As a result of several clinical studies, RSV has been shown safe and well-tolerated within dose 0.5–2 g per day during 52 weeks (Turner et al., 2015). It improves memory function and cognition in diabetic patients with subclinical cognitive impairment (Witte et al., 2014), but does not show any significant effect in AD patients (Turner et al., 2015). The protective potential of other stilbenoids against neurodegeneration have not been yet tested in clinic.

2.2.3.2 Cerebral ischemia

The neuroprotective properties of RSV against I/R injury are mostly attributed to its direct antioxidant effects and thus reducing oxidative stress during an ischemic cascade. However, several studies described its intervention into molecular mechanisms responsible for neuronal survival. At the preclinical level, Ren et al., 2011, demonstrated on the model of rat focal cerebral ischemia (MCAo), that 15 mg/kg/day and 30 mg/kg/day of RSV 7 day-pretreatment may reduce the infarct size, caspase-3 activation and neuronal apoptosis, improve the oxidative damage by upregulated expression of transcription factor Nrf2 and HO-1 in dose-dependent manner (Ren et al., 2011). Similarly, the intraperitoneal administration of RSV (30 mg/kg) for 7 days before inducing global cerebral ischemia in

rats by the four-vessel occlusion significantly attenuates neuronal death. The neuroprotective effects of RSV consists in parallel modulation of both glycogen synthase kinase-3 β (GSK-3 β) and CREB through PI3-K/Akt pathways, leading to neuronal survival and increase of blood glucose concentration, mechanism desirable after cerebral ischemia (Simão et al., 2012). Other studies enregistered the effect of RSV on the modulation of matrix metalloprotease-2 (MMP-2) and vascular endothelial growth factor (VEGF) levels inducing angiogenesis in MCAo mice (Dong et al., 2008), suppression of the excitatory synaptic glutamate transmission (Gao et al., 2006). Some studies pointed out also possible RSV therapeutic effects *via* the interaction with Sirtuin 1 (SIRT-1) (Della-Morte et al., 2009; Pallàs et al., 2009).

The study using a transient rat MCAo model has proven that oxyresveratrol (10 mg/kg and 20 mg/kg) reduces the volume of an infarction by 54% and 63%, respectively, improves neurological deficits induced by the ischemia/reperfusion (I/R) injury and inhibits the activation of caspase-3 (Andrabi et al., 2004).

In a recent study, pinosylvin showed neuroprotective effects in both *in vitro* and *in vivo* assays. In *in vitro* model of OGD/R injury, pinosylvin 10 μ M reduces mitochondrial dysfunction in PC12 cells and cell damage by inhibition of both necrosis (detected by LDH release) and apoptosis (decrease of TUNEL positive cells). In the same study, pterostilbene but not pinostilbene and 4-methoxystilbene, also slightly protects against OGD-induced cell damage (Xu et al., 2020). Pinosylvin further enhances Bcl-2 (anti-apoptotic marker) expression and decreases Bax (apoptotic marker) expression, indicating that pinosylvin may act through reducing the mitochondrial damage induced by OGD/R. In the rat MCAo model, pinosylvin ameliorates the oxidative stress-induced mitochondrial dysfunction by activating the Nrf2 pathway and induced phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1)/Parkin mediated mitophagy. This indicates that pinosylvin could induce cell mitophagy to remove the damaged mitochondria to eliminate their harmful effects (Xu et al., 2020).

To date, the intervention of other stilbenoids than RSV and pterostilbene has not been studied *in vivo* on the model of cerebral ischemia and no stilbenoid has been investigated in clinic.

2.3 Particular interest as treatment

Before stilbenoids could become a treatment, several pharmacological parameters are necessary to be determined, including pharmacokinetics and pharmacodynamics. Whereas the role of pharmacokinetics is to define the fate of administered molecule in the living organisms since administration until its excretion, the principal role of pharmacodynamics is to determine how a drug affects organisms. Several modes of action were described in previous chapters defining stilbenoids as agents with multitargeted action. Anyway, the pharmacodynamics are often referred to as the interaction of a pharmacologically active substance with a specific receptor, including dose-response effect with this target site, and biochemical and physiological consequences of these interactions (Hallworth, 2014).

2.3.1 Pharmacodynamics

Whereas larger, more polar molecules such as sugars, amino acids, peptides, and nucleosides need the membrane transporters to pass into cells, small, moderately polar and lipophilic molecules are able to passively diffuse across the cell membrane. The efficient membrane permeation is likely necessary for bioavailability, the important factor of interest for drug treatments. Therefore, several mechanisms, such as Lipinski's rule of 5 have been developed for the assessment of lipophilicity of compound and prediction of its membrane permeability (Yang and Hinner, 2015).

Despite many proven biological effects of RSV and some other stilbenoids, the detailed mechanism of action stays unclear. Nalli et al., 2016 studied on genetically modified cells HEK293 the ability of RSV and its related stilbenoids to modulate the activity of transient receptor potential ankyrin 1 (TRPA1) and transient receptor potential vanilloid 1 (TRPV1). Depending on substitution of the core by hydroxy- and methoxy-groups, stilbenoids were able to differently inhibit TRPA1-induced calcium influx, but any tested stilbenoid was able to significantly modulate TRPV1 channels. Similarly to other phenol-derived molecules and due to a non-electrophilic nature of stilbenoids, the binding to a hydrophobic pocket of TRPA1 thought a non-covalent mechanism has been proposed (Nalli et al., 2016). TRPA1 is a member of the TRP superfamily of channels, located on the plasma membrane of many human and animal cells. It is considered as a mechanical and chemical stress sensor. TRPA1 is expressed on the nociceptive primary afferent

neurons of the trigeminal, vagal, and dorsal root ganglia. These nociceptors are responsible for the production of neuropeptides substance P and calcitonin gene-related peptide (CGRP), which mediate neurogenic inflammatory responses (Nassini et al., 2014). TRPA1 can be activated by numerous exogenous (temperature, environmental irritants, some natural molecules) and endogenous inflammatory agents from non-neuronal cells in the area of tissue injury or disease, such as oxidative and nitrative stress. This activation may occur through a covalent binding of the molecule with cysteine residue or non-covalent binding that is typical for some non-electrophilic natural phenolic agents such as thymol or menthol. Therefore, it was proposed by several studies, that TRPA1 antagonists could be beneficial in the treatment of inflammatory and neuropathic pain (Nassini et al., 2014). Although TRPA1 may seem as an attractive target for the development of anti-inflammatory and analgesic drugs, differences between human and rodent TRPA1 homologs may complicate TRPA1-targeted drug discovery (Nassini et al., 2014).

On the other hand, phenolics containing one or more prenyl groups are lipophilic compounds, therefore prenylated stilbenes are supposed to pass the phospholipid cell membrane without the necessity of membrane transporter (Araya-Cloutier et al., 2018).

2.3.2 Pharmacokinetics

The pharmacokinetics describes how the body deals with an administered molecule. This involves four main processes - absorption, distribution, metabolism, and excretion. Drug liberation is sometimes considered as fifth (or first) main pharmacokinetic process, which depends on the type of drug formulation, such as tablet, capsule, or different types of nanocarriers (Hallworth, 2014). Primary goals of clinical pharmacokinetics target also enhancing efficacy and decreasing toxicity of drug therapy, what can be achieved by targeted drug delivery systems, such as liposomes (Sharma et al., 2006).

The absorption phase represents a process during which a drug enters the blood circulation. The percentage of drug that reaches the systemic circulation is called the bioavailability. Although the absorption of the most studied RSV is relatively high (70–75%), its bioavailability has been shown very low (<1%) due to the extensive and rapid hepatic first-pass metabolism (Sergides et al., 2016; Walle et al., 2004). Phase I clinical trials have shown that resveratrol up to a daily dose of 5 g is well tolerated by healthy individuals (Boocock et al., 2007b). The pharmacokinetic analysis suggests that

ingestion of RSV at a dose from 0.5–5 g/day (equivalent to the amount contained in several hundred bottles of red wine) produces C_{\max} plasma concentrations of 0.3–2.4 μM , which is markedly in contrast with RSV concentrations tested in the most of *in vitro* studies ranging from 3 to 1000 μM (Gambini et al., 2015). When considering data obtained in humans for other phenolic phytochemicals, such as epigallocatechin gallate from green tea, the poor bioavailability, and extensive pre-systemic elimination are comparable to these of RSV (Chow et al., 2001). On the other hand, hydroxylated and methylated RSV analogs have been shown to be more bioavailable. Study comparing the oral bioavailability of stilbenoids have shown that pterostilbene is more bioavailable than resveratrol, gnetol, piceatannol, and oxyresveratrol, with rates of 80%, 25%, 6.59%, 50.7%, and 9.13%, respectively (Akinwumi et al., 2018; Kapetanovic et al., 2011; Lin et al., 2020). Moreover, the poor bioavailability of stilbenoids may be increased by their incorporation into nanoparticles, such as liposomes or cyclodextrins. Moreover, liposomal forms represent a variety of advantages, including their possible coupling with specific antibodies and thus their organ-specific targeting, or even liposomal forms designed for passage through BBB. Recently, liposomes have been proposed as a promising strategy for drug delivery in stroke (Bruch et al., 2019).

When focusing on the distribution, the maximal RSV concentration is attained rapidly in rodents, up to 10 min post-dose (Asensi et al., 2002), and parent compound together with its metabolic conjugates are undetectable after 1 h post-dosing (Yu et al., 2002). In humans, RSV seems to be quickly absorbed, with maximal peak concentrations (C_{\max}) between 0.83 and 1.5 h post-dose (Boocock et al., 2007a).

RSV, as well as other stilbenoids, undergoes fast metabolization, which firstly involves glucuronidation in the intestine. Therefore, RSV absorption from the intestine occurs mainly in the form of glucuronide (Kuhnle et al., 2000). The remaining RSV, absorbed as the aglycone, is further metabolized in the liver to 3-sulfates and glucuronides as well (Boocock et al., 2007b). The presence of a second peak of RSV in the plasma drug concentration-versus time profile observed *in vivo* (Marier et al., 2002) and *in clinic* (Walle et al., 2004), together with the predominant amount of RSV compared with its metabolites in the feces indicate that RSV may undergo the enterohepatic recirculation. Stilbenoids are predominantly excreted as the metabolites in the urine, but partly in feces (less than 1%) (Boocock et al., 2007a).

2.4 *Macaranga stilbenoids*

While RSV could be found in several plants and food and is well documented, other plants like these from *Macaranga* spp. may be an interesting source of stilbenes, even allowing the discovery of more active stilbene derivatives. *Macaranga* Thou. (Euphorbiaceae) is a large genus that comprises over 300 species widespread in the tropics of Africa, South-Eastern Asia, Indonesia, Australia and Oceania. The phytochemical screening of different *Macaranga* species demonstrated the presence of over 190 secondary metabolites, among them isoprenylated and geranylated flavonoids, prenylated stilbenes, terpenes, tannins, coumarins identified mostly in leaves and bark (Magadula, 2014). Stilbenes and flavonoids have been found in high amounts and are responsible for several biological activities, such as antioxidant (Lim, 2009), anti-inflammatory (Phommart et al., 2005), anti-microbial (Lim, 2009), anti-plasmodial (Zakaria et al., 2012) cytotoxic (Péresse et al., 2017), and anti-cholinesterase effects (Thanh et al., 2012). Apart from a small number of reported studies, some species of *Macaranga* genus, such that of *M. siamensis* S.J.Davies (Fig. 13) remain very low investigated to date. Therefore we gave us a task to investigate more in detail this unknown species, recently (2001) identified in Thai forest.

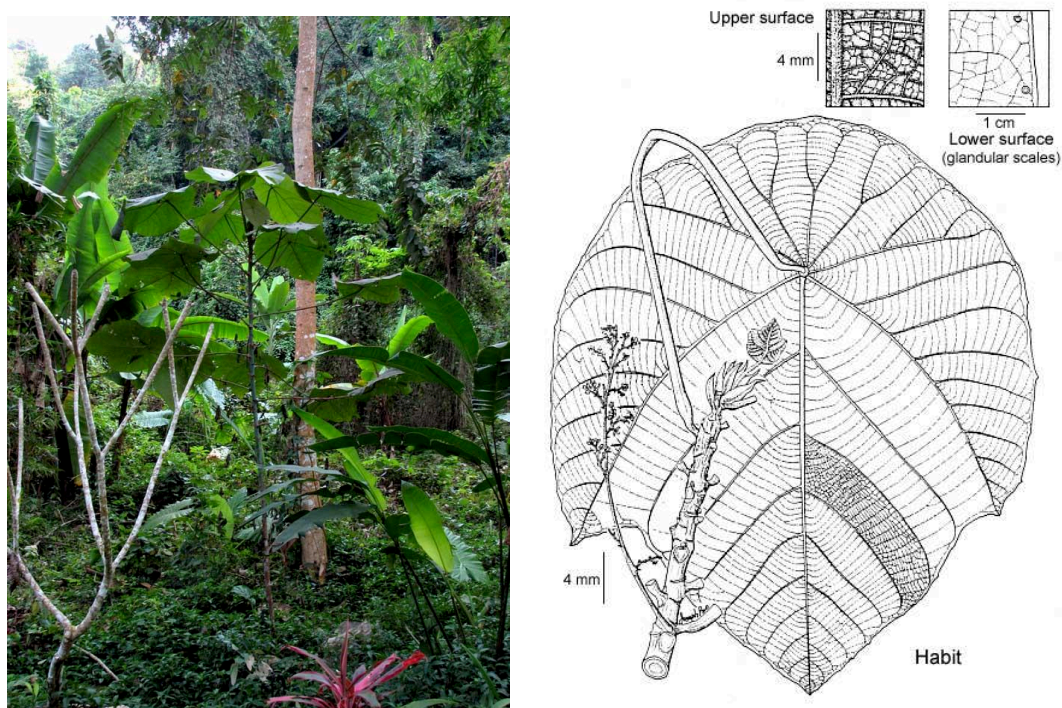


Fig. 13: *Macaranga siamensis* S.J.Davies (Euphorbiaceae), from (van Welzen and Chayamarit, 2020)

2.4.1 *Traditional use of Macaranga spp.*

Although *Macaranga* is the genus needed to be explored by scientists, it is well known and used in traditional medicine by indigenous people from native areas of these species. First scientific evidence about *Macaranga* use by aborigine people at islands in Oceania is dated to the years 1980 and 1983 when European expeditioners recorded the use of various species for the treatment of dysentery, diarrhea, and stomach complaints. *M. aleuritoides* F.Muell. fruits and seeds have been chewed to relieve abdominal pain (Holdsworth et al., 1983; Holdsworth, 1980). *M. harveyana* (Müll.Arg.) Müll.Arg. has been used in Tonga folk medicine for the treatment of gynecological and obstetric problems and *M. sampsonii* Hance has been administered for the treatment of cuts, sores, and swellings (Quynh et al., 2018).

However, the use of *Macaranga* is not only the subject of foregone practice, but several species are used also nowadays. The decoction from the root of *M. tanarius* (L.) Müll.Arg. is drunk in Malaysian and Thai folk medicine to treat fever and cough. Fresh leaves are applied for covering of wounds as a prevention and treatment of inflammation (Phommart et al., 2005). The young shoots are even eaten as a vegetable in Thailand, Indonesia, and Central Africa. In China, this plant is cultivated for manufacturing of commercial product, such as health drinks, extracts for toothpaste and herbal tea. Other species like *M. gigantea* (Rchb.f. & Zoll.) Müll.Arg. and *M. pruinose* (Miq.) Müll.Arg. are used for a treatment of fungal infections (Grosvenor et al., 1995).

The multiple-use in traditional Eastern medicines and mostly the use of *Macaranga* as a potent anti-inflammatory treatment aroused our interest to investigate the potential antiphlogistic effects of up to date very low investigated species of *M. siamensis* S.J.Davies, identified as a rich source of prenylated stilbenoids (Pailee et al., 2015). Among tested stilbenoids from this species on the model of systemic inflammation (Hošek et al., 2019) we found a promising candidate macasiamenene F (MF) for anti-inflammatory treatment.

Nevertheless, some stilbenoids have been identified at a preclinical level as potential treatments of CNS pathologies linked with inflammation, such as AD, traumatic brain injury, or ischemic stroke. Some stilbenes, such as RSV, pterostilbene, and pinosylvin have shown the ability to intervene in several molecular mechanisms of the

ischemic cascade and thus slow down the I/R-induced damage of brain tissue (Liu et al., 2020; Lopez et al., 2015; Xu et al., 2020). The growing evidence of studies in the last decade pointing out the neuroprotective potential of stilbenes in the brain pathologies motivated us to study the effects of this particular stilbene at CNS level, primarily its intervention into neuroinflammation and mechanisms of ischemic cascade.

3. ISCHEMIC STROKE

From all above-mentioned nervous system disorders linked with inflammation, stroke represents one of the most alarming health problems having an enormous clinical, social and economic impact. The incidence and burden of stroke has increased substantially over the last two decades because of the expanding and aging population. Despite decades of academic and industrial research, stroke remains a major cause of mortality and disability, annually affecting around 13.7 million people worldwide (Johnson et al., 2019). Approximately 30% of people die and another 30% stay permanently disabled. The high frequency of cases and the devastating consequences represent a high burden for the medical care system (Bourourou et al., 2016). The acute ischemic stroke that represents about 70–80% of cases is caused by vessel occlusion by a blood clot and leads to the lack of supply of oxygen and glucose into the affected area. The blood clot may be a result of thrombus in a brain vessel due to atherosclerosis or embolus coming from the systemic circulation (Fig. 14). In clinical practice, one of the most common types of severe ischemic stroke is the occlusion of the middle cerebral artery (MCA), therefore such an animal model was introduced in experimental methods. After MCA occlusion (MCAo), the ischemic damage will be more rapid and severe in the center of the infarcted territory called the ischemic core.

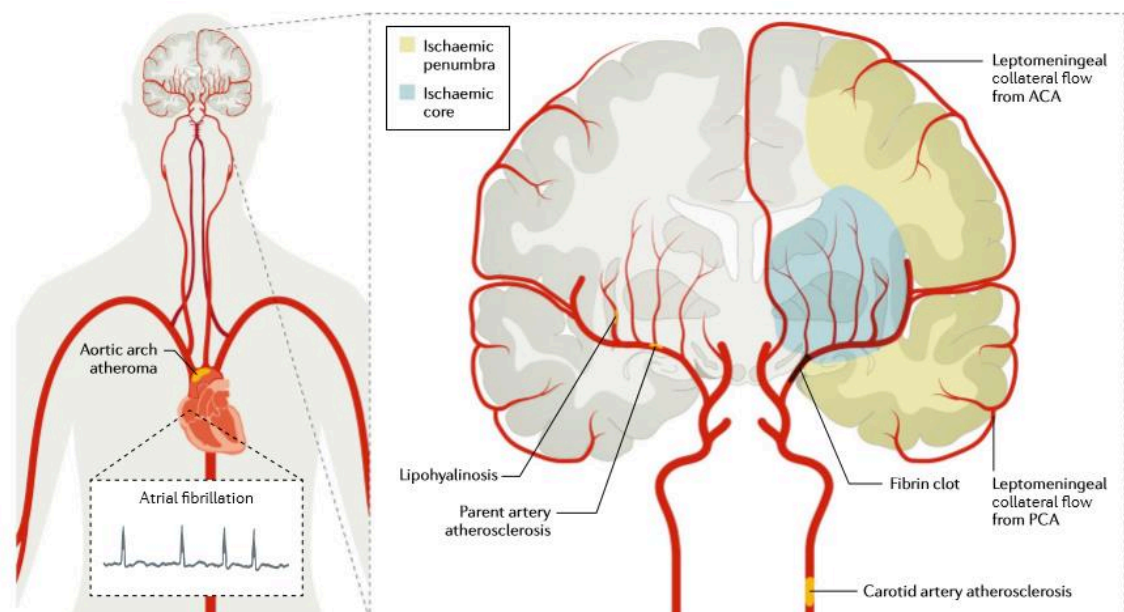


Fig. 14: Illustration of possible ischemic stroke mechanisms, e.g. cardioembolic stroke, carotid artery atherosclerosis, middle cerebral artery occlusion, and small vessel disease due to lipohyalinosis and parent vessel atherosclerosis. Regions without collateral blood flow become irreversibly injured. Adapted from (Campbell et al., 2019).

In the core, a bioenergetic failure develops after a few seconds of arterial occlusion, while ischemic cascade propagates into the periphery of the core region, called the penumbra. The neuronal damage in penumbra develops more slowly because blood flow from adjacent vascular territories (collateral flow) provides a barely acceptable cerebral perfusion, which is enough to avoid immediate cell death (Sas et al., 2017). Remaining 20–30% is restricted to hemorrhagic stroke due to a vessel rupture or leakage. The cause can be a trauma, uncontrolled high blood pressure, aneurysm, overdose with anticoagulants, or also ischemic stroke may lead to hemorrhage (Rymer, 2011).

3.1 Sequels of ischemic stroke

Stroke is the leading cause of disability worldwide. The localization and the volume of the infarct correlate with the long-term disability of over 80 million stroke survivors globally. These stroke survivors represent a high-risk population that also necessitates the prevention by secondary treatment strategies (Campbell et al., 2019). Clinically, many stroke victims suffer a variety of focal deficits and impairments on different levels, such as sensory, motor, cognitive, perceptual, and language functions (Mercier et al., 2001).

Among musculoskeletal complications, we understand possible hemiplegia (unilateral paresis of one entire side of the body) and reduced mobility, such as limb ataxia (loss of voluntary muscle coordination), numbness, weakness of the face muscles, ptosis (drooping of the eyelid), decreased reflexes (swallowing, pupil reactivity to light), and sensation. Typically, the symptoms are unilateral, affecting only one side of the body that is opposite the side of the brain lesion (Katan and Luft, 2018). This neuromuscular dysfunction can further cause pain syndromes, limb spasticity, tremor, and incontinence. Musculoskeletal pain affects mostly the shoulders, hips, but also other parts of the body. Incontinence of the bladder or bowel caused by hyperreflexia and urgency is also a frequent and significant indicator of poor clinical outcome that impacts the quality of life of stroke patients and may signalize the need for institutionalization (Patel et al., 2001; Weimar et al., 2002). However, case studies confirm that regular rehabilitation may lead to radical improvement of functional outcome and prognosis of patients after ischemic stroke (Paolucci et al., 2003).

Ischemic stroke can cause also sensory deficits, such as visual problems. Hemispatial neglect is one of them. It is defined as an inability of a person to process and

perceive stimuli on one side of the body or environment, in general contralaterally to the damaged hemisphere (Parton et al., 2004).

Cognitive impairment is another most common consequence of ischemic stroke. It includes apraxia, what is the inability to carry out the learned and purposeful activity. Apraxia is common for the left hemisphere stroke. It is estimated that approximately 30% of patients with left hemisphere stroke have apraxic impairment (Donkervoort et al., 2006). Dysarthria and aphasia are other common complications. Aphasia is characterized as an inability to comprehend or formulate language because of damage to specific brain regions. In the case study performed on 135 patients after ischemic stroke, approximately 18% of patients admitted to the rehabilitation unit suffered global aphasia. Complications with orientation, attention, and problem solving have been also unregistered (Paolucci et al., 2003).

Ischemic stroke affects patients also at the psychosocial level, causing anxiety and depression. Clinically significant post-stroke depression occurs in more than 30% of stroke patients. This depression is a consequence of cognitive impairments, but also physical disabilities and necessitates a secondary drug intervention (Paolucci et al., 2003).

Last but not least, several studies reported that patients after ischemic stroke are susceptible to post-stroke seizures and epilepsy. Whereas seizures are described as single or multiple convulsive episodes after stroke, post-stroke epilepsy is determined as recurrent seizures following stroke with a confirmed diagnosis of epilepsy. Myint et al., 2006, reported in their literature review the long-term risk of seizures and epilepsy. After first in life stroke, 11.5% of stroke survivors are at risk of developing post-stroke seizures by five years, while 3-4% of patients develop epilepsy (Burn et al., 1997; Myint et al., 2006). Hauser and colleagues studied the incidence of epilepsy and all unprovoked seizures in patient cases from 1935 to 1984, the cerebrovascular disease accounted for 11% of cases (Hauser et al., 1991).

3.2 Molecular mechanisms

The vessel occlusion and cerebral ischemia launch several interconnected molecular mechanisms what quickly result in ionic and metabolic disorders in neurons. The ischemic cascade is rapidly initiated within seconds to minutes after a loss of blood

flow in the specific area of the brain. This includes a series of subsequent biochemical events leading to neuronal death in the center (core) of the infarction and hypoperfused surrounding area called the penumbra. Ischemic cascade is swiftly diffusing and comprises several mechanisms, starting with a massive release of glutamate from post-synaptic neurons leading to excitotoxicity, following the oxidative stress caused by ROS, inflammatory response characterized by the release of pro-inflammatory cytokines and activation of apoptotic mechanisms resulting in neuronal death (Fig. 15). Additionally, destruction of microvessels and disruption of BBB accompanies all this detrimental process.

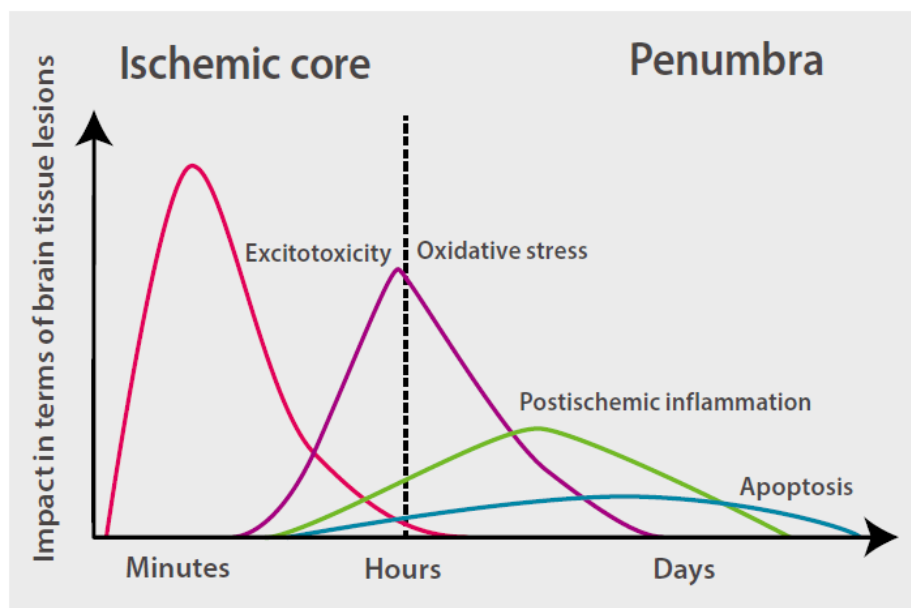


Fig. 15: The spatio-temporal evolution of the mechanisms involved in ischemic cascade from (Velly et al., 2018).

3.2.1 Excitotoxicity

Although the brain counts only 2% of the body weight of an adult, it consumes around 20% of the total energy produced. In comparison with muscles and liver, the brain is able to store only a very small amount of glycogen, predominantly in astrocytes, and therefore necessitates the constant supply of the energy in the form of glucose delivery by the bloodstream. In the further process of aerobic metabolism, the brain produces from glucose energy in the form of adenosine triphosphate (ATP) that is used for the right functioning of brain functions and mechanisms, including Na^+/K^+ -ATPase pump and $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) in the outer membranes of neurons. Transmission of a neuronal

signal is entirely dependent on the movement of Na^+ , K^+ , Cl^- and Ca^{2+} ions through cell membranes during an action potential. Under normal conditions, the concentration of Na^+ , Cl^- and Ca^{2+} is higher outside the cell, while K^+ ions are more concentrated inside the cell. This keeps a negative potential inside a cell. At the beginning of action potential, Na^+ ions enter into the cell through a sodium-channel during the phase called depolarization. This is followed by repolarization when K^+ ions flow out from the cell through a potassium-channel until the hyperpolarization. For the restoration of electrochemical potential, the Na^+/K^+ pump provides the efflux of Na^+ and the influx of K^+ . Because of a lack of oxygen during ischemia, neurons are not able to create ATP through aerobic metabolism. Without ATP, the Na^+/K^+ pump stops working, what leads to the accumulation of Na^+ and water in cells and results in cytotoxic edema (Brassai et al., 2015; Lai et al., 2014). An abrupt redistribution of ions across the membrane leads to ionic disruption and shutdown of neural activity (Lee et al., 2000).

Calcium is an important second messenger of the cells, its balanced concentration is necessary for the right functioning of signal transduction. In the absence of ATP, NCX normally bringing Na^+ into a cell and Ca^{2+} out of a cell, stops working as well. This flows up into the accumulation of Ca^{2+} inside the cell and causes the release of glutamate from presynaptic neurons. Glutamate as a primary excitatory neurotransmitter plays a critical role in the ischemic stroke. The massive release of glutamate from neurons (up to millimolar concentrations) is neurotoxic and causes the overactivation of ionotropic glutamate receptors, predominantly the *N*-methyl-D-aspartate (NMDA) receptor, but also α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptors. This leads to the excitation of other neurons and intracellular calcium overload. The elevated Ca^{2+} concentrations also activate degradative enzymes, such as proteases and lipases destroying cytoskeleton and cell membranes. This provokes the release of ROS and inflammation. Moreover, mitochondria activated by the environment start to produce apoptotic factors and calcium-dependent death-signaling proteins what flows in final to neuronal death (Blondeau, 2016; Lai et al., 2014).

Several studies suggest that stilbenes could attenuate neurotoxicity initiated by the overstimulation of NMDA receptors. In the HT22 neuronal cell model, RSV seems to exert the cytoprotective effect against glutamate-induced cytotoxicity in a dose-dependent manner (Kim et al., 2012). Li et al. (2010), demonstrated that chronic RSV treatment

(30 mg/kg intraperitoneally for 7 days) intensively inhibits the release of extracellular excitatory glutamate and aspartate during I/R in MCAo rats. Moreover, the basal levels of the inhibitory neurotransmitters GABA and glycine are enhanced during treatment with RSV, which can importantly contribute to protection against the excitotoxicity in following I/R period (Li et al., 2010).

3.2.2 Oxidative damage

Oxidative stress is the second major cause of brain damage after ischemic stroke. Deprivation of the brain from oxygen in ischemic stroke destroys every minute around 1.9 million neurons and ~14 million synapses. The lack of oxygen makes mitochondria unable to reduce O_2 to H_2O that supports ATP synthesis, the inevitable energy for a brain (Cobley et al., 2018). Under normal conditions, cells possess a complex antioxidative system comprising a variety of antioxidant enzymes and low molecular weight antioxidants located in mitochondria. Manganese superoxide dismutase (Mn-SOD, SOD2) was identified as a first-defense antioxidant enzyme of the mitochondrial matrix, that catalyzes the reduction of superoxide ($O_2^{\bullet-}$) radical to molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Another very important H_2O_2 generator in the cytosol and intermembrane mitochondrial space is a copper/zinc superoxide dismutase (Cu/Zn-SOD, SOD1) (Fukai and Ushio-Fukai, 2011). Superoxide radicals can be also removed by intermembrane cytochrome c playing role in the electron transport chain of mitochondria. Produced H_2O_2 can be neutralized to H_2O through glutathione (GSH) peroxidase (GSHPx), with the help of glutathione reductase (GR) regenerating GSH, and nicotinamide adenine dinucleotide phosphate (NADP⁺)/NADPH redox system (Ruszkiewicz and Albrecht, 2015). During ischemia, reactive molecules containing oxygen (ROS) and nitrogen (RNS) are produced and react with proteins, lipids, and DNA, which in turn activate apoptotic pathways (Wu et al., 2012).

There are several reasons why the brain is so fragile to oxidative stress. Firstly, the brain possesses less anti-oxidant enzymes than other organs. Secondly, there is plenty of easily oxidizable lipids in the brain, such as polyunsaturated n-3 fatty acids (notably DHA), that are very sensitive to lipid peroxidation leading to the generation of RO• (alkoxyl) and ROO• (alkoperoxyl) radicals (Allen and Bayraktutan, 2009). The brain is rich in several redox-active transition metals such as iron (Fe^{2+}) or copper (Cu^{2+}) that act as

enzyme co-factors. Iron is essential for myelin synthesis in oligodendrocytes (Connor and Menzies, 1996), $\text{Fe}^{2+}/\text{Fe}^{3+}$ system ensures the catalytic activity of several enzymes, including cytochrome P450. Cu^+ seems to be involved in cell signaling and neuronal excitability (Chang, 2015; Opazo et al., 2014), and copper is also an essential co-factor of enzymes cytochrome c oxidase (Ekici et al., 2014) and Cu/Zn-SOD (Harris, 1992).

During ischemia, mitochondrial Ca^{2+} overload induces $\text{O}_2^{\bullet-}/\text{H}_2\text{O}_2$ generation. Moreover, the excessive glutamate-induced Ca^{2+} release triggers nNOS-mediated NO^\bullet formation and further $\text{O}_2^{\bullet-}/\text{H}_2\text{O}_2$ generation (Fig. 16). Worthy to say, that neurotransmitters from the group of catecholamines, such as dopamine, adrenalin or serotonin may also under some circumstances auto-oxidize what renders the brain particularly sensitive to oxidative damage (Cobley et al., 2018).

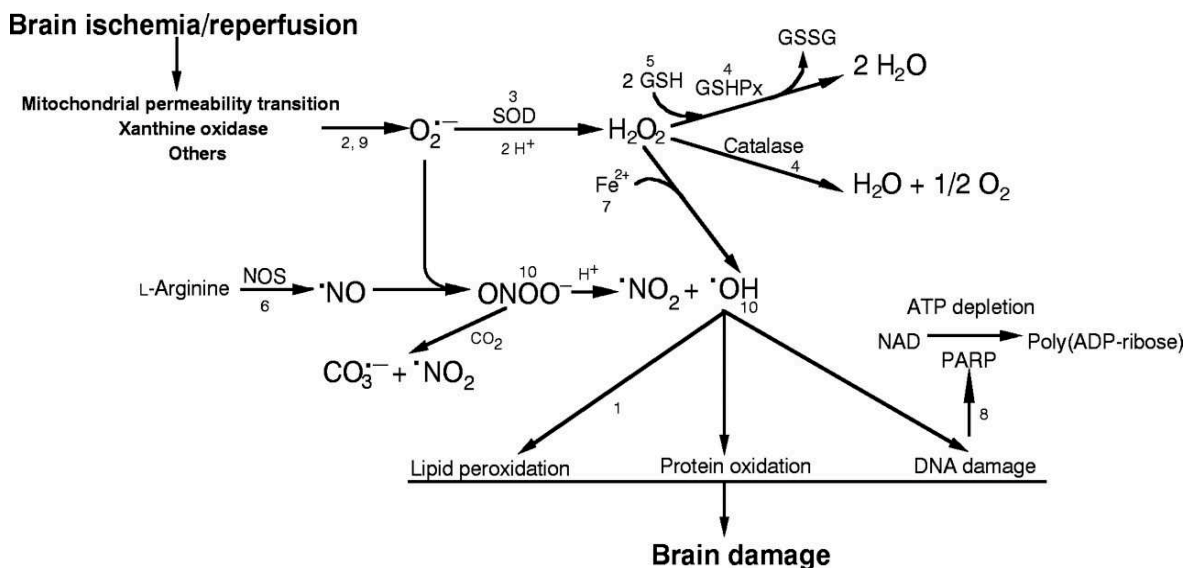


Fig. 16: Ischemia/reperfusion (I/R)-induced oxidative stress and site-specific targets for therapeutic intervention: 1/ inhibition of lipid peroxidation; 2/ inhibition of xanthine oxidase; 3/ superoxide dismutase (SOD), 4/ glutathione peroxidase (GSHPx), catalase, and 5/ glutathione (GSH) mimetics; 6/ nitric oxide synthase (NOS) inhibition; 7/ metal chelators; 8/ poly(ADP-ribose) polymerase (PARP) inhibitors; 9/ inhibitors of mitochondrial permeability transition; 10/ spin traps and peroxynitrite scavengers. $\text{O}_2^{\bullet-}$, superoxide; OH^\bullet , hydroxyl radical; H_2O_2 , hydrogen peroxide; GSSG, glutathione disulfide; NO_2^\bullet , nitrogen dioxide; NO^\bullet , nitric oxide; $\text{CO}_3^{\bullet-}$, carbonate radical; ONOO^- , peroxynitrite; NAD, nicotinamide adenine dinucleotide; from (Warner et al., 2004).

Unluckily, microglia can be also a source of free radicals, such as superoxide ($\text{O}_2^{\bullet-}$) ion generated via NADPH oxidase (NOX) isoforms (principally NOX2). NOX is a membrane-bound enzyme, abundantly expressed in phagocytic cells, including microglia

(Bedard and Krause, 2007). NOX-mediated ROS production may have evolved as a defense mechanism against invading bacteria, but it non-selectively damages also cells such as neurons (Dringen, 2005). NOX and ROS have been reported to be involved in the pathogenesis of cerebral ischemia injury, simultaneously NOX2 or NOX4 knockout mice exhibited lower brain injury after stroke (Walder et al., 1997; Wu et al., 2012).

RNA is equally susceptible to oxidation as DNA, undergoing analogous reactions. For example, 8-oxo-guanine is a principal outcome of both DNA and RNA oxidation (Hofer et al., 2005). Glucose can be also a factor having an impact on oxidative stress in the brain. When the brain lacks oxygen, anaerobic metabolism of glucose leads to the formation of lactate. Neurons constitutively degrade the glycolytic enzyme, phosphofructokinase, to preferentially use glucose to power the pentose phosphate pathway (PPP), that later leads to the formation of potentially toxic methylglyoxal which is more reactive than glucose and can glycate proteins, RNA, and DNA. Protein glycation is further linked with the formation of advanced end glycation products (AGE) which can trigger oxidative stress by stimulating inflammation, impairing protein and mitochondrial function (Cobley et al., 2018; Rabbani and Thornalley, 2008).

Altogether, several mechanisms have been identified to be implicated in oxidative and nitrate stress during cerebral ischemia what renders the brain extremely susceptible to oxidative damage. Therefore, an effective anti-oxidant strategy in the case of cerebral ischemia would be highly reasonable. There is numerous evidence about the antioxidant nature of stilbenoids (Frombaum et al., 2012; Trembl et al., 2019) being the main feature of compounds belonging to this group. Stilbenoids have been shown to affect several antioxidant mechanisms, including activation of Nrf2, SOD1/2, HO-1, or PPAR γ coactivator 1 α (PGC-1 α) modulating the anti-oxidant enzymes SOD2, thioredoxin, and glutathione peroxidase 1 (GPX1) (Lopez et al., 2015). More specifically, the studies on the rat model of focal ischemia showed that RSV inhibits the brain damage *via* regulation of nitric oxide mechanisms (Tsai et al., 2007). Another study on rat MCAo model showed the ability of RSV to upregulate the Nrf2/antioxidant response element (ARE) signaling pathway and expression of HO-1 and ameliorate the oxidative damage (Ren et al., 2011). Pinosylvin also improves I/R-induced mitochondrial dysfunction and decreases oxidative stress through Nrf2 and SOD upregulation, and inhibition of lipid peroxidation (Xu et al., 2020). All this scientific evidence credits stilbenoids as promising antioxidant agents

which could find interest in the prevention and as supplementary treatment of ischemic events in the brain.

3.2.3 *Inflammatory cascade*

Inflammation develops in the first minutes of cerebral ischemia and is initiated by massive ROS/RNS release and as a consequence of excitotoxic events in the area affected by vessel occlusion. Among all brain cells, neurons are much more vulnerable than glial or vascular cells and are first susceptible to become dysfunctional and die (Coyle and Puttfarcken, 1993). Cerebral ischemia triggers the activation of both, the innate (directly after) and adaptive immune system response in several days after vessel occlusion (D'Ambrosio et al., 2001). Post-ischemic inflammation is characterized by the activation of microglia, resident CNS macrophages, as well as the infiltration of systemic immune cells through disrupted BBB. The activity of these cells starting in initial stages of the lesion can hold over several days after the ischemic event (Wimmer et al., 2018). Moreover, ROS stimulate the pro-coagulant state comprising platelet, complement, and endothelial cells activation. Oxidative stress and inflammatory mediators cause increased permeability of BBB which starts to breakdown within 4-6 h after infarction (Lakhan et al., 2009).

Microglia represent the first line of defense, their role will be detailed later. In response to ischemia, microglia together with astrocytes develop an inflammatory phenotype and start to release the inflammatory factors such as cytokines, chemokines, ROS, and NO that will attract neutrophils, monocytes and lymphocytes. This is accompanied by up-regulation of cellular adhesion molecules (CAMs), especially intercellular adhesion molecule 1 (ICAM-1), P- and E-selectins, and enable the invasion and extravasation of the systemic immune cells into the brain (Sas et al., 2017).

BBB permeability allows the entrance of leukocytes that react on early changes of activated microglia, starting with the invasion of phagocytic neutrophils within the first 30 min, reaching a peak at 1-3 days. Post-mortem study on 16 patients confirmed the high concentrations of neutrophils in leptomeningeal and perivascular spaces, but a rare presence in the infarcted area (Zrzavy et al., 2018). Neutrophils are responsible for the recruitment of the other immune cells. As phagocytic cells, they possess the anti-microbial, pro-oxidants, and proteolytic enzymes which may contribute to brain damage (Planas,

2018; Wang et al., 2019). Their role in ischemic stroke was described mostly as detrimental, associated with larger infarct volume (Buck et al., 2008), and BBB breakdown (Jickling et al., 2015). Moreover, neutrophils can produce neutrophil extracellular traps (NETs) promoting clot formation (Martinod and Wagner, 2014). Their adhesion to the inflamed endothelium after I/R injury is also linked with a no-reflow phenomenon or microvascular obstruction which can lead to a significant reduction of perfusion and contribute to the extension of the ischemic lesion (del Zoppo et al., 1991).

Other important phagocytic cells infiltrating CNS within 24 h since the beginning of ischemia are systemic monocytes. Their maximal concentration in the ischemic area is detected in mouse/rat on the 4th day. Some monocytic populations persist for weeks and acquire features of tissue macrophages (Planas, 2018). Their role will be also detailed later.

Lymphocytes participate in ischemic cascade as well. The number of T cells grows up within the first 24 h after ischemia onset and may be present for a long time after. Their contribution to the ischemia has been described as mostly deleterious. In an effort to facilitate the adhesion of platelets and leukocytes to the vessels endothelium, they cause a “thromboinflammation” exacerbating the brain lesion (Kleinschnitz et al., 2010). Additionally, a subset of T cells, so-called gamma delta ($\gamma\delta$) T cells, has been identified as pathogenic in the experimental brain I/R because of secretion of IL-17 exacerbating the inflammatory response (Shichita et al., 2012). Other types of lymphocytes, such as regulatory T and B lymphocytes, display immunomodulatory and immunosuppressive functions. However, scientific reports do not coincide about their contribution to ischemic stroke (Planas, 2018).

Natural killers (NK) are other immune cells also participating in the ischemic cascade. Their number rapidly elevates after ischemic insult with a peak at 3 h and quickly declines. Studies report about their inflammation-promoting effects, but also about the production of IFN- γ improving post-stroke survival (Liu et al., 2017).

The above-mentioned immune cells contribute in different ways to the production and release of inflammatory mediators, such as cytokines and chemokines. TNF- α , IL-1 β , IL-6, IL-10, IL-20, and TGF- β are the most investigated cytokines associated with inflammation in acute ischemic stroke. Whereas TNF- α and IL-1 β appear to aggravate cerebral injury, TGF- β and IL-10 may be neuroprotective (Garcia et al., 2017; Zhu et al.,

2002). Nevertheless, the disproportional overproduction of pro-inflammatory factors than the anti-inflammatory ones is typical for severe infarctions leading to poorer clinical outcome. TNF- α produced by neuronal and glial cells is elevated in the infarcted area for hours up to days after stroke, depending on the severity of cerebral ischemic event and contributing to the neuronal tissue damage (Watters and O'Connor, 2011).

The increased IL-1 β mRNA expression is reported within the first 15-30 min after permanent MCAo with elevated IL-1 β protein expression few hours later, remaining up to 4 days and worsening cerebral infarction (Caso et al., 2007a). IL-1 β further induces IL-20, which in turn induces the production of IL-6 (Chen and Chang, 2009). In patients, the serum concentration of IL-6 has been correlated to the brain infarct volume (Acalovschi et al., 2003). Contrarily, IL-10 as the anti-inflammatory cytokine decreases IL-1 and TNF- α by suppressing expression of cytokine receptors and their activation (Lakhan et al., 2009). Monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein-1 α (MIP-1 α) are the most studied chemokines increasing leukocyte infiltration. They have been identified to have a detrimental effect on focal ischemia (Kim et al., 1995; Lakhan et al., 2009). The MMPs, especially MMP-2 and 9, proteins regulating several functions related to inflammation were identified to be involved in ischemic stroke as well. Park et al., 2009, demonstrated the up-regulated levels of MMP-9 in the serum of patients at 12 h after ischemic onset (Park et al., 2009).

3.2.4 Implication of monocytes and microglia

Stroke is no exception to the previously described rule of DAMPs activation of macrophages such as microglia and monocytes *via* their PRRs, including TLR2 and TLR4 that seem to play an inevitable role in cerebral ischemia. *In vivo* studies demonstrated much smaller brain infarctions in mice knock-out for TLR4 receptors, better outcomes, and less inflammatory response after the ischemic insult (Caso et al., 2007b; Gesuete et al., 2014; Sansing et al., 2011). Clinical trial involving 110 patients with ischemic stroke found out that a high expression of TLR2 and TLR4 at 24 h, 72 h, and 7 days after stroke were associated with a poor clinical outcome and correlated with higher serum levels of TNF- α , IL-1 β , and IL-6. Moreover, the same study showed the correlation between TLR4 expression and the volume of infarct. Patients with reduced expression of TLR4 exhibited lower volume of infarct and better outcome (Brea et al., 2011).

Microglia as resident immunocompetent and phagocytic cells of CNS are activated and recruited to the affected area directly after the ischemic onset. Their massive accumulation has been observed after 72 h persisting to 30 days in the core of infarction, contralateral hemisphere, and the peri-infarct zone of stroke patients (Price et al., 2006). The activated microglia undergo a series of morphological and functional changes. They proliferate, retract their ramified extensions, become motile and more amoeboid in shape, and develop the ability to phagocytize debris (Stence et al., 2001). The activated microglia, according to their environment, adapt the pro- (M1) or anti-inflammatory (M2) phenotype, as described previously. Their role in the ischemic stroke seems to be double-edged – protective and deleterious. Whereas M2 phenotype is present in the ischemic area mostly in the early state of stroke, this one is rapidly replaced by M1 phenotype contributing to the ischemic injury (Hu et al., 2012). NF- κ B plays an important role in the activation of M1 phenotype (Yu et al., 2020). Ischemia-activated microglia release a plethora of substances many of which are cytoprotective and/or cytotoxic and through which they can influence neuronal functions and viability (Ransohoff and Perry, 2009). M1 microglia are responsible for the production and release of cytokines such as TNF- α , IL-1 β , and IL-6, as well as other potential cytotoxic molecules including NO, ROS, and prostanoids (Lucas et al., 2006). Moreover, cytokine generation may represent an important factor in the suppression of neurogenesis (Monje et al., 2003). Nevertheless, microglia may display neuroprotective action by producing neurotrophic molecules such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) supporting neuronal survival (Lakhan et al., 2009). In comparison with other cells in CNS, microglia are major source of insulin-like growth factor 1 (IGF-1), which promotes neuronal survival, inhibits M1 microglial neurotoxic phenotype, promotes M2 microglial repair/regenerative phenotype, as well as M1 transition to M2 and the astrocytic response to inflammatory stimuli of M1 microglia (Labandeira-Garcia et al., 2017). Microglia and astrocytes are able to partly counteract the glutamate-mediated neurotoxicity by expressing the glutamate transporter 1 (GLT-1) removing excess glutamate from the extrasynaptic space (Ouyang et al., 2014; Persson et al., 2005; Shaked et al., 2005).

The co-culturing of activated microglia with ischemia-injured neurons results in the elevated release of proinflammatory cytokines, such as TNF- α , NO and H₂O₂ generation, while the inhibition of microglial activation results in the protection of neurons after

ischemic insult. These results indicate that inhibitors of microglial activation could represent a potential treatment of cerebral ischemia (Ma et al., 2017; Xu et al., 2016). Nonetheless, increasing evidence has shown that activated microglia may display also beneficial effects by attenuating neuronal apoptosis, enhancing neurogenesis and promoting functional recovery after cerebral ischemia. The dual role of microglia is dependent of the timeframe after stroke (Yu et al., 2020).

Importantly, the disrupted BBB may give access to chemokine-mediated systemic monocytes infiltration into the brain parenchyma, as described more in detail in the chapter 1.5 (Inflammatory mediators) (Planas, 2018). In the experimental ischemia model, monocytes-derived macrophages may help to the resolution of inflammation, but also promote inflammation. The highly elevated accumulation of circulating monocytes is recorded in patients with stroke-associated infection (SAI), what represents 23–65% of stroke patients. These bacterial infections, especially pneumonia, have been linked with an extent immunosuppression attributed to the stress hormones release during and after stroke. Those patients are more susceptible to post-stroke disability or even death (Chamorro et al., 2006; Vargas et al., 2006; Vermeij et al., 2009; Vogelgesang et al., 2014). This also underlines a thin boundary and possible crosslink of sterile (aseptic) and septic inflammation.

Other phagocytosing cells, monocytes, fighting an ischemia-induced inflammation, differentiate to macrophages and similarly to microglia undergo a classical (M1) pro-inflammatory or alternative (M2) anti-inflammatory phenotype depending on the environmental stimuli (Anrather and Iadecola, 2016). M1 phenotype is associated with elevated anaerobic glycolysis and activation of the hypoxia-inducible factor-1, while the energy production in M2 phenotypes rather relies on fatty acid oxidation. In M1 activated macrophages, arginine metabolism occurs through inducible nitric oxide synthase (iNOS) leading to generation of ROS and RNS that damage proteins, lipids, and DNA. Contrarily, M2 macrophages metabolize arginine through Arg-1 generating polyamines involved in cell division and collagen synthesis without ROS/RNS production (O'Neill et al., 2016). However, the factors contributing to the time-dependent differentiation to M1/M2 phenotype in the ischemic stroke has not been well clarified yet. Study on CD11b-DTR transgenic mice demonstrates that macrophages infiltrating the ischemic lesion contribute to neuroprotection in the early stage after the ischemic insult by adopting M2 phenotype

and depletion of macrophages exacerbates the pro-inflammatory state and ischemic lesion (Perego et al., 2016). Later study on humans having suffered ischemic stroke suggests that recruited macrophages primarily display proinflammatory features that with lesion maturation transform into intermediate pro- and finally anti-inflammatory phenotypes (Zrzavy et al., 2018). Monocytes also participate in tissue repair, as well as repair of the neurovascular unit. In acute stroke, their number in peripheral blood increases, leading to such phenotypic changes as attenuated expressions of antigen-presenting molecules, and lower production of pro-inflammatory TNF- α , while production of anti-inflammatory IL-10 stays unchanged. Their function also consists in the clearance of debris and damaged cells at later stages being part of a regenerative process (Harms et al., 2008; Klehmet et al., 2009).

Worthy to mention that monocytes/macrophages play a decisive role at all stages of atherosclerosis, the main risk factor of cerebral ischemia. They take part in the formation of unstable atherosclerotic plaques by phagocytosis and the ingestion of the oxidized low-density lipoproteins. They further maintain a local inflammatory response by secreting pro-inflammatory cytokines IL-1 β , TNF- α , and TGF- β , chemokines, and ROS. This leads to endothelial dysfunction. Dying macrophages are further responsible for the formation of necrotic core in progressing plaques (Bobryshev et al., 2016; Chiba and Umegaki, 2013).

3.2.5 Cellular death by necrosis and apoptosis

After cerebral infarction, cells in the affected area die by both mechanisms, necrotic and apoptotic cell death. Whereas severe insults lead to the necrosis observed in the early states, mild injuries trigger the activation of apoptotic factors, and cell programmed death occurs in the latest period. The percentage of cells dying by apoptosis vs. necrosis depends on several factors including the severity of insult, localization, and duration of ischemia (Bramlett and Dietrich, 2004; Gobbel and Chan, 2001).

Mitochondria have been described to be involved in both the necrotic and apoptotic pathways. In most cases, severe cerebral ischemia renders the mitochondria completely dysfunctional for ATP production. As described previously, this leads to glutamate excitotoxicity, which is the main cause of necrotic cell death. Necrosis occurs chiefly in the ischemic core suffering a severe insult and is associated with swelling of cells and organelles leading to plasma membrane failure (Lee et al., 2000).

Most of the apoptotic cell death takes place during the spread of the damage in the penumbra, starting with oxidative stress signaling directly after cerebral infarction. ROS generated within mitochondria cause the release of cytochrome c to the cytosol by mechanisms that may be related to the Bcl-2 family proteins, such as Bcl-2, Bax, etc. Released cytochrome c binds to the apoptotic protease activating factor 1 (Apaf-1) and caspase-9 to form a complex that subsequently activates caspase-3 and other caspases, such as caspase-2, -6, -8, and -10. Activated caspase-3 is known to cleave several nuclear DNA repair enzymes, including PARP and to activate the caspase-activated DNase (CAD), which then leads to nuclear DNA damage without repair, resulting in apoptosis (Sugawara et al., 2004).

Some scientific evidence suggests that RSV could affect also apoptotic/survival signaling pathways. RSV prophylactic treatment (30 mg/kg intraperitoneally during 7 days) prevented hippocampal neurons against I/R injury in rats that underwent global ischemia by parallel modulation of GSK-3 β and CREB through PI3-K/Akt pathways (Simão et al., 2012).

3.3 Medical and paramedical interventions

Ischemic stroke is highly severe and complex pathology, its onset may be triggered by an abrupt event such as sports injury, but in most of the cases it is a consequence of long-term ongoing associated diseases that may be caused by an unhealthy and stressful lifestyle. Therefore, the prevention and treatment of stroke necessitates a plethora of non-medical approaches and medical interventions, respectively. These approaches could be divided as precautions to prevent the stroke onset (primary prevention), to treat stroke (interventional therapy), and prevent the recurrence of stroke (secondary prevention).

3.3.1 *Primary prevention*

The control of major risk factors such as hypertension, atherosclerosis, DM, and high cholesterol levels is important to prevent ischemic stroke. This can be partially achieved by healthy lifestyle and balanced nutrition (Bourourou et al., 2016). Avoiding smoking, excessive alcohol and sugar consumption, as well as reducing daily stress count among preventive actions. Healthy nutrition plays a chief role in the prevention of major stroke risk factors – DM2, high LDL cholesterol, and atherosclerosis. Moreover, befitting

physical activity minimizes a risk of obesity that could contribute to hypertension, another important risk factor of stroke (Campbell et al., 2019).

3.3.2 *Interventional treatments*

Ischemic stroke is a pathology that needs to be treated without delay. Treatment of acute ischemic stroke (AIS) necessitates a very complex and individual medical approach aiming at the restoration of blood flow and the prevention of severe sequels. The intravenous administration of tissue plasminogen activator (tPA) is the only FDA-approved AIS therapy. However, the majority of AIS patients does not receive thrombolytic treatment because of a large infarct development linked with a late arrival to emergency and increased risk of hemorrhage after thrombolysis or other possible contraindications (Agyeman et al., 2006; Reiff and Michel, 2017; van den Berg and de Jong, 2009). Approximately, only 15–20% of patients are eligible for thrombolysis and even lower percentage receive the treatment (Campbell et al., 2019). Management of high blood pressure, control of respiratory functions, maintaining normoglycemia and euthermia of the hospitalized patient are further necessary matter-of-course steps (Goldstein, 2014).

tPA is an enzyme catalyzing the conversion of plasminogen to plasmin, which further degrades fibrin and dissolves the thrombus. Plasmin is rapidly inactivated by antiplasmin and, therefore, has a short half-life outside the thrombus. tPA is commercially manufactured using recombinant biotechnologies and referred to as recombinant tPA (rtPA), includes alteplase and tenecteplase. The time window for thrombolysis by alteplase is 4.5 h after vessel occlusion (Lees et al., 2010). It is generally carried out as an initial bolus followed by an one hour infusion. Some reports emphasize the combination therapy of thrombolytics with neuroprotectants, such as NMDA (Zivin and Mazzeo, 1991) and AMPA receptor antagonists (Meden et al., 1993), calcium channel blockers (Horn J. and Limburg M., 2001), MMPs inhibitors (Sumii and Lo, 2002), anti-oxidant agents, citicholine (Andersen et al., 1999), or topiramate (Yang et al., 2000). These molecules could have synergistic or additive effects, possibly reduce the dose of both combinational treatments, thus minimizing the side effects (Lo et al., 2003).

Tenecteplase, another type of rtPA, is FDA-approved treatment of myocardial infarction. Its advantage is greater fibrin specificity and longer half-time than alteplase what allows the administration as a single bolus. Its efficacy and safety had been evaluated

to be similar or superior to alteplase (Coutts et al., 2018). Moreover, clinical studies have shown improved reperfusion and better clinical outcome (Bivard et al., 2017; Campbell et al., 2018). This treatment is now licensed for the treatment ischemic stroke in India only, but there are seven ongoing clinical assays ending between 2020 and 2025 which should confirm/disprove its usefulness in the treatment of ischemic stroke (Campbell et al., 2019).

To improve time-sensitive stroke care delivery, mobile stroke units (MSUs) were developed to revolutionize the emergency stroke care. Such an innovative approach is already available for patients in the US, Germany, and Australia. MSUs are prehospital ambulances, fully equipped to perform a patient examination and diagnostic testing, including tomography/angiography, that are necessary to diagnose and initiate thrombolysis within the “golden hour” (first 60 min) prior to transporting to the closest medical establishment (Ehnholt et al., 2020; Phan et al., 2019; Reimer et al., 2020).

Endovascular thrombectomy (ET) is another procedure accepted as standard care for patients with proximal anterior artery occlusion. This approach comprises a surgical removal of a thrombus by a stent retriever by image-guided surgery. ET can be provided within 12 h after ischemic stroke. It has very low contraindications and risks. Moreover, ET is convenient also for elderly patients ineligible for *i.v.* thrombolysis due to a risk of systemic bleeding (Campbell et al., 2019). ET supplementary treatment with neuroprotective agents such as bioactive stilbenoids could represent a reasonable strategy preventing post-stroke extensive neuronal damage and improving recovery.

Although the early reperfusion of the occluded vessel can reduce the brain damage, its effectiveness is constrained by a bulk of secondary mechanisms triggered in the ischemic penumbra. All these mechanisms triggered by excitotoxic, oxidative and inflammatory state, initiated after the oxygen and glucose deprivation continue to impact the affected area and may results in tardive neuronal death (Ahmad and Graham, 2010). Therefore, minimization of deleterious effects of cerebral ischemia by an efficient neuroprotectant is highly demanded and opens a window for development of a supplementary neuroprotective agent. Neuroprotection is defined as whatever strategy that could protect CNS and antagonize or at least slow down detrimental effects caused by an acute or chronic brain injury, as well as promote a brain repair (Heurteaux et al., 2013;

Vajda, 2002). For some proselyte insiders it represents only strategies directed at targeting the neuronal cells (Moskowitz, 2010; Tuttolomondo et al., 2009).

More than one thousand molecules have been tested in preclinical assays during the last 60 years, from which around 120 proceeded to clinical trials, and most of them failed. The reasons are numerous: poorly reproducible preclinical experiments, failure of the drugs to penetrate the BBB and heterogeneous clinical trial populations with unpredictable timing, dosing, and extent of reperfusion (Moskowitz, 2010). Several reports highlight that translation of results from experimental models to the clinics is highly nonlinear and unpredictable (Lo, 2008). Therefore, guidelines for preclinical experiments, so-called Stroke Therapy Academic Industry Roundtable (STAIR) preclinical recommendations were introduced in 1991 and later updated to improve the quality of preclinical experimental procedures. STAIR recommendations include dose-response assessment, definition of maximal tolerated dose, description of the time window in well-characterized models, evaluation of histological outcomes and behavioral changes in acute state and long-term (at least 2 weeks and longer) period, and point out on reproducibility of assays in different laboratories before proceeding to clinical trials. Published articles should detail calculations of sample size, inclusion/exclusion criteria, randomization, and others, to overcome the neurocentric view of cell death and consider also the response of other brain cells on ischemic injury affecting the neuronal survival and death (Fisher et al., 2009; Stroke Therapy Academic Industry Roundtable (STAIR), 1999).

The interfering peptide nerinetide (NA-1) is the only neuroprotectant which advanced to Phase III of clinical studies over the last 20 years. Nerinetide has been shown as promising agent dissociating *in vitro* and *in vivo* the NMDAR-PSD95-nNOS complex, thus interrupting downstream glutamate-induced neurotoxic signaling without impairing a normal NMDAR synaptic transmission or calcium influx (Ballarin and Tymianski, 2018). Unfortunately, very recent results of the study on 1105 patients undergoing ET (eventually with alteplase treatment) did not prove better functional outcomes in patients with a single dose of 2.6 mg/kg nerinetide supplementation (<12 h) in comparison with the placebo group (Hill et al., 2020). However, this exploratory study disclosed also that 40% of patients without alteplase treatment but treated with nerinetide achieved better functional independence and lowered mortality in comparison with the placebo group. Pharmacokinetics studied on a small group of patients revealed reduced plasma levels of

nerinetide in patients with alteplase treatment, what can be explained by the feature of alteplase to cleave peptide bonds not only in fibrin but possibly also in nerinetide molecule (Hankey, 2020).

One of the other auspicious molecules, free radical scavenger NXY-059 has been also showed ineffective in clinical trials (Shuaib et al., 2007). Another radical scavenger edaravone is used in Asia, but there is missing a high-quality clinical evidence in non-Asian populations (Feng et al., 2011).

Despite all, stroke remains intricate human pathology, and targeting only one mechanism does not have to be sufficient. An effective stroke neuroprotectant should be ideally able to counteract several pathways of ischemic cascade, such as release of excitotoxic agents, oxidative stress, and inflammation which lead to neuronal damage and death. It is generally assumed that such a neuroprotective therapy must be started within hours after stroke onset before neuronal tissue becomes irreversibly damaged (Heurteaux et al., 2013; Neuhaus et al., 2017).

3.3.3 Secondary prevention

Secondary prevention means a medical treatment administered to prevent the recurrent stroke after the first-in-lifetime stroke. Study on humans has revealed that the risk of the recurrent stroke is near 6% up to 1 year and almost 17% up to 5 years after stroke. However, the origin of this recurrence is multifactorial, depending also on other comorbidities (Hillen Thomas et al., 2003). Patients after ischemic stroke have a particularly high risk of deep venous thrombosis, pulmonary embolism, and urinary infections which occur in 30–60 % of patients (Goldstein, 2014). Pharmacological prophylaxis with antiplatelets agents such as acetylsalicylic acid, clopidogrel, or anticoagulants in injection or oral form, like unfractionated and low molecular weight heparins, warfarin, dabigatran, apixaban, and rivaroxaban reduces the risk of venous thromboembolism. Blood pressure-lowering agents together with statins are other major prescribed drugs of the secondary prevention (Bansal et al., 2013; Campbell et al., 2019).

In any case, the change in lifestyle is an unavoidable step to be undertaken to minimize the risk of recurrent stroke that should not be underestimated. This includes primarily a radical change in the human diet, but also the restrain of alcohol intake, smoking, and increase of physical activity. Several authors suggest MedDiet modest on

saturated fatty acids, but rich on omega-3 and -6, vitamins, minerals, and polyphenols, like stilbenoids as a pattern for a healthy lifestyle, cardiovascular health, and longevity. MedDiet has been proposed also as a balanced nutrition for the prevention of stroke, whereas two studies on humans confirmed that low adherence to MedDiet is associated with a higher incidence of stroke (Paterson et al., 2018; Tsivgoulis et al., 2015). Stilbenoids are natural polyphenols present in food to be a part of MedDiet, such as grapes, peanuts, berries, beverages (white tea, red wine), and many medicinal plants. Stilbenoids were shown to be potent antioxidants and anti-inflammatory agents. There is scientific evidence about their vessel endothelium protective and anti-inflammatory effects in atherosclerosis, one of the major risk factors of stroke (Akinwumi et al., 2018). Moreover, stilbenes possess cardioprotective and neuroprotective properties which may represent an important approach in the prevention of cardio- and cerebrovascular diseases linked with strong inflammatory component and oxidative stress (Dvorakova and Landa, 2017). The most known stilbene RSV is commercially produced nutraceutical available in Asiatic countries and Europe as well. RSV use is recommended mostly for protecting the cardiovascular system, lowering cholesterol, and protecting against blood clots formation (due to its anti-COX-1 effect) which may be a cause of heart attack and stroke. Its potential health benefits have been attributed to its potent antioxidant and anti-inflammatory potential, although its beneficial effect in humans has been questioned (Akinwumi et al., 2018; Salehi et al., 2018). Several reports identified plant-based (chia, flaxseed, vegetal oils) omega-3 polyunsaturated alpha-linolenic acid (ALA) as a very potential nutraceutical to protect the brain against I/R induced injury. Its effects are pleiotropic, including direct neuroprotection, vasodilation of brain arteries, promotion of neuroplasticity, enhancement of motor coordination and cognitive functions (Blondeau et al., 2009; Bourourou et al., 2016; Heurteaux et al., 2006).

Plant and animal extracts contained in NeuroAiD (MLC601/MLC901) formulae have also shown *in vivo* pharmacological efficiency consisting in the prevention of neuronal death, lowering infarct size, facilitation of post-stroke recovery and stimulation of neurogenesis (Heurteaux et al., 2010, 2013). Clinical studies confirmed the safe profile of NeuroAiD in the long-term use (Young et al., 2010), and efficiency of 3-months MLC601 treatment embodying in achievement of better functional independence at long duration

points of 6 months persisting up to 18 months after stroke (Venketasubramanian et al., 2015).

Both ALA and NeuroAiD emphasize the interest and the potential of plant-derived molecules. Stilbenoids are another group of natural compounds arousing interest. They are not only powerful antioxidants and antiphlogistic agents, but some compounds from this group, such as RSV, display also potential to antagonize glutamate-induced damage, prevent mitochondrial dysfunction, reduce apoptotic neuronal cell death, as well as diminish lipid peroxidation and neuroinflammation (Fig. 17) (Singh et al., 2013).

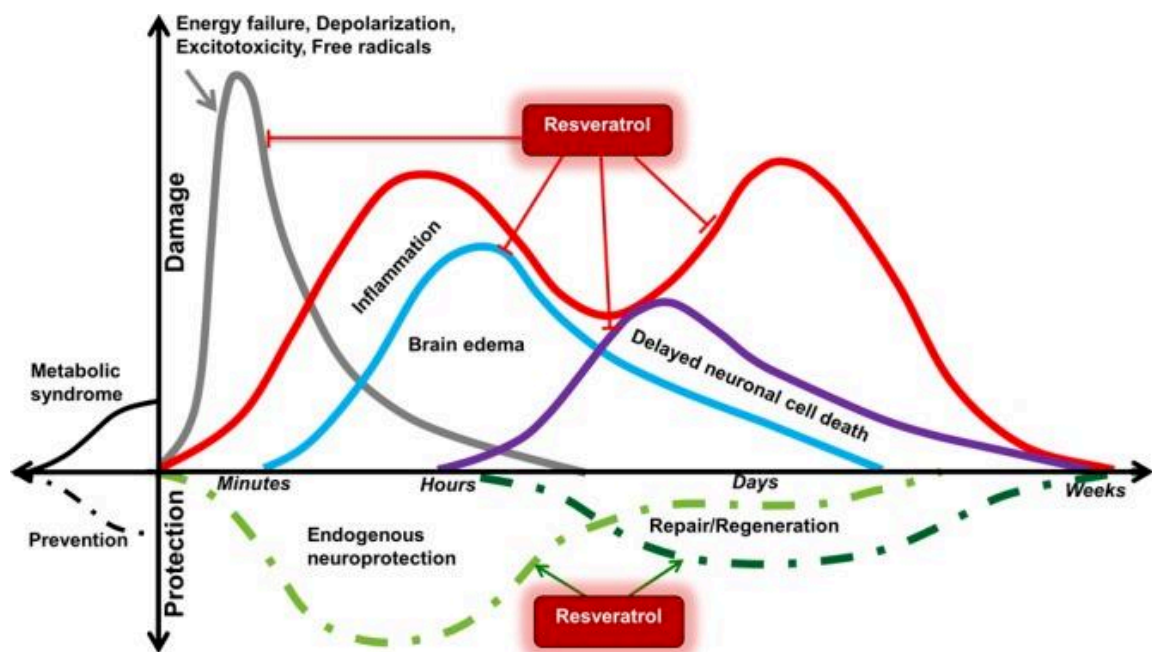


Fig. 17: Intervention of RSV in the several mechanisms of ischemic cascade from (Singh et al., 2013)

This led us to explore another low investigated particular stilbene macasiamenene F (MF) originated from Thai plant *M. siamensis*. In set of *in vitro* and *in silico* experiments performed by our group, this stilbene exerts greater anti-inflammatory effects than RSV itself and comparable to better effects than commercially widely used corticoid drug prednisone. These encouraging results motivated us to investigate if MF could counteract also other pathways of the ischemic cascade, such as oxidative stress and glutamate-induced neuronal death, as well as interrupt the detrimental effects of oxygen-glucose deprivation observed during stroke.

Despite very promising preclinical results, low RSV bioavailability associated with its poor solubility in water complicates the drug development and questions its efficacy. Unfortunately, this is the case of approximately 40% of molecules on the market and around 90% of compounds in drug discovery are poorly soluble in water (Kalepu and Nekkanti, 2015), and MF is not an exception. This may represent the reason why promising molecules failed in preclinical and latter clinical trials. Moreover, in the case of brain disorders, the limited entry into the brain parenchyma and adverse side effects linked with a nonspecific distribution are other complicating factors. Nanocarriers, such as liposomes may represent a solution for both – improvement of molecule solubility and targeted delivery to the desired area, including circumventing BBB and reducing adverse effects. The poor water solubility of MF could complicate the *in vivo* administration, we therefore decided to prepare liposomal formulations as a way of MF solubilization and targeted delivery to the brain.

4. LIPOSOMAL STRATEGIES – perspective in treatment of stroke

Liposomal nanocarriers represent a smart strategy for encapsulation of water-insoluble or poorly soluble molecules (Lee, 2020) and targeted drug delivery in the desired body area. Some liposomal formulations, such as anticancer drugs, vaccines, or vitamins are already marketed. Thanks to their numerous advantages, liposomes have been proposed also as future drug delivery systems for the treatment of stroke (Bruch et al., 2019).

Liposomes can be formulated in different size, composition, and lamellarity according to the target of their application. According to the type of used lipid, liposomes may have neutral character, or be positively or negatively charged. Realizing that eukaryotic cell membrane is negatively charged due to anionic phospholipids such as phosphatidylserine, this feature importantly influences the passage of liposomes into cells (Platre and Jaillais, 2017). Their surface can be further variably modified, coupled with specific antibodies and ligands for organ-specific targeting. They may accommodate both hydrophilic drugs in their aqueous core and hydrophobic drugs within their lipid bilayer (Fig. 18) (Zylberberg and Matosevic, 2016).

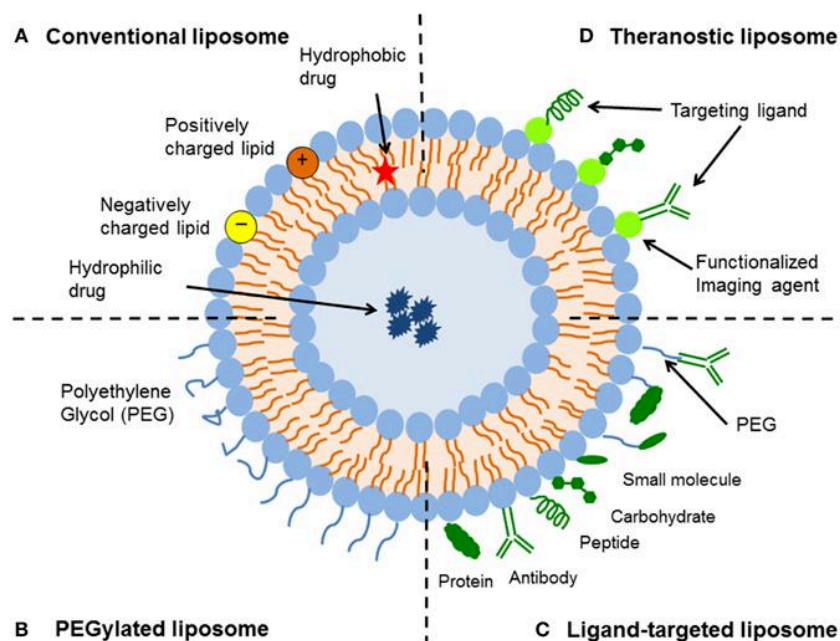


Fig. 18: Schematic presentation of the different types of liposomes. A. Conventional - consists of neutral, cationic, or anionic phospholipids and cholesterol forming lipid bilayer. B. PEGylated - coated with hydrophilic polymer polyethylene glycol (PEG) that changes liposome features and behavior in vivo. C. Ligand-targeted liposome- contains attached specific ligands (e.g., antibodies, peptides,...) for tissue-specific targeting. D. Theranostic - hybrid liposome for cell therapy and bioimaging, contains a therapeutic and imaging component; from (Sercombe et al., 2015).

Among various types of nanoparticles (e.g. micelles, polymer carriers,...), liposomes have been the most extensively studied due to their low toxicity, biocompatibility, and biodegradability. Other advantages are improved stability and protection of the drug from degradation in a biological environment, increased circulation time in the bloodstream, and accumulation in the target tissue. They are also able to efficiently mask therapeutic compounds and decrease their toxicity. Altogether, this leads to enhanced efficacy of drugs and improved therapeutic index. The most important factor in our case is possible passage of liposomes through the BBB (Bruch et al., 2019). Surface modification with polyethylene glycol (PEG) masks the recognition and phagocytosis by systemic macrophages what results in the prolonged systemic circulation (Niidome et al., 2006). Moreover, PEG-liposomes up to 200 nm have been recognized by several studies to accumulate in the ischemic tissue, what can represent a promising strategy for the treatment of cerebral ischemia triggered by vessel occlusion in stroke (Fukuta et al., 2016).

Passive transport of conventional liposomes with incorporated polymers such as PEG, polyvinylpyrrolidone, polyacrylamide, through BBB is based on the enhanced permeability and retention (EPR) effect which is linked with pathophysiological conditions, such as increased BBB permeability after stroke. Polymers incorporated within a lipid bilayer provide a protective and hydrophilic surface that prevents the recognition of liposomes by opsonins, extends the liposomal half-life, and reduces their clearance by the reticuloendothelial system (RES) of macrophages (Fukuta et al., 2016, 2015; Huwyler et al., 2008). For active transport through BBB, the coupling of small-molecule ligands, peptides, monoclonal antibodies (mAbs) on the liposomal surface, such as mAb OX26 targeting of transferrin receptors on the endothelium of brain capillaries is necessary (Jefferies et al., 1984).

Liposome reaching the intended diseased area interacts with the cell surface and may directly fuse with cell membrane releasing the entrapped drug. Another possible mechanism of delivery based on pH is called diffusion, or liposome may enter to cell also by targeting endocytosis in phagocytic cells. In this case, macrophages engulf liposome forming phagosome that fuses with a lysosome, creates phagolysosome inside which the liposome is lysed and the drug is then released (Chatin et al., 2015; Yang et al., 2016).

A number of liposomal formulations have found their application in human use, mostly in the treatment of cancer: chemotherapeutics doxorubicin, daunorubicin in the treatment of ovarian cancer and AIDS-related Kaposi's sarcoma, respectively, combination therapy of fluorouracil and leucovorin in the treatment of pancreatic adenocarcinoma, or injection form of cytarabine (Depocyt®) for intrathecal treatment of lymphomatous meningitis, actually available in the US (Bulbake et al., 2017; Salehi et al., 2019). Other drugs, such as encapsulated amphotericin B, are actually marketed for the treatment of fungal infections, as well as some analgesics, vaccines against hepatitis and influenza, or dietary supplements like vitamin C. Extensive research is being carried out using these nanocarriers also for the delivery of anti-inflammatory drugs and therapeutic genes (Bulbake et al., 2017). Drug liposomal formulations have been tested also for the treatment of neurodegenerative disorders, including AD (Yang et al., 2013) and PD (Xia et al., 2008).

Several promising molecules have been already encapsulated and also tested on *in vivo* models of stroke. Thrombolytic drug tPA, which loaded in anti-actin-targeted immunoliposomes and administered intravenously to rats with focal ischemia, reduces intracerebral hemorrhagic complications linked with thrombolysis (Asahi et al., 2003). Similarly, intravenous administration of PEG-liposomes charged with neuroprotective agent tacrolimus directly after reperfusion significantly suppresses neutrophil infiltration, reduces cerebral cell death, and improves motor function deficits in tMCAo rats (Ishii et al., 2013). Fasudil, a neuroprotective Rho-kinase inhibitor, and approved drug for cerebral vasospasm after subarachnoid hemorrhage was tested for the treatment of ischemic stroke. Phase III clinical trials showed fasudil usefulness and safety, but poor clinical efficacy, short permanence in the bloodstream and difficulty to penetrate the BBB. These results decrease the usefulness of this molecule in clinic (Fukuta et al., 2016; Shibuya et al., 2005). Interestingly, fasudil encapsulated in PEG-liposomes significantly reduces the volume of damaged brain tissue, and improves motor functions in MCAo rats, much better than free fasudil (Fukuta et al., 2016). Vascular endothelial growth factor (VEGF) charged in pegylated liposomes coupled with transferrin confers neuroprotection, promotes neurogenesis, and cerebral angiogenesis (Zhao et al., 2011).

Therefore, liposomes should offer new perspectives in the treatment of stroke. Playing with a liposome composition allows circumventing frequent problems linked to the

passage of drugs through BBB, their low bioavailability, systemic elimination, or heterogeneous distribution in the brain.

In order to prepare the most suitable formulation of conventional and particular liposomes for the incorporation of stilbenoid macasiamenene F, we reviewed the existing formulations that were tested on different models of stroke (Tab. 3). We were interested mostly in their lipid composition, size of particles, and charge (zeta potential), being identified as the main features to cross BBB and accumulate in the ischemic tissue. Prepared formulations are part of “Material and methods chapter”.

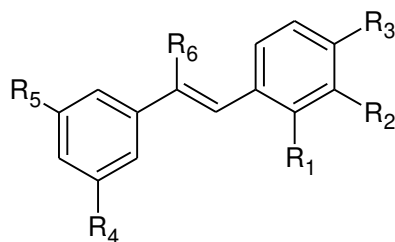
Drug	Formulation	Particle size (nm)	Zeta potential (mV)	Tested model	Ref.
ZL006	T7-DSPE-PEG, Mal-DSPE-PEG	72-76	16 to 17	2 h MCAo rat model, <i>i.v.</i> post-treatment	(Wang et al., 2015)
SOD	EPC, CHOL, mPEG2000-DSPE, and Mal-PEG2000-DSPE, (55:39:4:2)	151-169	-45 to -56	1 h MCAo mouse model, intracarotid bolus injection, post-treatment	(Yun et al., 2013)
CDP-choline	PE, CHOL, DCP, p-aminophenyl α -D-mannoside	60-90	-	30 min BCCAo rat model of global transient ischemia, <i>i.v.</i> 3 h pre-treatment	(Ghosh et al., 2010)
VEGF	POPC, DDAB, lipid PEG2000-DSPE, and Mal-PEG2000-DSPE	100	30	1 h MCAo rat model, post-treatment	(Zhao et al., 2011)
tPA	EPC, CHOL, fluorescent dye rhodamine-phosphatidyl ethanolamine (7:3:0,1)	200-250	-	rat model of the embolic focal stroke, <i>i.v.</i> administration of tPA at 6 h after the ischemic onset	(Asahi et al., 2003)
Fasudil	DSPC, CHOL, PEG2000	94-110	-5 to -2	1 h MCAo rat model, <i>i.v.</i> post-treatment	(Fukuta et al., 2016)

Table 3: Liposomal forms of drugs tested on models of cerebral ischemia. CDP-choline, cytidine 5'-diphosphocholine; VEGF, vascular endothelial growth factor; tPA, tissue plasminogen activator; DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; PEG, polyethylene glycol; Mal-, maleimide-derivatized; EPC, phosphatidylcholine; CHOL, cholesterol; PE, phosphatidylethanolamine; DCP, dicetylphosphate; POPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine, DDAB, didodecyldimethylammonium bromide; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; BCCAo, bilateral common carotid artery occlusion; MCAo, middle cerebral artery occlusion.

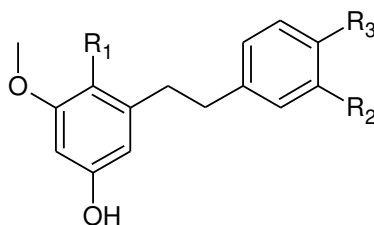
METHODS AND MATERIAL

1. Isolation/synthesis of compounds and treatments

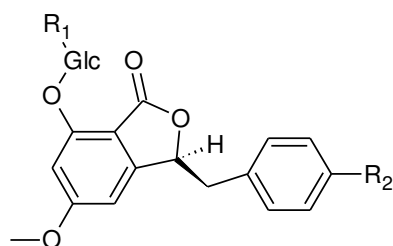
Most of thirty-seven tested stilbenoids were compounds naturally occurring in food and medicinal plants. Some of them were obtained commercially (**1-7, 15, 17, 18, 22, 23**) from Sigma-Aldrich (Germany), others isolated from plants *Morus alba* L. (Moraceae; **8**), *Tragopogon tommasinii* Sch.Bip (Asteraceae; **9-11, 24, 25**), *Artocarpus heterophyllus* Lam. (Moraceae; **26**), *A. altilis* (Parkinson ex F.A.Zorn) Fosberg (**27**), and *M. siamensis* S.J.Davies (**28-34, 37**). Compounds **8** and **27** were isolated at Dpt of Natural Drugs, MUNI using chromatographic methods (column chromatography; thin-layer chromatography, TLC; and high-performance liquid chromatography, HPLC). They were identified using spectral methods, including ultraviolet-visible (UV-Vis) spectroscopy, high resolution electrospray ionization mass spectrometry (HRESIMS), and nuclear magnetic resonance (NMR) spectroscopy, as described previously (Hošek et al., 2019; Leláková et al., 2019). Compound **27** was described as novel stilbenoid compound. (Hošek et al., 2019). Other compounds from *T. tommasinii* were provided by Dpt of Pharmacognosy and Molecular Basis of Phytotherapy, Medical University of Warsaw, Poland (Granica et al., 2015), stilbene **26** by Research Center of Natural Resources of Chinese Medicinal Materials and Ethnic Medicine, Jiangxi University of Traditional Chinese Medicine, China, and *M. siamensis* compounds **28-34** and **37** were provided by Chulabhorn Research Institute in Thailand, all isolated according previously published procedures (Jayasinghe et al., 2004; Pailee et al., 2015). Compounds **28, 29** and **37** were isolated as a mixture of enantiomers at C-2''' of the prenylated moiety in a 1:1 ratio as determined by chiral HPLC analysis. The values of the optical rotation were also close to zero, which indicate that the modification of a prenyl moiety to a dihydrofuran or a hydroxypyran ring system may be non-stereospecific in this plant (Pailee et al., 2015). Some of compounds were synthesized at Dpt of Chemical Drugs MU, Brno (**20, 21**), including novel molecules **34-36**, their synthesis is part of our publications (Hošek et al., 2019; Leláková et al., 2019) and resting compounds **12-14, 16, and 19** were of commercial synthetic origin obtained from Sigma-Aldrich (Germany). The purity of isolated and provided compounds was verified to be more than 98% using HPLC. The stability of compounds was regularly controlled by analytical HPLC measurements during the period of biological activity testing.



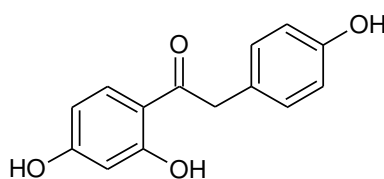
Compound	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶
<i>trans</i> -resveratrol (1)	H	H	OH	OH	OH	H
pterostilbene (2)	H	H	OH	OCH ₃	OCH ₃	H
piceatannol (3)	H	OH	OH	OH	OH	H
pinostilbene (4)	H	H	OH	OH	OCH ₃	H
piceatannol-3'- <i>O</i> -β-glucopyranoside (5)	H	<i>O</i> -Glc	OH	OH	OH	H
thunalbene (7)	H	OH	H	OH	OCH ₃	H
oxyresveratrol (8)	OH	H	OH	OH	OH	H
<i>trans</i> -stilbene (12)	H	H	H	H	H	H
<i>cis</i> -stilbene (13)	H	H	H	H	H	H
4-stilbenecarboxylic acid (14)	H	H	COOH	H	H	H
pinostilbenoside (15)	H	H	<i>O</i> -Glc	OH	OCH ₃	H
<i>trans</i> -α-methylstilbene (16)	H	H	H	H	H	CH ₃
pinosylvin-monomethylether (17)	H	H	H	OH	OCH ₃	H
isorhapontigenin (18)	H	OCH ₃	OH	OH	OH	H
2,4,3',5'-tetramethoxystilbene (19)	OCH ₃	H	OCH ₃	OCH ₃	OCH ₃	H
3,4',5-trimethoxy- <i>trans</i> -stilbene (20)	H	H	OCH ₃	OCH ₃	OCH ₃	H
3'-bromo-3,4',5-trimethoxy- <i>trans</i> -stilbene (21)	H	Br	OCH ₃	OCH ₃	OCH ₃	H
3,5-dimethoxystilbene (22)	H	H	H	OCH ₃	OCH ₃	H



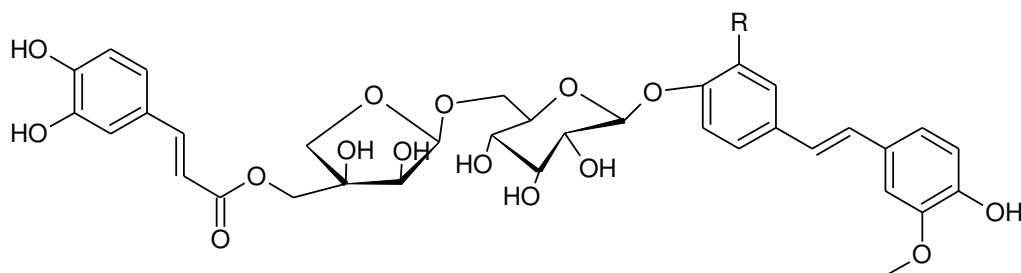
Compound	R ¹	R ²	R ³
batatasin III (6)	H	OH	H
2-carboxyl-3- <i>O</i> -methyl-4'-β-D-glucopyranosyl-dihydroresveratrol (10)	COOH	H	<i>O</i> -Glc



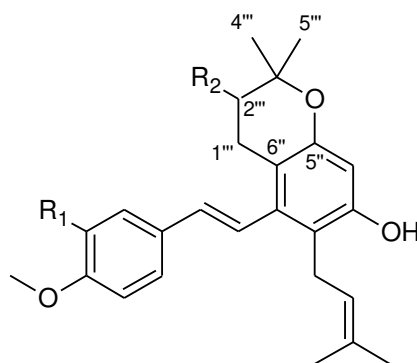
Compound	R ¹	R ²
7-β-glucopyranosyloxy-3-(4-hydroxybenzyl)-5-methoxyphthalide (9)	H	OH
7-(1→6)-α-rhamnosyl-β-glucopyranosyloxy-3-(4-hydroxybenzyl)-5-methoxyphthalide (11)	Rha	OCH ₃



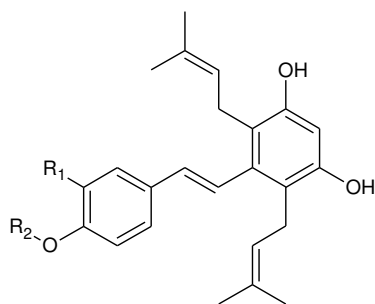
1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)ethanone (23)



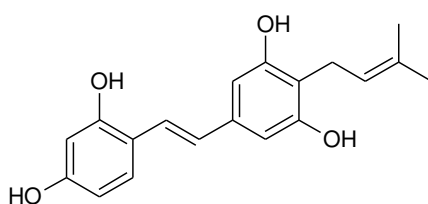
Compound	R
3- <i>O</i> -caffeoyl-(9→5)-β- <i>apiosyl</i> -(1→6)-β- <i>glucopyranosyl</i> -3'- <i>O</i> -methyl-dihydrochalcone (24)	OH
3- <i>O</i> -caffeoyl-(9→5)-β- <i>apiosyl</i> -(1→6)-β- <i>glucopyranosyl</i> -5,3'- <i>O</i> -dimethyl-dihydrochalcone (25)	OCH ₃



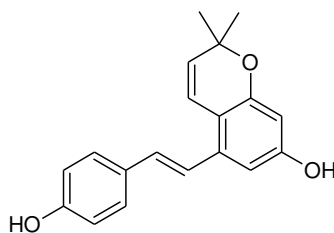
Compound	R ₁	R ₂
macasiamenene L (28)	H	OH
macasiamenene K (29)	OH	H



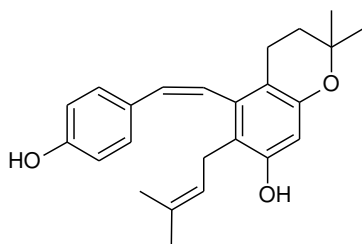
Compound	R ₁	R ₂
2,6-diprenylresveratrol (31)	H	H
macasiamenene A (32)	H	CH ₃
macasiamenene B (33)	OH	CH ₃



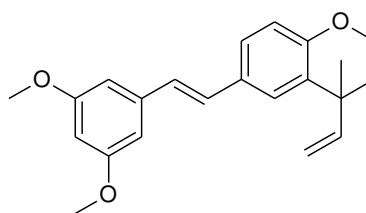
4-prenyl-oxyresveratrol (26)



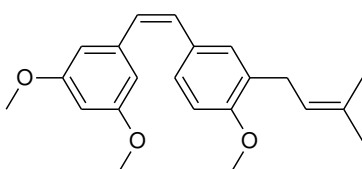
4-[(1E)-2-(7-hydroxy-2,2-dimethyl-2H-1-benzopyran-5-yl)ethenyl]-1-benzenol (27)



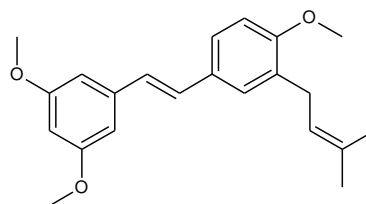
macasiamenene P (30)



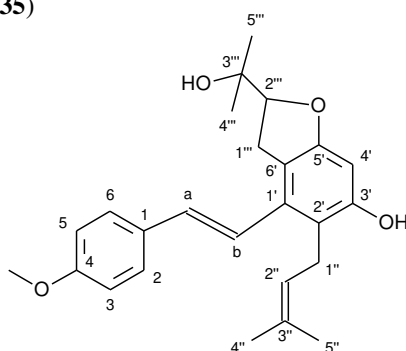
trans-3,4',5-trimethoxy-3'-(1,1-dimethyl-2-propen-1-yl)-stilbene (34)



cis-3,4',5-trimethoxy-3'-prenyl-stilbene (35)



trans-3,4',5-trimethoxy-3'-prenyl-stilbene (36)



macasiamenene F (37)

2. Pharmacological treatments

All tested compounds were dissolved in dimethyl sulfoxide (DMSO), which was used as a vehicle in all experiments, apart from *in vivo* studies. The concentration of DMSO in cell cultures never exceeded previously established safe concentration 0.1% (v/v) in the case of cell lines and 0.05% (v/v) in the case of primary cultures of microglia and neurons. LPS from *E. coli* 0111:B4 (Sigma-Aldrich) freshly dissolved in PBS was used in all assays at a concentration of 1 µg/mL (Taka et al., 2015; Triantafilou et al., 2001). NMDA (Sigma-Aldrich) was dissolved in ultra-pure water (Invitrogen) and used at a concentration of 50 µM (Sinor et al., 2000). Treatments with 2',7'-dichlorodihydrofluorescein-diacetate (DCFH₂-DA; Sigma-Aldrich) dissolved in DMSO [the final concentration in the medium 0.1% (v/v)] were performed at a concentration of 25 µM and 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH; Sigma Aldrich) dissolved in ultra-pure water was used at a concentration of 600 µM (Trembl et al., 2019). The saline solution (NaCl 0.9%) was used as a vehicle for MF liposomal formulations and their respective controls.

3. Animals and *ex vivo* models

Animals used for preparation of primary cell cultures and *in vivo* studies were housed under controlled conditions according to the FELASA guidelines and recommendations at 22°C with a 12 h light-dark cycle and free access to water and standard rodent diet. All animal care and use were performed in accordance with the policies of European Community Directive 86/609/EEC. The *ex vivo* experiments were approved by the ethical local committee. The *in vivo* experiments were approved by French ministry of higher education and scientific research and CIEPAL Nice (Protocol No. CHJM-AP-150). Every effort was made to minimize the number and suffering of experimental animals.

Primary microglial cultures

Microglial cells were isolated from the brains of adult seven week-old male C57BL/6JRj mice (Janvier, France) using the Adult Brain Dissociation Kit (Miltenyi Biotec, USA) according to the manufacturer's instructions. Brain homogenates were filtered through 70 µm cell strainers (BD Biosciences) and centrifuged (10 min, 2.000 rpm). The supernatant was removed, cell pellets resuspended and washed with PBS at pH 7.2 containing 0.5% BSA and 2.5 mM EDTA. Microglia and CNS associated macrophages were then labeled with CD11b⁺ MicroBeads (Miltenyi Biotec, USA), incubated for 15 min

at 4°C and isolated using LS columns (Miltenyi Biotec, USA) in a magnetic field. Brain-sorted microglia were then seeded into 96-well poly-D-lysine-covered plates in complete DMEM medium (Gibco) at a density of 7×10^4 cells/well. Microglia were then cultured at 37°C for the period of experiment being 18 h (Leláková et al., 2020).

Primary cortical neurons

Cortical neurons were isolated from 14-days old embryos of pregnant C57BL/6JRj mice (Janvier, France). Brains were dissected and placed in Petri dish with Ham's Nutrient Mixture F-12 medium (Gibco) at 4°C. The cortices were dissected under horizontal laminar flow using binocular microscope. The olfactory bulbs and eventually meninges were removed. The cortices were then cut to small pieces and placed into gentleMACS tube C (Miltenyi Biotec, US), slightly centrifuged (300 rpm, 1 min) with a removal of supernatant. Neural Tissue Dissociation Kit (P) (Miltenyi Biotec) was used for dissociation of neural tissue according to manufacturer's protocol. It means, for 400 mg of neural tissue, 50 µL of enzyme P (papain) were mixed with 1900 µL of buffer X and added to cortices, followed by addition of enzyme mix 2 (10 µL of enzyme A in 20 µL of buffer Y). The dissociation itself was carried out using the gentleMACS™ Dissociator (Miltenyi Biotec, US) at 37°C during 22 min. Dissociated tissue was then filtered using 40 µm cell strainers (BD Biosciences) and centrifuged (1000 rpm, 5 min). The supernatant was removed, pellet resuspended in 3 mL of Neurobasal medium (Gibco), and cells counted manually. Then, neurons in Neurobasal medium, supplemented with GlutaMAX 0.5 mM (Gibco), B-27 supplement (Gibco), and antibiotics [100 U/mL penicillin and 100 mg/mL streptomycin (Biosera)] were seeded into 24-well Corning® poly-D-lysine BioCoat™ plates at a concentration of $2.5\text{--}3.0 \times 10^5$ cells/well. The next day, neuronal cultures were treated with 5-fluoro-2'-deoxyuridine (FdU, Sigma) and uridine (U, Sigma) at a concentration of 2 µM for each to suppress the proliferation of other non-neuronal cells potentially occurring in the culture. Every third day, half of medium was carefully aspirated from each well and replaced by the fresh medium. Cortical neurons were cultivated for 12 days until they displayed an extensive neurite outgrowth and complete expression of NMDA receptors before proceeding with experiments (Bourourou et al., 2020).

4. Culture of cell lines

THP-1 monocytes and THP1-Xblue™-MD2-CD14 cells

The THP1 human monocytic leukemia cell line was purchased from the ECACC (Salisbury, UK). THP1-Xblue™-MD2-CD14 cells (Invivogen, San Diego, CA, USA) were derived from THP-1 and stably express the NF-κB and AP-1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. Moreover, THP1-Xblue™-MD2-CD14 cells stably express CD14, macrophage-specific differentiation antigen that interacts with several TLRs, and an accessory molecule essential for LPS-induced TLR4 response, MD2. Upon TLR stimulation, NF-κB/AP-1-activation leads to SEAP secretion that can be easily detectable using QUANTI-Blue™ reagent (Invivogen, San Diego, USA), thus this cell line allows to indirectly measure the NF-κB/AP-1 activity. THP-1 and THP1-Xblue™-MD2-CD14 cells were cultured in RPMI 1640 medium (Biosera, France) supplemented with antibiotics [100 U/mL penicillin and 100 mg/mL streptomycin (Biosera)] and 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) at 37°C. Both cell lines were passaged approximately once or twice per week during three months of experimental part performed on passages 3–16. To avoid the effect of hormones and cytokines present in serum, all experiments were performed in serum-free medium, replaced 2 h before starting experiment, as described previously (Leláková et al., 2019).

Differentiation to macrophages

The stimulation with phorbol myristate acetate (PMA) at a final concentration of 50 ng/mL was used for differentiation of THP-1 monocytes to macrophages. The PMA-stimulated suspension of cells (500.000 cells/mL) in complete RPMI 1640 medium was split into 96-well plates (5×10^4 cells/well) and cultivated for 24 h. The culture medium of adherent macrophages was then replaced with fresh complete medium. After another 24 h, the cells were washed with PBS, put into serum-free medium for 2 h and then used for subsequent experiments (Brezani et al., 2018).

BV-2 microglia

The BV-2 murine microglia were generated from primary microglia by infection with v-raf/v-myc oncogene carrying J2 retrovirus (Blasi et al., 1990) and cultured in DMEM medium (4.5 g/L glucose, 4 mM L-alanyl-glutamine, and w/o sodium pyruvate (Sigma), supplemented with 10% FBS (Gibco) and antibiotics [100 U/mL penicillin and 100 µg/mL

streptomycin (Gibco)]. Cells were grown at 37°C in a water-saturated atmosphere containing 5% CO₂. Microglia were passaged approximately in 3 days intervals when the culture reached 80–90% of confluence. Experiments were performed on passages ranging from 3 to 16. All experiments were performed in serum-free medium, replaced 2 h before starting experiment (Leláková et al., 2020).

5. Evaluation of cellular viability and mortality

WST-1 assay

The viability of THP-1 and THP1-XBlue™-MD2-CD14 cells was determined by WST-1 assay. Undifferentiated monocytes in serum-free medium placed in 96-well plates at a density of 5×10^4 cells/well were treated with the test compounds dissolved in DMSO at increasing concentrations (0.125–20 µM). Measurements were taken after 24 h incubation. Cell viability was determined using the Cell Proliferation Reagent kit WST-1 (Roche Diagnostics, Basel, Switzerland) by spectrophotometric measurement at 440 nm with the reference wavelength 630 nm. The resultant viability curves were used to calculate the IC₅₀ values using four parameters logistic (4PL) analysis (Leláková et al., 2019).

MTS assay

The effect of compounds on the viability of adherent BV-2 cells and cortical neurons in culture was determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, France). In the case of BV-2 cells, the dose-response assays were carried out in exponential phase of growth pre-determined in a pilot study in 24-well plates. Briefly, BV-2 cells were seeded in the evening in complete DMEM medium at a density of 1.5×10^5 cells/well, the next day early in the morning washed by PBS, and the medium was replaced by DMEM serum-free medium. Cells were treated after 2 h with MF at concentrations ranging from 1 to 15 µM. The viability corresponding to the mitochondrial activity of cells was evaluated after 24 h by spectrophotometric measurement at 492 nm (Leláková et al., 2020). The viability of cortical neurons treated with MF free/liposomal 0.1–15 µM was evaluated in a similar way after 1, 2, 6, or 24 h of incubation depending on the type of experiment.

LDH assay

Cell mortality of BV-2 microglia and primary cortical neurons was assessed from supernatants simultaneously with measurements of mitochondrial activity using the

Cytotoxicity Detection Kit (LDH; Roche), according to manufacturer's protocol. LDH activity was determined spectrophotometrically at 492 nm after 1, 2, 6, or 24 h of incubation according to the type of experiment. The percentage of mortality was calculated as the amount of lactate dehydrogenase (LDH) released by damaged cells, compared to control cells.

Cell counting

In proliferation assays, cells were counted manually using a Trypan blue (Gibco) exclusion method. Counting of pyknotic nuclei in OGD assay was carried out using The Cytation 5 Cell Imaging Multireader software (Biotek, France). Cells washed with PBS were fixed with PAF 4% and stained with fluorescent Hoechst dye (1:2000; Invitrogen). Pyknotic nuclei of dead cells were recognized as condensed, highly fluorescent cells, in comparison with nuclei of healthy cells. Dead cells were scored by counting several randomized subfields for each well and condition measured in sextuplicates. The ratios of numbers of pyknotic nuclei *vs.* total cell numbers were then calculated using Image J software.

6. Determination of NF- κ B/AP-1 activity

The NF- κ B/AP-1 activity was measured on THP-1-XBlue™-MD2-CD14 cells stably expressing the NF- κ B and AP-1-inducible SEAP, which was detected by QUANTI-Blue™ reagent. Cells in serum-free medium RPMI 1640 medium were seeded into 96-well plates at a density of 5×10^4 cells/well and incubated for 2 h at 37°C. Compounds, together with vehicle DMSO (0.1 % (v/v)) and prednisone used as a positive control were added at their non-toxic concentration of 2 μ M for non-prenylated stilbenes **1–25** and 1 μ M for prenylated ones **26–37** (apart from compound **28** where concentration of 0.1 μ M was used due to toxicity). After 1 h of incubation, the cells were treated with LPS (1 μ g/mL) to stimulate TLR4-mediated activation of NF- κ B/AP-1 and SEAP production. The non-LPS-stimulated cells treated only with compounds were used as controls. After 24 h, the plates with cells were centrifuged (300 rpm, 3 min), 20 μ L of the supernatant was mixed with 175 μ L of Quanti-Blue™ reagent (Invivogen, San Diego, USA) and incubated for 30–40 min at 37°C. Spectrophotometric measurements at 655 nm were carried out in the Fluostar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany). Compounds without LPS stimulation did not increase the NF- κ B/AP-1 activity. Therefore, the percentage of inhibition in LPS-stimulated groups was calculated and related to the vehicle treated cells (Brezani et al., 2018).

7. Gene expression analysis

Isolation of RNA and quantitative RT-PCR

Total RNA was extracted using TRI Reagent[®] (Sigma-Aldrich), according to producer's protocol. The pellets were then resuspended in RNase-free water. The presence of nucleic acids was verified on 1% agarose gel with ethidium bromide, together with checking their purity on Nanodrop. To remove DNA, samples were further treated with TURBO DNase (Invitrogen) according to producer's protocol. To prevent the contamination of samples with DNA, classic PCR using GoTaq G2 Green Master Mix (Promega, M782A) and GAPDH primers (Eurogentec, France) was performed. The cDNA sample from previous assays was used as a positive control and nucleic acid-free H₂O as negative control. The absence of DNA in RNAs samples was verified by agarose gel electrophoresis with UV detection. First-strand cDNAs were then synthesized from 0.5 µg of total RNA using 200 U/µL Superscript[™] IV Reverse transcriptase (Invitrogen) in reaction buffer in the presence of 2.5 µM oligo d(T)₂₀ primers (Eurogentec, France), 0.5 mM deoxyribonucleotide triphosphate (dNTP) mix, and 5 mM dithiothreitol (DTT). Two samples without adding the reverse transcriptase were used as negative controls. The reaction started with 5 min incubation at 65°C, then for 10 min at 50°C, and was finally inactivated at 80°C for 10 min. After the reverse transcription, a classic PCR using GoTaq G2 Green Master Mix (Promega, M782A) and agarose gel electrophoresis were performed to check the presence of cDNA in samples and verification of successful reverse transcription. Finally, quantitative RT-PCR was performed using the LightCycler[®] 480 SYBR Green I Master with a LightCycler[®] 480 sequence detector (Roche Diagnostics). RPLO and GAPDH, pre-determined as genes with a stable expression in our samples, were used as reference genes for normalization. The following sequences of primers were used: mRPLO (forward primer 5'-ACTGGTCTAGGACCCGAGAAG-3', reverse primer 5'-TCCCACCTTGTCTCCAGTCT-3'), mGAPDH (forward primer 5'-CCAGTGAGCTTCCCGTTCA-3', reverse primer 5'-GAACATCATCCCTGCATCC-3'), mL-1β (forward primer 5'-TGGTGTGTGACGTTCCCAT-3', reverse primer 5'-CAGCACGAGGCTTTTTTGTG-3'), and mTNF-α (forward primer 5'-CATCTTCTCAAAATTCGAGTGACAA-3', reverse primer 5'-TGGGAGTAGACAAGGTACAACCC-3') (Eurogentec, France). Each assay was performed at least 3 times in sextuplicates (Leláková et al., 2020).

8. Protein expression analysis of proinflammatory cytokines

Immunodetection by ELISA

The protein expression of proinflammatory cytokines in THP-1 differentiated macrophages was determined by ELISA assays. Briefly, macrophages were pretreated for 1 h with the selected test compounds or prednisone dispersed in 0.1 % DMSO at concentration of 2 μ M (non-prenylated **1-25**) and 1 μ M (prenylated stilbenes **26-37**, apart from **28** for which 0.1 μ M). The 0.1% DMSO in serum-free medium was used as the vehicle group. After 1 h of incubation, the cells were treated with LPS (1 μ g/mL) to stimulate the NF- κ B/AP-1-mediated expression of proinflammatory cytokines. The release of TNF- α and IL-1 β was evaluated from supernatants after 24 h, using Human TNF- α and IL-1 β ELISA kits (Diacclone, France) and spectrophotometric measurements at 450 nm with the reference wavelength 620 nm, according manufacturer's recommendations (Hošek et al., 2019).

Immunodetection by AlphaScreen

The protein expression of TNF- α and IL-1 β in BV-2 cells was determined from cell contents and supernatants. Briefly, the medium of cells plated (1.5×10^4 /well, 24-well plate) preceding evening was replaced by serum-free medium for 2 h before experiment. Then, cells were treated with MF 1 μ M in DMSO or DMSO 0.1% alone (Vehicle) 1 h before (pre-treatment), simultaneously (co-treatment protocol) with LPS 1 μ g/mL. Post-treatment protocol consisted in the treatment of cells by MF 3 h or 6 h after LPS-stimulation. TNF- α and IL-1 β expression was determined using AlphaLISA mIL1 β (AL503C) and mTNF α (AL505C) kits (PerkinElmer, MA, USA), according to manufacturer's protocol. Detection was carried out by fluorometric measurements using EnVision-Alpha Reader (PerkinElmer, USA). The total quantities of cytokines produced were calculated and normalized to the amounts of total proteins determined by Bradford protein assay (Biorad, France) (Leláková et al., 2020).

Immunodetection by Western blotting

THP-1-XBlueTM-MD2-CD14 cells seeded in 6-well plates at a density of 3×10^6 cells/well were treated with selected test compounds or the vehicle (Veh). After 1 h, LPS (1 μ g/mL) was added to stimulate TLR4-mediated activation of I κ B/NF- κ B and MAPKs pathways. Cells without LPS treatment represented the control (Ctrl). The kinetics of I κ B and MAPKs activation was pre-established at 15, 30 and 45 minutes with selection of the

second time point optimal for testing the activation of these pathways. Therefore, after 30 min, the cells were collected, washed in cold PBS, and homogenized in cold lysis buffer (50 mM Tris-HCl, pH 7.5; 1 mM EDTA; 1 mM EGTA; 1 mM sodium orthovanadate; 5 mM sodium pyrophosphate; 50 mM sodium fluoride; 270 mM sucrose) with protease inhibitors (Roche Diagnostics, Basel, Switzerland). The equal amounts of proteins (9 µg) were separated in 12% PAGE gel and transferred to the polyvinylidene fluoride (PVDF) membrane. The membrane was then incubated overnight at 4°C with the following monoclonal primary antibodies: anti-IκB-α (1:500; Cell Signaling, USA), anti-β-actin (1:5000; Abcam, Cambridge, UK), anti-SAPK/JNK (1:1000; Sigma-Aldrich, USA), antiphospho-p44/42 MAPK (p-ERK1/2, 1:2000), anti-p44/42 MAPK (ERK1/2), anti-phospho-p38 MAPK, and anti-phospho-SAPK/JNK (1:1000; Cell Signaling, MA, USA) and polyclonal antibody: anti-p38 MAPK (1:1000; Cell Signaling, MA, USA). Each membrane was then probed with the appropriate secondary peroxidase-conjugated antibodies (1:2000, from Sigma-Aldrich). The ECL signal (Bio-Rad, USA) was detected using Syngene PXi4 chemiluminescence imaging system (Cambridge,UK). Densitometric analysis was performed using AlphaEaseFC 4.0.0 software (Alpha Innotech, USA). The relative effects of compounds and vehicle (Veh) on IκBα and MAPKs were compared after normalization to β-actin, as we described previously (Leláková et al., 2019).

Immunohistochemistry

The effects of MF on the expression of proinflammatory cytokines were observed by epifluorescence microscopy. BV-2 cells plated on coverslips were pretreated with MF 1 µM and LPS 1 µg/mL during 24 h, then washed with PBS, fixed by PAF 4%, washed twice with PBS, incubated with polyclonal primary rabbit anti-TNF-α and anti-IL-1β antibodies (Abcam), then labeled with goat anti-rabbit IgG Alexa Fluor 594 or Alexa Fluor 546 (Invitrogen) secondary antibodies. DAPI (1:2000) was used for the staining of cell nuclei. The coverslips were mounted on slides using Mowiol mounting medium and observed using Zeiss Axioplan 2 Imaging system.

The observations of MF incorporation into cells were based on its autofluorescence properties which come from its structure. The excitation and emission spectra were determined using spectrofluorometer Chronos DFD (ISS, US). The MF incorporation in THP-1 cells in suspension after 24 h of incubation with MF was observed at VRI Brno (CZ) using a super-resolution live cell imaging SP8 Lightning (Leica), while BV-2 cells

were fixed before imaging. In brief, BV-2 cells were cultured on poly-D-lysine covered coverslips and treated in the same manner described above. After 24 h, cells were washed by PBS, fixed with PAF 4% in PBS for 10 minutes at room temperature, then washed twice with PBS. The coverslips were mounted on slides using Mowiol mounting medium and observed by Zeiss LSM 980 microscope.

9. Cellular Antioxidant Assay (CAA)

The antioxidant activity of tested compounds was measured according to Wolfe and Liu method with some modifications (Wolfe and Liu, 2007). BV-2 microglia were pre-incubated for 1 h in serum-free DMEM medium containing 25 μM DCFH₂-DA (Sigma-Aldrich) dissolved in DMSO 0.1% (v/v) at 37 °C. Then, the cells were centrifuged, washed with PBS, resuspended in serum-free medium and seeded into 96-well black plate (5×10^4 cells/well). Then, cells were incubated with MF in DMSO at a concentration range from 0.5 to 6 μM or DMSO alone for 1 h. After that, AAPH (Sigma-Aldrich) at a concentration of 600 μM was added to cells to trigger the ROS generation. Directly after, the plate was placed in FLUOstar Omega microplate reader (BMG Labtech) at 37 °C and the level of oxidized fluorescent 2',7'-dichlorofluorescein (DCF) was measured every 5 min during 1 h with excitation at 485 nm and emission at 538 nm. Cells treated with DCFH₂-DA and oxidant AAPH represented the control and cells treated with DCFH₂-DA without oxidant were used as a blank. Well known antioxidant quercetin (Q) 0.5–2 μM was used as a positive control. After blank subtraction from the fluorescence readings, the area under the curve (AUC) of fluorescence versus time was integrated to calculate the CAA value of test compounds: $\text{CAA unit} = 100 - (\int\text{SA} / \int\text{CA}) \times 100$, where $\int\text{SA}$ is the integrated area under the sample fluorescence versus time curve and $\int\text{CA}$ is the integrated area from the control curve, as we recently described in Malaník et al. (Malaník et al., 2020).

10. NMDA-induced Excitotoxicity Assay

Primary mouse cortical neurons plated in poly-D-lysine-coated 6-well plates (Corning® BioCoat™, Germany) at a density of 250.000 cells/well in growth Neurobasal media (Gibco), supplemented with B-27 neuronal cell culture supplement (Gibco), GlutaMAX 0.5 mM, and antibiotics [100 U/mL penicillin and 100 mg/mL streptomycin (Biosera)] were maintained at 37°C in 5% CO₂. At day 12, cells were carefully washed with Neurobasal medium and culture medium was replaced by the fresh one before experiment. Cells were pretreated with MF or Vehicle and incubated for 1 h. Then, the excitotoxic state

was induced with NMDA 50 μ M during 28 h to observe the NMDA-mediated toxicity and cell death (Sinor et al., 2000). Control cells were not exposed to NMDA treatment. The cell viability and LDH release were determined in time intervals of 2, 6, 20 and 28 h using MTS and LDH assay, respectively. The viability and mortality of cells treated with test compounds or vehicle were compared to NMDA non-treated cells for every time point (Blondeau et al., 2002; Bourourou et al., 2020).

11. *In vitro* model of stroke - Oxygen Glucose Deprivation (OGD) model

Primary mouse cortical neurons were cultivated until day 12 for the complete expression of NMDA receptors, then used for OGD assays. The day of experiment, the culture medium of neurons was carefully replaced by the fresh Neurobasal medium containing glucose (6 g/L), Glutamax 1X, B-27 supplement, and antibiotics (AB). Treatments with Vehicle (Veh., DMSO 0.05%, determined as safe) or MF 5 μ M were applied 1 h before OGD, kept during OGD, and added at the end of OGD with fresh medium for next 24 h. Each condition had its own control without OGD challenge. The flowchart illustrating the experimental design is given in Fig. 19. After 1 h of pre-incubation of cells with treatments, the cells were washed with afore prepared and filtered HBSS medium (NaCl 1.4 M, KCl 5.4 mM, MgSO₄ 1.2 mM, CaCl₂ 1.2 mM, NaHCO₃ 25 mM, NaH₂PO₄ 1.25 mM per 1 L of sterile H₂O) without glucose. The HBSS medium without glucose including MF 5 μ M/Veh. treatment was replaced in the case of neurons undergoing OGD challenge. The cells were placed into hypoxia chamber with 1.2% O₂/ 93.8% N₂/ 5% CO₂ for 1 or 2 h. The medium of control cells was replaced by Neurobasal medium (containing glucose, Glutamax, B-27, AB), together with MF/Veh treatment and placed under normoxic conditions (21% O₂/ 74% N₂/ 5% CO₂) at 37°C. After OGD challenge, the mitochondrial activity, and neuronal death by necrosis and apoptosis were evaluated in both OGD and non-OGD groups by MTS assay, LDH assay and counting of the pyknotic cell nuclei, respectively. The counting of dead cells was described above. The medium of resting cells from OGD-group was replaced by oxygenated Neurobasal medium (containing glucose, Glutamax, B27, AB) with MF/Veh treatment and placed under the normoxic conditions (21% O₂/ 74% N₂ / 5% CO₂) at 37°C as control groups for next 24 h. Then, the mitochondrial activity and cell mortality were assessed again in the same manner for both OGD- and non-OGD groups (Fig. 19). The effects of treatments against OGD-triggered

damage were compared to their respective non-OGD controls for each time point (Bourourou et al., 2020; Moha Ou Maati et al., 2012).

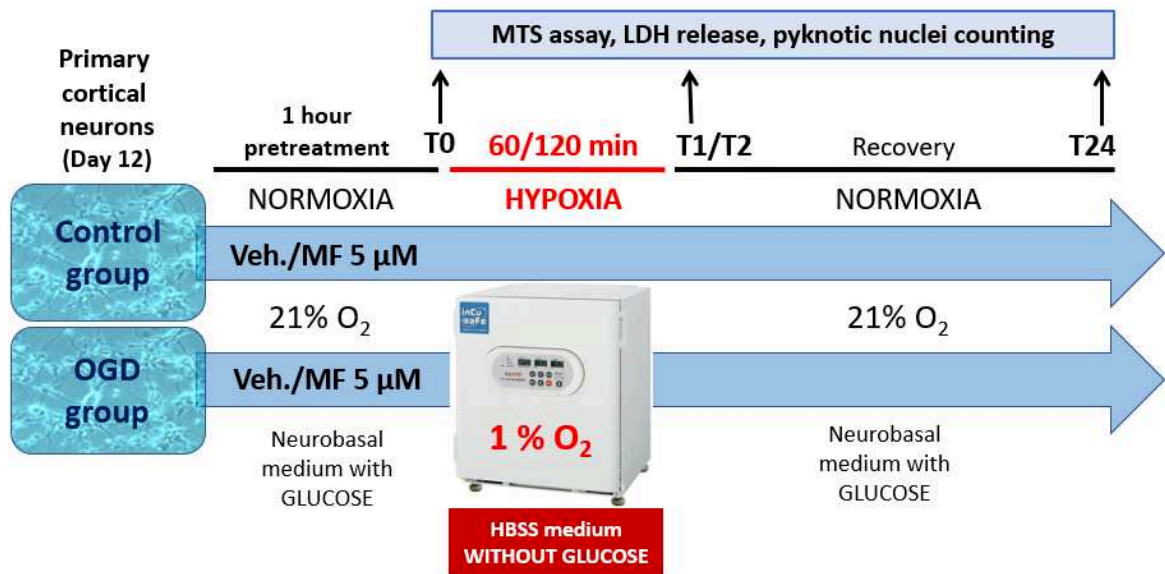


Fig. 19: *In vitro* model of stroke – Oxygen Glucose Deprivation (OGD) model. Primary mouse cortical neurons were pretreated for 1 h with MF 5 μM or vehicle (Veh.), the treatment was maintained during the whole experiment. Cells were exposed to 1 h or 2 h OGD. Mitochondrial activity (MTS assay), LDH release, and pyknotic nuclei rate were evaluated at T0 (before starting OGD), T1/T2 (directly after OGD), and after 24 h (T24).

12. Preparation of liposomes

Liposomes were prepared by PharmDr. Josef Mašek, PhD. and PharmDr. Eva Vohlídalová at Veterinary Research Institute (VRI) Brno, Czech Republic. For different compositions displayed in Table 4, following lipids were used: phosphatidylcholine (EPC), cholesterol (CHOL), cationic 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-CHOL), anionic palmitoyl-oleoyl-phosphatidylglycerol (POPG), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000). Lipid components with MF or without (controls) were dissolved in chloroform. The organic solvent was then evaporated on the rotary vacuum evaporator and followed by rehydration of the film in an aqueous solvent (0.9 % NaCl) and sonication. Liposomes were prepared by manual membrane extrusion through 200 nm polycarbonate filter (Whatman Nuclepore, Sigma Aldrich, Czech Republic) using manual extruder (LiposoFast, Avestin, Canada), and homogenized. The size and distribution were determined using dynamic light scattering (DLS) technique (Zetasizer Nano ZSP, Malvern, Great Britain). The charge of

liposomes was determined as value of ζ -(zeta) potential (Zetasizer Nano ZSP, GB). The content of MF in liposomes represented 5 molar percent, established as stable composition meeting all quality requirements. The stock solutions of liposomes were prepared at a concentration of MF 1 mM, then diluted in saline for desired concentrations used in dose-response studies. Control groups represented liposomal formulations without MF, used in the same concentration of lipids than tested MF incorporated. The encapsulation of MF was verified using spectrofluorometric measurements (spectrofluorometer Chronos DFD, ISS, US) and confocal microscopy (Leica TCS SP8 MP, Leica Microsystems, Germany) based on autofluorescence properties of MF (ex. max. at 309 nm, em. max. at 422 nm). Morphology of liposomes was determined using transmission electron microscopy (TEM microscope Philips 208S Morgagni, FEI, Czech Republic). Polydispersity index of all formulations was <0.2. The stability of liposomes was verified once per week using Zetasizer Nano during all the period of biological activity testing. To determine the optimal composition and charge of liposomes for testing MF incorporated on *in vivo* models of stroke, the MF compositions together with their respective controls displayed in Table 4 were prepared.

Sample Name	Type of liposome	Composition	Size (nm)	ζ -potential (mV)
I	Anionic	30% POPG, 5% MF, 65% EPC	137.5	-28.1
II	Anionic	20% POPG, 5% MF, 75% EPC	156.6	-23.2
III	Anionic	10% POPG, 5% MF, 85% EPC	139.6	-14.6
IV	Neutral	5% MF, 95% EPC	188.3	-2.4
V	Cationic	10% DC-CHOL, 5% MF, 85% EPC	167.3	20.1
VI	Cationic	15% DC-CHOL, 5% MF, 80% EPC	160.8	22.7
VII	Cationic	20% DC-CHOL, 5% MF, 75% EPC	159.1	25.9
VIII	Cationic (combined)	15% DC-CHOL, 25% CHOL, 5% MF, 55% EPC	168.2	31.9
PEG-I	Pegylated I	30% POPG, 5% MF, 60% EPC, 5% DSPE-PEG 2000	146.6	-3.4
PEG-VIII	Pegylated VIII	15% DC-CHOL, 25% CHOL, 5% MF, 50% EPC + 5% DSPE-PEG 2000	159.7	0.2

C/I	Anionic	30% POPG, 70% EPC	124.3	-26.9
C/II	Anionic	20% POPG, 80% EPC	125.4	-20.9
C/III	Anionic	10% POPG, 90% EPC	131.8	-14.4
C/IV	Neutral	100% EPC	193.5	-1.61
C/V	Cationic	10% DC-CHOL, 90% EPC	146.6	19.5
C/VI	Cationic	15% DC-CHOL, 85% EPC	138.0	21.3
C/VII	Cationic	20% DC-CHOL, 80% EPC	137.4	25.7
C/VIII	Cationic (combined)	15% DC-CHOL, 25% CHOL, 60% EPC	166.1	27.9
C/ PEG-I	Pegylated 1	30% POPG, 65% EPC, 5% DSPE-PEG 2000	145.3	-3.1
C/ PEG-VIII	Pegylated 8	15% DC-CHOL, 25% CHOL, 55% EPC + 5% DSPE-PEG 2000	155.7	0.2

Table 4: Tested liposomal formulations of MF I-VIII, PEG-I, PEG-VIII, and respective controls C/I–C/VIII, C/PEG-I, C/PEG-VIII. The % of used lipids represents the percentual ratios of their molar concentrations. Prepared at VRI Brno.

13. *In vivo* model of stroke – mouse middle cerebral artery occlusion (MCAo)

Induction of focal transient ischemia

Focal ischemia was induced by occlusion of the left middle cerebral artery (MCA) in 7 weeks old C57BL/6J male mice (Janvier, France) using an intraluminal filament technique (Ding-Zhou et al., 2003) based on modifications of the original rat model (Longa et al., 1989). After a midline neck incision was performed, the left common and external carotid arteries were isolated and ligated with a 4-0 silk suture (Ethicon, Belgium). A temporary Yasargil aneurysm clip (BMH31, Aesculap, Germany) was temporarily placed on the internal carotid artery. Then, a 6-0 nylon monofilament (Ethicon) was introduced through a small incision into the common carotid artery and 13 mm distal to the carotid bifurcation for occlusion of the MCA origin. Animals were kept at 37°C for one hour. Then, the filament was carefully withdrawn to allow reperfusion of the MCA territory. The regional cerebral blood flow (rCBF) was monitored by laser Doppler flowmetry (Perimed, France) to control the severity of MCAo. Mice with reduced CBF under 80% were excluded from the study (<10%). Sham-operation was performed by inserting a thread into

the common carotid artery without advancing it to occlude the MCA (Heurteaux et al., 2010). Treatments with saline, MF free and MF liposomal (PEG-VIII) at a concentration of 0.117 mg/kg (n=5 per condition), calculated on the mouse volume of blood according to the concentration used in assays on primary mouse cortical neurons, were administered intravenously in penile vein 10 min after the end of MCAo. Then, the animals were allowed to regain full consciousness on a heating pad. The efficiency of a single-dose was evaluated after 24 h (Heurteaux et al., 2010).

Determination of infarct volume

The survival rate of mice was observed at 24 h after MCAo, dead mice were removed from the analysis. Mice were then killed by cervical dislocation. Brains were quickly extracted and fresh frozen in isopentane at -40°C , and frozen sectioned into 20 μm thick coronal slices (12 slices per brain) on a cryostat (Phymep, France), then stained with a solution of 1% cresyl violet in 0.25% acetic acid (Pietri et al., 2019). The whole surface of infarction was determined on each section using a computer image analysis system and corrected for brain edema according to Golanov and Reis (Golanov and Reis, 1995). Infarct volume (in mm^3) was calculated by a linear integration of the corrected lesion areas (Bourourou et al., 2016).

14. Statistical analyses

Statistical analysis was carried out using GraphPad Prism 6.01 software. Data were expressed as the Mean \pm SEM. Statistical analyses of differences between groups were performed using the parametric ($n > 30$) Student's t-test (for comparisons of 2 groups) and ANOVA with Bonferroni's test (for more than 2 groups), and non-parametric ($n < 30$) Mann-Whitney (for 2 groups), and Kruskal-Wallis analysis, together with Dunn's post-hoc test (for more than 2 groups). Values of P less than 0.05 were considered statistically significant. n=number of values. N=number of repetitions at least in triplicates.

Results

PART I – Evaluation of anti-inflammatory effects of non-prenylated and prenylated stilbenes against systemic inflammation

Thirty-seven compounds of both natural and synthetic origin sharing the same stilbene skeleton were selected for the evaluation of their *in vitro* anti-inflammatory properties with a focus on TLR4-mediated activation of NF- κ B/AP-1 signaling in THP-1 human monocytes. According to the substitution of stilbene core, this panel of molecules was divided into two groups of “non-prenylated stilbenoids” found largely in food (berries, nuts, etc.) and “prenylated stilbenoids” containing specific prenyl (3-methylbut-2-en-1-yl) unit, occurring preferentially in medicinal plants. Prenyl substituent increases the lipophilicity of compound and may potentially influence its passage through the cell membrane, as well as its biological effect (Zhang et al., 2019). Therefore, biological activities of tested compounds were compared separately for each group.

A. Non-prenylated stilbenoids

Cell viability – dose-response relationships

Determination of the dose-response effect of each compound on the viability of THP-1 was the first indispensable step for the selection of a safe concentration which could be used for the evaluation of their antiphlogistic potential. Therefore all compounds within a concentration range of 0.125–20 μ M were applied to human monocytes for 24 h. No tested compound from the group of non-prenylated stilbenes affected the cell viability neither at a high concentration of 20 μ M. The only exception was piceatannol (**3**) whose IC_{50} value was 6.7 ± 1.0 μ M for THP-1 cells (Fig. 20).

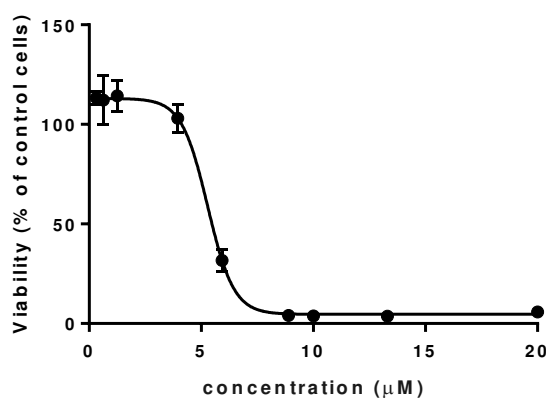


Fig. 20: Dose-response effect of piceatannol on the viability of THP-1 cells

According to bioavailability data obtained from studies on humans, the maximal plasma concentration of RSV that can be achieved after oral administration of 5 g RSV is approximately 2.4 μM (Boocock et al., 2007a; Smoliga et al., 2011). Therefore, to carry out a clinically relevant study, we selected a common concentration of 2 μM for following *in vitro* studies. This concentration has been shown safe for all compounds **1-25**, including piceatannol (**3**), and allowed us to compare the biological activities of tested compounds.

Anti-inflammatory potential of non-prenylated stilbenoids

THP-1-XblueTM-MD2-CD14 human monocytes derived from THP-1 cells by insertion of NF- κ B and AP-1 inducible SEAP reporter were selected for testing of TLR4-activated NF- κ B/AP-1 signaling. The activity of NF- κ B/AP-1 was measured indirectly by detection of SEAP in cell supernatants using QUANTI-BlueTM reagent. Monocytes pre-treated for 1 h with compounds at pharmacologically relevant concentration of 2 μM were stimulated by LPS 1 $\mu\text{g}/\text{mL}$. The NF- κ B/AP-1 activity detected after 24 h was then compared to vehicle (**V**)- and prednisone (**P**)-treated cells (Fig. 21).

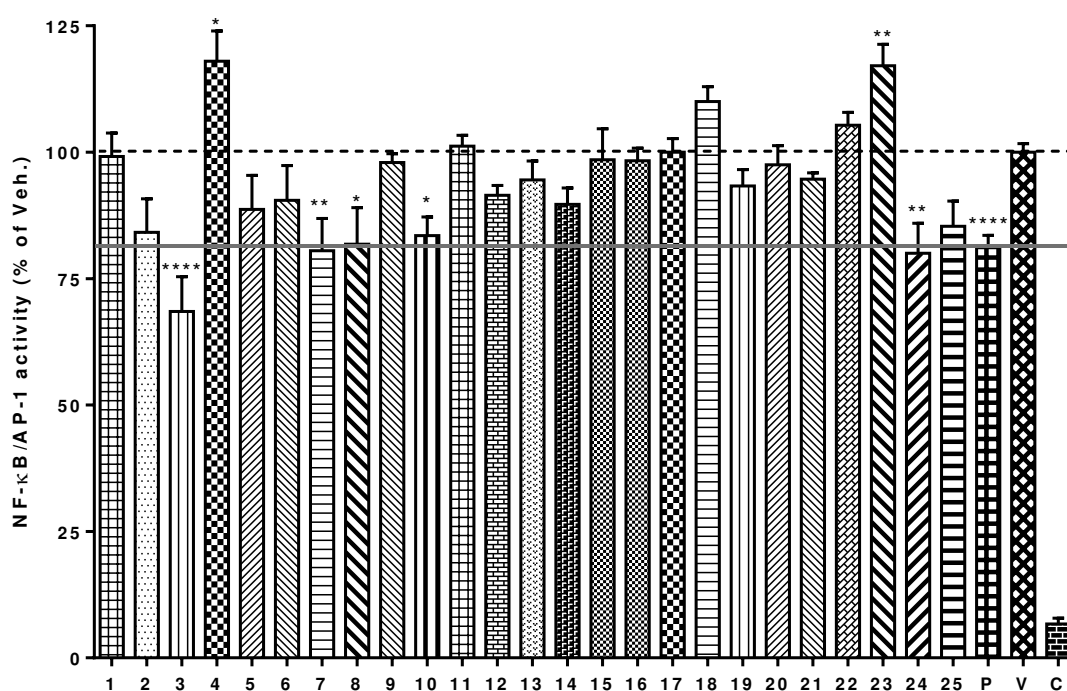


Fig. 21: Effects of compounds 1-25 on LPS-induced NF- κ B/AP-1 activity in THP-1-XblueTM-MD2-CD14 cells. The NF- κ B/AP-1 activity was compared to this Vehicle (V; dashed line) and prednisone (P; grey line). C=LPS-non-stimulated control. * indicates $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. Mean \pm SEM. N=6. Analyzed by ANOVA with Bonferroni's test.

Piceatannol (**3**), thunalbene (**7**), oxyresveratrol (**8**), 2-carboxyl-3-*O*-methyl-4- β -D-glucopyranosyl-dihydroresveratrol (**10**) and dihydropiceatannol glycoside (**24**), but not RSV (**1**) itself, have shown the ability to significantly dampen the NF- κ B/AP-1 activity, as compared to commercially available corticoid drug prednisone (**P**), used as a positive control (Fig. 21). Contrarily, pinostilbene (**4**) and 1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-ethanone (**23**) markedly induced the NF- κ B/AP-1 activity in comparison with only vehicle-treated cells. When looking more in detail on structure-activity relationships, piceatannol (**3**) and oxyresveratrol (**8**), differing from RSV (**1**) only in one additional aromatic hydroxyl group, have shown much greater inhibitory activity. This could indicate that hydroxylation of aromatic rings potentiates the inhibitory effect against NF- κ B/AP-1 activation. Furthermore, compounds **3** and **7** with hydroxyl groups in *meta*-position of ring B, showed greater inhibitory effect on NF- κ B/AP-1 activity than their structural analogues **8** and **4** with a hydroxyl group in *ortho*- and *para*- position, respectively. Interestingly, hydroxyl group in *para*-position seems to enhance the NF- κ B/AP-1 activity in the case of compound **4** compared to **7**. These results indicate that position of hydroxyl groups is a critical factor influencing the activity of stilbenes in order *para* < *ortho* < *meta*. On the other hand, methylation of aromatic hydroxyl groups could decrease activity of stilbenes, like it was observed in the case of compound **25**, *O*-methylated derivative of **24**. Other modifications, such as hydrogenation of ethylene bridge in the case of batatasin III (**6**) caused a decrease in activity in comparison with thunalbene (**7**). Furthermore, substitution of ethylene bridge, (*e.g.* compound **23** referred to **12**) may also influence the NF- κ B/AP-1 activity.

In the next step, the most active compounds **3**, **4**, **7**, **8**, **10**, **24**, and **25**, together with piceatannol glycoside (**5**) and batatasin III (**6**) which are structurally very similar derivatives of piceatannol (**3**) and carboxylated dihydroresveratrol glycoside (**10**) were respectively selected for the next evaluation of their effects on the protein expression of prototypical inflammatory cytokine TNF- α in PMA-differentiated THP-1 macrophages. The effects of the most investigated compounds RSV (**1**) and pterostilbene (**2**) on TNF- α had already been known (Silva et al., 2014; Yao et al., 2018), however, RSV (**1**) as the most representative stilbene was selected for the next assay as positive control together with prednisone (**P**). The effects of compounds **3** and **25** on the secretion of TNF- α were similar to these of our positive controls prednisone (**P**) and RSV (**1**), respectively. In

accord with a previous assay, aglycone piceatannol (**3**) was more active than its glycoside (**5**). Interestingly, it was not the case of batatasin III (**6**) and its carboxylated glycoside (**10**), which both, together with compound **24** inhibited TNF- α in a greater manner than P and RSV, approximately by 40%. On the other hand, pinostilbene (**4**)-induced NF- κ B/AP-1 activity was accompanied by increased TNF- α secretion from THP-1 cells (Fig. 22).

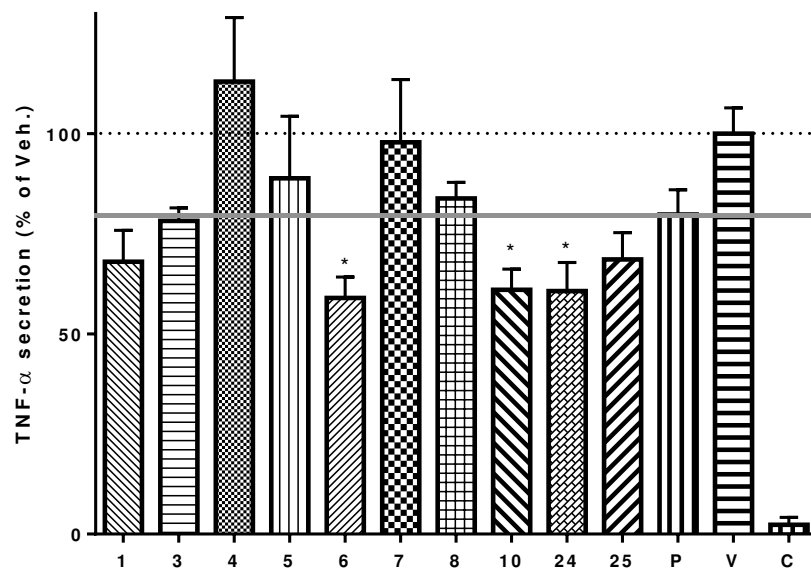


Fig. 22: Effects of selected stilbenes **1**, **3-8**, **10**, **24**, and **25** on LPS-stimulated TNF- α secretion in PMA-differentiated THP-1 macrophages. Cells pre-treated by compounds and prednisone (P) at a concentration of 2 μ M, or vehicle (V; DMSO 0.1%) were stimulated by LPS 1 μ g/mL. C=LPS-non-stimulated control. TNF- α was detected by ELISA at 24 h. * indicates a significant difference $P < 0.05$ vs. Veh (dashed line). Mean \pm SEM. $N=6$. Analyzed by ANOVA with Bonferroni's test.

We further focused on NF- κ B/AP-1 up-stream signaling which includes I κ B α , direct inhibitor of NF- κ B, and MAP kinases ERK1/2, p38, and JNK mostly responsible for AP-1 activation. The most active compounds **3**, **4**, **6**, **7**, **10**, and **24** which showed a significant effect on NF- κ B/AP-1 activity and/or downregulated TNF- α expression were therefore selected for further analysis of up-stream signaling pathways. Apart from compound **24**, all selected non-prenylated stilbenes have shown the ability to inhibit the LPS-stimulated phosphorylation (activation) of ERK1/2 and JNK. Moreover, stilbenes **3**, **4**, **7**, and **10** attenuated also the activation of the p38 pathway, but no compound dampened the I κ B α degradation at tested concentration of 2 μ M (Fig. 23). Contrarily, it seems that compound **7** decreased the I κ B α level. It can be hypothesized that its inhibitory effect on NF- κ B/AP-1 activity tends to act *via* MAPKs/AP-1 pathway, rather than through I κ B α . It is noteworthy that pinostilbene (**4**) enhanced the NF- κ B/AP-1 activity and slightly increased TNF- α secretion, but showed the ability to dampen MAPKs activation. It can be

hypothesized that this effect could have a connection with previously demonstrated potential of pinostilbene to radically inhibit the activity of inducible COX-2 (Leláková et al., 2019), whose gene expression is transcriptionally regulated by these pathways (Allport et al., 2000). Compound **24** did not influence the level of MAPKs neither I κ B α , possibly because of the bigger size and shape of molecule which would not fit into the active centers of these proteins. Anyway, this compound could affect the NF- κ B/AP-1 activity *via* another mechanism, such as inhibition of NF- κ B/AP-1 nuclear translocation or DNA binding, and thus reduce TNF- α expression in cells. The inhibitory effect on the activation of MAPKs ERK1/2, p38, and JNK was described also for RSV (Oh et al., 2009), as well as for pterostilbene, which affected the MAPKs/c-Jun-induced expression of pro-inflammatory cytokines (Paul et al., 2009).

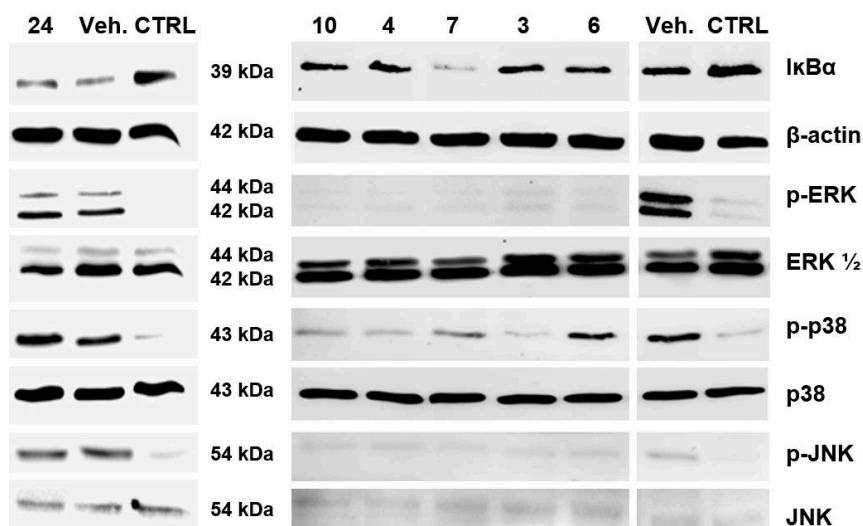


Fig. 23: Effects of selected stilbenes **3**, **4**, **6**, **7**, **10**, and **24** on I κ B α and MAPKs signaling in THP1-XBlue™-MD2-CD14 cells. Cells were pre-treated by compounds at a concentration of 2 μ M, or vehicle (Veh.; DMSO 0.1%) and stimulated with LPS 1 μ g/mL. C=LPS-non-stimulated control. The levels of I κ B α , β -actin, and MAPKs in cell lysate were detected 30 min after LPS stimulation by Western blot and immunodetection. Representative blot images of 3 independent experiments.

B. Prenylated stilbenoids

Cell viability – dose-response relationships

Prenylated phenolics in comparison with their non-prenylated derivatives display a different spectrum of biological activities, including antimicrobial (Bruijn et al., 2018) or antifungal (Ioset et al., 2001) properties. However, these effects are linked with some level of cytotoxicity which can be partly attributed to enhanced lipophilicity of these compounds due to the presence of one or more prenyl units and thus easier passage through cell membranes (Šmejkal, 2014). Therefore it was firstly necessary to verify their effects on the viability of THP-1 monocytes. Stilbenes from *Artocarpus* spp. (**26** and **27**), synthetic compounds **34-36**, and compound **31** from *M. siamensis* have shown relatively safe profiles with IC₅₀ values over 10 μM. Other *M. siamensis* compounds (**28-30**, **32**, **33**, **37**) affected the viability of THP-1 cells to certain extent, their IC₅₀ values were <10 μM (Table 5).

Compound	IC ₅₀ [μM]	Compound	IC ₅₀ [μM]
26	>10	32	2.7±1.1
27	>10	33	3.9±1.1
28	1.8±1.1	34	>10
29	3.3±1.0	35	>10
30	4.9±1.9	36	>10
31	>10	37	5.7±1.1

Table 5. The effects of compounds 26-37 on viability of THP-1 human monocytes. The data are displayed as IC₅₀ values, expressed as the mean ± SEM for several measurements in triplicates using at least five-point concentration curves.

Anti-inflammatory potential of prenylated stilbenoids

According to results from the previous assay, we selected for the evaluation of anti-inflammatory effects a pharmacologically relevant common dose of 1 μM for all prenylated compounds (0.1 μM for compound **28**) which have been shown safe for all compounds without any harmful effect on the viability of THP-1 and THP-1-XBlueTM-MD2-CD14 monocytes. Again we first evaluated the effect of prenylated stilbenes on LPS-induced NF-κB/AP-1 activity in genetically modified THP-1-XblueTM-MD2-CD14 cells. Most of tested compounds severely dampened the NF-κB/AP-1 activity, stilbenes **27**, **31**,

32, 34, 35, and 37 in a greater manner than commercially used prednisone (approximately by 25% vs. 20%). Contrarily, compound **26** slightly increased the NF- κ B/AP-1 activity in comparison with vehicle-treated cells (Fig. 24). Compounds themselves did not increase the basal level of NF- κ B/AP-1 activity in LPS-non-stimulated cells. Due to little set of prenylated compounds and heterogeneity of their structures, it was impossible to provide any conclusion on structure-activity relationships.

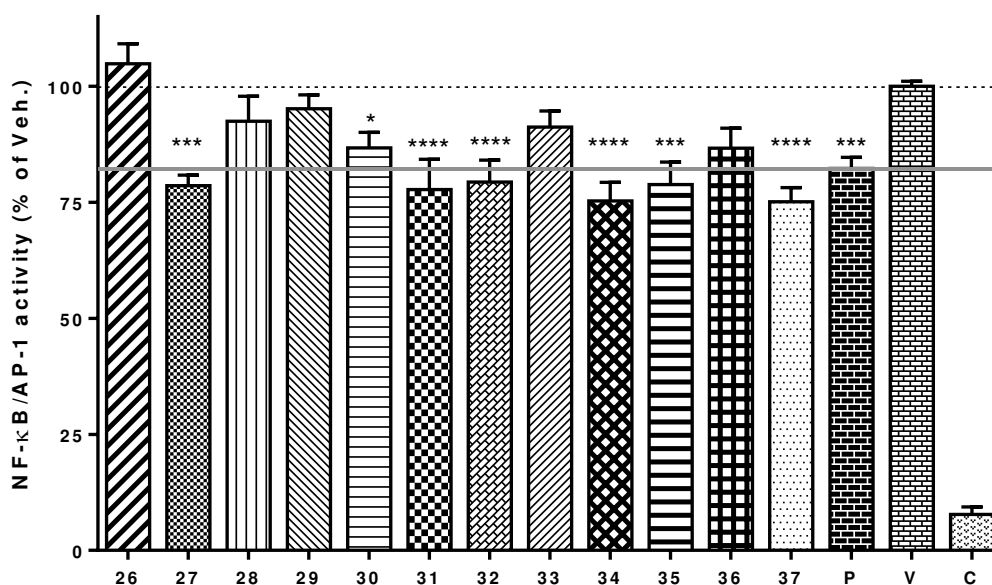


Fig. 24: Effects of compounds 26-37 on LPS-induced NF- κ B/AP-1 activity in THP-1-XblueTM-MD2-CD14 cells. Cells were pre-treated with compounds 26-37 or prednisone (P) at a concentration of 1 μ M (0.1 μ M for compound 28), or vehicle (V). Inflammatory response was stimulated by LPS 1 μ g/mL after 1 h, apart from control cells (C). * indicates a significant difference of * P <0.05, *** P <0.001, **** P <0.0001 vs. Vehicle (V; dashed line). Grey line compares the effect of compounds with prednisone (P). Mean \pm SEM. $N=5$, measured in triplicates. Analyzed by ANOVA with Bonferroni's test.

Furthermore, we carried out the ELISA assays focused on the intervention of prenylated stilbenes into protein expression of pro-inflammatory TNF- α being under the NF- κ B/AP-1 transcriptional control. Synthetic compound **34** and natural *Macaranga* stilbene **37** reduced the secretion of TNF- α in THP-1 macrophages comparably to the positive control prednisone. The inhibitory activity of compound **27** from *A. altilis* was significantly higher than this of prednisone (Fig. 25). In spite of great NF- κ B/AP-1 activities, only small portion of prenylated stilbenes significantly reduced TNF- α release. Therefore, we undertook to assess their possible effect on another ubiquitously expressed inflammatory cytokine under NF- κ B/AP-1 control, such as IL-1 β .

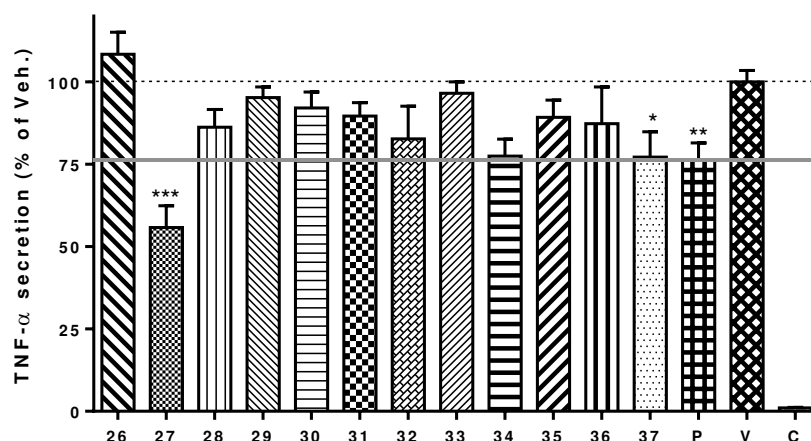


Fig. 25: Effect of prenylated stilbenes 26-37 on LPS-stimulated TNF- α secretion in THP-1 macrophages. Cells were pretreated with compounds at a concentration of 1 μ M (0.1 μ M for compound 28), prednisone (P) 1 μ M, or vehicle (V), and stimulated by LPS (1 μ g/mL) 1 h later, apart from control cells (C). TNF- α was detected by ELISA at 24 h. * indicates a significant difference * P <0.05, ** P <0.01, *** P <0.001. Mean \pm SEM. N =4, measured in triplicates.

Macaranga compounds 31 and 37, together with synthesized stilbene 36 reduced the LPS-stimulated secretion of IL-1 β in the way similar to this of prednisone (Fig. 26). Interestingly, compound 32 inhibiting markedly NF- κ B/AP-1, only slightly decreased TNF- α and had no effect on IL-1 β . The effect on other pro- and anti-inflammatory cytokines under NF- κ B/AP-1 cannot be excluded. On the other hand, stilbene 28 moderately dampened the NF- κ B/AP-1 activity and secretion of TNF- α , but enhanced the expression of IL-1 β , which may be explained by its interactions with some post-transcription or post-translation regulatory mechanisms.

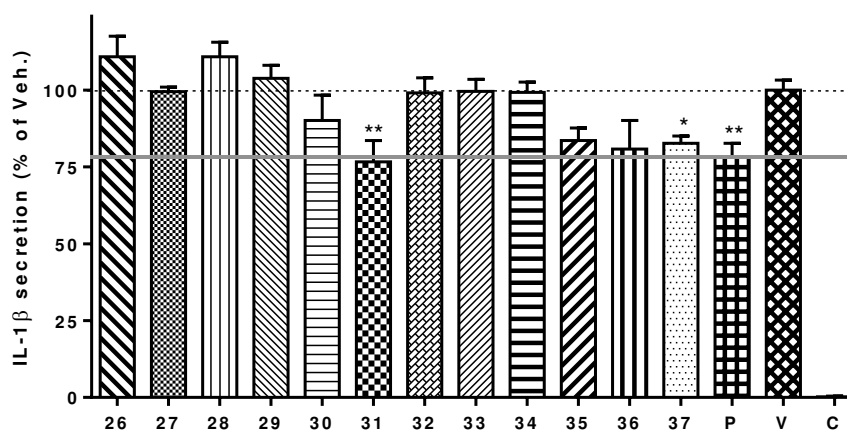


Fig. 26: Effect of prenylated stilbenes 26-37 on LPS-stimulated IL-1 β secretion in THP-1 macrophages. Cells were pre-treated with compounds at a concentration of 1 μ M (0.1 μ M for compound 28), prednisone (P) 1 μ M, or vehicle (V), and stimulated by LPS (1 μ g/mL) 1 h later, apart from control cells (C). IL-1 β was detected by ELISA at 24 h. * indicates a significant difference * P <0.05, ** P <0.01. Mean \pm SEM. N =3, measured in triplicates.

Newly identified compound **27** and recently described *Macaranga* stilbene **37** that were not or very low investigated had been selected for more detailed analysis of events up-stream. Both significantly attenuated the NF- κ B/AP-1 activity and TNF- α expression in human macrophages. Moreover, stilbene **37** reduced also the expression of IL-1 β . The anti-inflammatory effect of another promising compound **31** had been already partly described (Verotta et al., 2009). Therefore, for better comparison we included his active structural analog **32** in the next analysis. Stilbenes **27** and **37** showed to effectively inhibit the I κ B α degradation and any compound affected the activation of MAPKs signaling (Fig. 27). Compound **32** reduced neither I κ B α degradation nor MAPKs activation. Thereby it can possibly interfere with another mechanism, such as NF- κ B/AP-1 nuclear translocation or DNA binding. *In silico* analysis later showed its potential to bind to p50/p65 complex with a high binding affinity ($\Delta G = -6.6$ kcal/mol) and dampen NF- κ B DNA binding (Leláková et al., 2020).

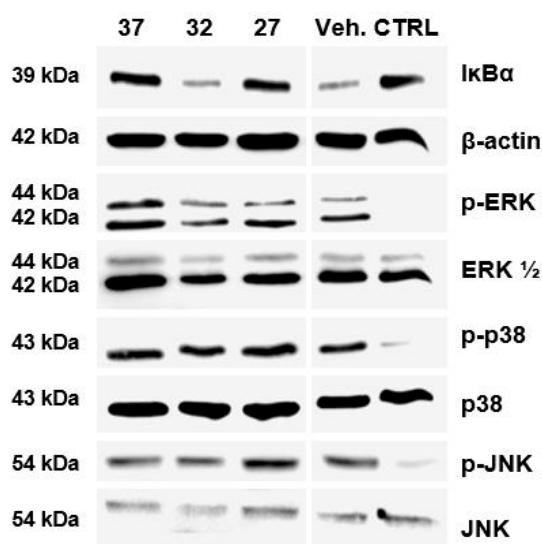


Fig. 27: Effects of selected stilbenes **27**, **32**, and **37** on I κ B α and MAPKs signaling in THP1-XBlue™-MD2-CD14 cells. Cells were pre-treated with compounds at a concentration of 1 μ M, or vehicle (Veh.) and stimulated with LPS (1 μ g/mL), except of control cells (C). The levels of I κ B α , β -actin, and MAPKs in cell lysate were detected 30 min after LPS stimulation by Western blots and immunodetection. Representative blot images of 3 independent experiments.

When comparing between two tested groups, non-prenylated stilbenes showed very little effect on cellular viability and thus safer profile. Prenylated stilbenes displayed some level of cytotoxicity, but also greater anti-inflammatory effects. About one fifth of tested non-prenylated stilbenes (**3**, **7**, **8**, **10**, and **24**) at a concentration of 2 μ M inhibited the NF- κ B/AP-1 activity in a statistically significant manner (approximately by 20%, compound **3**

by 33%), while most of the prenylated stilbenes dampened the NF- κ B/AP-1 by 20–25% at a twice lower concentration of 1 μ M. Non-prenylated stilbenes **6**, **10**, and **24** and prenylated **27**, **34**, and **37** importantly reduced TNF- α secretion in a way comparable or greater than prednisone. Moreover, stilbenes **31**, **36**, and **37** reduced also IL-1 β secretion in THP-1 macrophages. While non-prenylated stilbenes have shown to regulate MAPKs ERK1/2, JNK, and p38 activation at tested concentrations, the prenylated ones reduced the I κ B α degradation. From the most active stilbenes inhibiting the LPS-induced inflammatory-like response in THP-1 human monocytes, we could depict piceatannol (**3**) and oxyresveratrol (**8**) which belong to the most studied stilbenes. Furthermore, thunalbene (**7**), *T. tommasinii* compound **24**, and structurally similar batatasin III (**6**) and its glycoside (**10**) with hydrogenated ethylene bridge were demonstrated as most promising candidates counteracting the TLR4-mediated activation of NF- κ B/AP-1 activation. Our investigation revealed also several active prenylated stilbenes, primarily newly identified compound **27** from *A. altilis*, newly synthesized stilbenes **34** and **35**, and several low investigated *M. siamensis* compounds **30–32**, and **37**. All these compounds were identified as interesting agents for further *in vitro* and *in vivo* investigations of their intervention into inflammatory systemic diseases. This part of our work showed that stilbenes in bioactive foods and medicinal plants possess an auspicious antiphlogistic potential and thus their consumption could be potentially associated with some health benefits in the prevention of inflammatory diseases. Certainly, further *in vitro* and *in vivo* studies are needed.

Anyhow, some stilbenes, such as RSV or oxyresveratrol displayed in previous studies the beneficial effects also at the CNS level in disorders accompanied by mild to a strong inflammatory reaction, including AD and stroke, respectively (Akinwumi et al., 2018). This motivated us to undertake the investigations of stilbenes anti-inflammatory effects at the CNS level. As monocytes in the periphery, microglia together with CNS-associated macrophages play an inevitable role in the control and maintaining of homeostasis in the brain. Thus, we established *in vitro* and *ex vivo* models of LPS-stimulated microglia to observe the inflammation-protective effects of the most active stilbenes against peripheral inflammation. Novel particular prenylated stilbenes from *A. altilis* and *M. siamensis* as natural no/low-investigated compounds aroused our interest because of their promising antiphlogistic potential. However, a low-isolated amounts of compound **27** led us to focus primarily on *Macaranga* stilbenes.

PART II – Stilbenoids in the combat of neuroinflammation

Peripheral and CNS immune systems are closely connected and their abundant crosstalk during the inflammatory event should not be overlooked. The excessive long-term inflammation at the periphery, as well as a severe brain injury or microbial infection may cause the BBB breakdown and drive the infiltration of peripheral immune cells into brain resulting in worsening of inflammatory state and brain injury (Skaper et al., 2018). Neuroinflammation underlines several brain pathologies and activated microglia are its main hallmark. Therefore, discovery of a safe molecule with a strong anti-inflammatory potential able to calm down the inflammation at the periphery, as well as to dampen the activation of microglia would be highly useful in both systemic and CNS inflammatory pathologies.

1. Selection of the most promising molecule

To investigate if stilbenes could mitigate the neuroinflammation, we first compared the effects of 7 tested *Macaranga* compounds (Fig. 28A) on systemic inflammation with the aim to select the most promising agent. Macasiamenene F (**MF**) together with 2,6-diprenyl-resveratrol (**31**) displayed the safest profiles, with IC_{50} values $5.7 \pm 1.1 \mu\text{M}$ and $12.8 \pm 2.4 \mu\text{M}$ for THP-1 cells, respectively (Fig. 28B). MF at concentration of $1 \mu\text{M}$ exhibited a potential to effectively reduce both TNF- α (Fig. 28C) and IL-1 β (Fig. 28D) release by 20%. MF together with compounds **31** and **32** inhibited up-stream the activity of NF- κ B/AP-1 (Fig. 28E), in a greater manner than corticoid prednisone. Anyway, the fact that the anti-inflammatory action of **31** was partly known (Verotta et al., 2009) and stilbene **5** has a limited window of concentrations to be tested due to its IC_{50} $2.7 \pm 1.1 \mu\text{M}$ for THP-1 monocytes, resulted in the selection of MF. We were the first to describe MF multitargeted action and the ability to strike inflammation at several points of the inflammatory cascade, including the reduced degradation of I κ B α (Fig. 28F), a direct inhibitor of NF- κ B. Nevertheless, MF did not show to influence the activation of MAPKs. Therefore it's tempting to believe that MF acts predominantly through I κ B α /NF- κ B pathway, rather than *via* MAPKs/AP-1. Moreover, MF displayed the anti-inflammatory potential at pharmacologically and clinically relevant concentration which was proved to be achievable in human plasma after oral administration of the most known stilbene RSV (Boocock et al.,

2007b). Worthy to mention that most of studies showing the biological effects of stilbenes were carried out using much higher concentrations up to 1 mM (Smoliga et al., 2011).

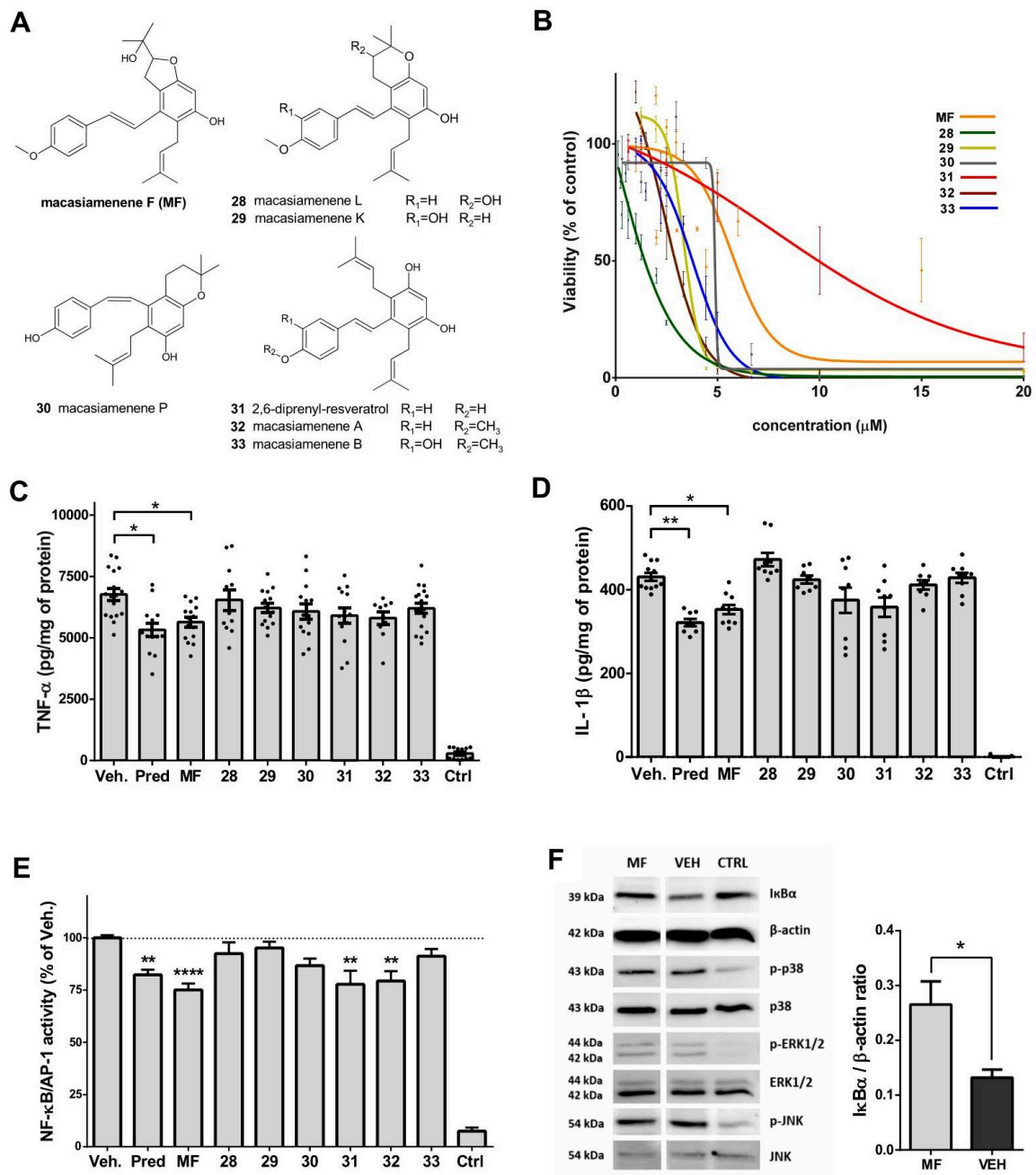


Fig. 28: Effects of prenylated *M. siamensis* stilbenoids on IκBα/NF-κB signaling in LPS-stimulated THP-1 macrophages and THP-1 XBlue™-MD2-CD14 monocytes. **A**, Tested *Macaranga* stilbenoids; **B**, Dose-response curves showing viability of THP-1 after a 24 h incubation; **C**, Effect on the secretion of TNF-α protein; **D**, Effect on the secretion of IL-1β protein; **E**, Activity of NF-κB/AP-1 after 24 h; **F**, MF upstream regulation of IκBα. Mean±SEM, N=3, *P<0.05, **P<0.01, ****P<0.0001 vs. vehicle treated cells (Veh), analyzed by the non-parametric tests Kruskal-Wallis in the case of C-E, and the Mann and Whitney for F.

Considering that the inflammatory response of NF- κ B is guided by its nuclear translocation and its DNA binding activity, intervening with one of these steps or eventually with both may count for MF anti-phlogistic effect. Because the primary role of I κ B α is to prevent the translocation of NF- κ B to the nucleus, our results showing MF potential to inhibit the I κ B α degradation suggest that MF could interfere with NF- κ B nuclear translocation. Therefore, in collaboration with Dr. Václavík from Dpt of Natural Drugs MU, we further aimed to investigate whether MF could also directly interact with NF- κ B to prevent its DNA binding. Using molecular ligand-protein docking *in silico* simulations we studied the interactions of MF with NF- κ B p65/p50 heterodimer (Fig. 29A) according to the method by Trott and Olson (Trott and Olson, 2010). MF displayed several interactions with p50 subunit. The hydroxyl groups of MF tended to create the classical hydrogen bonds (O-H \cdots O) with residues Thr502 and Ser508, and non-classical hydrogen bond (N-H \cdots π) with Leu507. The alkyl hydrophobic bonds were observed with residues His441, Lys444, Val447, and Leu507. Moreover, two phenyl rings of MF typical for stilbene skeleton tend to form π -alkyl hydrophobic interactions with Lys444 and Leu507 (Fig. 29B). Altogether, MF showed a high affinity ($\Delta G = -6.7$ kcal/mol) for NF- κ B DNA binding pocket comparable to other biologically active stilbenes inhibiting the NF- κ B activity (Leláková et al., 2019). Although binding activities of *M. siamensis* compounds were similar (close to $\Delta G = -7$ kcal/mol), MF exerted the highest extension to DNA binding site and thus provided the highest steric interactions with DNA binding pocket. Overall, these results indicate that MF may not only inhibit the NF- κ B activity by indirectly preventing its translocation through decreased I κ B α degradation but also directly by interfering with NF- κ B DNA binding (Leláková et al., 2020).

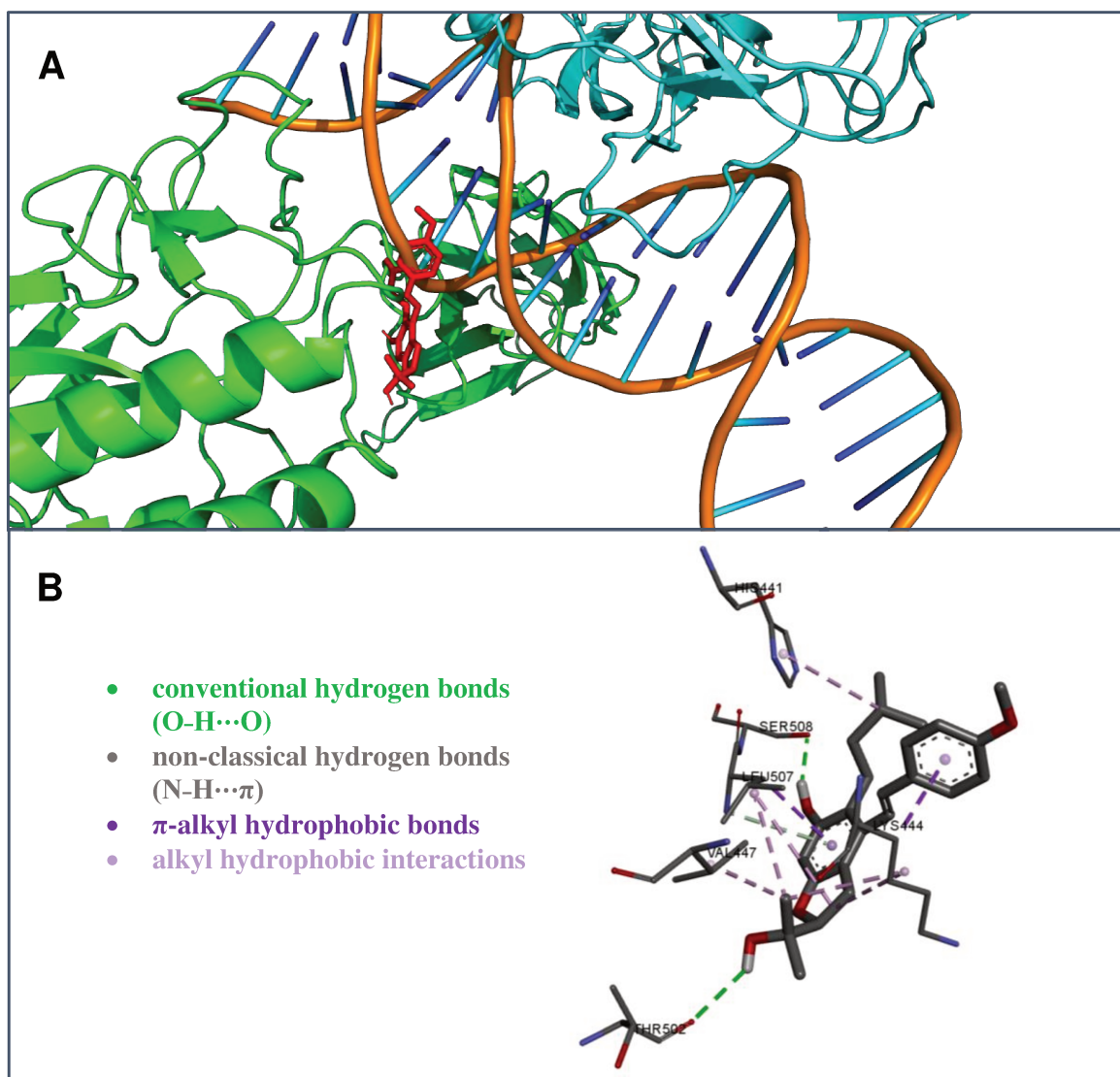


Fig. 29: Molecular docking simulation of MF interactions with NF- κ B DNA binding site (PDB 3GUT). A, The bond of MF to p50 subunit of NF- κ B DNA binding pocket. Red - MF, green ribbon - p50 subunit, blue ribbon - p65 subunit, orange double helix with colored sticks - DNA fragment; B, Interactions of MF with p50 NF- κ B depicting hydrogen bonds (green-classical, grey-non-classical) with p50 residues Thr502, Ser508, and Leu507, and hydrophobic interactions (dark and light purple) with His441, Lys444, Val447, and Leu507.

2. Determination of MF dose-response relationships in microglia

Then we tried to extrapolate the same inflammatory set up for microglial cells, immune brain cells involved in the first line of CNS defense, equivalent to macrophages on the periphery. To perform the anti-inflammatory assays on the safe side, we firstly evaluated the dose-response effect of MF on cell proliferation and mitochondrial activity of BV-2 mouse microglia, which are two main indicators of microglial proper cellular functioning.

Mitochondria are generators of energy for cellular processes, involved in maintaining the cell cycle, growth, and differentiation, as well as cell death. Their altered activity reflects an imbalance in cell physiology and is often associated with a switch from physiological to a pathological state. Moreover, changes in mitochondrial activity may induce an inflammatory response (Meyer et al., 2018). Therefore it was important to identify whether and which dose of MF could affect the BV-2 vital functions. While the dose of 6 μM did not affect the proliferation of BV-2 cells, neither their mitochondrial activity, the dose of 15 μM had some negative effect (Fig. 30A+B).

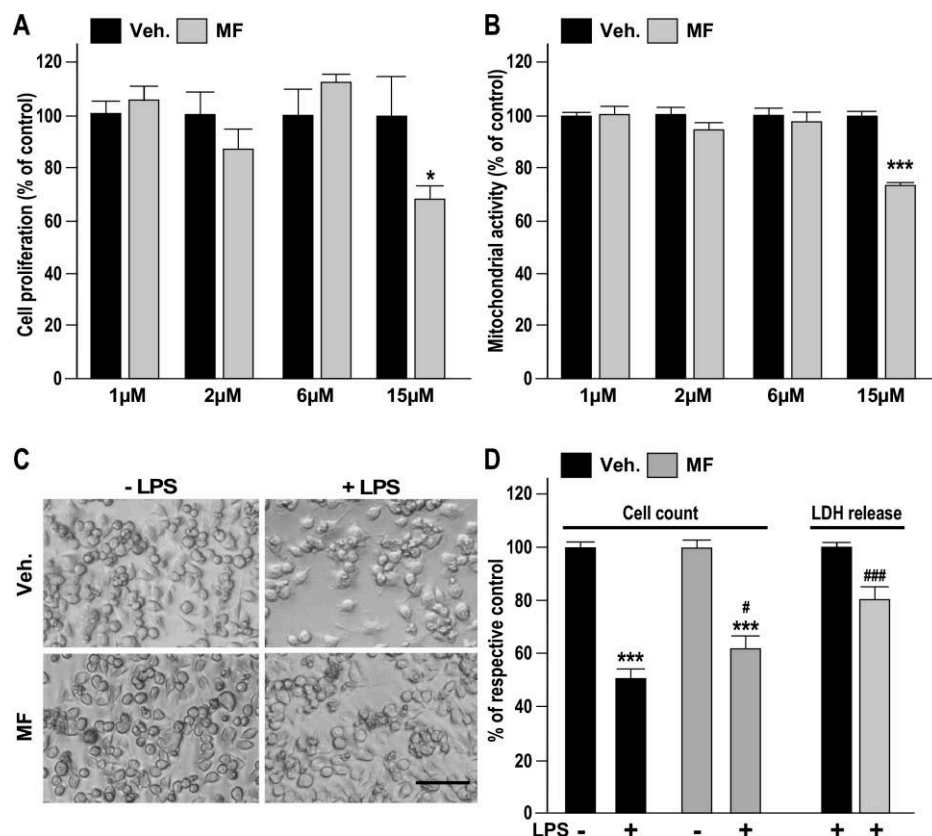


Fig. 30: Evaluation of MF dose-limiting toxicity and its cytoprotective action against inflammation-challenged BV-2 cells. **A**, Effect of MF at concentrations of 1, 2, 6, and 15 μM on the proliferation of cells assessed by cell counting after 24 h, * indicates $P < 0.05$, significant difference in comparison with only the vehicle (Veh.) treated cells; **B**, Effect of MF treatment on the mitochondrial activity of BV-2 cells at 24 h, *** indicates $P < 0.001$ compared to Veh.; **C**, Representative pictures (Scale bar = 100 μm) showing the MF-induced improved survival of BV-2 cells injured by a 24 h-LPS challenge; **D**, Quantitative evaluation of the beneficial effect of MF on BV-2 cells injured by a 24 h-LPS challenge assessed by cell counting and the extent of LDH release, *** indicates $P < 0.001$ compared to the respective LPS non-stimulated control, # $P < 0.05$ and ### $P < 0.001$ indicate significant differences between MF+LPS vs. Veh.+LPS. Mean \pm SEM; N=6 measured in sextuplicates, analyzed by Student's *t*-test.

To provide an equivalent study, we therefore selected the previously identified anti-inflammatory dose of 1 μ M to study the anti-inflammatory potential of MF on LPS-triggered inflammation of mouse microglia. At this concentration, the 24 h application of MF attenuated the LPS-triggered loss of microglia by 20% in comparison with the vehicle-treated cells (Fig. 30C+D). This cytoprotective action was supported by a reduction in the release of LDH by one fifth in cells pretreated with MF, as compared to vehicle-treated cells (Fig.30D).

3. Pre-treatment by MF for prevention of neuroinflammatory diseases

In the next part of our study, we evaluated whether a pre-treatment of microglia with MF 1 μ M could have some anti-inflammatory properties (Fig. 31).

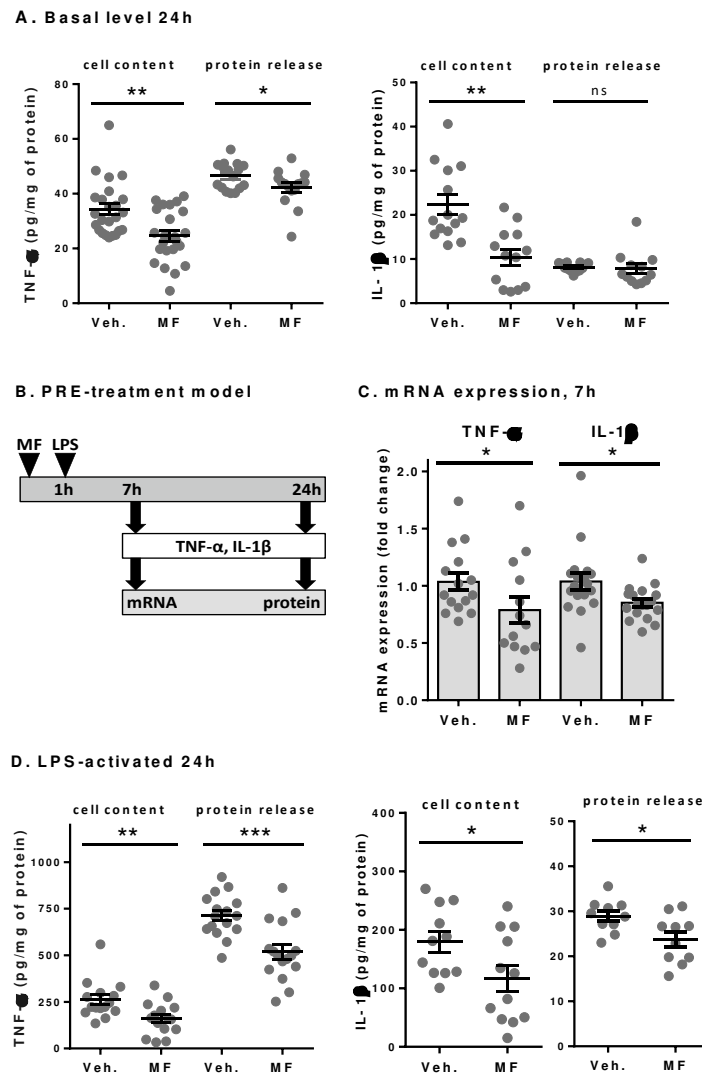


Fig. 31: Anti-inflammatory effects of MF pre-treatment on the expression of TNF- α and IL-1 β . A, MF reduced the basal expression and release of TNF- α and IL-1 β in BV-2 cells; B, Schema

illustrating the protocol of pre-treatment of BV-2 cells with MF (1 μ M) and the timing of evaluation of its effect on the mRNA and protein expression: **C**, MF reduced the LPS-stimulated expression of TNF- α and IL-1 β mRNA; **D**, MF reduced the LPS-induced expression and release of TNF- α and IL-1 β by BV-2 cells. * P <0.05, ** P <0.01, *** P <0.001 vs. Veh. group. Data are expressed as the mean \pm SEM, $N=3$, measured in sextuplicates, analyzed by the non-parametric Mann and Whitney test for two independent groups.

We firstly observed that MF reduced already the basal level of IL-1 β and TNF- α without a harmful stimulus (Fig. 31A). Then, MF pre-application to LPS-stimulated microglia resulted in reduction of TNF- α and IL-1 β protein levels detected from cell lysates and supernatants after 24 h (Fig. 31D). This was correlated with decreased levels of mRNA expression for both studied cytokines, whose reduction represented about 26% and 20% for TNF- α and IL-1 β , respectively (Fig. 31C). Dampened intracellular levels of TNF- α and IL-1 β at 24 h were also detected using epifluorescence microscopy, as displayed on representative pictures in Fig. 32 and Fig. 33, respectively.

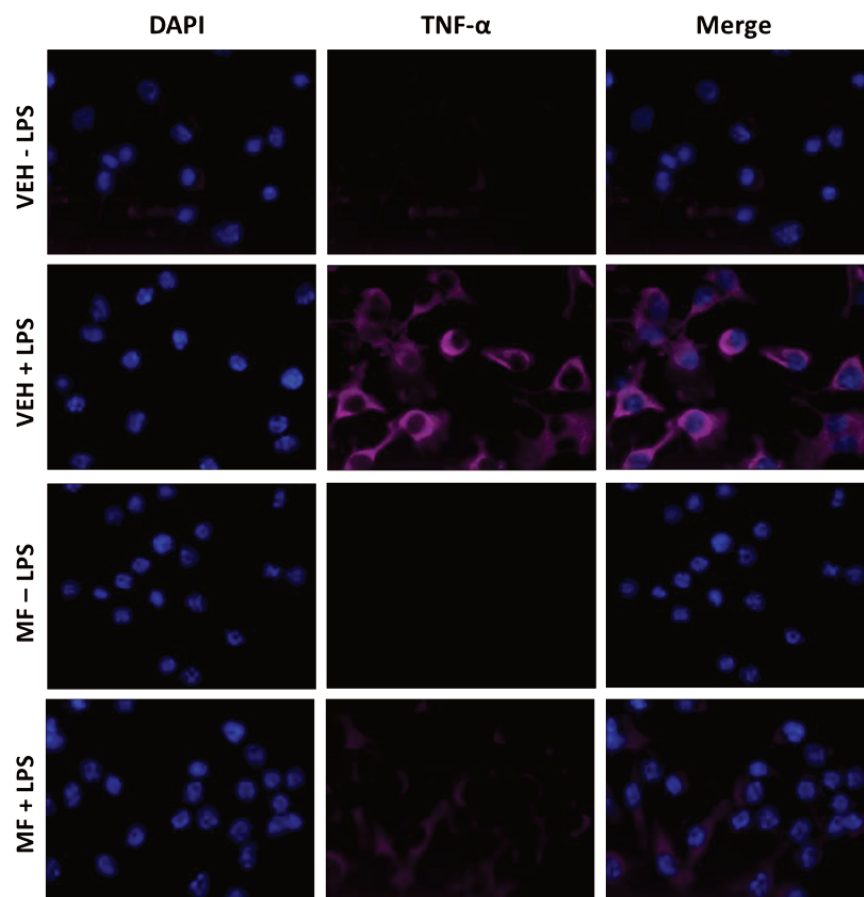


Fig. 32: Representative pictures of the effect of MF pre-treatment on the expression of TNF- α . BV-2 microglia pre-treated with MF 1 μ M and stimulated by LPS were stained at 24 h with Alexa Fluor 594-labeled anti-TNF- α antibody and DAPI, then observed by epifluorescence microscopy (magnification 40x).

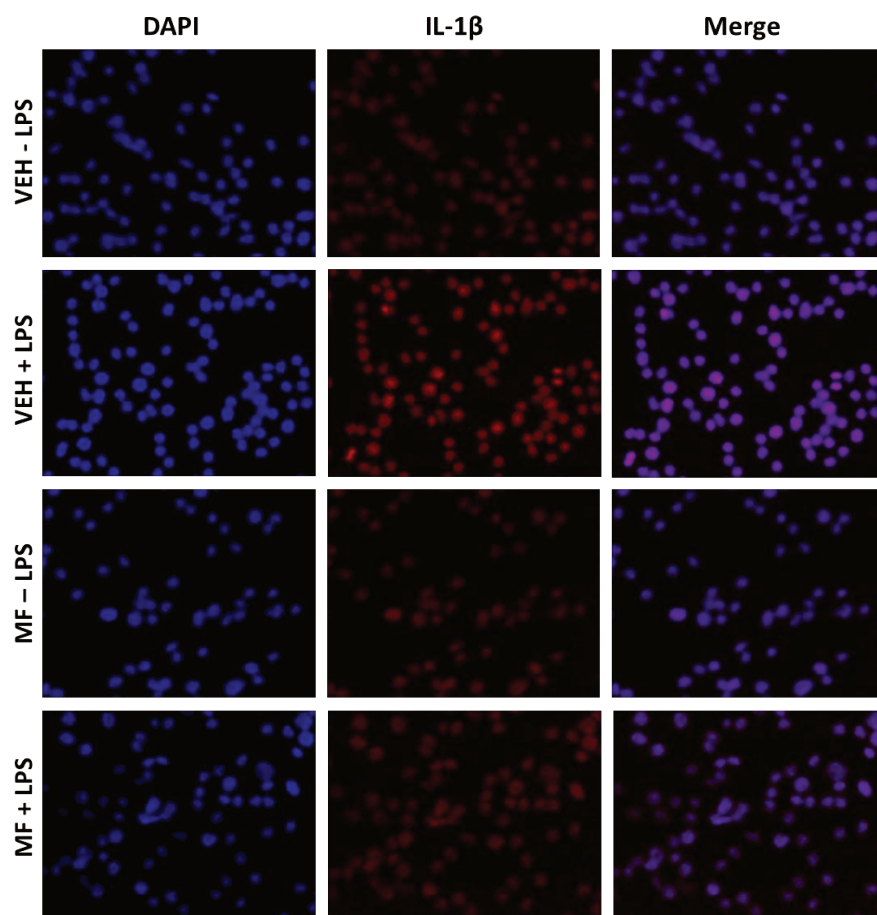


Fig. 33: Representative pictures of the effect of MF pre-treatment on the expression of IL-1 β . BV-2 microglia pre-treated with MF 1 μ M and stimulated by LPS were stained at 24 h with Alexa Fluor 546-labeled anti-IL-1 β antibody and DAPI, then observed by epifluorescence microscopy (magnification 20x).

4. Interventional treatment by MF for slowing down the inflammatory response

The antiphlogistic effect of MF pre-treatment would be interesting in the prevention of brain inflammatory diseases and the development of nutraceuticals and functional foods. However, the fact that this treatment should be applied pre-emptively limits its clinical usefulness, mostly for deleterious events emerging without warnings, such as traumatic brain injury or ischemic stroke. Hence, we studied a possible therapeutic use of MF as interventional treatment during (co-treatment protocol; Fig. 34) or after (post-treatment protocol; Fig. 35) the induction of inflammation. To investigate the ability of MF to intervene in the early phase of inflammation, we assessed the cellular content and protein release of TNF- α and IL-1 β in BV-2 cells after 6 h and 24 h of co-application of MF with LPS.

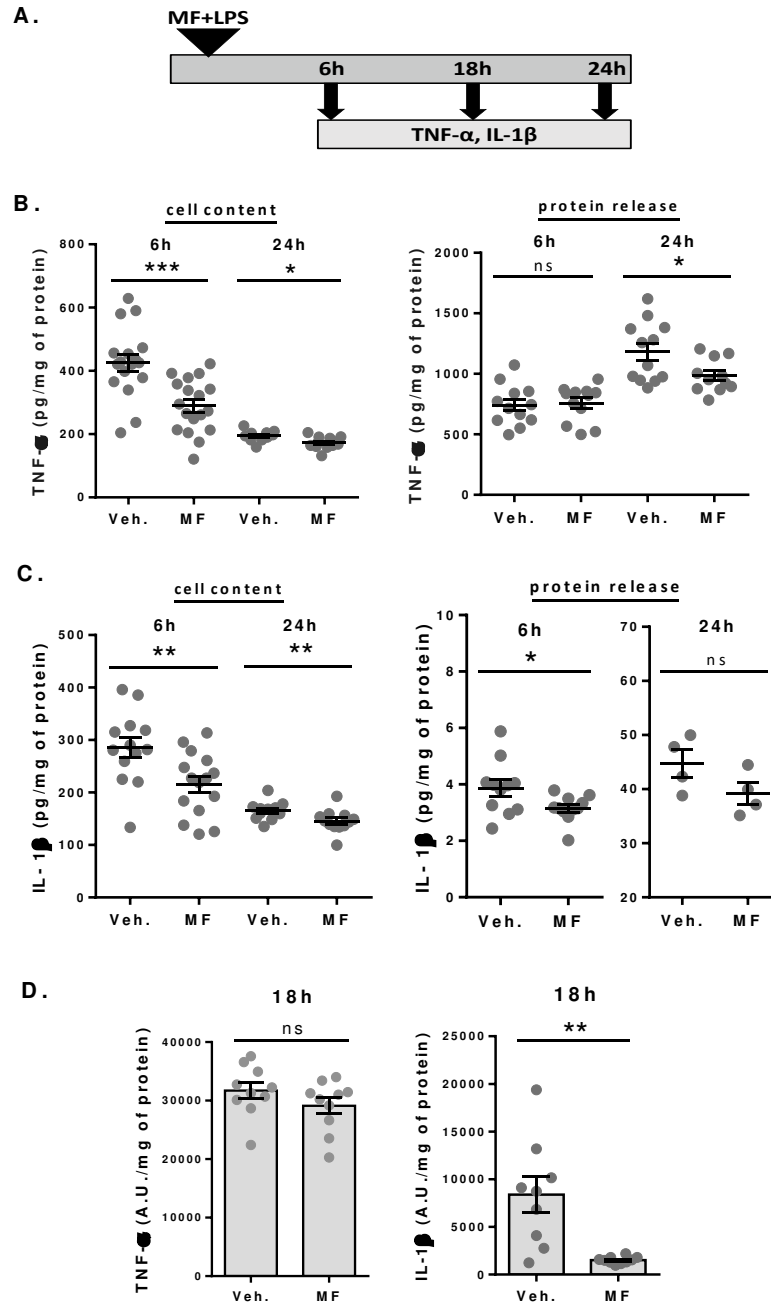


Fig. 34: Anti-inflammatory effects of MF co-treatment with LPS in the BV-2 cells and the ex vivo cultured microglial cells. **A**, Schema displaying the co-treatment of BV-2 cells and primary mouse microglia with MF (1 μ M) co-treatment with LPS (1 μ g/ml) together with time points at which the cytokines were measured: 6 h and 24 h for the BV-2 cell line, and 18 h for the brain-sorted microglia; **B**, Expression of TNF- α in the cell content and protein release from the BV-2 cells at 6 and 24 h; **C**, Expression of IL-1 β in cell content and protein release from BV-2 cells at 6 and 24 h; **D**, TNF- α and IL-1 β protein contents in brain-sorted mouse microglia after 18 h of co-treatment of MF or Veh. with LPS. Data are expressed as the mean \pm SEM, N=3, measured in sextuplicates, analyzed by the Mann-Whitney test. *P<0.05, **P<0.01, ***P<0.001 vs. Veh. group.

Co-treatment with MF markedly dampened both TNF- α and IL-1 β intracellular levels and release induced by LPS. MF reduced by 33% the LPS-stimulated intracellular up-regulation of TNF- α after 6 h of co-treatment, which resulted in a decrease of TNF- α protein release at 24 h, approximately by 20% (Fig. 34B). Very similar inhibitory effect of MF was observed also in the case of LPS-induced IL-1 β protein expression in BV-2 cells (Fig. 34C). With the aim to slightly approach to more clinically transferable data, we next determined the effect of MF and LPS co-application on the model of *ex vivo* cultured primary mouse microglia. While the level of TNF- α protein release did not seem to be significantly decreased, MF dampened the IL-1 β protein expression by factor of five in comparison with the vehicle (Fig. 34D).

Afterward, we evaluated the efficiency of MF on ongoing inflammation as anticipated step toward clinical translation. Applied as post-treatment, anti-inflammatory drugs are supposed to resolve the inflammatory state by dampening the levels of pro-inflammatory cytokines such as TNF- α and IL-1 β . The expression of such molecules is initiated within the first 3–6 h after the LPS challenge. This correlates with their expression observed *in vivo* in severe acute cerebrovascular diseases accompanied by a massive inflammatory reaction, like stroke or trauma (Le Thuc et al., 2016). The inhibition of inflammation is of interest to avoid deleterious effect of an ischemic event and increase chances for improved stroke outcomes, essentially when the treatment is initiated after the therapeutic window for thrombolysis with rtPA, thus after 3–4.5 h since the stroke onset (Hacke et al., 2008). For this reason, we evaluated if MF applied 3 and 6 h after the inflammation onset could change the course of an ongoing inflammatory reaction at 6 and 24 h, respectively (Fig. 35). MF applied 3 h after LPS reduced the levels of TNF- α and IL-1 β approximately by 20% observed at 6 h (Fig. 35B). When microglia were treated with MF 6 h after the inflammatory onset, MF was still able to reduce TNF- α cellular content and release observed at 24 h. The amounts of IL-1 β stayed unchanged at 24 h, as compared with vehicle-treated cells (Fig. 35C). This may be explained by the fact that IL-1 β expression occurs in high amounts during the first hours after LPS stimulation (Kwon et al., 2010). Thus, a late post-treatment, together with a low dose of MF would be too strict conditions to exert any anti-inflammatory effect. So, increasing the dose and/or shortening the time of application may be anticipated options for further investigations aiming to improve the MF anti-inflammatory effect on this particular cytokine.

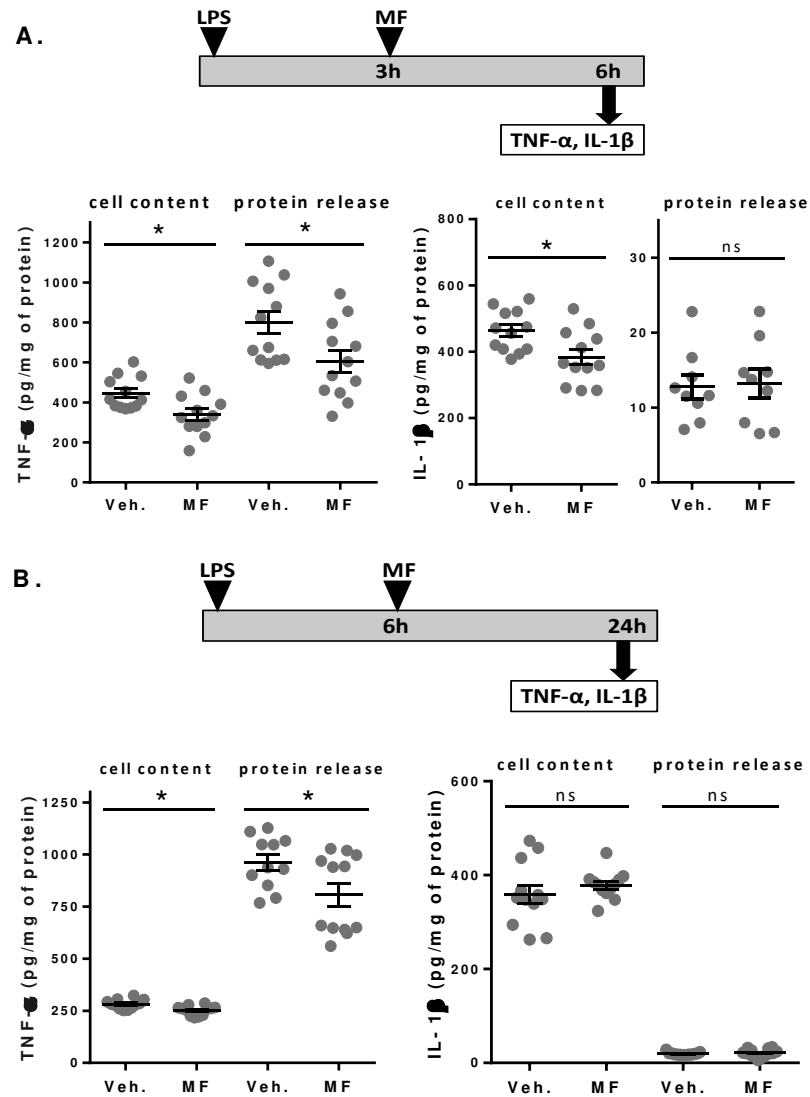


Fig. 35: Anti-inflammatory effects of MF post-treatment in BV-2 cells. Schemas illustrating the treatment protocol by inducing inflammation with LPS (1 $\mu\text{g}/\text{mL}$) in BV-2 cells and treating them 3 h (A) or 6 h later (B) by applying MF post-treatment (1 μM). A, MF post-treatment applied 3 h after LPS reduced levels of both the protein content and release of the TNF- α and the IL-1 β evaluated at 6 h after LPS stimulation. B, MF post-treatment applied at 6 h after LPS reduced TNF- α protein content as evaluated at 24 h after the application of LPS, but not influenced the IL-1 β protein expression, which probably occurred entirely before the post-treatment application. Data are expressed as the mean \pm SEM, N=3, measured in sextuplicates, analyzed by the Mann-Whitney test. * P <0.05 vs. Veh. group.

PART III – Effects of MF on other mechanisms of ischemic cascade

Neuroinflammation is undoubtedly one of the major factors in stroke pathobiology and its outcome. Nevertheless, ischemic stroke launches also other highly shattering mechanisms, counting oxidative stress and glutamate-induced excitotoxicity. Hence, the intervention of MF into one or both of these devastating mechanisms would count for its neuroprotective effects.

1. Effect of MF on the oxidative stress produced by microglia

Microglia are one of major producers of free radicals contributing to oxidative damage of other brain cells, including neurons (Guruswamy and ElAli, 2017). Therefore we decided to gain some insights if MF could lessen the oxidative stress produced by BV-2 microglia and exert some antioxidant effects. To establish a model of oxidative stress, it is important to select an appropriate source of free radicals. An extensively reported generator of free radicals 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) is often employed in the study of lipid peroxidation, which is the main harmful oxidative process in cerebral ischemia. The decomposition of AAPH produces one mole of nitrogen and two moles of carbon radicals which further react with molecular oxygen and generate peroxy radicals, maintaining a constant rate of free radical production (He et al., 2013). The cellular antioxidant assay (CAA) is considered as biologically more relevant method than numerous simple antioxidant activity assays (Trembl et al., 2019). The antioxidant activity of MF was shown to be poor in comparison with known flavonoid antioxidant quercetin (Q) used as a positive control. MF 1 μ M showed a capability to slightly (by 13%) lower the oxidative stress in BV-2 cells unlike the potent antioxidant effect of quercetin 1 μ M whose inhibition of ROS 1 h after AAPH stimulation represented 41% (Fig. 36B). To verify if this inhibitory effect of MF could be dependent on the concentration as it is known for quercetin, we further tested MF in concentration range of 0.5–6 μ M. While the effect of quercetin was dose-dependent, MF displayed a very similar antioxidant activity at all tested concentrations (Fig. 36A-C). Obtained curves further served for the calculations of AUCs of fluorescence *vs.* time correlations and evaluation of CAA units as marker of antioxidant activity. The antioxidant effect of quercetin 0.5, 1, and 2 μ M represented 26, 39, and 47 CAA units, while this of MF 0.5, 1, 2, and 6 μ M only 6, 7, 7, and 10 CAA units, respectively (Fig. 36D).

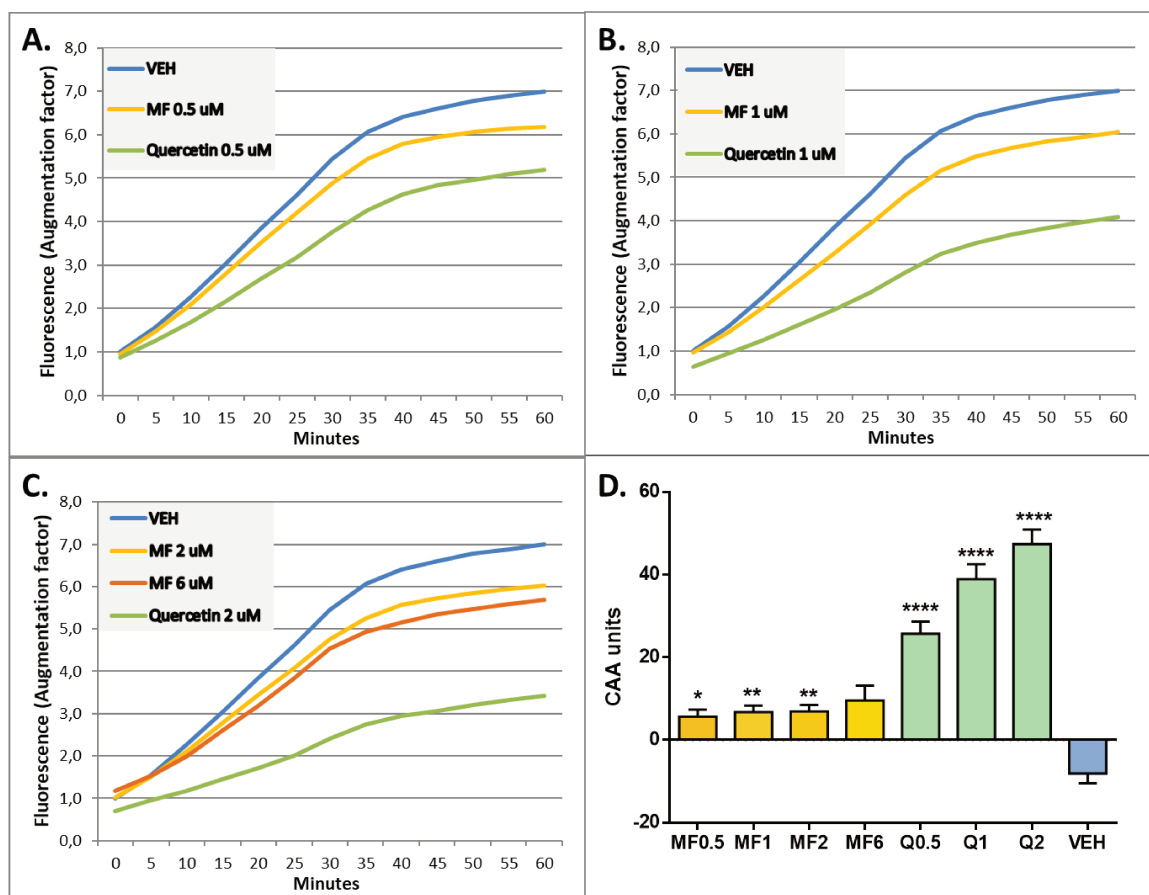


Fig. 36: Cellular antioxidant activity of MF in comparison with quercetin (Q). A.-C. Effect of MF 0.5 - 1 - 2 - 6 μM and quercetin 0.5 - 1 - 2 μM on AAPH-induced oxidative stress in BV-2 cells. Obtained time dependent curves A-C further served for calculations of CAA units, based on AUCs of fluorescence versus time correlations. Mean \pm SEM, N=3, measured in sextuplicates, analyzed by the Kruskal-Wallis test. * P <0.05, ** P <0.01, **** P <0.0001 vs. Veh., evaluated by Kruskal-Wallis analysis.

These results indicate that the antioxidant potential of MF is limited which is due to MF structure. Stilbenes with several hydroxyl groups displayed a remarkable antioxidant activity. Quantitative structure-activity relationships (QSAR) comparisons revealed that 3,4-dihydroxy or 3,4,5-trihydroxy substitution of stilbene core is very important for enhanced antioxidant action of stilbenes (Murias et al., 2005). It has been demonstrated in several studies that RSV (*trans*-3,4',5-trihydroxystilbene) and its related stilbenes, such as piceid (*trans*-5,4'-dihydroxystilbene-3-*O*- β -D-glucopyranoside) exhibit a high radical scavenging activity in biological membranes undergoing lipid peroxidation (Fabris et al., 2008; Tadolini et al., 2000). Study comparing the antioxidant effects of RSV and its five hydroxylated derivatives on superoxide ($\text{O}_2^{\bullet-}$) radicals, which are precursors of radicals involved in lipid peroxidation revealed that the most hydroxylated analogues piceatannol

(3,3',4',5-tetrahydroxystilbene) and 3,3',4,4',5,5'-hexahydroxystilbene display much stronger superoxide radicals removal than RSV and the efficient antioxidant α -tocopherol. Moreover, piceatannol and 3,4,4',5-tetrahydroxystilbene were shown as significant scavengers of diphenylpicrylhydrazyl (DPPH[•]), which is often used to assess the ability of polyphenols to transfer labile H-atoms to lipid peroxy radicals (Murias et al., 2005). MF structure comprises only one free hydroxyl in *meta*- position of B ring and one hydroxyl is cyclized by prenyl moiety. Although prenyl moiety comprises also one hydroxyl substitution, possibly it has only a low impact on MF antioxidant effects. This result support other studies showing that modification of stilbene core and mostly substitution of phenolic hydroxyl groups (*e.g.* their methylation) tend to decrease the antioxidant potential of hydroxystilbenes (Trembl et al., 2019). We hypothesize that a similar antioxidant effect could be observable also in neuronal cells.

2. Effect of MF against NMDA-induced excitotoxicity in cortical neurons

While the antioxidant potential of MF seems poor, MF may display other beneficial effects like counteracting the glutamate-induced excitotoxicity which could support its potential protective effect against an ischemic insult. Hypoxic neuronal injury is linked with abnormal activation of postsynaptic glutamate receptors and the toxicity of glutamate was attributed to the lethal Ca²⁺ influx, mostly through dysregulated NMDA receptors (NMDAR) (Lauritzen et al., 2000). Thus, we further investigated if MF could protect against NMDAR-mediated toxicity in primary mouse cortical neurons using *N*-methyl-D-aspartic acid (NMDA) that is a specific agonist of NMDAR mimicking the excitatory action of glutamate. Primary cortical neurons were pretreated with MF 1 and 5 μ M or Vehicle, then exposed to NMDA at a concentration of 50 μ M (Zhou et al., 2013) which progressively triggered the LDH release from damaged neurons and neuronal death. The cell leakage was continuously growing in time and found to be increased by a factor of five at 28 h (Fig. 37). This was accompanied by a gradual decrease of cellular viability (Fig. 38). MF 1 μ M did not show any significant effect on neuronal survival and only moderate decrease in LDH release (data not shown). However, MF 5 μ M effectively dampened the NMDA-induced LDH release at 20 h and 28 h approximately by 25% for both time points (Fig. 37). The viability of neuronal cultures treated with MF was assessed to be 41% vs. 27% for the vehicle-treated cells at 20 h. Small protection was found also at

28 h, although the NMDA 50 μ M treatment during 28 h represented lethal conditions for neuronal culture and MF did not promote any radical effect at 28 h (Fig. 38).

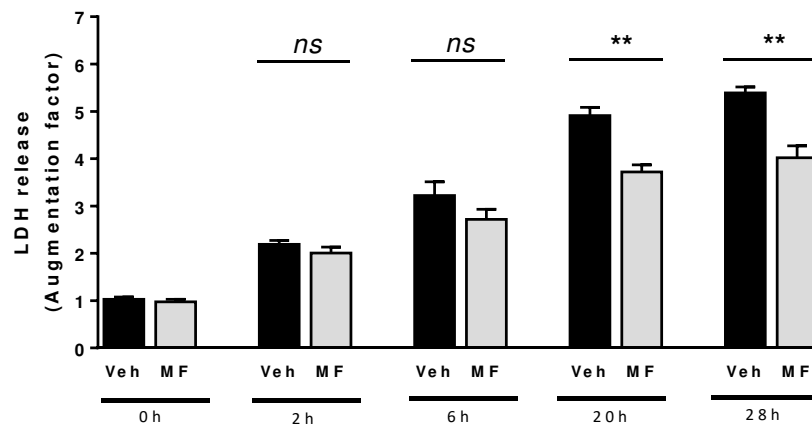


Fig. 37: Effect of MF on LDH release in NMDA-challenged cortical neurons. Primary cortical neurons were treated with MF 5 μ M or Vehicle (Veh) alone for 1 h. The excitotoxicity was then induced with NMDA 50 μ M. Increase in LDH release was calculated in relation to basal levels of non-stimulated respective controls. Data are expressed as mean \pm SEM, measured in sextuplicates, analyzed by the Mann-Whitney test. ** P <0.01 vs. Veh. group.

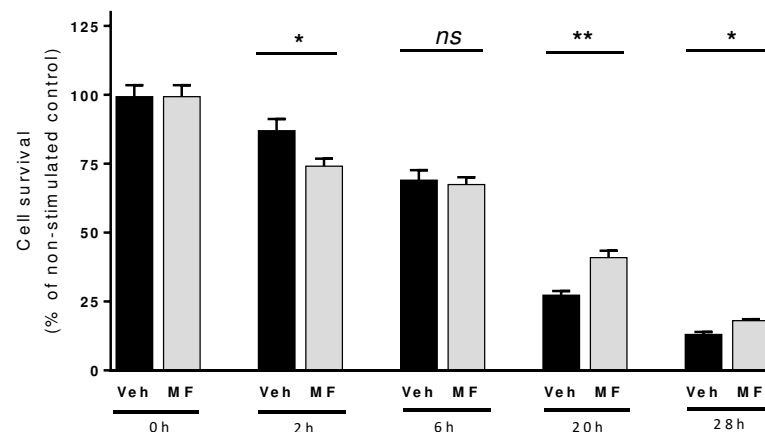


Fig. 38: Effect of MF on cell survival against NMDA-challenged excitotoxicity in cortical neurons. Primary cortical neurons were treated with MF 5 μ M or Vehicle (Veh) alone for 1 h, then, the excitotoxicity was then induced using NMDA 50 μ M. Cell survival rate was calculated as the percentage of NMDA-non-stimulated cells of respective controls for each time point. Data are expressed as mean \pm SEM, measured in sextuplicates, analyzed by the Mann-Whitney test. * P <0.01, ** P <0.01 vs. Veh. group.

These results indicate that MF at a higher concentration of 5 μ M could prevent neuronal damage induced by the excitotoxic state and to some extent promote neuronal survival. Nevertheless, other investigations would be necessary to describe how MF exert such an effect, *e.g.* study of its effect on Ca²⁺ levels, or implication in post-synaptic

glutamatergic transmission through NMDARs. NMDA excitotoxicity being the driving force of ischemic cell death, our previous result suggested MF could antagonize or at least slow down the deleterious effects induced by the privation of oxygen and glucose occurring during ischemia. Thus, in the following assays, we evaluated MF effects against oxygen-glucose deprivation (OGD)-challenge in primary cortical neurons.

3. Effect of MF on OGD-challenged primary cortical neurons

Altogether, to investigate if MF could counteract the deleterious effects of the ischemic injury and prevent the neuronal damage, we selected an *in vitro* model of stroke – primary cortical neurons in culture exposed to oxygen-glucose deprivation (OGD). Briefly, neurons treated for 1 h with vehicle (Veh) or MF 5 μ M which has been shown efficient in the previous assay were exposed to 1 h (lethal) or 2 h (supralethal) OGD challenge according to the method described previously (Maati et al., 2013). Then, the mitochondrial activity corresponding to the percentage of viable cells and cellular death were determined directly after 1 h/2 h OGD (T1/T2, respectively) and 24 h after OGD. Necrotic cell death was determined as the amount of LDH release from damaged cells, while the percentage of cells dead by apoptosis was evaluated by counting of pyknotic nuclei stained by Hoechst. Unlike normal healthy cells, pyknotic nuclei were recognized as small condensed nuclei with high fluorescence, calculated using Image J software, then served for evaluation of pyknotic nuclei/total nuclei number ratios. While the cellular viability corresponding to the metabolically active cells exposed to 1 h OGD was not affected directly after the OGD challenge, its harmful effect was observable at 24 h (Fig. 39A). The percentage of cell survival at 24 h in OGD-exposed cells was similar in both groups – treated and non-treated with MF. Nevertheless, we observed a radical decrease in metabolic activity also in non-OGD-exposed cells treated with the vehicle (Fig. 39A). We supposed that this harmful effect could be attributed to the aging of neuronal culture, as well as manipulation (even very careful) during experiment which included several necessary rinsing steps (before Veh/MF treatment, then before and after OGD) of these highly susceptible cells. Surprisingly, MF significantly protected neurons against these deleterious effects (Fig. 39A). One hour of OGD severely induced the necrotic cell death observed as a massive release of LDH directly after OGD (T1h+OGD) and at 24 h (T24h+OGD; Fig. 39B), while the number of counted apoptotic nuclei in OGD-treated groups was not significantly

elevated at 1 h neither 24 h, in comparison with respective non-OGD controls (Fig. 39D). In accord with previously observed effect on cell survival, we observed the reduced LDH release in non-OGD group treated with MF at 24 h. Lower LDH release was observed also in OGD-exposed cells treated with MF, but this effect did not differ from the Vehicle-treated group when compared both Veh/MF to their respective non-OGD controls (Fig. 39B). The deleterious effect of OGD at 24 h is displayed in Fig. 39C. The similar trend was observed also in the case of pyknotic nuclei counting, where MF showed a significant effect on apoptotic cell death in non-OGD-treated cells at 24 h (Fig. 39D).

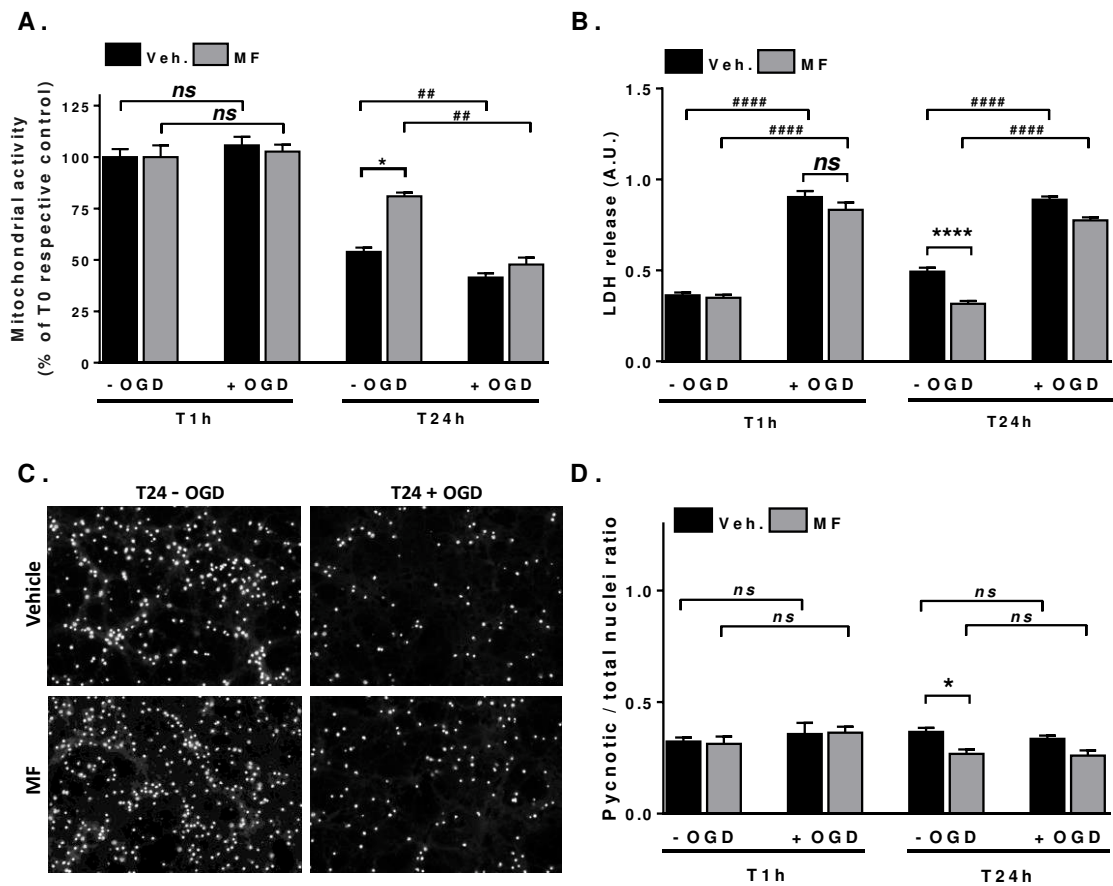


Fig. 39: Effect of MF on primary cortical neurons challenged by 1 h OGD. Primary cortical neurons were treated with MF 5 μ M or Vehicle (Veh) for 1 h, then exposed to 1 h OGD conditions. **A**, Effect of MF 5 μ M on mitochondrial activity of neurons directly after (T1h) and 24 h after (T24h) OGD, expressed as a percentage of basal activity of respective controls before (T0) OGD challenge. **B**, Effect of MF on LDH release at 1 h and 24 h, compared to basal activity of respective controls before (T0) OGD. **C**, Representative photomicrographs of neuronal cells stained by Hoechst depicting the density of neurons in culture after 1 h OGD challenge. Magnification 20x. **D**, Effect of MF on apoptotic neuronal death at 1 h and 24 h, compared to the basal levels of respective controls at T0, calculated as a number of pyknotic/total nuclei ratios.

*Data are expressed as mean±SEM, measured in sextuplicates. *P<0.05, ****P<0.0001 compared to Vehicle-treated group, ##P<0.01 and ####P<0.0001 compared to respective non-OGD controls.*

Since the 1 h OGD triggered mostly necrotic cell damage and its harmful effects were observable at 24 h, we exposed neurons to 2 h (supralethal) OGD challenge to observe its short-term (T2) and long-term (T24) effects on neuronal survival and death (Fig. 40). Unlike 1 h of OGD, two hours of OGD decreased the mitochondrial activity in both vehicle and MF treated neurons by 23% and 45% observed at 2 h (Fig. 40A). This was correlated with radical increase in LDH levels at 2 h, higher in MF treated group (Fig. 40B). Results from this assay confirm the previously described effects of manipulation and aging of neuronal culture observed in non-OGD groups at 24 h (Fig. 40A+B). Again, MF displayed the ability to protect neurons against these negative effects and protect cell survival (Fig. 40A), as well as reduce LDH release (Fig. 40B) in non-OGD-treated groups at 24 h. Unlike 1 h OGD, two hours of OGD challenge induced markedly the apoptotic cell death, observed in the form of elevated numbers of pyknotic nuclei at 2 h and 24 h (Fig. 40C+D), while LDH release at 24 h in OGD-exposed cells was much lower than after 1 h OGD (Fig. 40B). Lower number of pyknotic nuclei was counted at 2 h and 24 h in cultures treated with MF (Fig. 40D).

The protective effect on cell survival, dampened LDH release, and lower number of counted pyknotic nuclei found at 24 h in both assays in non-OGD-challenged groups indicate potential neuroprotective effect of MF against the aging of neuronal culture and harmful effects related to the manipulation, while it complicates the analysis of OGD assay and evaluation of MF effects against OGD itself. Therefore, a modification of the experimental set-up would be desirable to prove this protective effect of MF. For instance, perform treatments with Veh/MF directly before OGD to reduce on rinsing step or avoiding rinsing after OGD and working on pure ischemia without reperfusion would be some of possible options.

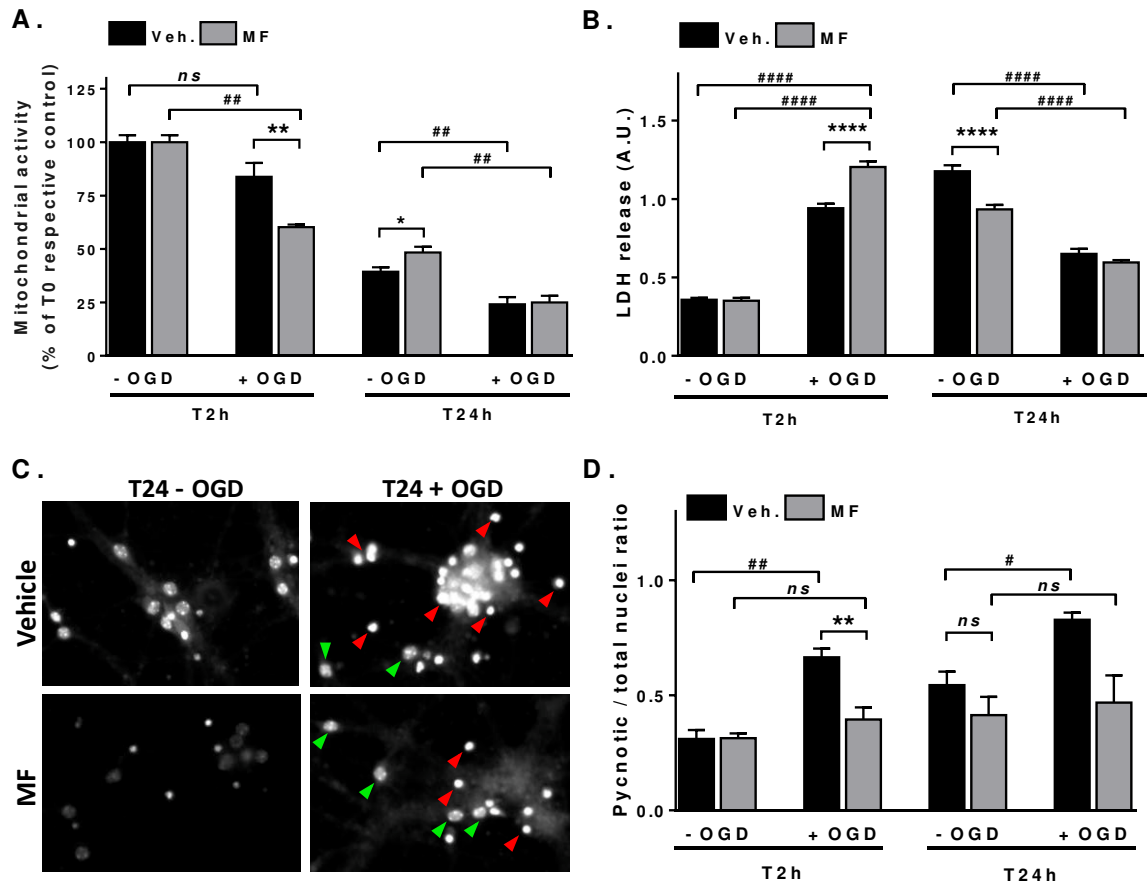


Fig. 40: Effect of MF on primary cortical neurons challenged by 2 h OGD. Primary cortical neurons were treated with MF 5 μ M or Vehicle (Veh) for 1 h, then exposed to 2 h OGD conditions. **A**, Effect of MF 5 μ M on mitochondrial activity of neurons directly after (T2h) and 24 h after (T24h) OGD, expressed as a percentage of basal activity of respective controls before (T0) OGD challenge. **B**, Effect of MF on LDH release at 2 h and 24 h, compared to basal activity of respective controls before (T0) OGD. **C**, Representative photomicrographs of neuronal nuclei stained by Hoechst. Green flashes point out the normal healthy nuclei and red flashes pyknotic (apoptotic) nuclei with condensed chromatin and high fluorescence. Magnification 40x. **D**, Effect of MF on apoptotic neuronal death at 1 h and 24 h, compared to basal level (T0) observed before experiment, calculated as a number of pycnotic/total nuclei ratios. Data are expressed as mean \pm SEM, measured in sextuplicates. * P <0.05, ** P <0.01, **** P <0.0001 compared to Vehicle-treated group, # P <0.05, ## P <0.01, #### P <0.0001 compared to non-OGD respective control.

PART IV – Liposomes as a smart solution for MF brain delivery

More than 40% of the new drugs in pharmaceutical development are faced with water insolubility which is often the cause of their low bioavailability. This requires some physical or chemical modifications of drugs, such as salt formation, complexation, etc. (Savjani et al., 2012). Poor water solubility being around 0.3 mg/L was described also in the case of RSV and concerns its related stilbenes. It is supposed that solubility of stilbenes with a lipophilic prenyl group could be even lower. Stilbenes are well soluble in ethanol, DMSO, eventually in polyethylene glycol (PEG), which indeed complicates their administration *in vivo*. Moreover, fast metabolization described for RSV and some derivatives contributes to the poor bioavailability (Amri et al., 2012). Therefore, to avoid the use of chemical vehicles and anticipate possible failure of MF in *in vivo* studies because of above-mentioned factors, we decided to design an optimal liposomal MF formulation as a smart solution to overcome MF poor water solubility. Such a formulation would further serve for *in vivo* assays on the models of systemic or CNS inflammatory diseases. With the aim to select the right formulation, several neutral, negatively, and positively charged liposomal formulations with surface modification were prepared at the Veterinary Research Institute (VRI, Brno) in collaboration with Dr. Josef Mašek and Dr. Eva Vohlídalová. Size between 100–200 nm and compositions of prepared formulations were selected after a detailed literature review for intravenous administration, with possible delivery to the brain and application in the treatment of brain injuries, including an ischemic insult. The liposomal charge is one of the predominant factors of a liposomal formulation determining a passage of liposome into cell, thereby we undertook to test several types of liposomes: **anionic (I–III)** with charge ranging from -28.1 to -14.4 mV, **neutral (IV; -2.4 mV)**, **cationic (V–VII; 20.1–25.9 mV)**, and **cationic combined (VIII; 31.9 mV)** (Fig. 41).

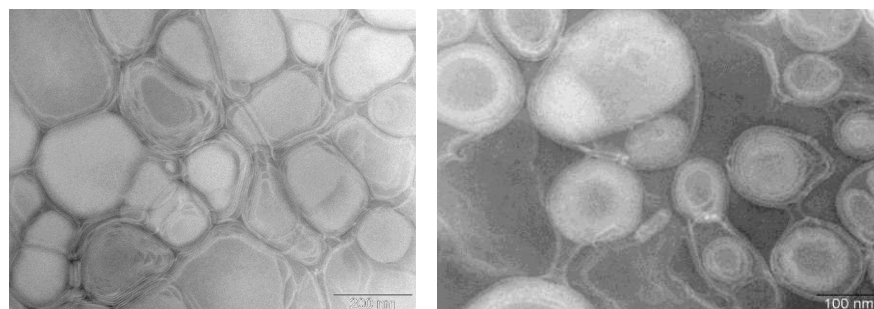


Fig. 41: TEM images of cationic VI (left) and anionic II (right) liposomes (89000x). Pictures obtained at VRI Brno, Czech Republic (2019).

The surface of liposomes **I** and **VIII** was later modified, as will be described in the next chapters. The samples **I–VIII** were assorted according their ζ (zeta)-potential, it means a charge at the interface of the surface being in contact with a liquid medium, ranging from -28.1 mV to +31.9 mV. The content of MF incorporated represented 5 molar percent of liposome which was pre-determined stable formulation for all types of liposomes. The efficiency of MF encapsulation and the incorporation of MF liposomal into cells were determined using fluorescent spectrometry based on autofluorescence properties of MF. The excitation maximum of MF was defined to be at 309 nm, and emission maximum at 422 nm (Fig. 42). The toxicity and biological effects of different liposomal formulations were then tested on THP-1 monocytes, BV-2 microglia, and primary cortical neurons, in a similar manner which had been described for testing of MF free (non-incorporated) with the aim to find a formulation with equivalent or eventually better properties than MF free.

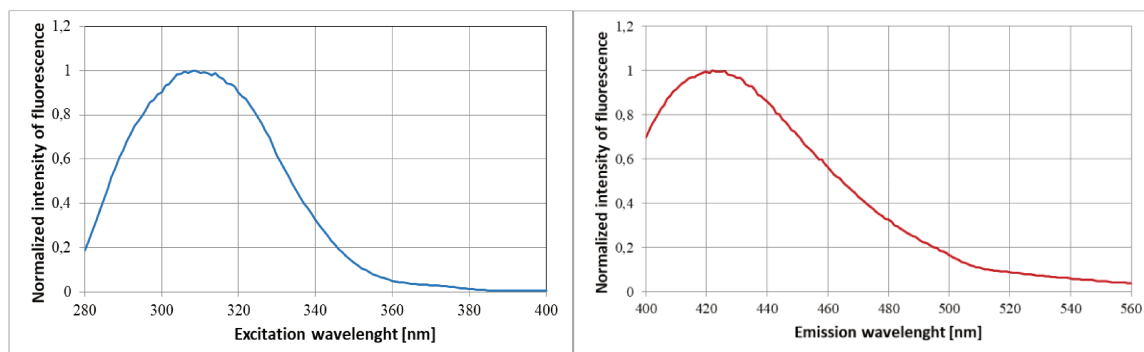


Fig. 42: Excitation (left) and emission (right) fluorescent spectra of MF in 96% EtOH. Determined at VRI Brno, Czech Republic (2019).

1. Effect of MF liposomal on THP-1 and THP-1-XBlueTM-MD2-CD14 monocytes

Since the safety of a drug is the most important factor, we firstly tested the dose-response effect of all prepared MF liposomal formulations **I–VIII** at concentrations of MF 0.1–15 μ M and their respective controls **C/I–C/VIII** without MF at the same concentrations of lipids on metabolic activity and LDH release (cell damage) in THP-1 and THP-1-XBlueTM-MD2-CD14 cells. Any of liposomal MF formulations, neither their controls without MF did not significantly affect the viability of THP-1 and THP-1-XBlueTM-MD2-CD14 monocytes Their IC_{50} values were over 15 μ M for both cell lines, as displayed in Fig. 43A+B for one representative of each group. Considering that IC_{50} of MF free is 5.7 ± 1.1 μ M for THP-1 and 5.4 ± 1.3 μ M for THP-1-XBlueTM-MD2-CD14 cells, these results suggest that incorporation of MF into liposomes could decrease its toxicity as it has been

described for other commercially available drugs (Sercombe et al., 2015). Moreover, cationic (VI and VIII) liposomes at tested concentrations 0.1 – 1 – 5 μ M of MF incorporated, as well as liposomes alone, attenuated the basal LDH release in the range from 20% to 33% for THP-1 cells (Fig. 43C+D) and samples VI and VII in the range of 18–40% for THP-1-XBlue-MD2-CD14 cells in comparison with MF free which reduced the LDH only by 6%.

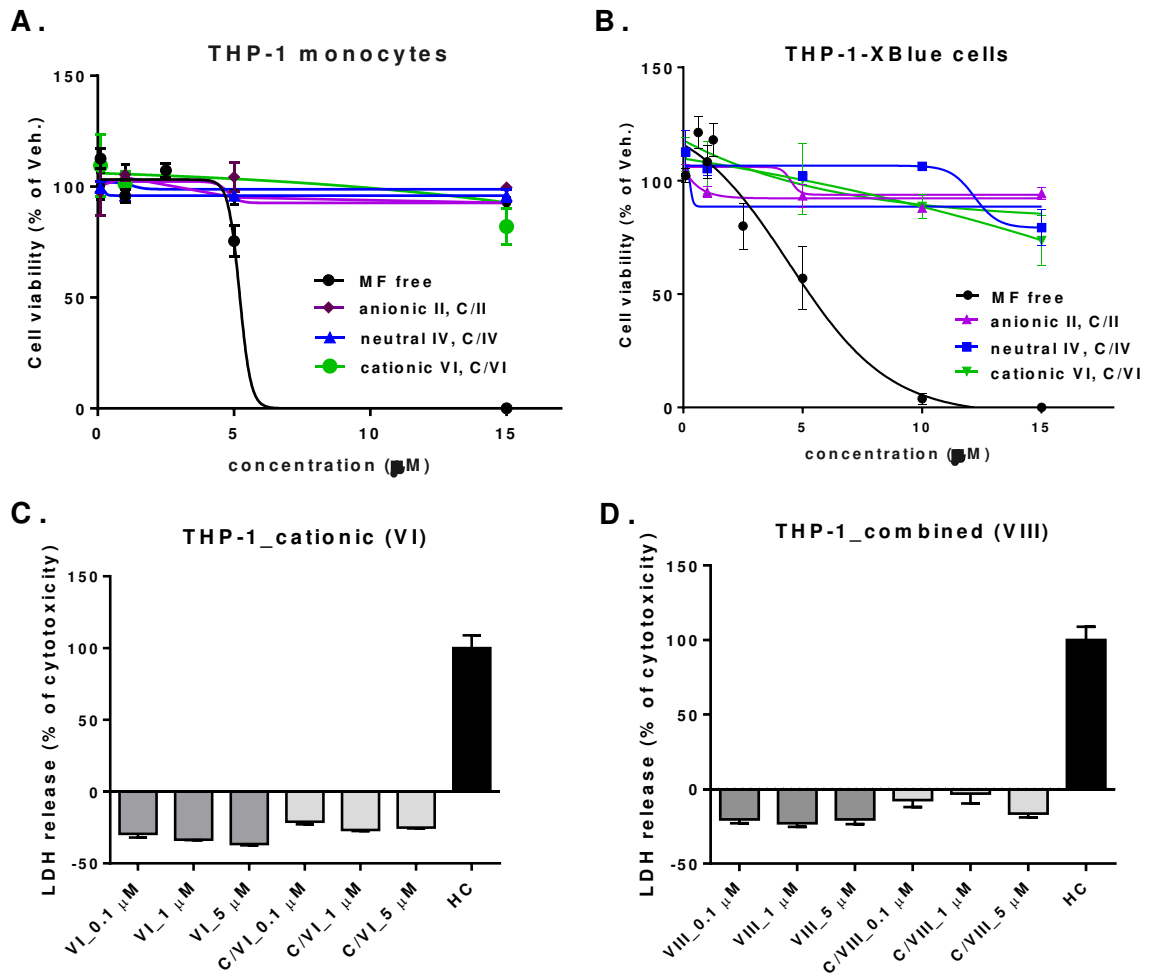


Fig. 43: Effect of MF liposomal on cell viability and LDH release in THP-1 and THP-1-XBlue-MD2-CD14 monocytes. **A**, Effect of one representant from each group of anionic (II), neutral (IV), and cationic (VI) MF liposomal and their respective controls C/II, C/IV, and C/VI on cell viability of THP-1 cells corresponding to metabolically active cells determined by WST-1 assay. **B**, Effect of one representant from each group of anionic (II), neutral (IV), and cationic (VI) MF liposomal and their respective controls C/II, C/IV, and C/VI on cell viability of THP-1-Xblue-MD2-CD14 cells. **C**, Effect of cationic MF liposomal (VI) on LDH release in THP-1 cells. **D**, Effect of cationic combined MF liposomal (VIII) on LDH release in THP-1 cells. % of cytotoxicity was calculated as ratio $[\text{LDH sample} - \text{LDH low (non-treated control)}] / [\text{LDH high control (Triton 2\%)} - \text{LDH low control}]$. Mean \pm SEM, N=3. HC – high control.

In general, tested liposomes alone or with MF incorporated showed a safe character on human monocytes, liposomal forms with higher charge (up to 21 mV) even promoted beneficial effect on basal level in the form of decreased LDH release.

Other important feature of MF liposomal would be its ability to dampen the LPS-mediated activation of NF- κ B pathway, as we showed in previous assays. Hence, we tested in the same manner the effects of liposomal formulations **I–VIII** at a concentration of MF 1 μ M, together with the effect of their respective controls **C/I–C/VIII** at the same concentration of lipids to observe and compare their effect on the activation of NF- κ B/AP-1 factors in THP-1-XBlue-MD2-CD14 cells. Surprisingly, negatively charged liposomes **C/I–C/III** and cationic **C/V** displayed by themselves a great inhibitory activity of NF- κ B/AP-1 (Fig. 44A). This inhibitory potential may be attributed to different phospholipid contents as the anti-inflammatory potential of some anionic phospholipids such as POPG (Kuronuma et al., 2009), and cationic ones (Filion and Phillips, 1997) has been already described. Contrarily, neutral liposomes (**C/IV**) and cationic with higher ζ -potential (**C/VI–C/VIII**) did not show alone any significant effect on NF- κ B/AP-1 activity (Fig. 44A). Anionic liposomes **I–III** with MF incorporated have shown a similar (**I**) or lower (**II–III**) inhibitory potential than liposomes alone. Neutral MF liposomal (**IV**) and cationic **V** did not exert any significant effect and positively charged liposomes **VI–VIII** displayed the NF- κ B/AP-1 inhibitory activity increasing with the ascending charge (Fig. 44B). Compared to their respective controls (Fig. 44C), MF liposomal **II** and **III** seem to induce the NF- κ B/AP-1 activity, while samples **I**, **IV**, and **V** exhibited no effect compared to their respective controls. On the other hand, positively charged MF liposomes (**VI–VII**) slightly reduced NF- κ B/AP-1 activity, while MF in cationic combined liposomes **VIII** displayed the greatest inhibitory effect, slightly better than MF free (Fig. 44C).

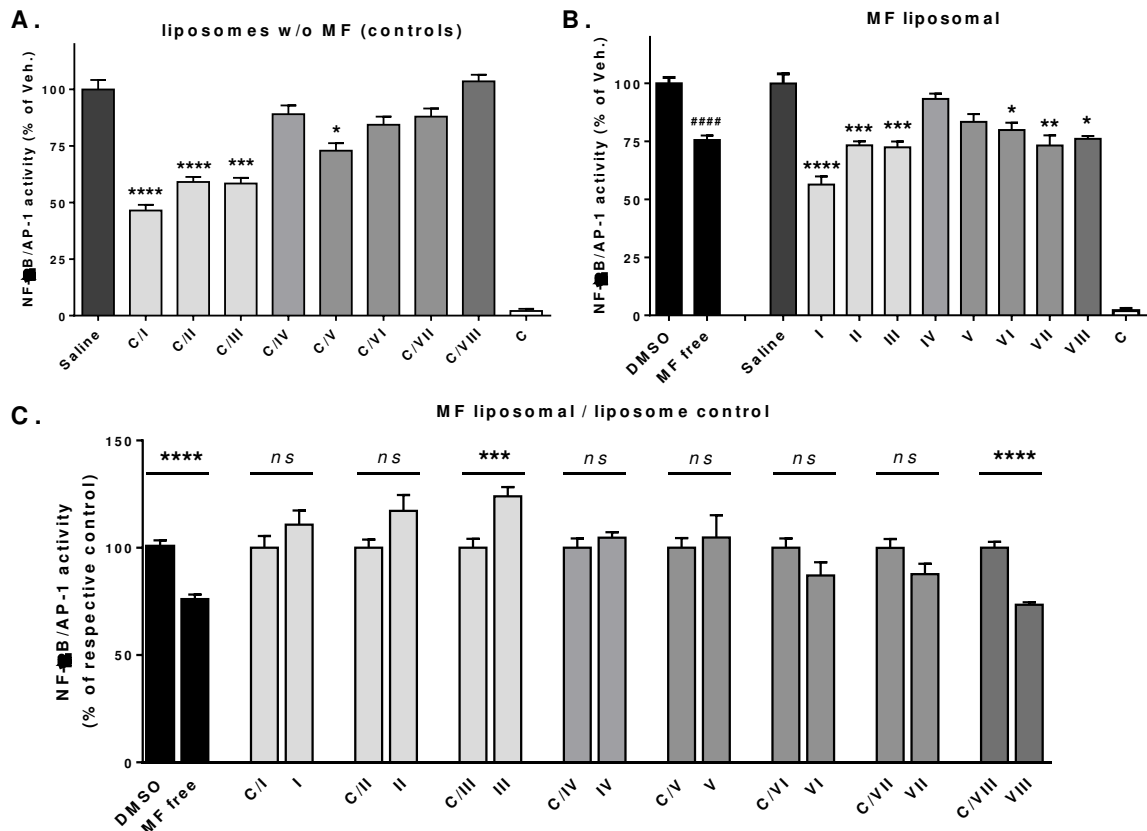


Fig. 44: Effect of liposomes alone and MF liposomal formulations on NF-κB/AP-1 activity in THP-1-XBlue-MD2-CD14 monocytes. **A**, Effect of 1 h pre-treatment by liposomal controls C/I-C/VIII (without MF) on LPS-stimulated NF-κB/AP-1 activity detected after 24 h. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ compared to vehicle (Saline). **B**, Effect of MF free and MF liposomal (I-VIII) at concentration of 1 μM on LPS-stimulated NF-κB/AP-1 activity. #### $P < 0.0001$ compared to DMSO, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to Saline. **C**, Effect of MF liposomal I-VIII (1 μM) compared to their respective controls (C/I-C/VIII) without MF, and effect of MF free 1 μM compared to its vehicle (DMSO 0.1%). *** $P < 0.001$, **** $P < 0.0001$ compared to respective control. Mean \pm SEM, $N = 2$, measured in sextuplicates. Data were analyzed by Mann and Whitney test for comparison of two independent groups and Kruskal-Wallis test for more than 2 independent groups.

Since a charge (ζ -potential) of the liposome can influence its passage into a cell whose membrane is negatively charged, we were interested in what portion of MF liposomal enters the cell in comparison with MF free and if different passage could possibly stay behind diverse biological effects. Thus, we incubated THP-1 monocytes with MF free or MF liposomal at a concentration of 1 μM for 24 h. Then, cells were washed with PBS, lysed and the fluorescence of MF in cell lysates was measured. The lower fluorescence corresponding to a lesser amount of MF in cells was detected in cells treated with anionic I–III liposomes, while liposomes with positive charge seem to pass more

easily into THP-1 cells. Moreover, in the case of samples **II–III** and **V–VII**, we could say that the passage rises with increasing ζ -potential. This could possibly have a link with the effect on NF- κ B/AP-1 activity. The anionic MF liposomes **II** and **III** did not display an inhibitory effect since the concentration of MF in cells is lower in comparison with MF free, while the growing positive effect of cationic liposomes **V–VII** may have a connection with their easier passage to cells (Fig. 45).

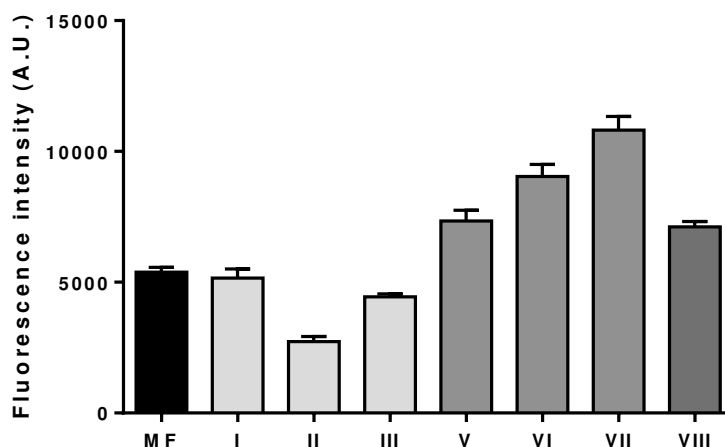


Fig. 45: Passage of MF free (MF) and MF liposomal I–III, V–VIII in THP-1 cells. Detected by fluorescence measurements of cell lysates. Mean \pm SEM, N=2, measured in sextuplicates.

According to photos obtained from confocal microscopy, we suppose that MF free as a lipophilic molecule can enter the cell by passive diffusion and accumulate in lipidic organelles, preferably in the cell membrane, while MF liposomal is rather actively phagocytized by macrophages. It can be supposed that phagocytized liposomes will accumulate in phagosomes, and then in phagolysosomes will be degraded (Hirayama et al., 2017). We observed that MF free was more uniformly distributed in the cell membrane, whereas condensed spots with high fluorescence were detected in cells treated with MF liposomal (Fig. 46). Certainly, this theory needs to be confirmed by additional assays with other organelle-specific staining.

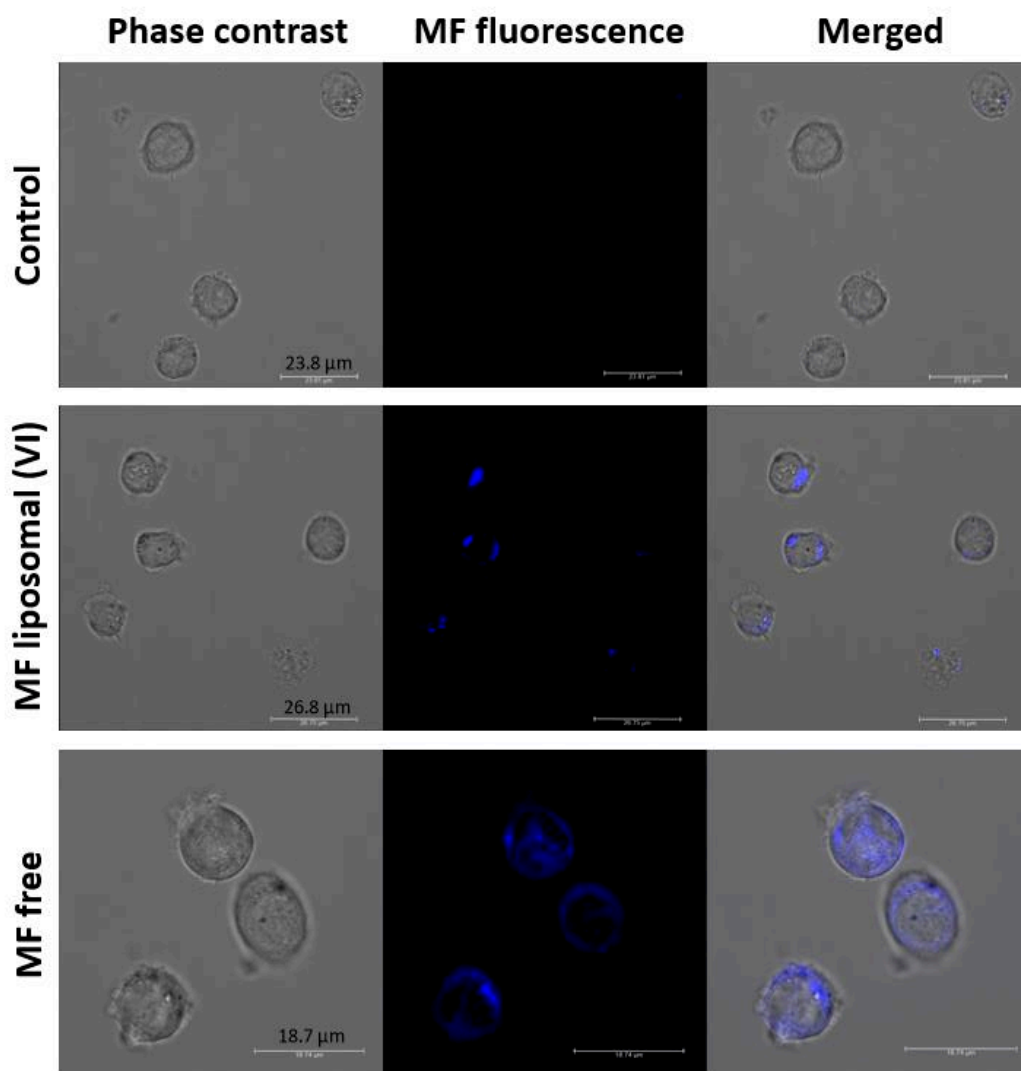


Fig. 46: Representative photos of MF free and MF liposomal in THP-1 cells. Detected by live cell imaging at VRI Brno (2019) using SP8 Lightening technology (Leica) after 24 h of incubation of THP-1 cells with MF free or MF liposomal VI at concentration of 1 μ M, based on autofluorescence properties of MF.

For the next studies at CNS level, we further selected two different liposomal formulations which showed the most desirable properties, thus combined cationic liposomes **VIII** which exhibited a safe character, similar inhibitory effect on NF- κ B/AP-1 activation and better passage to the cell than MF free. Moreover, the liposomal control **C/VIII** alone did not display the effect on THP-1 basal activity, thereby the effect of MF liposomal (**VIII**) was well observable. The next selected candidate was anionic formulation **I** which seemed to be safe, passed to cells in similar proportion than MF free, and **C/I** had by itself a great NF- κ B/AP-1 inhibitory activity, that could possibly result in synergic effects of MF and liposome alone or prolonged effect in long-term assay initiated by the anti-inflammatory

action of liposome, followed by the effect of MF after liposome degradation. The surface of these two types of conventional liposomes was further modified with polyethylene glycol (PEG), since the pegylated anionic and cationic liposomes with similar composition and size have been previously described as a promising way of drug delivery through the BBB (Bruch et al., 2019). Moreover, Fukuta et al. (2019) have described the ability of pegylated liposomes up to 200 nm to accumulate in the ischemic tissue. Thereby such type of liposomes could be further used for *in vivo* assays on the models of ischemic stroke, *e.g.* mouse MCAo model (Fukuta et al., 2019). Therefore, the safety of selected liposomal formulations **I** and **VIII**, as well as their pegylated forms **PEG-I** and **PEG-VIII** was further verified on brain cells, including mouse microglia BV-2 and primary cortical neurons. Together with safety we further evaluated the ability of these forms to enter the cells which has been shown to be an important factor to achieve the biological effect. The safe liposomal form keeping the anti-inflammatory and/or cytoprotective properties of MF free would be further studied *in vivo* on the model of focal ischemia in MCAo mice.

2. Effect of MF liposomal on BV-2 mouse microglia

Similarly as for THP-1 cells, BV-2 mouse microglia were incubated with MF free and MF liposomal **I**, **PEG-I**, **VIII**, and **PEG-VIII** at a concentration of MF 5 μ M or 15 μ M. The incorporation of MF was measured at 6 h for both concentrations and at 24 h only for 5 μ M concentration which had been assessed as safe for 24 h incubation. Contrarily to previously tested suspension of THP-1 human monocytes, adherent BV-2 cells exerted only a weak uptake of negatively charged liposomes **I**, as well as their pegylated forms **PEG-I** at 6 h and 24 h. The increase in concentration up to 15 μ M did not influence this effect (Fig. 47). We assume that THP-1 monocytes in cell suspension more actively phagocytize liposomal particles than fully differentiated adherent microglia. On the other hand, the uptake of positively charged liposomes **VIII**, together with their pegylated form **PEG-VIII** was similar or slightly higher than this of MF free 5 μ M at 6 h. After 24 h, the uptake of **VIII** and **PEG-VIII** was 90% and 78%, respectively. The augmentation of concentration by three times did not influence the uptake of **VIII**, while **PEG-VIII** was phagocytized only by 66% in comparison with MF free 15 μ M (Fig. 47).

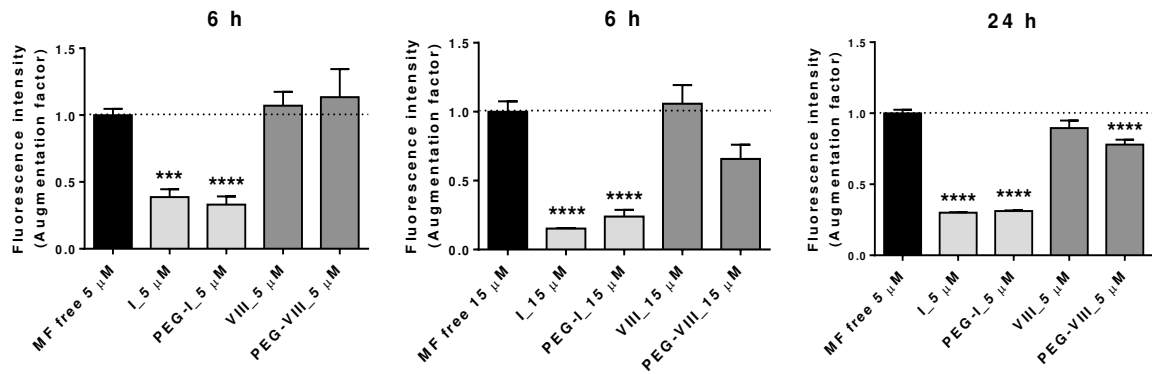


Fig. 47: The uptake of MF liposomal and MF free by BV-2 cells. Determined from cell lysates by measurements of MF fluorescence. Mean±SEM, N=3, measured in quadruplicates.

Moreover, the observations from confocal microscopy confirmed our results obtained from measurements of fluorescence from cell lysates. Fluorescence of MF in BV-2 cells treated by MF in anionic liposomes **I** at concentration 5 μM detected at 6 h was minor in comparison with cells treated with **MF free**, **VIII** and **PEG-VIII** 5 μM (Fig. 48).

Based on these results, we assumed that it would be highly challengeable to achieve any anti-inflammatory effect in microglia using anionic **I** and **PEG-I** as only a small portion of these nanoparticles enter the cells and thus it is less probable to obtain some beneficial effects. In consequence, we decided to carry out the following assays by testing only positively charged liposomes **VIII** and their pegylated forms **PEG-VIII**. In the next step, we verified their safety by testing their effects on LDH release and mitochondrial activity. Hence, BV-2 microglia were treated with positively charged MF liposomal **VIII**, its pegylated form **PEG-VIII**, together with **MF free** at concentration of 5 μM previously showed as safe, and 15 μM which could affect the cell viability. Fig. 49 displays that MF free up to 5 μM did not affect the cellular integrity since LDH release was maintained at its basal level after 24 h of treatment. Contrarily, MF 5 μM seemed to improve a native state of cells reducing basal levels of LDH (Fig. 49A). However, MF at concentration of 15 μM had a negative effect causing cellular damage (Fig. 49A). Moreover, this effect was accompanied by dampened mitochondrial activity of microglial cells (Fig. 49B). Interestingly, MF liposomal **VIII** and **PEG-VIII**, as well as their respective controls **C/VIII** and **C/PEG-VIII** did not enhanced the LDH expression in BV-2 cells at 15 μM concentration. **PEG-VIII** 5 μM and 15 μM rather reduced the basal levels of LDH (Fig. 49E).

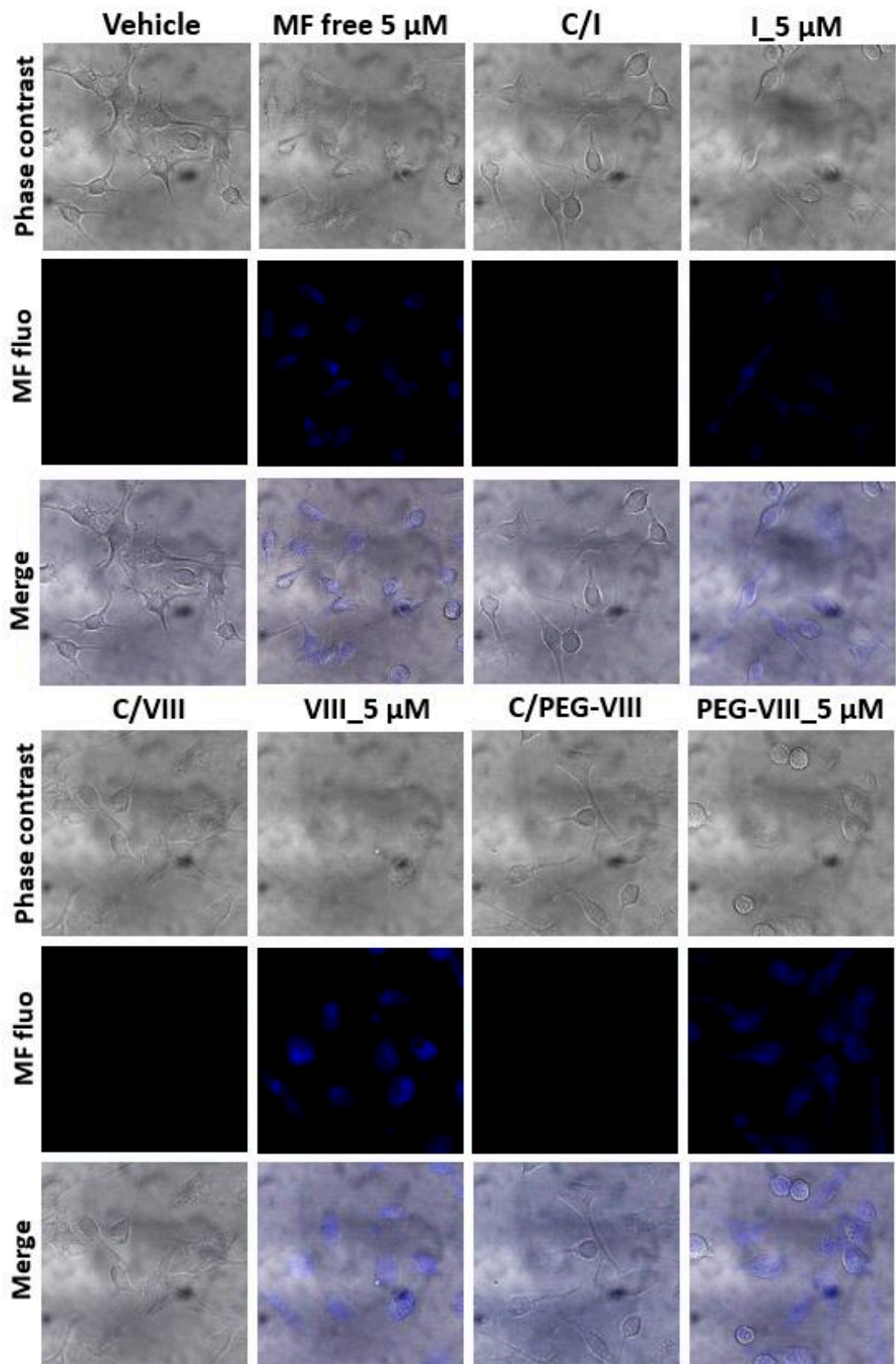


Fig. 48: Representative photos of MF liposomal and MF free in BV-2 cells. Detected after 6 h of incubation with, MF free and MF liposomal I, VIII, and PEG-VIII at concentration of 5 μ M. Magnification 40x.

Although empty liposomes or with MF incorporated did not cause a direct damage of microglia, both **VIII** and **PEG-VIII** affected at 15 μM the mitochondrial activity of cells as compared to MF free 15 μM , which is a sign of impaired energy homeostasis the use of this concentration should be avoided (Fig. 49D+F).

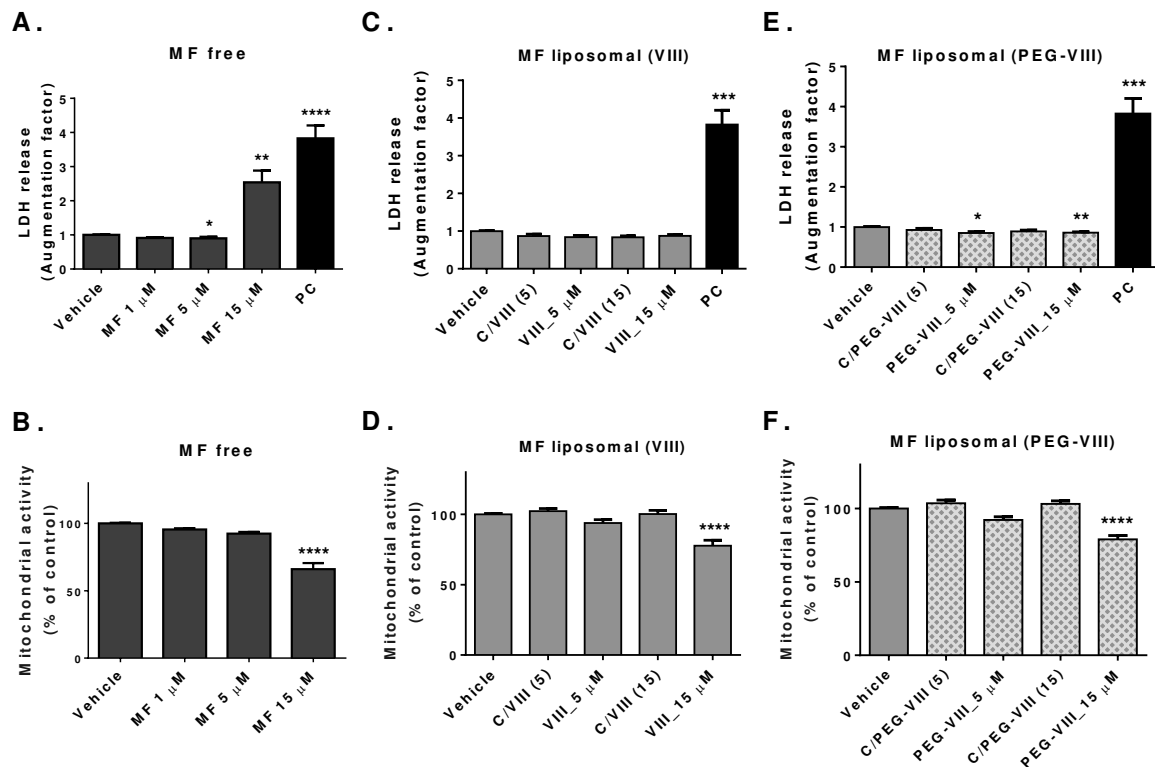
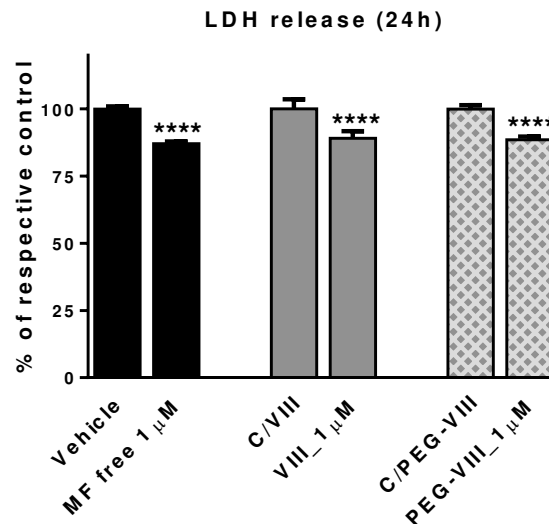


Fig. 49: Effect of MF free and MF liposomal VIII and PEG-VIII on LDH release and mitochondrial activity of BV-2 cells. **A**, Effect of MF free 1, 5, and 15 μM on LDH release compared to vehicle-treated cells. **B**, Effect of MF free 1, 5, and 15 μM on mitochondrial activity compared to vehicle-treated cells. **C**, Effect of MF liposomal VIII at 5 and 15 μM , and their respective controls (C/VIII; w/o MF) with the same concentration of lipids. **D**, Effect of VIII at 5 and 15 μM , and their respective controls C/VIII on mitochondrial activity of BV-2 cells. **E**, Effect of pegylated MF liposomal PEG-VIII at 5 and 15 μM , and their respective controls (C/PEG-VIII; w/o MF) with the same concentration of lipids. **F**, Effect of PEG-VIII at 5 and 15 μM , and their respective controls on mitochondrial activity of BV-2 cells. PC=positive control (Triton 2%). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Mean \pm SEM, $N=3$, measured in sextuplicates, evaluated by Kruskal-Wallis test.

In previous assays, we determined the ability of **PEG-VIII** to inhibit the NF- κ B/AP-1 signaling in THP-1-XblueTM-MD2-CD14 cells, thereby we were further interested if PEG-VIII could exert also cytoprotective effects previously described for MF free. Therefore, we exposed BV-2 cells pre-treated with **MF free**, **VIII**, or **PEG-VIII** at concentration of 1 μM to the LPS (1 $\mu\text{g}/\text{mL}$) challenge during 24 h. Then, the amounts of LDH release

corresponding to the cellular damage were detected. MF liposomal **VIII**, as well as **PEG-VIII** reduced significantly the levels of LDH in culture medium in comparison with cells treated by their respective controls without MF. This cytoprotective effect was similar to this of MF free (Fig. 50).



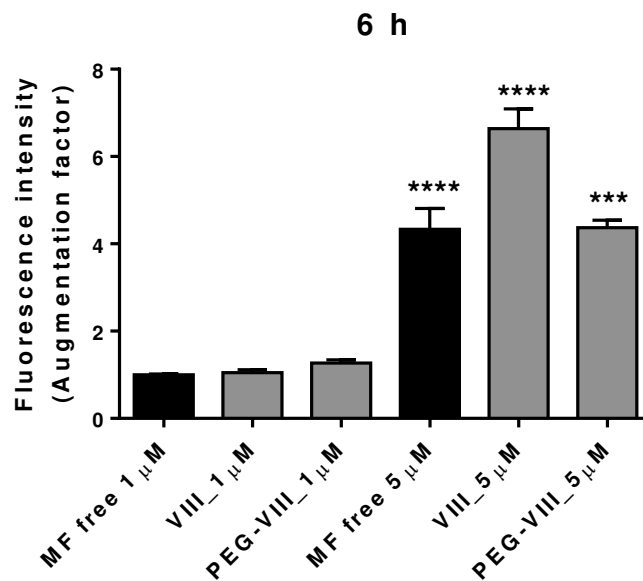
*Fig. 50: Cytoprotective effects of MF free, MF liposomal VIII and PEG-VIII against inflammatory challenge in BV-2 cells. BV-2 cells were pretreated for 1 h with MF free, VIII, and PEG-VIII at concentration 1 μ M before stimulation with LPS 1 μ g/mL. The LDH levels were detected after 24 h and the effects of MF free/liposomal were compared to their respective controls. **** P <0.0001 vs. respective control. Mean \pm SEM, N =3, measured in sextuplicates, evaluated by Mann-Whitney test for two independent groups.*

The last step to verify a possible use of selected liposomal formulations before testing their intervention in the inflammatory and ischemic conditions *in vivo*, would be to control their safety for the most sensible brain cells – neurons. Thus, we investigated the effects of MF free and MF liposomal on viability of neurons, together with their ability to enter the cells which is a necessary step to effectuate their previously demonstrated biological effects.

3. Effect of MF liposomal on primary mouse cortical neurons

Neurons, unlike monocytes and microglia are not phagocytic cells, thus the mechanisms of entry of liposomes into cells will differ. Fluorescence measurements provided by Azzazy et al. (1995) suggest that liposome label incorporates into membrane either by fusion of liposomes with neuronal membranes or by phospholipid transfer

between liposomes and neurons (Azzazy et al., 1995). Therefore, it was important to determine if a passage of MF liposomal differs quantitatively in these cells and thus could influence its biological effect. We observed that the capability of both MF liposomal **VIII** and **PEG-VIII** at a concentration of 1 μM to enter cortical neurons was comparable to this of **MF free** 1 μM after 6 h. When the concentration of MF treatment was increased up to 5 μM , the passage of positively charged **VIII** through cell membrane was much higher than this of **MF free** and **PEG-VIII** which showed a similar effect (Fig. 51).



*Fig. 51: Quantitative analysis of passage of MF free, MF liposomal VIII and PEG-VIII into primary neurones in culture. Cortical neurones were treated with MF free, VIII, and PEG-VIII at a concentration of 1 μM or 5 μM , their passage to cells was quantitatively evaluated based on detected fluorescence intensity of cell lysates. *** $P < 0.001$, **** $P < 0.0001$ vs MF free 1 μM . Mean \pm SEM, $N=3$, measured in sextuplicates, evaluated by Kruskal-Wallis test.*

Next we verified if different passage of compounds into cells could have some impact on mitochondrial function, the crucial indicator of neuronal metabolic rate, whose right functioning is essential for maintaining neuronal activity, establishment of membrane excitability, and execution of the complex processes of neurotransmission and plasticity (Kann and Kovács, 2007). Hence, we evaluated again the effect of MF free/liposomal at same concentrations ranging from 1 μM to 15 μM on mitochondrial activity and LDH release in neurons after 24 h of treatment. We observed that contrarily to monocytes and microglia, **MF free** up to 15 μM did not cause a direct cellular damage since the LDH level was not increased in neuronal medium. Moreover, **MF free** in the range from 1 μM to 10 μM moderately reduced basal levels of neuronal LDH release by 14–16% (Fig. 52A).

The lowered basal LDH was accompanied by a slight increase of metabolically active cells treated with MF free 1–10 μM (Fig. 52B). Together with lowered LDH amounts, this result could indicate an improved native state and survival of aging neuronal culture in comparison with the only vehicle-treated cultures. On the other hand, MF free 15 μM rapidly decreased the metabolic activity of neurons. This effect corresponded to that observed on microglia, and indicated that this concentration may jeopardize neuronal viability (without triggering necrosis as shown by the absence of LDH) (Fig. 52A). Surprisingly, positively charged liposomes (C/VIII) alone, as well as MF liposomal VIII at 5 μM and 15 μM exerted deleterious effects on neuronal cultures affecting neuronal integrity and causing cellular damage (Fig. 52C).

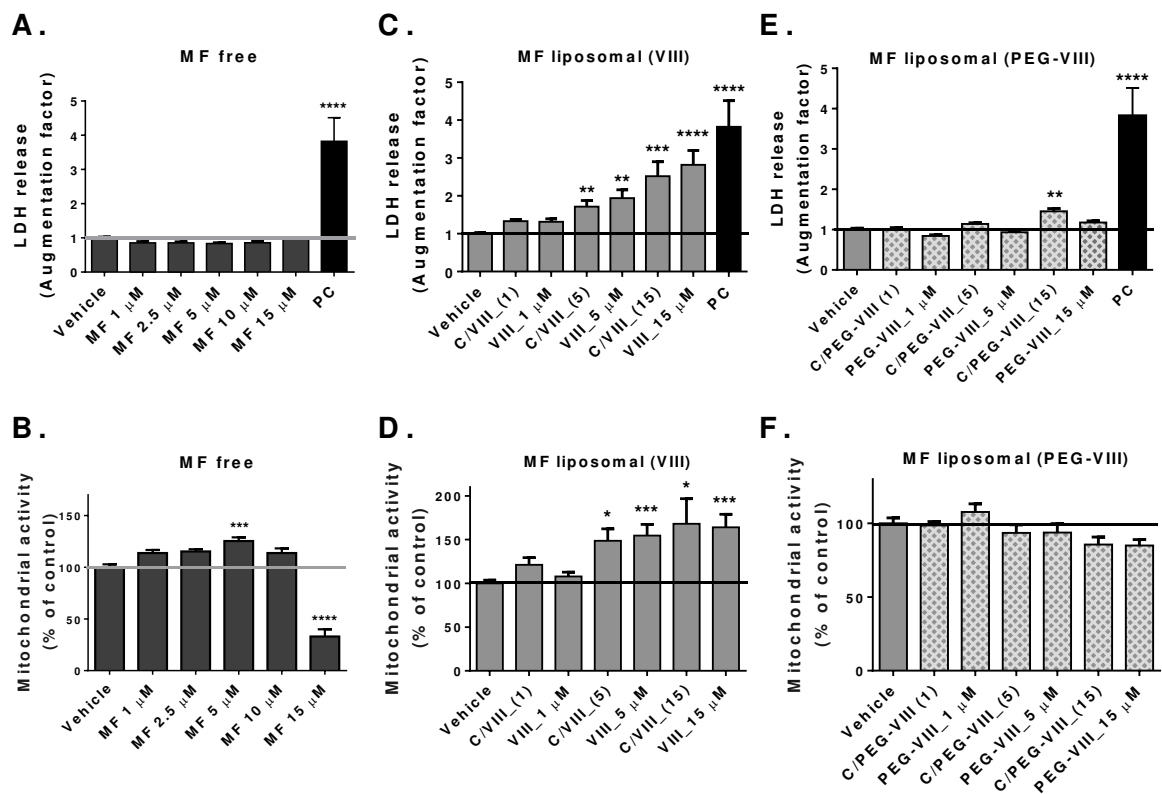


Fig. 52: Effect of MF free, MF liposomal VIII and PEG-VIII on LDH release and mitochondrial activity of primary cortical neurons. **A,** Effect of MF free 1–15 μM on LDH release compared to vehicle (DMSO 0.1%)-treated cells. **B,** Effect of MF free on mitochondrial activity compared to vehicle-treated cells. **C,** Effect of MF liposomal VIII at 1–15 μM , and their respective controls (C/VIII; w/o MF) on LDH release, compared to vehicle (Saline). **D,** Effect of VIII at 1–15 μM , and their respective controls on mitochondrial activity. **E,** Effect of pegylated MF liposomal PEG-VIII at 1–15 μM , and their respective controls (C/PEG-VIII; w/o MF) on LDH release. **F,** Effect of PEG-VIII at 1–15 μM , and their respective controls on mitochondrial activity of neurons. PC=positive control (Triton 2%). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs Vehicle. Mean \pm SEM, N=3, measured in sextuplicates, evaluated by Kruskal-Wallis test.

This was accompanied by radically elevated mitochondrial activity (up to 160% compared to the vehicle) in the case of neurons treated with **C/VIII** and **VIII** (Fig. 52D). Microscopic observations confirmed detrimental effects of **C/VIII** on neuronal culture (Fig. 53). These unforeseen results could be attributed to the used amounts of positively charged phospholipids. Azzazy et al. (1995), have observed that use of cationic phospholipids below 150 μM of total lipids should maintain normal electrical activity without damage of neurons, but higher concentrations could trigger major alterations in electrical activity of neurons affecting their electrophysiological behavior (Azzazy et al., 1995). This could explain our observations and indicate that use of higher concentrations of cationic **VIII** phospholipids should be avoided for neuronal cells. The situation was different for pegylated liposomes **C/PEG-VIII**, because PEG effectively covers a positive charge of liposome (their charge is 0.2 mV compared to 27.9 mV for **C/VIII**) and allows the safe entry to cells. At 24 h, there was no harmful effect observed for **PEG-VIII** 1–5 μM on LDH release (Fig. 52E) and mitochondrial activity (Fig. 52F). Control **C/PEG-VIII** at concentration of lipids corresponding to MF 15 μM slightly increased LDH release, which was dampened to basal level by MF liposomal **PEG-VIII** 15 μM (Fig. 52E). No alterations in neurons treated with pegylated liposomes were observed under microscope (Fig. 53). Thus, **PEG-VIII** seems to be safe for neuronal cultures up to concentration of MF incorporated 5 μM , as well as for previously tested cells and could be possibly used for *in vivo* assays on the specific models of systemic and neuroinflammation. We therefore selected **PEG-VIII** as the appropriate MF liposomal formulation for a pilot *in vivo* study on the mouse model of middle cerebral artery occlusion (MCAo).

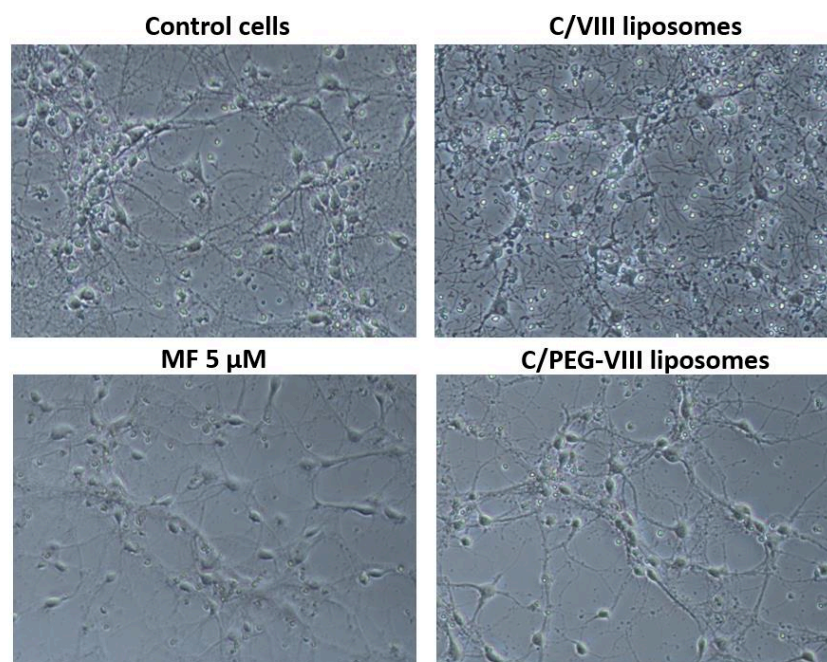


Fig. 53: Representative photomicrographs of cortical neurons treated with cationic liposomes C/VIII and C/PEG-VIII, in comparison with control cells and MF free 5 μ M. Phase contrast pictures. Magnification 40x.

4. *In vivo* model of focal ischemia

After the characterization of MF effects *in vitro* on several mechanisms of ischemic cascade and selection of the safe and efficient MF liposomal formulation, studies on an animal model would be the next step toward finding possible MF therapeutic usefulness. To test MF on an *in vivo* model of stroke, we selected the mouse MCAo model, which is a commonly used model in preclinical stroke assays (Mergenthaler and Meisel, 2012) and standardly practiced model in our laboratory. Pilot *in vivo* study was initiated at the time of writing this thesis. Focal ischemia was induced in 7 weeks old male mice (n=5 per condition) by left MCA occlusion for 1 h. Treatments with saline, liposomal control (C/PEG-VIII), MF liposomal (PEG-VIII), and MF free at a concentration of 0.117 mg/kg ($\approx 3 \mu\text{g}/\text{mouse}$), calculated on the volume of blood according to the concentration used in OGD assays on primary mouse cortical neurons, were administered intravenously 10 min after the end of MCAo. Infarct volumes were determined after 24 h. Mice treated with MF liposomal (PEG-VIII) and MF free (MF) showed better survival in comparison with mice treated with liposomal control (C/PEG-VIII) and saline, respectively. The most of animals did not reperfuse after MCAo, which was not dependent on the post-MCAo administered treatments. For the moment, it is not possible to make some conclusion on the efficiency of

treatments, as the number of animals per group was too low to get any significant results. Nevertheless, we observed that there was a tendency of decrease of the infarct volume in the reperfused animals by MF (Fig. 54). Undoubtedly, this study needs to be repeated several times, on a higher number of animals per group in the case of reperfusion issues. Unfortunately, because of *SARS-CoV-2* epidemiological situation, it was impossible to repeat the *in vivo* experiments up to date of writing this dissertation.

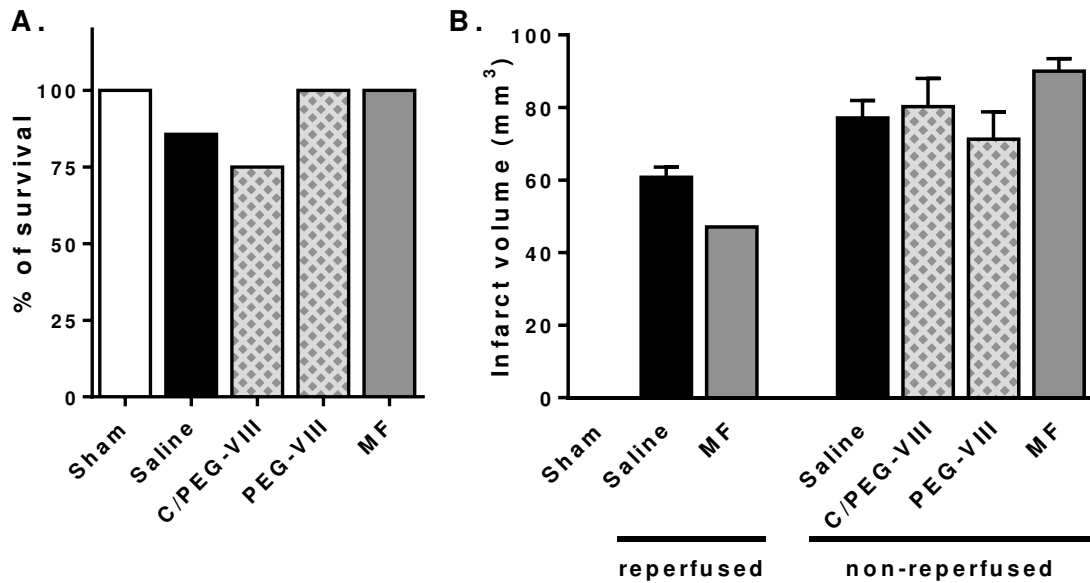


Fig. 54: Effect of MF post-treatment on survival and infarct volume in MCAo mice. **A,** Effect of liposomal control (C/PEG-VIII), MF liposomal (PEG-VIII), and MF free (MF) post-treatment on survival rate. Results are given in percentage of control, corresponding to sham group. **B,** Effect of liposomal control (C/PEG-VIII), MF liposomal (PEG-VIII), and MF free (MF) post-treatment on infarct volume in mice subjected to 1 h MCAO ($n=3$ (1 for MF) for perfused, $n=3$ for non-reperfused experimental group). Volumes of infarcts in perfused and non-perfused animals were evaluated 24 h after MCAo. Saline, C/PEG-VIII, and PEG-VIII post-treatment was administered intravenously in penile vein (0.117 mg/kg) 10 min after the end of MCAo. Data are expressed as means \pm SEM.

DISCUSSION

Chronic diseases are estimated by WHO to be the greatest threat to human health. Most of them are underlined by long-lasting mild inflammatory response of the immune system and thus are ranked as “inflammatory disorders” causing worldwide death of 3 patients of 5. Chronic respiratory diseases, heart disorders, cancer, obesity, and diabetes are all categorized into this group (Pahwa et al., 2020). Nevertheless, many acute disorders, primarily these of microbial character, such as bacterial meningitis, but also aseptic ones, such as stroke and traumatic brain injury are accompanied by a severe inflammatory reaction as well. Although the primary role of inflammation is to destroy a harmful agent and restore homeostasis, its switch to uncontrolled and non-resolved long-lasting inflammatory response may have deleterious effects worsening these pathologies.

Stroke is undoubtedly one of the worst devastating diseases worldwide with an enormous economic and social impact affecting annually about 13.7 million people worldwide. Ischemic stroke represents the most cases, but the scale of possible medications is strictly limited and despite decades of research, there is no effective neuroprotective treatment that would antagonize the destructive mechanisms of ischemic cascade and improve stroke outcome. Neuroinflammation is assuredly one of the major factors in stroke pathology, together with glutamate-induced excitotoxicity, and oxidative stress. Neuroinflammation is represented by rapid activation of resident microglia and the production of proinflammatory mediators, which trigger the infiltration of other inflammatory cells in the ischemic brain area and contribute to the damage of the most important brain cells - neurons. Therefore discovering novel anti-inflammatory molecules would help to counteract these inflammatory events assisting in the ischemic brain injury and offer new opportunities in stroke management. Despite relatively wide scale of commercially available anti-inflammatory drugs, most of them are connected with severe adverse effects, such as NSAIDs or corticosteroids, especially when used in the long-term. Other treatments called “biological”, among them inflammatory cytokines antibodies, are costly and not available to all patients.

Stilbenoids are natural agents found in food (edible berries, grapes, nuts, etc.) and several medicinal plants, those anti-inflammatory and antioxidant properties arouse interest in scientific circles. Stilbenoids as natural molecules attract also by their relative safety described in numerous studies. Moreover, many foods comprising stilbenes content are

part of human diet called the Mediterranean which represents a gold standard in preventive medicine combining elements with antioxidant and anti-inflammatory properties (Martinez-Gonzalez and Martín-Calvo, 2016). The effects of MedDiet have been confirmed in humans demonstrating an improvement of inflammatory state in metabolic syndrome (Richard et al., 2013), promoting insulin homeostasis (Bédard et al., 2015), and showing the importance of MedDiet in the prevention of cardiovascular diseases (Becerra-Tomás et al., 2020), as well as reduction of stroke incidence in patients with cardiovascular diseases (Paterson et al., 2018).

In our study, we demonstrated that stilbenoids, such as piceatannol, pterostilbene, oxyresveratrol, or pinostilbene, found widely in foods, such as different edible berries, grapes, peanuts, rhubarb, and beverages like red wine or white tea, have a remarkable *in vitro* anti-inflammatory potential. From 25 tested non-prenylated stilbenes, piceatannol (**3**), thunalbene (**7**), oxyresveratrol (**8**), and RSV and piceatannol glycosides (**10** and **24**) significantly inhibited the NF- κ B/AP-1 activity in LPS-stimulated THP-1-XblueTM-MD2-CD14 human monocytes, in a way comparable to clinically used corticoid drug prednisone. *In silico* analysis using molecular docking simulations of interactions of stilbenes with p50/p65 complex carried out within the same study confirmed the interactions of the most active compounds with NF- κ B complex. Compounds **3**, **7**, **8**, **20**, and **24** displayed to bind to NF- κ B with high binding energies (ΔG around -7.0 kcal/mol). The last two showed direct interactions with NF- κ B DNA binding site (Leláková et al., 2019). Moreover, stilbenes **3**, **10**, **24**, **25**, together with RSV (**1**) and batatasin III (**6**) at a pharmacologically relevant concentration of 2 μ M also effectively reduced TNF- α secretion in PMA-differentiated THP-1 human macrophages. When looking on up-stream signaling, selected non-prenylated stilbenes upregulated the activation of MAPKs, including the inhibition of ERK1/2, JNK, and p38 phosphorylation. Moreover, our co-workers demonstrated within the same study that stilbenes **1**, **2**, **4**, **17**, and **18** are effective inhibitors of COX-1 and COX-2, however more preferential to COX-1. Compounds **2-4**, **14**, and **23** also significantly inhibited 5-LOX, of these, piceatannol (**3**) in the manner comparable to clinically used zileuton (Leláková et al., 2019). This is in accord with previous studies showing the ability of the most studied RSV (**1**), pterostilbene (**2**), and piceatannol (**3**) to intervene into NF- κ B/AP-1 signaling, as well as COX-1/2 and 5-LOX pathways (Dvorakova and Landa, 2017; Eräsalo et al., 2018). Although it was shown that stilbenoids undergo fast metabolization by colon microbiota, their corresponding metabolites might

show different or potentially stronger biological activity than their parent molecules (Aires et al., 2014; Jarosova et al., 2019). Additionally, a recent study using human fecal fermentation colon model provided by our collaborators within the same project demonstrated that stilbenoids could create favorable colonic conditions. This study investigating the effect of six stilbenoids (RSV, piceatannol, thunalbene, batatasin III, oxyresveratrol, and pinostilbene,) at a physiological concentration of 10 µg/mL on the composition of gut microbiota showed that the first four mentioned stilbenes positively modulate the bacterial composition of the gut microbiome in humans (Jaimes et al., 2019). All these findings suggest that stilbenoids as dietary phenolics are compounds with multitargeted action and their regular consumption in food may have health-promoting and inflammatory diseases preventing effects.

We next showed that prenylated stilbenes, such as those of Moraceae or Euphorbiaceae family, are even more active compounds. Prenylated stilbenes were found preferentially in medicinal plants, but some of them were isolated also from fungus-elicited peanut (*Arachis hypogaea*) as defense metabolites, which in following assays exerted great antimicrobial properties against methicillin-resistant *Staphylococcus aureus* (MRSA) (Bruijn et al., 2018). Tested compound **26** was originated from *A. heterophyllus* in Asia known as jackfruit and new prenylated stilbene **27** isolated for the first time from plant source by our group was obtained from *A. altilis*, called as breadfruit, whose fruits are used as a staple food in several tropical regions of Southeastern Asia and Latin America (Badrie and Broomes, 2010). Generally, the biological activities of prenylated stilbenes were much less described. In comparison with their non-prenylated derivatives, they display some level of toxicity and the safety of some compounds from this group is limited in higher concentrations, which was confirmed also by our study (Hošek et al., 2019). Nevertheless, most of twelve studied prenylated stilbenes **26-37** significantly inhibited the LPS-stimulated NF-κB/AP-1 activity in human monocytes at safe and a twice lower concentration of 1 µM in a similar or greater way than prednisone. Novel compound **27**, MF (**37**) from *M. siamensis* and synthetic **34** effectively reduced TNF-α secretion and *Macaranga* compounds **31** and **37**, together with synthetic ones **35** and **36**, significantly dampened the IL-1β expression in THP-1 macrophages. Unlike non-prenylated stilbenes, the most active prenylated compounds **27** and **37** showed to reduce IκBα degradation, the direct inhibitor of NF-κB, but not affected the MAPKs activation. Thereby, it is tempted to believe that the action of these compounds is driven through the IκBα/NF-κB pathway,

rather than *via* MAPKs/AP-1. Similarly to their non-prenylated analogs, prenylated stilbenes **29** and **31-33** displayed even greater COX-1 and COX-2 inhibition, with a high COX-1 specificity (Hošek et al., 2019). Although COX-1 inhibitors are often linked with gastrointestinal complications characteristic for non-selective NSAIDs, COX-1-dependent synthesis of prostaglandins is also involved in pathological processes such as atherosclerosis, endothelial dysfunction, or neuroinflammation, therefore, selective COX-1 inhibitors could find usefulness in these pathologies (Perrone et al., 2010). Additionally, stilbenes **27**, **31-33** exhibited also remarkable 5-LOX inhibition (Hošek et al., 2019).

Our study focused on the comparison of anti-inflammatory effects of selected stilbenoids showed that stilbenes in bioactive foods and medicinal plants possess a propitious antiphlogistic potential, which could have health promoting effects in the prevention of inflammatory diseases. We have also revealed a hidden anti-inflammatory potential of prenylated stilbenes which have been studied very little to date. Next, this wide pharmacological screening led to the identification of the most promising compounds from stilbenoid group which deserve being investigated in further *in vitro* and *in vivo* studies on specific models of inflammatory diseases at systemic or CNS level.

Neuroinflammation, characterized by activation of resident microglia and elevated levels of pro-inflammatory cytokines, is one of the main components in stroke pathobiology determining its outcome. To investigate if stilbenes could lessen the inflammatory state at CNS level, we undertook to investigate the effects of the most promising agent from previously performed pharmacological screening - macasiamenene F (MF) - using *in vitro* and *ex vivo* models of LPS-stimulated microglia. In previous screening, MF displayed the greatest potential to effectively downregulate TNF- α and IL-1 β protein release in THP-1 macrophages, and dampened up-stream the activity of NF- κ B/AP-1 in a greater manner than corticoid prednisone. We were the first to describe its multitargeted action and the ability to strike inflammation at several points of the inflammatory cascade, including the reduced degradation of I κ B α (direct inhibitor of NF- κ B) and interaction with p50/p65 DNA binding. Nevertheless, MF did not show to influence the activation of MAPKs, thus we suppose that MF could act predominantly through the I κ B α /NF- κ B pathway. Moreover, MF exhibited the antiphlogistic potential at pharmacologically and clinically relevant concentration of 1 μ M which was proved to be achievable in human plasma after oral administration of the most known stilbene RSV

(Boocock et al., 2007b), while data on bioavailability after oral administration of MF are not available. It's noteworthy to mention that most of studies showing the biological effects of stilbenes were carried out using much higher concentrations up to 1 mM (Smoliga et al., 2011). Unlike some other tested stilbenes which were showed to be potent preferential COX-1 or/and 5-LOX inhibitors (Hošek et al., 2019; Leláková et al., 2019), MF exerted neither anti-5-LOX nor anti-COX-1 and anti-COX-2 activity (unpublished data), which could be possibly responsible for gastrointestinal adverse effects in the case of COX-1 inhibition (Cryer, 2003) or renal and cardiovascular problems which are linked with selective COX-2 inhibition (Sharma and Jawad, 2005). Although MF affected to a certain extent the viability of THP-1 cells (IC_{50} value $5.7 \pm 1.1 \mu M$), its safety was previously described for A549, HepG2, HuCCA-1, and MOLT-3 cells (Pailee et al., 2015). At CNS level, MF displayed a relatively safe profile for therapeutic use, with low cytotoxic effects on BV-2 microglia and primary cortical neurons. Moreover, MF showed also cytoprotective effects against LPS-induced loss of microglia. The 24 h application of MF diminished also the LDH release and reduced the cell damage by 20%. Furthermore, MF decreased the basal levels of inflammatory cytokines TNF- α and IL-1 β in BV-2 cells. Under inflammatory conditions, MF dramatically inhibited the LPS-stimulated gene and protein expressions of both TNF- α and IL-1 β , whatever the stage of treatment (pre-, co-, and post-treatment). The greatest reduction of cytokines (up to 43%) was achieved when microglia were pretreated with MF before induction of inflammation. These results arouse interest for the potential development of MF as a nutraceutical or dietary supplement, aimed at the prevention of any type of systemic and/or CNS inflammatory disease, maintaining or improving organ function, preventing chronic disorders, or even delaying the process of aging (Nasri et al., 2014; Tauskela et al., 2016).

Although MF potential in the prevention of inflammation is quite impressive, the fact that MF should be applied pre-emptively limits its usefulness in disorders arising without warning such as ischemic stroke. Therefore we undertook to investigate if MF could counteract the ongoing inflammation by the establishment of models of co- and post-treatment with MF, applying MF during the onset of inflammation or after it, respectively. Co-treatment with MF remarkably reduced both TNF- α and IL-1 β intracellular levels and their release induced by LPS in BV-2 cells at early (6 h) and later (24 h) time points. MF also radically dampened the IL-1 β protein expression in brain-sorted mouse microglia. All

these data demonstrate the ability of MF to intervene in the early phase of inflammation. This ability of MF to downregulate TNF- α and IL-1 β may be of particular interest in chronic CNS inflammatory disease, such as AD, PD, or HD, because these cytokines are known to be involved in the activation of resident microglia and astrocytes (Norden et al., 2016). Their dysregulated and sustained activity can drive a toxic circle of elevated inflammatory mediators which endanger the functioning and survival of neurons (Ramesh et al., 2013). Reducing neuroinflammation could be a way to slow down the progress of these diseases (Sastre et al., 2014). Some stilbenoids, such as *trans*-resveratrol, isorhapontigenin, gnetol, and pinosylvin, have shown a significant potential to inhibit butyrylcholine esterase (BChE), one of the causal enzymes of AD (Sermboonpaisarn and Sawasdee, 2012). Oxyresveratrol exerts neuroprotective effects against Parkinsonian mimetic 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in neuroblastoma cells (Chao et al., 2008). Our results point out the interest in MF as potential supplementary compound used against chronic inflammatory diseases that would correspond to the so called “early phase of inflammation” in the present work.

Both TNF- α and IL-1 β are potent regulators of the innate immune system important for the host defense, moreover IL-1 β has a prominent role in acute neuronal injuries (Allan and Rothwell, 2003). Our data show that MF could find usefulness also in acute brain disorders, such as cerebral ischemia, where the level of cytokines is highly elevated as demonstrated on post-treatment model. Applied as post-treatment, MF proved the ability to improve the inflammatory state by dampening the levels of both TNF- α and IL-1 β when applied 3 h after LPS challenge and only TNF- α when applied 6 h after induction of inflammation. The application of an anti-inflammatory treatment initiated after 3–4.5 h therapeutic window determined for thrombolysis could help to avoid the destructive effects of inflammation and improve stroke outcomes (Hacke et al., 2008; Le Thuc et al., 2016). Although the protective effects of stilbenes against ischemic events have been very little investigated to date, some studies report about the ability of pterostilbene to protect against myocardial ischemia-reperfusion (I/R) injury by reducing oxidative/nitrosative stress and the inflammatory response to it (Yu et al., 2017), reduce the size of a myocardial infarction (Wu et al., 2017), and reduce *in vivo* inflammation in high fat-induced atherosclerosis which is a risk factor of both myocardial and cerebral infarction (Zhang and Zhang, 2016). At the CNS level, pterostilbene protected against hyperglycemia- (Yang et al., 2017) and

glutamate-induced neuronal oxidative injury (Wang et al., 2016). Oxyresveratrol reduced the infarction and thus prevented neurological deficits induced by the ischemia/reperfusion (I/R) injury in rat MCAo model, inhibited the cytochrome c release, and thus prevented the activation of caspase-3 (Andrabi et al., 2004). This suggests that stilbenoids as potent antioxidant and anti-inflammatory agents could be effective in the treatment of I/R-injury.

Despite decades of a tremendous academic and clinical research, this devastating pathology stays without a neuroprotective therapeutic strategy. One of the reasons is the complexity of interconnected deleterious mechanisms triggered by an ischemic event. Inflammation is only one factor of this neuropathology, however, oxidative stress and glutamate-induced excitotoxicity are other striking processes of ischemic cascade contributing to neuronal damage. Therefore we decided to look into the possible intervention of MF in the last two mentioned mechanisms. MF structure does not predict this compound to be a strong antioxidant agent, as the antioxidant properties of stilbenes have been attributed to the presence of several hydroxy groups on stilbene skeleton, preferably with catechol (3,4-dihydroxyphenyl) or pyrogallol (3,4,5-trihydroxyphenyl) pattern (Murias et al., 2005). Although MF comprises a 3,5-dihydroxy substitution of ring B, one hydroxyl is cyclized by prenyl moiety which is further hydroxylated at C-3'', and the fourth hydroxyl on A ring is methylated. Our investigation of MF antiradical effects in AAPH-induced oxidative stress in BV-2 microglia confirmed our assumptions. In spite of this, MF showed limited antioxidant action, however, this effect was much lower than the known potent antioxidant quercetin. Although the antioxidant properties of MF are poor, its possible ability to protect neurons against excitotoxic injury would count for MF therapeutic potential. Therefore, we also tested the ability of MF to interfere into NMDA-induced toxicity in primary mouse cortical neurons. MF (5 μ M) effectively dampened the NMDA-induced neuronal damage (LDH release) by 25% and promoted neuronal survival, the most significantly at 20 h after the excitotoxic insult. Nevertheless, other investigations would be indispensable to determine at which stage MF slows the glutamate or NMDA action (*e.g.* by verifying post-synaptic glutamatergic transmission through NMDAR or effects on Ca^{2+} levels). Instead of going more in detail in the study of separate mechanisms, we were interested if MF could oppose the harmful effects of the whole ischemic cascade and protect the survival of neurons exposed to 1 h or 2 h of oxygen-glucose deprivation (OGD). Whereas the 1 h OGD triggered mostly necrotic cell death and

its harmful effects were observable at 24 h, an additional massive apoptotic cell death was induced by a 2 h OGD, observed in the form of increased numbers of pyknotic nuclei. Protective effect on cell survival, reduced LDH release, and lowered numbers of pyknotic nuclei were found in both assays at 24 h mostly in non-OGD-exposed cells treated with MF. These results indicated a potential neuroprotective effect of MF against the aging of neuronal culture and harmful effects related to the manipulation with these highly susceptible cells, but complicated the analysis of assay for MF-treated OGD-challenged neurons. Therefore, a modification of the experimental set-up would be desirable to prove this protective effect of MF. For example, treatments with Veh/MF performed directly before OGD to reduce one rinsing step or avoiding rinsing after OGD and working on pure ischemia without reperfusion would be some of possible options.

After the characterization of MF effects *in vitro* on several mechanisms of ischemic cascade, studies on animal models would be the next step to approach its therapeutic usefulness. The selection of an appropriate stroke model, as well as measured parameters, and statistical analyses, is necessary for latter translation of basic research findings into eventual clinical trials. Before testing MF *in vivo* in the mouse MCAo model which is a commonly used model of preclinical stroke (Mergenthaler and Meisel, 2012), we had to handle a complication typical for 40% of molecules on the market and even higher percentage of molecules in preclinical research. MF poor solubility in water disables the MF administration in water solutions. Moreover, low water solubility is often the cause of low bioavailability of drugs. To prevent a possible failure of MF in *in vivo* assays due to mentioned reasons, we decided to incorporate MF into conventional and PEG-modified liposomes as a way for possible intravenous administration. Liposomes are sophisticated nanocarriers comprising numerous advantages such as improved stability and bioavailability, decreased toxicity, as well as targeted transport and passage through BBB. With the aim to find the most suitable liposomal formulation, MF was incorporated into eight different types of liposomes with size between 150-200 nm, differing in their charge (ζ -potential). We tested 1 type of neutral, 3 types of negatively charged, and 4 types of positively charged liposomes. All prepared liposomal formulations fulfilled the quality requirements and proved the stability of MF incorporated. Their safety was firstly verified on the model of THP-1 monocytes. All formulation proved the safe character, moreover, decreased MF toxicity at a concentration of 15 μ M and improved basal state of cells.

Surprisingly, liposomes by themselves showed the ability to attenuate the NF- κ B/AP-1 activity, with the most significant effect in the case of negatively charged liposomes. MF liposomal **VIII** compared to its respective control showed the most propitious inhibitory effect comparable to MF free and thus was selected for following assays together with negatively charged liposomes **I** with the highest activity by itself. According to previous studies on stroke models (Fukuta et al., 2019), the liposomes under 200 nm covered by polyethylene glycol (PEG) were able to pass the BBB and accumulate in the ischemic tissue. For this reason, we decided to prepare the pegylated forms of these two bioactive formulations to test their effect at the CNS level. Pegylated and non-pegylated liposomes **I** and **VIII** displayed a relatively safe character for BV-2 microglia, but unlike monocytes, the passage of negatively charged liposomes into cells was poor. On the other hand, positively charged (**PEG-VIII** and **VIII**) liposomes protected against LPS-induced damage of microglia similarly to MF free. The effect of the last two was tested also on primary neuronal cultures where cationic non-pegylated liposomes **VIII** showed a harmful effect at higher concentrations, but pegylated ones (**PEG-VIII**) displayed a safe character. Additionally, when compared to MF free, **PEG-VIII** 15 μ M improved the mitochondrial function in neurons. The incorporation rate of **PEG-VIII** was comparable to this of MF free. This part of our investigations allowed us to identify the most suitable MF liposomal formulation for further *in vivo* assays on the mouse MCAo model. The pilot *in vivo* study was initiated at the time of writing this thesis. While additional studies on a higher number of animals should be carried out to obtain enough data that would enable us to make some significant conclusion of MF effects *in vivo*, our preliminary data are encouraging indication for pursuing this path.

CONCLUSION AND PERSPECTIVES

This work demonstrates a remarkable anti-inflammatory potential of stilbenes in bioactive foods and medicinal plants at both systemic and CNS levels. The safe character of stilbenes would allow their potential use in the prevention of inflammatory diseases and development of stilbene nutraceuticals or functional foods promoting health or even delaying the aging process. Moreover, some stilbenes, such as MF from *M. siamensis* have shown the ability to lessen the ongoing inflammation in activated microglia, which is the hallmark of brain pathologies. This suggests that stilbenes could possibly find usefulness also in acute brain disorders, such as cerebral ischemia.

Wide pharmacological screening of natural compounds is one of the four main tools in the pharmaceutical discovery of novel biologically active molecules. Nevertheless, it is only the initial step and perspective of the bioactive stilbenes successfully identified in this selective study lies in their further testing on more specific *in vitro* and *in vivo* models of inflammatory diseases (*e.g.* inflammatory bowel diseases). Although some stilbenes, including RSV, piceatannol, or pterostilbene, have been studied more in details, clinical trials to investigate their potential in humans are still needed. Other no or very little investigated stilbenes stand in a queue for their discovery. MF would be among the most promising thanks to its ability to effectively counteract both peripheral and CNS inflammation at pharmacologically relevant concentrations, in a way comparable to clinically used drug prednisone displaying a wide variety of side effects. Moreover, MF exerts cytoprotective effects reducing the inflammation-induced loss of microglia and NMDA-induced excitotoxic damage of cortical neurons. To determine how MF exerts such an effect, the studies of its action *e.g.* on the release of glutamate from presynaptic neurons, effect on Ca²⁺ levels, or implication in post-synaptic glutamatergic transmission through NMDA receptor, would be needed for detailed characterization of MF neuroprotective effects.

This work dealt also with the evaluation of safety and anti-inflammatory effects of different MF liposomal formulations *in vitro*. This part led to the selection of a safe formulation that keeps the best the properties of MF unincorporated. Although MF liposomal formulations have been designed with the aim to sophisticatedly deliver MF to the brain ischemic tissue, it would be worthy to verify the passage of MF liposomal through BBB, which is one of the planned assays on the list. Further modifications of liposomal

surface for even more specific brain delivery would be also possible by coupling liposomes with a brain-specific antibody, or preparation of PEG pH-sensitive liposomes which would release a molecule in the acidic environment of ischemic penumbra. To determine the dose-response effect and long-term safety of MF *in vivo* is another indispensable step.

Anyway, to evaluate if selected MF liposomal could antagonize deleterious effects of I/R injury, additional studies on different *in vivo* models of stroke in accordance with STAIR recommendations (*e.g.* focal ischemia induced by the insertion of clots, photothrombosis, or electrocoagulation, model of global ischemia, or possible usage of other animals, such as rat, pig, or primate) would be surely appropriate (Fisher et al., 2009). The window of possible studied factors at physiological, molecular, and behavioral level is enormously large, starting with a determination of infarct volume, effects on activation or inhibition of ion channels and Akt pathway involved in neuroprotection, behavioral (motor and cognitive) tests such as accelerated rotarod test, Morris water maze test, or force swim test for post-stroke depression would be accurate parameters to evaluate the efficiency of treatment against I/R injury. Besides the assessment of neuroprotective action, studies of MF contribution in neuroregeneration processes, such as an increase of BDNF expression, neurogenesis, or synaptogenesis, would be other possible options (Heurteaux et al., 2013). Up to date, studies on several mouse and rat models of focal and global ischemia emphasize the efficiency of RSV and pterostilbene to reduce the brain volume infarction, modulate microglial activation, suppress inflammation, and improve I/R-induced neurological deficits (Akinwumi et al., 2018; Liu et al., 2020). These data encourage to study also other stilbenes with modified stilbene core, such as MF. Our non-exhaustive study identifies MF as a promising candidate for the treatment of inflammation-related pathologies, including secondary brain injury following stroke, for which a therapeutic opportunity is severely lacking. However, these are only preliminary findings which should be supported by other more extensive studies directed on the discovery of novel therapeutic agents based on natural molecules.

It was the epidemiological studies in developed countries which revealed the impact of diet on health and disease in humans. These studies also identified a need of nutriments, such as omega-3 and polyphenols in human diet, which led to the development of several nutraceuticals. However, this is not an invention of the modern pharmacotherapy. Already Hippocrates considered nutrition as one of the main tools that

a doctor can apply. His quote: “Let food be thy medicine and medicine be thy food” should be a reminder also for today. The balanced nutrition *e.g.* in the form of MedDiet rich on omega-3 and polyphenols has been shown in clinic to possess several preventive and health-promoting benefits. Nevertheless, there is a long path to prove the efficiency of various nutriments, including stilbenes, contained in MedDiet, allowing to answer several questions. Could regular intake of stilbenoids in nutraceuticals really improve health and inflammatory diseases? Could the incorporation of stilbenes, such as MF, into liposomes increase their bioavailability? May increasing of their intake lead to the enhancement in the plasma concentrations? And would it be correlated with improved health?

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Hošek, J.[#], Leláková, V.^{#,*}, Bobál, P., Pížová, H., Gazdová, M.*, Malaník, M., Jakubczyk, K., Veselý, O., Landa, P., Temml, V., Schuster, D., Prachyawarakorn, V., Pailee, P., Ren, G., Zpurný, F., Oravec, M., Šmejkal, K.*, 2019. Prenylated Stilbenoids Affect Inflammation by Inhibiting the NF-κB/AP-1 Signaling Pathway and Cyclooxygenases and Lipoxygenase. *Journal of Natural Products*, 82(7), 1839–1848. doi: 10.1021/acs.jnatprod.9b00081

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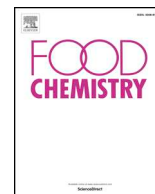
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Analytical Methods

Parallel *in vitro* and *in silico* investigations into anti-inflammatory effects of non-prenylated stilbenoids

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ABSTRACT

Stilbenoids represent a large group of bioactive compounds, which occur in food and medicinal plants.

Twenty-five stilbenoids were screened *in vitro* for their ability to inhibit COX-1, COX-2 and 5-LOX. Piceatannol and pinostilbene showed activity comparable to the zileuton and ibuprofen, respectively.

The anti-inflammatory potential of stilbenoids was further evaluated using THP-1 human monocytic leukemia cell line. Tests of the cytotoxicity on the THP-1 and HCT116 cell lines showed very low toxic effects. The tested stilbenoids were evaluated for their ability to attenuate the LPS-stimulated activation of NF-κB/AP-1. Most of the tested substances reduced the activity of NF-κB/AP-1 and later attenuated the expression of TNF-α.

The effects of selected stilbenoids were further investigated on inflammatory signaling pathways. Non-prenylated stilbenoids regulated attenuation of NF-κB/AP-1 activity upstream by inhibiting the phosphorylation of MAPKs.

A docking study used to *in silico* analyze the tested compounds confirmed their interaction with NF-κB, COX-2 and 5-LOX.

1. Introduction

Stilbenoids form a wide group of naturally occurring compounds, the best-known member of which is *trans*-resveratrol (1) (Giacomini, Rupiani, Guidotti, Recanatini, & Roberti, 2016; Shen, Wang, & Lou, 2009). Stilbenoids are phenolic compounds displaying two aromatic rings linked by an ethylene bridge, structurally characterized by the presence of a 1,2-diarylethylene nucleus with hydroxyl substitution on the aromatic rings (Fresco, Borges, Diniz, & Marques, 2006). In some plants, including food and medicinal plants, they are very variable substituted, mainly with hydroxy, methoxy, methyl, or prenyl groups. The diarylethylene core can be further modified to form 2-arylbenzofurans or lactone ring containing phtalides.

Stilbenoids are distributed in higher plants in the form of monomers or oligomers, and are commonly present as glycosides (Han, Shen, & Lou, 2007). *Trans*-resveratrol (1), the most investigated stilbene, is present in a variety of berries, peanuts, and medicinal plants together with its glycoside piceid in common grapes (*Vitis vinifera*), and particularly in grapes infected by *Botrytis cinerea* (used for production of Tokaji wines), or in grapes of *V. rotundifolia* used for manufacturing of muscadine wine. The high content of resveratrol (1) can be found also in peanut butter and dark chocolate (Baur & Sinclair, 2006). Pterostilbene (2) is primarily originated from blueberries (*Vaccinium* spp.), its content can vary depending to the type of berry (McCormack & McFadden, 2013). Moreover, the presence of stilbene derivatives is also observed in many medicinal plants (Sut, Maggi, & Dall'Acqua, 2017;

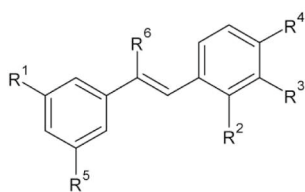
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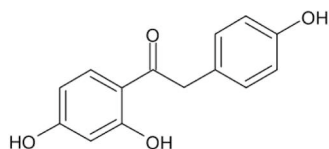
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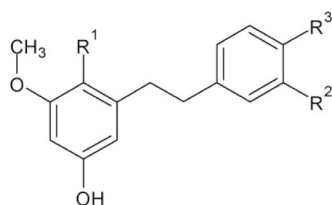


	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶
<i>Trans</i> -resveratrol (1)	OH	H	H	OH	OH	H
Pterostilbene (2)	OCH ₃	H	H	OH	OCH ₃	H
Piceatannol (3)	OH	H	OH	OH	OH	H
Pinostilbene (4)	OCH ₃	H	H	OH	OH	H
Piceatannol-3'- <i>O</i> -β-glucopyranoside (5)	OH	H	<i>O</i> -Glc	OH	OH	H
Thunalbene (7)	OCH ₃	H	OH	H	OH	H
Oxyresveratrol (8)	OH	OH	H	OH	OH	H
<i>Trans</i> -stilbene (12)	H	H	H	H	H	H
<i>Cis</i> -stilbene (13)	H	H	H	H	H	H
4-Stilbenecarboxylic acid (14)	H	H	H	COOH	H	H
Pinostilbenoside (15)	OCH ₃	H	H	<i>O</i> -Glc	OH	H
<i>Trans</i> -α-methylstilbene (16)	H	H	H	H	H	CH ₃
Pinosylvin-monomethylether (17)	OCH ₃	H	H	H	OH	H
Isorhapontigenin (18)	OH	H	OCH ₃	OH	OH	H
2,4,3',5'-tetramethoxystilbene (19)	OCH ₃	OCH ₃	H	OCH ₃	OCH ₃	H
3,4',5-trimethoxy- <i>trans</i> -stilbene (20)	OCH ₃	H	H	OCH ₃	OCH ₃	H
3'-bromo-3,4',5-trimethoxy- <i>trans</i> -stilbene (21)	OCH ₃	H	Br	OCH ₃	OCH ₃	H
3,5-dimethoxystilbene (22)	OCH ₃	H	H	H	OCH ₃	H

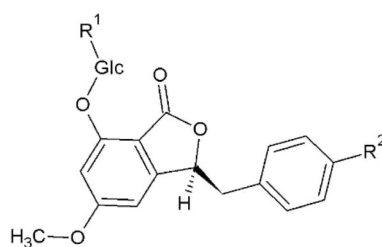


1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-ethanone (23)

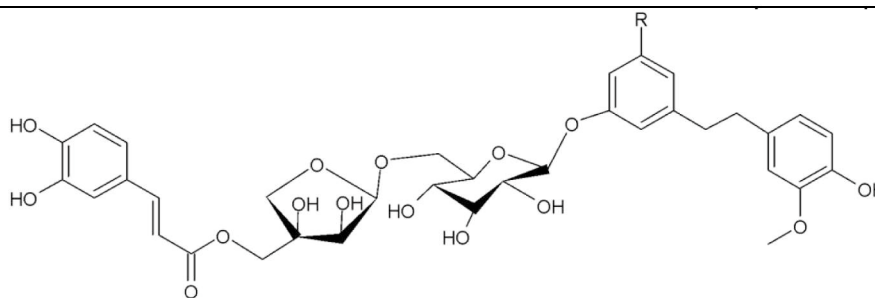
Fig. 1. Structures of the test compounds 1–25.



	R ¹	R ²	R ³
Batatasin III (6)	H	OH	H
2-carboxyl-3- <i>O</i> -methyl-4'-β-D-glucopyranosyl-dihydroresveratrol (10)	COOH	H	<i>O</i> -Glc



	R ¹	R ²
7-β-glucopyranosyloxy-3-(4-hydroxybenzyl)-5-methoxypteralide (9)	H	OH
7-(1→6)-α-rhamnosyl-β-glucopyranosyloxy-3-(4-hydroxybenzyl)-5-methoxypteralide (11)	Rha	OCH ₃



	R
3- <i>O</i> -caffeoyl-(9→5)-β-apiosyl-(1→6)-β-glucopyranosyl-3'- <i>O</i> -methyl-dihydroresveratrol (24)	OH
3- <i>O</i> -caffeoyl-(9→5)-β-apiosyl-(1→6)-β-glucopyranosyl-5,3'- <i>O</i> -dimethyl-dihydroresveratrol (25)	OCH ₃

Fig. 1. (continued)

Xiao et al., 2008).

After consumption and absorption into blood stream, stilbenoids interact with different plasma and body proteins and this interaction could influence their pharmacokinetic profile and biological effect

(Cao, Shi, Jia, Xiao, & Chen, 2016; Tang et al., 2017). There are numerous studies reporting the positive effects of stilbenoids supplemented as dietary agents or nutraceuticals, mostly on cardiovascular health and cancer chemoprevention (Chachay et al., 2011). The

presence of stilbenoids in food and medicinal plants is therefore associated with the multiple health benefits, including anti-inflammatory activity.

Inflammation is a natural reaction of an organism, generally directed against any type of damage, such as infection, irritation, or injury. It is characterized more or less specifically by activation of the immune system, which leads to vasodilatation and increased blood flow, release of inflammatory mediators, elevated cellular metabolism and the influx of various immune-system cells into the tissues (Brezáni, Šmejkal, Hošek, & Tomášová, 2018; Medzhitov, 2008). Upon activation, cells involved in the inflammatory reaction produce and release various inflammatory mediators to control the reaction of the organism. Under normal circumstances, the reaction is limited to the affected tissue and is strictly controlled. In some cases, connected with so-called civilization diseases, the inflammation expands from the acute phase, and becomes uncontrolled and continuous, switching into a chronic phase (Murakami & Hirano, 2012). Well-known examples of diseases associated with such chronic inflammation include diabetes mellitus (type 2), certain types of cancer, inflammatory bowel diseases, and some neurodegenerative disorders like Alzheimer's disease and Parkinson's disease (Bontempo et al., 2015; Chen, Zhang, & Huang, 2016; Khansari, Shakiba, & Mahmoudi, 2009; Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). Consumption of a well-balanced diet rich in natural phenolic compounds, such as stilbenoids, flavonoids, anthocyanins and others, can represent an approach resulting in health-promoting effects and prevention against different types of inflammatory and cancer diseases (Bontempo et al., 2015; Pandey & Rizvi, 2009).

There is no certain way to cure chronic inflammation. Several pharmacotherapeutic methods act on different levels. In general, non-steroidal anti-inflammatory drugs (NSAIDs) can be distinguished from steroids (corticosteroids), and so-called biological treatments such as anti-TNF- α antibodies are also available (Tabas & Glass, 2013). Anti-inflammatory therapy based on NSAIDs involves inhibiting enzymes of the arachidonic acid metabolic pathway (cyclooxygenase, COX; lipoxigenase, LOX; phospholipase A, PLA), or reducing the production of various pro-inflammatory proteins (cytokines), like IL-1 β , IL-6 or TNF- α , whose expression is controlled by NF- κ B (Nuclear Factor kappa-light-chain-enhancer of activated B cells) transcriptional factor (Golan-Goldhirsh & Gopas, 2014). Anti-inflammatory therapy is usually associated with problems caused by side effects of the drugs used in long-termed therapeutic schemes or with pleiotropic effects of some drugs. Scientists are therefore looking for new compounds with anti-inflammatory potential, which target different cellular proteins, such as transcriptional factors responsible for cytokines release and which intervene into their signaling pathways. The inhibition of the release of enzymes and mediators from neutrophils, macrophages and mast cells, inhibition of inducible nitric oxide synthase (iNOS) expression, nitric oxide (NO) and reactive oxygen species (ROS) release represent also

possible targets of new anti-inflammatory molecules (Brezáni et al., 2018; Krakauer, 2004; Laveti et al., 2013).

Information about the anti-inflammatory activity of stilbenoids is available, especially as it concerns *trans*-resveratrol (1), but it is well-nigh impossible to compare the activities of compounds using different assays (Dvorakova & Landa, 2017). For this reason, we systematically analyzed the ability of 25 stilbenoids to inhibit COX-1, COX-2, 5-LOX and the production of pro-inflammatory cytokines in lipopolysaccharide (LPS)-activated THP-1 macrophages. These effects were also evaluated *in silico* by molecular docking simulations of the ability of tested compounds to interact with active sites of COX-2 and 5-LOX, and to NF- κ B.

2. Experimental section

2.1. General experimental procedures

Twenty-five tested stilbenoids (1–25) (Fig. 1) were obtained commercially (1–7, 12–19, 22–23), isolated from plant sources (8–11, 24, 25) or synthesized by our group (20, 21). Most of them are naturally occurring compounds (1–11, 15, 17, 18, 20, 22–25) (Dvorakova & Landa, 2017; Granica et al., 2015; Li et al., 2015; Moodie et al., 2017; Pan and Lungren, 1996; Weng et al., 2016; Woo, Min, & Ryoo, 2010; Xiao et al., 2008), the resting compounds are of a synthetic origin (12–14, 16, 19, 21). Compounds 9–11, 24 and 25 are atypical compounds obtained from *Tragopogon tommasinii* Sch.Bip. (Asteraceae, Cichorieae) (Granica et al., 2015), oxyresveratrol (8) was isolated from *Morus alba* L. (Moraceae) root bark and identified by comparison of its spectral data with literature (Qiu et al., 1996). Reagents and solvents used for isolation and synthesis were purchased from Sigma-Aldrich (Steinheim, Germany) and used as received, if not stated otherwise. Anhydrous solvents and reagents were absolutized as usual and distilled prior to use. The progress of the reactions and the purity of the intermediates were monitored using TLC plates with a normal phase silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany).

Melting points were measured using a Bötetius apparatus (Franz KüstnerNachf. KG, Germany) and were not corrected. IR spectra were recorded on a SmartMIRacle ATR Zn/Se for Nicolet Impact 410 FT-IR spectrometer (Thermo Scientific Corp., MA, USA). NMR spectra were collected on a JEOL ECZR-400 MHz instrument (Jeol Corp., Japan). All of the NMR measurements were done at 25 °C. Chemical shifts δ are reported in parts per million (ppm) and J values in Hz. The signal of TMS or the residual solvent signals of CDCl₃ or DMSO-*d*₆ were used as an internal reference.

We have used the modified procedures described by Davis and Groshens (2012) to prepare trimethoxystilbenes, as shown in Fig. 2. The total syntheses of 3,4,5-trimethoxy-*trans*-stilbene (20) and 3-bromo-3,4,5-trimethoxy-*trans*-stilbene (21) were carried out in a four-step

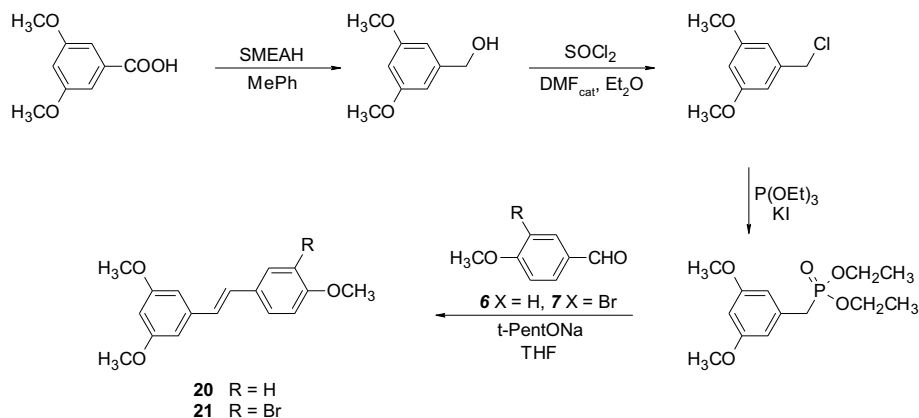


Fig. 2. The total synthesis of (*E*)-3,4,5-trimethoxystilbene (20) and (*E*)-3-bromo-3,4,5-trimethoxystilbene (21).

linear sequence to provide the products in satisfactory overall yields. Direct reduction of 3,5-dimethoxybenzoic acid by sodium bis(2-methoxyethoxy)aluminumhydride (SMEAH) in toluene yielded benzyl alcohol. Chlorination of the alcohol with thionyl chloride in diethyl ether provided an intermediate which upon Michaelis–Arbuzov reaction with triethylphosphite yielded the key phosphonate. Finally, Horner–Wadsworth–Emmons reaction of the intermediate with 4-methoxybenzaldehyde or 3-bromo-4-methoxybenzaldehyde using sodium *tert*-pentoxide as a base produced 3,4,5-trimethoxy-*trans*-stilbene (**20**) or 3-bromo-3,4,5-trimethoxy-*trans*-stilbene (**21**) in 32% and 54% yields, respectively.

2.2. COX-1 and COX-2 assays

An enzymatic *in vitro* assay was used to test inhibitory activity of the stilbenoids. COX-1 (1 unit/reaction) from ram seminal vesicles and human recombinant COX-2 (0.5 unit/reaction; both from Sigma-Aldrich, USA) were added to 180 μ L of a mixture consisting of 100 mM Tris buffer (pH 8.0), 5 μ M porcine hematin, 18 mM L-epinephrine, and 50 μ M Na₂EDTA. Tested compounds were dissolved in DMSO (pure DMSO as blank) to obtain 20 μ M concentration for an initial screening. Subsequently, 0.032, 0.16, 0.8, and 4 μ M concentrations were prepared for selected compounds. Ten microliters of the compounds to be tested were incubated with reaction mixture for 5 min at room temperature. The reaction was then initiated by adding 5 μ L of arachidonic acid (10 μ M) and the mixture was incubated for 20 min at 37 °C. The reaction was stopped by adding 20 μ L of formic acid (10% v/v). (S)-(+)-ibuprofen (Sigma-Aldrich, USA) was used as a reference inhibitor. The inhibition of COX enzymes was evaluated as the potential to reduce the synthesis of prostaglandin E₂ (PGE₂), quantified using a Prostaglandin E₂ ELISA kit (Enzo Life Sciences, NY, USA). Samples were diluted 1:15 in the assay buffer provided in the kit, before being used with the kit according to the manufacturer's instructions. The concentration of PGE₂ was estimated according to the absorbance measured at 405 nm by a Tecan Infinite M200 microplate reader (Tecan Group, Switzerland). The inhibitory activity was expressed as percent inhibition compared to the blank. The experiments were repeated at least two times with at least two replicates in each test.

2.3. 5-LOX assay

In case of the LOX assays, the inhibitory potential of each stilbenoid was tested using an enzymatic *in vitro* assay. Human recombinant 5-LOX (Cayman Chemical, MI, USA) was added (1 unit/reaction) to 180 μ L of an incubation mixture consisting of phosphate buffer saline (pH 7.4), 1 mM Na₂EDTA, and 200 μ M ATP. Tested compounds were dissolved in DMSO (pure DMSO as blank) to obtain 5 μ M concentration for initial screening. Subsequently, 0.008, 0.04, 0.2, and 1 μ M concentrations were prepared for selected compounds. Ten microliters of the compounds to be tested were incubated with reaction mixture for 10 min at 4 °C. Then, 5 μ L of 80 mM CaCl₂ was added, the reaction was initiated by adding 5 μ L of arachidonic acid (800 μ M) and the mixture was incubated for 10 min at 37 °C. The reaction was stopped by adding 20 μ L of formic acid (10% v/v). Zileuton (Farmak a.s., Czech Republic) was used as a reference inhibitor. Inhibition of the 5-LOX enzyme was evaluated as the potential to reduce the synthesis of leukotriene B₄ (LTB₄). LTB₄ was quantified using a Leukotriene B₄ ELISA kit (Enzo Life Sciences). Samples were diluted 1:15 in the assay buffer provided in kit before being used with the kit according to the manufacturer's instructions. The measurement of the absorbance and the determination of the inhibitory activity were the same as for the COX assays.

2.4. Cell culture and differentiation to macrophages

The THP-1 human monocytic leukemia cell line and HCT116 colon carcinoma cell line were purchased from the European Collection of

Authenticated Cell Cultures (Salisbury, UK). The THP-1-XBlue™-MD2-CD14 cell line was supplied by Invivogen (San Diego, CA, USA). THP-1 and THP-1-XBlue™-MD2-CD14 cells were cultured in RPMI 1640 medium containing stabilized 2 mM L-glutamine (Biosera, France), whereas HCT116 cells were cultured in DMEM high glucose medium w/ L-glutamine and w/sodium pyruvate (Biosera, France), both supplemented with antibiotics [100 U/mL penicillin and 100 mg/mL streptomycin (Biosera, France)], and 10% FBS (HyClone, UT, USA). Cells were kept in an incubator at 37 °C in a water-saturated atmosphere of air containing 5% CO₂. The suspensions of THP-1 and THP-1-XBlue™-MD2-CD14 cells were passaged approximately twice a week, adherent HCT116 cells were passaged in 2–3 days intervals when the culture reached 80–90% of confluence.

To differentiate the THP-1 monocytes to macrophages, cells were split into microtiter plates at a concentration of 500 000 cells/mL and stimulated by phorbol myristate acetate (PMA), as described previously (Hanáková et al., 2017) with some modifications. Briefly, after differentiation by PMA and 24 h incubation, the culture medium of adherent macrophages was replaced with a fresh complete medium. After another 24 h, the cells were washed with PBS, put into serum-free medium for 2 h, and then used for subsequent experiments.

2.5. Cytotoxicity testing

The undifferentiated floating THP-1 cells (500 000 cells/mL) were transferred into a serum-free RPMI 1640 medium and seeded into 96-well plates (100 μ L/well) in triplicates. After 2 h, the test compounds (1–25) dissolved in DMSO were added in increasing concentrations (0.313, 0.625, 0.988, 1.25, 1.48, 2.22, 2.50, 3.33, 5, 6, 10, 15, and 20 μ M). Measurements were taken after 24 h incubation with each of the test compounds. The adherent HCT116 cells (500 000 cells/mL) transferred into a serum-free DMEM high glucose medium were treated in the same manner described above. The tested concentrations of compounds (1–25) for this cell line were 5 μ M and 10 μ M. The maximum concentration of DMSO in the assays never exceeded 0.1% (v/v). Cell viability was measured using the Cell Proliferation Reagent kit WST-1 (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instruction. The amount of formazan created (which corresponds to the number of metabolically active cells in the culture) was calculated as a percentage of the control cells, which were treated only with DMSO, assigned as 100%. The resultant viability curves were used to calculate the IC₅₀ values using four parameters logistic (4PL) analysis.

2.6. Determination of the NF- κ B/AP-1 activity

The activity of NF- κ B/AP-1 was evaluated on the THP-1-XBlue™-MD2-CD14 cell line (Invivogen; San Diego, CA, USA) as described previously (Miláčková, Kapustová, Mučaji, & Hošek, 2017) and slightly modified. Briefly, cells were transferred into serum-free medium (500 000 cells/mL) and the test compounds (1–25) at the concentration of 2 μ M were added 2 h later. After a 1 h of incubation of the treated cells with the compounds, cells were stimulated by LPS (1 μ g/mL). LPS stimulation of the Toll-like 4 receptors in this cell line induced signaling cascades leading to the activation of NF- κ B/AP-1 and the subsequent production of the reporter protein SEAP was evaluated by a QUANTI-Blue™ assay. After 24 h of incubation, 20 μ L of cultivation medium was mixed with 175 μ L of Quanti-Blue™ reagent (Invivogen, San Diego, USA) and incubated at 37 °C for 30–40 min according to the manufacturer's instructions. The activity of NF- κ B/AP-1 was determined spectrophotometrically in a Fluostar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany) and compared to vehicle.

2.7. Signaling pathway analysis

The level of I κ B was determined on the THP-1-XBlue™-MD2-CD14 cell line, in a way similar to that described previously (Miláčková et al.,

2017). The mitogen-activated protein (MAP) kinase pathways mediated by p38, ERK1/2, and JNK were measured. Briefly, cells cultivated in serum-free medium were pretreated with the test compounds (3, 4, 7, 10, and 24) at a concentration of 2 μ M. One hour later, 1 μ g/mL of LPS was added and after 30 min the cells were collected into ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 270 mM sucrose) with protease inhibitors (Roche Diagnostics, Basel, Switzerland). Cell lysates were centrifuged 3000 g/5 min/4 °C and supernatants were mixed with 5 \times Laemmli buffer [250 mM Tris-HCl pH 6.8, 10% (w/v) SDS, 30% (v/v) glycerol, 5% (v/v) β -mercaptoethanol, 0.04% (w/v) bromophenol blue] and incubated 5 min/70 °C. To separate the proteins, 9 μ g of the proteins were loaded onto a 12% polyacrylamide gel and then transferred electrophoretically to PVDF (polyvinylidene fluoride) membranes that were subsequently blocked using 5% BSA dissolved in TBST buffer [150 mM NaCl, 10 mM Tris base, and 0.1% (v/v) Tween-20]. The membranes were incubated at 4 °C for 16 h with primary mouse anti-I κ B- α antibody and primary rabbit anti-phospho-SAPK/JNK, anti-p44/42 MAPK (ERK1/2), anti-phospho-p44/42 MAPK (ERK1/2), anti-p38 MAPK, and anti-phospho-p38 MAPK antibodies (Cell Signaling, Danvers, MA, USA; products No. 4814, 4668, 4695, 4370, 9212 and 4511) at a dilution of 1:500 (anti-I κ B- α), 1:2000 (anti-phospho-p44/42 MAPK) and 1:1000 (other antibodies). Two other primary mouse antibodies were used, anti- β -actin (Abcam, Cambridge, UK; product No. 8226) at a dilution of 1:5000 and anti-SAPK/JNK (Sigma-Aldrich, product No. SAB4200176) at a dilution of 1:1000. After washing, the secondary anti-mouse and anti-rabbit IgG antibodies (Sigma-Aldrich, products No. A0168 and A0545) were diluted 1:2000 and applied to the membranes, which were then incubated for 1 h at the laboratory temperature (~22 °C). The amount of bound secondary antibody was determined using ECL reagent (Bio-Rad, USA). Chemiluminescence was detected using a Syngene PXi4 chemiluminescence imaging system (Cambridge, UK).

2.8. Evaluation of cytokine secretion

Differentiated THP-1 macrophages, prepared as described above, were pretreated for 1 h with 2 μ M solutions of the test compounds or prednisone dissolved in DMSO 0.1% or with a 0.1% DMSO solution alone (the vehicle). The concentrations of the test compounds (1–25) used did not have cytotoxic effects (cell viability \geq 95%). An inflammatory-like response was triggered by adding 1 μ g/mL of LPS isolated from *Escherichia coli* 0111:B4 (Sigma-Aldrich, USA) and dissolved in water to the pretreated macrophages; the control cells were cultured without LPS treatment. After 24 h, the medium was collected and the concentration of TNF- α was measured using a Human TNF α ELISA Kit (Diacclone, Besançon, France) according to the manufacturer's instruction. Each experiment was performed in triplicate.

2.9. Molecular docking

The simulations of ligand-protein docking on NF- κ B were performed with AutoDockVina (Trott & Olson, 2010) using a PyRx virtual screening tool. Molecular dynamics were performed with a CUDA version of NAMD (Phillips et al., 2005), and VMD was used to prepare the molecular visualization and protein preparation. Docking simulations were performed using system with Core i7 and NVIDIA GTX 850. The docking simulations on 5-LOX and COX-2 were carried out in Gold 5.2 (CCDC, www.ccdc.cam.ac.uk, 2015). Docking poses and protein-ligand interactions were visualized in LigandScout 4.1 (Inte:Ligand, <http://www.inteligand.com/>, 2017).

In case of 5-LOX, the docking simulation was carried out on a structure based on PDB entry 3O8Y (Gilbert et al., 2011), which was virtually mutated to wild type 5-LOX and energetically minimized in Discovery Studio 4.0 (Bioviva). The binding site was defined in an 8 Å radius around the γ 2 carbon atom (CG2) of ILE167. ChemScore Kinase

was selected as a configuration template and CHEMPLP was selected as a scoring function. These settings were optimized in a previous SAR study on 5-LOX (Pein, Ville, Pace, Temml, Garscha, Raasch, Alsabil, Viault, Dinh, Guilet, Troisi, Neukirch, König, Bilancia, Waltenberger, Stuppner, Wallert, Lorkowski, Weinigel, Rummler, Birringer, Roviezzo, Sautebin, Helesbeux, Séraphin, Mosig, Schuster, Rossi, & Richomme, 2018).

For the docking on COX-2 the X-ray crystal structure 5IKV (Orlando & Malkowski, 2016), a structure of human COX-2 co-crystallized with flufenamic acid was employed. The ligand from the A chain was extracted and re-docked to validate the workflow (RMSD to the original ligand was 0.295 Å). The binding site was defined in a radius of 6 Å around the extracted ligand (A_1; A_1:5IKV). ChemScore Kinase was selected as a configuration template and CHEMPLP was selected as a scoring function.

The procedure described previously (Liu et al., 2015) was used for a protein energy minimization calculation. Besides, an additional GPU accelerator with NAMD software package was applied for the acceleration of the computation process. The crystallographic coordinates of NF- κ B bound to DNA were obtained from the RCSB Protein Data Bank with ID 3GUT. Only chain A (p65) and B (p50) were used for simulations and all other coordinates were removed. These two protein chains were minimized using molecular dynamics simulation with an NAMD package. The system of proteins was neutralized and the concentration of NaCl was set to 0.15 mol/L. Minimization used 5000 steps (Liu et al., 2015). After minimization, all heteroatoms were removed from the file and the proteins were subjected to docking using a PyRx docking tool with AutoDockVina with the exhaustiveness set to 20. All tested compounds meet the conditions of Lipinski rule of five (except of compounds 24 and 25, which possess higher molecular weight) suggesting that tested compounds can penetrate cell membranes, pass into cell compartments, and have several chemical groups forming hydrogen bonds. All compounds formed complexes on various binding sites of target protein structure with high binding energy ($\Delta G < -7.0$ kcal/mol). In case that binding site of best hit from docking result competed with DNA binding into DNA protein complex, the non-specific result was treated as positive.

2.10. Statistical evaluation

Statistical analyses were carried out using GraphPad Prism 6.01 software (San Diego, CA, USA). The data were graphed as the mean \pm SEM. Comparisons between groups were made using a one-way ANOVA test, followed by a Bonferroni's multiple comparisons test.

3. Results and discussion

3.1. COX/LOX studies

All of the compounds (1–25) were tested for their potential to inhibit the catalytic activity of COX-1, COX-2, and 5-LOX *in vitro*. Cell-free assay using human recombinant enzymes was used for the primary screening of activity and the reaction products were quantified using ELISA kits. Based on the results of screening, IC₅₀ values were calculated for the most promising compounds (Table 1). Only 5 of the 25 tested compounds showed significant activity against COX-1 and/or COX-2. *Trans*-resveratrol (1) showed even more activity against both COX-1 and COX-2 than ibuprofen used as a standard, although it was much more selective for COX-1 than COX-2 (COX-1/COX-2 selectivity ratio 0.22). The inhibitory activity of stilbenoids against COX enzymes is well-known. A report describing the activity of *trans*-resveratrol (1), pterostilbene (2), and piceatannol (3) on COX-1, COX-2, and 5-LOX enzymes accords with the observations we present here (Kutil et al., 2015). Comparison of the compounds described here shows that any big change in the substitution pattern of *trans*-resveratrol (1), such as methoxylation, glycosylation, or modification of the carbon linkage

Table 1

Calculated IC₅₀ values for COX-1, COX-2, and 5-LOX, and the COX-1/COX-2 selectivity ratio for selected stilbenoid compounds and corresponding standards, as determined from a series of measurements in triplicates.

Compound/Assay	COX-1 IC ₅₀ [μM]	COX-2 IC ₅₀ [μM]	COX-1/COX-2 selectivity ratio	5-LOX IC ₅₀ [μM]
<i>Trans</i> -resveratrol (1)	0.20 ± 0.1	0.90 ± 0.32	0.22	Inactive
Pterostilbene (2)	5.99 ± 1.57	6.67 ± 2.21	0.90	1.63 ± 0.44
Piceatannol (3)	Inactive	Inactive	–	0.39 ± 0.09
Pinostilbene (4)	1.37 ± 0.55	0.25 ± 0.06	5.48	4.01 ± 0.58
4-stilbenecarboxylic acid (14)	Inactive	Inactive	–	5
Pinosylvin monomethylether (17)	1.66 ± 0.19	5.86 ± 1.25	0.28	Inactive
Isorhapontigenin (18)	8.77 ± 1.23	10.01 ± 3.15	0.88	Inactive
1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)ethanone (23)	Inactive	Inactive	–	5
Ibuprofen	6.92 ± 2.74	2.03 ± 1.11	3.41	Not tested
Zileuton	Not tested	Not tested	–	0.11 ± 0.06

connecting rings diminishes or eliminates the activity. On the other hand, pinostilbene (4) demonstrated the greatest selectivity for COX-2 (COX-1/COX-2 selectivity ratio 5.48), even greater than ibuprofen (COX-1/COX-2 ratio 3.41). A noteworthy is also the effect of pinosylvin monomethylether (17) and isorhapontigenin (18) against both COX-1 and COX-2 enzymes. However, a high selectivity to COX-1 (COX-1/COX-2 ratios 0.28 and 0.88, respectively), comparable with that of *trans*-resveratrol (1), is often associated with side effects like gastrointestinal damage, characteristic for traditional non-selective NSAIDs. Although the attention is aimed mostly at the development of selective COX-2 inhibitors, Perrone, Scilimati, Simone, and Vitale (2010) highlight the important role of COX-1 in the organism under normal and pathological conditions and suggest that selective COX-1 inhibition might be highly relevant for several diseases.

A similar situation was observed for the anti-5-LOX effect, where piceatannol (3) and pterostilbene (2) (and to limited extent also pinostilbene (4)) showed promising activity, comparable to the zileuton used as a positive control (Table 1). The specific activities of 4-stilbenecarboxylic acid (14) and 1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-ethanone (23) against 5-LOX are described here for the first time.

In *in silico* simulation carried out on COX-2 the investigated compounds were docked to the substrate binding site. The docking studies in general confirmed the results of *in vitro* assays. The most active compounds all showed a clear binding pattern that involves hydrogen bonds with key residues of COX-2. The hydroxyl groups of the most active compound pinostilbene (4) form hydrogen bonds with Tyr355, Tyr385 and Ser530 which act as a carboxyl binding site for arachidonic acid (AA) (Fig. 3A) (Kiefer et al., 2000).

The compounds were also docked in an allosteric binding site on 5-LOX that has been shown to accommodate phenolic 5-LOX inhibitors (Schaible et al., 2016). It has lately been shown that some 5-LOX inhibitors do not act in competition with AA, but instead inhibit the enzyme independently of substrate concentration (Mohamed et al., 2014;

Srivastava et al., 2016; Cheungal et al., 2018). We therefore docked the compounds to the allosteric binding site that is currently being investigated. The most active compounds dock to the binding site with good scores and form hydrogen bond interactions that would suggest a stable placement. The hydroxyl groups of the most active compound piceatannol (3) are predicted to form hydrogen bonds with Phe402 and Arg138 (Fig. 3B).

3.2. Determination of cytotoxicity

The THP-1 cell line was used to further evaluate the anti-inflammatory potential of compounds 1–25. Moreover, the human colon carcinoma cell line HCT116 was selected to verify the non-toxic effect of the tested stilbenoids. A WST-1 assay was used to measure the viability of cells after treatment with increasing concentrations of stilbenoids 1–25 in order to determine the tolerable non-toxic concentrations for use in subsequent *in vitro* experiments and potentially calculate the values of IC₅₀. These assays showed relatively low toxic effects of tested compounds, with IC₅₀ values greater than 10 μM for both cell lines (the viability was > 70% for THP-1 at the concentration of 20 μM and for HCT116 it was > 75% at 10 μM). The only exception was piceatannol (3) with an IC₅₀ value of 6.7 ± 1.0 μM for the THP-1 cell line. The viability of the HCT116 cell line was not markedly affected by the tested compounds. Cytotoxicity assays showed relatively safe profiles for all of the compounds tested which enabled us to continue our study of cell-based anti-inflammatory activity. The low toxicity of the stilbenoids we tested may be related to the presence of several hydroxyl groups attached directly on ring A and/or ring B. The absence of lipophilic substituents (Sirerol et al., 2016) that could increase the ability of the compounds to penetrate the membranes of cells and interact with the internal cell environment might be also a factor (Hanáková et al., 2017), but subsequent experiments showed that at non-toxic concentrations stilbenoids are able to interact well with intracellular mechanisms. Based on the cytotoxicity assays, the following bioactivity

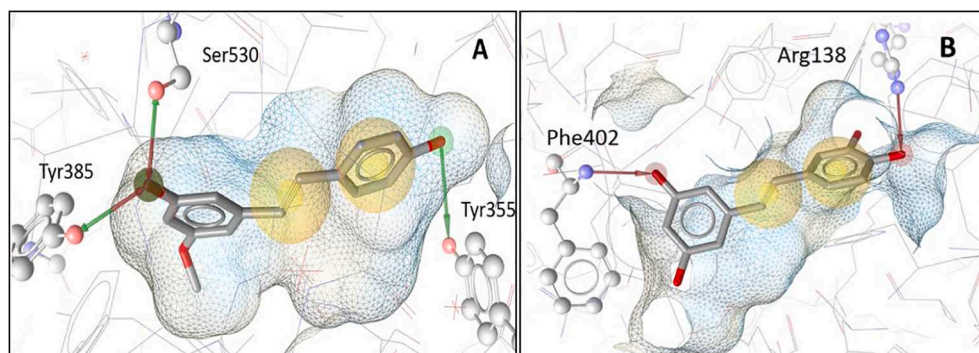


Fig. 3. Docking pose of pinostilbene (4) in the substrate binding site of COX-2 (A), piceatannol (3) in the allosteric binding site of 5-LOX (B).

tests were performed on each of the compounds 1–25 at non-toxic concentrations of 2 μ M.

3.3. Anti-inflammatory potential

Although the anti-inflammatory effect of stilbenoids is well-known, only a few compounds have been in detail evaluated for this activity (Dvorakova & Landa, 2017), *trans*-resveratrol (1), piceatannol (3), and pterostilbene (2) having been studied the most. Our study evaluated the activities of 25 stilbenoids all at the same pharmacologically relevant concentration of 2 μ M and all under the same conditions in order to provide a reliable comparison of their effect. All of stilbenoids 1–25 were first evaluated for their ability to attenuate the LPS-stimulated activation of NF- κ B/AP-1 in THP1-XBlue™-MD2-CD14 cells in a primary screening for anti-inflammatory effects. LPS stimulation of the TLR4 receptors in this cell line induced signaling cascades that led to the activation of both the NF- κ B and AP-1 transcription factors. Most of the tested compounds reduced the activity of NF- κ B/AP-1 when the cells were stimulated by LPS (Fig. 4A). Piceatannol (3), thunalbene (7), oxyresveratrol (8), 2-carboxyl-3-*O*-methyl-4- β -D-glucopyranosyl-dihydroresveratrol (10) and 3-*O*-caffeoyl-(9 \rightarrow 5)- β -apiosyl-(1 \rightarrow 6)- β -glucopyranosyl-3-*O*-methyl-dihydroresveratrol (24) affected the activity of NF- κ B/AP-1 in a statistically significant manner, comparable to that of the conventionally used drug prednisone. These data indicate that hydroxylation of aromatic rings increases the inhibition activity against NF- κ B/AP-1. Piceatannol (3) and oxyresveratrol (8), differing from *trans*-resveratrol (1) only in one additional aromatic hydroxyl group, have shown greater activity against NF- κ B/AP-1 than 1 itself. Likewise, compound 24 has been more active than his *O*-methylated analogue (25). The position of hydroxy groups influences also the activity of stilbenes in order *para* < *ortho* < *meta*. Compounds 3 and 7, having a hydroxyl group in *meta*-position of ring B, showed greater inhibitory effect on NF- κ B/AP-1 activation than their analogues 8 and 4, having a hydroxyl group in *ortho*- and *para*- position, respectively. Interestingly, hydroxyl group in *para*-position seems to enhance the NF- κ B/AP-1 activity in the case of compound 4 compared to 7. On the other hand, modifications of ethylene bridge, for example its substitution (compound 16, 23 referred to 12), attenuates the inhibition of activity against NF- κ B/AP-1. Similarly, the hydrogenation of ethylene bridge in the case of batatasin III (6) caused a decrease of activity in comparison with thunalbene (7). The most active compound piceatannol (3), in agreement with previous studies, showed a greater effect on the inhibition of the NF- κ B/AP-1 pathway than resveratrol (1) and oxyresveratrol (8) (Ashikawa et al., 2002; Liu, Kim, Park, Na, & Surh, 2009; Son et al., 2010). Furthermore, this is the first report to describe the attenuating effects of thunalbene (7) and compounds (10 and 24) from *T. tommasinii* on the NF- κ B/AP-1 activity.

Compounds possessing significant effect on NF- κ B/AP-1 activity, piceatannol (3), pinostilbene (4) (due to its observed effect on LPS-stimulated NF- κ B activity), thunalbene (7), 2-carboxyl-3-*O*-methyl-4- β -D-glucopyranosyl-dihydroresveratrol (10), and 3-*O*-caffeoyl-(9 \rightarrow 5)- β -apiosyl-(1 \rightarrow 6)- β -glucopyranosyl-3-*O*-methyl-dihydroresveratrol (24), were selected for more detailed analysis of their effect on NF- κ B and on the signaling pathways of mitogen-activated protein kinases (MAPKs). MAPK cascades, represented by p38, ERK, and JNK kinases, can be triggered by phosphorylation events in their activation loops during inflammation or in response to an extracellular stimulus, such as LPS. This activates the AP-1 transcription factor. As for MAPKs, extracellular stimuli activate transcription factor NF- κ B by inducing degradation of its inhibitor I κ B α (Lee & Kim, 2007). Piceatannol (3), pinostilbene (4), thunalbene (7), 2-carboxyl-3-*O*-methyl-4- β -D-glucopyranosyl-dihydroresveratrol (10) inhibited the phosphorylation of MAPKs p38, ERK1/2, and JNK (Fig. 4B). Only compound 24 did not affected MAPKs, possibly because the size and shape of molecule would not fit into an active center of these proteins. Several previous studies have demonstrated attenuation of MAPKs phosphorylation by stilbenoids, for example

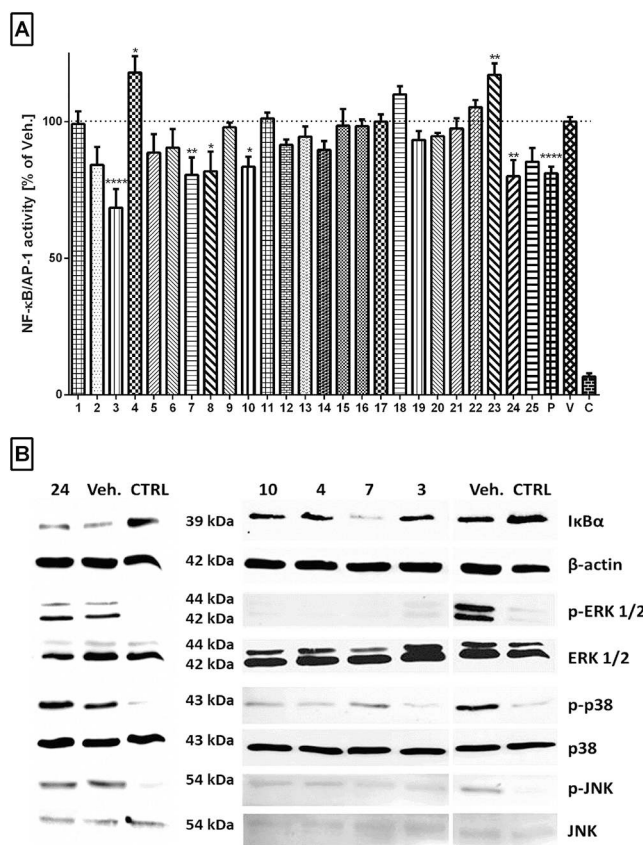


Fig. 4. Effects of compounds 1–25 on LPS-stimulated NF- κ B/AP-1 activity (A) and effect of compounds 3, 4, 7, 10 and 24 on the signaling cascade in the THP1-XBlue™-MD2-CD14 cell line (B). (A) Cells of the THP1-XBlue™-MD2-CD14 cell line were pre-treated with the test compounds (2 μ M, dissolved in DMSO), or prednisone (P; 2 μ M, dissolved in DMSO) for 1 h. Subsequently, LPS (1 μ g/mL) was added [except for the control cells (C or CTRL)] to trigger the activation of NF- κ B/AP-1 signaling. After 24 h, the NF- κ B/AP-1 activity was evaluated based on the spectrophotometric measurement of amount of alkaline phosphatase secreted. The results are expressed as the mean \pm S.E. for six independent experiments. (B) The level of MAPKs, I κ B and β -actin in cell lysate was detected 30 min after LPS stimulation by Western blot and immunodetection. The blots shown represent the results of three independent experiments. V or Veh. represent the vehicle treated cells. * Indicates a significant difference of $p < 0.05$ in comparison with cells treated only with the vehicle, ** indicates a significant difference of $p < 0.01$ in comparison with cells treated only with the vehicle, and **** indicates a significant difference of $p < 0.0001$ in comparison with cells treated only with the vehicle.

pterostilbene (2) (Paul et al., 2009), desoxyrhapontigenin (Choi, Chun, Khan, & Kim, 2014), *trans*-resveratrol (1) (Oh et al., 2009), or piceatannol (3) (Ko et al., 2013). However, it is worth noting that most of these studies applied much greater concentrations of stilbenoids than 2 μ M used in current study, levels that do not correspond to concentrations likely to be found in living organism, e.g., for resveratrol (1) the maximal plasma concentrations were \approx 2 μ M after oral administration (Cottart, Nivet-Antoine, Languillier-Morizot, & Beaudeau, 2010; Walle, Hsieh, DeLegge, Oatis, & Walle, 2004). None of the tested compounds influenced the degradation of I κ B α at a concentration of 2 μ M, moreover, compound 7 notably decreased its level. Similar negative results have been found for pterostilbene (2) using different models (Paul et al., 2009) and at low concentrations (< 10 μ M) for piceatannol (3) (Liu et al., 2009) and *trans*-resveratrol (1) (Oh et al., 2009). It can be hypothesized that if thunalbene (7) attenuates NF- κ B activity, but the level of I κ B is low, 7 could directly interact with activated NF- κ B and inhibit its activity. Several studies report about targeting the NF- κ B signaling pathway by small natural molecules at

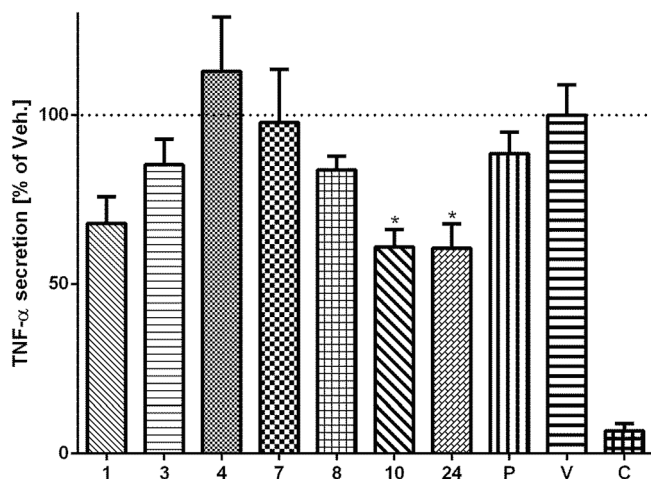


Fig. 5. The effect of compounds **1**, **3**, **4**, **7**, **8**, **10** and **24** on the secretion of TNF- α . THP-1 macrophages were pre-treated with each of the compounds (2 μ M, dissolved in DMSO) or prednisone (P; 2 μ M, dissolved in DMSO) for 1 h. LPS (1 μ g/mL) was then added [except for the control cells (C)] to trigger the secretion of the pro-inflammatory cytokine TNF- α . After 24 h, the amount of TNF- α was evaluated by ELISA. The results are expressed as the mean \pm S.E. for six independent experiments. * Indicates a significant difference, $p < 0.05$ in comparison with the cells treated only with the vehicle (V).

various levels, e.g. p65 subunit acetylation, methylation and DNA binding. Similarly, NSAIDs have been found to block NF- κ B, but their effects are highly pleiotropic (Gupta, Sundaram, Reuter, & Aggarwal, 2010).

These experiments show that some stilbenoids attenuate the activity of NF- κ B and AP-1 by inhibiting the MAP kinases p38, ERK1/2, and JNK, rather than by inhibiting the degradation of I κ B α . Interestingly, compound **24** affected neither the MAPKs nor I κ B α , but was nevertheless able to attenuate the activity of NF- κ B/AP-1 and the secretion of TNF- α . To investigate another possible mechanism of action, all of the test stilbenoids were docked into the DNA binding pocket of the NF- κ B heterodimer p65/p50 (Fig. S1). The bond of compound **24** to NF- κ B had one of the highest binding energies ($\Delta G = -6.8$ kcal/mol) (Table S1).

A mechanism of competitive inhibition has been described for *trans*-resveratrol (**1**) and its derivatives (Mulakayala et al., 2013) and may explain why **24** inhibits NF- κ B/AP-1 activity, even though does not affect MAPKs or NF- κ B/AP-1 signaling cascades. Interestingly, compound **25**, which differs from **24** only by a single methoxy group, had a great binding energy ($\Delta G = -7.3$ kcal/mol), but its inhibitory activity against NF- κ B/AP-1 was slightly lower [$14.7 \pm 5.0\%$ for **25** vs. $20.0 \pm 6.0\%$ for **24**]. Piceatannol-3-O-glucoside (**5**) had the same binding energy ($\Delta G = -7.3$ kcal/mol), but its inhibition of NF- κ B/AP-1 was relatively weak ($11.3 \pm 6.8\%$).

The next experiments were focused on the influence of stilbenoids on LPS-activated TNF- α expression, which is under the control of NF- κ B transcription. Piceatannol (**3**), thunalbene (**7**), oxyresveratrol (**8**), 2-carboxyl-3-O-methyl-4- β -D-glucopyranosyl-dihydroresveratrol (**10**) and 3-O-caffeoyl-(9 \rightarrow 5)- β -apiosyl-(1 \rightarrow 6)- β -glucopyranosyl-3-O-methyl-dihydroresveratrol (**24**) were selected for this analysis based on their significant effects on the inhibition of NF- κ B/AP-1. Pinostilbene (**4**) and resveratrol (**1**) were added to the experimental set because they were the most potent COX and 5-LOX inhibitors, as seen from previous experiments. Compounds **1**, **3**, **8**, **10**, and **24** diminished the production of TNF- α more than the prednisone that was used as a positive control, but only compounds **10** and **24** influenced the LPS-stimulated TNF- α secretion in a statistically significant way ($p < 0.05$) (Fig. 5). These results substantiate the previous study of Granica et al. (2015), in which compounds **10** and **24** significantly attenuated the secretion of TNF- α from human neutrophils. The ability of *trans*-resveratrol (**1**),

piceatannol (**3**) and oxyresveratrol (**8**) to diminish the TNF- α expression has been already described in several studies (Dvorakova & Landa, 2017; Mouihate, Horn, & Pittman, 2006). Interestingly, *trans*-resveratrol (**1**) did not influence the NF- κ B activity, which indicates that it interacts with some post-transcription and/or post-translation regulatory mechanism. In contrast, thunalbene (**7**) reduced the activity of NF- κ B/AP-1, but did not influence the expression of TNF- α . Possible interaction of **7** with other pro-inflammatory cytokines being under NF- κ B transcription control, would be a subject of further investigation. It is worth noting that pinostilbene (**4**) moderately increased the production of TNF- α , probably because of its greater effect on NF- κ B/AP-1. Compound **4** showed the greatest inhibitory effect on COX-2 and moderate influence on 5-LOX.

All of the above-mentioned mechanisms - direct inhibition of the NF- κ B/AP-1 activity and/or MAPKs signaling cascades, the interaction with the DNA binding pocket of NF- κ B, the inhibition of the secretion of TNF- α , and the inhibition of the COX and LOX enzymes - could contribute to overall anti-inflammatory effect of stilbenoids. This study confirms that stilbenoids should be considered as potent multi-target anti-inflammatory agents and perspective candidates for further drug discovery.

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Conflict of interest

Authors do not have any conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2019.01.128>.

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Prenylated Stilbenoids Affect Inflammation by Inhibiting the NF- κ B/AP-1 Signaling Pathway and Cyclooxygenases and Lipoyxygenase

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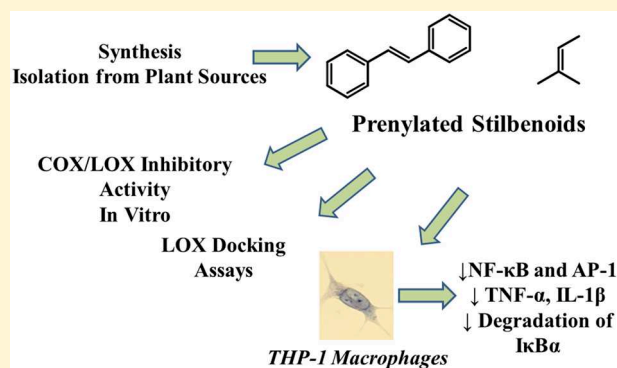
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Supporting Information

ABSTRACT: Stilbenoids are important components of foods (e.g., peanuts, grapes, various edible berries), beverages (wine, white tea), and medicinal plants. Many publications have described the anti-inflammatory potential of stilbenoids, including the widely known *trans*-resveratrol and its analogues. However, comparatively little information is available regarding the activity of their prenylated derivatives. One new prenylated stilbenoid (**2**) was isolated from *Artocarpus altilis* and characterized structurally based on 1D and 2D NMR analysis and HRMS. Three other prenylated stilbenoids were prepared synthetically (**9–11**). Their antiphlogistic potential was determined by testing them together with known natural prenylated stilbenoids from *Macaranga siamensis* and *Artocarpus heterophyllus* in both cell-free and cell assays. The inhibition of 5-lipoxygenase (5-LOX) was also shown by simulated molecular docking for the most active stilbenoids in order to elucidate the mode of interaction between these compounds and the enzyme. Their effects on the pro-inflammatory nuclear factor- κ B (NF- κ B) and the activator protein 1 (AP-1) signaling pathway were also analyzed. The THP1-XBlue-MD2-CD14 cell line was used as a model for determining their anti-inflammatory potential, and lipopolysaccharide (LPS) stimulation of Toll-like receptor 4 induced a signaling cascade leading to the activation of NF- κ B/AP-1. The ability of prenylated stilbenoids to attenuate the production of pro-inflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) was further evaluated using LPS-stimulated THP-1 macrophages.



Inflammation of tissues occurs when the immune system is activated to combat an infection, an irritation, or a wound.¹ Immune-system cells generate and release a variety of mediators that affect the development of the inflammatory

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response. Macrophages, which play an important role in acute as well as in chronic inflammation, produce a number of endogenous mediators, including the vasoactive amines histamine and serotonin, metabolites of arachidonic acid, the complement system, the kinin system, the fibrinolytic system, platelet-activating factor (PAF), neuropeptides, hydrolytic enzymes, adhesion molecules, reactive oxygen species (ROS), and inflammatory cytokines.^{2,3} Studies describing the bioactivity of prenylated phenols have shown that they may potentially affect the production of these substances.²

Prenylated phenolics are an interesting class of plant secondary metabolites,² because their structures combine the common structural features of a phenolic skeleton with terpenoid side chains, most commonly isopentenyl and geranyl units.^{2,4} Prenylated phenols are classified into several groups, according to the type of phenolic skeleton.^{2,5,6} The 1,2-diphenylethylene core characteristic for stilbenoids is formed in plants through the shikimate pathway via cinnamoyl-CoA or *p*-coumaroyl-CoA, and the reaction is catalyzed by stilbene synthase.⁷ The prenyl unit is synthesized via the isoprenoid mevalonate or deoxyxylulose pathway, and prenylation is mediated by stilbenoid-specific prenyltransferase.^{4,7} Many of the plants containing prenylated phenolic constituents that are used in traditional medicine show a variety of biological effects, such as anti-inflammatory, antioxidant, antimicrobial, or cytotoxic activities.^{2,4,8} The many possible structural combinations of a prenyl group with a phenolic skeleton seem to be especially auspicious for achieving such beneficial biological activities.⁹ Moreover, the prenyl moiety could facilitate the permeation of cell membranes and thus enhance bioavailability.^{10,11} Study comparing the pharmacokinetics of two prenylated stilbenes, *trans*-arachidin-1 and *trans*-arachidin-3, with their nonprenylated analogues, *trans*-piceatannol and *trans*-resveratrol, respectively, suggested that improved bioavailability of prenylated stilbenes could be achieved by their slower metabolism.¹¹

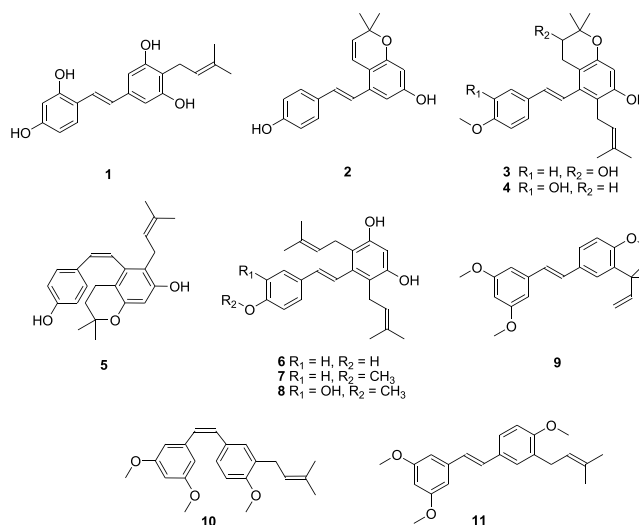
Although stilbenoids have been described to be relatively widespread in food and medicinal plants, the number of studies describing the distribution of prenylated stilbenoids in the plant kingdom is limited to date. Like prenylated flavonoids and benzofurans, prenylated stilbenoids are typically isolated from plants of the Moraceae family, such as *Morus* or *Artocarpus* species.^{12,13} They have also been found in several *Macaranga* species, belonging to the family Euphorbiaceae.^{14,15} Three species from Fabaceae, *Arachis hypogea*,¹⁶ *Cajanus cajan*, and *Lonchocarpus chiricanus*, have also been described as sources of prenylated stilbenoids.^{17,18} Some studies have reported their isolation from various exotic propolis samples.¹⁹

Recently, the in vitro anti-inflammatory potential of several nonprenylated stilbenoids was described. The results indicate that stilbenoids are agents able to target several inflammatory pathways.²⁰ The activities of prenylated stilbenoids have been reported as mostly antimicrobial¹⁶ and cytotoxic,¹⁵ but due to their structural similarity to prenylated flavonoids, it is assumed that they may also have anti-inflammatory activity. However, the anti-inflammatory potential of prenylated stilbenoids has not been reported yet. To fill this gap, the in vitro anti-inflammatory effects of several prenylated stilbenoids obtained from *M. siamensis*, *A. altilis*, and *A. heterophyllus* were evaluated. Because the number of prenylated stilbenoids in plants is limited, a method using prenylation was developed to extend the series of compounds tested. Moreover, the synthesis of

other C-prenylated analogues could facilitate the comparison of influence of structural aspects on tested biological activity.

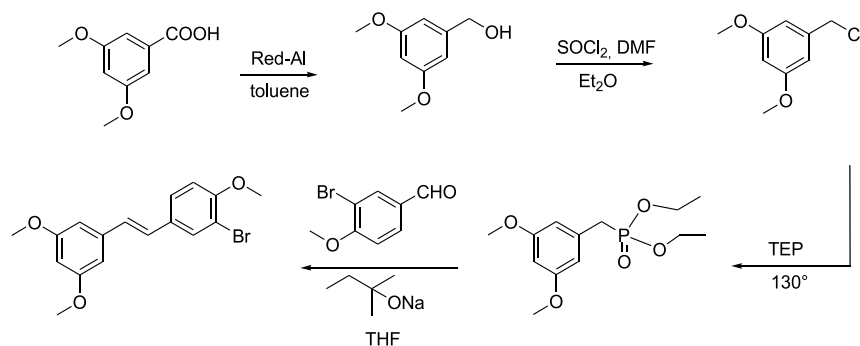
RESULTS AND DISCUSSION

Isolation of Compounds and Elucidation of Structures. Eleven prenylated stilbenoids (1–11) were tested for their anti-inflammatory potential. Eight of these (1–8) occur in Nature. Compound 1 was obtained from *A. heterophyllus*, and compound 2 from *A. altilis*. The isolation and identification of compounds 3–8 from *M. siamensis* has been described previously,^{14,21} and reported here are the syntheses of the new compounds 9–11.

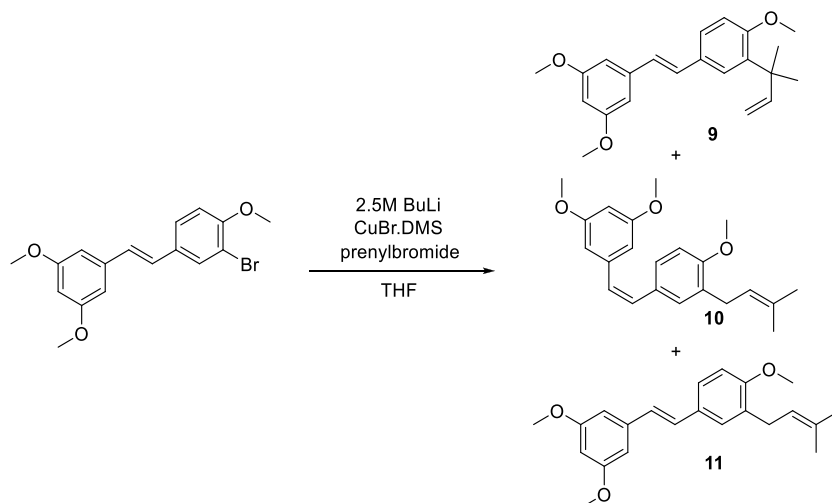


The new compound 2 was isolated by column chromatography on silica gel and reversed-phase semipreparative HPLC in the form of a pale yellow, amorphous powder. Its molecular formula (C₁₉H₁₈O₃) was determined by HRMS, which gave a pseudomolecular ion peak at *m/z* 293.1198 [M – H][–], calcd 293.1178. The ¹H NMR spectrum showed signals for *trans*-olefinic protons at δ_H 6.81 and 6.98 ppm (each 1H, d, *J* = 16.0 Hz) and six aromatic protons [δ_H 6.44 (1H, s), 6.60 (1H, ov), 6.84 (2H, d, *J* = 8.5 Hz), and 7.37 ppm (2H, d, *J* = 8.5 Hz)], suggesting the presence of a *trans*-stilbenoid structure. Furthermore, two *cis*-olefinic protons at δ_H 5.60 and 6.63 ppm (each 1H, d, *J* = 10.0 Hz) and two methyl groups at δ_H 1.44 ppm (6H, s) were observed, indicating the presence of a 2,2-dimethylpyran group attached to one of the stilbenoid rings. That the 2,2-dimethylpyran unit is attached to ring A of the stilbenoid at positions C-2 and C-3 was deduced from the heteronuclear multiple bond correlation (HMBC) spectrum. The proton at δ_H 6.63 ppm (C-1'') correlated with a carbon NMR signal at δ_C 153.0 ppm (C-3) and the quaternary carbon signal at δ_C 75.9 ppm (C-3''), suggesting a linkage to oxygen. Accordingly, 2 was assigned as 7-[(1*E*)-2-(4-hydroxyphenyl)ethenyl]-2,2-dimethyl-2*H*-1-benzopyran-5-ol and was described as obtained for the first time from a natural source.

Synthesis of Prenylated Stilbenoids. Prenylated stilbenoids 9–11 were prepared from 1-[(*E*)-2-(3-bromo-4-methoxyphenyl)ethenyl]-3,5-dimethoxybenzene via direct lithiation followed by the addition of prenyl bromide in the presence of a cuprous bromide dimethyl sulfide complex.²² The bromostilbenoid used for these syntheses was itself synthesized from commercially available 3,5-dimethoxybenzoic acid by standard methods. The carboxylic acid function was reduced selectively using Red–Al and chlorinated with thionyl

Scheme 1. Synthesis of a *trans*-3'-Bromo-3,5,4'-trimethoxystilbene Intermediate

Scheme 2. Prenylation Method Yielding a Variety of Products



chloride.²³ The phosphonate derivative was prepared by the Michaelis–Arbuzov method using triethylphosphite, because it produced a better yield and purity than the Michaelis–Becker procedure with diethyl phosphite used in previous work.²⁴ The bromostilbenoid was prepared from the phosphonate intermediate by the base-catalyzed Horner–Wadsworth–Emmons reaction with 3-bromo-4-methoxybenzaldehyde²⁵ (Scheme 1).

Three compounds were isolated following the prenylation step: the expected *trans*-isomer of the prenylated stilbenoid (**11**), its *cis*-isomer (**10**), and a product of allylic rearrangement (**9**) (Scheme 2). The structures of all isolated compounds were determined by spectroscopic methods, as described in the Experimental Section.

Inhibition of COX and LOX. Compounds **1–11** were tested for their ability to inhibit the catalytic activity of cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and 5-lipoxygenase (5-LOX) in vitro in a cell-free assay using human recombinant enzymes. Primary screening of activity was performed at a concentration of 20 μM . Based on the results of this screening, IC_{50} values were calculated for the most promising compounds. Several of the test compounds showed some activity against COX-1 and/or COX-2. Compounds **6**, **7**, and **8** were the most active; the submicromolar IC_{50} values for their anti-COX-1 effect were greater than that of ibuprofen, used as a positive control (Table 1). However, these compounds were much more selective for COX-1 than for COX-2 (COX-1/COX-2 selectivity ratio 0.06–0.17). Commonly used nonsteroidal anti-inflammatory drugs (e.g., ibuprofen or naproxen) are nonselective COX

Table 1. Calculated IC_{50} Values for COX-1, COX-2, and 5-LOX and the COX-1/COX-2 Selectivity Ratio for Selected Stilbenoid Compounds and Corresponding Standards, as Determined from Serial Measurements in Triplicate

compound/ assay	COX-1 IC_{50} [μM]	COX-2 IC_{50} [μM]	COX-1/ COX-2 ratio	5-LOX IC_{50} [μM]
2	13.3 \pm 7.59	>20		2.4 \pm 0.44
4	12.6 \pm 4.98	35.5 \pm 12.57	0.36	inactive
6	0.4 \pm 0.03	5.7 \pm 3.67	0.07	0.9 \pm 0.31
7	0.8 \pm 0.41	4.7 \pm 1.39	0.17	1.1 \pm 0.54
8	0.9 \pm 0.43	14.7 \pm 5.55	0.06	0.9 \pm 0.59
ibuprofen	6.9 \pm 2.74	2.0 \pm 1.11	3.4	
zileuton				0.1 \pm 0.06

inhibitors. They inhibit activity of the inducible COX-2 isoform to a similar extent as the activity of constitutive COX-1. COX-1 plays a cytoprotective role in the gastrointestinal system, and its suppression can result in erosions, ulcers, bleeding, and perforation.²⁶ Therefore, selective COX-2 inhibitors (with their side effects) were developed to avoid such complications. However, selective inhibition of COX-1 could be useful as well, because COX-1-dependent prostaglandin synthesis is involved in pathological processes such as atherosclerosis, endothelial dysfunction, neuroinflammation, preterm labor, pain, and cancer. Therefore, the development of selective COX-1 inhibitors was initiated.^{27–29}

The ability of certain stilbenoids to inhibit COX enzymes is well known. However, information on the anti-COX activity of

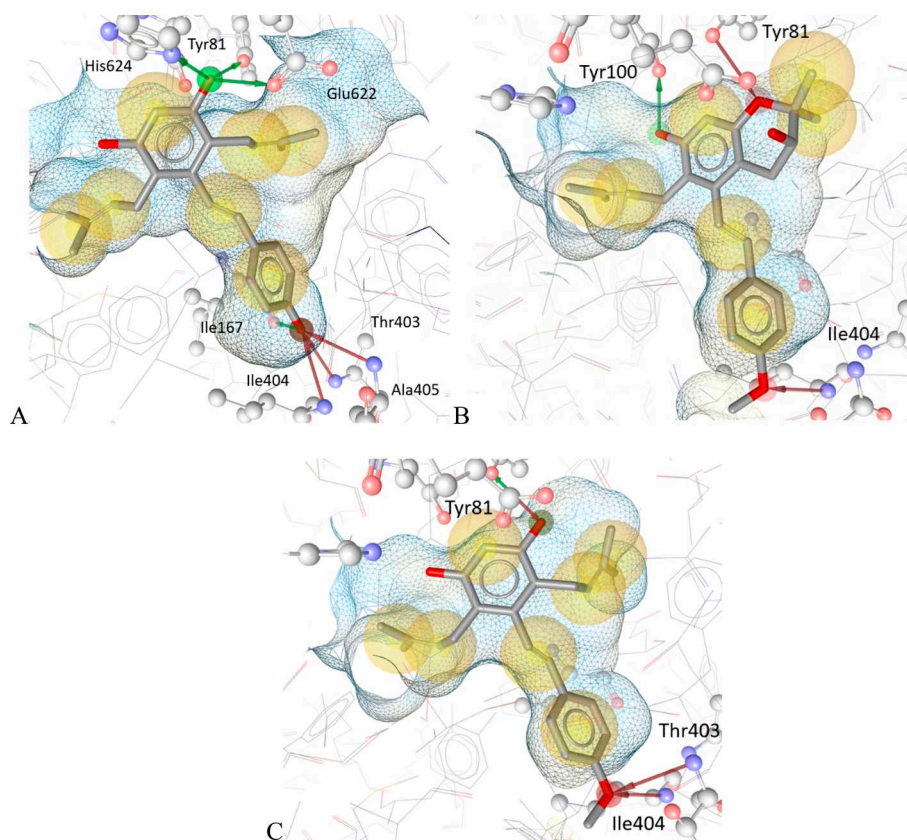


Figure 1. (A) Compound 6 shown in the crystal structure of 5-LOX. It binds to the residues Ile167, Thr403, Ile404, and Ala405, which act as hydrogen bond interaction partners to the *para*-hydroxy group and to Tyr81, Tyr100, Glu622, and His624, forming interactions with one of the *meta*-hydroxy groups on the other phenyl moiety. (B) Compound 3 in 5-LOX, forming an interaction between Ile404 and the methoxy group, while the hydroxylated chromane interacts with Tyr100 and Tyr81. (C) Compound 7 in 5-LOX, which also binds to Tyr81, Thr403, and Ile404. Red arrows signify interactions with a hydrogen bond acceptor; green arrows with a hydrogen bond donor. Yellow spheres represent hydrophobic contact with the binding pocket.

prenylated stilbenoids is scarce. One study showed the propensity of the prenylated stilbenoid carexane to decrease the COX-2 expression in HspB-transfected AGS cells,³⁰ and three prenylated stilbenoids from *A. dadah*³¹ were selectively active for COX-1 when compared to COX-2. Prenylation of a stilbenoid does not seem to strongly increase anti-COX activity, but the activities of prenylated stilbenoids 6, 7, and 8 against COX-1 are noteworthy. Further modifications of the prenyl moiety, like *O*-cyclization, diminished the anti-COX/LOX activity, as evident from the comparison of the activities of 4 and 8 (Table 1). One report describing the activities of *trans*-resveratrol, pterostilbene, and piceatannol against COX-1, COX-2, and 5-LOX has been presented,³² and another relevant publication has appeared recently.²⁰ The activities of these nonprenylated stilbenoids were similar to those of the prenylated stilbenoids described herein, with the exception of pinostilbene, which showed a greater selectivity for COX-2 than for COX-1. Prenylated stilbenoids 2 and 6–8 showed also promising anti-5-LOX effects (Table 1).

Molecular Docking Studies. To rationalize the activity of the prenylated stilbenoids observed in vitro against LOX, docking studies of selected compounds (2–8) were performed to elucidate their interaction with the target enzyme. The prenylated stilbenoids were docked into an allosteric binding site of 5-LOX located between the catalytic and the membrane binding domains that was identified for AA-noncompetitive inhibitors of a similar size by a previous study.³³ Compound 6,

the most active compound, docked with a score of 41.76. It interacted with the two major anchor sites within the binding pocket (see Figure 1A). One of these was formed by Ile167, Thr403, Ile404, and Ala405, which serve as partners for hydrogen bonding to the *para*-hydroxy group. The second site has formed by Tyr81, Tyr100, Glu622, and His624, which interact with one or the other *meta*-hydroxy group on the other phenyl moiety. The other highly active compound, 8, was found to bind to these two sites with a score of 39.82. Compound 7, which only differs from 6 by having a methoxy group instead of hydroxy substituent, also followed this binding pattern, but with a significantly lower score of 27.27 (Figure 1C). This suggests that the hydroxy group fits into the binding site slightly better. The inactive compound 4 would be turned around in the binding pocket and hence did not interact with either of the two sites. Compound 3 formed key interactions and docked with a score of 39.72 (see Figure 1B). Compound 5 is inverted and did not form any key interactions.

Cell Viability Testing. Before the anti-inflammatory potential of a compound in a cellular system may be assessed, its cytotoxicity should be determined. The THP-1 cell line was used to evaluate the effect of each compound on cell viability. The chemopreventive and cytotoxic potential of the test stilbenoids obtained from *M. siamensis* for a panel of cell lines have been reported.¹⁴ Lipophilic substituents that increase the ability of a compound to penetrate the cellular membranes and interact with the internal cell environment may be responsible

for cytotoxic effects.^{34,35} For previously untested compounds, a WST-1 assay was performed, which measures cell viability on the THP-1 model of human leukemic monocytes, and IC₅₀ concentrations were determined (Table 2). Compounds 3, 4,

Table 2. Effects of Compounds 1–11 on Cell Viability, Expressed as IC₅₀ Values Calculated for THP-1 Cell Line^a

compound	IC ₅₀ [μ M]	compound	IC ₅₀ [μ M]
1	>10	7	2.7 \pm 1.1
2	>10	8	3.9 \pm 1.1
3	1.8 \pm 1.1	9	>10
4	3.3 \pm 1.0	10	>10
5	4.9 \pm 1.9	11	>10
6	12.8 \pm 2.4		

^aThe data are expressed as the means \pm SE for serial measurements in triplicate mode using at least five-point concentration curves.

5, 7, and 8 affected the viability of THP-1 cells; their IC₅₀ values were less than 10 μ M. On the other hand, compounds 1 and 2 and synthetic stilbenoids 9, 10, and 11, with IC₅₀ values greater than 10 μ M, showed relatively safer profiles. No detailed structure–activity relationships were deduced, but prenylation increased the cytotoxicity based on a comparison of these results with those obtained for nonprenylated stilbenoid analogues.²⁰ Subsequent experiments showed that at nontoxic concentration the test stilbenoids interacted well with the intracellular environment of cells and affected their metabolism. These assays formed the basis of a series of bioactivity assays to evaluate the anti-inflammatory potentials of compounds 1–11. The nontoxic concentration of 1 μ M was used for all compounds except 3, for which the concentration employed was 0.1 μ M.

Anti-inflammatory Potential in Cell-Based Models.

The ability of prenylated stilbenoids to attenuate the

lipopolysaccharide (LPS)-stimulated activation of NF- κ B/AP-1 was evaluated using THP1-XBlue-MD2-CD14 cells. The stimulation of TLR4 receptors using this cell line by LPS activates signaling cascades that lead to an increase in both NF- κ B and AP-1 transcription factors and the resulting production of the reporter protein SEAP, the activity of which may be measured with a QUANTI-Blue assay.²⁰ Most of the test compounds attenuated the activities of NF- κ B and AP-1 as much as or even more than prednisone, a widely used anti-inflammatory drug (Figure 2). Stilbenoids 6, 7, and 9 were the most active compounds, and their inhibitory effects on the activity of NF- κ B/AP-1 were statistically significant and greater ($p < 0.0001$) than that of prednisone ($p < 0.001$). Marked activity, comparable to the effect of prednisone, was demonstrated also by compound 2, newly isolated from *A. altilis*, and the synthetic compound 10. The activity of prenylated stilbenoids in mediating the activation of NF- κ B/AP-1 is reported herein for the first time.

The effects of compounds 1–11 on the secretion of pro-inflammatory cytokines IL-1 β and TNF- α , which are transcriptionally controlled by NF- κ B and AP-1, were assessed using a phorbol myristate acetate (PMA)-differentiated human macrophage model. The release of these two cytokines is characteristic of certain chronic inflammatory disorders, such as rheumatoid arthritis.³⁶ The influence of compounds 1–11 on the LPS-triggered inflammatory-like response of THP-1 macrophages was evaluated using ELISA. The test compounds were more effective against the secretion of TNF- α , where their activity correlated with the effect observed on NF- κ B/AP-1, rather than IL-1 β (Figures 3 and 4). Compounds 2 and 9 reduced the secretion of TNF- α as much as or more than the reference drug prednisone. However, neither of these compounds suppressed the release of IL-1 β . Conversely, compound 6 suppressed the secretion of IL-1 β , but had only a moderate effect on TNF- α . Compounds 5, 6, 7, and 10

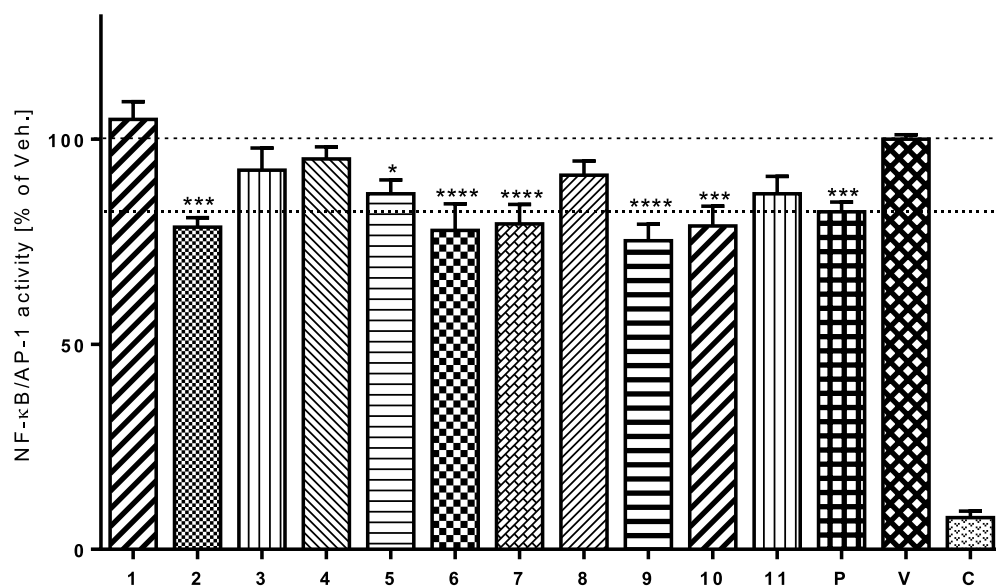


Figure 2. Effects of compounds 1–11 on NF- κ B/AP-1 activity. THP-1-XBlue-MD2-CD14 cells were pretreated for 1 h with the test compounds (1 μ M dissolved in DMSO, 0.1 μ M for compound 3), prednisone (P; 1 μ M dissolved in DMSO), or the vehicle (DMSO) only (V and C). Subsequently, LPS (1 μ g/mL) was added [except for the control cells (C)] to trigger the activation of NF- κ B/AP-1. After 24 h, the activity of NF- κ B/AP-1 was measured spectrophotometrically. The results are expressed as the means \pm SE for five independent experiments measured in triplicate (* indicates a significant difference in comparison with the vehicle-treated cells (V), $p < 0.05$, *** indicates $p < 0.001$, and **** corresponds to $p < 0.0001$).

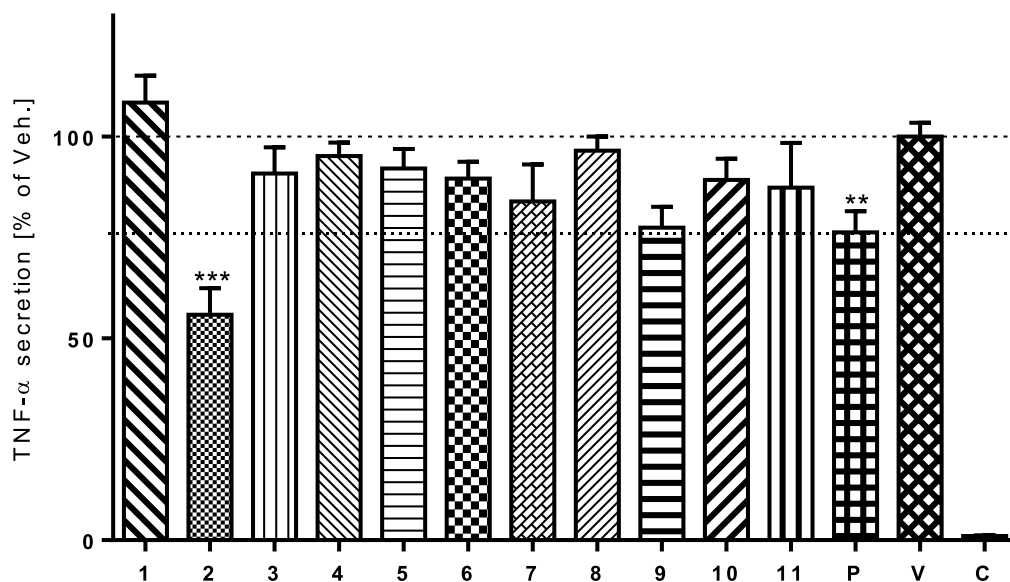


Figure 3. Effects of compounds 1–11 on the secretion of TNF- α . THP-1 macrophages were pretreated with the test compounds [$1 \mu\text{M}$ ($0.1 \mu\text{M}$ for compound 3), dissolved in DMSO], prednisone (P; $1 \mu\text{M}$, dissolved in DMSO), or the vehicle (DMSO) only (V and C) for 1 h. Subsequently, LPS ($1 \mu\text{g}/\text{mL}$) was added [except for the control cells (C)] to trigger the secretion of the pro-inflammatory cytokine TNF- α . After 24 h, the amount of TNF- α was evaluated by ELISA. The results are expressed as the means \pm SE for four independent experiments measured in triplicate (** indicates a significant difference in comparison with the vehicle-treated cells (V), $p < 0.01$, *** indicates $p < 0.001$).

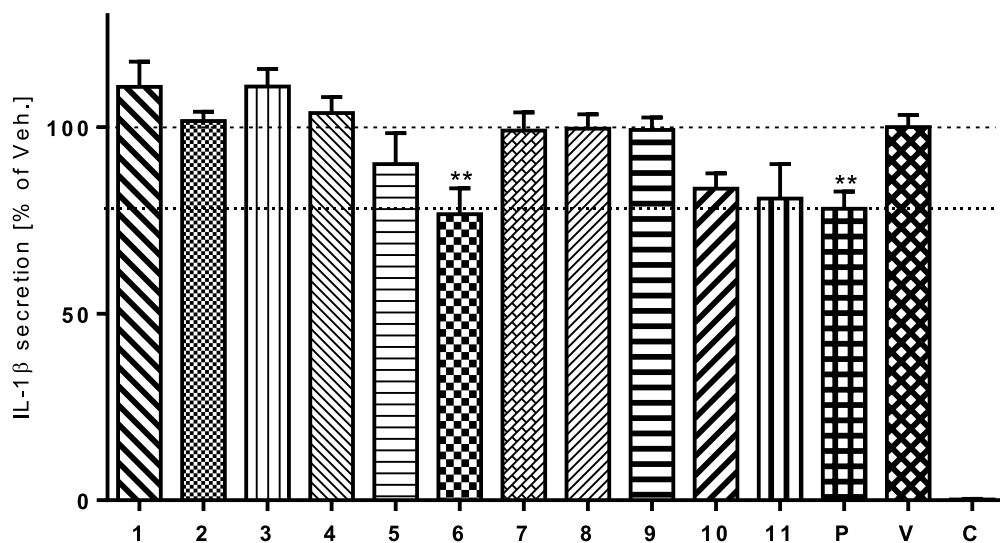


Figure 4. Effects of compounds 1–11 on the secretion of IL-1 β . THP-1 macrophages were pretreated with the test compounds [$1 \mu\text{M}$ ($0.1 \mu\text{M}$ for compound 3), dissolved in DMSO], prednisone (P; $1 \mu\text{M}$, dissolved in DMSO), or the vehicle (DMSO) only (V and C) for 1 h. Subsequently, LPS ($1 \mu\text{g}/\text{mL}$) was added [except for the control cells (C)] to trigger the secretion of the pro-inflammatory cytokine IL-1 β . After 24 h, the amount of IL-1 β was evaluated by ELISA. The results are expressed as the means \pm SE for three independent experiments measured in triplicate (** indicates a significant difference in comparison with the vehicle-treated cells (V), $p < 0.01$).

inhibited the activity of NF- κB /AP-1, but reduced the secretion of TNF- α only slightly. Synthetic compounds 10 and 11 moderately inhibited the secretion of IL-1 β . Compound 1 slightly increased the secretion of both pro-inflammatory cytokines, what was congruent with its ability to activate NF- κB /AP-1. Interestingly, compound 3 decreased the activity of NF- κB /AP-1 moderately as well as the secretion of TNF- α , but slightly increased that of IL-1 β . This effect might result from a further interaction of this compound with a post-transcription or post-translation regulatory mechanism for both. It is worth noting that because of its cytotoxic effect, compound 3 was tested at a concentration of only $0.1 \mu\text{M}$.

Hence, its observed effect on the production of TNF- α and IL-1 β could be caused by an unknown mechanism that also causes the toxicity of this compound (Table 2).

Compound 2, which attenuated the activity of NF- κB /AP-1 and markedly reduced the subsequent NF- κB -controlled secretion of TNF- α , was selected for more detailed analysis of the events that occur upstream. The activation of NF- κB is mediated by the phosphorylation of its inhibitory kappa B ($\text{I}\kappa\text{B}$) protein, which subsequently degrades.³⁷ Activated NF- κB subsequently translocates from the cytosol into the nucleus, where it binds to DNA and controls the transcription of genes implicated in inflammation, including those for TNF- α and IL-

1 β . The activation of AP-1 is up-regulated by mitogen-activated protein kinases (MAPKs). MAPK cascades, represented by the kinases p38, ERK, and JNK,³⁸ can be triggered by phosphorylation events in their activation loops during inflammation or in response to extracellular stimuli, such as LPS. Moreover, MAP kinases can participate in the regulation of NF- κ B transcriptional activity. Stilbenoid 2 showed an inhibitory effect on NF- κ B by reducing the degradation of I κ B α (Figure 5). Unlike the nonprenylated stilbenoids

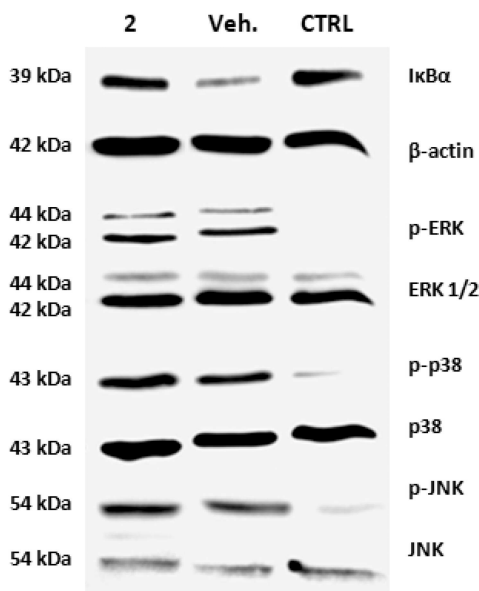


Figure 5. Effects of stilbenoid 2 on the LPS-stimulated NF- κ B and I κ B α signaling cascade using the THP1-XBlue-MD2-CD14 cell line. The cells were pretreated with the compound (1 μ M) or the vehicle (DMSO) only. After 1 h of incubation, the inflammatory response was triggered by LPS [except for the control cells (CTRL)]. The level of proteins was detected in cell lysates by Western blot and immunodetection after 30 min. Each blot represents the results of three independent experiments.

pinocastanol, pinostilbene, and thunalbene, which diminish the activity of NF- κ B/AP-1 by reducing the phosphorylation of MAPKs p38, ERK1/2, and JNK,²⁰ compound 2 did not affect the pathways of MAPKs. It is indicated that 2 specifically inhibits the NF- κ B pathway via attenuation of I κ B degradation. This ability is relatively rare among natural compounds, and it was described, for example, for the diterpenoid incensole acetate, isolated from *Boswellia* resin, a well-known anti-inflammatory natural drug.³⁹ Observed effects could be caused by inhibition of either the I κ B kinase (IKK) cascade or proteasome, which is responsible for I κ B clearance.⁴⁰ However, any other mechanisms of action could not be excluded.

Prenylated stilbenoids showed promising anti-inflammatory potential. Both natural and synthetic compounds with this structure have shown this type of activity. A synthetic method for achieving prenylation with satisfying yields would certainly lead to the discovery of many more active substances. The direct inhibition of the activity of NF- κ B/AP-1 and/or the I κ B α signaling cascade, the inhibition of the secretions of TNF- α and IL-1 β , and the inhibition of the COX and LOX enzymes, including interactions with the binding pocket of 5-LOX, could contribute to the overall effects of these compounds. The present study has shown that prenylation can favorably affect activity and increase anti-inflammatory

potential and suggest that these compounds deserve additional attention as potential multitargeting anti-inflammatory compounds. However, more studies concerning the prenylation and modification of the prenyl moiety would be done to elucidate the exact influence of such modifications on anti-inflammatory activity.

EXPERIMENTAL SECTION

General Experimental Procedures. The reagents and solvents used for isolation and synthesis were purchased from Sigma-Aldrich and used as received, if not stated otherwise. Anhydrous solvents and reagents were distilled prior to use. Melting points were measured using a Bötetius apparatus (Franz Küstner Nachf. KG) and were not corrected. IR spectra were recorded on a SmartMIRacle ATR ZnSe for a Nicolet Impact 410 FT-IR spectrometer (Thermo Scientific Corp.). 1D and 2D NMR spectra were obtained on a Bruker Avance III 400 Ultrashield spectrometer with a superconducting 400 MHz magnet system or a JEOL ECZR-400 MHz instrument (JEOL Corp.). All of the NMR measurements were done at 25 °C. Chemical shifts δ are reported in parts per million (ppm), and J values in Hz. NMR spectra were acquired in CD₃OD, CDCl₃, or DMSO- d_6 with tetramethylsilane (TMS) as an internal standard. The signal of TMS or the residual solvent signals of CD₃OD, CDCl₃, or DMSO- d_6 were used for reference. Norell StandartSeries 5 mm NMR tubes were utilized. MS analysis was performed on a Mariner ESITOF spectrometer or an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher Scientific). Mass spectra were obtained using electrospray ionization (ESI) in the positive- or negative-ion mode. A solution of each test compound was prepared in a mixture of methanol and water (50/50 v/v) with 0.2% ammonia and injected directly into the spectrometer. The isolation process, progress of the reactions, and purity of intermediates were monitored using TLC plates with normal-phase silica gel 60 F₂₅₄ (Merck). HPLC analysis was performed using a DionexUltiMate 3000 liquid chromatography pump coupled to a DionexUltiMate 3000 diode array detector. Merck Kieselgel 60 silica gel (70–230 mesh particle size) was used for column chromatography. All fractions were screened on silica gel thin-layer chromatography (TLC) plates (225 μ m thickness, 60 A silica gel medium) with the compounds detected using fluorescent light.

Plant Material. The wood and bark of *Artocarpus altilis* were collected from the Botanical Garden Teplice, Teplice, Czech Republic. The original plant in Botanical Garden Teplice was acquired as *A. altilis* from the Thai company Meyer & Mongkol in 2002. The plant was identified based on Flora Malesiana⁴¹ by Dr. Filip Zpurný (Botanical Garden Teplice), and a voucher specimen (*A. altilis*/2013) has been deposited in the herbarium of the Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno.

Extraction and Isolation. The wood (both heartwood and sapwood) and bark from *A. altilis* was air-dried until the weight was constant (2287 g) and coarsely ground. Extraction was performed by maceration in 95% ethanol (3 \times 7 L) at room temperature. The ethanolic extract was concentrated under vacuum at 40 °C using a rotary evaporator to afford a residue of 70.86 g. This residue was suspended in 80% MeOH and partitioned with petroleum ether, to produce a petroleum ether portion (26.84 g). The 80% MeOH layer was then concentrated under vacuum, redissolved in CHCl₃, and partitioned successively with H₂O and EtOAc to afford CHCl₃ (14.2 g), H₂O (26.65 g), and EtOAc (3.16 g) portions, respectively. The chloroform-soluble part was selected for subsequent column chromatography on silica gel (elution with C₆H₆–EtOAc in different proportions). All necessary personal protective equipment and principles of personal hygiene were used, as stated in the Safety Data Sheet. All work with C₆H₆ was carried out in a laboratory hood with permanent air extraction. Column chromatography was followed by TLC screening and purification using preparative HPLC on an RP-Amide column (Ascentis RP-amide 250 \times 10 mm, particle size 5 μ m, Supelco) to yield compound 2 (for a scheme of separation see the Supporting Information).

7-[(1E)-2-(4-Hydroxyphenyl)ethenyl]-2,2-dimethyl-2H-1-benzopyran-5-ol (**2**): pale yellow, amorphous powder; $^1\text{H NMR}$ (MeOD- d_4 , 400 MHz, 303 K) δ_{H} 7.37 (2H, d, $J = 8.5$ Hz, H-2', H-6'), 6.98 (1H, d, $J = 16.0$ Hz, H-7'), 6.84 (2H, d, $J = 8.5$ Hz, H-3', H-5'), 6.81 (1H, d, $J = 16.0$ Hz, H-7), 6.63 (1H, d, $J = 10.0$ Hz, H-1''), 6.60 (1H, ov, H-6), 6.44 (1H, s, H-4), 5.60 (1H, d, $J = 10.0$ Hz, H-2''), 1.44 (6H, s, CH₃); $^{13}\text{C NMR}$ (MeOD- d_4 , 101 MHz, 303 K) δ_{C} 154.9 (C-4'), 153.0 (C-3), 150.0 (C-5), 130.0 (C-1'), 128.9 (C-7', C-2''), 127.8 (C-2', C-6'), 126.0 (C-7), 125.7 (C-2), 116.1 (C-1''), 115.4 (C-3', C-5'), 108.2 (C-1), 106.7 (C-6), 105.5 (C-4), 75.9 (C-3''), 28.1 (C-4'', C-5''); HRESIMS (negative) m/z 293.1198 [$\text{M} - \text{H}$]⁻ (calcd for C₁₉H₁₈O₃, 293.1178).

Compound **1** from *A. heterophyllus* and compounds **3–8** from *M. siamensis* were isolated and identified according to published procedures.^{14,21}

Synthesis of Compounds 9–11. The synthesis of compounds **9–11** involved first synthesizing the reactants required (see *Scheme 1*). To prepare 3,5-dimethoxybenzyl alcohol, 27.7 g (0.0892 mol) of 60% Red-Al was added dropwise to a mixture of 5.00 g (0.0274 mol) of 3,5-dimethoxybenzoic acid in 100 mL of dry toluene. The reaction mixture was held at 80 °C and stirred for 1.5 h under an atmosphere of argon. The reaction was monitored by TLC (ethyl acetate–hexane, 2:3). When the starting material had disappeared, the mixture was cooled to 0 °C and 75 mL of water was added slowly. The reaction mixture was then acidified with 1 M HCl and extracted three times with toluene (3 × 100 mL). The organic layer was separated, washed twice with water, and dried with anhydrous MgSO₄. The solvent was evaporated under reduced pressure to produce 3.79 g (82%) of white crystals of 3,5-dimethoxybenzyl alcohol: $^1\text{H NMR}$ (400 MHz, DMSO- d_6) δ 6.48 (2H, d, $^4J = 2.3$ Hz), 6.34 (1H, t, $^4J = 2.3$ Hz), 5.17 (1H, t, $^3J = 6.0$ Hz), 4.43 (2H, d, $^3J = 6.0$ Hz), 3.72 (6H, s); $^{13}\text{C NMR}$ (101 MHz, DMSO- d_6) δ 160.3, 145.2, 103.9, 98.5, 62.8, 55.0.

3,5-Dimethoxybenzyl chloride²⁵ was produced by dissolving 3.79 g (0.0225 mol) of 3,5-dimethoxybenzyl alcohol in dry diethyl ether (70 mL), cooling this mixture in an ice bath, and adding dimethylformamide (DMF) (1 mL), followed by a mixture of SOCl₂ [5.36 g (0.0451 mol)] in dry diethyl ether (10 mL). The reaction mixture at 25 °C was stirred overnight under a condenser equipped with a calcium chloride closure to exclude moisture. The reaction was monitored by TLC (ethyl acetate–hexane, 2:3). When the starting material had disappeared, the mixture was cooled and water (50 mL) was added slowly. It was then extracted three times with diethyl ether (3 × 50 mL). The organic layer was washed with water (50 mL) and then brine (50 mL) and dried with anhydrous MgSO₄. The solvent was evaporated under reduced pressure, and the product was purified by column chromatography (ethyl acetate–hexane, 2:3) to yield 3.08 g (73%) of yellowish crystals of 3,5-dimethoxybenzyl chloride: $^1\text{H NMR}$ (400 MHz, CDCl₃) δ 6.55 (2H, d, $^4J = 2.3$ Hz), 6.40 (1H, t, $^4J = 2.3$ Hz), 4.53 (2H, s), 3.81 (6H, s); $^{13}\text{C NMR}$ (101 MHz, CDCl₃) δ 160.9, 139.5, 106.4, 100.4, 55.4, 46.3.

Diethyl 3,5-dimethoxybenzylphosphonate²⁵ was prepared by mixing 2.70 g (0.0145 mol) of 3,5-dimethoxybenzyl chloride, 1.00 g (0.01445 mol) of triethyl phosphite, and 0.20 g of potassium iodide and stirring for 3 h at 130 °C under a condenser equipped with a calcium chloride closure to exclude moisture. The same amounts of triethyl phosphite and potassium iodide were again added, and the mixture was again stirred for 3 h at 130 °C. The reaction was monitored by TLC (ethyl acetate–hexane, 2:3). The reaction mixture was cooled, water (10 mL) was added, and the mixture was extracted three times with ethyl acetate (3 × 10 mL). The organic layer was washed with brine (10 mL) and dried over anhydrous MgSO₄. The solvent was evaporated under reduced pressure to produce 4.10 g (98%) of yellow oil, diethyl 3,5-dimethoxybenzylphosphonate, which was used in the next step without purification: $^1\text{H NMR}$ (400 MHz, CDCl₃) δ 6.45 (2H, t, $^4J = 2.3$ Hz), 6.35 (1H, q, $^4J = 2.1$ Hz), 4.07–3.99 (4H, m), 3.77 (6H, s), 3.09 (2H, d, $^2J_{\text{PH}} = 22.0$ Hz), 1.26 (6H, t, $^3J = 7.1$ Hz); $^{13}\text{C NMR}$ (101 MHz, CDCl₃) δ 160.7, 133.5, 107.8, 99.0, 62.2, 55.2, 33.9, 16.4.

trans-3'-Bromo-3,5,4'-trimethoxystilbene²⁴ was prepared by dropwise addition of a solution of 4.63 g (0.0161 mol) of diethyl 3,5-

dimethoxybenzylphosphonate and 3.45 g (0.0161 mol) of 3-bromo-4-methoxybenzaldehyde in dry THF (20 mL) to a solution of 1.77 g (0.0161 mol) of sodium *tert*-pentoxide in dry THF (100 mL), with the reaction mixture refluxed and stirred under an inert atmosphere. The reaction was monitored by TLC (ethyl acetate–hexane, 1:2). Water was (20 mL) added, and the mixture was extracted into ethyl acetate three times (3 × 50 mL). The organic layer was washed with brine (50 mL) and dried over anhydrous MgSO₄. The solvent was evaporated under reduced pressure. Recrystallization from ethanol gave 2.87 g (51%) of *trans*-3'-bromo-3,4',5'-trimethoxystilbene as colorless crystals: $^1\text{H NMR}$ (400 MHz, CDCl₃) δ 7.74 (1H, d, $^4J = 2.0$ Hz), 7.39 (1H, dd, $^3,4J = 8.6$, 1.9 Hz), 6.95 (1H, d, $^3J = 16.2$ Hz), 6.88 (1H, d, $^3J = 16.2$ Hz), 6.87 (1H, d, $^3J = 8.6$ Hz), 6.65 (2H, d, $^4J = 2.2$ Hz), 6.40 (1H, t, $^4J = 2.2$ Hz), 3.92 (3H, s), 3.84 (6H, s); $^{13}\text{C NMR}$ (101 MHz, CDCl₃) δ 161.0, 155.4, 139.2, 131.4, 131.0, 127.9, 127.2, 126.9, 112.1, 111.9, 104.4, 99.9, 56.3, 55.3.

Prenylation Procedure.²² A 2.30 g (6.5861 mmol) aliquot of *trans* 3'-bromo-3,5,4'-trimethoxystilbene was dissolved in dry THF (100 mL) and cooled to –80 °C. Exactly 4.0 mL (9.8792 mmol) of a 2.5 M solution of *n*-butyllithium was added dropwise, and the solution was stirred for 1 h at –80 °C. The reaction mixture was then warmed to –40 °C, 0.68 g (3.2931 mmol) of CuBr·DMS at –40 °C was added all at once, and the mixture was stirred at this temperature for 1 h. The reaction mixture was again cooled to –80 °C, with 1.47 g (9.8792 mmol) of prenyl bromide added dropwise, and the resulting mixture was then stirred at this temperature for 1 h. HPLC analysis showed that the reaction yielded 7.64% *trans*-3'-(2-methylbut-3-en-2-yl)-3,4',5'-trimethoxystilbene **9**, 10.26% *cis*-3'-prenyl-3,4',5'-trimethoxystilbene **10**, 63.42% *trans*-3'-prenyl-3,5,4'-trimethoxystilbene (**11**), and 18.69% *trans*-3, 5,4'-trimethoxystilbene. A saturated solution of ammonium chloride (50 mL) was slowly added to the reaction mixture, which was then extracted three times with diethyl ether (3 × 100 mL). The organic layer was washed with 1 M HCl (50 mL) and dried over anhydrous MgSO₄. The solvent was evaporated under reduced pressure, and the crude product was purified using a short silica gel column (10% ethyl acetate in hexane) to produce 1.46 g (67%) of a mixture of products.

This mixture of products was dissolved in MeOH and separated by semipreparative HPLC using a Dionex UltiMate 3000 instrument with an Ascentis RP-amide (250 mm × 10 mm, particle size 5 μm) column (Supelco) and gradient elution with a MeOH and H₂O mixture. The flow rate was 5 mL/min, the column temperature was 40 °C, and the separation was monitored using UV detection at 254 and 350 nm. The mobile phase was removed using a rotavapor followed by lyophilization. A yellowish precipitate was obtained.

trans-3'-(2-Methylbut-3-en-2-yl)-3,5,4'-trimethoxystilbene (**9**): $^1\text{H NMR}$ (400 MHz, CDCl₃) δ 7.44 (1H, d, $^4J = 2.3$ Hz), 7.38 (1H, dd, $^3,4J = 8.2$, 2.3 Hz), 7.06 (1H, d, $^3J = 16.0$ Hz), 6.90 (1H, d, $^3J = 16.0$ Hz), 6.88 (1H, d, $^3J = 8.2$ Hz), 6.67 (2H, d, $^4J = 2.3$ Hz), 6.38 (1H, t, $^4J = 2.1$ Hz), 6.23 (1H, dd, $^3,3J = 17.4$, 11.0 Hz), 5.02–4.94 (1H, m), 3.86–3.80 (9H, m), 1.50 (6H, s); $^{13}\text{C NMR}$ (101 MHz, CDCl₃) δ 161.0, 158.2, 147.9, 139.8, 136.7, 129.3, 126.3, 125.8, 125.6, 112.1, 110.0, 109.8, 104.3, 99.6, 55.4, 55.2, 40.6, 27.2; IR ν_{max} (cm⁻¹) 2928, 1593, 1499, 1460, 1253, 1204, 1155; HRMS m/z [$\text{M} + \text{H}$]⁺ 339.1961 (calcd for C₂₂H₂₇O₃, 339.1955).

cis-3'-Prenyl-3,5,4'-trimethoxystilbene (**10**): $^1\text{H NMR}$ (400 MHz, CDCl₃) δ 7.12–7.05 (2H, m), 6.72 (1H, d, $^3J = 8.2$ Hz), 6.52 (1H, d, $^3J = 11.9$ Hz), 6.45 (2H, d, $^4J = 2.3$ Hz), 6.42 (1H, d, $^3J = 11.9$ Hz), 6.32 (1H, t, $^4J = 2.3$ Hz), 5.20–5.13 (1H, m), 3.81–3.78 (3H, m), 3.67 (6H, s), 3.21 (2H, d, $^3J = 7.3$ Hz), 1.68 (3H, s), 1.62 (3H, s); $^{13}\text{C NMR}$ (101 MHz, CDCl₃) δ 160.5, 158.5, 139.7, 132.4, 130.5, 130.1, 129.6, 129.2, 128.3, 127.7, 122.2, 109.8, 106.5, 104.3, 99.6, 55.4, 55.2, 28.2, 25.7, 17.59; IR ν_{max} (cm⁻¹) 2930, 1589, 1498, 1453, 1332, 1250, 1202, 1152, 1121, 1063, 1031, 835, 782; HRMS m/z [$\text{M} + \text{H}$]⁺ 339.1960 (calcd for C₂₂H₂₇O₃, 339.1955).

trans-3'-Prenyl-3,5,4'-trimethoxystilbene (**11**): $^1\text{H NMR}$ (400 MHz, CDCl₃) δ 7.36–7.29 (2H, m), 7.04 (1H, d, $^3J = 16.0$ Hz), 6.90 (1H, d, $^3J = 16.0$ Hz), 6.84 (1H, d, $^3J = 8.2$ Hz), 6.67 (2H, d, $^4J = 2.3$ Hz), 6.38 (1H, t, $^4J = 2.3$ Hz), 5.38–5.30 (1H, m), 3.90–3.81 (9H, m), 3.35 (2H, d, $^3J = 7.3$ Hz), 1.78 (3H, s), 1.75 (3H, s); $^{13}\text{C NMR}$

(101 MHz, CDCl₃) δ 160.9, 157.2, 139.8, 132.6, 130.3, 129.5, 129.1, 127.6, 126.2, 125.4, 122.3, 110.3, 104.2, 99.5, 55.4, 55.3, 28.5, 25.8, 17.8; IR ν_{\max} (cm⁻¹) 2931, 1593, 1500, 1459, 1253, 1204, 1154, 1066, 1032, 860, 827; HRMS m/z [M + H]⁺ 339.1955 (calcd for C₂₂H₂₇O₃, 339.1955).

Cell Culture and Differentiation to Macrophages. The THP-1 human monocytic leukemia cell line and THP-1-XBlue-MD2-CD14 cells were purchased from the ECACC (Salisbury, UK) and Invivogen (San Diego, CA, USA), respectively. Both cell lines were cultured in RPMI 1640 medium (Biosera, France) supplemented with antibiotics [100 U/mL penicillin and 100 mg/mL streptomycin (Biosera)] and 10% fetal bovine serum (HyClone, Logan, UT, USA) at 37 °C. To differentiate the THP-1 monocytes to macrophages, cells were split into 96-well microtiter plates at a concentration of 5×10^5 cells/well and stimulated by phorbol myristate acetate, as described previously^{35,42} with some modifications.

Cell Viability Testing. Undifferentiated floating THP-1 cells were transferred into serum-free medium at a density of 5×10^5 cells/well in 96-well plates. After 2 h, the test compounds dissolved in DMSO were added at increasing concentrations (0.125–20 μ M). Measurements were taken after a 24 h incubation with each of the test compounds. Cell viability was measured using the Cell Proliferation Reagent kit WST-1 (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. The resultant viability curves were used to calculate the IC₅₀ values using four parameters logistic (4PL) analysis.

Evaluation of Cytokine Secretion. Differentiated THP-1 macrophages were pretreated for 1 h with test compounds **1**, **2**, and **4–11** at a nontoxic concentration of 1 μ M (0.1 μ M for compound **3**) or 1 μ M prednisone dissolved in DMSO or DMSO alone (the vehicle). An inflammatory-like response was triggered by adding 1 μ g/mL of LPS isolated from *Escherichia coli* 0111:B4 (Sigma-Aldrich). The control cells were cultured without LPS treatment. After 24 h, the medium was collected and the concentration of TNF- α was measured using a human TNF α ELISA kit (Diacclone, Besançon, France), according to the manufacturer's instructions. Experiments were performed in triplicates and repeated at least three times.

Determination of the NF- κ B/AP-1 Activity. The activity of NF- κ B/AP-1 was evaluated using the THP-1-XBlue-MD2-CD14 cell line as described previously,⁴³ with slight modifications. Briefly, cells (5×10^5 /well in 96-well plates) were pretreated with test compounds or prednisone at a concentration of 1 μ M (0.1 μ M for compound **3**). After a 1 h incubation, cells were stimulated by LPS (1 μ g/mL). After 24 h of incubation, the activity of NF- κ B/AP-1 was determined using Quanti-Blue reagent (Invivogen, San Diego, CA, USA) according to the standard protocol, and measurement of OD at 655 nm was performed in a Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany) and compared with a vehicle (100%).

Signaling Pathway Analysis. The level of I κ B and mitogen-activated protein (MAP) kinases p38, ERK1/2, and JNK was determined on the THP-1-XBlue-MD2-CD14 cell line, in a similar way to that described previously.²⁰ Briefly, cells were pretreated with test compound **2** at a concentration of 1 μ M and stimulated with 1 μ g/mL LPS 1 h later. After 30 min, the cells were collected and cell lysates were prepared. The equal amounts of proteins (9 μ g) were separated in 12% PAGE gel, transferred to PVDF membranes, blocked in 5% bovine serum albumin, and incubated with primary mouse anti-I κ B- α antibody and primary rabbit anti-phospho-SAPK/JNK, anti-p44/42 MAPK (ERK1/2), anti-phospho-p44/42 MAPK (ERK1/2), anti-p38 MAPK, and anti-phospho-p38 MAPK antibodies (Cell Signaling, Danvers, MA, USA; product nos. 4814, 4668, 4695, 4370, 9212, and 4511, respectively) at dilutions of 1:500 (anti-I κ B- α), 1:2000 (anti-phospho-p44/42 MAPK), or 1:1000 (the other antibodies). Two other primary mouse antibodies were used, anti- β -actin (Abcam, Cambridge, UK; product No. 8226) at a dilution of 1:5000 and anti-SAPK/JNK (Sigma-Aldrich, product no. SAB4200176) at a dilution of 1:1000. Appropriate secondary IgG antibodies (1:2000; Sigma-Aldrich, products Nos. A0168 and A0545) were applied to the membranes, followed by ECL reagent (Bio-Rad),

and the chemiluminescence was measured using a Syngene PXi4 imaging system (Cambridge, UK).

COX-1 and COX-2 Assays. The inhibition of COX enzymes was determined in the same manner as described previously.²⁰ Samples were dissolved in DMSO, pure DMSO was used as blank, and ibuprofen was used as positive control. COX-1 from ram seminal vesicles and human recombinant COX-2 (Sigma-Aldrich) were used, and the concentration of PGE₂ created in catalytic reaction from arachidonic acid was quantified with the aid of a prostaglandin E₂ ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA), followed by a measurement of OD at 405 nm using a Tecan Infinite M200 microplate reader (Tecan Group, Männedorf, Switzerland). The results were expressed as a percentage of inhibition compared to a blank.

5-LOX Assay. The inhibition of human recombinant 5-LOX enzyme (Cayman Chemicals, Ann Arbor, MI, USA) was assessed as the potential to reduce the synthesis of leukotriene B₄ (LTB₄) created in catalytic reaction from arachidonic acid. As described previously,²⁰ samples were dissolved in DMSO, pure DMSO was used as blank, and zileuton (Farmak a.s., Olomouc, Czech Republic) was used as positive control. LTB₄ was quantified using a leukotriene B₄ ELISA kit (Enzo Life Sciences); measurements and analyses were the same as for the COX assays.

Molecular Docking. The docking simulations on 5-LOX were carried out using the program Gold Suite 5.2 (CCDC, www.ccdc.ac.uk, 2015). Docking poses and protein–ligand interactions were visualized in Ligandscout 4.1 (Inte:Ligand, <http://www.inteligand.com/>, 2017). The docking simulation for 5-LOX was carried out on a structure based on pdb entry 3O8Y,⁴⁴ which was mutated virtually to wild-type 5-LOX and energetically minimized in Discovery Studio 4.0 (Biovia, San Diego, CA, USA, 2015). The binding site was defined within an 8 Å radius around CG2 of ILE167. ChemScore Kinase was selected as the configuration template, and CHEMPLP as the scoring function.

Statistical Evaluation. Statistical analyses were carried out using GraphPad Prism 6.01 software (San Diego, CA, USA). The data were graphed as the means \pm SEM. Comparison between groups was made using a one-way ANOVA test, followed by a Bonferroni's multiple comparisons post hoc test.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.9b00081.

Figures illustrating the NMR spectra (Figures S1–S14) (PDF)

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Notes

The authors declare no competing financial interest.

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Therapeutic potential of prenylated stilbenoid macasiamenene F through its anti-inflammatory and cytoprotective effects on LPS-challenged monocytes and microglia

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ABSTRACT

Ethnopharmacological relevance: *Macaranga* Thou. (Euphorbiaceae) is a large genus that comprises over 300 species distributed between Western Africa and the islands of the South Pacific. Plants of this genus have a long-standing history of use in traditional medicine for different purposes, including the treatment of inflammation. Fresh and dried leaves of certain *Macaranga* species (e.g. *M. tanarius* (L.) Müll.Arg.), have been used to treat cuts, bruises, boils, swellings, sores and covering of wounds in general. Several reports described *Macaranga* spp. being a rich source of polyphenols, such as prenylated stilbenoids and flavonoids, mostly responsible for its biological activity. Similarly, an abundant content of prenylated stilbenes was also described in *M. siamensis* S.J.Davies, species recently identified (2001) in Thailand. While the respective biological activity of the prenylated stilbenes from *M. siamensis* was poorly investigated to date, our recent study pointed out the interest as the natural source of several novel anti-inflammatory stilbenoids isolated from this species.

Aim of the study: This work investigated the potential anti-inflammatory effects of the stilbenoid macasiamenene F (MF) isolated from *M. siamensis* S.J.Davies (Euphorbiaceae) on the lipopolysaccharide (LPS)-induced inflammation-like response of monocytes and microglia, major cells involved in the peripheral and central inflammatory response, respectively.

Materials and methods: LPS-induced stimulation of TLR4 signaling led to the activation of inflammatory pathways in *in vitro* models of THP-1 and THP-1-XBlue™-MD2-CD14 human monocytes, BV-2 mouse microglia, and an *ex vivo* model of brain-sorted mouse microglia. The ability of the stilbenoid MF to intervene in the IκB/NF-κB and MAPKs/AP-1 inflammatory cascade was investigated. The gene and protein expressions of the pro-inflammatory cytokines IL-1β and TNF-α were evaluated at the transcription and translation levels. The protective effect of MF against LPS-triggered microglial loss was assessed by cell counting and the LDH assay.

Results: MF demonstrated beneficial effects, reducing both monocyte and microglial inflammation as assessed *in vitro*. It efficiently inhibited the degradation of IκBα, thereby reducing the NF-κB activity and TNF-α expression in human monocytes. Furthermore, the LPS-induced expression of IL-1β and TNF-α in microglia was dampened by pre-, co-, or post-treatment with MF. In addition to its anti-inflammatory effect, MF demonstrated a cytoprotective effect against the LPS-induced death of BV-2 microglia.

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Conclusion: Our research into anti-inflammatory and protective effects of MF has shown that it is a promising candidate for further *in vitro* and *in vivo* investigations of MF interventions with respect to acute and chronic

Abbreviations

A β	amyloid beta
AD	Alzheimer's disease
AP-1	activator protein 1
BBB	blood-brain barrier
COXs	cyclooxygenases
DAMPs	damage-associated molecular pattern molecules
HD	Huntington's disease
I κ B	inhibitor of NF-kappa B transcription factor
IL-1 β	interleukine 1 beta
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAPKs	mitogen-activated protein kinases
NF- κ B	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NSAIDs	non-steroidal anti-inflammatory drugs
PAMPs	pathogen-associated molecular patterns
PD	Parkinson's disease
RA	rheumatoid arthritis
SEAP	secreted embryonic alkaline phosphatase
TLR4	toll-like receptor 4
TNF- α	tumor necrosis factor alpha

inflammation, including potentially beneficial effects on the inflammatory component of brain diseases such as stroke and Alzheimer's disease.

1. Introduction

Inflammation represents an adaptive reaction of body tissues to invading pathogens, injuries, or endogenous signals from stressed, damaged, or dead cells. It is a physiological process that is beneficial, well-coordinated, and self-regulated. However, when the response to inflammatory stimuli becomes uncontrolled and continuous, chronic inflammatory conditions may develop and lead to severe pathologies (Medzhitov, 2008). Non-resolving inflammation contributes to the pathogenesis of both peripheral (neuropathic pain) and central nervous system diseases, such as stroke, traumatic brain injury, multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), or depression (Degan et al., 2018; Miller et al., 2009; Skaper et al., 2018). Moreover, a prolonged exposure to pathogens or an ongoing systemic inflammation of the kind observed in metabolic diseases such as diabetes or obesity can lead to a chronic state of neuroinflammation and ultimately result in neuronal cell death (Nicolas et al., 2017; Skaper et al., 2018).

Unrecovered and repeated acute inflammation, as well as long-term moderate exposure to noxious stimuli at the central or systemic level may result in a chronic state of disease. Nowadays, chronic low-grade inflammation is often related to diseases of modern civilization, such as metabolic syndrome, which is often characterized as a cluster of conditions such as hypertension, hyperglycemia, insulin resistance, obesity, and high levels of serum triglycerides, that increase the risk of diabetes mellitus type 2, cerebrovascular disease, and stroke (Lopez-Candales et al., 2017; Minihane et al., 2015). Some inflammatory autoimmune disorders, such as rheumatoid arthritis (RA), are also based on chronic inflammation (Smolen et al., 2018). Systemic and CNS immune system crosstalk during inflammatory response is a critical

consideration not to be overlooked (Minihane et al., 2015). Systemic macrophages and brain microglia together with CNS-associated macrophages play a crucial role in surveillance of the internal environment and control of homeostasis (Ginhoux and Jung, 2014; Yin et al., 2017). Through toll-like receptors (TLRs), they transmit a signal during bacterial infection, injury, or brain damage, including that triggered by hypoxia/ischemia (Lehnardt et al., 2003).

Acute inflammation is often associated with a microbial infection that exemplifies itself in a form such as meningitis (Hoffman and Weber, 2009), bronchitis (Wark, 2008), pneumonia (Percopo et al., 2019), myocarditis (Blauwet and Cooper, 2010), or cystitis (Flores-Mireles et al., 2015). Moreover, Gram-negative bacteria such as *Klebsiella pneumoniae*, *Escherichia coli*, or *Pseudomonas* sp., possess in their outer membrane the endotoxin lipopolysaccharide (LPS), responsible for their pathogenic potential and massive clinical manifestations in humans (Sperandio et al., 2013). High peripheral concentrations of LPS can disrupt the blood-brain barrier (BBB) and promote neuroinflammation (Banks et al., 2015). LPS triggers an innate immune system response through TLR4 receptors linked with several inflammatory signaling pathways leading to the production of cytokines and the onset of inflammation (Pålsson-McDermott and O'Neill, 2004). We therefore used LPS from *E. coli* to induce TLR4-mediated inflammatory cascades in our experimental set up.

Stilbenoids are natural phenolic compounds found in foods (grapes, peanuts, berries, passion fruit, rhubarb), beverages (wine, white tea), and medicinal plants, including *Morus* spp., *Artocarpus* spp., and the less well known *Macaranga* spp. (Dvorakova and Landa, 2017; Hošek et al., 2019). Structurally, they share a common 1,2-diphenylethylene core and differ in its substitution, which may include prenyl group. Stilbenoids serve the plant as phytoalexins, protecting it against infection or harm. The strong anti-oxidant and anti-inflammatory properties of

stilbenoids have multiple health benefits. Cardioprotective, neuroprotective, anti-diabetic and cancer preventive properties have been reported (Akinwumi et al., 2018; Dvorakova and Landa, 2017; Eräsalo et al., 2018; Hornedo-Ortega et al., 2018). The neuroprotective potential of stilbenoids has been tested mostly on neurodegenerative disorders, such as AD. For instance, verapenol, *cis*-scirpusin A and oxyresveratrol, stilbenes obtained from *Smilax* spp., showed significant inhibitory effects *in vitro* against β -secretase, the enzyme crucially responsible for the amyloid beta (A β) formation in AD (Jeon et al., 2007). Other than the most widely known and structurally simple stilbene *trans*-resveratrol, and its hydroxyderivatives oxyresveratrol and piceatannol (found, e.g. in grapes), whose antioxidant properties and therapeutic potential in cardiovascular diseases arouse great interest, have been studied very little to date. Little or no information is available about the biological activities of less-known stilbenoids, including those of the genus *Macaranga* (Hošek et al., 2019).

The genus of *Macaranga* comprises around 300 species and is native to Africa, Australia, Asia, and various islands of Indian and Pacific Oceans. Some species, such as *M. tanarius* (L.) Müll.Arg., in Thailand known as “Mek”, have been used in traditional Thai medicine as anti-inflammatory treatment. Whereas the fresh leaves have been used to cover wounds, a decoction of the dried root has been administered against cough and fever (Kamarozaman et al., 2018; Phommart et al., 2005; Phupattanonpong and Wongprasert, 1987). *M. sampsonii* Hance has been used for the treatment of swellings, cuts and sores (Quynh et al., 2018). The European expeditioners surveying the traditional medicinal plants of Papua New Guinea islands recorded the use of *M. aleuritoides* F. Muell. fruits and seeds by aborigine people to relieve abdominal pain. Various species of *Macaranga* were used for the treatment of diarrhea, constipation and stomach complaints in several islands, such as Tonga, Fiji, Java, Philippines, Bougainville Island and Malaysia (Holdsworth et al., 1983; Nick et al., 1995). *M. harveyana* (Müll.Arg.) Müll.Arg. in Tongan language known as “Loupata” was used in Tonga folk medicine for treatment of obstetric and gynecological disorders, such as post-partum hemorrhage and secondary amenorrhea (Singh et al., 1984).

Several studies trying to describe the phytochemical profile of *Macaranga* species identified this genus as a rich source of polyphenols such as prenylated and geranylated stilbenoids and flavonoids, terpenes, tannins, steroids, and coumarins. Most of the described biological activities are attributed to stilbenes and flavonoids found in high amounts (Magadula, 2014; Ngoumfo et al., 2008).

M. siamensis S.J.Davies is a large-leaved plant, common in central and northern Thailand, firstly identified in 2001. The first phytochemical investigation provided by Pailee et al. (2015) demonstrated the high content of novel stilbenes, including macasiamenenes A, B, F, K, L, and P that have been isolated from dichloromethane extract of leaves and twigs. But, little information about their biological effects has been reported. Based on our previous study suggesting the anti-inflammatory properties of several prenylated stilbenoids contained in *M. siamensis* (Hošek et al., 2019), by the present work, we undertook to compare the anti-inflammatory effects of seven compounds obtained from *M. siamensis* to identify the therapeutic potential value of the most promising candidate, macasiamenene F (MF), for treatment of both systemic and central inflammation. The characterization of MF led us to describe for the first time its anti-inflammatory effects on microglial inflammation.

2. Materials and methods

2.1. Animals

Seven week-old male C57BL/6JRj mice (Janvier, France) were housed at 22 °C with a 12-h light-dark cycle and free access to water and food. All animal care and use were performed in accordance with the policies of European Community Directive 86/609/EEC. The *ex vivo*

experiments were approved by the local ethical committee. Every effort was made to minimize the number of animals used and their suffering.

2.2. Isolation of compounds and treatments

Pailee et al. (2015) isolated the macasiamenene F (MF) along with L (1), K (2), P (3), A (5), B (6) and 2,6-diprenyl-resveratrol (4) from leaves and twigs of *M. siamensis* S.J.Davies (Euphorbiaceae) by chromatographic methods and identified them by spectroscopic methods, including NMR analysis. The voucher specimen (No. CRI 466) was deposited in the Laboratory of Natural Products, Chulabhorn Research Institute, Thailand (Pailee et al., 2015). The plant name has been checked with <http://www.theplantlist.org>. Compound 4, 2,6-diprenyl-3,5,4'-trihydroxystilbene (4) was already known (Verotta et al., 2009), but compounds MF, 1–3, 5, and 6 were isolated for the first time. The purity of compounds was confirmed to be more than 98% using HPLC (Fig. S1). Dimethyl sulfoxide (DMSO) was used as a vehicle; its concentration in cell culture never exceeded 0.1% (v/v). Apart from the dose-response studies, treatments were performed using appropriate serum-free medium at a concentration of 1 μ mol/L (0.1 μ mol/L for compound 1), which would not affect cell viability. At these concentrations, the viability measured by WST-1 was 100% compared to non-treated cells (Fig. 1B). LPS from *E. coli* O111:B4 (Sigma-Aldrich) freshly dissolved in PBS was used at a concentration of 1 μ g/mL (Taka et al., 2015; Triantafilou et al., 2001).

2.3. Cell culture and differentiation

The THP-1 human monocytic leukemia cell line was purchased from the European Collection of Authenticated Cell Cultures (Salisbury, UK). The BV-2 murine microglial cells were generated from primary microglial culture by infection with *v-raf/v-myc* oncogene carrying J2 retrovirus (Blasi et al., 1990). The THP-1 and THP-1-XBlue™-MD2-CD14 (Invivogen, San Diego, CA, USA) cells were cultured in RPMI 1640 medium containing stabilized 2 mmol/L L-glutamine (Biosera, France), whereas the BV-2 cells were cultured in DMEM (4.5 g/L glucose, 4 mmol/L L-alanyl-glutamine, and w/o sodium pyruvate (Sigma). All of the cultures were supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotics [100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco)]. Cells were grown in a water-saturated atmosphere of 5% CO₂ at a temperature 37 °C. The experiments were performed on passages ranging from 3 to 16 for all types of cell lines. The THP-1 monocytes were differentiated into macrophages by adding 50 ng/mL of phorbolmyristateacetate (PMA) (Leláková et al., 2019). All subsequent experiments with all cell lines were performed after 2 h of incubation in serum-free medium.

2.4. Sorting of brain immune cells

Immune cells were isolated from the brains of adult mice using an Adult Brain Dissociation Kit (Miltenyi Biotec, USA) according to the manufacturer's instructions. Brain homogenates were filtered using 70 μ m cell strainers (BD Biosciences) and centrifuged (10 min, 2000 rpm). The resulting cell pellets were then re-suspended and washed with PBS at pH 7.2 containing 0.5% BSA and 2.5 mM EDTA. They were then labeled with CD11b⁺ MicroBeads (Miltenyi Biotec, USA) and incubated for 15 min at 4 °C. CD11b⁺ mouse microglia and CNS-associated macrophages were isolated using LS columns (Miltenyi Biotec, USA) in a magnetic field according to the manufacturer's instructions.

2.5. Ex vivo culture of microglia

Brain-sorted microglia were seeded into 96-well tissue culture plate in DMEM (Gibco) containing 10% fetal bovine serum (FBS, Gibco) and antibiotics [100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco)] at a density of 7×10^4 cells/well. Microglial cells were cultured on poly-

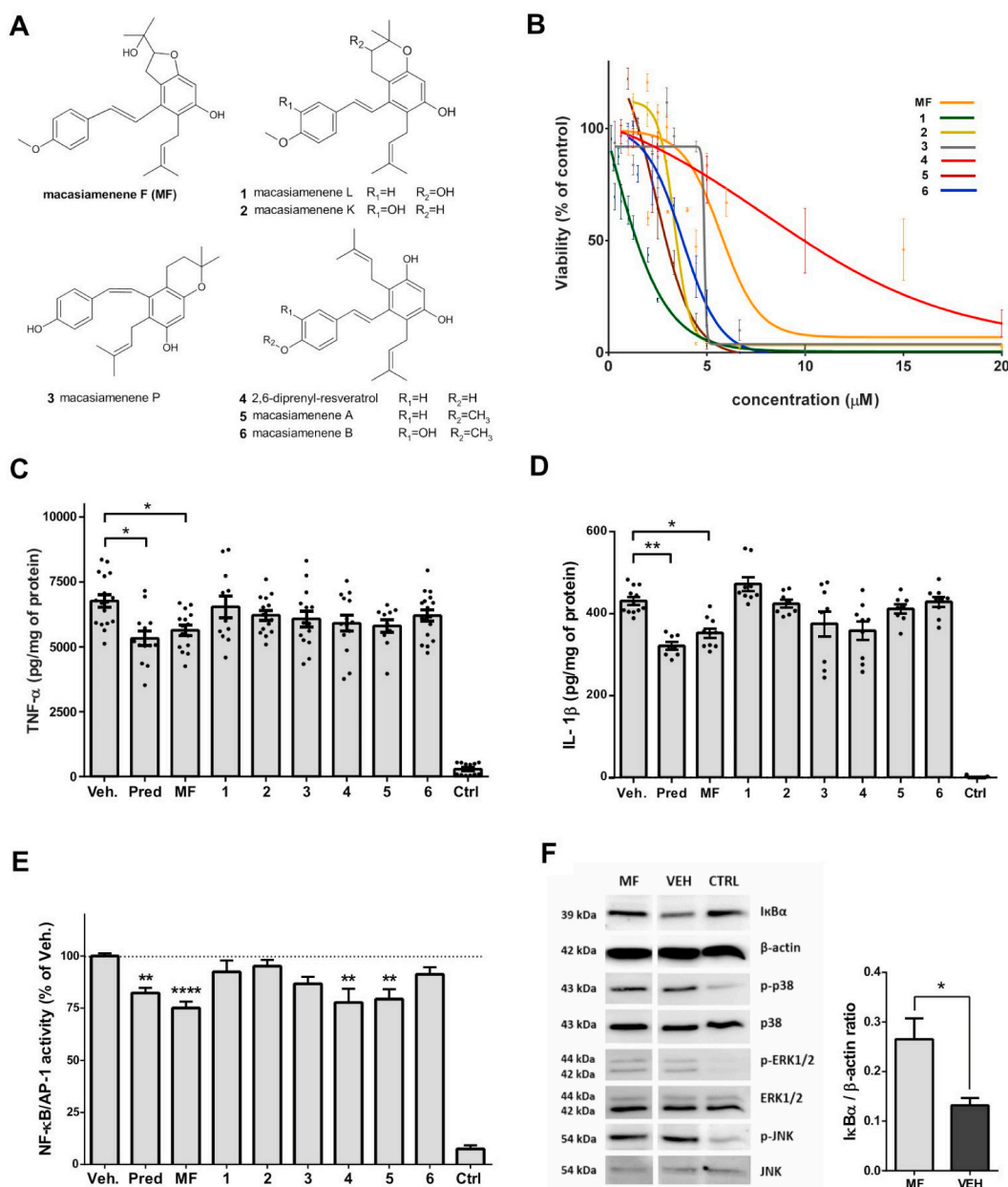


Fig. 1. Effects of prenylated *M. siamensis* stilbenoids on NF-κB signaling of LPS-stimulated THP-1 macrophages and THP-1 XBlue™-MD2-CD14 monocytes. **A**, Test stilbenoids MF, 1–6; **B**, Dose-response curves showing viability of THP-1 after a 24 h incubation with compounds MF, 1–6; **C**, Effect on release of TNF-α protein; **D**, Effect on release of IL-1β protein; **E**, Activity of NF-κB/AP-1 after 24 h; **F**, MF upstream regulation of IκBα. Data are presented as the mean ± SEM, n = 3, *indicates a significant difference of **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 versus only with the vehicle treated cells (Veh.) analyzed by the non-parametric tests Kruskal-Wallis in the case of C-E, and the Mann and Whitney for F.

D-lysine-covered plates at 37 °C in an atmosphere containing 5% CO₂ for 18 h. Thirty minutes after cell plating, MF at a concentration of 1 μmol/L, which has been determined by our group to be non-toxic for both monocytes and microglia, was added together with LPS (1 μg/mL). The supernatants and cell contents were collected and used subsequently to measure the levels of cytokines after 18 h.

2.6. Cell viability: dose-response relationships

Undifferentiated floating THP-1 cells in serum-free RPMI 1640 medium were seeded into 96-well plates (5 × 10⁴ cells/well). After 2 h, compounds MF, and 1–6 were added at concentrations ranging from 0.125 to 20 μmol/L. The cell viability was assessed 24 h later using Cell Proliferation Reagent WST-1 kit (Roche Diagnostics, Basel,

Switzerland). Based on the resulting viability curves, the IC₅₀ values were calculated according to four parameters logistic (4PL) analysis. In the case of adherent BV-2 cells, the dose-response assays were carried out in exponential phase of growth established by a pilot study in 24-well plates. Briefly, BV-2 cells were seeded in the evening in complete DMEM medium at a density of 1.5 × 10⁵ cells/well, the next day washed by PBS, and the medium was replaced by serum-free medium. Cells were treated after 2 h with MF at concentrations ranging from 1 to 15 μmol/L. The mitochondrial activity and cell proliferation were evaluated after 24 h of incubation using a Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (MTS; Promega, France) and manual counting of the living cells excluded from Trypan Blue (Sigma) staining, respectively. Cell mortality was assessed after 24 h using a Cytotoxicity Detection Kit (LDH; Roche) and represented as the percentage of lactate

dehydrogenase (LDH) released by damaged cells compared to cells treated with only the vehicle.

2.7. Determination of NF- κ B/AP-1 activity

The activity of nuclear factor κ B (NF- κ B) and activator protein-1 (AP-1) was measured on THP-1-XBlue™-MD2-CD14 cells (Invivogen, CA, USA) derived from THP-1 human monocytes. This cell line stably expresses the NF- κ B and AP-1 inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene, the activity of which was detected using QUANTI-Blue™ reagent (Invivogen, San Diego, USA). Cells in serum-free RPMI 1640 medium were seeded (5×10^4 cells/well, 96-well plate) and incubated for 2 h at 37 °C. Compounds MF, 2–6, and prednisone (P) used as a positive control, were added at the non-toxic concentration of 1 μ mol/L (0.1 μ mol/L was used for compound 1), together with vehicle DMSO (0.1% (v/v)). After 1 h of incubation, the cells were treated with LPS (1 μ g/mL) to activate NF- κ B/AP-1 and produce SEAP. After 24 h, 20 μ L of the supernatant was withdrawn, mixed with 175 μ L of QUANTI-Blue™ reagent and incubated for 30 min at 37 °C. Spectrophotometric measurements at 655 nm were carried out in a Fluostar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany).

2.8. Molecular docking

All of the simulations of ligand-protein docking on NF- κ B were performed with AutoDockVina (Trott and Olson, 2010) using a PyRx virtual screening tool. Molecular dynamics were performed with a CUDA version of NAMD (Phillips et al., 2005) and VMD was used to prepare the molecular visualization and protein preparation. Docking simulations were performed using system with Core I7 and NVIDIA GTX 850 in the same manner we described previously (Leláková et al., 2019). The crystallographic coordinates of NF- κ B bound to DNA were obtained from the RCSB Protein Data Bank with ID 3GUT. Only chain A (p65) and B (p50) were used for simulations. All tested compounds meet the conditions of Lipinski rule of five, suggesting that tested compounds can penetrate cell membranes, pass into cell compartments, and have several chemical groups forming hydrogen bonds. All compounds formed complexes with target protein structure with high binding affinity (Δ G) around -7.0 kcal/mol.

2.9. Western blot analysis of TLR4-mediated signaling pathways

THP-1-XBlue™-MD2-CD14 cells in 6-well plates at a density of 3×10^6 cells/well were treated with MF or the vehicle (Veh). After 1 h, LPS (1 μ g/mL) was added to the cells. Cells not stimulated with LPS represented the control (Ctrl). After 30 min, the cells were collected, washed in PBS, and homogenized in cold lysis buffer (50 mmol/L Tris-HCl, pH 7.5; 1 mmol/L EDTA; 1 mmol/L EGTA; 1 mmol/L sodium orthovanadate; 5 mmol/L sodium pyrophosphate; 50 mmol/L sodium fluoride; 270 mmol/L sucrose) with protease inhibitors (Roche Diagnostics, Basel, Switzerland). Equal amounts of proteins (9 μ g) were separated in 12% polyacrylamide gel and transferred to a PVDF (polyvinylidene fluoride) membrane. This membrane was then incubated overnight at 4 °C with the following monoclonal primary antibodies: anti-I κ B α (1:500; Cell Signaling, USA, product No. 4814), anti- β -actin (1:5000; Abcam, Cambridge, UK, No. 8226), anti-SAPK/JNK (1:1000; Sigma-Aldrich, USA, SAB4200176), antiphospho-p44/42 MAPK (p-ERK1/2, 1:2000, No. 4370), anti-p44/42 MAPK (ERK1/2, No. 4695), anti-phospho-p38 MAPK, and anti-phospho-SAPK/JNK (1:1000; Cell Signaling, MA, USA, No. 4511 and 4668) and polyclonal antibody: anti-p38 MAPK (1:1000; Cell Signaling, MA, USA, 9212). Each membrane was then probed with the appropriate anti-mouse or anti-rabbit IgG secondary peroxidase-conjugated antibodies (1:2000, from Sigma-Aldrich, USA, No. A0168 and A0545). The ECL signal (Bio-Rad, USA) was detected using a Syngene PXi4 chemiluminescence imaging system (Cambridge, UK). Densitometric analysis was performed using AlphaEaseFC 4.0.0

software (Alpha Innotech, USA). The relative effects of MF and vehicle (Veh) on I κ B α were compared after normalization to β -actin.

2.10. Isolation of RNA and qRT-PCR

Total RNA was extracted using TRI Reagent® (Sigma), followed by treatment with TURBO DNase (Invitrogen). First-strand cDNAs were synthesized from 0.5 μ g of total RNA using 200U/ μ L Superscript™ IV Reverse transcriptase (Invitrogen) in reaction buffer in the presence of 2.5 μ mol/L oligo d(T)₂₀ primers (Eurogentec, France), 0.5 mmol/L deoxyribonucleotide triphosphate (dNTP) mix, and 5 mmol/L dithiothreitol (DTT). The reaction was incubated for 5 min at 65 °C, then for 10 min at 50 °C, and it was finally inactivated by raising the temperature to 80 °C for 10 min. Quantitative RT-PCR was performed using a LightCycler® 480 SYBR Green I Master with a LightCycler® 480 sequence detector (Roche Diagnostics). RPLO and GAPDH were used as reference genes for normalization. The following sequences of primers were used: mL1- β (forward 5'-TGGTGTGTGACGTTCCCAT-3', reverse 5'-CAGCACGAGGCTTTTGTG-3'), mTNF- α (forward 5'-CATCTTCTCAAATTCGAGTGACAA-3', reverse 5'-TGGGAGTAGACAAGGTACAACCC-3'), mRPLO (forward 5'-ACTGGTCTAGACCCGAGAAG-3', reverse 5'-TCCCACCTGTCTCCAGTCT-3'), and mGAPDH (forward 5'-CCAGTGAGCTTCCCGTTCA-3', reverse GAACATCATCCCTGCATCC-3') (Eurogentec, France).

2.11. Immunodetection of inflammatory cytokines

Differentiated THP-1 macrophages were pretreated for 1 h with the test compounds or prednisone 1 μ mol/L (in the case of compound 1 0.1 μ mol/L) dispersed in 0.1% DMSO or with a 0.1% DMSO solution alone (the vehicle). The release of TNF- α and IL-1 β was evaluated by testing the supernatant after 24 h, using Human TNF- α and IL-1 β ELISA kits (Diacclone, France). For BV-2 cells, the expression of TNF- α and IL-1 β was determined by testing cell contents and supernatants, using AlphaLISA mL1 β (AL503C) and mTNF α (AL505C) kits (PerkinElmer, MA, USA). The Amicon® Ultra 0.5mL filters (10 K) for protein concentration (Merck Millipore, France) were used to concentrate supernatants before detection of IL-1 β released from BV-2 cells. The total quantities of cytokines produced were calculated and normalized to the amounts of total proteins determined by Bradford protein assay (Biorad, France).

2.12. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6.00 software. Data were expressed as the mean \pm SEM. Statistical analyses of differences between groups were performed using the parametric ($n > 30$) Student's t-test (for comparisons of 2 groups) and non-parametric ($n < 30$) Mann-Whitney (for 2 groups), and Kruskal-Wallis analysis, together with Dunn's post-hoc test (for more than 2 groups). Values of p less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of Macaranga stilbenoids on the viability of THP-1 cells

The prenylated stilbenoid MF has been found in the plant *M. siamensis* S.J.Davies, isolated by chromatographic methods, and identified by NMR analysis and other spectral methods (Pailee et al., 2015). We are the first to evaluate its effects on the viability of THP-1 human monocytes. Fig. 1B shows that MF displays one of the safest profiles. Its IC₅₀ was calculated at 5.7 ± 1.1 μ mol/L. The IC₅₀ values of compounds 1–6 were 1.8 ± 1.1 , 3.3 ± 1.0 , 4.9 ± 1.9 , 12.8 ± 2.4 , 2.7 ± 1.1 , and 3.9 ± 1.1 μ mol/L, respectively (Hosek et al., 2019).

According to previous studies on humans evaluating the impact of oral supplementation of stilbenoids on their circulating concentration in plasma, targeting the concentration of 1 μ mol/L that is accepted for the

most studied stilbene resveratrol as lowest dose with a significant anti-inflammatory effect on peripheral cells (Chen et al., 2018) is a pharmacologically and clinically relevant (Boocock et al., 2007; Smoliga et al., 2011). Therefore to identify the most interesting anti-inflammatory candidate at this dose, we performed a non-exhaustive screening of the anti-inflammatory activities on stimulated macrophages of the seven compounds isolated from the plant *M. siamensis*.

3.2. MF reduces the secretion of inflammatory cytokines by acting on the $I\kappa B\alpha$ /NF- κB pathway

We first evaluated and compared to prednisone, a corticosteroid used in the management of inflammatory conditions or diseases, the effect of the seven compounds isolated from the plant *M. siamensis* at the concentration of 1 $\mu\text{mol/L}$ on the release of the pro-inflammatory cytokine TNF- α (Fig. 1C) and IL-1 β (Fig. 1D) in PMA-differentiated THP-1 macrophages. Interestingly, MF reduced the secretion of TNF- α from macrophages stimulated by LPS by 20%, which was more than any of the other prenylated *Macaranga* stilbenoids (range of 3–16%) tested by Hošek et al. (2019). This anti-inflammatory effect of MF was comparable to that of prednisone (24%), a routinely used corticoid anti-inflammatory drug (Fig. 1C). Moreover, a similar anti-inflammatory effect of MF was also observed on the secretion of IL-1 β from macrophages stimulated by LPS (Fig. 1D).

We next focused on the regulation of TNF- α and IL-1 β upstream, particularly on the transcription factors NF- κB and AP-1, which concomitantly control the expression of several pro-inflammatory cytokines, including TNF- α and IL-1 β . The canonical inflammatory pathway that was triggered by TLR4 and led to overexpression of TNF- α involved NF- κB /AP-1 activation (Lawrence, 2009; MacIntyre et al., 2014). Again in this case MF showed one of the strongest inhibitory effects of the tested prenylated *M. siamensis* stilbenoids (Fig. 1E), with a therapeutic potential greater than that of prednisone. Overall at the

clinically transferable dose of 1 $\mu\text{mol/L}$, the anti-inflammatory activities of compounds 4 and 5 are weaker than MF justifying it to be the most promising candidate for further evaluation of anti-inflammatory and protective effects. Then, to gain some mechanistic insights we investigated MF effect on the upstream activation of NF- κB /AP-1 which includes $I\kappa B\alpha$ (Lawrence, 2009) and mitogen-activated protein kinases (MAPKs) (MacIntyre et al., 2014). Interestingly, MF reduced the degradation of $I\kappa B\alpha$, a direct inhibitor of NF- κB , but did not inhibit the activation (phosphorylation) of MAPKs known for leading to the activation of AP-1 (Fig. 1F). Therefore it is tempting to believe that MF could inhibit the inflammation in LPS-stimulated macrophages primarily through $I\kappa B\alpha$ /NF- κB signaling, rather than by acting via the MAPK/AP-1 pathway.

Since inflammatory response of NF- κB is driven by its nuclear translocation and its binding activity, interfering with one of these steps or with both may account for MF anti-inflammatory effect. Since $I\kappa B\alpha$ primary role is to bind to NF- κB for preventing its translocation to the nucleus, our results showing MF potential to inhibit $I\kappa B\alpha$ degradation suggests that it could interfere with NF- κB nuclear translocation. Therefore we further investigated whether MF could also directly interfere with NF- κB to dampen its binding activity.

Thus, we evaluated the interaction of MF with NF- κB DNA binding site by simulating a ligand-protein docking into DNA binding pocket of the NF- κB heterodimer p65/p50 according to the method by Trott and Olson (Trott and Olson, 2010) (Fig. 2A). The hydroxyl groups of MF tend to form conventional hydrogen bonds (O–H...O) with Thr502 and Ser508 residues. Non-classical hydrogen bond (N–H... π) was observed with Leu507. The alkyl hydrophobic interactions were detected with residues His441, Lys444, Val447, and Leu507. Two phenyl rings of MF typical for stilbene core tend to form π -alkyl hydrophobic bonds with Lys444 and Leu507 (Fig. 2B and C). Overall MF displayed a high affinity ($\Delta G = -6.7$ kcal/mol) for NF- κB DNA binding site similar to other biologically active stilbenes inhibiting the NF- κB /AP-1 pathway (Leláková et al., 2019). In comparison with other tested *Macaranga*

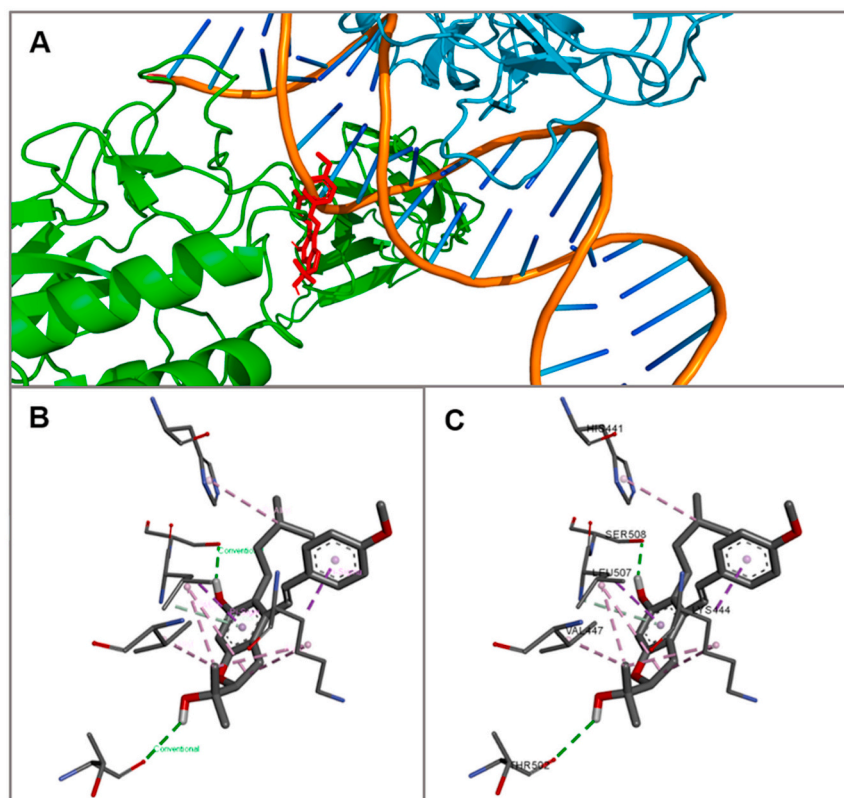


Fig. 2. Molecular docking pose of MF interactions with NF- κB (PDB 3GUT). A, The bond of MF to p50 subunit of NF- κB DNA binding pocket. Red - MF, green ribbon - p50 subunit, blue ribbon - p65 subunit, orange double helix with colored sticks - DNA fragment; B, Interactions of MF with p50 NF- κB depicting hydrogen bonds (green—classical, grey—non-classical) and hydrophobic interactions (dark and light purple); C, MF forms interactions with p50 residues Thr502, Ser508 (classical hydrogen bonds), Leu507 (non-classical hydrogen bond and hydrophobic interactions), His441, Lys444, and Val447 (hydrophobic interactions).

compounds (Table S1), MF had the highest extension to DNA binding site and thus provided the highest steric interaction with DNA binding site. Altogether our results suggest that MF may not only inhibit NF- κ B activity by indirectly targeting its translocation preventing I κ B α degradation but also directly by interfering with the DNA binding site of NF- κ B.

3.3. Anti-inflammatory dose of MF does not alter the proliferation and viability of BV-2 microglial cells

We then tried to determine if this anti-inflammatory effect observed on monocytes could be extended to microglia, the immune cells of the brain. To evaluate whether the anti-inflammatory effect of MF could be obtained on the safe side within a concentration range similar to the active one used for monocytes, we first performed a dose-response study to assess whether MF had an effect on the proliferation of BV-2 microglia (Fig. 3A) and their corresponding mitochondrial activity (Fig. 3B), that are accepted indicators of BV-2 vital cellular functioning. Not only changes in mitochondrial activity reflect an alteration of the energy homeostasis of the cell physiology often associated with a switch from physiological to pathological states, but mitochondria alteration is

known for triggering inflammation and to play a central role in pro-inflammatory signaling and inflammatory response (Meyer et al., 2018). Therefore it was crucial to know whether and at which dose MF may alter the physiological functioning of BV-2. The high dose of 15 μ mol/L altered the proliferation and activity of BV-2, but doses below 6 μ mol/L had no negative effect (Fig. 3A and B).

We therefore chose the concentration 1 μ mol/L, previously identified and used to reduce the LPS-induced inflammatory response in monocytes, and used it for all subsequent experiments to evaluate the protective and anti-inflammatory potential of MF on microglia challenged by LPS (Kacimi et al., 2011). At this concentration, pretreatment with MF decreased the LPS-stimulated loss of microglia by 20% relative to the vehicle after 24 h (Fig. 3C and D). This cellular protection was accompanied by a 20% reduction in the release of LDH by cells pretreated with MF as compared to cells treated only with the vehicle (Fig. 3D).

3.4. Pretreatment with MF dampens the LPS-induced pro-inflammatory response of BV-2 microglial cells

The important anti-inflammatory properties of MF at 1 μ mol/L, established on LPS-stimulated monocytes, along with its protective

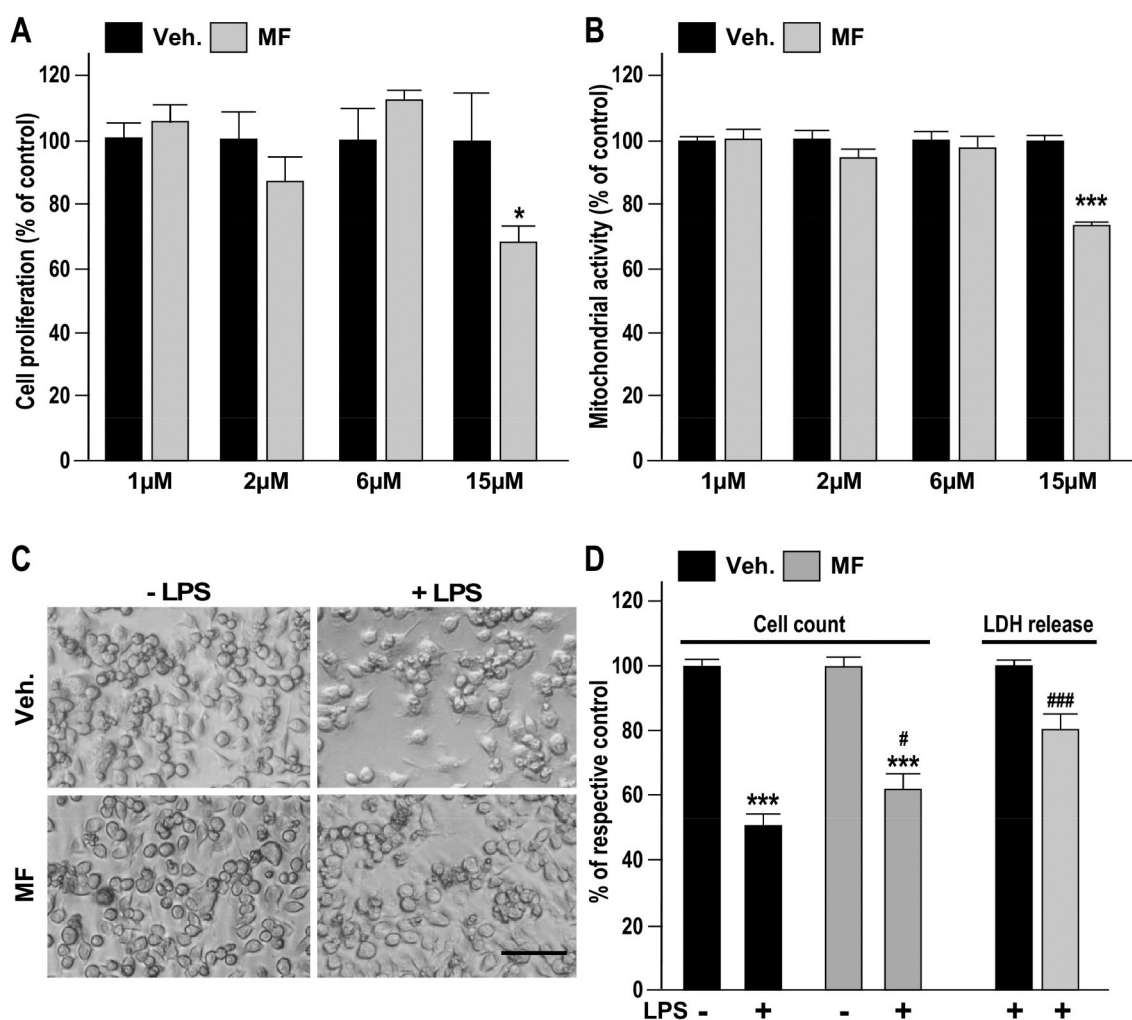


Fig. 3. Determination of the dose-limiting toxicity of MF and its protective effect against inflammatory challenge in BV-2 cells. **A**, Effect of a 24 h-application of MF at concentrations of 1, 2, 6, and 15 μ mol/L on the proliferation of cells assessed by cell counting, * indicates $P < 0.05$, significant difference in comparison with BV-2 cells treated only with the vehicle (Veh.); **B**, Effect of a 24 h-application of MF on the mitochondrial activity, *** indicates $P < 0.001$ compared to Veh.; **C**, Representative snapshots (Scale bar = 100 μ m) showing the MF-induced improved survival of BV-2 cultures injured by a 24 h-LPS challenge; **D**, Quantitative assessment of the beneficial effect of MF on BV-2 cultures injured by a 24 h-LPS challenge, as determined by cell counting and the extent of LDH release, *** indicates $P < 0.001$ compared to the respective LPS non-stimulated control, # $P < 0.05$ and ### $P < 0.001$ indicate significant differences between MF+LPS vs. Veh.+LPS. Data are presented as the mean \pm SEM; $n = 6$ measured in sextuplicates, and analyzed by Student's t-test.

action for BV-2 brain immune cells, led us to investigate its anti-inflammatory action in reducing the production of IL-1 β and TNF- α . MF reduced the TNF- α and IL-1 β protein expression in both intracellular content and release of proteins at the basal level, with no harmful stimuli (Fig. 4A).

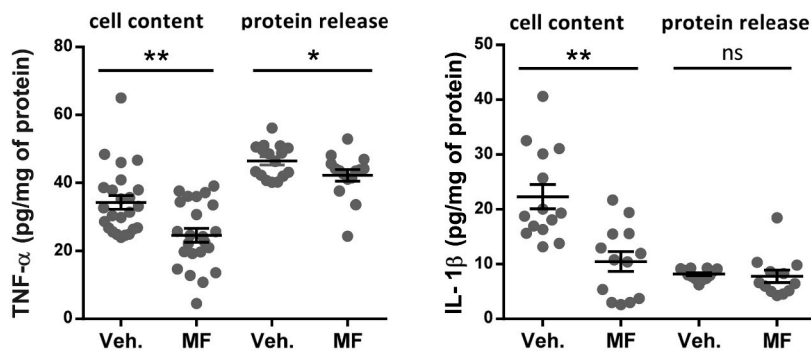
Moreover, BV-2 cells pretreated with MF showed an anti-inflammatory effect in the form of dampened BV-2 response to LPS-induced inflammation (Fig. 4D). Indeed, the intracellular content and release of both cytokines were also substantially reduced in cells pretreated with MF as compared to those treated only with the vehicle (Fig. 4D). This effect was correlated with a reduced level of gene expression for both pro-inflammatory cytokines: 1 h of pretreatment of BV-2 cells with MF significantly reduced the expression of LPS-stimulated TNF- α and IL-1 β mRNA by 26% and 20%, respectively (Fig. 4C).

3.5. MF dampens the early stage of inflammation in a co-treatment with LPS

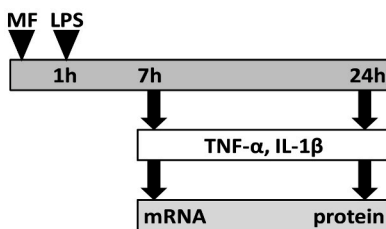
The anti-inflammatory effect of pretreatment with MF is of great interest for nutritional intervention and the development of functional foods. But the fact that pretreatment with MF would need to be applied pre-emptively limits its clinical usefulness, particularly for the situations arising without warning. Therefore, we investigated the possible therapeutic use of MF as an anti-inflammatory drug during (a cotreatment protocol) or after (a post-treatment protocol) the establishment of inflammation.

To study the effect of MF in the early phase of inflammation, we assessed the cellular content and protein release of the inflammatory cytokines TNF- α and IL-1 β in a culture medium of BV-2 cells after 6 h and 24 h of cotreatment with MF and LPS. Co-application of MF and LPS

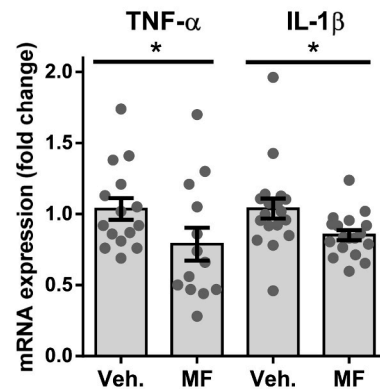
A. Basal level 24h



B. PRE-treatment model



C. mRNA expression, 7h



D. LPS-activated 24h

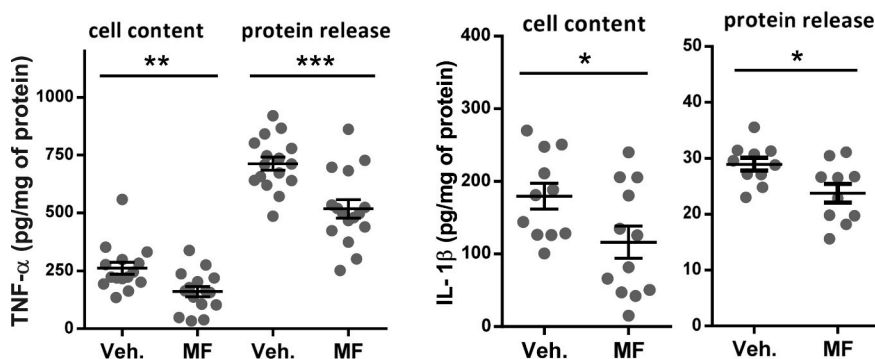


Fig. 4. Anti-inflammatory effects of MF pre-treatment on the expression of TNF- α and IL-1 β . A, MF reduced the basal expression and release of TNF- α and IL-1 β by BV-2 cells; B, Schema illustrating the protocol of pre-treatment of BV-2 cells with MF (1 μ mol/L) and the timing of evaluation of its effect on the mRNA and protein expression; C, MF reduced the LPS-stimulated expression of TNF- α and IL-1 β mRNA; D, MF reduced the LPS-induced expression and release of TNF- α and IL-1 β by BV-2 cells. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. Veh. group. Data are expressed as the mean \pm SEM, n = 3, measured in sextuplicates, analyzed by the non-parametric Mann and Whitney test for two independent groups.

reduced both the intracellular level and the release of TNF- α which are normally induced by the application of LPS (Fig. 5). Marked inhibition (33%) of the LPS-induced TNF- α up-regulation was already observed after 6 h of cotreatment. This anti-inflammatory effect clearly influenced the level of TNF- α release, which was decreased by 20% after 24 h (Fig. 5B). Similarly, severe inhibition of the IL-1 β response to LPS was reflected in both the cell content and the culture medium after 6 and 24 h of cotreatment (Fig. 5C). We then determined the extent to which the beneficial effects observed in the BV-2 cell line could be extended to brain-sorted primary microglia (Fig. 5D). Whereas under the applied experimental conditions cotreatment with MF seemed not to be efficiently reduce the level of TNF- α in a statistically significant manner.

Cotreatment with MF lowered the level of IL-1 β in primary microglia by factor of five, as compared to the vehicle (Fig. 5D).

3.6. Post-treatment with MF for resolving LPS-induced inflammation

Establishing the proof of the anti-inflammatory MF efficiency as post-treatment is a mandatory step toward clinical translation. Given as post-treatment, anti-inflammatory drugs are expected to resolve an ongoing inflammatory state by lowering the levels of cytokines such as TNF- α and IL-1 β . After LPS challenge, the expression of such pro-inflammatory molecules is initiated within a 3–6 h time window, that also correlates with their *in vivo* expression, observed in severe

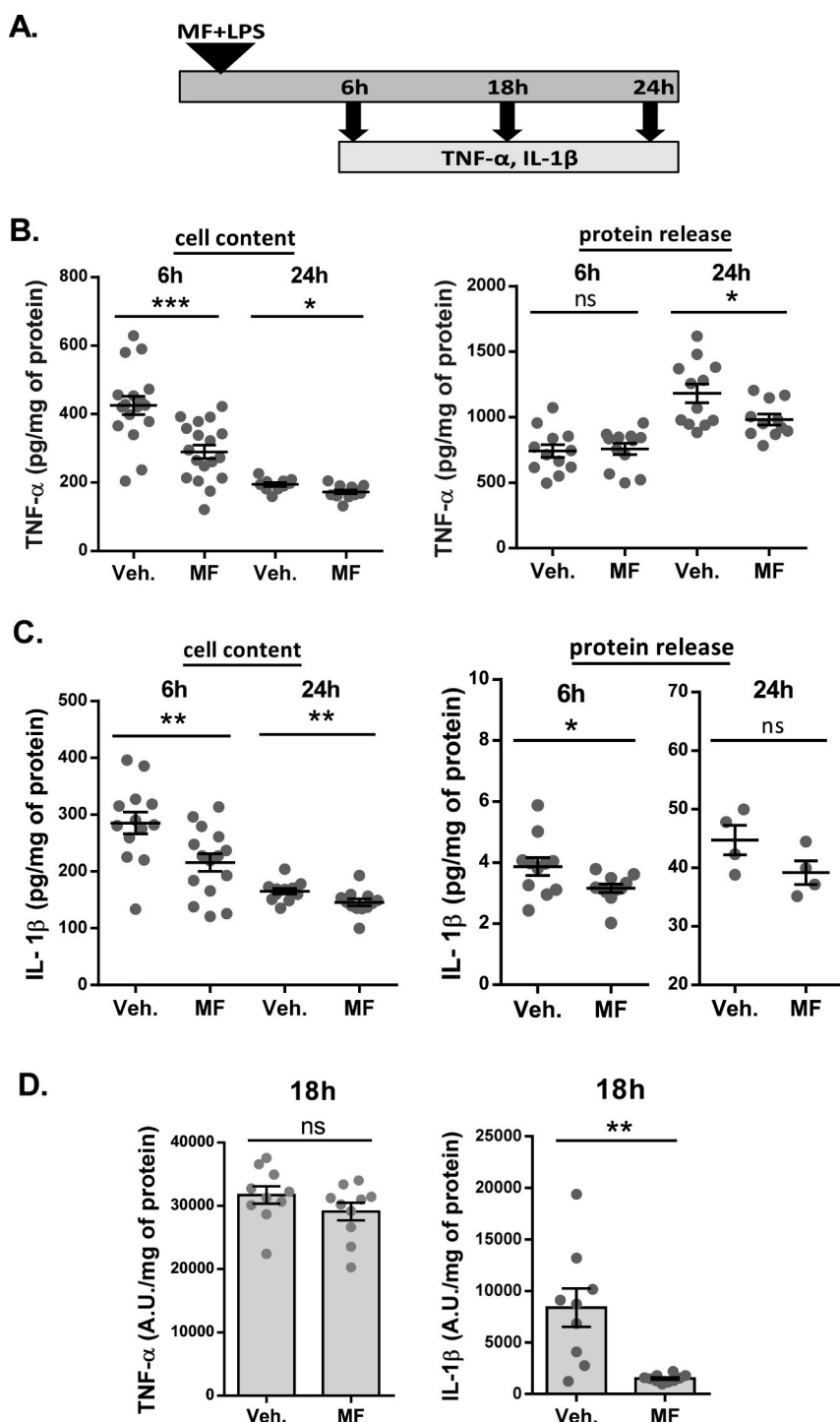


Fig. 5. Anti-inflammatory effects of MF cotreatment with LPS in the BV-2 cell line and the *ex vivo* culture of microglia cells. **A**, Schema illustrating the cotreatment of BV-2 cells and primary mouse microglia with MF (1 μ mol/L) co-treatment with LPS (1 μ g/mL) together with the times at which the cytokines were measured: 6 h and 24 h for the BV-2 cell line, and 18 h for the *ex vivo* culture of microglia cells; **B**, TNF- α expressed in the cell content and protein release from the BV-2 cells at 6 and 24 h; **C**, IL-1 β expression in cell content and protein release from BV-2 cells at 6 and 24 h; **D**, TNF- α and IL-1 β protein contents in brain-sorted mouse microglia after 18 h of cotreatment of MF with LPS or only the vehicle. Data are expressed as the mean \pm SEM, $n = 3$, measured in sextuplicates, analyzed by the Mann-Whitney test. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. Veh. group.

acute CNS pathologies with a massive inflammatory reaction, like stroke (Le Thuc et al., 2016; Nilupul Perera et al., 2006). As inhibition of inflammation is of interest to change the course of the pathology and improve stroke outcome, especially if starting the treatment after the recommended therapeutic window for thrombolysis with tPA (after 3–4.5 h after the onset of stroke) (Hacke et al., 2008), we therefore investigated the effects of administering MF as a post-treatment, 3 or 6 h after the onset of LPS-induced inflammation (Fig. 6A and B, respectively) on TNF- α and IL-1 β inflammatory response, observed after 6 h or 24 h, respectively.

The amount of TNF- α was found to be reduced by approximately 20% in both the cellular content and external medium, whether MF was applied 3 or 6 h after LPS (Fig. 6A and B). Treating the inflammation with MF 3 h after its induction reduced the IL-1 β response to LPS found after 6 h, but treatment delayed by 6 h did not influence IL-1 β levels 24 h after the onset of inflammation (Fig. 6B). This result may be explained by the fact that as IL-1 β is known to be expressed rapidly during the first hours after LPS stimulation (Kwon et al., 2010), the association of late post-treatment and a low dose of MF would be too stringent conditions to show any anti-inflammatory effect. Therefore, increasing the dose

and/or shorten the timing of administration may be anticipated options to further investigate for revealing and improving the MF anti-inflammatory effect on this particular cytokine. Moreover, while several groups have shown that BV-2 cells produce a detectable quantity of interleukin, in our settings, IL-1 β was hardly induced and secreted as compared to TNF- α at the selected measurement times (6 and 24 h). In these stringent settings, it is most likely that the difference in MF effects at the dose of 1 $\mu\text{mol/L}$ on TNF- α and IL-1 β could be explained by the timing and level of the protein release in response to LPS challenge.

4. Discussion

Inflammation accompanies many acute and chronic pathologies at both the peripheral and CNS levels. Acute inflammatory diseases are mostly infectious, but inflammation may also be a part of non-infectious pathologies, such as stroke, traumatic brain injury (Skaper et al., 2018), and hemorrhage (Goerge et al., 2008). Chronic inflammation is usually characterized as a slowly progressing mild inflammatory reaction that is linked with several long-term disorders, including atherosclerosis (Libby et al., 2002), inflammatory bowel diseases (Vočyánová et al., 2015),

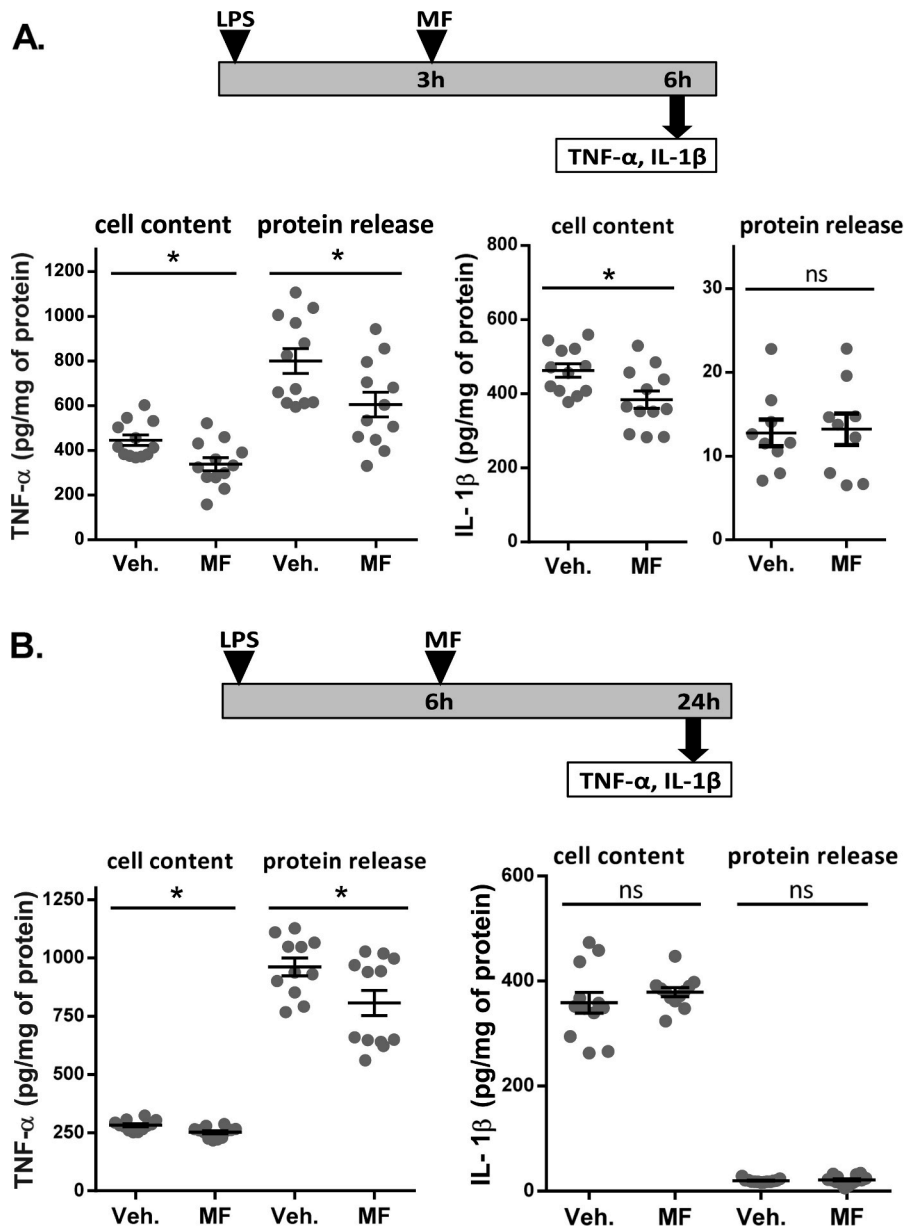


Fig. 6. Anti-inflammatory effects of post-treatment with MF. Schema illustrating the protocol of inducing inflammation with LPS (1 $\mu\text{g/mL}$) in BV-2 cells and treating them 3 h (A) or 6 h later (B) by applying MF post-treatment (1 $\mu\text{mol/L}$). A, Effect of MF applied 3 h after the induction of inflammation by LPS. MF post-treatment reduced both the protein content and release of the TNF- α and the IL-1 β as evaluated 6 h after the application of LPS. B, When the MF post-treatment was effectuated at 6 h after the induction of inflammation by LPS, the anti-inflammatory effect of reduced TNF- α protein content was still found 24 h after the application of LPS, but that of the IL-1 β protein was not. That transitory expression of IL-1 β apparently occurred entirely before the post-treatment was applied. Data are expressed as the mean \pm SEM, $n = 3$, measured in sextuplicates, analyzed by the Mann-Whitney test. * $P < 0.05$ vs. Veh. group.

chronic obstructive pulmonary disease (Barnes et al., 2015), osteoarthritis (Robinson et al., 2016), or autoimmune disorders, such as allergic asthma (Barnes et al., 2015) or RA (Smolen et al., 2018).

Monocytes and microglia are major phagocytes of the innate immune system, providing the first line of defense. The presence of TLRs enables them to respond to different noxious stimuli, exogenous pathogen-associated molecular patterns (PAMPs) and some endogenous damage-associated molecular pattern molecules (DAMPs). TLR4 is a major receptor implicated in the inflammatory response recognizing bacterial LPS (Zhu and Mohan, 2010). Activation of TLR4 signaling leads to the expression of several pro-inflammatory cytokines, including TNF- α and IL-1 β , mediated by the activation of NF- κ B signaling pathway (Pálsson-McDermott and O'Neill, 2004). Both TNF- α and IL-1 β are potent regulators of the innate immune system important for host defense. Especially, there is an increasing evidence pointing out a prominent role of IL-1 β in acute neuronal injuries and many chronic brain diseases (Allan and Rothwell, 2003). Targeting the TLR4/NF- κ B pathway therefore represents a potential therapeutic strategy for both acute and chronic inflammation-based diseases that are linked with abnormal secretion of cytokines (Kuzmich et al., 2017).

Plant-derived stilbenoids have demonstrated a promising ability to attenuate peripheral (Dvorakova and Landa, 2017; Eräsalo et al., 2018; Leláková et al., 2019) and CNS inflammation (Akinwumi et al., 2018; Hornedo-Ortega et al., 2018) *in vitro* and *in vivo*. However, clinical trials to investigate their potential in humans are still needed. Pterostilbene, a natural derivative of *trans*-resveratrol, that has frequently been investigated, has shown the ability to inhibit platelet aggregation *ex vivo* (Messina et al., 2015), improve high fat-induced atherosclerosis inflammation via NF- κ B signaling *in vivo* (Zhang and Zhang, 2016), protect against myocardial ischemia-reperfusion (I/R) injury by reducing oxidative/nitrosative stress and the inflammatory response to it (Yu et al., 2017), and reduce the size of a myocardial infarction (Wu et al., 2017). At the CNS level, pterostilbene protects against hyperglycemia- (Yang et al., 2017) and glutamate-induced neuronal oxidative injury (Wang et al., 2016). In contrast, the stilbenoids of *Macaranga* spp. have not yet been thoroughly investigated. Among these compounds, MF displays a safe profile for therapeutic use, with low cytotoxic effects on THP-1 cells and on the BV-2, MOLT-3, HepG2, HuCCA-1, and A549 cell lines (Pailee et al., 2015). We focused on the activity of the transcriptional factors NF- κ B and AP-1, both of which have been shown to concomitantly control the expression of various inflammatory cytokines during inflammatory response (MacIntyre et al., 2014). Whereas NF- κ B is mostly responsible for the gene and protein expressions of TNF- α and IL-1 β and is up-regulated by its direct cytoplasmic inhibitor I κ B α (Lawrence, 2009), AP-1 is controlled predominantly by ERK1/2, p38, and JNK, members of the family of MAPKs (Zhou et al., 2018). In the present work, we reveal the promising potential of MF against peripheral inflammation by demonstrating its ability to: 1) intervene in the I κ B α /NF- κ B pathway and inhibit the degradation of I κ B α , which leads to decreased activation of the transcriptional factor NF- κ B, and 2) reduce secretion of inflammatory cytokines TNF- α and IL-1 β , which are under the transcription control of NF- κ B. This result is in agreement with the ability of non-prenylated stilbenoids to reduce the phosphorylation of MAPKs (Leláková et al., 2019), whereas prenylated stilbenoids have been shown to affect the degradation of I κ B α (Hošek et al., 2019).

Like monocytes at the peripheral level, microglia and CNS infiltrated monocytes are responsive to neuronal damage. Their activation is an alarming sign of the brain pathology that can occur in conditions such as microbial infection, traumatic brain injury, or stroke, and also degenerative disorders of the CNS (Dheen et al., 2007). In this work, we demonstrate the ability of MF to improve the *in vitro* native state of microglia by reducing the basal release of cytokines. This beneficial effect is associated with protection against LPS-induced loss of microglia.

Moreover, under inflammatory conditions, MF dramatically inhibits the LPS-stimulated gene and protein expressions of both TNF- α and IL-

1 β , whatever the stage of treatment (pre-, co-, and post-treatment). The greatest reduction of cytokines (up to 43%) is achieved when the microglia are pretreated with MF before inflammation is induced. Although the up-stream signalization, such as level of NF- κ B and I κ B was not investigated in BV-2 cells, due to the phenotypic similarity of monocytes and microglia (Orihuela et al., 2016), it is tempting to anticipate that the response in mouse microglia may be similar. These results rivet attention on the potential for developing MF as a nutritional or dietary supplement, aimed at preventing any type of systemic and/or CNS inflammatory disorder, and thereby maintaining or improving organ function, preventing chronic disorders, promoting good health, or even delaying the process of aging (Nasri et al., 2014; Tauskela et al., 2016).

In the acute and chronic states of inflammatory disease, the ability of MF to reduce the secretion of the cytokines TNF- α and IL-1 β may be of particular interest, because these cytokines are known to be involved in the activation of resident microglia and astrocytes during infection, inflammation, and the development of brain injury (Norden et al., 2016). Their dysregulation and sustained activation can create a toxic circle, elevating the release of inflammatory factors that endanger the functioning and survival of neurons (Ramesh et al., 2013).

Our results show, that MF could be interesting as a supplementary compound used against chronic inflammatory diseases that would correspond to the so called "early phase of inflammation" in the present work. Low-grade systemic inflammation is like CNS inflammation (neuroinflammation), a pathological process that accompanies many persistent diseases and also the normal aging process nicknamed "inflammaging". Inflammaging is possibly related to chronic activation of the innate immune system and changes in the functions of monocytes (Benedetto et al., 2017). Altered activation of microglia, characterized by elevated levels of TNF- α and IL-1 β , is associated with neurodegenerative disorders, such as AD, PD, and HD, and is a reaction to the presence of cellular aggregates and the misfolded proteins β -amyloid, α -synuclein, and huntingtin, respectively. Reducing neuroinflammation could be a way to slow the progress of these diseases (Sastre et al., 2014). Inflammation underlying neurodegeneration is also present in multiple sclerosis, amyotrophic lateral sclerosis, systemic lupus erythematosus, neuromyelitis optica, diabetes mellitus, chronic bacterial meningitis, Lyme neuroborreliosis and HIV-1 encephalitis (Amor et al., 2010; Ramesh et al., 2013). Some stilbenoids, such as *trans*-resveratrol, isorhapontigenin, gnetol and pinosylvin, have shown significant potential to inhibit butyrylcholine esterase, one of the causal enzymes of AD (Sermbonpaisarn and Sawasdee, 2012). Oxyresveratrol exerts neuroprotective effects against Parkinsonian mimetic 6-hydroxydopamine-induced neurotoxicity in neuroblastoma cells (Chao et al., 2008). Studies of the "Mediterranean diet", which is based on vegetables, fruits, whole grains, nuts, fish, and seafood, healthy fats, and olive oil, with moderate consumption of red wine, and is rich in polyphenols, including stilbenoids, have reported beneficial effects, mitigating age-related and other neurodegenerative pathologies (Hornedo-Ortega et al., 2018).

The anti-inflammatory effects of MF may also be of interest for acute conditions such as cerebral ischemia, in which reduced blood flow and oxygen supply (hypoxia) lead to cerebral infarction. This condition triggers an intravascular inflammatory cascade that extends into the surrounding tissues of the injured brain, and is characterized by elevated levels of inflammatory cytokines, chemokines, adhesion molecules, reactive oxygen species, nitric oxide, extracellular proteases, and other substances flowing into and accumulating in immune cells, such as microglia (Le Thuc et al., 2015). A therapeutic strategy that intervenes in the inflammatory process of cerebral ischemia might slow down and limit the brain damage caused by ischemia. The potential of stilbenoids to act against cerebral ischemia has been, however not thoroughly, investigated. It is noteworthy that a study using a transient rat middle cerebral artery occlusion model has shown that oxyresveratrol reduces the infarction and thus prevents neurological deficits induced by the ischemia/reperfusion (I/R) injury, inhibits the release of cytochrome c,

and prevents the activation of caspase-3 (Andrabi et al., 2004). This indicates that as strong anti-oxidant and anti-inflammatory agents, stilbenoids could be effective in the treatment of I/R-injury, a devastating pathology without therapeutic opportunity.

Despite their relatively wide range, most of the anti-inflammatory drugs available on the market are linked with many side effects when used in the long term. The therapeutic and adverse effects of the most commonly prescribed non-steroidal anti-inflammatory drugs (NSAIDs) are related to the inhibition of cyclooxygenases (COXs). Their gastrointestinal, renal, and cardiovascular toxicity, together with numerous drug interactions, represent a risk especially for elderly patients who take multiple medications (Wongrakpanich et al., 2018). Steroidal drugs can have pleiotropic and severe adverse effects (Aljebab et al., 2017). Biological treatment in the form of anti-TNF- α and anti-IL-1 β antibodies has been shown to be highly effective for chronic disorders such as RA, especially when treatment is started in the early phase of the disease. However, this treatment represents an enormous financial burden for the health care system and is not accessible to all patients (Dinarello et al., 2012; Monaco et al., 2015). Thus, nutritional intervention and the development of functional food based on natural molecules that are not known to have profound side effects alter promise of preventing inflammation caused by several chronic and acute diseases. Many good candidates may be hidden in natural sources. MF would be among the most promising thanks to its ability to counteract both peripheral and CNS inflammation. MF is structurally a small molecule, effective at a pharmacologically relevant concentration. Synthesizing it in the laboratory could lower its production cost as compared to existing commercial treatments.

MF and its derivatives could fulfill the goal of finding a novel anti-inflammatory substance that is potent in prevention and/or supplementary treatment of inflammation-based pathologies of both systemic and central character and safe for long-term use.

5. Conclusion

Our current study shows that stilbenoid macasiemenene F isolated from a plant *M. siamensis* has significant anti-inflammatory effects. It is therefore an interesting candidate for further investigation using *in vivo* models where inflammation in the brain plays a major role, e.g., cerebrovascular diseases. The increasing prevalence of chronic inflammatory disorders and more frequent occurrence of acute inflammatory diseases make the need for safe and effective anti-inflammatory therapeutic strategies. Other promising candidates may yet be found in natural sources, but with respect to the current state-of-the-art in nutraceuticals, MF is the most likely to prove being effective for the prevention and treatment of inflammation-related pathologies, including secondary brain injury following a stroke, for which a therapeutic opportunity is severely lacking.

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Author contributions

VP and PP isolated the studied molecules. VL, CH, NB, JH and KŠ designed the research project and interpreted the study data; VL, JV performed the experiments and collected the data; SBD, CW, TC and JM assisted the animal experiments. CH and NB share the position of last author. All authors critically revised the manuscript and approved the final version of the article, including the authorship list.

Declaration of competing interest

The authors declare to have no conflict of interest related to this publication and there has been no financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2020.113147>.

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